Edited by CONSTANTINOS A. GEORGIOU
and GEORGIOS P. DANEZIS

FOOD AUTHENTICATION

Management, Analysis and Regulation



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Food Authentication

Management, Analysis and Regulation

Edited by Constantinos A. Georgiou and Georgios P. Danezis

Agricultural University of Athens, Greece

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Preface

Food Authentication: Management, Analysis and Regulation has been written by an international group of peers with high academic and research credentials. As well as providing a reference for researchers working in food authentication, it will serve as an essential source of information for analytical and food scientists in the field in who have an interest in analytical approaches. The book is also a companion for under- and post-graduate students in their study of food authentication, and aims to be useful to researchers in universities and research institutions.

We would like to acknowledge the generous contribution of all the chapter authors. The remarkable effort of the contributors that are leading scientists in their field is greatly appreciated. Further, we acknowledge Wiley staff for their helpful assistance through the development of this project. It has been a pleasure to work with all of you. Last, but not least, we thank our families for their support.

To our families,

Constantinos A. Georgiou
and Georgios P. Danezis
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December 2016

Part A

Introduction and Status

1

Introduction, Definitions and Legislation

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1.1 Introduction

Consumers have always wanted to know where their food came from and what it consisted of. In recent days, especially in more economically developed countries (MEDC), consumers have demanded to know without any doubt the origin and content of the food and whether it is safe to eat; in certain cases, consumers are willing to pay more for specific quality attributes (Aprile *et al.* 2012). Apart from being assured of the quality of the product they purchase, there is also strong interest in adulteration and fraud which, as well as financially damaging, can represent significant health hazards.

The proper description of food (and beverages) and its ingredients is enforced by labeling legislation which aims to reassure the consumer by giving them all the available information needed by issuing guidelines on the proper description of food. In order to enforce this legislation, state inspection bodies use various scientific methods to certify that the food products available in the market fully comply with label description.

Authenticity means the quality of being authentic or genuineness (www.dictionary.com). This word originates from the Greek word *authentikós* which means original, primary, at first hand, equivalent to authént (ēs) one who does things himself (aut- aut- + -hentēs doer). Similar definitions identify authentication with genuineness and truthfulness of origins, attributions, commitments, sincerity, and intentions; if something is authentic, it is not a copy or forgery.

The concept of authentic, defined by something original, true, undoubted, etc. applied to food, certifies that these products are of a certain origin in concordance with standards and force rules and with the inscriptions of the presentation label (Pascu 2013). Authenticity is of great concern for producers, manufacturers, traders, consumers, and state inspection bodies.

Food authentication is the process by which a food is verified as complying with its label description. Labeling and compositional regulations, which may differ from country to country, have a fundamental place in determining which scientific tests are appropriate for a particular issue (Dennis 1998). This is a brief definition of the principle of "food authentication", but food authentication includes or may include many different attributes that give each food product its unique character.

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1.2 Definitions

1.2.1 Food Origin

This may refer to the place or region in which the food or its ingredients were produced. It also refers to the botanical species and/or raw material used. In the case of meat products, this includes the wild or farmed species. A tool for covering these prerequisites is traceability. Traceability in most developed countries is a legal obligation of the production and marketing, but is also a major part of quality management schemes and standards (i.e., HACCP, ISO 22000:2005, FSSC 22000, EurepGAP, BRC, etc.). "Traceability" means that the path that led the product and its ingredients from its initial origin to the point that it became available to the final (or the intermediate) consumer(s) is known. All the information of the pathway that the product and its ingredients followed is recorded to the final point of sales. However, any systems involved in defining traceability are only as strong as their weakest link. In the EU, the main tool which enforces traceability is Regulation 178/2002 which came into force in 2005, although since 1993 the legislation (Directive 93/43) had made mandatory the existence of an active hazard analysis and critical control point (HACCP) plan in most enterprises that handled food, and traceability is *sine qua non* in any such scheme.

1.2.2 Label

The description of any food product is presented on its label. Although the main role of the label is to give the purchaser all the description details of the product needed to make an informed choice, it is often connected with legal aspects concerning the protection of the specific brand of the company that handles the product such as: brand protection (from counterfeit/fraud, misbranding); trademark; trade dress; trade secret; and liability of the production or marketing company(ies). Other data that the label may provide are nutritional properties, date of production (or year of harvest), date of manufacture, expiration date, and the type of technology used to produce or process the food. When a company addresses its products to a specific target group of consumers, specific labeling often combined with certain certifications and logos can be used. Examples of this practice include: related religion practices (i.e., kosher for Jews, musbooh and halal for Muslims, no beef for Hindu); groups of people with special dietary needs or health concerns (i.e., diabetics, high cholesterol, infants, children, lactose intolerant, vegan, vegetarian, gluten free, potential allergens); and lifestyle choices (organic, biodynamic agricultural products, low carbon footprint, fair trade, sugar free, low calorie content, low fat content).

1.2.3 Adulteration and Fraud

Fraud of foods or food ingredients is usually defined as a deliberate alteration of the product in order to increase profit. The UK's Food Standards Agency (FSA) describes two main types of fraud: (1) the sale of food which is unfit and potentially harmful; and (2) the deliberate misdescription of food (FSA 2015). Although the misdescription of the product results in financial loss for the consumer, in most cases it rarely intends to harm (Brereton 2013; Braden 2014). Nevertheless, it can reduce consumer confidence and raise obstacles in authentic products sales. In 2009 the US Food and Drug

Administration (FDA) adopted the following working definition of fraud or economically motivated adulteration (EMA) (Braden 2014):

> fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production, i.e., for economic gain. EMA includes dilution of products with increased quantities of an already-present substance (e.g., increasing inactive ingredients of a drug with a resulting reduction in strength of the finished product, or watering down of juice) to the extent that such dilution poses a known or possible health risk to consumers, as well as the addition or substitution of substances in order to mask dilution.

Adulteration is generally described as the addition of inferior ingredients. Adulteration is not a new practice; it has been carried out since antiquity and probably since the beginning of trade. Pliny the Elder (AD 23–79) describes the ways in which traders used to adulterate wine, pepper, and balsam, all commodities of high added value and price (Rackham 1960). Such practices continued throughout the Middle Ages and until the present days. In 1820, German chemist Fredrick Accum (1769–1838) published his book *"A Treatise on Adulterations of Food and Culinary Poisons"* in which he described methods of adulteration and analytical techniques to detect them, focusing on valuable commodities of his era such as tea, coffee, bread, beer, and pepper. He also emphasized the quality of the water used for food and drug production (Accum, 1820), a key element even in the quality and safety schemes of the present day. His book was a best seller and was reprinted in Germany and USA; some consider it as milestone in the history of adulteration (Wilson 2008; Shears 2010).

Tähkäpää *et al.* (2015) describe food frauds/adulterations cases published in the EU Rapid Alert System for Food and Feed (RASFF) as well as the notifications of recalls published by the Finnish Food Safety Authority (Evira) during the period 2008–2012. Most cases (92%) originated outside EU, although the suggested reason for this is that the RASFF system "is tailored especially to report non-EU cases of frauds or adulterations." Another reason for this conclusion is that many EU regulations regarding the organization of the common market lay provisions of sampling checks of products on the internal market; on the other hand, checks for imported products from third countries are obligatory for almost all products with some exceptions (e.g., if a third country has requested European Commission approval and received certification; such approval is published in the C series of the Official Journal of the European Union).

1.3 Geographical Indications

When we are discussing the origin of food, one of the main issues is the link between origin and the particular geographical area where specific goods (and services) are originated. This link is covered by the generic terms "geographical indications (GI)" or "indications of geographical origin (IGO)", depending on the international and regional law. Such a link could give food added value reflected in its price.

According to the WTO (article 22.1 of the Trade-Related Aspects of Intellectual Property Rights, TRIPS Agreement 1994), geographical indications are: "indications

which identify a good as originating in the territory of a Member, or a region or locality in that territory, where a given quality, reputation or other characteristic of the good is essentially attributable to its geographical origin" (WTO 1994).

Similar definitions are found in other treaties. There are four global international treaties which provide regulation of GIs, namely the Paris Convention (1883), the Madrid Agreement (1891), the Lisbon Agreement (1958), and the TRIPS (1994), but none of these treaties may be considered as a treaty providing for uniform protection of GIs (Blakeney 2014; Mantrov 2014).

Keblová (2014) describes three general categories of GIs protection: (1) the trademarks system, followed by 56 countries including the USA, Australia, Canada, and Japan; (2) the *"suis generis"* system, followed by 111 countries including the EU; and (3) through legal regulation of unfair competition or other nonspecific legislation (Keblová 2014).

1.3.1 PDO, PGI, and TSG

In order to protect foods that were produced in certain member states or regions, in the early 1990s the European Economic Community (EEC; since 1993 European Union, EU) created a specific legal framework by introducing the terms "protected designation of origin" (PDO) and "protected geographical indication" (PGI), enacted with Council Regulation (EEC) No. 2081/92 and Council Regulation 2082/92, This was the first legal protection of geographical indications and designations of origin for agricultural products and foodstuffs linked to a territory or a particular production method, and an attempt to provide a set of rules for harmonization of European food production (with the exception of wines and spirits). For example, in France the terms *"appellations d' origine"* (AO) existed since 1919 and *"appellations d' origine contrôlée"* (AOC) since 1935 (Marie-Vivien *et al.* 2015).

In 2006, following the third reform of the Common Agricultural Policy (CAP) of 2003 which was aimed at quality as well as quantity (Swinnen 2010), the EU replaced Council Regulation (EEC) No. 2081/92 by Council Regulation (EC) No. 510/2006. This also remedied disputes USA and Australia had raised to the World Trade Organization (WTO) against the previous regulations in 1999 (Carcea and Melini 2013, p. 6), and led to the 2005 decision of the WTO Dispute Settlement Body (WTO 2005). At the beginning of 2013, Regulation (EC) No. 510/2006 was repelled by Regulation (EU) No. 1151/2012, the main legislation act that governs GIs in EU.

According to Regulation (EU) No. 1151/2012 (under Article 5 paragraph 1), a "designation of origin":

> is a name which identifies a product:
>
> a) originating in a specific place, region or, in exceptional cases, a country;
> b) whose quality or characteristics are essentially or exclusively due to a particular geographical environment with its inherent natural and human factors; and
> c) the production steps of which all take place in the defined geographical area.

where "geographical indication" (under Article 5 paragraph 2)

> is a name which identifies a product:
>
> a) originating in a specific place, region or country;

b) whose given quality, reputation or other characteristic is essentially attributable to its geographical origin; and
c) at least one of the production steps of which take place in the defined geographical area.

Both definitions describe how the production of a food is linked to a specific geographical area which can vary from a small island to a whole country. The difference between a PDO and PGI is that in the former all production steps take place in the designated area; for the latter at least one stage in the chain production must take place in the designated area to qualify a food product as PGI. The same regulation under Article 18 defines the criteria that a specific product or foodstuff must meet in order to be eligible to be registered as "traditional speciality guaranteed" (TSG), a term introduced by Council Regulation No. 509/2006. In all cases (PDO, PGI, and TSG), a product must first be registered in order to take advantage of the protection of the aforementioned legislation.

In the EU, PDOs, PGIs, TGIs, and organic products are labeled with special signs which are not trademarks and are strictly defined by relative legislation enforced by inspection bodies of the member states. Figure 1.1 depicts different versions of the EU official logos for the existing categories of geographical indications.

To date, in the EU there are 618 PDOs, 702 PGIs, and 54 TSGs registered products; 46 PDOs, 72 PGIs, and 6 TSGs are currently pending approval as presented at DOOR, the official database for registration of food products of the European Commission, initially enacted with Council Regulation (EEC) No. 2081/92 and Council Regulation 2082/92. These products number 1411 in total. As the above numbers show and Tosato (2013) concludes, the TSG scheme has experienced limited success so far due to structural flaws in the legislation and has not yet reached full potential (Keblová 2014).

Figure 1.2 shows the number of products registered according to Regulation (EU) No. 1151/2012 per country. As can be seen, most applications concern countries of the Mediterranean basin. One reason for this could be that countries such as Italy, France, Spain, Greece, and Portugal already had existing GI legislation schemes, mainly based on the AOC concept influenced by local traditions and autochthones species for many years before the first adoption of PDOs and PGIs in 1992 by EU. For example, even in 1990 the volume of AOC cheese production was more than 10% of total cheese



Figure 1.1 Versions of the official GIs logos of the European Union (PDO, PGI and TSG) according to Commission Implementing Regulation (EU) No. 668/2014.

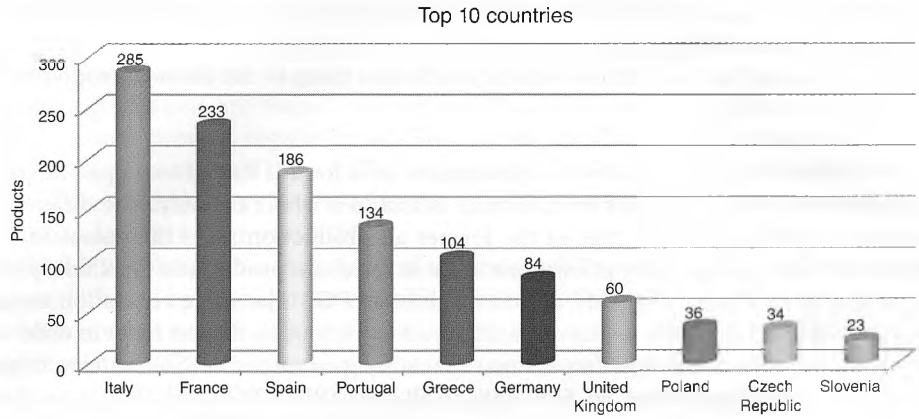


Figure 1.2 Collective number of PDO, PGI and TSG products per EU country. Top ten as in DOOR (EC 2016).

production in France and 49% in Italy (Bertozzi and Panari 1993). In Figure 1.3 the registered products are presented as categorized by the official classes of Regulation (EU) No. 1151/2012.

Regulation (EU) No. 1151/2012 also introduced the concept of "optional quality terms" suggesting two new categories: "mountain product" and "product of island farming." As stated, "Operating quality schemes for producers which reward them for their efforts to produce a diverse range of quality products can benefit the rural economy". It is suggested that "The optional quality term 'mountain product' ... will add value to the product on the market." Although only a general description is given for the term "product of island farming," the "mountain product" under article 31 (paragraph 1) is defined as follows:

> The term 'mountain product' is established as an optional quality term. This term shall only be used to describe products intended for human consumption listed in Annex I to the Treaty in respect of which: (a) both the raw materials and the feedstuffs for farm animals come essentially from mountain areas; (b) in the case of processed products, the processing also takes place in mountain areas.

1.3.2 Wines

The wine sector is a special case. It is often connect with tradition and, as an alcoholic beverage, has always been of special interest of taxation. A pre-existing geographical designation system in the EEC was the legislation regarding Quality Wines Produced in Specified Regions (quality wines p.s.r. or QWPSR) which were established with Regulations (EEC) No. 816/70 and No. 817/70 of the council which specified down "additional provisions for the common organisation of the market in wine" and "special provisions relating to quality wines produced in specified regions," respectively.

In 2008, the EU acknowledged that existing legislation had not " proved effective in steering the wine sector towards a competitive and sustainable development". In order to achieve the required objectives – "increasing the competitiveness of the Community's wine producers; strengthening the reputation of Community quality wine as the best in

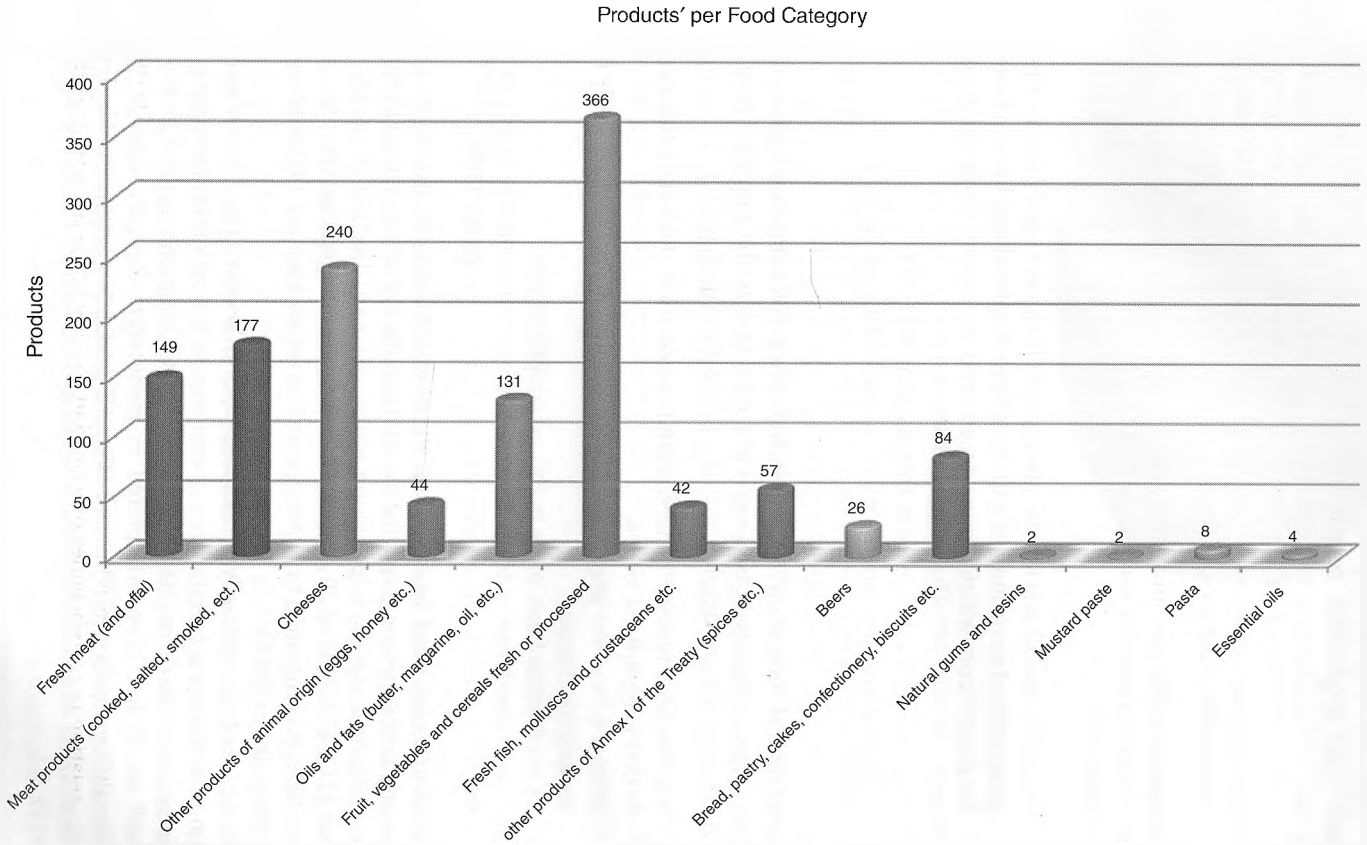


Figure 1.3 Registered agricultural products and foodstuffs per category from DOOR (EC 2016).

the world; recovering old markets and winning new ones in the Community and worldwide; creating a wine regime that operates through clear, simple and effective rules that balance supply and demand; creating a wine regime that preserves the best traditions of Community wine production, reinforcing the social fabric of many rural areas, and ensuring that all production respects the environment" – the EU issued Council Regulation (EC) 479/2008 of 29 April 2008 on the common organization of the market in wine. Following the framework of regulation 510/2006, the terms PDO and PGI were introduced within the wine sector. Under Article 34 of Regulation 479/2008:

> "designation of origin" means the name of a region, a specific place or, in exceptional cases, a country used to describe a wine that complies with the following requirements:
>
> i) its quality and characteristic are essentially or exclusively due to a particular geographical environment with its inherent natural and human factors;
> ii) the grapes from which it is produced come exclusively from this geographical area;
> iii) its production takes place in this geographical area;
> iv) it is obtained from vine varieties belonging to Vitis vinifera"

and

> "geographical indication" means an indication referring to a region, a specific place or, in exceptional cases, a country, used to describe a product referred to in Article 33(1) which complies with the following requirements:
>
> i) it possesses a specific quality, reputation or other characteristics attributable to that geographical origin;
> ii) at least 85% of the grapes used for its production come exclusively from this geographical area;
> iii) its production takes place in this geographical area;
> iv) it is obtained from vine varieties belonging to Vitis vinifera or a cross between the Vitis vinifera species and other species of the genus Vitis.

This approach continued by including wines (except specific products, e.g., spirit drinks, aromatized wines, or grapevine products) in the provisions set out by Regulation (EC) No. 1151/2012, the main legislation framework regarding GIs in the EU. Council Regulation (EC) 479/2008 of 29 April 2008 and Council Regulation (EC) No. 491/2009 of 25 May 2009, accompanied by 10 implementing regulations, are the basic regulations of the wine sector in the EU.

A special case of GI, especially concerning wine, is "*terroir*". This French word relates the origin of a wine to a specific area, sometimes to a single farm consisting of a few acres. According to Barham (2003) "The French AOC system is guided by the concept of '*terroir*," which has been interpreted in many ways through time. In 2010 The International Organisation of Vine and Wine (Organisation Internationale de la Vigne et du Vin or OIV) issued Resolution 333/2010 giving "*terroir*" the following definition (OIV 2010):

> Vitivinicultural "*terroir*" is a concept which refers to an area in which collective knowledge of the interactions between the identifiable physical and biological

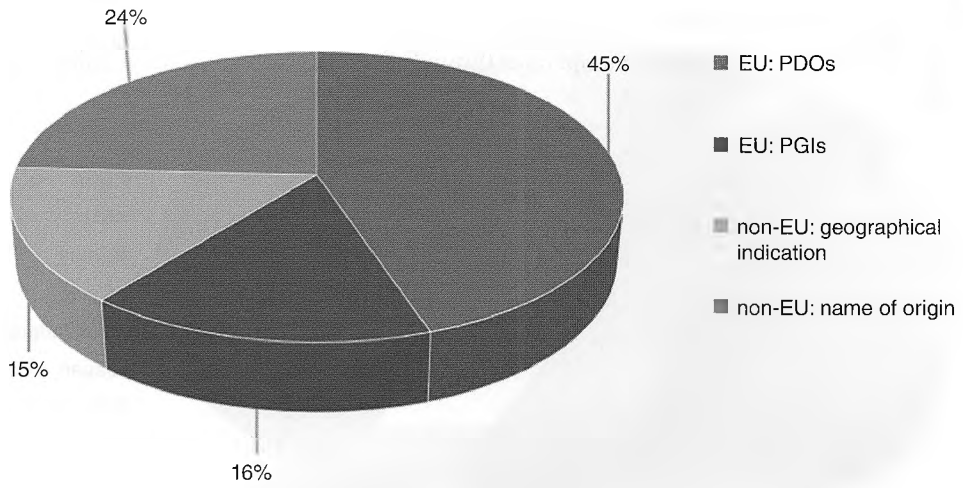


Figure 1.4 Wines registered in EU in accordance with Regulation (EU) No. 1308/2013. Data from E-Bacchus.

environment and applied vitivinicultural practices develops, providing distinctive characteristics for the products originating from this area. "*Terroir*" includes specific soil, topography, climate, landscape characteristics and biodiversity features.

Presently, the number of registered wines with geographical identifications in the EU is 2883 (E-Bacchus: database of GIs for wines in EU and third countries, in accordance with Regulation (EU) No. 1308/2013; EC 2013) and are categorized in Figure 1.4. It is interesting to note that only two non-EU wines are registered as PDOs, forming a distinct category (one from Napa Valley, USA and one from Vale dos Vinhedos, Brazil).

1.4 Organics

The Research Institute of Organic Agriculture (FiBL) and International Federation of Organic Movements (IFOAM) estimate that the size of the global organic market increased to US$ 72 billion in 2013, an estimation based on data provided by 170 countries (FiBL & IFOAM 2015). A total of 90% of these sales were made in Northern America and Europe, the leading markets in the organic sector. The European market share is worth €24.3 billion, the EU share €22.3 billion, and the North America market is almost €27 billion (since the data are collected from many countries and different currencies, FiBL and IFOAM survey uses the average exchange rate of 2013: 1 Euro = 1.3281 US dollars, according to the Central European Bank). Figure 1.5 shows the largest markets in terms of retail sales shares.

According to the Organic Trade Association (OTA, https://www.ota.com/) of the USA, in 2014 the sales of organic products in the USA exceed $39 billion with $35.9 billion worth of organic food representing almost 5% of total US food sales.

The organic production in the EU is mainly regulated by Council Regulation (EC) No. 834/2007 of 28 June 2007, which repealed its predecessor Council Regulation

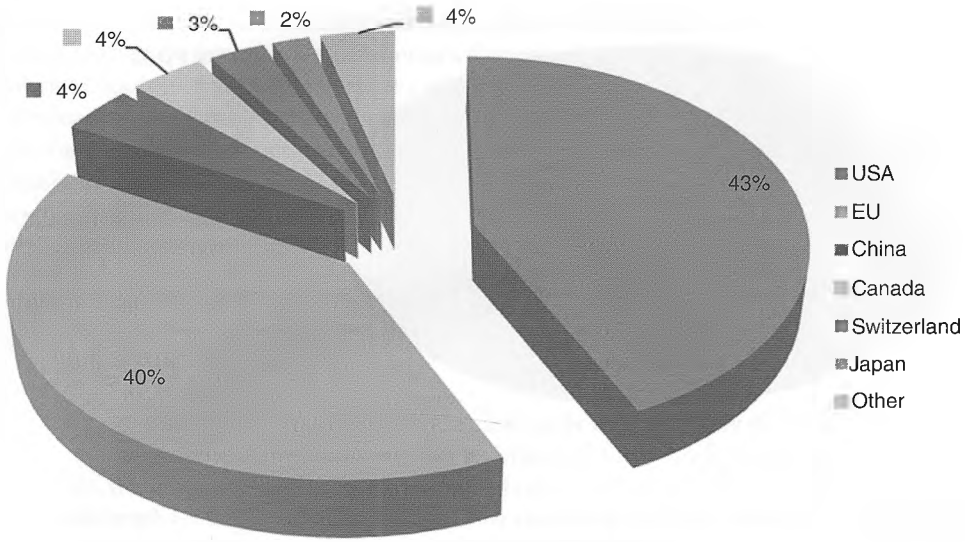


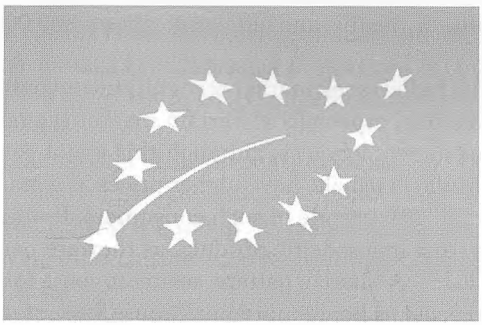
Figure 1.5 Global market of Organic Food: Distribution of Retail sales by single markets 2013 (FiBL & IFOAM 2015).

(EEC) No. 2092/91. Commission Regulation (EC) No. 889/2008, which provides the main legal framework, details production rules and the implementation of Commission Regulation (EC) No. 1235/2008. Regulation (EU) No. 271/2010 of 24 March 2010 amended Regulation (EC) No. 889/2008, laying down detailed rules for the implementation of Council Regulation (EC) No. 834/2007. As far as the organic production logo of the European Union is regarded, the presence of "Euro-leaf" organic logo is obligatory on all packaged organic products of EU that are placed in the market, in accordance with the requirements of Regulation (EEC) No. 2092/91 and its implementing regulations or Regulation (EC) No. 834/2000 since July 2010. The previous and presently used organic logos according EU legislation are shown in Figure 1.6.

In the USA, agricultural products labeled as organics have to be certified under the scheme of USDA's National Organic Program (NOP). NOP is authorized under the Organic Foods Production Act (OCFPA) of 1990, as part of the 1990 Farm Bill (Winter and Davis 2006; Braden 2014). National Organic Program Standards were announced in 2000 and became effective on 21 October 2002. All products marketed as organic must contain at least 95% organically produced ingredients; the source of the other 5% must be included on the national List of Approved Substances (Winter and Davis 2006). Figure 1.7 depicts the organic seal of USDA that agricultural products may use when production and handling meets all the requirements of the USDA organic regulations. In most cases, in order to use the seal in Figure 1.7 or relative claims of organic production, the producer, handler, or processor must be accordingly certified; there is an exemption from certification rule for some operations, for example for organic farmers who sell $5000 or less.



(a)



(b)

Figure 1.6 (a) Versions of the old official EU organic logo (used until July 2010, according to Regulation (EC) No. 889/2008). (b) Versions of the official "Euro-leaf" organic logo of EU (http://ec.europa.eu/agriculture/organic/downloads/logo/index_en.htm).



Figure 1.7 Versions of the organic logo in USA (NOP Regulations, §205.311 USDA Seal).

1.5 Conclusion

People need to be reassured about the quality and safety of the food they consume. Any loss of consumer confidence in a product has impact on markets, trade, and probably even on taxes that governments collect. For example, at the beginning of 2013, the Food Safety Authority of Ireland announced the results of a study that had found horse DNA in frozen beef burgers placed in supermarkets, the prelude to the "horsemeat scandal". The incident attracted much publicity, affected many countries, and resulted in the immediate decline of sales for relative products, especially in the supermarket chains involved in the initial findings (Yamoah & Yawson 2014) although there was no health safety issues involved (Lees & Toutain 2013). Another incident of what could probably be described as a combination of both adulteration and fraud is the case of the addition of melamine to powdered milk intended for infants in China in 2008, resulting in about 52 000 hospitalizations, 6 deaths and affecting about 300,000 infants (Motarjemi *et al.* 2014).

Producers (farmers included) seek market recognition and better prices and GI certification seems to enable this, especially in certain market segments. The origin of the food plays a role in consumers' choices, although this is not easily measured (Profeta *et al.* 2012). Organic products usually have higher prices as a result of the more favorable willingness-to-pay (WTP) attitude of such consumers. Products with organic logos obtain the consumer's trust in a specific product, as they are perceived to be of higher quality (Zanoli *et al.* 2015). A similar pattern seems to exist for products with health claims, as they are perceived as being more nutritious (Stolz *et al.* 2011). In general, all added-value claims by food producers create new challenges for the respective food authentication authorities.

The conformity of a food or drink to its actual description is checked by state authorities using various analytical techniques. Methods range from simply weighing to more sophisticated state-of-the-art combined analysis using expensive instruments, aided by chemometrics and specialized computer software to process and analyze the accumulated data. Sometimes a macroscopic examination or a tasting panel can change the validation status of a product based on chemical analysis, just by a simple tasting test. For example, in the case of wine and olive oil an organoleptic panel test is a key element procedure included in quality characterization. Another problem are the limits of detection that certain analytical methods have in qualitative or quantitative terms. For example, in certain cases existing methods cannot verify that a product is 100% GMO free due to limits of detection. Finally, even if the perfect analytical protocol could be developed, in order to be effective in practice it has to be accepted in court; in most cases this means being adopted by the existing legislation.

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2

Food Authentication by Numbers

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2.1 Introduction

Globalization and the growing complexity of the food chain, combined with recent food scares such as the horsemeat scandal, the melamine-adulterated milk and numerous others, have raised consumer awareness regarding the quality and authenticity of the food they consume. Authenticity has probably always been a major concern of many consumers, and it is gaining increasing importance. In Europe, origin is one of the main authenticity issues for food products. The recent trend in European legislation is the protection of "mountain products" and "products of island farming" (EU Regulation 1151/2012). These two labels are the very recent companions to PDO and PGI labels that will be implemented in EU legislation in the near future. The information includes, beyond others, a characterization of the morphology of the geographic region, stemming from the consumer perception of special quality attributed to mountain and island products. In the case of cultivated species, the EU indicates that a reference should be made to the country in which the product undergoes the final development stage. High-quality products with geographical indications and designations of origin are generally high priced and bring a higher benefit to the producers than other similar products. There is therefore a need to protect such products by detecting possible commercial frauds. These products are defined by geographical origin, know-how, certain farming methods (e.g. organic foods) and, in some cases, by feeding diet and animal breed.

2.2 Research Trends

Figure 2.1 depicts the major publication media. As shown, more than 80% of publications are original work published in research journals while review articles and conference proceedings account for around 7–8% each. Due to increased public and scientific interest, various book chapters/books have been published totaling 5%. Most have been published since 2006.

Nowadays, the determination of geographical origin, adulteration, mislabeling, and food safety are among the main aspects in food control. As depicted in Figure 2.2, the

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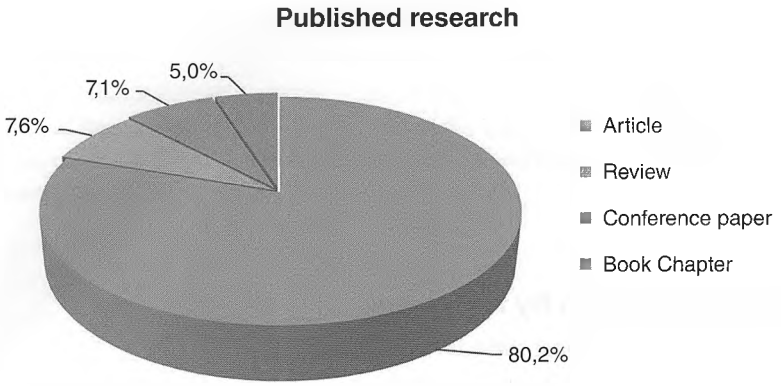


Figure 2.1 Published research on food authentication as depicted in Scopus (6–2015).

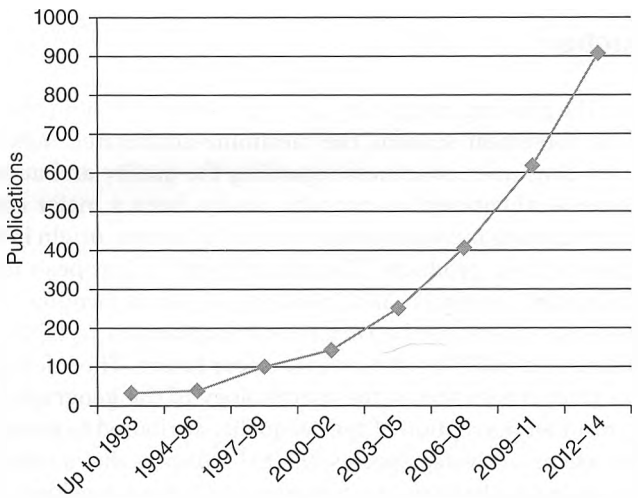


Figure 2.2 Temporal evolution of food authentication publications, 3-year period.

volume of published research has grown exponentially since 2000. It is interesting to note that 409 articles were published during 2006–2008, while 907 were published for the period 2012–2014.

2.3 Analytical Techniques

Determining the authenticity could involve a range of verification approaches depending on the level of sophistication of the fraud itself. This section provides insight into analytical techniques needed to verify the origin of our food in terms of regulatory and, more recently, consumer and industry requirements. Concerning geographical origin determination, analytical methods rely largely on the determination of chemical composition, which may be quite similar even when the same materials come from different geographical areas. Attempts have been made to determine some components

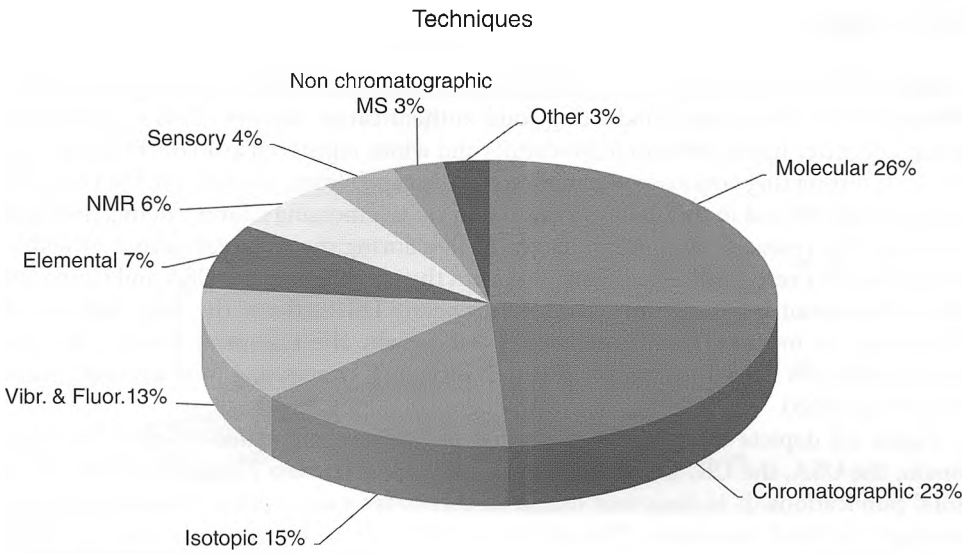


Figure 2.3 Publications distributed over different techniques.

as typical for certain areas or production methods or using elemental pattern. Other methods that are applied include molecular methods when different strains/breeds of organisms are involved in production.

Various analytical techniques have been assessed for their suitability for food authentication studies throughout the years. As shown in Figure 2.3, chromatographic and molecular are the two major groups of techniques that are used for that. Almost half of research articles fall within these two groups, followed by isotopic, vibrational, UV and fluorescence spectroscopy, elemental techniques and nuclear magnetic resonance (NMR). Some other concepts that should not be ignored are sensory analysis, non chromatographic mass spectrometry (MS), and immunological techniques. Further, it is very interesting to note that chromatographic, molecular, vibrational and fluorescence spectroscopy, sensory and non chromatographic MS techniques have increased in popularity in the last few years. The most extensive use of molecular techniques is for the determination of species and botanical origin, while the others mainly deal with geographical origin determination and adulteration cases.

Mass spectrometry is a frontline technology which has replaced others in many fields of food science. This trend also extends to food authentication literature, due to the unsurpassed advantages such as high sensitivity, selectivity, throughput and multi-analyte capabilities of MS techniques (Georgiou and Danezis 2015). It is important to note that multi-analyte capabilities are essential for food authentication studies, as more descriptors facilitate better classification (Borràs *et al.* 2015). We have therefore arrived at a point of a vast volume of data which we have to handle. To this end, various techniques described either under the term chemometrics or data mining are crucial for successful prediction models, utilizing the data.

2.4 Countries

As can be observed in Figure 2.4, south European countries such as Italy, Spain, France, Portugal and Greece are conducting food authentication studies. This is normal as these countries had a plethora of foodstuffs and wines registered as PDO, PGI, etc. It is also interesting that countries with high scientific capabilities, for example the USA, are likely to stay behind in this field. Further, China is an emerging country in this field and increased its research output by a factor of five during the five-year period of 2009–2014. Another remarkable finding is that, with the exception of the USA and China, all the other countries in the top 10 are European. This reflects the high interest of Europeans in foodstuffs authenticity and is also why the European Union (EU) has announced new calls for scientific research programs concerning food authentication in Horizon 2020.

Figure 2.5 depicts the temporal evolution of food authentication research for Italy, Spain, the USA, the UK, Germany, France and China, the top 7 countries in terms of total publications. It is clear that Italian scientists are more active, while Chinese are strongly involved nowadays. This could be correlated to the high number of Italian authentic food products; as shown in Figure 1.2, Italy produces more PDO-PGI-PGI food products than all others. A possible explanation for the intensively growing activities in China could be attributed to the growth of the gross domestic product per capita and the interest associated with quality food products such as olive oil (Euromonitor). All other countries also show increased activities on food authentication. Spain has also produced a great number of authentic food products (Fig. 1.2).

A different index that provides useful insight into the research efforts in food authentication of individual countries (the top 10 countries in publications) is the number of

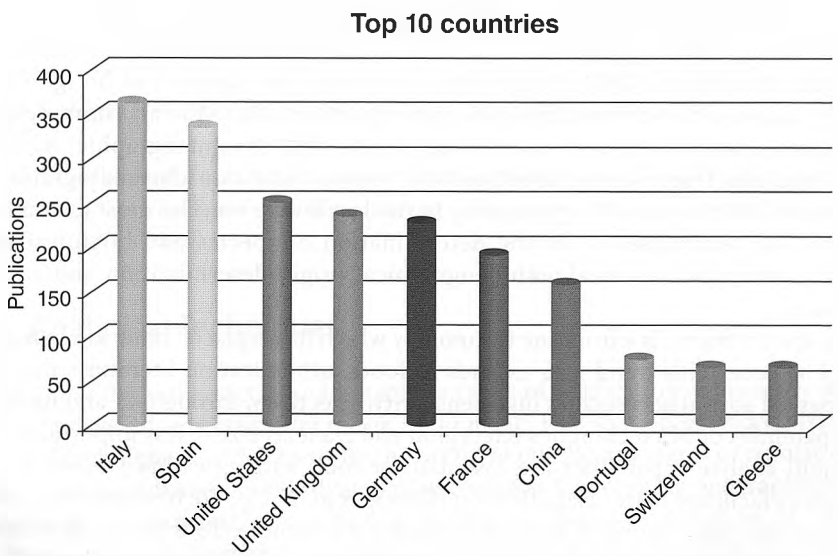


Figure 2.4 Top 10 countries on food authentication publications.

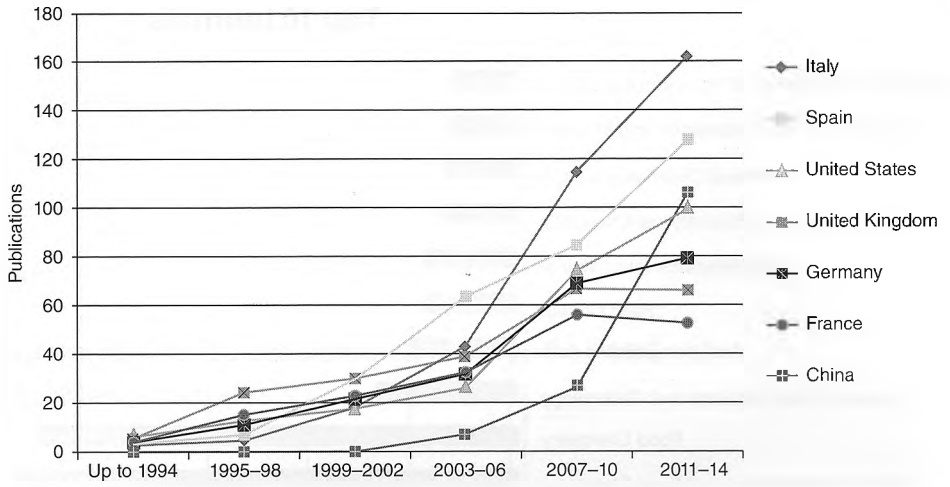


Figure 2.5 Research activity progress on food authentication in different countries.

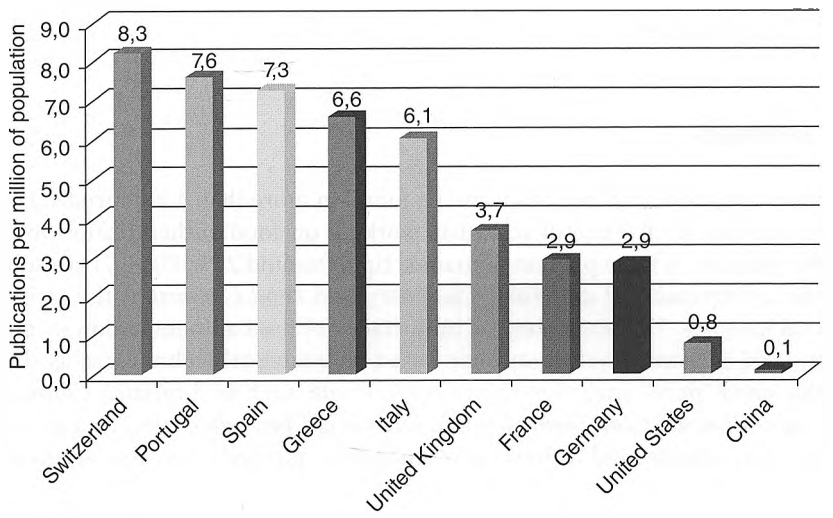


Figure 2.6 Food authentication publications per million capita of the top 10 countries.

relevant publications per million of population, as shown in Figure 2.6. Data presented this way are normalized relative to size. Note that mostly European countries are active concerning quality food products, followed by the USA and China. According to this index, south European countries are more active than northern European countries and this is attributed to the quality food products produced there. It is important to note here that northern European countries have larger biodiversity and unique climatic features. Noticeable is the leading position of Switzerland. Could that mean that Switzerland is the most interested in quality food, and could that be correlated with the high wealth of the country?

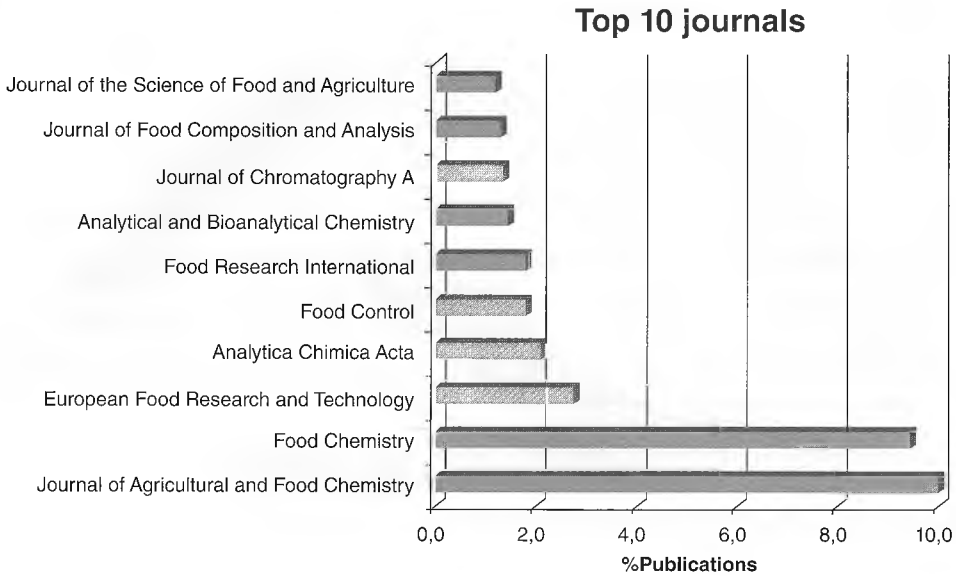


Figure 2.7 Top 10 journals on food authentication publications.

2.5 Journals

Publications on food authentication can be found in more than 150 journals. However, there is a certain preference of scientists working on food authentication in specific scientific journals. A large portion of manuscripts (around 20%; Fig. 2.7) are submitted to *Journal of Agricultural and Food Chemistry* and *Food Chemistry*, the most highly cited food journals. This indicates the importance of food authentication studies. The fact that food authentication is strongly based on analytical technologies is shown by the highly cited "pure" analytical chemistry journals, such as *Analytica Chimica Acta*, *Analytical & Bioanalytical Chemistry*, and *Journal of Chromatography A*. This indicates the need for reliable and robust new analytical methods for the verification of authenticity.

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Part B

Consumer Attitudes Towards Authentic Food and Market Analysis

3

The Concept of Authenticity and its Relevance to Consumers: Country and Place Branding in the Context of Food Authenticity

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3.1 Introduction: The Challenge of Authenticity

Resulting from a paramount interest in the strategic thinking that supports the 'global' against the 'local', the notion of authenticity has long been approached with considerable scepticism. Some maintain that authenticity has lost its meaning; that contemporary ultra-global cultures are responsible for the creation of a world where the historical characteristics of places have lost their importance; and that societies have in a way progressed in a post-authenticity era. This chapter intends to show that authenticity actually remains of vital strategic importance, and even supports the view that in a market over-saturated with offerings originating in all too distant destinations, authenticity has become even more important due to its ability to segregate the 'general' from the 'specific' (Pine and Gilmore 2007). Accordingly, authenticity must be part of every branding strategy and continue, if used properly, to constitute a source of essential value creation.

3.1.1 The Origin of Authenticity and its Marketing Relevance

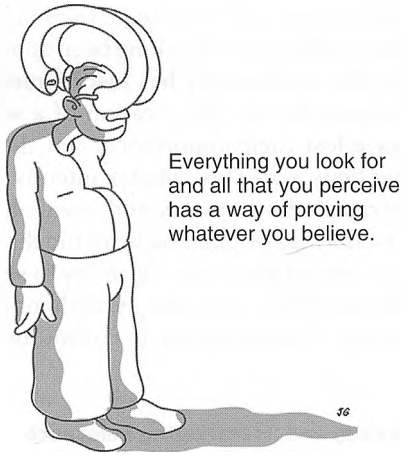
The word 'authenticity' comes from the Latin word *auctor*, which means 'founder' or literally the 'one who causes [something] to grow'. The word shares its etymology with the Greek *poetes*, which means 'creator, author or poet' from the verb *poein*, 'to create or compose'. Authenticity is therefore what sources and continues to be from an initial act of creation. In a business context, the notion of authenticity has always been an issue of consideration for branding strategies. In the field of branding, there is a strong interest in everything that withstands time, therefore is classical and 'true'.

In a way, authenticity is the *raison d'ëtre* of branding: it supports its rationale departing from the basic hypothesis that what is successful in the market must always be the most 'real', honest, essential and in itself precious. In reality, what the marketing theory considers to be the pioneer signs of branding have been initiated from the anxiety to 'authenticate' the genuine. The premature marketing campaigns have aimed at certifying the (oftentimes local) origin of a product. Commodities became 'original' branded goods in order to communicate in this way a certain symbolic quality guarantee.

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Kellogg's Corn Flakes is a good example of this (Hornskov 2011): in the early 20th century Kellogg's launched the message '*original and best*' in order to position itself apart from the multitude of common variations of the same generic product. Consumers have been encouraged to think that Kellogg's were in reality of higher quality because they were actually the first, and therefore the only authentic. They were the 'prototype', those that reserved the essential properties of the first-in-time archetype (and ever since, are believed to be simply *better*).

Despite the fact that the scientific theory and practice maintain that authenticity is all about verifying product properties, there is every reason to emphasise that it is human agents who 'create' authenticity (Knudsen and Waade 2010), since '... it is the belief that a product is authentic what makes it so' (Hornskov 2011, p.106). From this essential realisation streams the strategic interest in authenticity. The challenge of authenticity is therefore the fulfilment of the strategic aim to communicate the territory-relevant, thus enduring quality properties of products. It is to narrate around the world how stories of things that originate in a place constitute a unique and lasting value source. The challenge of authenticity therefore finds its foundation in a special passion for the historical origin of things, and how their origin-related character transforms constantly over time.



The hypothesis that the prototype is inherently better than its copies also implies that if we can imprint the experience of authenticity in the memory of consumers (along with tourists, investors, conventioneers, etc.), we have created a constant source of real, attractive narratives about a place. This implies an emphasis on a series of outcomes every time we talk about authenticity, when we characterise things as 'authentic' and when we try to make others see them as such (e.g. like a 'commitment' to help consumers overcome everyday experiences and cut through the bulk of trivial marketing messages which, compared to the promise of authenticity, leave consumers at best indifferent; Hornskov 2011).

In this respect, authenticity is closely connected to tangible, earthy things such as historical findings, monuments and production sites that function as proofs of authenticity. Our experiences of authenticity depend on them. Accordingly, material goods and their manufacturing practices, first materials, ingredients, recipes, architecture and design come into light. The physical environment and the dimensions of a place and the

narratives that go together become critical areas of strategic concern (Graham *et al.* 2000; Olins 2004). Authenticity therefore brings about a special form of performance: if we perceive things as authentic, this makes them different and for this difference we like to talk with enthusiasm. Authenticity is therefore not static. It may seem given, such as the authentic Corn Flakes recipe, but it is always open to new interpretations as it constantly reinvents itself.

3.1.2 The Philosophy of Authenticity

Besides business and social sciences, authenticity and the interrelated concept of authorship is an on-going topic of interest in arts and humanities. The German philosopher Walter Benjamin touched upon the issue of authenticity in his article 'The work of art in the age of mechanical reproduction' (1935). The objective of Benjamin was to understand what happens when, with the outburst of (at the time modern) means of printing techniques, authorship at the hands of the creator ceases to be an endogenous characteristic of authenticity. In other words, what happens when a work is no longer defined as a special and sole artistic proof, but as a form of quality that can be copied and (re)distributed infinitely (i.e. an original painting that is reproduced in a number of 'authentic' copies, or the contemporary issue of authorship of digital music).

Benjamin's approach eventually became more relevant, as the issue of authorship developed to a very complicated one. Nowadays, with the appearance of the possibility for collective authorship through on-line media (i.e. Wikipedia), it becomes apparent that authorship is shared in many cases within many proprietors. This implies that the responsibility of the creator, but also his creativity, spreads and become shared with many protagonists. Moreover, as the Wikipedia case shows, modern communication means encourage the constant re-writing of messages.

The authenticity of art is therefore a complicated issue; the authenticity of a product that comes from a specific place is even more complicated, however. The distribution of the 'authorship' of the product among its creators here functions as a prerequisite, not just as a result. The true proprietorship of a product cannot belong to one and only 'author', due to the narratives that accompany this product from its birth, and belongs to many 'poets'. The making of authenticity is a more dynamic process than the creation of a communication message or even of artworks that withstand time. In the conception of branding based on the origin of a product, the messages and the narratives that go with it are created and recreated continuously in the course of generations.

The design and implementation of an authenticity-based strategy can therefore take place at two levels. First, the strategy gathers the wider possible 'audience' of authenticity through markets and communication channels (what Hornskov 2011 calls the 'immaterial' part of the strategy). It then concentrates in maximising the performance of the material, quality dimension of authenticity that can be communicated through 'immaterial' means. Consequently, authentic branding strategies often refer to the physical environment as a reality that supports or even lends credibility to the goods that grow or originate in this environment. This might explain why the concept of (cultural, physical or knowledge) heritage becomes so important in the promotion of a product as authentic (Koolhaas 2004).

Heritage and the importance of the physical environment in addition underline the intense demand for cross-disciplinary collaboration in devising an authenticity campaign.

While a purely immaterial strategy (i.e. a marketing campaign, for instance the empowerment of consumers to talk in social media about and indeed co-create authenticity) might be applied alike by scientists or practitioners, an authenticity-founded strategy that would involve the physical environment calls for collaboration among various scientific fields and different local and national stakeholders, each of which will offer their own points of view and contribute their own stories to the narrative of a place and its products.



The following sections intend to bring forth the country- and the territory-related dimensions of physical environment and its impact on authenticity-based destination branding. In particular, Section 3.2 discusses the aspect of destination branding at the country level and emphasises the effect of country names of our purchase choices. Section 3.3 touches upon the place-level aspect of destination branding and examines various aspects of authenticity in relation to food products that originate in specific places.

3.2 Countries as Brands: The Country-of-Origin (COO) Effect on Product Choices

3.2.1 Do Places get the Reputation they Deserve?

Products developed and manufactured in remote and formerly improbable parts of the world have nowadays become worthy global competitors, stealing positions from formerly mighty leaders. Examples are the Korean Hyundai and Kia, the Indian Tata Motors and the Chinese Chery Automotive in car industry; the Chinese Lenovo and the Taiwanese Aser in electronics; the Korean LG in appliances; or the Brazilian Embaer in aircraft industry. On the other hand, thousands of small and medium enterprises (SMEs) find ways to approach consumers using novel distribution and communication channels (e.g. on-line shopping), creating entirely new business models in both business-to-business (B2B) and business-to-consumer (B2C) markets. Likewise, globalised conditions also allow small communities, unfamiliar cities, remote regions and formerly improbable nations to compete, and be considered by global stakeholders (i.e. consumers, investors, tourists, conventioneers, etc.). In this context, any community, city, state, region or nation has a possibility to create brand equity, making place marketing and place branding a decisive tool to success.

However, changing people's perceptions about places is indeed incredibly difficult. The Anholt's Nation Brand Index suggests that the reputation of nations is remarkably stable, with only a few exceptions (e.g. surge in the popularity of the USA after Obama's election in 2008, or in Brazil's reputation after the undertaking of the World Cup 2014). Despite an upsurge in academic research and a similar explosion in the number of consultancy firms specialised in country/place image building, we are still far from a clear appreciation of what place marketing and place branding means. One of the main reasons for this is that most of relevant terms, that is, 'authenticity', are contested and remain elusive. The prevalent approach to place branding focuses on the link between country-of-origin (COO) and consumer attitude formation and buying behaviour. Kotler and Gertner (2011) claim that '... every time the name of a country is mentioned, there is opportunity to add value to this country brands' equity' (p. 36).



3.2.2 Countries as Brands

A brand is defined as a 'distinctive and legally protected name, term, sign, symbol or design, or a combination of them, intended to identify the goods and services of one seller and to differentiate them from those of competition' (American Marketing Association). Brands allow marketers to differentiate their products from those of competitors by building unique associations with the product or service in the hearts and minds of prospective customers. Brands represent a promise of value and performance. Besides utilitarian benefits, brands have social and emotional value, brands 'have personality and speak to consumers' (Kotler and Gertner 2011, p.36). Brands have the ability to add to the perceived value of a product or service, and command premium prices from consumers. Brands have equity, which expands into many dimensions, such as performance, social image, trustworthiness and identity. Accordingly, brand equity translates into customer preference and loyalty, and therefore into long-term competitive advantage and profitability for the brand 'author' (i.e. owner).

The term 'country equity' refers to the emotional value resulting from consumers' perceptions about a (positive) brand–country association. Country names behave like brands by helping consumers evaluate products originating in different countries to make purchasing decisions. Early empirical evidence (e.g. Wang and Lamb 1983;

Cordell 1993; Agbonifoh and Eliminian 1999) suggests that consumers are more willing to buy products from industrialised nations as a result of country equity. For instance, consumers' evaluation of the same Toyota car model differ when the model is made in the US, Mexico or Japan (Chinen *et al.* 2000); or COO is still the most important parameter in Chinese consumers' choice of wine (Balestrini and Gamble 2006; Hu *et al.* 2008).



A country may therefore deliberately use its name to promote its products. For the last three decades, US consumers regard *Café de Colombia* as top-quality coffee. The relevant logo was created in 1981 by the National Federation of Coffee Growers of Colombia and introduced to advertising in 1982. The character of Juan Valdez helped to designate the country name 'Colombia' (i.e. Designation of Origin) as a guarantee of high quality. The logo has been extensively used in advertising, promotional material and coffee packages, and in 2011 received the World's Best Logo award. The campaign has paid off: Colombia is the leading exporter of coffee to the US and Café de Colombia holds over 40% of the coffee market in the US; 80–90% of Americans associate Juan Valdez with coffee without any descriptive words, while 50% correctly identify it as a Colombian coffee brand (Kotler and Gertner 2011).



But even when a country does not consciously manage its name as a brand, people still hold images of countries (i.e. country stereotypes) that can be activated by simply voicing their name. A country's image derives from its geography, history, culture, famous citizens etc. Country images are likely to influence people's purchasing decisions, for example positive country images create country equity. Categories such as perfumes, wines, cars, software, electronics, watches, food, etc. strongly identify with certain countries, as do are social issues such as epidemics, political unrest, human rights violations, environmental degradation, racial conflict, corruption, poverty, hunger or crime. Different persons hold different images of nations; however, stereotypes are widespread and pervasive, shared by members in a given social group or society.



3.2.3 Impact of Country Names on Attitudes Towards Products

Country images, or simple knowledge about a place, are commonly used as shortcuts for information processing and consumer decision. People, especially in low-involvement/low-knowledge situations, resist changing their opinions or adjusting prior knowledge. Instead, they prefer to fit what they see to what they know. People are also more likely to focus on information that confirms their expectations, disregarding information that challenges established knowledge. Images (i.e. country stereotypes) can therefore be long lasting and difficult to change. However, country–product associations can be assessed and measured, and may be managed and influenced by marketers. COO effects come as a result of country equity (i.e. the creation of positive match between a brand and its 'made-in' country). Findings consistently support the theory that consumers use COO information to make inferences about quality. COO became an integral part of the repertory of extrinsic cues to product evaluations, along with price, brand name, packaging, etc. Manipulation of the COO or 'made-in' label has long been observed to influence people's attitudes (see a brief review of research areas relevant to COO in Table 3.1), even when consumers are given the chance to see, feel, smell or taste the very same physical product (e.g. Nagashima 1970; Terpstra 1988; Hong and Wyer 1990; Wall *et al.* 1991; Johansson *et al.* 1994; Jaffe and Martinez 1995; Liefeld *et al.* 1996; Li *et al.* 1997).









Table 3.1 Research areas on COO effect.

Area	Examples
Multi-COO effect, for example effects of the country of design, country of manufacture, country of assembly and country of brand	Han (1990); Ahmed and d'Astous (1995, 1996, 1999); Hulland (1999)
Top-down approach: impact of COO on attitudes towards global brands; and Bottom-up approach: impact of other brand quality indicators on COO, for example strong brand could counterbalance negative COO effect	Garvin (1987); Helsop and Liefeld (1988);Chao (1989); d'Astous and Ahmed (1992); Tse and Lee (1993); Rosenbloom and Haefner (2009)
Ethnocentrism, patriotism and hostility towards a foreign nation (ethnocentrism/patriotism and hostility operate differently, as they concern own-country and foreign country, respectively)	Hirschman (1981); Klein *et al.* (1998); Liu and Johnson (2005); Chattalas *et al.* (2008)
COO effect moderators: motivation and involvement (low), expertise (high), cultural dimensions, individualism and collectivism, etc.	Gurhan-Canli and Maheswaran (2000); Pecotish and Ward (2007)
COO effect and ethnocentrism with respect to food choices in various national contexts	Brodowsky (1998); Orth and Firbasova (2003); Hamin (2006); Chryssohoidis *et al.* (2007); Ahmed and d'Astous (2008)

Case Study 3.1 Ethnocentric beliefs and country-of-origin (COO) effect: impact of country, product and product attributes on Greek consumers' evaluation of food products

The study by Chryssochoidis, Krystallis and Perreas (2007) was aimed at evaluating the level of consumer ethnocentrism (CE) and its implications on consumer evaluations of food product quality. The study also examined the level at which the country-of-origin (COO) effect is activated (i.e. country, product or attribute level) per consumer cluster of different level of CE.

Case Study 3.1 (Continued)

To attain the above aims, a questionnaire was developed and completed though personal interviews with 274 Greek respondents (average age 37.6 years, typically university graduates). The set of exemplary countries of origin selected encompasses Greece, Italy and Holland, since inclusion of more 'typical' foreign countries was necessary to ensure familiarity of consumers with food products originating in them. The same rationale is applied while selecting the set of exemplary products under consideration. Beer, ham products and yellow cheese are considered as representative of the above-mentioned countries, ensuring familiarity of consumers as well as satisfying the prerequisite for compatibility between the products and the countries of origin selected. All three products had to have at least one domestic (Greek) substitute and had to belong to different categories (namely alcoholic drinks, meat and dairy products) to allow for cross-category comparisons.

The questionnaire consisted of three parts. In the first part, respondents were asked to evaluate several product attributes using seven-point Likert-type agreement questions with end-points 1 ('totally agree') to 7 ('totally disagree'). The evaluation was carried out using the following paired combinations of products and countries: (a) Greek v. Dutch beer; (b) Greek v. Italian ham products; and (c) Greek v. Dutch yellow cheese. The evaluation criteria were derived from the set of 36 questions formulated by Steptoe *et al.* (1995), which is an inclusive set of all quality evaluation criteria that consumers bear in mind when purchasing food products. The second part of the questionnaire comprised the CET-SCALE (Shimp and Sharma 1987), which consists of 17 Likert-type questions with end-points 1 ('totally agree') to 7 ('totally disagree'). The CET-SCALE is the most widely used instrument to measure ethnocentric tendencies of various consumer cultures around the world. The scale was carefully translated and tailored to fit the Greek food market realities. The last section of the questionnaire consisted of questions regarding participants' purchasing habits for Italian and Dutch food products in general, as well as their demographic characteristics.

More than one in four respondents (27%) bought Italian products at least once per month, whereas 10.9% had neither bought nor considered buying them. The corresponding frequencies for Dutch products were 31.4% and 10.2%. Irrespective of their country of origin 20.8% of the sample purchased beer at least once a week, whereas non-beer drinkers were limited to 4.7%. In the case of ham and yellow cheese, the relevant figures were 34.7% for both (users at least once a week) and 2.2% and 2.6% respectively (non-users). Average scores of the items in the CET-SCALE highlighted that the sample could be characterised as marginally ethnocentric. Exploratory and confirmatory factor analyses justified the uni-dimensionality of CE. Cluster analysis allocated the sample into two clusters, the 'ethnocentric' and the 'non-ethnocentric' (Table 3.2).

Comparing the two clusters in terms of their attributes towards Greek and Dutch beer, the ethnocentric consumers evaluated the two products differently (statistically significant differences) in 26 out of 27 attributes (96.3%) (Table 3.3). On the contrary, only in 12 attributes (44.4%) were the two products perceived differently by the non-ethnocentric consumers. Regarding ham products from Greece and Italy, the ethnocentric evaluated them differently in 27 out of 33 attributes (81.8%), whereas the non-ethnocentric in only

(Continued)

Case Study 3.1 (Continued)

13 (37.5%). Correspondingly, the ethnocentric consumers perceived Greek yellow cheese as differing from its Dutch counterpart in 29 out of 32 attributes (93.5%) and the non-ethnocentric in only 15 out of 32 attributes (48.3%). The same pattern was observed when comparing two products from the same foreign country (i.e. beer and yellow cheese from Holland). The ethnocentric respondents perceived them differently in 22 out of their 27 (common) attributes (81.4%), whereas the non-ethnocentric evaluated them differently only in 14 attributes (51.8%).

Interestingly, for the overwhelming majority of attributes, both ethnocentric and non-ethnocentric consumers evaluated more favourably the Greek products over their foreign origin counterparts. More specifically, the ethnocentric evaluated Greek products as superior to foreign alternatives in all attributes apart from the additional chemical substances content in ham products. The non-ethnocentric also evaluated Greek products more favourably, albeit the differences between products of Greek and non-Greek origin were not as prominent as in the case of the ethnocentric consumers. Striking differences were observed in price, value for money, ease of consumption and availability/familiarity in beer and ham. Regarding Greek yellow cheese, price along with attributes related to health and safety were the most favourably evaluated by the non-ethnocentric consumers. A notable fact was that both the ethnocentric and the non-ethnocentric perceived the three Greek products as traditional ('are like the products I ate when I was a child'), which always attached greater emotional value to the domestic (Greek) alternatives.

These results not only show that ethnocentrism affects consumer beliefs, but also the way in which perceived quality of domestic and foreign products is evaluated, culminating in a COO effect. Examination of preferences of the ethnocentric consumers highlights the existence of statistically significant differences in the way they evaluate Greek and foreign products in favour of the former. These differences were observed for the vast majority of product attributes (26 out of 27 beer attributes, 27 out of 32 ham attributes and 28 out of yellow cheese attributes). Moreover, it is worth noting that in all three product categories the attribute of 'clear indication of the country of origin' was statistically evaluated differently, meaning that ethnocentric consumers do pay attention in the indication of the domestic country of origin in food products.

Discussion. From the study of Chryssohoidis *et al.* (2007) it can be concluded that in the case of ethnocentric consumers, the COO effect is activated at the initiatory level, namely that of the country of origin of a food product (country specific; Fig. 3.1), and not at the level of the product category (product specific) or product attributes (attribute specific), since in every domestic v. foreign comparison the Greek product was evaluated distinctively and overwhelmingly higher, with no variations per product or attribute. In particular, regarding the comparison between two products from the same foreign country (i.e. Dutch beer and Dutch yellow cheese) the ethnocentric evaluated differently 22 out of their 27 common attributes (81.4%), confirming that they perceive these two products as two different 'entities' regardless their country of origin. That leads to the conclusion that when the foreign country of origin is given, the COO effect for ethnocentric consumers is activated at the level of the product type (product specific).

Case Study 3.1 (Continued)

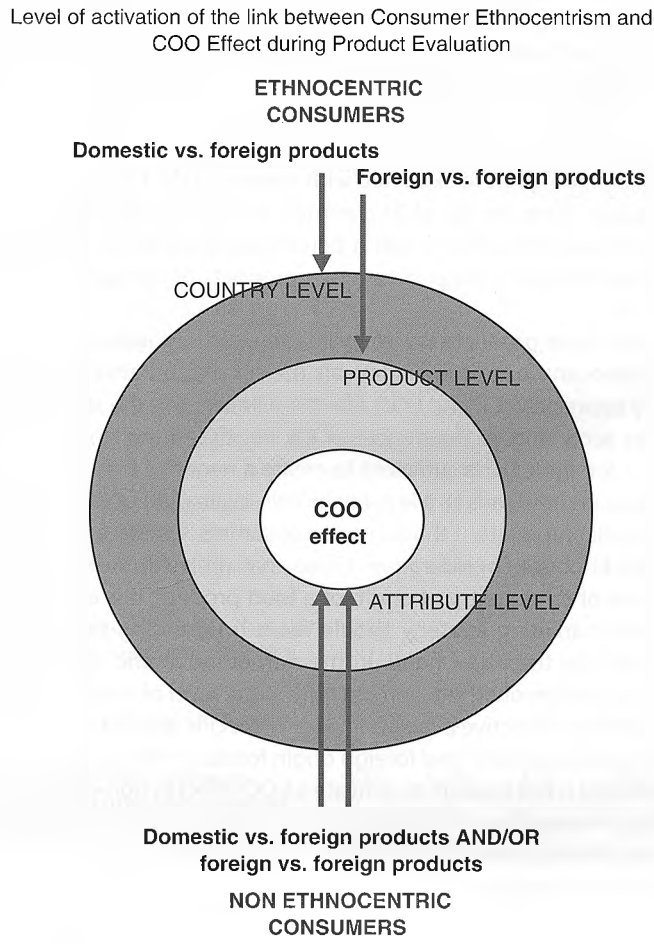


Figure 3.1 Level of activation of the link between consumer ethnocentrism and COO effect during product evaluation.

Concerning the non-ethnocentric consumers, there were also statistically significant differences in the way they evaluated Greek and foreign products, albeit to a lesser degree. The non-ethnocentric still evaluated somewhat more favourably the domestic products; unlike the ethnocentric, however, foreign products were not overall rejected: many of their attributes were evaluated as superior compared to the corresponding attributes of their Greek counterparts. The non-ethnocentric perceived the domestic and the foreign alternative as having significant differences in no more than 50% of their attributes. It is indicative that the attribute 'clear indication of the country of origin' was not statistically different. Given that differences were not observed for the majority of

(Continued)

Case Study 3.1 (Continued)

attributes, it cannot be claimed that country of origin has a distinctive effect over the evaluations of non-ethnocentric consumers. On the contrary, the partial differentiation in terms of only selected attributes implies that the COO effect becomes activated at the level of product attributes (attribute specific). The country of origin does not lead to an overall acceptance or rejection, but instead it affects the evaluation of specific product attributes. Similar findings were extracted with regards to the Dutch beer v. Dutch yellow cheese comparison. From the set of 27 common attributes, only 15 (55.6%) were found statistically significant. This rather modest percentage leads to the conclusion that non-ethnocentric consumers do not perceive two products of the same (foreign) origin as entirely different.

A focus on the three products under evaluation and separately for the ethnocentric and the non-ethnocentric consumers reveals the set of food attributes that consumers seem to mostly appreciate. CE and COO effect are linked, but the stimulus that activates their link differs according to the degree of CE. For the ethnocentric cluster, the mere mentioning of a foreign COO is sufficient to create a negative bias against foreign products, which subsequently leads to a less-favourable evaluation of their attributes as compared to domestic products. Ethnocentric consumers evaluate more favourably all domestic products under consideration. Given that their ethnocentric beliefs are activated at the level of the country of origin of a food product, it is easily understandable that a successful marketing strategy should focus on bringing the domestic origin of products forward. On the other hand, in the non-ethnocentric cluster the COO effect appears as an expression of ethnocentrism only at the level of product attributes, resulting in a favourable but selective evaluation of only specific attributes. Simple mentioning of the COO (between domestic and foreign origin foods) or the type of a product (within foreign origin foods) is not enough to activate a COO effect in non-ethnocentric consumers. A marketing strategy that will emphasise the country of origin of a product will most possibly not be effective, since the superiority of domestic products is limited to specific attributes per product category.

It would be interesting for future research to examine if the current findings apply to a broader set of products and countries of origin, including countries that differ significantly from Western countries in cultural and economic terms. Also, in light of the current results, future research should consider products with no domestic alternatives, as well as more than two foreign countries of origin for each product, in order to examine whether any given country is regarded as the 'prototypical' benchmark within a specific product category, or all foreign products are evaluated similarly. Another promising area would be the further examination of COO effect with reference to brand names in order to investigate their possibility of moderating or intensifying the phenomenon. Finally, it would be useful to approach the issue of COO effect considering more factors of psychological, political and technological nature, such as the perceived risk and level of development of the country of origin.

Table 3.2 Means, standard deviations and statistically significant differences of clusters, CET-SCALE (n=274).

CET-SCALE		Ethnocentric (n_1= 59.8%)		Non-ethnocentric (n_2= 40.2%)		t-test	
		mean	std. dev.	mean	std. dev.	t	sig
1	Greek people should always buy Greek-made products instead of imports.	2.10	1.092	4.53	1.379	16.2	**
2	Only those products that are unavailable in Greece should be imported.	2.02	1.315	4.95	1.319	18.0	**
3	Buy Greek-made products. Keep Greece working.	1.49	0.779	2.73	1.401	9.37	**
4	Greek products, first, last and foremost.	2.62	1.307	5.42	1.176	18.0	**
5	Purchasing foreign-made products is un-Greek.	4.15	1.641	6.33	0.756	12.9	**
6	It is not right to purchase foreign-made products, because it puts Greeks out of jobs.	3.01	1.414	5.65	0.982	16.9	**
7	A real Greek should always buy Greek-made products.	3.70	1.659	6.12	0.865	14.0	**
8	We should purchase products manufactured is Greece instead of letting other countries get rich out of us.	3.02	1.568	5.55	1.254	14.1	**
9	It is always better to purchase Greek products.	2.62	1.407	4.68	1.514	11.5	**
10	There should be very little trading or purchasing of goods from other countries unless out of necessity.	2.35	1.134	4.78	1.455	15.4	**
11	Greeks should not buy foreign products, because this hurts Greek business and cause unemployment.	2.82	1.388	5.28	1.142	15.4	**
12	Barriers should be put on all imports.	3.46	1.520	5.35	1.253	10.8	**
13	It may cost me in the long-run but I prefer to support Greek products.	2.76	1.253	4.27	1.596	8.7	**
14	Foreigners should not be allowed to put their products in our markets.	4.26	1.570	5.93	1.098	9.6	**
15	Foreign products should be taxed heavily to reduce their entry into Greece.	3.52	1.746	5.10	1.354	8.0	**
16	We should buy from foreign countries only those products that we cannot obtain within our own country.	2.71	1.383	5.00	1.465	13.1	**
17	Greek consumers who purchase products made in other countries are responsible for putting their fellow Greeks out of work.	3.91	1.756	6.11	0.817	12.2	**
	Mean ethnocentrism per cluster	2.97	1.407	5.16	1.225		

** Statistically significant for p<0.01.

(Continued)

Table 3.3 Differences per cluster between Greek and Dutch beer, Greek and Italian delicatessen and Greek and Dutch cheese (paired *t*-tests).

		Beer		Ham		Yellow cheese	
							
Characteristics of evaluation		**Ethnocentric**	**Non-ethnocentric**	**Ethnocentric**	**Non-ethnocentric**	**Ethnocentric**	**Non-ethnocentric**
1	Is easily consumable/edible	0.000**	0.0**	0.000**	0.003**	0.000**	0.330 (n.s.)
2	Contains no additives	0.000**	0.030*	0.857 (n.s.)	0.610 (n.s.)	0.000**	0.000**
3	Is low in calories	–	–	0.000**	0.741 (n.s.)	0.009**	0.164 (n.s.)
4	Tastes good	0.000**	0.595 (n.s.)	0.000**	0.834 (n.s.)	0.000**	0.029*
5	Contains natural ingredients	0.000**	0.006*	0.000**	0.092 (n.s.)	0.000**	0.000**
6	Is not expensive	0.000**	0.000**	0.000**	0.000**	0.001**	0.027*
7	Is low in fat	–	–	0.001**	0.320 (n.s.)	0.105 (n.s.)	0.141 (n.s.)
8	Is familiar	0.000**	0.000**	0.000**	0.000**	0.000**	0.017*
9	Is nutritious	–	–	0.001**	0.052 (n.s.)	0.000**	0.000**
10	Is easily available in shops and supermarkets	0.000**	0.000**	0.000**	0.000**	0.000**	0.001**
11	Is good value for money	0.000**	0.000**	0.000**	0.002**	0.000**	0.348 (n.s.)
12	Cheers me up	0.000**	0.735 (n.s.)	0.001**	0.253 (n.s.)	0.000**	0.510 (n.s.)
13	Smells nice	0.000**	0.360 (n.s.)	0.000**	1.000 (n.s.)	0.000**	0.390 (n.s.)
14	Can be consumed/eaten very simply	0.000**	0.089 (n.s.)	0.000**	0.038*	0.000**	0.348 (n.s.)
15	Helps me cope with stress	0.001**	0.083 (n.s.)	0.137 (n.s.)	0.707 (n.s.)	0.014*	0.025*
16	Helps me control my weight	–	–	0.082 (n.s.)	0.530 (n.s.)	0.044*	0.339 (n.s.)

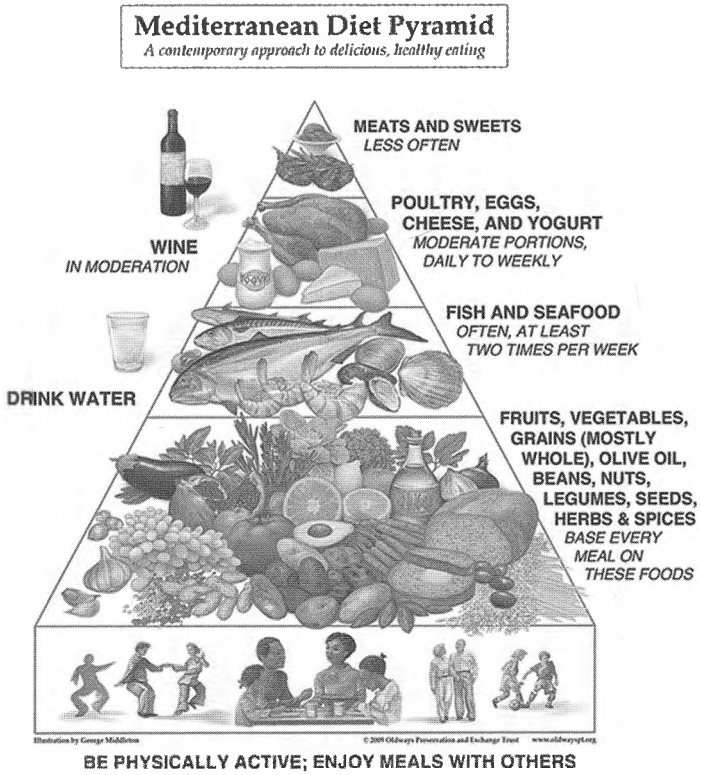
17	Has a pleasant texture	0.000**	0.283 (n.s.)	0.001**	0.294 (n.s.)	0.000**	0.777 (n.s.)
18	Is packaged in an environmentally friendly way	0.000**	0.139 (n.s.)	0.002**	0.200 (n.s.)	0.001**	0.804 (n.s.)
20	Are like the products I ate when I was a child	0.000**	0.002**	0.000**	0.000**	0.000**	0.000**
21	Contains no artificial ingredients	0.000**	0.071 (n.s.)	0.007**	0.031*	0.000**	0.000**
22	Keeps me awake/alert	0.003**	0.090 (n.s.)	0.145 (n.s.)	0.469 (n.s.)	0.008**	0.158 (n.s.)
23	Packaging looks nice	0.414 (n.s.)	0.000**	0.526 (n.s.)	0.011*	0.127 (n.s.)	0.004**
24	Helps me relax	0.000**	0.540 (n.s.)	0.001**	0.052 (n.s.)	0.010**	0.259 (n.s.)
25	Is high in protein	–	–	0.000**	0.264 (n.s.)	–	–
26	Takes no time to consume/eat	0.000**	0.400 (n.s.)	0.000**	0.117 (n.s.)	0.000**	0.852 (n.s.)
27	Keeps me healthy	0.041*	0.530 (n.s.)	0.000**	0.158 (n.s.)	0.000**	0.003**
28	Makes me feel good	0.000**	0.625 (n.s.)	0.000**	0.196 (n.s.)	0.000**	0.334 (n.s.)
29	Has the country of origin clearly marked	0.000**	0.302 (n.s.)	0.000**	0.221 (n.s.)	0.000**	0.927 (n.s.)
30	Is what I usually eat	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**
31	Helps me to cope with life	0.019*	0.863 (n.s.)	0.001**	0.274 (n.s.)	0.000**	0.235 (n.s.)
32	Can be bought in shop close to where I live/work	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**
33	Is cheap	0.000**	0.000**	0.000**	0.000**	0.000**	0.035*

Note: Question 19 '*Comes from country I approve of politically*' was omitted.
** Statistically significant differences for $p<0.01$; *: Statistically significant differences for $p<0.05$

3.3 Place Branding: Geographic Indication Labels and their Effect on Food Choice

3.3.1 Mediterranean Diet: A Typical Place Marketing Paradigm

The 1950's *7 Countries Study* by Keys *et al.* in 1968 (http://sevencountriesstudy.com/about-the-study) established that Cretan men had exceptionally low death rates from heart disease, despite moderate to high intake of fat. Scientists of that era attributed this to the dietary model of that part of the world, the so-called 'Mediterranean diet' (hereafter MD; Table 3.4). Ever since, scientific evidence in relation to a large number of health benefits of the traditional MD offers is abundant: it provides substantial protection against type II diabetes (Martínez-González *et al.* 2008); MD results in greater weight loss than low-fat diet (Shai *et al.* 2008); strict following of the MD reduces the risk of cancer and cardiovascular disease, as well as the risk of developing Parkinson's and Alzheimer's disease (Sofi *et al.* 2008); some components of the MD, such as low meat and meat product consumption, moderate alcohol consumption, high fruit and nut consumption and high legume consumption are again associated with lower risk of mortality (Trichopoulou *et al.* 2009).



Despite the unavoidable overexploitation of the MD idea and the strong divergence of contemporary dietary models of South European nations (e.g. spectacular rise in the total intake of calories and of calories from animal sources, fat and sugar in particular,

Table 3.4 Mediterranean diet (Rockefeller Foundation 1948, adapted from Helsing 1995). Sources of calories consumed (%) in Crete[a] and the US[b] 1948.

Food group	Crete, autumn 1948	US 1948–1949	Difference (%)
Cereals	39	25	+14
Potatoes	4	3	+1
Sugar	2	15	−13
Pulses and nuts	7	3	+4
Vegetables and fruits	11	6	+5
Meat, fish and eggs	4	19	−15
Dairy	3	14	−11
Oils and vegetable fats	29	15	+14
Wine, beer and spirits	1	n.a.	–
Total	100	100	

a) Based on a seven-day dietary recording.
b) Based on Food Balance Sheets 1950 supplement, FAO, April, Washington DC.

accompanied by a serious decrease of caloric intake form fruits and vegetables and plant-origin fat), South Europeans still show higher per capita consumption of cereals, fruits, vegetables and vegetable oils, and a lower consumption of dairy, meat and sugar than North Europeans (Askegaard and Madsen 1998).



Is contemporary MD still 'authentic'? Dietary health benefits are still readily apparent in South European populations and should be assigned to a large extent to the traditional foods that comprise the core of the MD. To produce traditional food, local-origin, authentic first material is usually used, processed locally in an artisanal way. Traditional Mediterranean foods are a retrospective culinary expression of the Mediterranean nations' cultural heritage. At the same time, (many) traditional foods have strong inherently healthy characteristics, since tradition seldom honours food that is not both tasty and healthy. This combination of healthy and tasty is very attractive to the food industry, so it is tempting to produce traditional/local food through mass production techniques. However, traditional or artisanal food, as many other expressions of ethical, sustainable or quality foods, comes predominantly from small-scale production, making small size beautiful!

Case Study 3.2 Consumer attitudes towards 'small-scale', sustainability aspects of food production: insights from the EU and China

The study by Krystallis *et al.* (2012) was aimed at analysing citizens' sustainability attitudes towards food production in two continents, using pork as an exemplary production system. The study collected empirical evidence in four European countries with higher-than-average per capita pork consumption (Belgium, Denmark, Poland and Germany) and in China. The overall objective of the study was to map the attitudes of people in these countries towards sustainable characteristics of pig production systems, and investigate whether these attitudes coincide with their general attitudes towards (environmental and socio-economic) sustainability on the one hand, and their consumption of specific pork products on the other. Selection of the above countries took into account their cultural diversity, as well as their important role in the global pork sector: China is the world's largest pig producer, and the EU follows as the second largest. Moreover, both the EU and China stand as first and fifth largest countries in global exports, respectively. Accordingly, the main research question of the study was as follows: do citizens who prefer more sustainable pig farming practices hold strong environmental or social concerns, and as a result consume common pork products less frequently? Further, in which way and to which extent does the quest for sustainable food production differ among specific social groups between Europe and China? In this respect, the work provided public policy insights as to the real extent to which citizens behave as consumers who consciously endorse sustainable food production.

Data collection took place through a questionnaire-based consumer survey with N=2410 consumers in the five above-defined countries (1931 EU individuals, approximately 480 in each of Belgium, Denmark, Germany and Poland; and 479 Chinese individuals, approximately 80 in each of six major cities of mainland China). In Europe, a professional research agency selected participants randomly from on-line panels. In China, data collection concentrated on major cities since this is where major changes in eating habits take place and most of the purchasing power concentration lies. Regional allocation then focused on the top provinces of China according to the China National Bureau of Statistics in terms of pork production and consumption. The questionnaire in all five countries included the following four sections, with some modifications between the European and the Chinese versions to grasp existing cultural differences: (a) general sustainability attitudes; (b) consumption frequencies of various pork products common in every country; (c) 15 written descriptions of various pig farms based on a conjoint design (see below); and (d) socio-demographic characteristics of the respondents. All the attitudinal scales were related to either the environmental (i.e. environment and nature, food and environment) or the socio-economic (i.e. industrial food production, technological progress, animal welfare) angle of sustainability.

A conjoint experiment aided in the investigation of attitudes towards pig meat production systems. Conjoint analysis models the nature of consumer evaluations of a concept in the form of consumer trade-offs among multiple attributes of the concept (the 'factors'). It allows the identification of the relative importance of each factor and the factor level combinations that are most preferred by respondents. In the Krystallis *et al.* (2012) survey, the relevant factors tapped essential aspects of pig farming that could be used as evaluation criteria. Accordingly, the factors had to reflect all components of

Case Study 3.2 (Continued)

sustainability in food production (environmental, social and economic), and take into consideration existing differences in the socio-cultural and economic background of the populations under study. Extensive consultation with pig production systems' expert and the relevant literature contributed to the identification of the factors in Table 3.5. In China in particular, due to certain unique conditions (i.e. China is the largest pig producer and consumer in the world, while 75–80% of pork meat comes from very small family farms or rural backyards, making food safety a major concern for urban Chinese consumers) the study emphasised the social and economic angles of sustainability, approaching food safety as a social sustainability attribute.

Based on the above rationale, the total number of combinations of the factors was 15 (conjoint 'profiles'), essentially corresponding to 15 different types of pig farming. Respondents in every country then had to look at written descriptions of each of the 15

Table 3.5 Conjoint factors and factor levels per country.

EU	China
1. Stocking density[1] Less than 100 sows About 400 sows 800 or more sows	6. Farm size[1] Small (family farm with 1–5 sows) Medium (large-scale family farm with up to 400 sows) Large (industrial pig farm with several thousands of sows)
2. Housing and floor type[2] Slatted floor Litter bedding Outdoors access	7. Animals' breed[6] Traditional Chinese European
3. Effort to protect soil, air, water[3] Minimal Some Maximum	8. Food safety efforts at the farm level[7] Not a special consideration Special attention (regular veterinary control and hygiene regulations) Maximum attention (strict veterinary control and hygiene regulations)
4. Fat content according to feed[4] Standard Lower Healthier	9. Meat type[8] Tasty Lean
5. Quality type of the product[5] Consistent Variant Demanded by key-customers	10. Quality type of the product[5] Consistent Variant

1) expression of economic and environmental sustainability.
2) expression of social and environmental sustainability.
3) expression of environmental sustainability.
4) expression of social and economic sustainability.
5) expression of economic and social sustainability.
6) expression of economic and social sustainability (in China).
7) expression of social sustainability (in China).
8) expression of economic and social sustainability (in China).

(Continued)

Case Study 3.2 (Continued)

conjoint profiles successively. The five attributes common to all production systems, with two or three alternatives for each attribute (the factor 'levels'), essentially defined each production system. Each respondent had to indicate how much (s)he liked the described system by assigning a score to each on a 11-point rating scale, with end points −5 ('dislike very much') to +5 ('like very much'). Before this task, respondents filled in the attitudinal and the pork consumption frequency parts of the questionnaire. After the task, they filled in the socio-demographic part. Conjoint analysis led to the results graphically depicted in Figure 3.2. In Europe, it was clear that 'housing and floor type' and 'efforts to protect soil,

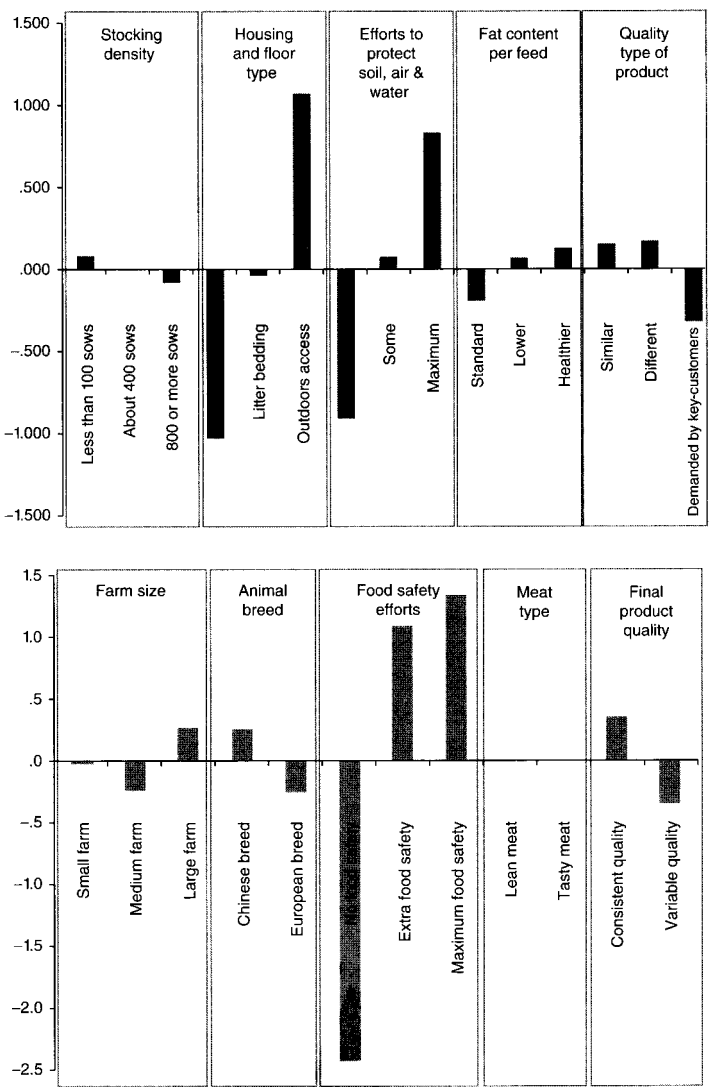


Figure 3.2 Conjoint analysis' part-worth utilities per factor level, EU and China (N=2410).

Case Study 3.2 (Continued)

air and water at the farm' had the strongest influence on the respondents' evaluation of pig production systems, with the most positive evaluations resulted from 'outdoor access' and 'maximum effort to protect soil, air and water' of the farm. In China, 'food safety efforts' had by far the strongest influence on respondents' evaluation, followed by 'quality of the final product', 'farm size' and 'animal breed', with the most positive evaluations resulting from 'maximum' and 'extra' food safety, followed by 'consistent' quality of the final product, 'Chinese breed' animal and 'large' farm size.

The second phase of the analysis was the implementation of a segmentation task through cluster analysis. Respondents' grouping criteria were the 15 preference variables emerging from the evaluation of the 15 farming systems above. In Europe, a 4-cluster solution was the most successful (Fig. 3.3). In China, a 3-cluster solution was the best (Fig. 3.4). Cross-tabulation, chi-square tests and Scheffe post-hoc ANOVAs substantiated the statistically significant differences among the clusters in the two areas in terms of their socio-demographic, attitudes and pork consumption profiles.

In Europe, cluster 1 (17.1% of the sample) put (almost exclusively) the strongest emphasis on the maximum effort to protect the environment at the farm level. Cluster 1 was therefore termed the 'environmental conscious' citizens. It comprised upper-class respondents from all four EU countries (better educated, well-off, more urban, more in managerial jobs). Cluster 1 held the second-most pessimistic attitude towards the environment and nature, and the strongest attitude towards the need for an environmentally friendly food production. Moreover, it included the second highest percentage of regular pork eaters. Cluster 2 (59.1%) presented the weakest evaluations of the pig farming characteristics; it was therefore termed the 'indifferent, ambivalent' citizens. They closely resembled the profile of the overall sample, holding the least strong sustainability attitudes and the highest percentage of regular pork eaters. Cluster 3 (12.3% of the sample) put the strongest emphasis on the type of housing and floor, favouring outdoor access; accordingly, it is named the 'animal welfare conscious'. It consisted of respondents mainly from Germany and Denmark, who held the second-strongest attitude towards animal welfare and the lowest or second-lowest regular consumption for most of the pork products examined. Finally, cluster 4 (11.5% of the sample) also put a very strong emphasis on the type of housing and floor by favouring outdoor access more than any other cluster, while they preferred all the attributes that characterise the extensive type of pig farming (e.g. small stock density, maximum effort to protect the environment, different quality of the final product); they were therefore termed the 'sustainability-oriented' citizens. They originated mostly in Denmark and Germany, held the most pessimistic attitude towards the environment, the strongest attitude towards animal welfare and the second-strongest attitude towards environmentally friendly food production.

In China, respondents in cluster 1 (31.5% of the sample) put almost exclusively the strongest emphasis on the special or maximum food safety efforts; they were therefore characterised the 'food safety conscious'. They expressed the strongest concerns towards environment, the most positive attitude towards technological progress and the strongest request for environmental friendly food production, while they exhibited the lowest regular pork consumption. Cluster 2 (44.1%) showed indifference (i.e. close-to-zero preference) towards all factors under examination; it was therefore named the 'indifferent,

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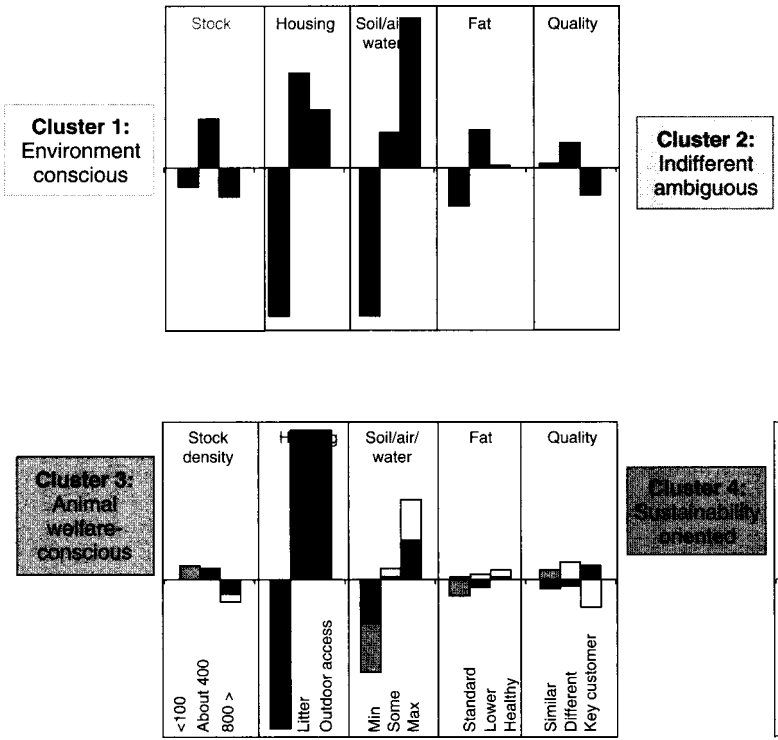
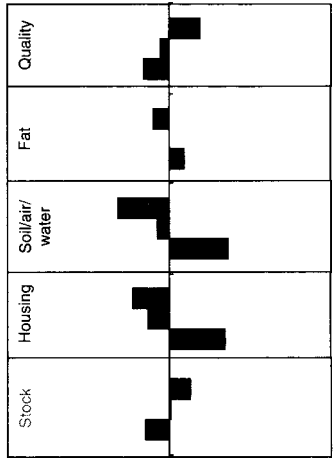
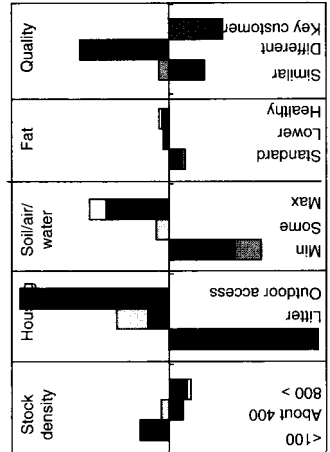


Figure 3.3 Cluster analysis results in Europe, four-cluster solution (*N*=1931).

Case Study 3.2 (Continued)





Case Study 3.2 (Continued)

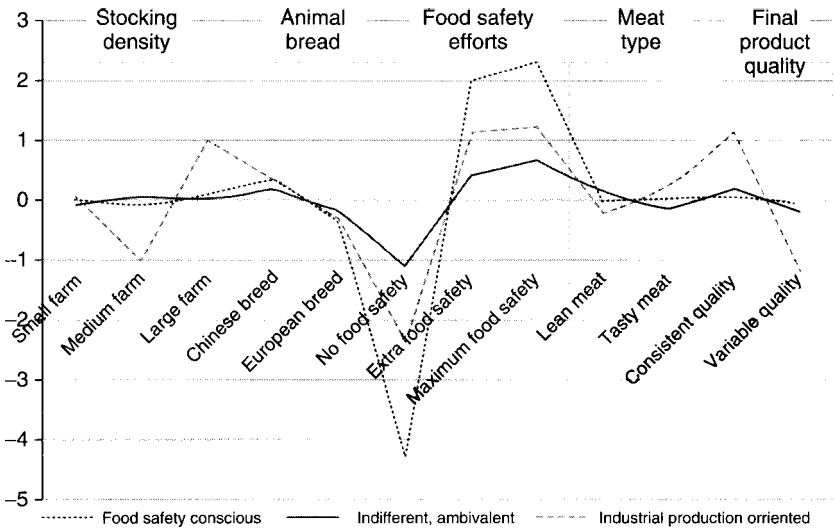


Figure 3.4 Cluster analysis results in China, three-cluster solution (N=479).

ambivalent'. They held the weakest attitudes towards all sustainability attitudes under consideration and exhibited the highest regular pork consumption across all pork types. Finally, cluster 3 (23.0%) mostly preferred industrial/extensive pig farming characteristics (i.e. large size and similar quality of the final product); it was therefore termed the 'industrial production oriented'. Their attitudinal profile and pork consumption were close to the overall sample.

Discussion. In both EU and China, peoples' attitudes towards the environment, food production, animal welfare and green food turned out to be rather moderately strong, contrary to numerous past surveys proclaiming the opposite. These rather weak attitudes may influence the way citizens see pig farming: the biggest clusters in both the EU and China, comprising more than half of the overall sample, had only weak opinions about differences between pig farming systems. At the same time, pork appears to play a substantial role as a part of the diet for most respondents in the five countries of this study.

In the EU, results showed that people assigned most importance to animal and environmental well-being as a criterion to discriminate between 'good' and 'bad' farming practices. In the absence of strong opinions, it is quite possible that EU citizens adopt politically correct stances that minimise the possibility of misjudgment. Possibly aided by the media, people often have clearer opinions about how production practices rather than end-product characteristics ought to be in general. Cluster analysis results, however, allowed for a more differentiated view. Small-sized, clear-cut segments of EU citizens emerged, paying attention to specific sustainable pig farming attributes (i.e. clusters 1, 3 and 4). It therefore seems that certain small but conscious social groups from cultures with relevant heritage would favour more ethically responsible types of farming.

(Continued)

Case Study 3.2 (Continued)

In China, both pork meat demand and supply have been rising. The small family farm raising a few pigs mainly for their own consumption, while still the dominant part in the sector, is giving way to large-scale industrial pig farms. At the same time there is a widespread concern about food safety, which reflects policymakers' characterisation of food safety as a social sustainability component. One may speculate that the three clusters which emerged represent stages in a diffusion process: there is a social group that is not (yet) concerned about food safety (stage 1); another social group has become concerned about the way pork is produced, because they see a safety issue (stage 2); and a third social group believes they see the solution to this issue, namely the transformation to large-scale industrial production (stage 3). This interpretation coincides with the process of long-term change and differentiation of consumer preferences in an emerging economy. Government favours industrialisation and industrial actors push it even further, so it is communicated as a socially beneficial development.

From a public policy point of view, the above results indicate that people's attitudes to pig production may be more important for food producers' long-term license to produce than for their sales. While people's attitudes influence purchase behaviour only weakly, they may still be expressed in the public debate and influence policy formation at regional, national and global levels. On the other hand, it cannot be ruled out that a stronger relationship between attitudes and consumer demand would develop if in the market there were more products clearly positioned with regard to various sustainability aspects, while retaining good eating quality and an attractive price. People's opinions about different ways of producing food not only impact their preference for certain types of products, but also shape the agenda of the public discourse; opinions therefore have an indirect impact on legislations, their implementation and their long-term market implications.

3.3.2 Regulatory Initiatives of Place Branding: Designation of Origin Labelled (DOL) Food and the PDO/PGI/TSG Schemes

Typical components of the Mediterranean diet constitute local/traditional foodstuffs and products, which often carry a Designation of Origin Label (DOL) as an authenticity certificate for their genuine local character. A DOL is a form of intellectual property (or 'authorship') equivalent to a trademark (the most commonly known intellectual property example of the kind), which ties a good with its physical environment (i.e. a geographical area) by origin, quality, production method, or any other measure that can link the product to the area. DOLs have been a matter of concern for the EU since the 1980s, when deceitful use of geographical indications was considered a threat to free competition.

The registration of local foods as DOL-certified (i.e. foods bearing the PDO/PGI/TSG labels; see Fig. 1.1) was first introduced in 1992 through EU Regulation 2081. The registration procedure was clearly described and has been adopted across the EU ever since. In order to register a local food as a DOL-certified product with the relevant EU Commission services, the following prerequisites need to be in place: a geographical designation included in its name; a description of the product's characteristics; an exact definition of the specific geographical area of origin of the product; evidence of the product's origin; evidence of the authentic local production and/or processing and

packaging methods; as well as scientific proof for the link between the quality of the product and its geographical origin. The two main DOLs, namely PDO and PGI, were introduced in 1993. Their description is as follows.

- PDO (Protected Designation of Origin) is used to differentiate the product by its place of origin. This means that the product is considered to have gained its fundamental traits essentially or exclusively due to its place of origin. Nature and the human factor are also considered important elements; the whole transformation from a raw good to a finished product therefore has to be carried out in the same geographical area.
- PGI (Protected Geographical Indication), on the other hand, imposes the requirement for a food product to possess only one quality pertaining to a certain area, not necessarily its place of origin. At least one of the production steps (typically production of the raw good) must take place in a well-specified geographic location, the location that is fundamental for the traits of the good.In 2007, Regulation 1216/2007 introduced a third kind of DOL.
- TSG (Traditional Speciality Guaranteed) indicates foods products that possess traits of traditional character that differentiate them from other products in the same category. In order to obtain a TSG certification, the producer must provide evidence of the exclusivity of the product, that is, traditional raw materials, production methods or place that differentiate the goods from others of the same area or category.

All DOL-certified foods (Fig. 3.5) are granted the same level of protection in all Member States (EC Regulation 510/2006). As stated in the regulation, all registered DOLs are protected against: (a) direct or indirect use of the name for commercial purposes by non-covered products, which are comparable to the registered product, or if these products damage the reputation of the protected name; (b) any misuse of the location or the registered name, even if it is applied indirectly to the non-registered good; (c) other indications on the packaging, advertisements or documents which can give a false impression of the product's origin; and (d) any other practice that can mislead consumer on the true origin of the product. As DOLs are a measure of property rights, they are also covered by TRIPS (trade-related aspects of intellectual property rights, an agreement by the WTO members). This allows third countries to apply geographical indications if the good is registered there.



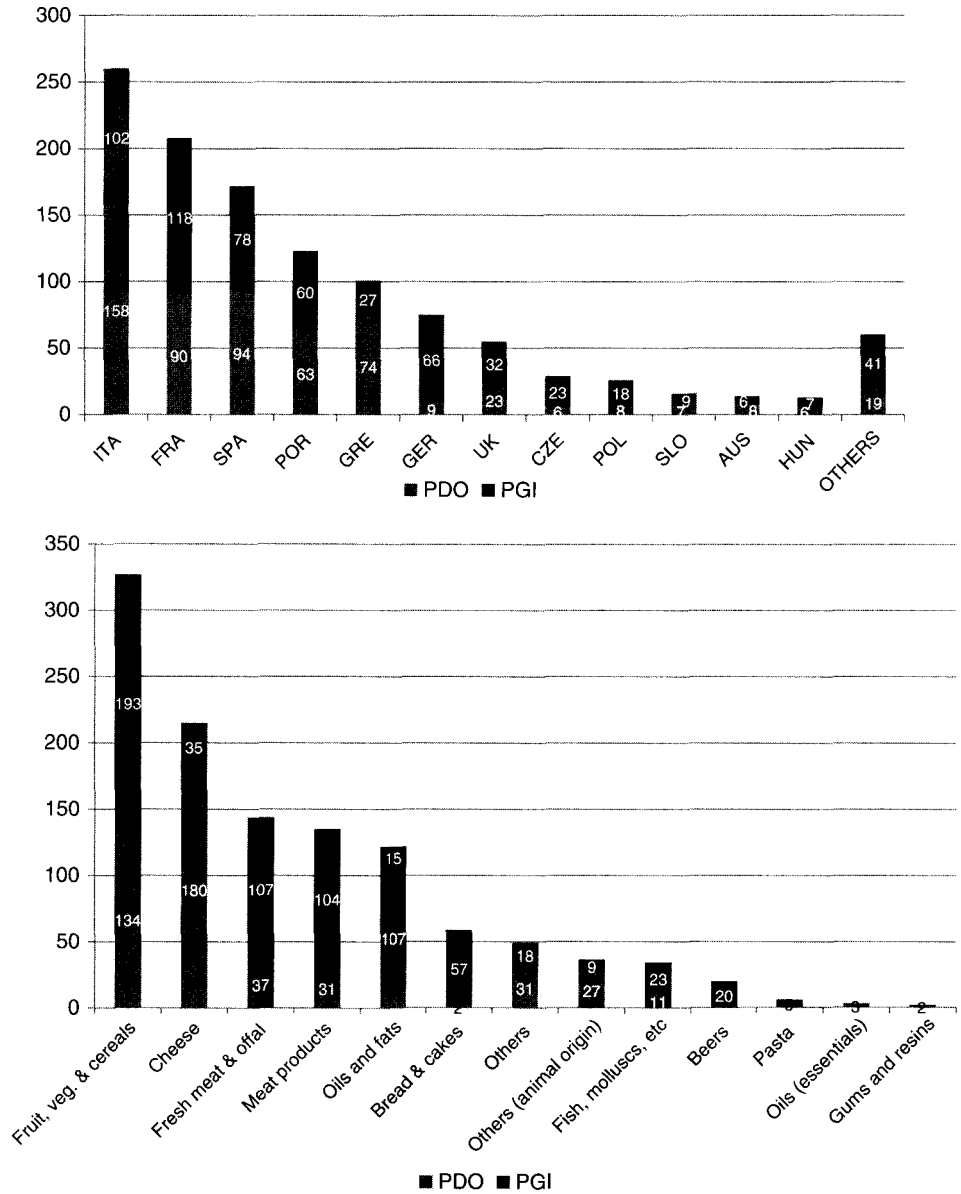
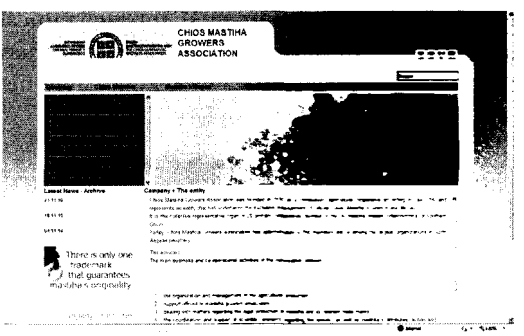


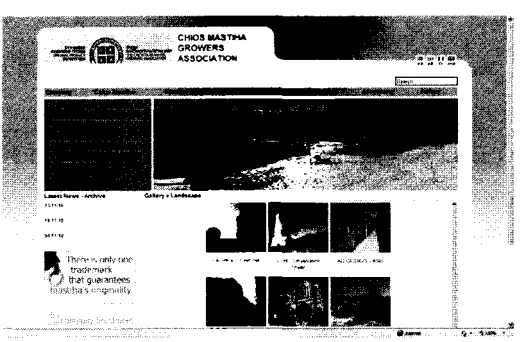
Figure 3.5 PDO-PGIs in the EU: 27 per country and category. Source: European Commission DOOR database.

DOLs therefore offer numerous advantages to the European agriculture and food SMEs, mostly due to their comprehensive scope of influence through legal organisations and treaties. The first benefit of the DOLs is higher involvement on behalf of the local niche markets. The labelling ensures that the customers are aware of the product's origin, which can be a useful tool for decision-making during the purchasing process (Fotopoulos and Krystallis 2003; Fotopoulos *et al.* 2011; Chrysochou *et al.*

2012). DOL foods are perfect examples of place-branding strategies that combine intrinsic (genuine, territory-related) characteristics with extrinsic quality cues (i.e. DOLs and PDO/PGI/TSG logos) to communicate credence quality (i.e. authenticity, naturalness, purity).

The 'genuine authentic' examples of place branding are numerous. The Chios mastic gum ('masticha') is the only natural, completely unprocessed gum that comes as a 'tear' from mastic trees that grow in the world only in 24 villages in the southern part of Chios, an island in the northeast side of the Aegean archipelago. The Chios Masticha Growers Association was founded in 1938 and represents the entity that has undertaken the exclusive management of natural Chios masticha internationally. Since 1997, Chios masticha has been certified as a PDO product. The product has a variety of health benefits extensively documented for the authenticity of the relevant health claims, including: antimicrobial, antibacterial and antioxidant activities of the mastic; dermatological and adhesive properties; and a protective effect against cancer. These health benefits are widely attributed to phytochemical properties of the mastic oil, that are the result of the unique association of the mastic tree with its natural environment.

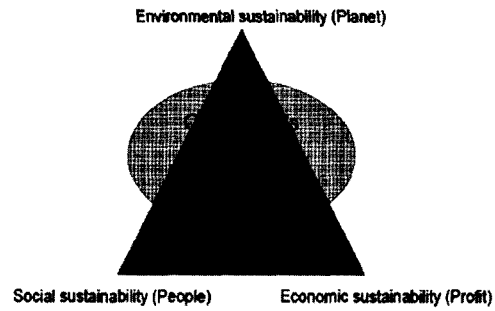




The commercial forms of the product are numerous, all similar to any other well-known gum brand. However, due to the naturally contained mastic oil, the product is also suitable for body care use. The company produces a rather steady turnover of approximately €15 million per year for the local mastic growers, more than 60% of which through exports. Its market presence is also very vivid through its own retail shop chain 'masticha shop®'.



In all, DOL products such as the Chios mastic gum are ideal examples of authentic products that satisfy all three dimensions of sustainability: economic, by safeguarding dissent income for local producers; environmental, through ethical and sustainable use of local natural resources implied from the 'teroirre', the local character of the product; and social, through the provision of scientifically substantiated health benefits and the satisfaction of wider ethical concerns of the society at large.



Case Study 3.3 Can consumer personal values explain quality food-related consumer behaviour?

The study by Fotopoulos *et al.* (2011) was aimed at enriching the understanding of the ability of personal values to explain consumer behaviour in the context of quality food, such as Organic and Designation of Origin Labelled (DOL) foods (i.e. Protected Denomination of Origin, PDOs). Personal 'values', such as achievement or security, have been defined as 'an enduring belief that a specific end-state of existence or specific mode of conduct is preferred to a different end-state or mode of conduct for living one's life' (Rokeach 1973). Fotopoulos *et al.* (2011) postulated that consumers who exhibit different purchasing behaviour towards organic and DOL food would also identify more strongly with different personal value domains; moreover, this identification with different values should allow for the emergence of a number of distinct and well-differentiated consumer types (i.e. clusters) with equally distinctive purchasing behaviour towards organic and DOL foods.

Case Study 3.3 (Continued)

The concept of personal values is frequently drawn upon to shed light on the attitudes and behaviour of individuals and the functioning of organisations, institutions and societies. However, systematic theories on the content and organisation of the value systems of individuals have been proposed and empirically validated only in the past 20 years, among which the 'Values Theory' (Schwartz 1992) has a prominent place. The Values Theory identifies what may be a comprehensive set of 10 different types of cross-cultural values (called 'value domains'), namely: benevolence, universalism, self-direction, stimulation, hedonism, achievement, power, security, conformity and tradition (Fig. 3.6). The postulated by Values Theory distinction between various types of values constitutes a platform of fundamental importance for the purposes of better understanding consumer value structures that act as possible drivers of quality food purchasing. For instance, when the environmentally friendly method of organic food production is the central motive of organic food purchasing, the importance of universalism is expected to be high. On the other hand, when organic selection is motivated by the protection of persons close to an individual (e.g. children, family and friends), the importance of benevolence is expected to be higher. In the same fashion, if the preservation of local identity is the central motive of PDO purchasing, one would expect tradition and/or security to be prominent.

A robust research instrument, the Portrait Value Questionnaire (PVQ) (Schwartz 1992), has been designed to measure the value orientation of various cultures. The PVQ is a 40-item

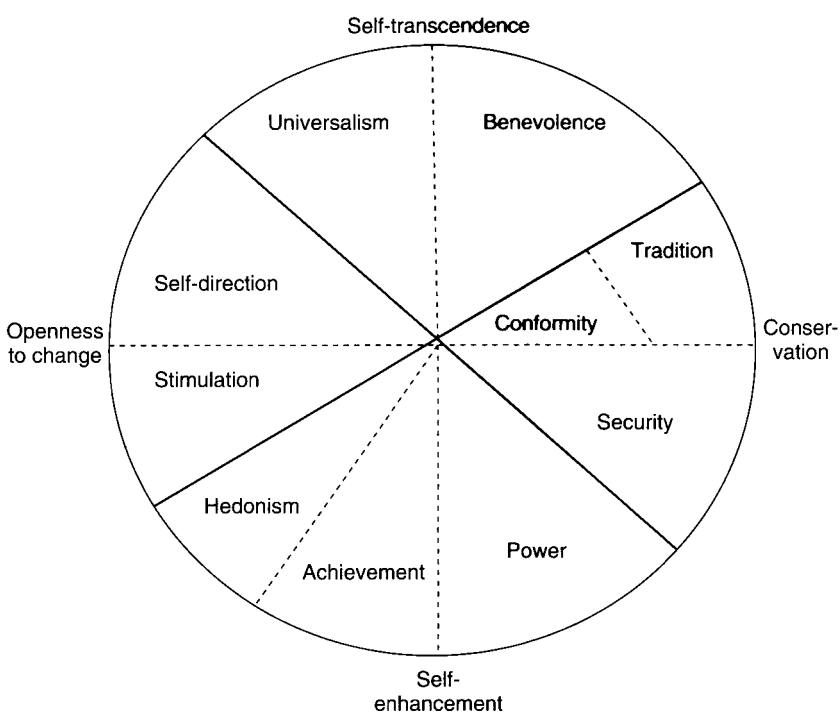


Figure 3.6 Schwartz's taxonomy of motivational value domains (Schwartz 1992).

(Continued)

Case Study 3.3 (Continued)

typology based on the 10 domains of Values Theory, with the form of short verbal portraits of 40 different people. Each portrait describes a hypothetical person's goals or aspirations that point implicitly to the importance of a value, e.g. 'He really wants to enjoy life. Having a good time is important to him/her' describes a person to whom hedonism values are important. For each portrait, respondents are asked: '... Think about how much each person is or is not like you'. Responses are: 'very much like me', 'like me', 'somewhat like me', 'a little like me', 'not like me' and 'not like me at all'. In asking respondents to compare the portrait to themselves rather than themselves to the portrait, they pay attention only to personality aspects that are portrayed. The similarity judgment is likely to focus only on these value-relevant aspects. The number of portraits for each domain reflects the breadth of its conceptual definition: 6 for universalism; 5 for security; 4 each for tradition, conformity, benevolence, self-direction and achievement; and 3 each for stimulation, hedonism and power. PVQ requires about 15 min to be completed in face-to-face or telephone interviews. Since its conception, PVQ has been validated by different scientific disciplines internationally in a broad range of settings to investigate the invariance, configuration and organisation of values, as well as to predict individual behaviour.

The survey of Fotopoulos *et al.* (2011) is based on a random sample of 997 Greek consumers. A south European country is an ideal exemplary area to study consumer value orientation and the relationship with quality food purchasing since in south European societies consumers are typically more involved in food purchasing and preparation, while the market shares of 'traditional' food products manufactured from local SMEs are typically much higher than in the north of Europe. Data were gathered by a professional research agency. The questionnaire that was developed for the purpose of data collection was self-administered, completed by a person aged 18 and above in each household, (co)responsible for grocery shopping in the household (sample mean age 42.9 years). The sample was nationally representative in terms of education, income and geographic distribution. The questionnaire comprised four multi-item parts. The first parts examined observed food purchasing behaviour, namely frequency and place of purchase for a total of 23 bulk, fresh, packed and/or processed 'conventional' and 30 quality food products (15 organic and 15 PDO foods). Given the nature of the domestic quality food market (i.e. product availability, price margins, etc.), the choice 'at least once per month' was designed to realistically express a type of regular purchasing of organic and/or PDO products. The second part examined consumers' self-reported awareness of the organic and PDO schemes. In this respect, after the question 'do you know what an organic (PDO) product is about?' ('Yes'/'No'), consumers were encouraged to provide their own definition of the relevant schemes. The third part measured consumers' monthly expenditure for in-home food preparation and self-reported willingness to pay (WTP) for organic and PDOs. The fourth part included the 40 PVQ value items (1 = 'very much like me' to 6 = 'not like me at all').

Preliminary descriptive analysis regarding the observed food purchasing variables for the 15 organic and the 15 PDO products offered interesting insights. Firstly, the organic products exhibited much lower purchasing frequencies compared to their conventional counterparts (Table 3.6). On the other hand, the situation for the PDOs was much different with purchasing frequencies being comparable to conventional products, at least for those fresh and packed/processed PDO products that were distributed nationally

Case Study 3.3 (Continued)

Table 3.6 Sample's purchasing frequencies (selection), %, *N*=997.

A: Organic products

Purchase frequency:	At least once/month	Less than once/month	Never
1. Tomatoes	5.2	6.0	88.8
2. Apples	2.1	2.5	95.4
3. Oranges	1.9	2.6	95.5
4. Lettuce	1.5	2.9	95.6
5. Chicken	1.4	2.3	96.3
6. Pasta	1.4	1.9	96.7
7. Eggs	1.4	1.3	97.3
8. Pork	1.2	2.3	96.5
9. Milk	1.1	0.8	98.1

B: PDO products

Purchase frequency:	At least once month	Less than once/month	Never
1. Olives	28.2	17.5	54.3
2. Apples	17.8	11.1	71.1
3. Red peppers	15.9	19.1	65.0
4. Potatoes	13.4	7.9	78.6
5. Small baked breads	12.9	9.2	77.8
6. White cheese	10.5	11.6	77.8
7. Strawberries	4.2	6.8	89.0

(e.g. PDO olives, apples, red peppers, potatoes, white cheese, etc.). In terms of purchasing channels, for organic products the specialty stores emerged as the primary place of purchase (especially for the most frequently purchased among them). The supermarkets seemed to play an important role as outlets for less-frequently purchased organic products and as alternative places of purchase in general. For PDOs, the primary purchase places were the same with conventional products, mainly supermarkets (i.e. processed or packed PDOs), open markets (i.e. PDO fruits/vegetables) and specialty stores (i.e. PDO wines and meat products).

Regarding knowledge of the organic and the PDO schemes, 80% of the overall sample answered correctly in relation to the organic product definition. A closer look at the definitions provided by those who claimed to be aware (e.g. 'without pesticides', 'don't get

(Continued)

Case Study 3.3 (Continued)

sprayed', etc.) revealed that this high percentage of self-reported knowledge was actually true. The latter observation did not hold for the definitions provided for the PDO scheme, for which self-reported knowledge was very low (only 20% of the overall sample said they knew what PDO products were). Finally, a significant percentage of consumers claimed to be willing to pay a 10% premium or higher over the price of conventional products to buy organics (52.6% of the sample) or PDOs (38.4% of the sample).

Based on the mean scores of the 40 PVQ items, the larger similarities were found within the values of *security* (2.60), *universalism* (2.65), *benevolence* (2.74), and *tradition* (2.74), while consumers found only weak similarities with *power* (3.30), *stimulation* (3.05) and *achievement* (3.01). A value-based segmentation task was implemented at the final stage of the analysis using the PVQ items to assign consumers into clusters. A five-cluster solution was the most statistically robust. Statistically significant differences among the five clusters were established using chi-square and one-way ANOVA tests. In order to develop the profiles of each cluster, a cross-tabulation process took place between the cluster membership variable and the remaining variables of the questionnaire (Tables 3.7, 3.8). It became apparent that the five clusters differed both in terms of mean similarity with the PVQ domains, their socio-demographic profile and their quality food purchasing patterns. A detailed description of the five clusters is as follows.

Cluster 1 (27.7%) had the strongest similarity of all clusters with the values of self-transcendence (benevolence, universalism) and security, and the second highest similarity with conformity, tradition and self-direction. It comprised more individuals of relatively older age, tertiary education, high and high-average income levels and city dwelling (Athens and other large and medium cities across the country). Furthermore, cluster 1 included consumers with the highest monthly expenditure for in-home food preparation, the highest and second-highest WTP and the second highest self-reported knowledge for PDO and organic foods. Based on its socio-demographic profile and quality food purchasing patterns, it was termed the 'urban upper class'. Cluster 2 (22.4%) did not show particular similarity with any value domain. Its socio-demographic profile did not portray any particular type of individuals either, but most of the relevant characteristics resembled those of the overall sample; it was therefore named the 'national middle class'. Cluster 3 (18.2%) expressed the strongest similarity to all the remaining value domains besides self-transcendence (mainly universalism). It included the highest percentages of average-income families, with small children, dwellers of large cities other than Athens and Thessaloniki, or of rural towns. It also exhibited the second-highest monthly expenditure for in-home food preparation, the second highest WTP for organic food but the second-lowest WTP for PDO foods, and the highest of all clusters self-reported knowledge about the organic and the PDO schemes; it was named the 'countryside class I'. Cluster 4 (20.8%) had the second-strongest similarity with self-enhancement values (hedonism, achievement, power) and stimulation. Its socio-demographic profile resembled that of the 'countryside class I' with some exceptions (i.e. mainly higher percentages of males and inhabitants of medium cities and rural areas). Moreover, it showed the highest and second-highest WTP for organic and PDO foods, respectively. However, compared to cluster 3, it exhibited relatively lower self-reported knowledge about the organic and the PDO schemes, one of the lowest of all clusters. Cluster 4 formed the 'countryside class II'.

Table 3.7 Cluster's quality food purchasing pattern (%), N=997. Bold font: highest value; italics font: lowest value.

Sample profile		Sig.	Cluster 1: 27.7% (n_1=276) urban upper-class	Cluster 2: 22.4% (n_2=223) national middle class	Cluster 3: 18.2% (n_3=182) countryside class I	Cluster 4: 20.8% (n_4=207) countryside class II	Cluster 5: 10.1% (n_5=110) urban low-class
1. Expenditure for in-home food preparation							
> €300/month	38.1	**	**47.5**	33.1	***42.0***	38.2	16.3
2. Willingness to pay (WTP) for organics							
0%	40.3	**	37.7	39.0	***43.1***	34.8	**55.5**
10% or higher	52.6		***53.6***	53.0 (1)	***53.6***	**57.1**	39.9
3. Willingness to pay (WTP) for PDOs							
0%	47.8	*	43.1	***49.8***	53.6	39.6	**61.8**
10% or higher	38.4		**41.6**	37.2	37.0	***41.1*** (2)	30.1
4. Self-reported knowledge about organics							
Yes, I know what an organic product is	76.4	**	***80.1***	79.4	**81.8**	75.4	54.5
5. Self-reported knowledge about PDOs							
Yes, I know what a PDO product is	20.0	**	***22.5***	18.4	**24.9**	22.2	4.5

**: χ^2 test, p<0.01; *: χ^2 test, p<0.05; (1): WTP at least +20% = 16.2%; (2): WTP at least +20% = 11.1%

(Continued)

Table 3.8 Cluster's quality food purchasing profiles (products with statistically significant difference among the clusters), %.

	Cluster 1		Cluster 2		Cluster 3		Cluster 4		Cluster 5	
Purchase frequency	**At least once/ month**	**Never**	**At least once/ month**	**Never**	**At least once/ month**	**Never**	**At least once/ month**	**Never**	**At least once/ month**	**Never**
Organic products										
1. Tomatoes (**)	4.3	89.1	5.4	86.1	***6.7***	87.7	**6.8**	89.4	1.8	***93.6***
2. Apples (**)	***2.1***	96.0	1.3	92.4	1.7	***97.8***	**3.4**	95.2	1.3	96.4
PDO products										
1. Olives (*)	***28.6***	55.4	17.4	63.7	22.7	65.7	**32.4**	56.5	12.7	***82.7***
2. Apples (*)	**22.8**	63.8	17.5	67.7	***21.5***	71.3	11.1	78.3	11.8	***82.7***
3. Red peppers (*)	**27.2**	52.5	13.4	65.5	***14.9***	66.3	10.7	67.6	4.5	***88.2***
4. Potatoes (*)	***19.2***	69.9	10.7	82.1	14.9	76.2	3.8	***88.9***	**20.0**	78.2
5. Small baked breads (*)	**21.7**	67.4	***11.2***	77.6	***11.1***	82.3	6.7	***85.5***	9.1	82.7
6. White cheese(*)	4.7	***83.0***	10.3	77.1	**16.1**	79.6	***13.1***	70.0	11.8	78.2
9. Beans (*)	3.6	92.4	***5.3***	83.4	0.6	***95.0***	1.5	95.7	**7.3**	86.4
10. Fava Beans (*)	**5.1**	89.1	***4.7***	91.9	1.1	96.1	0.5	***97.6***	***4.5***	86.4

**: χ^2 test, $p<0.01$; *: χ^2 test, $p<0.05$

Case Study 3.3 (Continued)

Finally, cluster 5 (10.1%) expressed weak similarity to all value domains. It comprised the fewest of all clusters: males, high educated members, with average, high-average and high incomes, and inhabitants of large cities (besides Athens). It also showed the lowest monthly expenditure for in-home food preparation and WTP for quality foods; it was termed the 'urban low class'.

Discussion. Despite the progress made since the late 1990s, the market for organic food products in Greece remains underdeveloped. Regular purchasing frequencies for a large variety of organic products are practically zero, and even the most popular among them (i.e. fresh fruits and vegetables) are purchased regularly only by consumer niches. What is different from past research, though, is the much broader knowledge of the organic scheme: nowadays, more than three in four consumers have an accurate knowledge of organic food. This, coupled with the particularly low purchase frequencies, carries a disappointing message for the real potential of the organic market in Greece. On the other hand, consumers exhibit multi-fold higher regular purchasing frequencies for PDOs. This observation could reflect the fact that many PDOs are nationwide-famous local specialties, with a long-standing history in the Mediterranean culinary culture. PDOs – usually priced not (much) higher than their conventional counterparts – are therefore better established as more representative types of quality foods in consumer perceptions. PDOs find their way to the market easier, since they are much more preferred to organic products; the latter are often accompanied by consumers' distrust, as well as strong negative beliefs of unreasonably high prices. However, despite their quite satisfactory regular-purchase frequencies, consumers' true knowledge about PDOs remains at a surprisingly low level. Many PDO purchases are therefore made 'unintentionally', whereas their quite high WTP estimates should be seen with caution. In all, the marketing efforts targeting the quality food products seem to have failed to meet their objectives: in the case of organic foods, they created a widespread awareness but failed to alter the strongly ambiguous attitudes of consumers; in the case of PDOs, the failure to create widespread knowledge prevents the DOL scheme from delivering marketing value and delivering the social benefits initially intended.

At the value-based segmentation level of the analysis, the trend of stronger consumer similarity with security, universalism and benevolence that appeared at the sample level re-emerged for the 'urban upper class' cluster, whereas higher than average similarity to those value domains appeared for the 'countryside class I and 'countryside class II' clusters. Taken together, these three clusters account for 66.7% of the overall sample and are the clusters with the most dynamic quality food purchasing profile: the 'urban upper class' exhibited substantial regular purchasing frequency for both organic and PDO products; consumers in the 'countryside class I' showed high regular purchasing frequency mainly for PDO products, whereas their purchasing frequency for organics was also substantial; and the 'countryside class II' showed high regular purchasing frequency mainly for organic products, whereas their purchasing frequency for PDOs was also substantial. Clusters with higher percentages of regular quality food buyers therefore show higher similarity to the *self*-transcendence/conservation dimensions. Additionally, the importance assigned to openness-to-change/self-enhancement values also played an important role: in the regular buyers of 'countryside class I' and 'countryside class II', the relevant

(Continued)

Case Study 3.3 (Continued)

values function complementary to the self-transcendence/conservation values as drivers of quality food purchasing. Quality food purchasing turns out to be the combined outcome of self-transcendence/conservation and openness-to-change/self-enhancement values, with universalism, benevolence, security and tradition on the one hand, and achievement and hedonism on the other interacting to formulate the value profile of the 'typical' food quality consumer.

Overall, despite clear evidence of the relationship between consumers' self-transcendence and security value similarity and higher-than-average frequency of quality food purchasing, quality food consumers did not form a separate and clearly diversified cluster if the PVQ inventory functions as a basis for segmentation. Since all value-based clusters contained a substantial percentage of regular quality food buyers for a variety of such products, the PVQ typology does not have especially strong discriminating power among regular buyers. Unsatisfactory perceived knowledge levels of the PDO scheme and no particularly positive attitude towards the organic scheme might be responsible for this result. However, the main reason behind this outcome might be that values cannot function as a sole and direct predecessor of consumer behaviour within the quality food context. Future models should incorporate values together with intermediate-level constructs (e.g. beliefs and/or attitudes) when attempting to predict consumer behaviour towards quality food products. Alternatively, future work must also show the adaptation capability of existing taxonomies, such as the PVQ, by demonstrating that tailor-making of the generic instrument of core-values could better reflect the motivations of consumers in specific decision-making contexts.

3.3.3 The Industry's Response: Is Place Branding a Panacea?

Although traditional or artisanal food comes predominantly from small-scale production, the fact that local food combines healthiness and tastiness, as well as their inclusive sustainability character, are very attractive to the industrial food cluster. As a response, the number of new food products launched with a privately assigned DLO claim has increased steadily since the mid-1990s. For instance, more than 7000 food products have been launched since 1996 across all European countries with the term 'Mediterranean' in the product description in categories, such as: sauces and seasonings; meals and meal centres; fish, meat and egg products; and dairy, bakery and snacks. During the same period and across the same categories, the number of new food products launched with the term 'Traditional' in their description exceeded 14 000 (Fig. 3.7), typically in all major food markets in Europe such as the UK, Germany, France, Spain, Italy and the Netherlands (Fig. 3.8).

However, the DOL is not the only privately assigned claim or logo found on the package of those designated brands. A multitude of other positioning claims accompany the DOL. Besides the term 'Mediterranean', one can find on the package of those new food products claims such as 'no additives/preservatives', 'microwavable', 'vegetarian', 'low fat', 'premium', 'easy to use' and 'organic', similar to the over-exploitation of all positioning claims for the new food products launched with the term 'Traditional' (Fig. 3.9). Such information clutter for a single product makes the authentication of destination-related

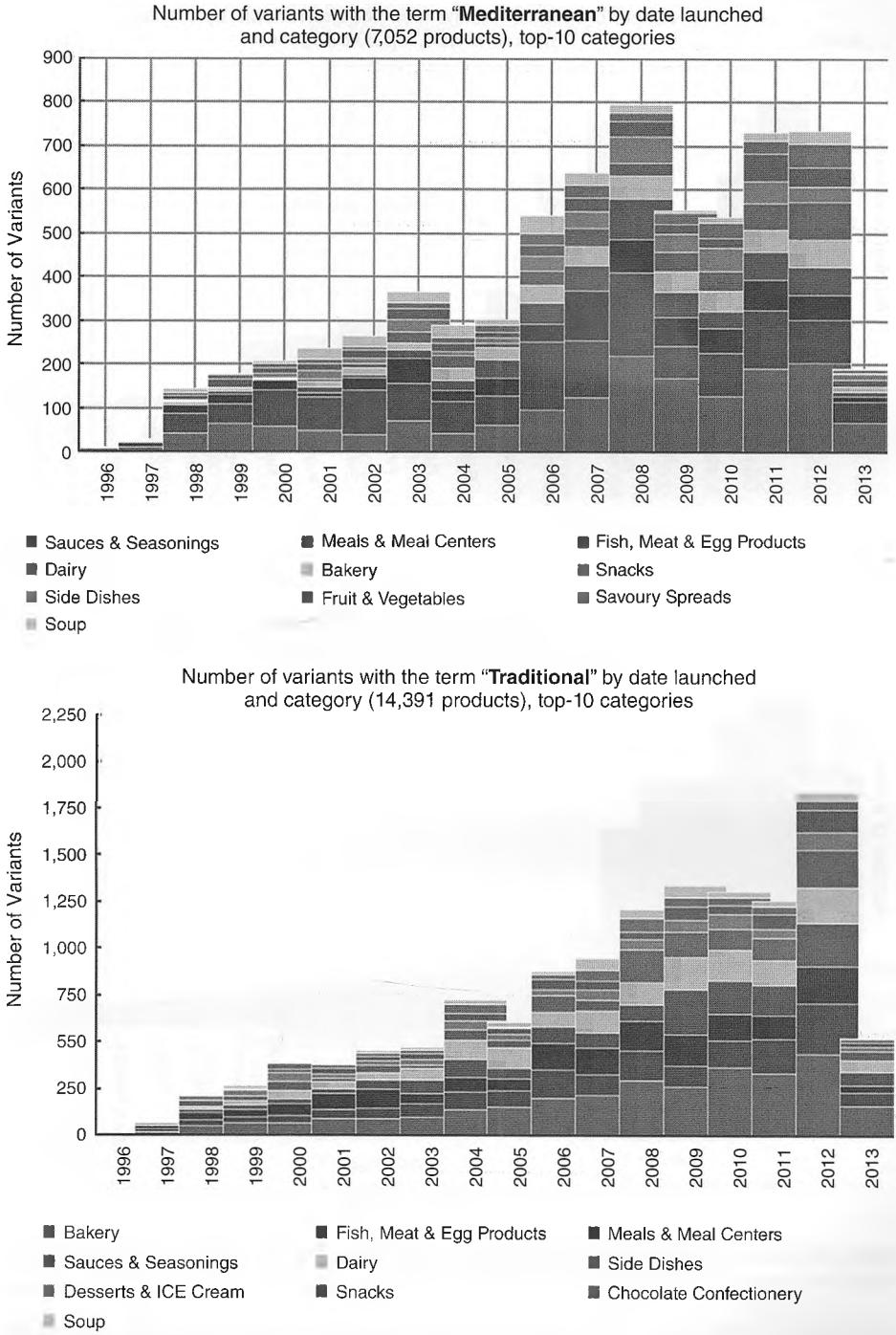


Figure 3.7 Number of new food products launched with destination brand traits (examples).
Source: Mintel GNPD database.

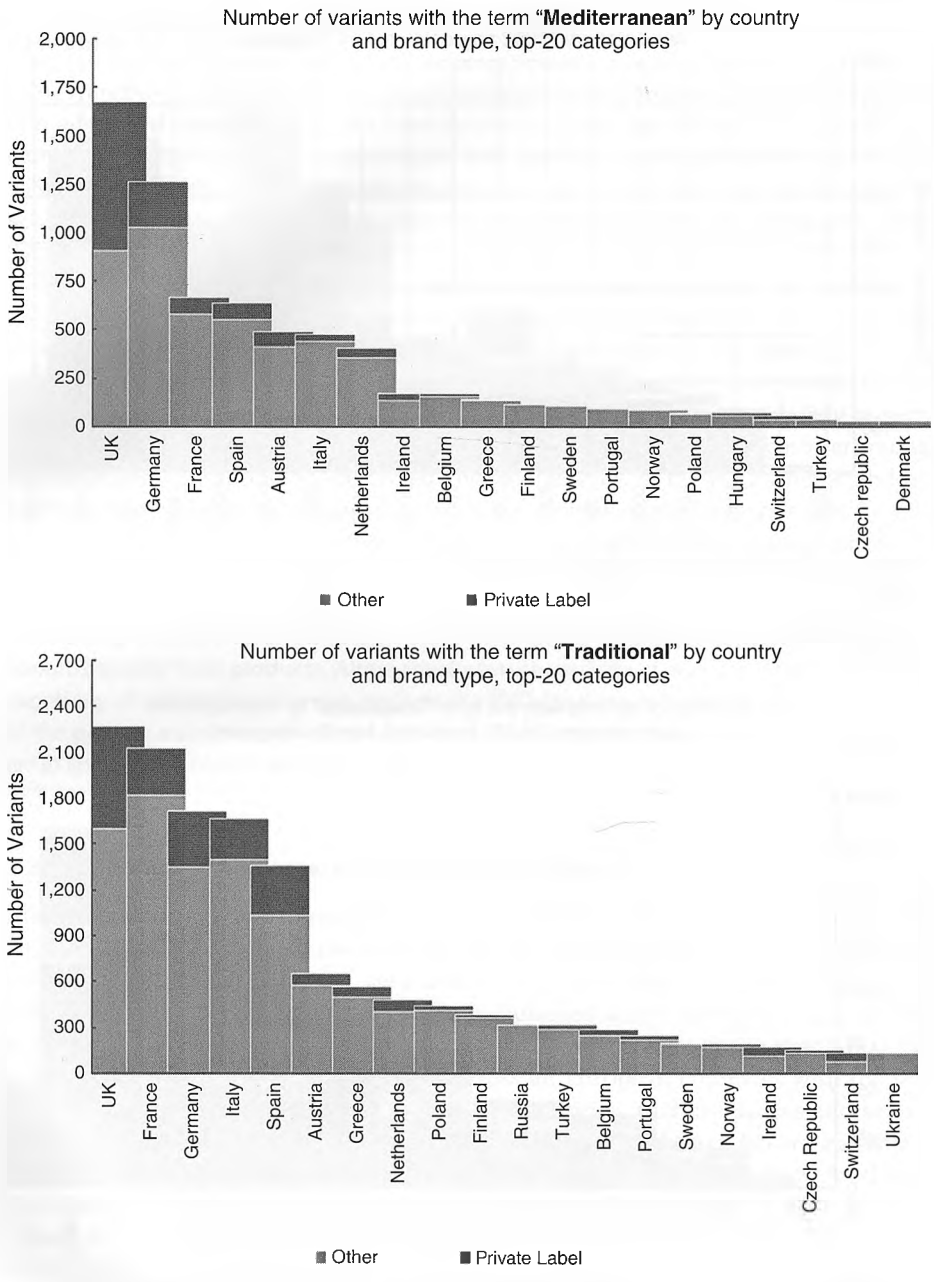


Figure 3.8 Country and brand type distribution of new food products launched with destination brand traits (examples). *Source:* Mintel GNPD database.

originality (i.e. Mediterranean) extremely complicated, if not impossible, and makes consumers sceptical in relation to the credibility and trustworthiness of those products and their manufacturers alike. The fact that a substantial part of the Mediterranean or 'traditional' claims concern private labelled product (retailer-branded; see Fig. 3.8)

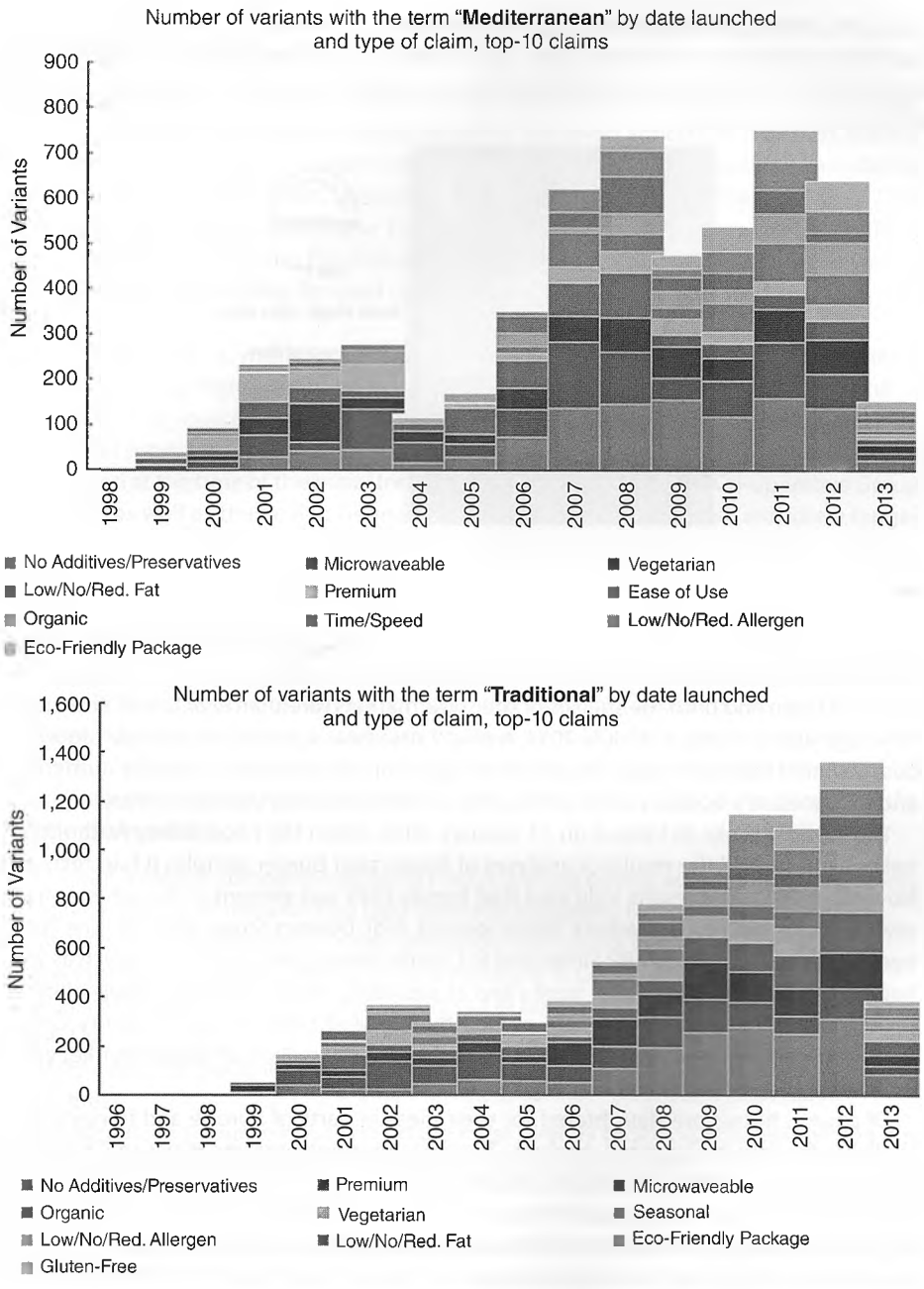
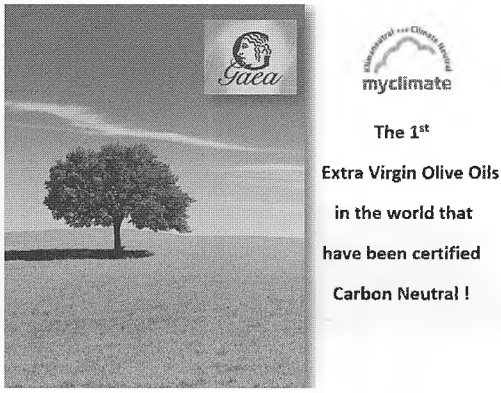


Figure 3.9 Other position claims found on the label of new food products launched with destination brand traits (examples). *Source:* Mintel GNPD database.

further indicates that authenticity messages through destination branding is today a viable strategic orientation for all parts of the supply chain (see Fig. 3.8), making the need of a regulated use of authenticity-positioning claims (i.e. 'Traditional') more than imperative.

Besides erosion of consumer trust and resulting concerns for hard to authenticate, fraudulent behaviour on behalf of the food industry, however, destination brand-based authenticity in the food sector remains dynamic, with new narratives accompanying old versions of authenticity.



Case Study 3.4 The European horsemeat scandal: network in disarray

The global headlines said it all: for several months during the first half of 2013 the whole of Europe was rocked with the news that unapproved horsemeat had found its way into the food chain and onto the shelves of the supermarkets (Brennan *et al.* 2014). The media coverage was enormous. By July 2013, typing 'horsemeat scandal' into Google produced over 228 000 hits from news organisations, government agencies, scientific authorities and food industry bodies, not forgetting the almost obligatory Wikipedia entry.

The scandal broke in Ireland on 14 January 2013, when the Food Safety Authority of Ireland announced the results of analyses of frozen beef burger samples it had tested in November 2012. The results indicated that horsey DNA was present in the samples from several major food supermarkets: Tesco, Iceland, Aldi, Dunnes Stores and Lidl. The Tesco beef burgers, supplied by ABP Silvercrest in County Monaghan, revealed as much as 29% horsemeat. Two other factories were cited as providing meat with lower levels of horse DNA: Dalepak in Yorkshire, UK, also owned by ABP; and Liffey Meats in County Cavan, Ireland. It is argued that the scandal knocked almost £300 million off Tesco's market value within 48 hours of the news breaking.

Of course, horses are slaughtered for their meat in parts of Europe and horsemeat is available for sale in European markets. The issue was not horsemeat *per se*. There was much confusion at first and debates raged about whether it was a food safety issue or a breach of consumer trust or both. Products were recalled, food was taken off the shelves of the supermarkets, and food standards and safety authorities set about trying to establish what had happened. Ultimately, at issue on this occasion was the fact that the meat in affected burgers and other products was actually intended to be beef and should not have contained traces of any other animal product. Indeed, analyses of some samples of beef indicated that pig DNA was also identifiable. The concerns became more about consumer trust than food safety. The lack of traceability, for example, meant that people seeking to avoid particular foodstuffs (for example, for religious observance reasons) found it very difficult to know what beef products they could trust.

Case Study 3.4 (Continued)

By the time the full extent of saga had been played out, 15 European countries had become involved. The European Union, including European agriculture ministers and the European Commissioner for Health and Consumer Policy, were involved for formulating responses at the highest levels. This included a very large program of DNA testing of 2250 processed beef samples across the European Union and further plans to bolster food safety legislation within the EU, including more money for food surveillance as well as stronger financial penalties for food operators that commit fraud or fail to comply with food safety laws.

Along the way, a whole collection of household names in food were affected. Horsemeat traces found their way into consumer food products either manufactured directly by – or sourced by the subcontractors of – Findus, Nestle and Birds Eye. Own-brand beef products sold by major supermarkets were also affected. In addition to those announced at the time of the initial Irish investigation, Asda and the Co-operative Group in the UK, as well as the Real in Germany and IKEA across Europe, also withdrew ranges of beef products in the wake of the scandal. Intermediaries such as food service companies and catering suppliers were not immune either. Brake Bros, a food service company which supplies the Whitbread Group of companies (which includes Premier Inn, Brewers Fayre pubs and Beefeater Grill restaurants) found traces of horsemeat in its beef lasagna and burgers. Catering companies Compass and Sodexo, which supply institutional markets as such as schools, hospital and prisons, also found horsemeat present.

There has been subsequent criticism of the extent to which the meat supply trade within Europe has become too complex. One example, relating to how horsemeat found its way into products such as Findus beef lasagna, shows how extended and complex the supply chain can be.

- Comigel, a French company supplying Findus, among others, asks its subsidiary, Tavola in Luxembourg, to manufacture beef products (including Findus beef lasagna).
- Tavola orders the beef from a meet processor, Spanghero, in the south of France.
- Spanghero subcontracts the supply to a company in Cyprus.
- The Cypriot company uses Draap Trading Ltd in Belgium to make the order of the meat itself.
- Draap uses the slaughterhouses Doly-Com & CarmOlimp in Romania for supplying the meat.
- The Romanian slaughterhouses send the meat directly to Spanghero, from where it is sent to Tavola, which makes the products for supply to Findus and retailers across Europe.

Some attribute this long and complex chain to the way pricing operates within the meat production industry overall, arguing that prices are squeezed more tightly over time by the powerful supermarkets, which means that animals are sourced from lower-cost countries for slaughter, creating greater opportunities for fraudulent activity in meat substitution. There seems to have been fraudulent behaviour in knowingly re-labelling horsemeat as beef, as well as incompetence in the network in failing to maintain product integrity throughout the entire chain.

(Continued)

Case Study 3.4 (Continued)

For the companies such as Tesco, the scandal has brought a salutary warning about relationships and networks. A company is only as good as the relationships it has (and this goes the whole way down the supply chain network). Other major supermarkets, such as Sainsbury and Waitrose in the UK, have not suffered the same negative consumer responses because their products did not have meat substitutions. Indeed, they have benefited at the expenses of those who were implicated, by arguing that they use local meat from known sources and with supply chains that are much shorter. Relationships bring burdens that mean they will always require attention to what is happening in the network overall.

The cost of monitoring relationships and maintaining the quality and integrity of the product have to be borne somewhere in the network. The economic climate may well be a driver for reducing costs overall, but the question becomes one of where the costs are born since they cannot be eliminated completely. Some of the typically very large companies further downstream relied heavily on the costs being born upstream, without any real basis for trusting all the actors in the network and/or effective means of identifying and protecting the value chain from fraudulent behaviour. Given what Håkansson and Snehota (1995) have called the 'unruliness and stickiness' of relationships when companies do not have control and are dependent on the associates of associates, in retrospect this appears to have been a risk that came back to bite them. Of course, it was possible for many of the big players to drop key suppliers quite quickly in the light of scandal as it unfolded. However, adverse consequences did occur, such as damage to reputation, likelihood of greater product assurance costs arising from the more stringent requirements of the food standards agencies, and the prospect of having to assume greater relationship costs in the future.

As well as ties with its natural environment, incorporation of modern social concerns in relation to the ethical dimension of production techniques within the story-telling of territory-bounded products allows for new interpretations of the authenticity concept. These new interpretations yield new examples of authentic products consumers are proud and enthusiastic to endorse. In parallel, the immaterial component of the authenticity concept (i.e. branding and marketing campaigns, on-line loyalty platforms or social media) remains an efficient channel of the distribution of product and place-related narratives across the world.

In all, there is a number of conditions for a place brand to thrive (Charters and Spielmann 2014) that manufacturing companies need to take into consideration. First, there has to be a strong tie with the territory which is responsible for the unique outcome of the production process that cannot take place anywhere else. Second, the place brand incorporates all individual proprietary brands in the territory: 'each brand that includes the territorial reference in its own brand name or on its label cannot opt out from the overarching territorial brand itself' (Charters and Spielmann 2014, p.1462). From this stems the frequent quest of place brands for legal recognition, in order to defend themselves in the marketplace against similar products that do not have the special relationship with the territory (Van den Land *et al.* 2001). Third, all individually owned brands under the umbrella of the place brand are equally dominant, otherwise

the most powerful of those would overwhelm the place brand, jeopardising its existence. Fourth, the conception and development of a place brand is not the decision or responsibility of a sole decision-maker; no-one can be credited with the creation of a place brand, which instead evolves over time through co-creation from a number of local yet well-separable actors.

Place brands offer strong strategic advantages to producers by impacting on consumers' decision-making process and multiplying the loyalty of buyers (Orth *et al.* 2012). However, as Case Study 3.5 will show us, this should not be taken for granted. To be successful, place brands require effective management. Companies engaged in place brand development must be meticulous with managing the quality of their inputs and output in order to maintain uniformly high standards as a key factor of collective success. At the very end, place brands must be in communication with each other, sharing a sense of responsiveness to challenges and an understanding of common opponents as a strategic advantage that helps them generate an unprecedented degree of collaboration among seemingly competing entities (Charters and Spielmann 2014).

Case Study 3.5 Quality assurance labels as drivers of customer loyalty in the case of traditional food products

The study by Chrysochou *et al.* (2012) was aimed at investigating the role of quality assurance labels as product attributes that drive customer loyalty in traditional food products (TFPs). TFPs are exemplary differentiated products forming a particularly fragmented market, characterised by many small brands produced by relatively small firms. TFPs are very common in Europe, whereas the EU has introduced quality assurance schemes known as Designation of Origin Labels (DOLs; e.g. Protected Designation of Origin or PDO; Protected Geographical Indication or PGI). Such a policy intervention aims to regulate the market of TFPs and give a competitive marketing advantage to the SMEs involved in the supply chain of TFPs (in the face of the inability of SMEs to build strong branded products on their own). However, it is not yet known whether DOLs accomplish their strategic mission, namely to assist TFP producers in finding markets for their products.

The study by Chrysochou *et al.* (2012) therefore addressed the following strategic question: do DOLs contribute in customer 're-attraction' (i.e. repetitive purchases) of the brands of TFPs that carry such labels? In this frame, customer re-attraction was used as an indicator of behavioural loyalty of consumers, meaning that consumers who buy DLO-certified TFPs more frequently that other TFP types should be doing so because they are loyal to the DLO certification. Answering the above question is relevant both from a managerial and a public policy perspective. Managers and producers of TFPs, as well as those who operate in fragmented markets in general, eager to invest in quality assurance labelling schemes need to know to which extent such schemes act as additional drivers of loyalty. From a public policy perspective, there is a need to know whether consumers respond to policy interventions of this type and, consequently, the extent to which such interventions affect their overall buying behaviour.

To answer the above strategic question, the study utilised data derived from a panel of 789 consumers who were loyalty card holders of a single retail chain in France. Purchases of 10 brands of the TFP dry-cured ham category available on the self-service shelves of

(Continued)

Case Study 3.5 (Continued)

the supermarkets of the specific chain were recorded for a period of one year. The total number of dry-cured ham units bought within the study period was 4674, with an average purchase rate of 6.0 units and an average annual transaction of €21.4 per shopper. Each brand was categorised according to three different attributes: brand type ('Private label'; 'Commercial'; 'No frills'); existence of a quality assurance label ('DOL'; 'No DOL'); and price tier ('High'; 'Medium'; 'Low'). 'Commercial' were private firm brands and 'Private label' were brands owned by the distributor. 'No frills' brands are those positioned as 'best price' offers by the retailer, and were therefore considered as a separate level of brand type. 'DOLs' were those brands which had a Designation of Origin Label, whereas 'No DOL' did not have any such label. For the specific product category, the DOL label was a PGI. Prior to categorising each brand on price tiers, the price of each pack was standardised based on the number of slices and total weight. Based on the standardised price, 'high price' brands were those with a price above €3.28, 'medium price' brands were those priced between €2.48 and €3.28, and 'low price' brands were those priced below €2.48. The price tiers were created after consultation with a product manager from the retail chain from which the data were derived (Table 3.9).

Specific brand performance measures (i.e. market share, purchase rate and penetration) were estimated, both at the attribute level (i.e. brand type, existence of a DOL and price) and the brand level (i.e. the specific 10 dry-cured brands used). Moreover, the polarisation idex φ (phi) was used as a measure of loyalty. Polarisation captures changes in the degree of heterogeneity of consumer choice as the probabilities of purchasing various brands also change. Polarisation has many advantages against other brand performance measures. Given that 'bigger' brands (i.e. brands of high market share) have more buyers who exhibit higher repeat purchase rates than for smaller brands (i.e. buy big brands more frequently than small brands), standard brand performance measures such as penetration and purchase frequency incorporate this bias. On the contrary, polarisation is unrelated to the effect of the brand size. Another advantage of polarisation is that it is easy to interpret, given that its value ranges from zero to one. Values of polarisation close to zero signify pure 'homogeneity' in consumer choice, and therefore indicate high probability of switching levels among competing brands within a product category. Values of polarisation close to one signify the existence of maximum 'heterogeneity', and therefore indicate high levels of loyalty within a product category. Polarisation has traditionally been utilised at the brand level. However, recent applications extend its use to account for loyalty at the product attribute level (Krystallis and Chrysochou 2010, 2011).

Overall, the category-level estimates (also called 'Dirichlet Multinomail Distribution' or DMD, from the name of the type of distribution of purchase incidences assumed for this type of data) offer a baseline that, in comparison to the brand attribute-level estimated (called 'Beta Bionomial Distriution' or BBD, a simpified type of DMD), can indicate different types of purchase behaviour for different brands within a category as a source of loyalty. For instance, the term 'reinforcing' brands refers to the purchase behaviour caused by big brands (i.e. those with a big market share), which show typically higher levels of loyalty (i.e. more frequent purchases by their buyers than small market share brand show). On the other hand, when brands exhibit lower levels of loyalty than their market

Table 3.9 Product categorisation and observed performance measures.

	Products	Brand type	Quality assurance label	Price tier	Market share (%)	Penetration (%)	Purchase frequency
1	Ham_2	Private label	No DOL	Medium	22.9	54.8	2.5
2	Ham_3	Private label	DOL	High	18.7	44.5	2.5
3	Ham_4	Commercial	No DOL	High	12.3	31.2	2.4
4	Ham_6	Private label	No DOL	Medium	11.0	28.9	2.3
5	Ham_5	Commercial	No DOL	High	9.5	29.3	2.0
6	Ham_8	Commercial	No DOL	High	7.4	20.4	2.2
7	Ham_7	Private label	No DOL	Medium	7.0	14.4	3.0
8	Ham_1	No frills	No DOL	Low	6.0	16.8	2.1
9	Ham_9	Commercial	DOL	High	3.7	11.7	2.0
10	Ham_10	Private label	No DOL	High	1.5	4.9	1.9

(Continued)

Case Study 3.5 (Continued)

share would justify (i.e. are purchased by many consumers but infrequently), they are characterised as 'change-of-pace', which is the outcome of variety-seeking purchase behaviour. Finally, brands that exhibit excess loyalty and have a small market share are characterised as 'niche', which is a very rare type of positioning for small brands (Fig. 3.10).

Figure 3.11 depicts the attribute-level (i.e. BBD) polarisation estimates, together with the respective market share for each brand of dry-cured ham. The category-level (i.e. DMD)

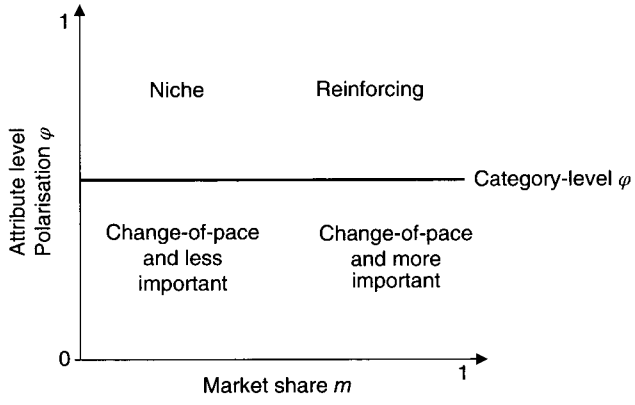


Figure 3.10 Schematic representation of reinforcing, niche and change-of-pace characteristics.

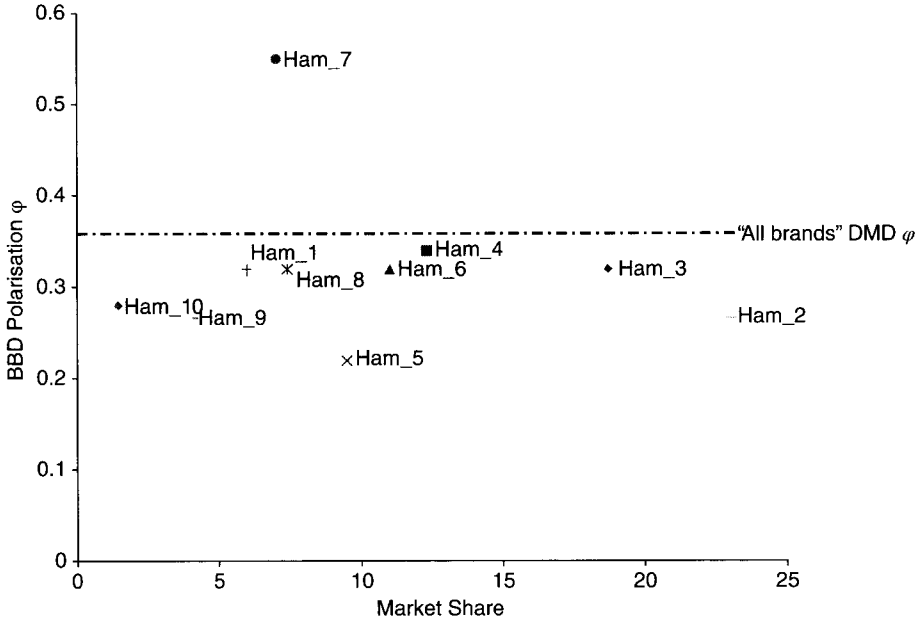


Figure 3.11 Category-level (DMD) and attribute-level (BBD) polarisation φ v. market share for all brands.

Case Study 3.5 (Continued)

Table 3.10 DMD (attribute) and BBD (attribute level) polarisation φ v. market share.

	φ	Market share
All Brands	0.30	
Brand type	0.41	
Private label	0.41	0.61
Commercial	0.43	0.33
No frills	0.32	0.06
Price tier	0.39	
High price	0.37	0.53
Medium price	0.42	0.41
Low price	0.32	0.06
Quality assurance label	0.24	
No DOL	0.23	0.78
DOL	0.31	0.22

polarisation estimate is also presented (φ=0.37), which helps in understanding which brands perform better in terms of loyalty. Ham_7 can be considered as a 'niche' brand given that it had a market share of 7.0% and the BBD polarisation estimate was 0.55. Ham_5 can be considered as a 'change-of-pace' brand given that it had a market share of 9.5% and the BBD polarisation estimate was 0.22. Regarding the rest of the brands, the BBD polarisation estimates were close to the category-level polarisation estimate (i.e. DMD); they can therefore be considered 'normal' in terms of their market share v. loyalty.

Table 3.10 presents the results from the polarisation estimates for each attribute and attribute level. DMD-level estimates explain which attributes were the most important drivers of loyalty. The polarisation estimate for brand type was higher (φ=0.41), followed by price (φ=0.39) and existence of a DOL (φ=0.24).Within brand type, 'Private label' and 'Commercial' had a high market share and a polarisation estimate similar to that of the category, whereas 'No frills' had a very low market share and a polarisation estimate that was lower than that of the category. Within quality assurance label, 'No DOL' had a high market share and the polarisation estimate was close to that of the category, whereas 'DOL' had a small market share and the polarisation estimate was higher than that of the category. Finally, within price tiers, 'High price' and 'Medium price' had high market share and polarisation close to the category, whereas 'Low price' had a small market share and polarisation lower than the category. Figure 3.12 depicts the relationship between market share, BBD and DMD polarisation estimates and allows for the understanding of the position of each attribute level.

The above results suggest that, at the attribute-level, brand type and price tiers showed higher loyalty in comparison to quality assurance labels. This result suggests that, for the specific TFP category, DOLs did not constitute a more important driver of loyalty for

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Case Study 3.5 (Continued)

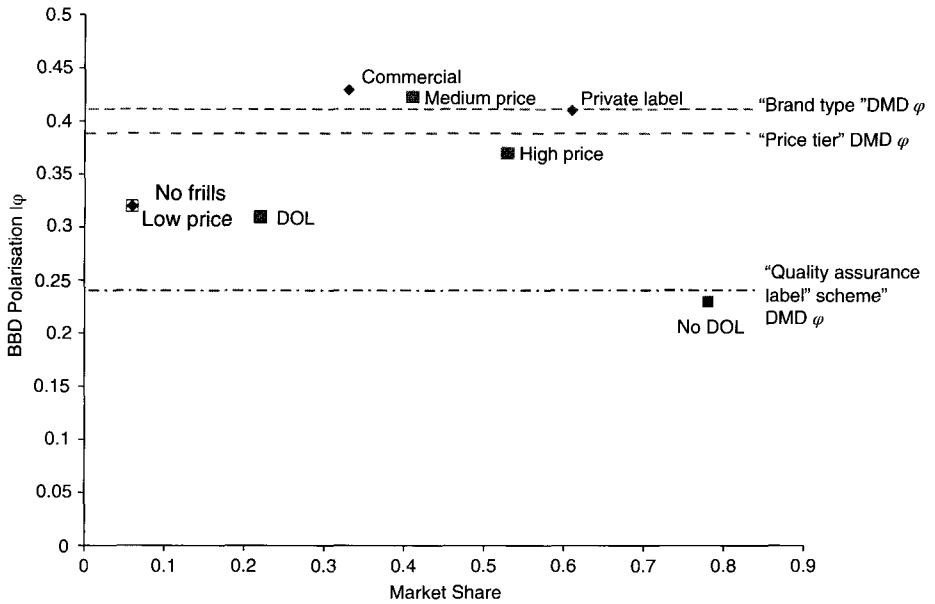


Figure 3.12 DMD and BBD polarisation φ v. market share for all attributes.

consumers in comparison to brand type and price tiers. There are two possible explanations. First, consumers are said to lack awareness and have limited understanding of the specific DOL scheme (see Case Study 3.3), which may have resulted in consumers attributing less importance towards DOLs as a choice-affecting product characteristic. Second, loyalty card holders are said to be inclined towards private label brands; as a result, loyalty towards 'brand type' may have been overestimated.

The polarisation scores obtained for each attribute level in comparison to their respective market shares and the polarisation score of each attribute yields further insights. In relation to brand type, commercial and private label attribute levels have similar loyalty, which was similar to the category and did not lead to safe conclusions as to whether they are 'change-of-pace' or 'reinforcing' attribute levels. However, the 'No frills' attribute level had a low market share with low loyalty, suggesting that promotion branding in TFP categories performs as a 'change-of-pace' characteristic of low importance. In relation to the quality assurance label, brands that did not carry any DOL had a higher market share but less loyalty, suggesting that the 'No DOL' is a 'change-of-pace' attribute level. On the other hand, brands carrying a DOL had a lower market share but greater loyalty, suggesting that DOL forms a 'niche' attribute level.

Discussion. Quality assurance labels can act as reinforcing product attributes, enhancing the overall loyalty exhibited towards a brand. In fragmented consumer markets, such as the market of TFPs, understanding which product attributes and attribute levels drive loyalty and sell the most is essential, since the marketing mix is often based on product attributes other than the brand name. In the case of TFPs, DOLs constitute less important

Case Study 3.5 (Continued)

drivers of loyalty in comparison to other extrinsic product attributes, such as price tiers and brand type. However, in comparison to brands that do not carry any label, brands carrying a DOL show greater levels of loyalty.

Substantial implications result from this study. From a managerial perspective, the study illustrates directions for the implementation of marketing strategies based on product attributes that induce loyalty, such as in the case of TFPs and other fragmented markets. As a result, levels of attributes that show greater loyalty require 'reinforcing' strategies, such as strong branding and heavy advertising. In contrast, levels of attributes showing 'change-of-pace' characteristics require variety-seeking strategies, such as sales promotion and featuring. In the case of TFPs, variety-seeking strategies based on the DOL should be avoided. Such strategies may only skim variety-seeking customers who will switch back again to other brands.

Managers responsible for the development of brand portfolios are always eager in properly selecting levels of attributes. In the case of TFPs, the question remains about whether managers should invest in building a brand that carries a DOL and, if yes, what price level would be more appropriate. However, the selection of attribute levels is limited by definition and the choice should be based on whether an attribute level is able to yield a higher return on investment. Under such circumstances, a general rule could be to invest on attribute levels that are able to generate greater loyalty. The Chrysochou *et al.* (2012) study suggests that the ideal combination of attribute levels that may generate greater loyalty is a commercial brand, carrying a DOL, and sold at an average price level. Overall, the above study supports the fact that DOLs act as reinforcing attributes, contributing towards creating a niche positioning for TFP brands. By definition, TFPs are produced within a specific geographical area, often with limited production capabilities; their market share therefore cannot increase substantially. Appropriate marketing strategies therefore need to be deployed so that products establish a loyal consumer base and achieve better price levels. From a public policy perspective, the introduction of quality assurance labels has been successful in this respect, since it helped producers to establish a niche position in the market of TFPs.

3.4 Conclusion: Towards a Definiton of Authenticity in a Business Context

The concept of authenticity remains elusive and only partially defined, reflecting its multi-dimensional character that calls for an interdisciplinary approach to understand it. 'Authenticity' is defined in the context of business and social sciences from many different points of view that all intend to evoke its various dimensions (Beverland 2005). The typical meaning assigned to the term is a reference to genuineness, honesty or sincerity of an object (e.g. of a product or brand). Authenticity induces perceptions of innocence, originality, naturalness and simplicity (Boyle 2003; Fine 2003). From a consumer perspective, authenticity is a perception and as such subjective. This implies that it is experienced and indeed defined differently by different people, who may use an extensive collection of extrinsic cues to evaluate it depending on their personalities, and

resulting involvement in and knowledge of the issue. Such evaluative cues of authenticity might include indexical cues (i.e. a factual connection between the object and time) or iconic cues (i.e. the extent to which an object or event is a reasonable reconstruction of the past) (Grayson and Martinec 2004). As a result, the authenticity evaluation outcome and the verdict of '(in)authentic' is strongly subjective, confounded within personal as well as social context parameters.

Conformity to traditions, artistry and production excellence, as well as a refutation of the importance of technology and the role of modern industrial production processes, can lend products a scent of authenticity. Consumers perceive authentic products as having a 'clarity of purpose' (Napoli *et al.* 2014), being manufactured by artisans (Beverland 2006), using epoch-withstanding traditions, hand-made production processes and natural ingredients (Caroll and Swaminathan 2000). Typical authentic products often remain loyal to their original design, carefully incorporating novelties as reflection of modern times instead of an abrupt surrender to radically new trends (Beverland *et al.* 2008) that disorientate consumers and create at best scepticism regarding the truthfulness of any claimed authenticity (Kozinets 2002; Brown *et al.* 2003). On the contrary, consumers may reject suspicions, re-conciliate with controversial cues and reconstruct them to recover the sense of authenticity with products that convey a 'touch' of history, have preserved their bridges with traditions, regions and local communities and are surrounded with nostalgia, hence possess a unique and indispensable identity (Brown *et al.* 2003).

Images of nostalgia may not always have a sincere basis (Chhabra *et al.* 2003; Beverland *et al.* 2008). Products that have built strong associations with heritage can eventually identify with cultural values of broader social groups, hence acquire meanings that transcend those originally intended by their 'poets'. Products that are authenticity symbols are legitimate ambassadors of the area and the people who conceived them in the first place. Consumers of these product-symbols will never wonder (or never care to find out) about the absolute truth of the authenticity claims on the grounds of a rock-solid faith and trust developed between themselves and the products (Eggers *et al.* 2012). In this respect, all legendary brands like Kellogg's cereals, Harley Davidson motorcycles, the VW Beatle, Omega watches or Disney's cartoon characters have been over the years incorporated into the wider cultural context of their era, acquiring sales, growth, legitimacy and institution-like status (Holt 2004; Eggers *et al.* 2012).

Accordingly, intellectual owners of such brands are often seen as motivated by a sense of duty to preserve the identity of their creations out of sheer virtue, integrity, professional dignity, commitment to excellence and genuine love of the product (Beverland *et al.* 2006, 2008). The studies of Beverland (2005) and Charters and Spielmann (2014) of fine wines and champagne show that an honest narrative consisting of demonstrable reference to the area, its people, their stories and their values are structural components of the authenticity perception. Thompson *et al.* (2006) emphasised the success of niche coffee brands in building authenticity images by consciously creating mental disassociations with large commercial brands like Starbucks, projecting their overall market concept closer to experiences of tradition, heritage and craftsmanship in coffee making. Irrespective of their size, brands can therefore function as conveyors and multipliers of authenticity. Napoli *et al.* (2014) define 'brand authenticity' as 'a subjective evaluation of genuineness ascribed to a brand by consumers' (Napoli *et al.* 2014, p. 1091). The same authors maintain that a brand authenticity construct must be multifaceted and built around perceptions of heritage, nostalgia, cultural symbolism, craftsmanship, quality commitment and design consistency.

While brands are assumed to hyper-dominate contemporary markets, with buyers striving to make a choice among competing brands that all claim to deliver a 'genuine' value, authenticity can be considered a means to confront dilemmas and surpass the market (Kozinets 2002; Fine 2003). The mere behaviour of consumer in the marketplace (i.e. selecting among 'true' or 'false' cues and endorsing or distant themselves from brands accordingly) can be considered a self-authentication act (Arnould and Price 2000). Napoli *et al.* (2014) claim that there is an emerging stream of social research that 'reconciles' branding and authenticity by acknowledging that consumers actually assign to brands degrees of authenticity that add status, equity and corporate prestige. Moreover, in their very inclusive review, the same authors state various aspects covered by extant literature that support this reconciliation process: consumers' quest for authentic 'experiences' and the search for authentic 'self'; cues and processes used to determine authenticity of various 'objects'; and the various 'forms' that authenticity can take. Ultimately, '... understanding the outcomes of authenticity for a consumer is important because the search for authenticity is part of a consumer's identity project and is thus goal-driven' (Napoli *et al.* 2014, p. 1090).

Special merit with regard to authenticity therefore goes to place branding. Contrary to the 'typical' brand that is the property of one or a group of companies under common ownership, there are cases where a number of independent entities share the intellectual property of a brand. This is often the case with place brands which originate from a specific well-defined territory, and from which they are inseparable and draw a collective yet unique identity (Charters and Spielmann 2014). As extensive parts of this chapter indicate, this character of place brands is particularly relevant to food products that carry geographic indications, such as Designation-of-Origin labels. Charters and Spielmann (2014) characterise this as a 'two-tier' branding, for example a food product brand owned by an individual food company and a geographic indication (e.g. a PDO collectively owned by all related firms, oftentimes family or micro-firms) located in the area and involved in the production of the food product. This two-tier character lies in the heart of place branding due to its 'intimate' relationship with the territory, and creates a multiplication effect in what concerns the benefits stemming from both branding schemes. Champagne (the product) from Champagne (the area), Brie cheese from Brie (area near Pairs) or Chios gum (from the island of Chios) are perfect examples. Ultimately, place brands do not follow a 'market orientation' trend in their conception; instead, they constitute classical examples of 'production orientation', whose core values source from the way they are produced and their interdependence with the territory in which they have been originally conceived and its narratives over the course of time.

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Part C

Geographical, Botanical, and Species Origin, Method of Production and Food Frauds Detection

4

Elemental Fingerprinting

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4.1 Introduction

The international food market has become increasingly complex with the combined demands of regulations and certifications required for each product, target market, and source of production, while consumers demand reassurance regarding the content and origin of their foods, fearing adulteration issues. These problems can occur for a variety of reasons, often linked to financial gain. Increases in profitability may be achieved by adulteration to improve the perceived quality of products, mimic an established brand, reduce production costs, or for product extension purposes. Manufacturers need to be able to confirm the authenticity of components of their products and comply with legislation.

Consumers are increasingly interested in the authenticity of the foodstuffs. Among other information, they want knowledge of the geographical origin of the consumed food products. The reasons for this interest in provenance include: specific cooking and organoleptic qualities associated with regional products; decreased confidence in the quality and safety of products produced outside their local region, country, or the EU or those of unknown provenance; concerns about animal welfare and production methods perceived as "environmentally friendly," more often adopted by smaller regional producers; and finally patriotism. Consequently, products from highly respected provenances could be sold at higher prices than similar products of other or unknown geographical origin (Rossmann and Brereton 2013).

Elemental food content is under thorough discussion as a quality parameter for nutritional and toxicological relevance. Multi-element techniques constitute key analytical tools for addressing a wide range of these questions in food and nutrition research. Elemental mass spectrometry has become the enabling technology for both identifying and quantifying low-level chemical residues and contaminants in one of the most complex biological matrices: food. Elemental analysis has been applied to several authentication issues such as determination of geographical origin, detection of economically motivated malpractices, and differentiation between organic and conventional products.

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This chapter presents the current state of the art and a future outlook of rare earth and trace-element fingerprints as authentication markers. It provides some basic principles, characteristics, perspectives of employed techniques in the field, and also a scientometric approach. Applications to various food types are also surveyed.

4.2 Elemental Techniques

Elemental fingerprinting is one of the most important analytical methods of food authentication. Even though we appreciate techniques such as electrothermal atomic absorption spectrometry (ETAAS) and graphite furnace atomic absorption spectrometry (GFAAS), the major techniques for authenticity assessment are inductively coupled plasma–mass spectrometry (ICP-MS) and inductively coupled plasma–atomic emission spectroscopy (ICP-AES) due to their ability to acquire multi-element measurements. Further, purchase cost for a basic ICP-MS or ICP-AES instrument has fallen tremendously during the last decade, making them affordable to many laboratories that could not consider these techniques in the past. Elemental mass spectrometry techniques in particular show great convenience and suitability for food authentication assessment due to their sensitivity, efficiency, high-throughput capability, accuracy, precision, robustness, and ease of automation.

4.2.1 ICP-MS

4.2.1.1 Operation Principle: Main Features

ICP-MS is the most versatile ultra-trace elemental analysis technique available today, and uses an ICP plasma source to create positively charged elemental ions from each sample. The ions are extracted from the plasma and passed into the mass spectrometer, where they are separated based on their atomic mass-to-charge ratio by a quadrupole or magnetic sector analyzer. The number of ions produced, combined with very low backgrounds, provides the best detection limits available for most elements, normally in the parts per trillion range. It is important to remember that detection limits can be no better than lab cleanliness allows. Quadrupole mass spectrometers are most common in ICP-MS, yet magnetic sector instrumentation fulfils exacting requirements that demand the ultimate detectability and isotope ratio measurement.

There is a number of different ICP-MS designs available today, which share many similar components such as nebulizer, spray chamber, plasma torch, and detector, but can differ quite significantly in the design of the interface, ion focusing system, mass separation device, and vacuum chamber. A schematic diagram of an ICP-MS instrument is shown in Figure 4.1.

ICP plasma source dissociates the sample into its constituent atoms or ions. Plasma is formed by the interaction of an intense magnetic field, produced by a radio frequency (RF) created by a copper coil, on a tangential flow of gas through concentrically structured quartz tube (torch). This has the effect of ionizing the gas and, when seeded with a source of electrons from a high-voltage spark, forms a very high temperature plasma discharge (*c.* 10 000 K) at the open end of the tube. This high temperature ensures almost complete decomposition of the sample into its constituent atoms. The ionization conditions within the ICP result in the highly efficient ionization of most

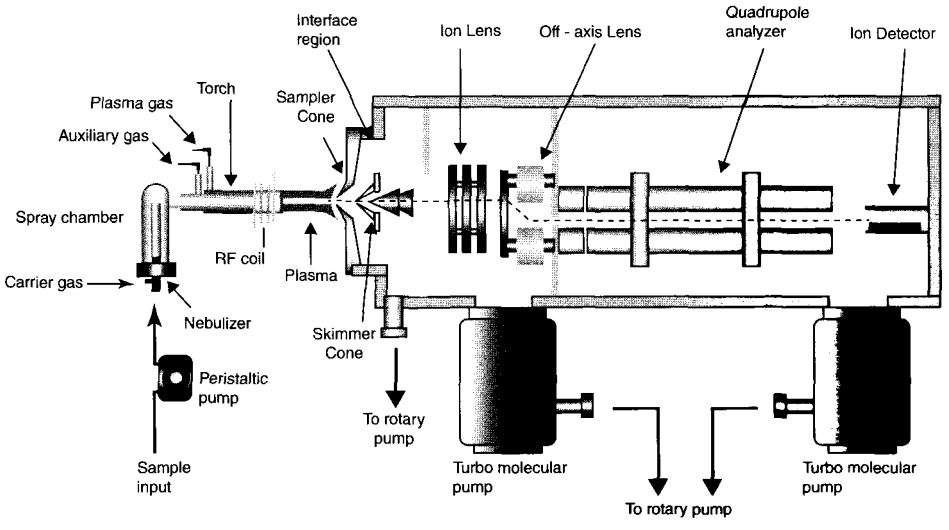


Figure 4.1 The basic components of an ICP-MS system.

elements in the Periodic Table and, importantly, these ions are almost exclusively singly charged.

The plasma torch is positioned horizontally and is used to generate positively charged ions and not photons. In fact, every attempt is made to stop the photons from reaching the detector because they have the potential to increase signal noise. The high number of ions produced, combined with very low backgrounds, provides the best detection limits available for most elements, normally in the parts per trillion (ppt) range. As we note in Figure 4.2, more than 85% of the elements that can be determined by a contemporary commercial ICP-MS have detection limits less than 1 ppt. After ion generation in the ICP ion source, the positively charged ions are extracted from the argon plasma via the differentially pumped interface, between the sampler and skimmer cones, into the high vacuum of the mass analyzers.

After the ions have been successfully extracted, they are directed into the main vacuum chamber by a series of electrostatic lens called ion optics. The purpose of the ion optics is to guide analyte ions to the ion separation devices where mass analysis takes place. The ion beam containing all the analytes and matrix ions exits the ion optics and passes into the heart of the mass spectrometer, where the ions are separated based on their atomic mass-to-charge ratio (Cubadda 2004; Thomas 2004; Nelms 2005; Becker 2008; www.perkinelmer.com).

4.2.1.2 Mass Analyzers

As an essential part of a mass spectrometer, the ion separation system has the task of separating ions with respect to their different mass-to-charge m/z ratios. The separated ion beams are then supplied to the ion detection system for spatial or time-resolved ion detection and registration. The mass spectrum is then the 2D representation of ion intensity as a function of the m/z ratio. Functionally, all mass analyzers perform two basic tasks: (1) they separate ions according to their mass-to-charge ratio; and (2) they measure the relative abundance of ions at each mass. These processes are achieved in a

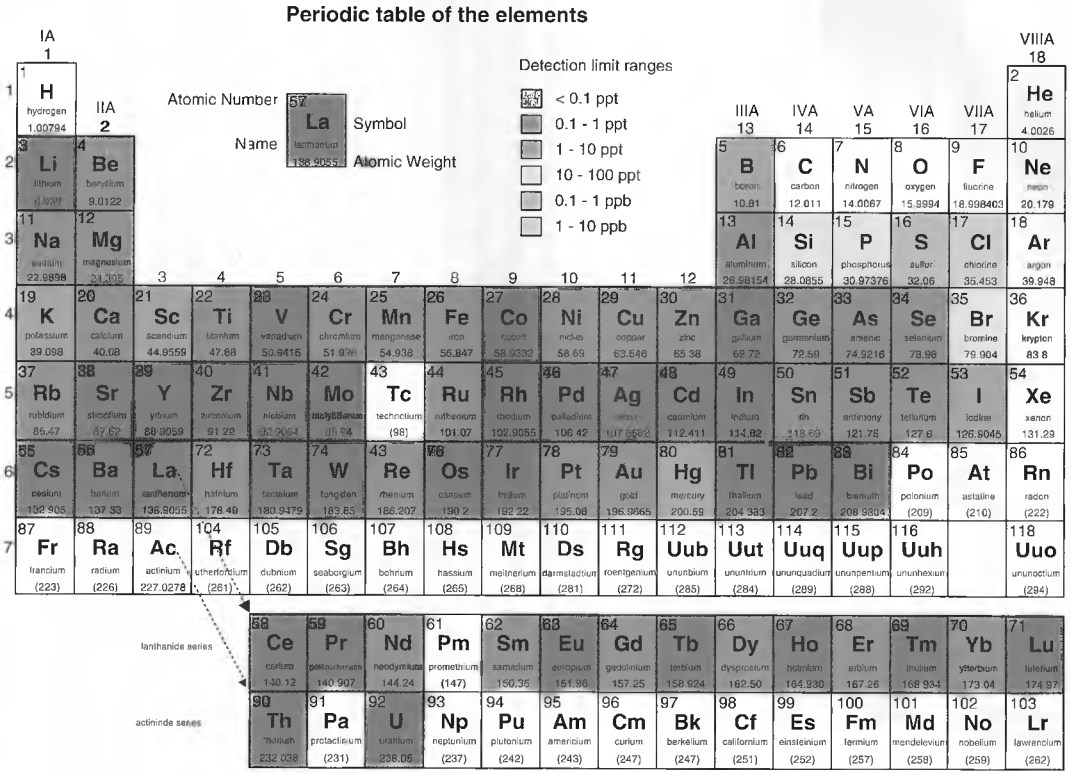


Figure 4.2 Detection limits of a contemporary ICP-MS.

number of different ways depending on the mass separation device, but they all have one common goal and that is to separate the ions of interest from all other nonanalyte, matrix, solvent, and argon-based ions (Becker 2008; Hill 2008).

There are many different mass separation devices. Commercially available instrumentation includes quadrupole-based, sector-based, and time-of-flight- (TOF-) based instruments. Depending on the design of the mass spectrometer, this is either a scanning process, where the ions arrive at the detector in a sequentially manner, or a simultaneous process, where the ions are detected at the same time (Nelms 2005).

Quadrupoles are the cheapest choice of the above-mentioned mass spectrometry devices. They are versatile and robust machines, and account for the majority of the ICP-MS instrument sales. Simple quadrupoles employ a mass filter and have a resolution of <1 amu (atomic mass unit). They are typically referred to as low-resolution instruments. Quadrupoles of commercial ICP-MS instruments can be operated in different modes. The entire mass range or selected ranges may be scanned in the scanning mode, or a selected number of masses can be measured in the peak hopping mode. Rapid semiquantitative scans on the entire mass range are typically performed in a few minutes (Picó 2007; Ammann 2007).

Due to limited resolution, spectroscopic interferences are a major problem in quadrupole ICP-MS. Among them, polyatomic interferences, caused by two or more atoms forming an ion detected at the same m/z as the analyte of interest, are particularly deleterious. To address this issue, collision and reaction cell technology has been developed. Multipole collision/reaction cells were originally designed for the study of organic molecules using tandem mass spectrometry. A collision cell facilitates reaction of polyatomic ions formed between the analyte, argon, and oxygen atom(s) with a gas such as high-purity CH_4, N_2, etc. releasing single analyte ions. The cells are used to promote reactive and non-reactive collisions, with resultant benefits in interference reduction (Ammann 2007).

Although quadrupole mass analyzers represent >85% of all ICP-MS systems installed worldwide, limitations in their resolving power has led to the development of high-resolution spectrometers based on the double-focusing magnetic sector design. Sector field (SF) are more expensive and require more expertise on the part of the operator. As previously mentioned, a quadrupole-based ICP-MS system typically offers a resolution of 0.7–1.0 amu. This is quite adequate for most routine applications, but has proved to be inadequate for many elements that are prone to argon-, solvent-, and/or sample-based spectral interferences. These ICP-MS instruments offer resolving power of up 10 000, compared to a quadrupole which is on the order of around 300. This dramatic improvement in resolving power allowed elements prone to isobaric interferences such as Fe, K, As, V, and Cr to be determined with relative ease, even in complex sample matrices such as food (Thomas 2004; Picó 2007).

The recent growth in ICP-TOF-MS has come about because of its unique ability to sample all ions generated in the plasma at exactly the same time, which is suited for multi-element determinations of rapid transient signals, high-precision ratio analysis, and rapid data acquisition. The fact that all masses in the spectrum can be monitored simultaneously is the main attraction. Even for simple multi-element analysis, scanning analyzers suffer from a decrease in sensitivity as the number of ions to be monitored is increased. The fact that over 30 000 simultaneous mass spectra per second can be obtained by the TOF analyzer has many advantages for monitoring transient signals,

such as those produced by laser ablation and chromatographic, capillary electrophoresis, and electrothermal vaporization sample introduction techniques. In addition, improved isotope ratio measurements have been reported because of the elimination of noise due to temporal fluctuations in the plasma and sample introduction systems.

However, the resolving power of commercial ICP-TOF-MS systems is typically in the order of 500–2000. In comparison, commercial high-resolution systems based on the double-focusing magnetic sector design offer resolving power up to 10 000, while commercial quadrupoles achieve 300–400. Also, as only <20% of ions are accelerated to the TOF tube, the detection limits are about one order of magnitude worse than those obtained by quadrupole-based instruments. As a result, TOF instruments are likely to find application in specific areas but are not expected to reach the popularity of quadrupole ICP-MS in the near future (Thomas 2004; Picó 2007; Becker 2008; Hill 2008).

Despite their high mass resolution, ion trap (IT) mass analyzers have not been widely applied in inorganic mass spectrometry and no commercial ICP-IT-MS is available on the analytical market. One reason could be the occurrence of space charge effects of the most abundant argon plasma ions in the ion trap analyzer, which results in in-trap collisions and scattering of the analyte ions of interest. As a result, a decrease in the sensitivity of analyte ions has been observed in comparison to ICP-MS with double-focusing sector field geometry. Ion traps have been applied in inorganic mass spectrometry for fundamental studies of ion – molecule reactions, especially for reducing disturbing isobaric interferences (e.g., of $^{90}Sr^+$, $^{90}Y^+$, and $^{90}Zr^+$ for $^{90}Sr^+$ determination for isotope ratio measurements) by collision-induced dissociation and chemical reaction of ions with collision gas atoms or molecules.

The combination of the ICP-MS and ion trap techniques has remarkable potential for elemental and isotopic analysis. The ICP source offers sample tolerance and higher, more uniform ion yields than any other inorganic ion source. The ion trap technique, equally applicable to the lower mass range relevant to elemental analysis, offers enhanced atomic detection capability (necessary for certain applications) and reduction of matrix and plasma related polyatomic interferences (Barinaga *et al.* 1994; Picó 2007; Hill 2008).

4.2.1.3 Interferences

Some common interferences and the responsible ions are shown in Table 4.1. Interferences in ICP-MS are generally classified into two major groups: spectroscopic (spectral) and non-spectroscopic (matrix- and physical-based) interferences. Each of them has the potential to be problematic in its own right, but modern instrumentation and good software combined with optimized analytical methodologies have minimized their negative impact on elemental determinations by ICP-MS. Although interferences are reasonably well understood in ICP-MS, it can often be difficult and time-consuming to compensate for them, particularly in complex sample matrices such as foods. Having prior knowledge of the interferences associated with a particular set of samples will often dictate the sample preparation steps and the instrumental methodology used to analyze them.

In multi-element analysis of food, it takes considerable effort to develop methods which account for all the possible matrices and any associated interference. The great variability of analytes and the constituent levels among different food commodities often cause a potential interference to be negligible in one matrix and not in another.

Table 4.1 Common ICP-MS interferences.

Interferences	Interfered analyte
$^{12}C^{15}N^+$, $^1H^{12}C^{14}N^+$, $^{13}C^{14}N^+$, $^{14}N_2$	^{27}Al
$^{38}Ar^1H^+$	^{39}K
$^{40}Ar^+$	^{40}Ca
$^{35}Cl^{16}O^+$, $^{34}S^{16}O^1H^+$, $^{35}Cl^{16}O^+$, $^{38}Ar^{13}C^+$, $^{36}Ar^{15}N^+$, $^{36}Ar^{14}N^1H^+$, $^{37}Cl^{14}N^+$, $^{36}S^{15}N^+$, $^{33}S^{18}O^+$, $^{34}S^{17}O^+$	^{51}V
$^{35}Cl^{16}O^1H^+$, $^{40}Ar^{12}C^+$, $^{36}Ar^{16}O^+$, $^{37}Cl^{15}N^+$, $^{34}S^{18}O^+$, $^{36}S^{16}O^+$, $^{38}Ar^{14}N^+$, $^{36}Ar^{15}N^1H^+$, $^{35}Cl^{17}O^+$	^{52}Cr
$^{38}Ar^{16}O^1H^+$, $^{40}Ar^{14}N^1H^+$, $^{39}K^{16}O^+$, $^{37}Cl^{18}O^+$, $^{40}Ar^{15}N^+$, $^{38}Ar^{17}O^+$, $^{36}Ar^{18}O^1H^+$, $^{37}Cl^{17}O^1H^+$, $^{23}Na^{32}S^+$, $^{36}Ar^{19}F^+$	^{55}Mn
$^{40}Ar^{16}O^+$, $^{40}Ca^{16}O^+$, $^{40}Ar^{15}N^1H^+$, $^{38}Ar^{18}O^+$, $^{38}Ar^{17}O^1H^+$, $^{37}Cl^{18}O^1H^+$	^{56}Fe
$^{40}Ar^{16}O^1H^+$, $^{40}Ca^{16}O^1H^+$, $^{40}Ar^{17}O^+$, $^{38}Ar^{18}O^1H^+$, $^{38}Ar^{19}F^+$	^{57}Fe
$^{40}Ar^{35}Cl^+$, $^{59}Co^{16}O^+$, $^{36}Ar^{38}Ar^1H^+$, $^{38}Ar^{37}Cl^+$, $^{36}Ar^{39}K$, $^{43}Ca^{16}O_2$, $^{23}Na^{12}C^{40}Ar$, $^{12}C^{31}p^{16}O_2{}^+$	^{75}As
$^{40}Ar_2{}^+$, $^{32}S^{16}O_3{}^+$	^{80}Se

A number of analytical solutions have been proposed to overcome spectral interferences caused by matrix-induced polyatomic species in food analysis. For polyisotopic elements the most obvious is the selection of an alternative isotope, provided it has a sufficient abundance for analyte detection with acceptable sensitivity. Suitable sample preparation protocols have been developed by several authors to cope with interferences affecting one or few "difficult" elements, such as K, Ca, V, Cr, Fe, As, and Se. Furthermore, sample digestion procedures which minimize C and Cl content of digestates have been described. The best and probably most efficient way to remove spectral overlaps is to resolve them away using a high-resolution mass spectrometer such as a double focusing magnetic sector mass analyzer. Use of mixed-gas plasma has also been used to reduce interferences of food matrices. Generally, the addition of N_2 to both the outer and the carrier Ar gas flow appears to be the most successful in the reduction of oxides and other polyatomic species such as ArO, ClO, and ArCl. In order to achieve signal deconvolution, correction equations are also used. Finally, another efficient way to cope with the problem of spectral interferences is through collision cell and dynamic reaction cell (DRC) systems, in which the interaction of the ion beam with a gas, or a mixture of gases, enables the chemical removal of polyatomic interferents through collisional dissociation and selective ion–molecule reactions (Cubadda 2004; Thomas 2004; Nelms 2005).

Although food scientists could probably consider data on interferences as something of esoteric value for just the analysts, the truth is that in order to draw conclusions from elemental analyses they should be completely aware of all limitations that could render analyses invalid, leading to wrong conclusions. A straightforward example is the determination of cadmium in food samples. There are eight stable isotopes of Cd which all experience multiple spectral interferences from Zn, Sr, Y, Zr, Mo, As, Se, and

Sn when these elements appear at high concentration. Moreover, all isotopes except for ^{106}Cd suffer spectral interferences from Mo isotopes. This makes a resolution of approximately 30 000–600 000 essential to overcome Mo spectral interferences, which cannot be accomplished with commercial ICP-MS. It is important to be aware of this interference as high concentrations of Mo are expected in cereals, potatoes, beans, nuts, seed, vegetables, and algae. To combat these problems, solid phase extraction combined with isotope-dilution has been proposed. Further, addition of NH_4OH solution into digested sample solutions followed by SPE was enough to remove interfering elements such as Mo, Zr, and Sn (Zhu and Chiba 2012). Except from Cd, another "problematic" element in food analysis is As in high-chlorine food samples. Direct determination of trace arsenic by ICP-MS is complicated by the presence of $ArCl^+$ interference. Moreover, the high first ionization energy of As also results in low analytical sensitivity. In order to eliminate $ArCl^+$ spectral interference, the use of ion–molecule reactions were successfully applied in a dynamic reaction cell (DRC) with methanol addition (Guo *et al.* 2011).

4.2.1.4 ICP-MS versus Other Techniques

ICP-MS is undoubtedly the fastest-growing trace-element technique today. There is no question that the major reason for its unparalleled growth is its ability to carry out rapid multi-element determinations at ultra-trace level. During the last 10 years, the technique has spread outside research laboratories and is used for control activities and in industry. A number of desirable features have contributed to this success, as listed in Table 4.2. These include high sensitivity, multi-element capability, wide linear dynamic range, high sample throughput, and the ability to discriminate between isotopes. With modern instruments and in the absence of spectroscopic interferences, the detection limits (DLs) of most trace elements are in the low nanogram per liter (ng L^{-1}) range when sample digestates are analyzed. Another advantage is the suitability of ICP-MS as a selective on-line detector in hyphenated methods for the determination of element species (Houk *et al.* 1980; Cubadda 2004; Thomas 2004). For a fair presentation of the technique its limitations, most of which concern cost of the analysis, should also be acknowledged.

The strengths and limitations of ICP-MS include: ultra-low detection limits (<ppt); wide elemental coverage (73 elements); fast analysis times (73 elements in 3 min); high-throughput analyses (160 samples × 73 elements = 11680 analyses in 8 hours); well suited for a high number of samples and/or elements; wide dynamic range (up to nine orders of magnitude); ability to provide isotopic data (e.g. MC-ICP-MS); fast semi-quantitative screening; and application by hybrid techniques (LA-ICP-MS when analyzing solids; GC-ICP-MS for speciation).

Advantages include easily interpreted spectra and rare Earth elements determination.

Limitations to the technique include: large initial capital cost (*c.* €180 000 for a quadrupole-based ICP-MS); large operation cost (*c.* €250/10 hours); some spectral interferences (although well defined); liquids analyzed should contain less than <0.2% dissolved solids; requirement for clean room conditions to achieve ultra-trace analysis; and a need for suprapur acids for wet digestion.

ICP-MS not only offers extremely low detection limits in the sub parts per trillion range, but also enables quantification up to the parts per million level. This unique capability makes the technique very attractive compared to other atomic spectrometric

Table 4.2 ICP-MS comparison with other spectroscopic techniques

Characteristic	Technique			
	AAS	**GF-AAS**	**ICP-AES**	**ICP-MS**
Detection limits	Very good for some elements	Excellent for some elements	Very good for most elements	Excellent for most elements
Sample throughput	10–15 s/element	3–4 min/element	1–60 elements in <3 min	73 elements in <3 min
Dynamic range	10^3	10^2	10^6	10^8
Precision (%RSD)				
Short-term	0.1–1.0	0.5–5	0.1–2	0.5–2%
Long-term	1–2	1–10	1–5	2–4
Interferences				
Spectral	Few	Few	Many	Few
Chemical, matrix	Many	Many	Few	Very few
Physical, matrix	Some	Very few	Few	Very few
Dissolved solids (%)	0.5–5	>20	0–2	0.1–0.4
Sample volume	Large	Very small	Small	Very small
Semiquantitative analysis	No	No	Yes	Yes
Isotopic analysis	No	No	No	Yes
Ease of use	Very easy	Moderately easy	Easy	Moderately easy
Method development	Easy	Difficult	Moderately easy	Difficult
Rare Earth elements analysis	No	No	No	Yes
Capital costs	Low	Low to medium	High	Very high
Running cost:				
One element	Low	Medium	High	High
Multiple elements	Medium	High	Low	Low

techniques including atomic absorption spectrometry (AAS) in its various forms such as electrothermal (ET-AAS), graphite furnace (GF-AAS), hydride generation (HG-AAS), and cold vapor (CV-AAS), and inductively coupled plasma atomic emission spectroscopy (ICP-AES), also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES). ICP-MS has clear advantages due to its multi-element characteristics, speed of analysis, detection limits, isotopic capability, and high versatility (e.g., hybrid techniques, LC-ICP-MS). Compared to ICP-AES, which is also a high-throughput multi-element technique, ICP-MS also provides isotopic information, much lower detection limits, and capability for rare Earth elements.

The main disadvantage of ICP-MS in comparison to AAS techniques is the cost of instrument purchase and the running cost in terms of consumables (i.e., argon and suprapur acids). Furthermore, trained personnel are obligatory to carry out analytical work. When complex analyses are performed, large amounts of data can be generated which require careful processing; such procedures can be very time-consuming. Purpose-written software (e.g., spreadsheet macros) can be very efficient for handling multi-element and multi-isotope data, although visual inspection of analytical results by an experienced specialist is essential. A brief comparison with other techniques is provided in Table 4.2 (Hou and Jones 2000; Thomas 2004; Picó 2007).

4.2.2 ICP-AES and Other Techniques

Although ICP-MS is nowadays the technique of choice for elemental analysis, ICP-AES is a remarkable alternative for high-throughput multi-element analyses mainly due to lower capital cost, facilitating the use for authentication studies (Cugnetto *et al.* 2014; Li *et al.* 2014; Osorio *et al.* 2015). In comparison to AAS techniques, ICP-AES is a fast multi-element technique with an extended dynamic linear range and moderate to low detection limits.

ICP-AES uses the same type of ICP source as is used in ICP-MS. After sample dissociation into the constituent atoms or ions, they are excited to a level where they can emit light of a characteristic wavelength. ICP-AES is characterized by remarkably rich spectra. The possibility of working with axial and radial view provides high sensitivity with low background, avoiding interferences and involving minimal ionization effects. ICP-AES also allows the possibility of monitoring wavelengths down to 165 nm, providing high sensitivity for phosphorous and sulfur. Spectral interferences will occur in this case even when just a small number of elements are present at moderate concentrations (Boss and Fredeen 1999; de la Guardia and Illueca 2013).

Another less used group of analytical methods is the atomic absorption spectrometry methods, from which the most commonly used techniques are GFAAS and FAAS (flame atomic absorption spectrometry). The concentrations of elements are measured with the use of the wavelengths of light specifically absorbed by an element, which corresponds to the energies needed to promote electrons from one energy level to another, higher, energy level. Graphite furnace atomic absorption spectrometry (GFAAS) is so sensitive that it can measure down to parts per billion. Other techniques, such as X-ray fluorescence (XRF), neutron activation analysis (NAA), and proton-induced X-ray emission have only been rarely used for authenticity studies, so are not covered in this chapter (Imai *et al.* 2012; Otaka *et al.* 2014).

4.3 Sample Preparation: Pretreatment

An important step in the analytical process is the sample pretreatment. Its selection depends mainly on the type of sample. Fresh foods have to be prepared using stainless steel knives and clean plastic chopping boards minimizes contamination problems. Domestic blenders, coffee grinders, and food processors fitted with stainless steel, Ti, or ceramic blades can be extremely effective at homogenizing food samples while minimizing contamination. If the suite of elements to be analyzed does not include volatile elements, for example Hg, then samples can be freeze-dried before homogenization. Ensuring that the test material has been fully homogenized is difficult, although performing measurements in duplicate can help with this (Hill 2008).

In view of the high-throughput measurements facilitated by the aforementioned techniques, the bottleneck for high-throughput elemental characterization is sample pretreatment before measurement. ICP-MS and ICP-AES were originally developed for the analysis of liquid samples; they therefore require that samples are analyzed in a liquid or dissolved state. Further, milk, wine, or juices (liquid foods with high organic content) need appropriate treatment in order to reduce matrix interferences. If the sample is not a liquid, some kind of sample preparation has to be applied to get it into solution. Collecting a solid sample, preparing it, and getting it into solution undoubtedly represent the most crucial steps in the overall elemental analytical methodology because of the potential sources of contamination from grinding, sieving, weighing, dissolving, and diluting the sample (de la Guardia and Illueca 2013).

4.3.1 Digestion

For solid foods, total element determination using conventional (e.g., liquid) sample introduction requires a dissolution step for analyte presentation to the instrument in order to achieve a separation of the target elements from the food matrix. The choice of the digestion method is made on the basis of several considerations, including efficiency in sample solubilization, potential for loss of volatile elements and contamination of samples, reagent-related interferences and matrix effects, sample throughput, detection limit requirements, and safety aspects. Microwave (MW) -assisted digestion with HNO_3, generally in combination with H_2O_2, is a widely used method for the dissolution of food samples. In particular, microwave digestion with closed vessel systems has gained popularity, being a simple and fast digestion technique that minimizes acid consumption, the risk of sample contamination, and the loss of volatile elements. HNO_3 is the acid of choice for ICP-MS analysis as it causes minimal interferences. Small amounts of other acids can be added for specific analytical purposes, for example HF for the solubilization of Si-bound elements (Al, Co, Cr, Ni, Th, U, and V) in vegetable matrices containing substantial amounts of silicon. Although this use of HF is not that common, the elemental concentrations determined in this way are not relevant to the bioavailable fraction and so quite useless (in our opinion). As previously stated, the use of HCl, $HClO_4$, and H_2SO_4, is generally excluded to avoid resulting Cl and S interferences or cone blockage by salt formation (H_2SO_4). However, mixed acids can be successfully used for the heavier elements where polyatomic interferences are less problematic. Alternative solutions to digestion with concentrated acids have also been reported for a rapid, multi-element screening of biological and

botanical materials, for example extraction with water-soluble tertiary amine solutions containing Ethylenediaminetetraacetic acid (EDTA) (Thomas 2004; Picó 2007; Nardi *et al.* 2009).

Solid foods in powder form can be analyzed directly by means of LA-ICP-MS or ETV-ICP-MS to eliminate time-consuming sample dissolution procedures. However, this requires the preparation of homogeneous powdered samples; the subsequent analytical determination is not as straightforward as that based on liquid sample introduction and requires matrix-matched solid samples. Another problem is the preparation of suitable calibration standards. These are quite difficult to prepare; one approach favored by the authors is to use samples previously analyzed through standard wet digestion procedures.

Traditional techniques for sample pretreatment are time-consuming and require large amounts of reagents which are expensive, generate hazardous waste, and might contaminate the sample. An approach that circumvents these in liquid foods is direct aspiration into the plasma following acidification or, alternatively, after dilution and addition of suitable reagents. Advances in sample pretreatment over the last few decades have been propelled by the advance of microwave-assisted acid digestion, ultrasound-assisted extraction and slurry preparation, and direct solid sampling analysis (Andrade Korn *et al.* 2008).

The type of acid used in the preparation procedure can have important consequences in the measurement step. The most commonly used reagent in all atomic spectrometric techniques is nitric acid. In spite of occasionally observed signal suppression in its presence, no severe analytical problems are encountered in practice with nitric acid at concentrations up to 10%, sometimes higher, as long as its concentration is similar in calibration and sample solutions. Added in mineralization procedures, hydrogen peroxide and hydrochloric acid are quite well accepted. Because of its high viscosity, utilization of sulfuric acid is usually avoided. Mineralization for ICP-MS increases the cost due to the requirement for suprapur materials; such materials are needed not only in ultra-trace analysis but also to protect the MS system from contamination.

Wet digestion methods are carried out in open vessels, in tubes, on a hot plate, in an aluminum heating block, or in closed vessels at elevated pressure (digestion bombs) with thermal or microwave heating. Microwave-assisted digestion is an attractive method, especially for small samples. Extreme care should be exercised in using sealed pressure vessels since there is much anecdotal evidence of these vessels occasionally rupturing during conventional or microwave-assisted digestion of organic materials. The applicability of this technique is strictly dependent on the type of food: carbohydrates are easily distracted with nitric acid at 180°C, while fats, proteins, and amino acids cause incomplete digestion due to the relatively low oxidation potential of nitric acid at 200°C. These samples require the addition of sulfuric and/or perchloric acid, which introduces further problems related to their use at high temperature and pressure. An alternative procedure is to decompose most of the organic matter using H_2O_2 followed by the addition of nitric and/or other acids. H_2O_2 has the advantage of being eliminated and not increasing the ion content of the sample digestate.

Microwave-assisted digestion procedures based on the use of concentrated acids are an efficient and safe way to decompose many kinds of samples for the determination of the elemental fingerprint. Microwave heating has several advantages over conventional heating on a hot plate, etc., as the energy is generated in the digestion

mixture and not transferred by conduction. Among the key advantages of MW-assisted digestion are the much shorter digestion times and the reduced need for aggressive reagents to obtain complete digestion. There are two different systems available for MW-assisted digestion: pressurized closed-vessel systems; and open focused-MW systems that work under atmospheric pressure. Microwave-assisted digestion in closed vessels under pressure has gained popularity as a simple and fast dissolution technique that minimizes acid consumption, the risk of sample contamination, and the loss of sample and volatile elements. One of limitations is the time required for cooling before the vessels can be opened, which may be hours depending on the type of equipment used. The main advantages of MW radiation are safety, versatility, control of microwave energy released to the sample, and the possibility for the programmed addition of solutions during the digestion. It should be noted that microwave digestion is an inherently batch technique that is not amenable to automation. This latter fact makes the technique of direct solid analysis, described in the following section, more attractive.

4.3.2 Direct Solid Sampling Analysis

Among the techniques that can be used for direct solid samples analysis are laser ablation (LA) and electrothermal vaporization (ETV). Direct solid sampling analysis offers a number of advantages such as: reduced sample preparation time and hence a faster analysis; higher accuracy, as errors due to analyte loss and/or contamination are dramatically reduced; higher sensitivity due to the absence of any dilution; and the absence of any corrosive or toxic waste. Although published literature is currently minimal, LA is described here as it could have the potential to circumvent the sample pretreatment bottleneck, resulting in real high-throughput elemental assessment. It should be stressed again that the requirement of matrix-matched standards hampers the use of LA-ICP-MS for food. However, this mode of ICP-MS could prove to be useful in the future, taking advantage of the ability to provide microscale information that could be extremely interesting for specific foods (Becker 2008; Nardi *et al.* 2009; Pedrero *et al.* 2009). X-ray fluorescence (XRF) is also convenient for minerals determination, but its lack of sensitivity along with the low concentration of most of the elements that are used as markers made the use of XRF non-effective for food provenance studies (de la Guardia and Illueca 2013).

4.3.3 Sampling Problems and Remedies

All ingredient measurements start at the analytical balance to accurately weigh 0.1 mg of the food sample. It is of special interest to analyze the bioavailable part of a food commodity.

4.3.3.1 Wines and Beverages

Many wines and most other beverages only need to be shaken well before subsamples are taken. Sparkling wines, beers, etc. should be de-gassed in an ultrasonic bath before digestion and those wines which form a precipitate, for example crusted port and some red wines, have to be decanted into another container before subsampling. If Pb is to be analyzed in wines, then care may be needed to ensure that no contamination arises where Pb foil caps have been used as these can leave deposits on the lip of the bottle.

Dilution is usually applied to reduce the ethanol content of wine before analysis. Internal standard addition, isotope dilution analysis, matrix-matching, or ethanol removal by heating have also been reported as viable solutions to deal with matrix effects in the analysis of alcoholic beverages. However, depending on the nebulizer used, not only ethanol but also other organic and inorganic components can produce significant matrix effects that need to be taken care of, especially when red wines are analyzed.

4.3.3.2 Food Waste Water

Although water is assumed to be an "easy" matrix regarding sample pretreatment its analysis can be challenging, especially for samples with a high mineral or bicarbonate content. In such cases, as well as with food waste waters, the addition of an appropriate internal standard is recommended.

4.3.3.3 Vegetables and Mushrooms

Analyses in support of dietary intake studies may require that root vegetables that are customarily eaten whole should not be peeled but are scrubbed before pretreatment and outer leaves which are not normally consumed should be discarded from leafy vegetables. However, peel and outer leaves may have to be analyzed in plant uptake studies, and removal of surface contamination should be undertaken using cleaning methods which approximate treatment prior to consumption as closely as possible. Dicing the prepared vegetables, either using a stainless steel knife or an automated dicer/chopper, can be helpful but further blending may be needed if smaller sample sizes are taken for analysis. Mushrooms should be brushed clean before analysis, bearing in mind that local custom can dictate whether or not the outer skin is removed prior to consumption and that fungi can absorb significant amounts of water during washing.

4.3.3.4 Fruits

The outer skin or peel of fruit can be a useful indicator of surface contamination and may have to be analyzed separately from the remaining edible portions of hard fruits such as apples and pears. Freezing the pulp, allowing it to partially thaw and mixing the iced slurry well can also help produce a representative sample of flesh and juice.

Fruit juices which have a complex organic matrix with a significant carbohydrate content can be analyzed either following dilution, with subsequent centrifugation/filtration, or after full sample digestion.

4.3.3.5 Cereals

Edible grains need to be separated from the chaff and analyses performed either on a subsample from a well-mixed bulk of individual grains or on a rough flour produced using a coffee grinder or equivalent.

4.3.3.6 Fats and Oils

Oils need to be shaken well before subsampling but fats can probably be dealt with most effectively by melting and subsampling from the stirred molten bulk (although some fats may separate on melting, which can cause difficulties). Most fats will have been effectively mixed during preparation and a representative subsample taken through the bulk will probably be adequate.

4.3.3.7 Meat

Inedible material, for example skin, hair, etc., should be removed and discarded before the edible flesh is sampled. Meat samples can be homogenized using a stainless steel rotating knife homogenizer blender. It is imperative that the blender is cleaned well between samples using dilute detergent followed by tap water and then Milli-Q water.

4.3.3.8 Seafood

The fish should be gutted, washed with pure water and portions of edible flesh taken for analysis, avoiding the head, fins, and larger bones. Whether to include the skin or not may depend on the type of fish and local dietary customs. Fish species that are normally eaten whole should be blended without deboning. Removal of edible flesh from shellfish can be awkward and time-consuming but, wherever possible, fresh samples which have been washed free of sand should be analyzed. If practicable, viscera should be removed. It should be noted that freezing some crustaceans can cause undesirable liquefaction of the flesh on thawing. This can cause problems, for example if brown and white crab meat needs to be analyzed separately. In all cases a food blender can be used to produce a satisfactory homogenate.

4.3.3.9 Dairy Products

Dairy products should be transferred to the laboratory in an ice box, properly labeled, and stored in the refrigerator. It is preferable for sample preparation to be done before the expiry date. As for other liquids, milk should be mixed by vigorous shaking. Fresh milk dilution in alkaline media and multi-element quantification with the standard addition method has been successfully applied without any digestion, even though in most studies milk and infant formulae are analyzed following full digestion. To improve the limits of detection (LODs) milk can be freeze dried; this can also help with longer-term storage.

Regarding cheese and yogurt samples, cheeses are cut into small cubes, mixed in plastic bags and homogenized in a homogenizer. All containers and equipment have to be made from either plastic or stainless steel. Containers and other equipment are usually washed with a solution of 2% sodium EDTA and 2% sodium citrate in order to remove ions. Finally, all surfaces should be washed with Milli-Q water before contact with samples.

4.3.3.10 Honey

Microwave-assisted acid digestion has been used as the method of choice. Methods were optimized in order to minimize acidity and residual carbon content of the resulting final aqueous solution (Hill 2008; Reykdal *et al.* 2011; Khan *et al.* 2014; Leme *et al.* 2014).

4.4 Applications

4.4.1 Elemental Fingerprinting Rational and Trends

The fingerprint of organic compounds could be characteristic of the geographic origin. However, the profile of organic compounds varies according to various agricultural and livestock parameters such as fertilization, climatic conditions, history of fields, and the

variety or species, as well as geographical location and soil characteristics, so it is sometimes difficult to be definitive about the origin of a foodstuff. Elemental fingerprinting, and especially rare Earth profiles, has proved an excellent alternative solution in these types of problems (de la Guardia and Illueca 2013).

Elemental fingerprinting could contain not only macro-elements (such as sodium, calcium, and potassium) and trace elements (such as copper, zinc, and selenium), but also rare Earth elements (such as lanthanum, cerium, and samarium) or other elements occurring only at very low abundance (such as holmium, iridium, and gold). The elemental content of rare Earth elements is indicative of certain geographical areas (Drivelos *et al.* 2014*b*). Generally, the high content of sodium or chlorine (always related to the data of the same product from different areas), may indicate marine origin; a high content of potassium may indicate a provenance from granitic or gneiss underlying rocks; and high calcium content may indicate a provenance from sedimentary or basaltic rocks. In the case of other rare elements (nickel, vanadium, or chromium), a typical signature might be a result of technological processes (Rossmann and Brereton 2013).

Plant food products derive their mineral content from the soil in which they have grown. There is no direct link with total soil mineral content as a descriptor for that detected in the plant; a better predictor is the bioavailable fraction of soil minerals. Environmental growing conditions also play a role. Trace-element availability depends on several factors such as soil pH, humidity, porosity, clay, humic complexes, etc. One question raised here is how we assess the bioavailable fraction of minerals from the soil. There is no universal solution providing a clear view for all elements; further investigation is required. The controversy is highlighted when considering the large range of media used for extracting the bioavailable fraction of minerals. Some of the media used to date are acetic acid, EDTA, ammonium acetate, calcium chloride, sodium nitrate, and ammonium nitrate. For some of these media relevant standard reference soil materials have been developed (e.g. BCR-483, BCR-484, BCR-700). The soil in a specific growing region along with the bioavailability of specific elements could prove unique as geographical origin markers for plant-derived food. Alkaline Earth metals, especially rubidium and cesium being easily mobilized in the soil and easily transported into plants, are good indicators of geographical identity. Although variations in fertilization, harvesting, botanical origin, soil type, and production year cause concentration variations in several elements, these variations are smaller than these observed between production areas when appropriate elements are selected. Rare earth elements have great potential for geographical origin determination as their fingerprint is directly linked to the geology of the area and could be minimally influenced by different agricultural practices and harvest year.

The multi-element composition of animal tissues reflects, to some extent, that of the vegetation they eat. The vegetation is the compositional reflection of the bioavailable and mobilized nutrients present in the underlying soils from which they were cultivated. Chemometric techniques are used for statistical analyses of the obtained ICP-MS data (Kelly *et al.* 2005; Laursen *et al.* 2011; Drivelos and Georgiou 2012).

The term rare earth elements (REE) comprises the elements scandium (21), yttrium (39), lanthanum (57), and the 14 chemical elements following lanthanum (58–71) called lanthanoids. Favoring the tripositive oxidation state, REEs present a high affinity for ionic bonding; a large number of both organic and inorganic rare earth salts may therefore be formed. Nevertheless, REEs may also form complexes, especially with chelating

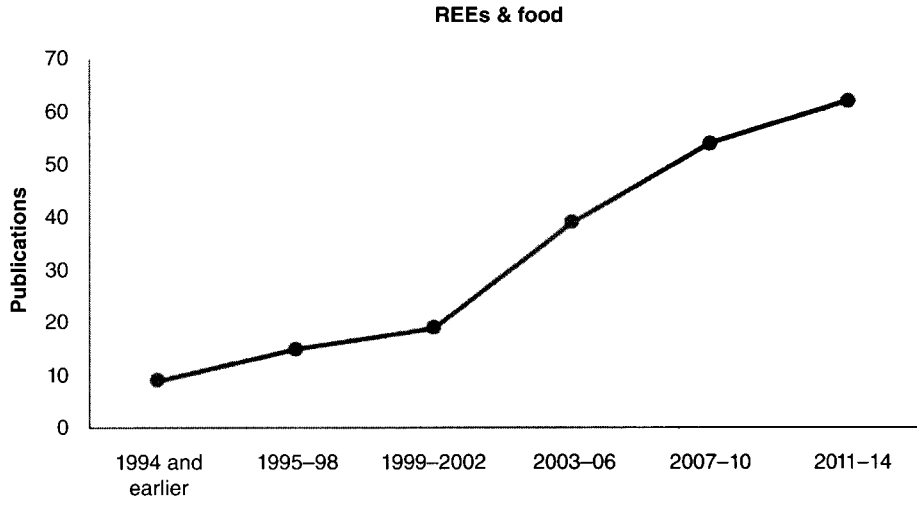


Figure 4.3 Temporal evolution REE research.

ligands. In nature, REEs occur in multiple minerals, such as bastnaesite and monazite which are mainly used for industrial production.

Although largely ignored before the 1990s, REEs are currently under the spotlight in food science and technology (Fig. 4.3). We feel that this growing trend will continue and expand in the near future, a catalyst of which could be the anticipated revelation of the involvement of REEs in human health. REEs could be the replacement/substitution for trace elements in important biochemical paths and systems, such as in enzymes. REEs have recently proved to be robust food authentication markers (Drivelos *et al.* 2014*a*). This relies on the fact that REE profiles are directly linked to the geology of the area and could be slightly influenced by different harvesting years and agricultural practices. REEs profile therefore represent the geographical characteristics of the production or cultivation area.

As shown in Figure 4.4, 10% of publications on elemental food analysis are for food authentication purposes. This is the most active field beyond toxic and nutrient elements assessment, while other fields such as food nanomaterials and migration studies are not that active. It should be noted (data assessed from Scopus 6-2015) that the volume of publications on food authentication utilizing elemental fingerprinting almost tripled between 2011 and 2014.

4.4.2 Elemental Fingerprinting Authenticates Various Food Products

The first step of a food authentication study is problem definition. A clear identification of the potential food fraud is indispensable. After that, an appropriate selection of samples (sampling plan) is needed along with a suitable analytical procedure. Analytical procedure concerns not only the selection of the analytical technique, but also the sample pretreatment level. The following steps include the analysis with a further statistical analysis to interpret the result. After these steps we should be able to answer in question of whether a unknown sample is authentic or non-authentic.

ICP-MS and ICP-AES can screen the authenticity of food products by the analysis of numerous inorganic elements and fingerprints of the element pattern. They have been

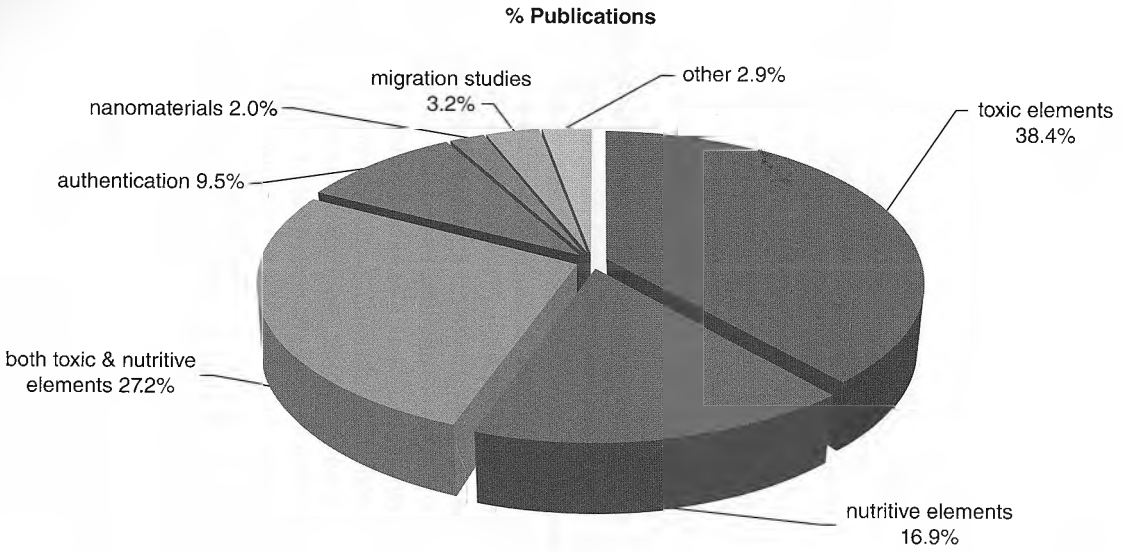


Figure 4.4 Percentage elemental analyses in different applications.

proven reliable techniques for provenance determination in all types of foods. Selected examples are described in the following sections.

4.4.2.1 Wines

The mineral profile is mainly used for geographical origin discrimination, as a result of the uniqueness of the cultivation area where the wine is produced. The determination of the authenticity of wine from its trace-element composition has allowed the identification of the region of origin of Spanish wines from three different regions. It was also possible to completely differentiate English and Spanish white wines (Baxter *et al.* 1997). The elemental profile of Okanagan Valley wines in Canada correlated strongly with vineyard of origin (Greenough *et al.* 1997). Isotope ratios and concentrations for lead measured by ICP-TOF-MS differentiated 20 wines from the five continents (Tian *et al.* 2000). $^{87}Sr/^{86}Sr$ ratio is also another good choice for regional origin determination of wines by SF-ICP-MS (Almeida and Vasconcelos 2001). Recently, intraregional classification of wine was achieved using the elemental profile with B, Ba, Cs, Cu, Mg, Rb, Sr, Tl, and Zn as suitable indicators. Results for the Stellenbosch wine district in the Western Cape Wine Region, South Africa, comprising an area of less than 1000 km^2, suggest that the classification of wines from different estates (120 wines from 23 estates) is indeed possible (Coetzee *et al.* 2014).

It is most obvious that the main application of elemental fingerprinting in wine is for authentication of geographic origin. The main factors influencing the mineral content, namely botanical origin, crop, climate, soil, and processing, result in a unique profile.

4.4.2.2 Beverages

There are several important food commodities included in the "beverage" group. Elemental concentrations and lead isotope ratio measured by SF-ICP-MS distinguish the origin of alcoholic beverages (Coetzee *et al.* 2014). Yerba mate (Ilex paraguariensis) is a widely consumed beverage in South America that is nowadays available worldwide. Classification of the country of origin is based on element concentrations using various chemometric tools (Marcelo *et al.* 2014). Elemental profile and $^{87}Sr/^{86}Sr$ ratios can reveal the geographical origin of ciders (García-Ruiz *et al.* 2007).

4.4.2.3 Fruits and Vegetables

Fruits and vegetables are a basic part of the daily diet worldwide and are crucial in providing a healthy lifestyle in developed countries, meaning that their authentication is of utmost importance. In this respect, growing high-quality fruits and vegetables is quite profitable, possibly making it a major research area in food authenticity. In terms of trace-element methods, studies began in the 1970s. Most of the first studies were based on AAS, including a very limited number of elements due to the multi-elemental limitations of this technique. The primary focus was therefore on essential plant nutrients and heavy metals (Cd and Pb) (De Vries *et al.* 1975). None of the AAS-based studies focused specifically on developing methods for authentication based on elemental analysis, but used AAS analysis to evaluate the impact of, for example, agricultural practice and fertilizer type on plant quality. Conclusions from these studies were that that the impact of geographical region, plant species, cultivar, and crop-rotation period is greater than agricultural practice. A few AAS-based studies focused on Cd and Pb in plants, showing a higher content of Cd in conventional plants. There are several reasons

for a potentially higher heavy metal content in conventionally produced plants, for example, fertilizer use, metal-based pesticides, contaminated water, industrial emission, transportation, harvesting processes, storage and/or sale. These factors, unrelated to agricultural practice, therefore also affect the content of heavy metals in plants. ICP-MS and ICP-AES methods were later adopted in several studies, although they were focused on a larger number of elements.

Ariyama *et al.* (2004) reported the elemental-profile-assisted distinction of Japanese and Chinese welsh onions from different regions. Differentiation according to their geographical origin was achieved for tomatoes and triple-concentrated tomato pastes from different Italian regions and China, Greece, and California. The origin of tomato fruits and the areas of production as either "Italy" and "non-Italy" of the triple-concentrated pastes were evaluated by three supervised pattern recognition procedures: linear discriminant analysis (LDA), soft independent modeling of class analogy (SIMCA), and K-nearest neighbors (KNN) (Lo Feudo *et al.* 2010). Determination of Tropea red onion (Allium cepa L. var. Tropea, PGI) origin by its elemental profile was achieved using LDA, SIMCA, and back-propagation artificial neural network (BP-ANN). Tropea red onion is among the most highly appreciated Italian products (Furia *et al.* 2011). Identification of species of the Euterpe genus by rare Earth elements and linear discriminant analysis was successful for açaí (Euterpe oleracea Mart) and juçara (Euterpe edulis Mart). These fruits are almost identical, rich in energy, minerals, vitamins, and natural compounds with antioxidant and anti-inflammatory properties. REEs content, in particular Sm, Th, La, Pr, Gd, and especially Ce and Nd, were the most important indices (Santos *et al.* 2014).

For the fruit commodity, research has been focused on geographical origin. For example, samples of a PGI product known as Clementine, a citrus fruit from specific areas of Calabria, a region located in southern Italy, were used to discriminate from similar products of different origin (e.g. Algeria, Tunisia, and Spain) with the use of chemometric models and trace-element analysis (Benabdelkamel *et al.* 2012). Perez *et al.* (2006) used ICP-OES and nitrogen and carbon stable-isotope analysis to determine the origin of fruit samples (e.g., strawberry, blueberry, and pear). Some 13 elements were determined, but only nine (i.e., Ca, Cu, Fe, Mg, Mn, K, Na, P, and Zn) were used for classifying fruits according their geographical origin. However, PCA and CDA approaches did not correctly classify these strawberry, blueberry, and pear samples (Gonzalvez *et al.* 2009). Samples of Navel oranges were gathered from three regions of China in order to discriminate their origin with the use of ICP-MS (Hu *et al.* 2014). Discrimination of the regional origin of orange juices and peel extracts by elemental fingerprinting has been related to differences in soil (Simpkins *et al.* 2000).

4.4.2.4 Oils

Olive oil is a food product of great interest, not only due to its importance in the Mediterranean diet but for its high nutritional value and its beneficial effects on the heart. For most olive oil products (especially PDO products), this has resulted in many European Directives indicating that the origin of olive oils should be clearly stated in the label. This becomes a fundamental task when organic production is considered. The organic production is usually confined within a well-determined area; any fraudulent addition of foodstuffs produced outside the defined area alters the quality and the safety of the product. In addition, a frequent troubling phenomenon is the adulteration of

high-quality oils such as olive oil with cheap oils. In the case of olive oil, which is the most studied edible oil, the International Olive Oil Council (IOOC) promotes olive oil around the world by tracking production, defining quality standards, and monitoring authenticity. Trace-element technology, from which the most significant is ICP-MS at the moment, is extensively used for this food commodity. However, what should be noted for this commodity is that sample treatments, such as wet, dry, or microwave digestions, are required in order to eliminate the organic matrix since direct analysis faces many drawbacks and usually causes the extinction of plasma. Another negative effect is represented by the injector blocking due to the carbon deposition from incomplete oxidation of organic matrix. These drawbacks have been overcome by the addition of oxygen to the plasma and by a decrease of the temperature inside the nebulization chamber (Benincasa *et al.* 2007; Gonzalvez *et al.* 2009; Drivelos and Georgiou 2012).

Discrimination of olive oils produced in four Greek regions according to their geographical origin was achieved using rare Earth elements content and supervised chemometric techniques. Different ANNs models, multi-layer perceptions (MLPs) and radial basis function (RBF), were developed and evaluated. Two additional supervised techniques, discriminant analysis (DA) and classification trees (CTs), were also applied for data pretreatment and comparison purposes (Farmaki *et al.* 2012). Multi-element analysis of virgin olive oils from different Italian regions by ICP-MS and the data procession by linear discriminant analysis (LDA) facilitated classification of unknown samples (Benincasa *et al.* 2007). Geographical origin identification of Styrian pumpkin seed oil, a highly priced local product which is protected by the European Union, was achieved through the rare Earth elements profile and discriminant analysis (Joebstl *et al.* 2010).

4.4.2.5 Cereals – Pulses

Discrimination between "Fava Santorinis" (PDO) and other yellow split peas using four classification methods, orthogonal projection analysis (OPA), Mahalanobis distance (MD), partial least squares discriminant analysis (PLS-DA) and K-nearest neighbors (KNN), was achieved fusing rare Earth and trace-element data (Drivelos *et al.* 2014*a*). Boron, holmium, gadolinium, magnesium, rubidium, selenium, and tungsten combined with $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios differentiate rice samples cultivated in the USA, Europe and Basmati regions (Kelly *et al.* 2002). Discrimination of Piedmont hazelnuts, Italy (PGI) from others was achieved through lanthanides fingerprint. The "Tonda Gentile delle Langhe" (TGL) variety is acknowledged all over the world as the best, and it is particularly appreciated when used to provide flavor in chocolate products. Authentication is a prerequisite to safeguard this variety against fraud, which can occur when the product is partially or totally substituted with hazelnuts of lower quality (Oddone *et al.* 2009). Classification of wheat from four major Chinese wheat-producing regions was through their elemental profile (Zhao *et al.* 2011).

4.4.2.6 Dairy Products

Dairy products are an important food commodity that have been investigated to differentiate between production regions based on the trace-element content. Although milk is an essential dairy product with wide consumption worldwide, cheese is the most-studied dairy product, majorly produced in the USA followed by Germany, France, and Italy. According to the Food and Agricultural Organization of the United Nations, over 18 million metric

tons of cheese are produced worldwide per year, more than the yearly production of coffee beans, tea leaves, cocoa beans, and tobacco combined. The interest of consumers in regional products, the quality of which is closely related to a specific geographical area and traditional methods of production, gives rise to the need to protect producers by accurately discriminating highly esteemed food from cheaper imitation products. In the case of cheese, a PDO cheese must be obtained from milk of animals bred in the PDO area and the geographical environment of the area of origin must determine its essential characteristics, in order to guarantee that it is not an adulterated product. In countries such as Greece PDO products are of high importance, for example the popular PDO cheese known as feta (Gonzalvez *et al.* 2009; Drivelos and Georgiou 2012). Multi-element profile (P, S, K, Ca, V, Cr, Mn, Fe, Co, Zn, Ga, Rb, Sr, Mo, Cs, and Ba) was instrumental for cow and buffalo milk differentiation (Benincasa *et al.* 2008). The sources of food and water available for consumption by the animals were also studied, and results showed no correlation between the elemental composition of the dietary components and milk (Benincasa *et al.* 2008).

4.4.2.7 Meat

As a result of recent concerns relating to bovine spongiform encephalopathy (BSE), human variant Creutzfeldt–Jakob disease (CJD), and its impact on the internal market the European Commission established far-reaching legislation concerning the labeling of beef. As a consequence of this legislation, the use of multi-isotopic and/or multi-element analyses to provide information on the provenance of beef is gaining wider acceptance. The European Union Protected Food Names Schemes came into force in 1992 (Council Regulation, EEC No. 2081/92) and offers an independent inspection and labeling system for the protection of food names on a geographical basis such as PDO lamb or PDO beef (e.g. PDO Orkney beef and PGI Welsh beef). The focus of multi-element measurements that have been employed allows the PDO of meat, mostly on lamb and beef products, to be determined. However, using these techniques to infer geographical origin is far from trivial. There are confounding factors, such as imported feed, animal movement prior to "finishing" and metabolic turnover times of animal tissues to consider. Nevertheless, there have recently been a small number of specific publications relating to the identification of beef geographical origin and production regime using and trace-element and multiple isotope analysis of the bioelements (H, C, N, O, and S) with varying degrees of success (Heaton *et al.* 2008; Drivelos and Georgiou 2012).

Determination of poultry and dried beef geographic origin by elemental concentrations was achieved through SF-ICP-MS: twenty-five poultry breast fillet samples originating from Switzerland, France, Germany, Hungary, Brazil, and Thailand, and 23 dried beef samples from M. biceps femoris and M. semitendinosus produced in Switzerland, Austria, Australia, USA, and Canada were classified. For both poultry meat and dried beef, differentiation of the origins was possible using the elements which vary significantly different between the countries: As, Na, Rb, and Tl in poultry; B, Ca, Cd, Cu, Dy, Eu, Ga, Li, Ni, Pd, Rb, Sr, Te, Tl, Tm, V, Yb, and Zn in beef (Franke *et al.* 2007). Determining the geographical origin of mutton from different regions of China was achieved by the elemental profile (Sun *et al.* 2011).

4.4.2.8 Fish and Fish Products

The distinction of commercial marine species from the East China Sea according to their geographical origin was achieved by their elemental fingerprint using multivariate

statistical analysis. It was proven that both PLS-DA and probabilistic neural network (PNN) precisely predict the origin of the marine species (Guo *et al.* 2013).

4.4.2.9 Honey

Honey is a high-value-added product, mostly for its valuable nutritional and medicinal qualities. These properties are determined, among others, by mineral components which depend on type and origin, thus attracting the interest of researchers in assessing its geographical origin. It is well known that some of the components of honey are due to maturation; others are added by the bees and some of them are derived from plants. One of the difficulties of analyzing honey is that it can come from different floral sources and that mixtures are not always well known. Also, honey samples of the same floral source can vary due to seasonal climatic variations.

One of the main methods of adulterating honey consists of diluting honey with water and adding sugars and syrups. In recent years however, there has been a rising presence of mono-floral honeys in markets; these are more expensive than multi-floral honeys, so the possible adulteration by honey mixtures must be controlled. As a result, there have been many attempts at using trace-element methods in order to examine and understand the mineral profile of honey for the purpose of its authenticity (Gonzalvez *et al.* 2009; Drivelos and Georgiou 2012).

The distinction between honeydew, buckwheat, and rape honey, and also between different areas in Poland, was achieved through the mineral profile with the aid of CA and PCA. CA showed three clusters corresponding to the three botanical origins of honey. PCA permitted the reduction of 13 variables to 4 principal components, explaining 77.19% of the total variance. The first most important principal component was strongly associated with K, Al, Ni, and Cd (Chudzinska and Baralkiewicz 2010).

4.4.2.10 Coffee and Tea

Being the most dominant beverage worldwide, coffee is exported by more than 50 countries, providing livelihood for over 100 million people worldwide. In terms of using trace-element methods for authenticating coffee products, it all started in 1993 when Krivan *et al.* made the first determination of the origin of coffee based on trace-element profile by using instrumental neutron activation analysis (INAA), ETAAS, and FAAS for the determination of 21 trace elements in samples from South America. Results from this study demonstrated that Mn was a selective variable to separate coffee production areas with 84% accuracy (Krivan *et al.* 1993). Distinction between 20 green coffee beans from different geographical origins was achieved through Sr and O isotope ratios. The final results allowed discrimination of local provenances investigated in this study by principal component analysis (PCA), demonstrating the potential to prove the authenticity of world coffees (Rodrigues *et al.* 2011).

Tea products are the second most popular types of beverages, with an estimated 18–20 billion cups consumed daily. Massive tea-producing countries include India, Sri Lanka, Indonesia, Malawi, Kenya, Zimbabwe, Tanzania, and China. Tea samples were classified according to geographical origin by elemental fingerprinting with principal component analysis (PCA) and cluster analysis (CA) as exploratory techniques, linear discriminant analysis (LDA), and soft independent modeling of class analogy (SIMCA) (Moreda-Piñeiro *et al.* 2003).

4.4.2.11 Spices – Food Ingredients

Although there are not as many studies as for other commodities, there has been significant progress in the authentication of spice via trace-element methods. The focus has mainly been on rare raw spices, for example PDO spices such as the saffron spice (Crocus sativus L. stigmas), Szegedi paprika, and Mastic gum (otherwise known as mastix or mastic). This is because the rarer a spice is, the higher are the chances of adulteration with other cheaper spices. Fortunately, specifically multi-element analysis has been a successful tool in authentication of spices for their geographical origin. Multi-elemental fingerprinting was an innovative approach for this food commodity (Drivelos and Georgiou 2012). Food ingredients or specific chemical compounds can allow the geographical origin of a food product to be determined. This can include crops such as wheat, the very soil of the production area, or even a specific chemical compound such as glycerol or ethanol.

An example of research in this field is the case of 27 saffron spices produced in three Italian regions – Abruzzo (L'Aquila), Umbria, and Sardinia – which were analyzed by inductively coupled plasma-mass spectrometry to test the potential of mineral composition in geographical traceability. A linear discriminant analysis (LDA) based on concentrations of 12 selected elements (Li, B, Na, Ga, Rb, Sr, Zr, Nb, Cs, Ba, Sm, and Hf) yielded better than 80% of correct predictions in leave-one-out cross-validation. Four elements (B, Na, Sr, and Rb), identified by step-wise LDA, provide more than 90% of correct prediction. Moreover, saffron spices cultivated in Sardinia and central Italy can be distinguished under more stringent validation conditions (20% of samples in the prediction set). The results indicated that mineral composition is a promising indicator of saffron geographical origin (D'Archivio *et al.* 2014).

Another study (Brunner *et al.* 2010) described the usefulness of combining strontium isotope ratios ($^{87}Sr/^{86}Sr$) with a multi-element pattern (Rb, Sr, Y, Zr, Mo, Cd, Ba, Pb, Th, U, Mg, Ca, Sc, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, and rare Earth elements) by means of ICP-MS to establish a unique fingerprint of authentic Szegedi (Füszer) paprika (PDO) and classify authentic and purchased paprika of different known, declared, and unknown geographical origin using multivariate statistical tools, principal component, and canonical discriminant analysis. Since paprika represents a processed spice, alterations in element and Sr isotopic composition throughout the production process were investigated. The Sr source in the final product was identified to stem from bioavailable Sr sources in soil. The ammonium nitrate extract of a soil is therefore sufficient to establish a Sr fingerprint for agricultural products of a region. As a consequence, the spice paprika can be traced back to its geographical origin even after processing (Brunner *et al.* 2010).

4.4.2.12 Organic Foods

Organic food is produced in a way that complies with organic standards set by national governments and international organizations. Currently, the EU, the USA, Canada, Mexico, Japan, and many other countries require producers to obtain special certification in order to market food as organic within their borders. Organic food production is a heavily regulated industry, distinct from private gardening. As a result, synthetic pesticides and chemical fertilizers are not allowed, although certain organically approved pesticides may be used under limited conditions. In general, organic foods are also not processed using irradiation, industrial solvents, or chemical food additives. The interest

of consumers in organic products mainly stems from health and environmental considerations, increasing organic farming popularity. However, for the time being, there may be some differences in the amounts of nutrients and anti-nutrients when organically produced food and conventionally produced food are compared. The variable nature of food production and handling makes it difficult to generalize results, and there is insufficient evidence to make claims that organic food is safer or healthier than conventional food. What is however important is that organic farming generally responds to site-specific farming and crop conditions by integrating cultural, biological, and mechanical practices that foster recycling of resources, promote ecological balance, and conserve biodiversity. Consequently, methods such as the use of trace elements for authentications based on geographical origin have certain importance in this field.

Elemental uptake of a specific crop and therefore its chemical composition are influenced by many factors, including plant species, cultivar, physiological age, soil fertility, climate, crop rotation, and fertilization strategy as well as pest and weed control management. Organic and conventional farming differ strongly in the last two factors. In particular, organic farming severely restricts the use of artificial fertilizers; instead, use is made of organic fertilizers and nitrogen-fixing plant species. The extent and rate of release of nutrients are therefore different in the two farming practices, and this has an impact on the overall crop chemical composition (Capuano *et al.* 2013). Organic wheat, barley, fava, and potato were characterized from their elemental fingerprinting. Crops were cultivated over two years at three different locations, each accommodating one conventional and two organic cropping systems (Laursen *et al.* 2011). Only eight of the minerals investigated, namely Zn, Se, Ba, U, Dy, Tl, Th, and Mo, were found important for the authenticity of organic coffee by elemental profile and the use of data mining techniques. Classification was achieved by multilayer perceptron (MLP), support vector machine (SVM) and naïve Bayes (NB) (Barbosa *et al.* 2014*a*). The authenticity of free-range pastured hens eggs was investigated using a 15-element (As, Ba, Cd, Co, Cs, Cu, Fe, Mg, Mn, Mo, Pb, Se, Sr, V, and Zn) database. The egg classification of free-range as opposed to battery eggs was carried out using two machine-learning algorithms, decision trees, and naïve Bayes (NB) (Barbosa *et al.* 2014*b*).

As observed, trace-element methods have a huge range of applications in the organic food commodity. There are two very interesting reviews (Capuano *et al.* 2013; Laursen *et al.* 2014) relevant to this task, which perfectly capture the advancement in this field.

4.4.3 Chemometrics

Results from elemental fingerprinting provide the basis for chemometric techniques. Although not a brand new scientific field (the term was coined during the 1980s), there are still various definitions of chemometrics. The most valid defines chemometrics as the science of extracting information from chemical systems by data-driven means, representing and displaying such information, and transforming it into other forms of data. However, the greater the need for the use of chemometrics, the more complex it becomes, resulting in various data patterns or statistical and mathematical models. In terms of authenticating food products via elemental fingerprinting, chemometrics are used mainly for evaluation of the results and for creating patterns with which authentication could be carried out in a more accurate way. To further understand the use of chemometrics we provide an example in the following paragraph. It should however be

noted that the key to understanding chemometrics is not necessarily understanding the mathematics of all of the different methods; rather, it is to know which model to use for a given analytical problem and properly applying it.

A recent attempt to discriminate a PDO product known as "Fava Santorinis" (Drivelos *et al.* 2014*a*), by using full REE profiles alone or fusing with full trace-element profiles, was made using yellow split pea samples from different parts of Greece and other countries. The measured ICP-MS data were evaluated using the chemometric techniques, orthogonal projection analysis (OPA), partial least squares discriminant analysis (PLS-DA), Mahalanobis distance (MD), and K-nearest neighbor (KNN) for the analysis of the two sets, namely Santorini and not Santorini (Fig. 4.5). The four classification methods

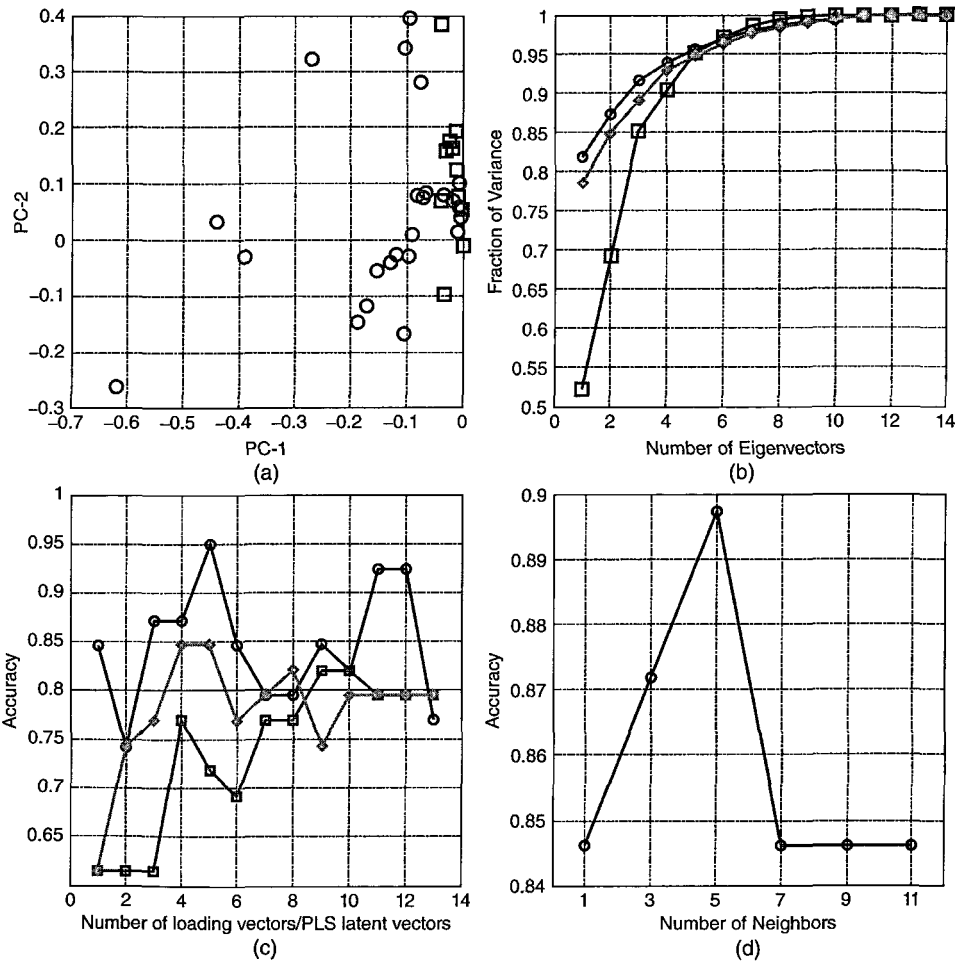


Figure 4.5 (a) Principle component analysis of all 39 samples based on the REE amounts using the first two principle components (PCs); Santorini samples (squares), and non-Santorini samples (circles). (b) Scree plots showing the respective cumulative fraction of the total variance for the full set of samples (diamonds), Santorini samples (squares), and non-Santorini samples (circles). (c) Accuracy values (above equation) using the classification methods OPA (circles), MD (squares), and PLSDA (diamonds). (d) Accuracy for KNN.

were then compared to each other based on their accuracy, with the use of a leave-one-out cross-validation (LOOCV) process. The performance of each classification method was determined based upon the counts of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). If a left-out sample in the LOOCV process is classified as belonging to a category and it belongs to that category, it is a TP; for example, a non-Santorini sample left-out is classified as non-Santorini. If a left-out sample is classified as not belonging to a category and it does not belong to that category, it is a TN. If a left-out sample is classified as belonging to a category and it does not belong to that category, it is an FP. Lastly, if a left-out sample is classified as not belonging to a category and it does belong to that category, it is an FN. These four terms are further used to calculate the accuracy value from the formulae: accuracy = (TP+TN)/(TP+TN+FP+FN).

The results of 100% accuracy for classification of the samples (according to orthogonal projection analysis or OPA) provide the best classification accuracy. A combination of trace-element techniques (in this case, REE fingerprinting) and chemometrics is extremely effective in terms of food authentication, especially in elemental fingerprinting (Drivelos *et al.* 2014*b*).

4.5 Conclusions and Outlook

Elemental techniques are currently the basic choice for food safety, quality, and authentication assessment. ICP-MS and ICP-AES are the techniques mostly used for elemental fingerprinting, due to their multi-element capability for a wide variety of food samples. ICP-MS has clear advantages in comparison to ICP-OES such as low detection limits, isotopic capability, and ability for rare Earth elements. Method detection limits reach the ppq (pg L^{-1}) range for elements with high m/z ratios, which makes the technique especially useful for determination of such low-abundant elements as REEs, actinide elements, and precious metals. Further, the recent surge in interest in REEs as authentication markers will increase in the near future. At present, knowledge of the elemental food fingerprint appears a plausible way to establish the geographical origin of food products. Finally, it would be an oversight if we did not mention here that authenticity assignment based on elemental fingerprint could be remarkably enhanced in fusing with isotopic and/or metabolomic fingerprint.

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5

Isotopic Fingerprinting

5.1

Light Isotopes

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5.1.1 Introduction

Stable isotope ratios analyses are intensively used in studies concerning food authenticity control. The use of stable isotope ratios in this domain allows both the establishment of geographic authenticity of foodstuffs and also identification of food adulteration with cheaper ingredients.

To establish an isotopic "profile" or "signature" for a material, the ratios of the stable isotopes of a number of elements such as $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$ should be measured. The possibility to assign the source of an organic substance stems from the isotopic content of the elements which comprise the material. Because the isotopic ratios of light elements such as hydrogen, oxygen, carbon, nitrogen, and sulfur can be locally enriched or depleted through a variety of kinetic and thermodynamic factors, the measurement of the isotope ratios can be used to differentiate between samples which otherwise share identical chemical compositions (Muccino and Jackson 2009).

In these types of studies the stable isotope composition of a substance is expressed in relation to an international reference standard in order to make it easier to handle the very small differences in absolute abundances, according to the relation:

$$\delta = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right),$$

where R is the ratio between the heavy and the light isotope, R_{sample} is the isotopic ratio of the sample, and R_{standard} is that of the reference material.

By definition standards have a δ value of 0. A positive δ value indicates that the amount of the heavy isotope is greater in the sample than in the standard (Georgi *et al.* 2005). The natural abundances of hydrogen, oxygen, carbon, nitrogen, and sulfur stable isotopes and also their corresponding internationally recognized standard are presented in Table 5.1.1.

From the four primary isotopic reference materials presented in Table 5.1.1, two are no longer available (SMOW and PDB). Consequently, the International Atomic Energy

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Table 5.1.1 Natural abundances of stable isotopes and international standards.

Element	Isotope	Abundance (%)	Primary reference materials	Isotopic ratio
Hydrogen	^{1}H	99.9855	Standard Mean Oceanic Water (SMOW)	0.00015576
	^{2}H	0.0145		
Carbon	^{12}C	98.892	Pee Dee Belemnite (PDB)	0.011237
	^{13}C	1.108		
Nitrogen	^{14}N	99.6337	Air (air nitrogen)	0.0036765
	^{15}N	0.3663		
Oxygen	^{16}O	99.7587	Standard Mean Oceanic Water (SMOW)	0.00200520
	^{17}O	0.0375		
	^{18}O	0.2039		
Sulfur	^{32}S	95.018	Canyon Diablo Troilite (CDT)	0.0450045
	^{33}S	0.750		
	^{34}S	4.215		
	^{36}S	0.02		

Agency (IAEA, Vienna) has calibrated alternative reference materials with isotopic values close to that of original primary reference.

5.1.2 Application of Stable Isotope Ratios in Food Control

5.1.2.1 Fruit Juices

Fruit juices are very popular beverages and the increasing demand for the required raw material all over the world has led to the appearance on the market of alternative cheaper solutions for their manufacture. For example, the partial substitution of a fruit juice concentrate by the much cheaper liquid sugars (beet or cane medium invert sugar or mixture of both) or the preparation of a single strength juice from concentrate (without proper labeling) could result in a remarkable cost advantage. Such adulteration does not usually affect consumer health, but such products mislead the consumer and disadvantage the honest producer (Rossmann 2001).

Determination of $\delta^{18}O$ and $\delta^{2}H$ values of water from fruit could differentiate between directly pressed and rediluted products made with tap water. The principle behind this detection is the fact that authentic juices have elevated $\delta^{18}O$ and $\delta^{2}H$ values of water as compared to tap water due to the evapotranspiration processes that take place during the plant life cycle. The rediluted products made using tap water, which is relatively depleted in heavy oxygen and hydrogen isotopes, will therefore have a distinct isotopic content as compared to single-strength juices (Koziet *et al.* 1993, 1995). Further, quantity and quality of the harvested fruits are subject to significant geographical and seasonal variations depending on climatic particularities in the area of production. For these reasons, the interpretation of such analysis requires a relevant number of authentic juices from different geographical areas and different fruit types.

In Rossmann's (2001) study on authentic apple juices from different European countries (Germany, Italy, Belgium, Poland, Austria, Hungary, Czech) during 1991–1994, the mean $\delta^{18}O$ value was about –4‰, showing a relatively large range of variation from –1‰ to –5‰. A recent study made by Bat *et al.* (2012) had shown for authentic Slovenian apple juices variations of $\delta^{18}O$ between –2.7‰ and –4.2‰ and δD between –45.4‰ and –28.5‰, depending on geographical origin. Magdas *et al.* (2012) found a mean value of $\delta^{18}O$ = –4.2‰ and δD ≈ –46.5‰ for authentic apple juices from Transylvania, with small differences among different geographical regions. The variation of the apple juice mean value of each investigated Transylvanian region was between –3.7‰ and –4.5‰ for $\delta^{18}O$ and between –45.5‰ and –49‰ for δD.

As well as the isotopic differences that appear among the same fruit species grown in different geographical, climatical, and meteorological conditions, the degree of isotopic enrichment varies from one fruit species to another, even from species that came from the same geographic area. In Figure 5.1.1 isotopic content of different fruit types (apples, pears, grapes, and plums) grown in Transylvania during 2010 are presented. It can be observed that the interpretation of isotopic results with respect to the authenticity of a fruit juice should also take into account the fruit species (Magdas and Puscas 2011).

Photosynthetic CO_2 assimilation via the C_3, C_4 and CAM pathways is of major importance in the use of carbon stable isotope ratio analysis in food authenticity control. The detection of commercial C_4 cane or corn-derived sugar syrups in C_3 agricultural products are therefore facilitated by characteristic differences in $\delta^{13}C$‰ values.

The technique is necessarily comparative, as it must take into account the natural variation of $\delta^{13}C$ values in authentic products due to environmental factors such as water availability and light intensity (Kelly 2003).

$^{13}C/^{12}C$ proved to be a good tool for characterizing geographical origin. Indeed, the $\delta^{13}C$ values of plant compounds are influenced by the availability of water, relative humidity, and temperature, which control stomata aperture and the internal CO_2 concentration in the leaf (O'Leary 1995). In food sciences, the $^{13}C/^{12}C$ ratio is a good probe for detecting the addition of cane sugar or maize glucose syrup to fruit juices (O'Leary 1981) due to the isotopic differences that appear between the two plants categories C_3 and C_4 plants. Stable carbon isotope ratios (expressed as $\delta^{13}C$) of C_3 and C_4 plants are different, with ranges from –11‰ to –14‰ for C_4 plants and those of C_3 plants varying between –24‰ and –32‰ (Schmidt & Winkler 1979; O'Leary 1981, 1988, 1995). Most

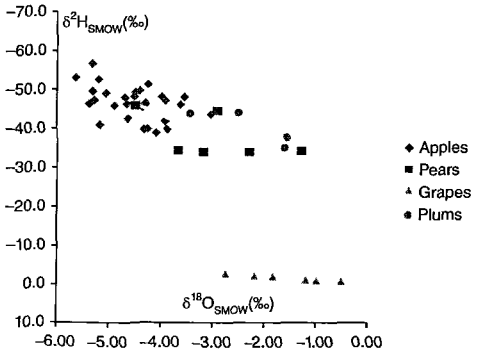


Figure 5.1.1 Isotopic content of authentic fruit juices from Transylvania from 2010 vintage.

fruits are included in the C_3 plants category except pineapple, while the C_4 plants category contain plants which produce the most inexpensive sugars available on the market such as maize, sorghum, and sugar cane.

The application of this technique to the detection of added C_4 sugars also requires a large database of authentic isotopic values with which test samples can be compared, especially in the case of a small quantity of C_4 added sugar. Differences in the mean $\delta^{13}C$ values reported by different authors for authentic apple juices exist due to the geographical region in which these fruits have grown.

For apple juices, Doner *et al.* (1980) observed that the mean $\delta^{13}C$ value of juices from different varieties of apple and different cultivation geographical locations was around −25.4‰, with no significant correlation between the variety of apple or geographical origin. Lee and Wrolstad (1988) found that the mean $\delta^{13}C$ value of whole apple juice was −24.2‰ and that apples from Argentina, Mexico, and New Zeeland did not differ significantly from those grown in the USA. For Transylvanian fruits, the reported results have shown a mean isotopic value of around $\delta^{13}C = -28.2‰$ (Magdas *et al.* 2012).

From these considerations is obvious that authentic products present a natural variation of their stable isotope content mainly due to seasonal or regional influences. In many cases, this variation is sufficiently wide to cover a small amount of adulteration which could remain undetected. One way to overcome this type of small adulteration is to use an internal reference approach by measuring the isotopic composition of two chemical components extracted from the same sample and to study their relationship in order to determine the authenticity (Bricout & Koziet 1987; Koziet *et al.* 1993). For the detection of exogenous sugars in fruit juices, Rossmann *et al.* (1997) proposed an internal reference method for the comparison of ^{13}C content of sugars with those of insoluble component of a juice, the pulp. In this case the $\delta^{13}C$ content of sugars and pulp will be at a constant ratio because they arise from closely related metabolic pools and were subjected to the same environmental influences (Rossmann *et al.* 1997). The isotopic content of the reference compound is therefore used to reduce the variability of authentic database and also to detect relatively small amounts of sugar addition even when the isotopic values of investigated sample are in the natural variation range of the fruits (Magdas *et al.* 2014).

5.1.2.2 Wines and Sparkling Wines

The authentication of wines involves several aspects such as geographical origin, year of vintage, grape cultivar, and quality. It is therefore necessary to base the proof of authenticity on wine origin-specific parameters which do not undergo alterations during vinification or which are difficult to adulterate. The effects of wine water supply, and in consequence the meteorological conditions, for example precipitation, mean temperature, relative humidity, rain and water stress, are important parameters which have to be taken into consideration in the case of evaluation and plausibility testing of geographical variations of stable isotope fractionation of carbon and oxygen in wine (Forstel *et al.* 1997; Tardaguila *et al.* 1997; Ogrinc *et al.* 2001; Gaudilliere *et al.* 2002; Christoph *et al.* 2003; Christoph *et al.* 2004).

The wine database that was established in 1990 (EU regulation 2676/90) collecting data of European reference wine samples is updated each year due to the remarkable seasonal and regional variability of isotopic data. For instance, extreme isotopic values

of wine samples from different regions were reported in literature. Christoph *et al.* (2003) reported for authentic Franconia wines, vintages 1992–2001, a range of variation of mean value for $\delta^{13}C$ from –29.4‰ to –26.3‰ and for $\delta^{18}O$ from –3.82‰ to 0.44‰, while for Lake Constance the $\delta^{18}O$ value can reach –5‰ and the mean value of $\delta^{13}C$ is around –28.5‰. Another extreme represents the isotopic values reported by Christoph *et al.* (2004) for Croatian wines: up to 9‰ for $\delta^{18}O$ values and –23.1‰ for $\delta^{13}C$. The explanation for all these extreme conditions is related to the climate. In the case of Lake Constance wines, the isotopic values are influenced by the special micro-climate that was, during that period, characterized by high quantities of precipitation in summer and before harvest; the higher isotopic values obtained for Croatian wines were directly linked to the extremely hot and dry climate at the coast of Adriatic Sea in 2000.

Sparkling wine is a beverage that originated in the Champagne region of France and that has now become popular globally, so that it is now produced in other regions of the world (Martinelli *et al.* 2003). The production of sparkling wines typically consists of two fermentation steps: a first fermentation that results in ethanol synthesis and a second short fermentation to produce CO_2. Carbon dioxide is a specific ingredient for sparkling wines (Calderone *et al.* 2007), which gives a more pleasant taste. Some aspects of CO_2 in beverages are regulated in EU regulations. The carbon dioxide may result from natural fermentation, either in a bottle as with the *méthode champenoise*, in a large tank designed to withstand the pressures involved (as in the Charmat process), or as a result of carbon dioxide injection (http://en.wikipedia.org/wiki/Sparkling_wine).

Dunbar (1982) and Gonzalez-Martin *et al.* (1997) presented their isotopic results of $\delta^{13}C$ from CO_2 in sparkling wines, obtaining values between –28‰ and –17‰ for CO_2 from C_3 plant sugars, and about –9‰ for sugars added from C_4 plants. A value of $\delta^{13}C$ above –7‰ or below –30‰ can suggest that the investigated sample is a gasified wine. The isotopic composition of ^{13}C of CO_2 in these products is therefore dependent upon the sugar type used in the second fermentation (C_3 or/and C_4) or on the isotopic value of the industrially added CO_2. This fact was also confirmed by the results of Calderone *et al.* (2007), who distinguished two kinds of sparkling wines according to the method of adding sugar at the second fermentation.

Martinelli *et al.* (2003) investigated the stable carbon isotopic composition of the wine and of the CO_2 bubbles produced during the second fermentation for many sparkling wines obtained in different countries around the world. The $\delta^{13}C$ values of Brazilian, American, and Australian sparkling wines were significantly heavier than the isotopic composition found in sparkling wines from South America and Europe. The isotopic composition of the CO_2 bubbles ($\delta^{13}C$-CO_2) followed similar trends. For most of the sparkling wines from Brazil or Argentina the average $\delta^{13}C$-CO_2 was –10.8‰, indicating that the carbon source for the second fermentation was sugar cane. Conversely, the average $\delta^{13}C$-CO_2 of most of the sparkling wines produced in Chile and Europe was –22.0‰, suggesting that a different sugar (most likely sugar beet) was used in the second fermentation. In many cases, the carbon isotope ratios of sparkling wine and CO_2 bubbles provide valuable information about the sugar sources.

5.1.2.3 Mineral Waters

Brencic and Vreca (2006) stipulated that bottled water is a food product that depends on the environment from which it originates, not only at the place where it is produced,

but also on the conditions in the recharge area of the wells or springs captured for bottling. Annual consumption of bottled water (e.g. well water, mineral water, or spring water) is increasing from year to year. Mineral water is natural water containing not less than 250 parts per million total dissolved solids. Mineral water is distinguished from other types of bottled water by its constant level and relative proportions of mineral and trace elements at the point of emergence from the source. No minerals can be added to this product (www.bottledwater.org). Bottled mineral waters present the potential for fraud and product misrepresentation (Spangenberg & Vennemann 2008) that may be physically or economically harmful to consumers (Bowen *et al.* 2005).

Bottled waters are regulated at the regional, national, and international levels. Authentication of these products represents a challenge to agencies that monitor and assure the quality, authenticity, and region of origin of food products around the world (Dennis 1998; Rossmann 2001). The naturally occurring stable isotopes of hydrogen and oxygen represent one possible tool to investigate the origin of mineral waters. The isotopic compositions of hydrogen and oxygen isotopes vary greatly across the globe and reflect natural processes in the hydrological cycle (Gat 1996; Clark & Fritz 1997). It is possible to investigate the fidelity of the isotopic signature of bottled water by comparing bottled water isotopic values (δ^2H and $\delta^{18}O$) with estimated mean annual precipitation isotope ratios for source or bottling locations (Raco *et al.* 2013). Additionally, the analysis of the $\delta^{13}C$ in the dissolved inorganic carbon (DIC) of carbonated bottled water allows detection of the origin of the CO_2 in bottles of sparkling water. This isotopic method can detect whether the carbon dioxide is naturally diluted in the water, or is of industrial origin and was added to the water in the bottling plant.

Bowen *et al.* (2005) presented a very complex study about the isotopic compositions (H and O) of the bottled waters of the world. They determined the isotope ratios for 234 samples, obtaining results between −147‰ to 15‰ for δ^2H and from −19.1‰ to 3‰ for $\delta^{18}O$. These data lie within a range of values typical of "normal" meteoric waters. Some samples from India illustrate the isotopic evidence of evaporative effects. Another example in this study displays the evidence for bottling of at least two distinct water sources, despite their labels indicating a single, common, bottling location from Argentina, on the basis of the isotopic results. The authors demonstrated that the isotopic signatures of source water are preserved in bottled water, and can provide information on the ultimate (recharge) and proximal (reservoir) sources of bottled water.

Brencic and Vreca (2006) investigated 58 bottled waters (15 foreign and 43 domestic brands) commercially available from supermarkets in Ljubljana (Slovenia). The isotopic results demonstrated the difference between a natural sparkling mineral water and artificially sparkling water by the carbon isotopic composition of DIC (dissolved inorganic carbon). δ^2H and $\delta^{18}O$ values in still waters were close to the Ljubljana LMWL and GMWL (Global Meteoric Water Line). They concluded that during the production process of still waters, the hydrogen and oxygen compositions remain similar to those of the parent water.

Dotsika *et al.* (2010) presented a study by IRMS of 25 domestic brands of Hellenic bottled still waters. The isotopic values ranged from −67.5‰ to −46.5‰ for δ^2H and from −9.9‰ to −6.9‰ for $\delta^{18}O$. By comparison of bottled water isotope ratios with natural spring water isotope ratios, the conclusion is that bottled water isotope ratios preserve information about the water sources from which they were derived, suggesting that in many cases bottled water should not be considered as an isotopically distinct

component of the human diet. This kind of study is useful in the determination of the natural origin of bottled water, and to indicate differences between the natural and production processes.

Similar studies were performed by Raco *et al.* (2013) for bottled waters (sparkling, still) available on the Italian market and by Bong *et al.* (2009) for the bottled mineral water samples on the South Korean market, suggesting that the stable isotope composition of bottled water can be used as a good quality control tool for market regulators.

5.1.2.4 Vanilla

Vanilla is an important flavor, and is always made from the vanilla bean. The vanilla bean is harvested as a long, thin, dark brown pod from the vanilla orchid. Vanilla is the second-most expensive spice after saffron, due to the extensive labor required to grow the vanilla seed pods. Vanilla extract contains high levels of vanillin which is an organic compound known as phenolic aldehyde, being the primary component of the extract of the vanilla bean. True vanilla extract contains hundreds of other volatile compounds which also contribute to the flavor, however. These can vary from bean to bean, and from variety to variety. Synthetic vanillin, instead of natural vanilla extract, is now more often used as a flavoring agent in foods, beverages, and pharmaceuticals. Because of the scarcity and expense of natural vanilla extract, there has long been interest in the synthetic preparation of its predominant component. The first commercial synthesis of vanillin began with eugenol. Today, artificial vanillin is made from guaiacol, lignin, curcumin or ferulic acid (http://en.wikipedia.org/wiki/Vanillin). Although more than 100 vanilla species are known, only three of them have mercantile importance: *Vanilla planifolia*, *Vanilla tahitienis*, and *Vanilla pompona*. It is necessary to employ powerful analytical techniques and tools to demonstrate the authenticity of the vanilla products and to protect honest food producers and consumers from adulteration of vanilla products (Hilmer *et al.* 2011). Isotope Ratio Mass Spectrometry (IRMS) is one of these techniques. Carbon analysis of vanillin isolated from vanilla extract has been used by IRMS to differentiate between natural and synthetic vanillin (Ashurst and Dennis 1997). After the vanillin is isolated from the extract by different procedures, it is combusted and the isotopic ratio $^{13}C/^{12}C$ is measured by IRMS. $\delta^{13}C$ values of vanillin from natural vanilla are about –20‰, while synthetic products from lignin, guaiacol, and eugenol range from –27‰ to –36‰ (see Table 5.1.2). In an inter-laboratory study, Guarino (1982) presents three tested methods for isolation of vanillin from vanilla extract for stable isotope ratio analysis. Each of the three methods provided vanillin which was useful for the intended purpose. The vanillin isolated by each of the three methods was suitable for stable isotope ratio analysis to determine whether the vanillin was of natural origin or was added to the extract.

Bensaid *et al.* (2002) had investigated 14 samples from different origins. To obtain more selective carbon and oxygen isotopic parameters by IRMS, the formyl group of vanillin was eliminated through the transformation of vanillin into guaiacol, which represents the aromatic moiety of vanillin. In the case of vanillin from natural precursors (beans, ferulic acid, lignin, and isoeugenol), the guaiacol molecule was slightly enriched in ^{13}C. In contrast, a slight depletion (–1‰) was observed for the fossil precursors.

Lamprecht and Blochberger (2009) had described an analytical method for the stable isotope ratio analysis of vanillin in ice cream and yoghurt. The $^{13}C/^{12}C$ ratio of the

Table 5.1.2 Stable carbon isotope values for authentic and synthetic vanillin.

Vanillin origin	$\delta^{13}C_{VPDB}$ (‰)	Source
Beans *V. planifolia* (Bourbon)	−21.5 ÷ −19.2	Scharrer and Mosandl (2002)
Beans *V. tahitienis* (Tahiti)	−19.7 ÷ −15.9	Gatfield *et al.* (2007)
Beans	−20.4 ÷ −20.2	Bensaid *et al.* (2002)
	−21.5 ÷ −16.8	Schmidt *et al.* (2007)
Guaiacol	−36.2 ÷ −29.0	Hoffman and Salb (1979)
Eugenol	−31.7 ÷ −29.9	Hoffman and Salb (1979)
Lignin	−28.7 ÷ −26.5	Schmidt *et al.* (2007)

vanillin fractions was determined by EA-IRMS. Their method was applied to 19 samples (12 yoghurt and related products samples and 7 ice cream samples) obtained from local food stores (Austria). The results had shown that three yoghurt samples contained synthetic vanillin. $\delta^{13}C$ values of vanillin isolated from yoghurt showed a range from −18.7‰ to −21.7‰ for authentic vanillin, and from −6.9‰ to −28.5‰ for synthetic vanillin.

5.1.2.5 Spices

Spices such as saffron, pepper, ginger, cinnamon, nutmeg, and cloves reach a high price; in consequence, they are vulnerable to adulteration. One of the powerful analytical techniques used for spices authentication is IRMS.

Maggi *et al.* (2011) presented a study by a multi-element (H, C, N) stable isotope analysis of 28 authentic saffron samples produced in Greece, Iran, Italy, and Spain from the same harvesting year (2006). The highest $\delta^{2}H$ values were obtained for Iranian samples (mean of −67.8‰), and the lowest for Greek samples (mean of −84.7‰). For Italian and Spanish saffron samples, $\delta^{2}H$ mean values were −72.4‰ and −75.1‰, respectively. $\delta^{13}C$ values reflect the C_3 photosynthetic pathway, ranging from −26.6‰ for Greek and Italian samples to −24.5‰ (mean value) for Iranian and Spanish samples. The lowest $\delta^{15}N$ values (1.2‰, and respectively 2.2‰) were obtained for Greek and Iranian saffron samples, suggesting the use of fertilization with synthetic nitrogen. $\delta^{15}N$ mean values of 5.3‰ and 4.2‰ respectively were obtained for Italian and Spanish samples, which are typical of materials derived from plants that have been fertilized with animal manures. The authors have made a three-dimensional scatter-plot of the combined isotopic results for the authentic saffron samples. This plot shows that the combined H, C, N stable isotope data has the ability to resolve the geographical origin of saffron.

5.1.2.6 Fish

In the category of food from animal origin, fish requires more authentication techniques than others due to the necessity of the certification of origin in labels (EU Regulation No. 104/2000) and to the great number of commercial frauds in this field. For certain foodstuffs (cheese, wines, ham) it is common to be described, or named, according to the particular region where they come from. Another reason to establish methods to

confirm the geographic origin lies in the quality or toxicity of the fish. European legislation establishes that the FAO area in which wild fish are caught should be part of the information available to consumers (EC regulation No. 2065/2001) (SEAFOODplus VALID report 2007). Until the publication of this report, there were no officially recognized methods. Stable isotope fingerprinting seems to be one of the most promising and suitable methods to authenticate livestock products. Analysis of stable carbon, nitrogen, and sulfur isotope signatures from fish tissues can provide temporally integrated information both on food sources and trophic position (SEAFOODplus VALID report 2007).

Dempson and Power (2004) used IRMS in fish authentication. They examined stable isotopes of carbon and nitrogen in wild and aquaculture-origin Atlantic salmon to evaluate their utility to identify farmed fish. Samples of muscle tissue obtained from wild salmon were significantly more enriched in nitrogen ($\delta^{15}N_{mean}$ = 12.75‰), but depleted in lipid corrected carbon ($\delta^{13}C_{mean}$ = −20.51‰) by comparison with aquaculture specimens ($\delta^{15}N$ = 10.96‰; $\delta^{13}C$ = −19.25‰), allowing the two groups to be completely classified.

Serrano *et al.* (2007) presented their isotopic results determined in wild and farmed gilthead sea bream (*Sparus aurata*) samples of white and red muscle, liver, gills, and gonads. In samples without lipid removal, for $\delta^{13}C$ values differences were found in all tissues studied apart from white muscle of wild fish (the tissue with the lowest lipid content). For $\delta^{15}N$ values, no differences were found in wild fish tissues. Liver from farmed fish showed lower $\delta^{15}N$ value after removal of lipid. $\delta^{13}C$ value of cultured fish tissues showed a mean depletion about of 3‰ compared to wild specimens (also after lipid removal), suggesting different sources of carbon in the diet, probably due to the feed used during sea-cage culture. Cultured gilthead sea bream tissues were significantly more enriched in nitrogen than wild specimens by an average of 1.5‰ in white muscle, indicating a slight increase in the trophic level. Determination of stable isotope signatures of gilthead sea bream tissues allows clear discrimination between wild and cultured sea bream, and characterization of differences in diet and feeding conditions in any tissue studied.

Oliviera *et al.* (2011) presented a study which discriminate among salted Atlantic cod and salted saithe using isotope ratio mass spectrometry (IRMS). They chose these species because Atlantic cod and saithe catches are approximately similar, although Atlantic cod is of greater value than saithe and also because of the large imports of salted products of these species in Brazil. Atlantic cod and saithe showed consistent differences in $\delta^{13}C$ and $\delta^{15}N$ mean values among body tissues. $\delta^{13}C$ mean values for cod muscle, bone, and skin were −19.4‰, −16.6‰, and −17.9‰, respectively. Instead, $\delta^{13}C$ mean values for saithe body tissues were −20.2‰, −7.4‰, and −18.5‰. $\delta^{15}N$ in tissues with lower turnover rates (bone and skin) and in tissues with greater turnover rates (muscle) can be used to authenticate the species of salted fish samples when distinguishing between Atlantic cod and saithe: $\delta^{15}N$ mean values for cod muscle, bone, and skin were 18.5‰, 19.2‰, and 18.9‰, respectively, and for saithe the isotopic composition of ^{15}N were 14.7‰, 13.2‰, and 12.9‰.

5.1.2.7 Beef

Multi-elemental stable isotope ratio analysis (SIRA) has the potential to assist in distinguishing organically from conventionally produced beef. Seasonal patterns in the

isotopic composition of beef exist, reflecting seasonality in animal feeding practices modulated by tissue turnover rates (Moloney *et al.* 2011).

Nakashita *et al.* (2008) investigated the isotopic composition of C, N, O of beef samples from Australia, Japan, and USA, suggesting that IRMS is a good technique to distinguish the provenance of beef not only among different countries, but also between different regions from the same country. The isotopic results for $\delta^{13}C$ were higher for the USA beef samples (from −13.6‰ to −11.1‰) than those from Japan (from −19.6‰ to −17.0‰) and Australia (from −23.6‰ to −18.7‰). $\delta^{18}O$ values were higher for Australian beef samples (ranging from 15.0‰ to 19.4‰) than those for Japanese samples (ranging from 7.3‰ to 13.6‰) and USA beef samples (ranging from 9.5‰ to 11.7‰). There were no notable difference for $\delta^{15}N$ values of beef samples from Australia, Japan, and USA. This would appear to indicate that no substantial differences exist in the bovine diet among these regions, knowing that $\delta^{15}N$ of animal tissue reflect that of cattle diet.

Similar results were obtained by Horacek and Min (2010) using stable isotopes (H, C, and N) to discriminate Korean beef from beef of other origin (USA, Mexico, Australia, and New Zealand).

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5.2

Heavy Isotopes

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5.2.1 Introduction

Before starting this chapter on the heavy elements isotope ratio for food traceability purposes, let us underline our thoughts about this currently important topic. In fact, it is fairly common to closely link the use of isotope ratios parameters, either of light-/bioelements or heavy elements, as tools against food counterfeiting. Furthermore, it is worth highlighting how these indicators have successfully tackled this task. Nevertheless, another important aspect, largely neglected, needs to be considered. Indeed, geographical traceability of food should be considered an opportunity to increase food quality. The objective possibility to link the food to its production territory (its "*terroir*") represents an opportunity of growth for both the producers and the consumers. For the former, the possibility to objectively certificate the origin of their products could be a way to gain a higher economical positioning on the market in a context of better "product quality". For the latter, the geographical traceability should be considered in a context of ethnicity and reputation of the producer, combined with greater transparency and confidence in the food web.

Although we are aware that food quality and food fraud might be considered as different sides of the same medal, in general we need to pay more attention to fraud and the associated risks; on the other side, as scientists we prefer to put our enthusiasm into developing and promoting the quality of the processes and products. Obviously, this is also the case for geographical traceability of food and food processes. At the same time, it would be foolish not to consider the capabilities and the use of these analytical indicators as tools against food sophistication and/or food adulteration, since the border between quality and fraud can be very thin.

In many cases, national and international research programs do not adequately support these topics since governments do not necessarily make a link between food quality and food adulteration/frauds. For this reason, much research does not address this specific issue and the obtained data are difficult to correlate due to their rare homogeneity.

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5.2.2 Quality vs. Geographical Traceability

Although the main aim of this chapter is not a detailed and exhaustive discussion on the food quality issues and how these are related to authenticity and traceability, few details will be reported here to help the readers to understand the importance of developing geographical traceability models for food and food commodity.

"Traceability" is a concept developed for industrial purposes and, at the very beginning, it was considered as a tool to ensure the quality of products and production processes (Wall 1994). Product tracing systems differentiate commodities for a number of reasons: food traceability systems allow supply chain actors and authorities to identify the source of a food safety or quality problem and promote procedures to remedy them (Commission of the European Communities 2000).

Through the adoption of Regulations (EEC) 2081/1992 and 178/2002, the European Union (EU) has laid the foundations for a common food policy between its members. Moreover, with the introduction of geographical indications, the EU has set specific quality requirements for food. Four geographical indications were introduced, namely: Protected Designation of Origin (PDO); Protected Geographical Indication (PGI); Organic Farming; and Traditional Geographical Specialties (TGS). These labels identify food commodities with specific characteristics deriving from a particular production area (origin PDO/PGI) and/or a farming method (TGS, Organic Farming). In this context, a geographical indication describes an agricultural product with specific characteristics related to its geographical origin or production process. The PDO denomination therefore represents the most stringent award for a food due to the localized origin constraints for the raw materials and production steps.

Despite patriotism, people around Europe (and increasingly around the world) are greatly interested in the origin of the food they eat. Authentic regional products, characterized by a restricted or well-defined production area, are preferred to food industry and many consumers are willing to pay an extra price for such products (Commission of the European Communities 2008; De Jonge *et al.* 2008; Van Rijswijk *et al.* 2008). On the other hand, due to the globalization of commercial exchange, the "European approach" towards food quality has been strongly challenged by the USA and Australia. Due to this opposition, activities to demonstrate the provenance and/or authenticity of food as objective criteria of choice only began a few years ago (Morgan *et al.* 2009).

With the aim of sustaining the declarations associated with the geographical indicator marks, the topics of food quality, geographical food origin, and food authenticity have gained increasing importance. Today, many researchers and institutions recognize the need to develop and improve systems for traceability (considering the more generalized meaning of the term) and authenticity for the most relevant food by using objective criteria (https://theanalyticalscientist.com/issues/1013/chewing-over-food-analysis/).

Besides the many different analytical approaches to food authenticity and origin issues, the use of isotope ratio of stable light elements and radiogenic elements, primary or direct indicators (Lee 2003), represent one of the more promising investigation and application fields.

Regarding the geographical traceability, it should be clarified that the analytical indicators of the provenance can be classified into two main categories (Lee 2003): direct or primary indicators; and indirect or secondary indicators. The former include variables

that can directly connect some chemical characteristics of the food with the same characteristics measured in the area, such as isotopic ratios of different elements and metals. The latter are related to the compositional characteristics of the food and to the transformation process. These indicators are based on an extensive characterization of the matrix (i.e., the determination of a large number of variables, chemical, chemical/physical, and microbiological, with different analytical techniques), allowing the identification of products of the same origin and provenance while differentiating and discriminating them from all the other "similar" products.

Regarding heavy elements (usually present in food at trace level concentrations, <1%), their isotopic analysis allows traceability models directly based on a "correlation" between the territory (soil from the area in which the life cycle of the plant is active) and the finished product (food or its raw materials) to be obtained. The link between food and its geological provenance is often studied by means of radiogenic isotopes of heavy elements, such as strontium (Sr; García-Ruiz *et al.* 2007; Swoboda et *al.* 2008; Marchionni *et al.* 2013; Petrini *et al.* 2014), lead (Pb; Mihaljevič *et al.* 2006; Manton 2010) and neodymium (Nd; Möller *et al.* 1998; Kraft *et al.* 2013), present in rocks and hence in the soil in limited quantities. Currently, data regarding the variation of the $^{87}Sr/^{86}Sr$ isotopic ratio provide the best results in the development of models of geographical traceability for different types of food matrices (Fortunato *et al.* 2004; Swoboda *et al.* 2008; Rummel *et al.* 2010; Ariyama *et al.* 2011; Baroni *et al.* 2011; Techer *et al.* 2011; Durante *et al.* 2013).

In fact, strontium isotopic ratio allows a precise mapping of the investigated geographical area: considering the same lithological composition, very ancient lands have higher values of this ratio with respect to geologically younger lands; the metal can be measured inside food since Sr has the property to be assimilated by living organisms as it actively takes part in the processes involved in their metabolism. Furthermore, during the intake of the minerals by plants, there is no significant isotopic discrimination of heavy elements present in the soil and their isotope ratio should remain unchanged passing from soil to food and acting as a fingerprint of provenance.

The isotopic maps obtained from the collected data can be used to generate more objective criteria to establish if a food belongs to a specific production area and/or a particular food class. In both the cases, this knowledge requires statistical-based approaches to obtain large-scale representative tracing models. Traceability can therefore: improve the management of risks related to food safety; improve product authenticity; and improve product quality.

However, to establish or demonstrate a relation between a food/food commodity and its provenance area represents a problem that might have multiple analytical technical approaches. Strictly speaking, the only way to state a link between the territory of provenance (soil) and the final product (food) is the geochemical approach.

The geochemical approach is a multi-step process starting from a deep geochemical characterization of the cultivated soil and the identification of a target variable/variables to be detected within the final food. Chemical indicators with these properties are the elements of the periodic system, with a special attention paid to the use of the rare Earth elements (REEs), and the isotopic ratio of particular elements such as strontium, lead, and neodymium. These indicators can be measured along the whole production chain and all the food matrices following a farm (the soil) -to-fork (the food) methodology.

5.2.3 The Isotopic Approach to Food Traceability

Isotopes are atoms of the same element that differ by mass. The term isotope is formed from the Greek for roots *"isos,"* namely equal, and *"topos,"* namely place: the "same place." Consequently, different isotopes of the same element have an equal number of electrons (and protons) but a different number of neutrons (different mass).

Isotopes are considered stable if their relative abundance does not vary with time (primordial nuclides), or radioactive when they are subject to decay processes by emitting particles (White 2013), varying their relative abundance as function of the half-life time value. Stable isotopes may be radiogenic if they are produced by the decay of a radioactive isotope. Examples include $^{87}Rb/^{87}Sr$ (isotope ratio $^{87}Sr/^{86}Sr$), $^{147}Sm/^{143}Nd$ (isotope ratio $^{143}Nd/^{144}Nd$), $^{232}Th/^{208}Pb$ (isotope ratio $^{208}Pb/^{204}Pb$), $^{238}U/^{206}Pb$ (isotope ratio $^{206}Pb/^{204}Pb$), $^{235}U/^{207}Pb$ (isotope ratio $^{207}Pb/^{204}Pb$), etc. (Banner 2004; White 2013).

As previously described, an important property of the isotopic pattern is the relative abundance between the isotopes. This parameter may vary due to the presence of fractionation mechanisms (this is the case for light elements such as H, B, C, O, N, and S) or due to decay of radiogenic isotopes).

In the case of heavy elements, fractionation phenomena are more or less negligible. However, with the recent advent of the newest spectrometers, mass-dependent fractionation (MDF) and mass-independent fractionation (MIF) mechanisms are supposed to be present for several isotopic patterns (Bigeleisen & Mayer 1947; Banner 2004; Johnson *et al.* 2004; Bergquist and Blum 2007; Schauble *et al.* 2009; Slovak and Paytan 2011).

The study of the variations in the isotopic composition of metal stable isotopes has provided important results in several application fields that may be focused on a range of issues: geochronology (Condon & Schmitz 2013); evolution of life on Earth (Burke *et al.* 1982; Von Blanckenburg *et al.* 2009); climate change (Berner 1991; Edmon *et al.* 1995; Berner and Berner 1996); people migration (Sillen *et al.* 1998; Price *et al.* 2002) environmental pollution (Bullen and Walczyk 2009; Foucher *et al.* 2009; Gehrke *et al.* 2011; Yin *et al.* 2013); biochemical and biomedical studies (Bullen and Walczyk 2009; Von Blanckenburg *et al.* 2009); archaeology and bioarchaeological studies (Slovak and Paytan 2011); and last, but not least, food traceability (Kelly *et al.* 2005; Oulhote *et al.* 2011; Drivelos & Georgiou 2012). Until today, the use of the Sr isotopic ratio ($^{87}Sr/^{86}Sr$) has provided the best results for geographical origin issues applied to different food commodities (Fortunato *et al.* 2004; Swoboda *et al.* 2008; Rummel *et al.* 2010; Ariyama *et al.* 2011; Baroni *et al.* 2011; Techer *et al.* 2011; Durante *et al.* 2013).

Sr has four natural stable isotopes with different relative isotopic abundances, namely ^{84}Sr (056 %), ^{86}Sr (9.86 %), ^{87}Sr (7.0 %), and ^{88}Sr (82.58 %); the ^{87}Sr specimen is radiogenic since its abundance increases by the beta radioactive decay (β decay) process of the ^{87}Rb isotope, as illustrated in the following:

$$^{87}\mathrm{Rb} \xrightarrow{\beta} {}^{87}\mathrm{Sr} + e^{-} + \bar{V}_e$$

$$n \xrightarrow{\beta} p + e^{-} + \bar{V}_e$$

In the β decay, a neutron n is converted into a proton p with the emission of an electron e^- and an electron-type antineutrino $\overline{V}_e$ (Banner 2004; White 2013).

^{87}Rb has a half-life of 48.8×10^9 years: three times the estimated age of the universe – $(13.798\pm0.037)\times10^9$ (Planck Collaboration and Tauber 2013) – and roughly 10 times the age of Earth (4.54×10^9 years; Manhes *et al.* 1980; Allègre 1995). There are therefore two main sources of ^{87}Sr contributing to the relative abundance of the isotope in materials: the former related to the initial amount of the element during the universe formation; and the latter formed by the radioactive decay of ^{87}Rb. If the relative isotopic abundance for ^{87}Rb changes over a geological timescale, the relative abundance for ^{87}Sr isotope varies depending on the type and age of the rocks and the Rb concentration (White 2013). In fact, starting from the radioactive decay law, it is possible to write the following equation:

$$\left(\frac{dN}{dt}\right) = -\lambda N \tag{5.2.1}$$

where λ is the decay constant and N the number of nuclides with a finite probability to decay in the time fraction dt.

The decay equation applied to the radiogenic ^{87}Rb/^{87}Sr system yields:

$$^{87}\mathrm{Sr} = {}^{87}\mathrm{Sr}_{t=0} + {}^{87}\mathrm{Rb}\left(e^{\lambda t} - 1\right). \tag{5.2.2}$$

The above equation is generally written in a normalized form with respect to a "non radiogenic" isotope which, in the case of Sr is usually ^{86}Sr. We therefore have:

$$\frac{^{87}\mathrm{Sr}}{^{86}\mathrm{Sr}} = \left(\frac{^{87}\mathrm{Sr}}{^{86}\mathrm{Sr}}\right)_{t=0} + \frac{^{87}\mathrm{Rb}}{^{86}\mathrm{Sr}}\left(e^{\lambda t} - 1\right) \tag{5.2.3}$$

which represents the fundamental geochemistry equation for Sr (Banner 2004; White 2013). In fact, the equation states that, in a closed system, the ratio ^{87}Sr/^{86}Sr depends on:

1) the isotopic ratio (IR) at time $t=0$ (Earth formation). In the present case, this value corresponds to the Basic Achondritic Best Initial value (Papanastassiou & Wasserburg, 1969)

$$\left(\frac{^{87}Sr}{^{86}Sr}\right)_{t=0} = 0.69899; \tag{5.2.4}$$

2) the ^{87}Rb/^{86}Sr ratio (this value can be approximate to the Rb/Sr ratio);
3) the λ value of ^{87}Rb; and
4) the elapsed time t since Earth formation.

On these bases, as stated by Gast (1960) in his work:

> The isotopic composition of a particular sample, whose history may or may not be known, may be the result of time spent in a number of such systems or environments. In any case the isotopic composition is the time-integrated result of the Rb/Sr ratios in all the past environments. Local differences in the Rb/Sr will,

in time, result in local differences in the abundance of ^{87}Sr. Mixing of material during processes will tend to homogenize these local variations. Once homogenization occurs, the isotopic composition is not further affected by these processes.

Due to its chemical and physical peculiarities, Sr is directly involved in the metabolism of many living organisms as Ca substitute. By comparing the Sr isotopic ratio along the food web, it is possible to observe that the numerical values do not show any meaningful variation; it is currently generally accepted that the element is transferred from the soil through the plant metabolism to the final food product without any detectable/measurable isotopic fractionation (i.e., variation of the relative isotopic abundance; Blum *et al.* 2000; Price *et al.* 2002).

Differences in the isotopic ratio values must therefore be related to the different geomorphological properties of the soils in terms of different type and age of rocks and elapsed time (Horn *et al.* 1993, 1998). We will return later to the topic of the isotopic fractionation issue related to the element uptake and the food chain processes.

5.2.3.1 Traceability Models Based on the Use of $^{87}Sr/^{86}Sr$

The $^{87}Sr/^{86}Sr$ ratio can be successfully used for provenance purposes (Horn *et al.* 1998; Fortunato *et al.* 2004; Swoboda *et al.* 2008; Rummel *et al.* 2010; Ariyama *et al.* 2011; Baroni *et al.* 2011; Techer *et al.* 2011; Durante *et al.* 2013). Among the different application fields, the agri-food sector for both plant and animal products has been at the centre of several studies aimed at identifying the geographical origin of the investigated products. In this case, several problems have to be considered in order to develop models for geographical origin of foods for their on-site application. In particular, accuracy/precision of the measured data, that is, the isotopic ratios, and the bioavailability of the element under investigation are two of the several aspects that probably play a fundamental role.

As far as the accuracy and precision of the measures are concerned with, it must be stressed that they might be dependent on several factors. On the one hand, there are problems arising from the instrumental reading procedure that can be summarized by the mass-bias term while, on the other hand, we have the problem of isobaric interference due to the presence of double-charged ions ($^{168}Yb^{2+}$, $^{168}Eu^{2+}$, $^{172}Yb^{2+}$, $^{174}Yb^{2+}$, etc.), charged polyatomic adducts ($^{42}Ca_2^+$, $^{44}Ca^{40}Ar^+$, $^{46}Ca^{40}Ar^+$, $^{44}Ca_2^+$, etc.), and other isotopes with the same mass to charge (m/z) ratio ($^{87}Rb^+$) that must be removed by chemical procedures. For the sake of clarity, we will only consider the inductively coupled plasma mass spectrometry instrumental technique with multi-collector detector (MC-ICP/MS) here, due to its high precision and accuracy of the data, in addition to the thermal ionization mass spectrometry (TIMS) technique (Smith 2000).

The fundamental prerequisite to perform isotopic ratio determination is to produce ions of the target element. For a long time, TIMS has been considered as the only analytical technique capable of providing a sufficient precision (and also accuracy) in evaluating a small variation of the relative abundance of heavy element isotopes. With this instrument, internal precision down to 0.005% RSD (relative standard deviation) can be attained for different isotopic patterns (Rehkämper *et al.* 2001; Balcaen *et al.* 2010). Although its low-energy dispersion of the formed ions during the element

ionization is a point of strength of TIMS in obtaining state-of-the-art data, its time-dependent fractionation process is a problem more complicated to solve than in MC-ICP/MS, where mass fractionation phenomena depend (in theory) only on the mass difference of the measured ions.

With the recent introduction of the modern multi-collector double-focusing ICP/MS instruments (www.nu-ins.com, www.thermoscientific.com/content/tfs/en/home.htlm), researchers can tackle problems involving the study of systems that undergo MDF or MIF processes with minimal variation in the ion relative abundances (Maréchal *et al.* 1999; Bergquist and Blum 2007; Yang *et al.* 2008) MC-ICP/MS instruments are able to simultaneously monitor the intensity of several ions with different mass-to-charge values generated by the plasma source that operates at a steady state, and therefore with no time-dependent fractionation of one ion relative to another. Furthermore, the coupling of an electric and a magnetic sector, double-focusing mass analyzer scheme, in addition to a multi-faraday cup as ion detector, allows isotope ratios to be determined with an internal precision down to 0.002% RSD (Rehkämper *et al.* 2001; Balcaen *et al.* 2010).

However, if the measurement precision is mainly related to the goodness of the hardware project, the accuracy of the analytical datum is conversely dependent on the instrument performance, the applied analytical method, and the mathematical equations used for mass bias and interference correction.

5.2.3.2 Mass Bias

The main characteristic of inductively coupled plasma (ICP) source mass spectrometry techniques is the huge mass bias of the transmitted ions. Mass bias is a phenomenon related to the preferential extraction and transmission of heavier ions (that are more focused within the ion-jet) with respect to the lighter ones (that are localized borderline in the ion-jet), Figure 5.2.1 (Tanner 1992; Niu & Houk 1996; Ingle *et al.* 2003), due to the presence of "space charge effects."

Space charge effects are responsible for the large mass fractionation observed in the transmission of the ions, and many authors have pointed out the importance of controlling this phenomenon during measurements (Vance & Thirlwall 2002; Archer & Vance 2004). In particular, space charge effects mainly take place in the plasma interface between the sample and skimmer cones due to the expansion of the neutral plasma (ions + electrons) and inside the electronic lenses system when only positively charged particles remain in the ion jet (Tanner 1992; Niu & Houk 1996; Ingle *et al.* 2003). Whatever the origin and entity of the instrumental mass bias, the induced mass fractionation must be carefully controlled (e.g., by maintaining as constant as possible the element concentration and the sample matrix for isotope ratio measurements) in order to obtain accurate and precise data. These latter considerations are of general use in analytical chemistry, but assume a peculiar relevance in mass spectrometry isotope ratio determinations (Ingle *et al.* 2003).

Furthermore, the entity of the instrumental mass fractionation is "instrument related;" it depends on the "age" of the different apparatus directly involved in the measurements such as the sampling and skimmer cones (generally the sensitivity decreases with increasing time of use of the cones), the detector performances (Faraday cup efficiency), and so on. In the case of long measuring sessions, particular strategies, such as the bracketing technique, should be adopted to compensate for the instrument drift as well (Albarède *et al.* 2004).

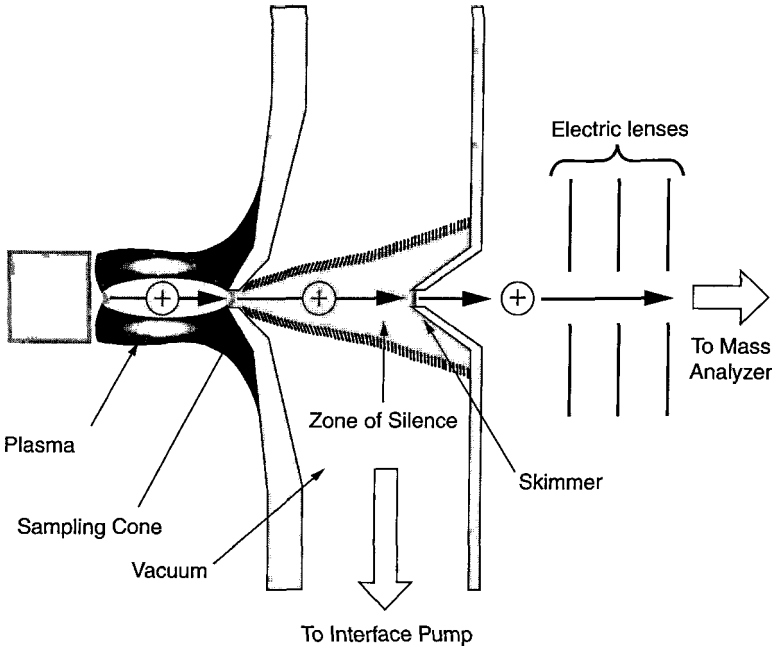


Figure 5.2.1 Schematic representation of the ICP sample introduction interface.

5.2.3.3 Correction of the Mass-Dependent Bias

To correct the isotopic ratio measurement for the mass-dependent instrumental bias, different approaches may to be adopted for the different isotopic systems, depending on for example the presence of isobaric interferences or the availability of any certified matrix/standard isotopic reference material. The correction of the mass bias can be determined from either the external or the internal standardization approaches (Ingle *et al.* 2003; Albarède *et al.* 2004).

The former method is applied when the isotope ratio of interest is measured in a standard solution and the calculated bias is then used to correct the same ratio in the sample. This approach allows the mass bias to be measured at the same masses as the analyte, but over a different time scale and on a different sample matrix.

Conversely, in the latter method the mass bias is determined in the sample solution either by adding a neighboring element with a known isotope ratio (isotopic standard) or, if possible, by using a couple of isotopes of the investigated analyte element that are invariant in nature. The mass-bias correction is input to a mathematical model optimized in order to calculate the analyte isotope ratio. The internal standardization provides a continuous monitoring of all the ions, so the mass bias is averaged over both the matrix and time. Note, however, that the internal standardization is not completely accurate since several inaccuracies might be introduced; for example, the masses of the isotopes used to calculate the bias may be different with respect to the target analyte or may have different ionization characteristic and/or isotopic abundances.

The mathematical models used for bias compensation and isotope ratio calculation based on linear, power, or exponential functions (Maréchal *et al.* 1999; Ingle *et al.* 2003;

Albarède *et al.* 2004). An in-depth discussion of these models is a topic beyond the scope of this manuscript and the reader is encouraged to approach these items by consulting the specialized literature (Taylor *et al.* 1995; Maréchal *et al.* 1999; Ingle *et al.* 2003; Albarède *et al.* 2004); here we only provide the basic equations for each model. The selection of one model over another is mainly related to the particular combination of isotopic system as well as the sample matrix rather than its mass-bias correction capabilities. Nevertheless, several studies have shown that the exponential law provides a more consistent correction than the linear or the power law (Russell and Papanastassiou 1978; Maréchal *et al.* 1999).

The starting equations for the three main models used to correct the mass-dependent fractionation phenomena are as follows:

$$\text{Linear model}: \frac{r_i}{R_k} = 1 + \delta(M_i - M_k) \tag{5.2.5}$$

$$\text{Power model}: \frac{r_i}{R_k} = g^{(M_i - M_k)} \tag{5.2.6}$$

$$\text{Exponential model}: \frac{r_i}{R_k} = \left(\frac{M_i}{M_k}\right)^{\beta} \tag{5.2.7}$$

where r_i and R_k indicate the measured and true isotopic ratio, M_i and M_k are the masses of the i and k isotopes ($M_i = M_k + \Delta m$) with the convention that the ratios have the reference isotope at the denominator. The terms δ, g, and β are the mass-bias factors for the linear, power, and exponential law, respectively. Equations (5.2.5–5.2.7) represent the starting point for developing the mathematical routines for the mass-bias correction using the internal/external standardization approaches.

The internal standardization procedure with the use of an isotope couple of the analyte of interest is widely applied to the Sr (Barbaste *et al.* 2002; Nowell *et al.* 2003; Fortunato *et al.* 2004; Balcaen *et al.* 2005) and Nd (Thermo Application Note 30111; Thirlwall 1991; Nowell *et al.* 2003) isotopic systematics. In the case of Sr, determination of $^{87}Sr/^{86}Sr$ is generally "normalized" by using the $^{88}Sr/^{86}Sr$ ratio that is assumed to be constant and therefore may be used for mass-bias correction (assuming that the fractionation factors for $^{87}Sr/^{86}Sr$ and $^{88}Sr/^{86}Sr$ are equal).

On the other hand, the conventional paradigm that no significant mass-dependent isotopic fractionation is expected for alkali and alkaline earth elements that commonly bond ionically (Johnson *et al.* 2004) or for elements that are heavy and where the mass difference between the heavier and the lighter isotope is small is undergoing a major shift. The currently accepted view is that the chemical, physical, and biological processes that take place at normal environmental conditions result in measurable variations in the isotopic ratios of heavy elements due to the increased performances of modern MC-ICP/MS instruments. For this reason, the assumption of the constancy of particular isotopic ratios is becoming a constraint that may reduce the accuracy of the isotopic determinations. For instance, regarding the Sr isotopic pattern, several

authors (Yang *et al.* 2008) have also reported variations in the $^{88}Sr/^{86}Sr$ ratio. In this the case, a different mass-bias correction procedure is required.

The simplest way to work out the non-constancy of the internal isotope ratio for the mass-bias correction is the use of couple of isotopes of another element which are added to the sample; for example, Zr is used to correct the mass bias for Sr (Yang *et al.* 2008) and Tl for Pb and Hg (White et *al.* 2000; Foucher and Hintelmann 2006; Bergquist & Blum 2009).

This procedure, as reported in earlier works, has some drawbacks since it is necessary to postulate or assume identical mass-bias correction factors between the analyte and the standards. Although this assumption has been widely used (Maréchal *et al.* 1999; White *et al.* 2000; Foucher and Hintelmann 2006; Cloquet *et al.* 2008; Yang *et al.* 2008; Bergquist and Blum 2009; Durante *et al.* 2013; Irrgeher *et al.* 2013), an alternative approach was recently proposed to bypass the former limits. Briefly, starting from the exponential model applied to both the analyte and the internal standard and, after a simple rearrangement, it is possible to write:

$$\ln r^{i/k} = \ln R^{i/k} + \left[\frac{\beta^{i/k}\ln\left(\frac{M_i}{M_k}\right)}{\beta^{j/l}\ln\left(\frac{M_j}{M_l}\right)}\right]\ln r^{j/l} - \left[\frac{\beta^{i/k}\ln\left(\frac{M_i}{M_k}\right)}{\beta^{j/l}\ln\left(\frac{M_j}{M_l}\right)}\right]\ln R^{j/l} \tag{5.2.8}$$

where $r^{i/k}$ is measured isotopic ratio of the analyte; $R^{i/k}$ is mass-bias-corrected isotopic ratio for the analyte; M_i and M_k are atomic masses of the analyte isotopes i and k; $r^{j/l}$ is the measured isotopic ratio of the internal standard; $R^{j/l}$ is the mass-bias-corrected isotopic ratio for the internal standard; M_j and M_l are the atomic masses of the analyte isotopes j and l; and $\beta^{i/k}$ and $\beta^{j/l}$ are the mass-bias factors for the analyte and the internal standard, respectively.

Equation (5.2.8) can be easily resolved by applying a linear regression model with the term $\ln r^{i/k}$ as the dependent variable Y and the term $\ln r^{j/l}$ as the independent variable X. By plotting the $\ln r^{i/k}$ term against the $\ln r^{j/l}$ it is possible to obtain the slope s as:

$$s = \frac{\beta^{i/k}\ln\left(\frac{M_i}{M_k}\right)}{\beta^{j/l}\ln\left(\frac{M_j}{M_l}\right)} \tag{5.2.9}$$

and the intercept *int* as:

$$\text{int} = \ln R^{i/k} - \left[\frac{\beta^{i/k}\ln\left(\frac{M_i}{M_k}\right)}{\beta^{j/l}\ln\left(\frac{M_j}{M_l}\right)}\right]\ln R^{j/l}. \tag{5.2.10}$$

Accuracy and precision of the data obtained by the regression method are closely related to the constancy of the $\beta^{i/k}/\beta^{j/l}$ term during the measuring session between sample and standards (Albarède *et al.* 2004; Yang 2009). Good results have been

obtained with this approach for several isotopic systems such as: Pb/Tl, Hg/Tl, Zn/Cu, Fe/Ni, and Sr/Zr (Maréchal *et al.* 1999; White *et al.* 2000; Arnold *et al.* 2004; Yang *et al.* 2008; Yang and Sturgeon 2009; Irrgeher *et al.* 2013).

Furthermore, in addition to the mass-dependent fractionation process occurring at the ICP sample interface, some mass-independent phenomena might also arise. Basically, mass-independent fractionation phenomena are due mainly to an incorrect instrumental set-up and/or aged instrument components. The most important causes may be summarized as: imperfect top peak due to incorrect torch position or front slit consumption; incomplete electron suppression; ion beams not perfectly focused; collision of ions in the flight tube; nonlinear response of the Faraday cups (cup + resistor + amplifier), etc. (Albarède *et al.* 2004). Although the existence of these problems may only have a minor influence on the accuracy of the IR data, it is of utmost importance to have a stringent control on the abovementioned variables when the acquisition of high-precision and -accuracy data is a priority.

Many MC-ICP/MS studies stress the importance of keeping the instrumental conditions such as gas flow, electronic lenses potentials, etc. as constant as possible to obtain constant mass fractionation factors (Rehkämper *et al.* 2001).

5.2.4 Bioavailability

The use of some isotopic systems such as Sr, Nd, and Pb for geographical traceability purposes is possible due to the properties of these elements in entering living systems and/or the food web. Among these elements, Sr appears to be the most useful (the isotopic system of Nd is related to that of Sr; for traceability purposes, Sr is preferred since the element concentration in the involved matrices, soil–plant–food, is much higher with respect to Nd).

However, in many cases the knowledge of the local bedrock geology represents only one of the first steps in the development of traceability models. In fact, it has been demonstrated that plants, with their metabolism, average out the $^{87}Sr/^{86}Sr$ isotopic ratio of the soil (Capo *et al.* 1998; Horn *et al.* 1998; Sillen *et al.* 1998; Frei *et al.* 2009; Durante *et al.* 2013).

It is today well known that these discrepancies may be due to the difference between the total strontium amount and its bioavailable fraction (Sillen *et al.* 1998; Blum *et al.* 2000; Price *et al.* 2002). In fact, only the biologically available strontium can be assumed to enter into the food web by the plants roots. To do this, mineral weathering processes are of fundamental importance to vehicle Ca, as a consequence Sr, in the hydrologic system in a bioavailable chemical form for the plant roots uptake. Depending on the starting chemical composition, minerals may weather by dissolving as a function of several variables such as the rock and soil type (thickness and porosity), temperature, and anthropogenic influences (Capo *et al.* 1998; Banner 2004).

Given the occurrence of these complex phenomena, the Sr budget in soils may vary by changing the environmental conditions; hence the evaluation of the bioavailable element fraction by simulating agents may become a quite complex and troublesome task (Tessier *et al.* 1979). Therefore, due to the difficulties arising in the evaluation of the bioavailable/exchangeable Sr fraction in soil, as detailed by other researchers (Capo *et al.* 1998; Sillen *et al.* 1998; Blum *et al.* 2000; Price *et al.* 2002; Durante *et al.* 2013),

reliable results in the development of geographical traceability models can be obtained switching from a passive approach of collecting and analyzing soils to a more active one where the plants do the sampling.

The latter approach presents some major advantages since: (1) the plants roots have direct access to the bioavailable elements reservoirs in soils; (2) the sample uptake of elements (nutrients) can be extended to the neighboring area of the plant; (3) the measured $^{87}Sr/^{86}Sr$ data are the result of the influence of several processes such as weathering reactions (Banner 2004), contaminations produced by the use of fertilizers (Böhlke & Horan 2000; Hosono *et al.* 2007) and the aerosol uptake of leaves; and (4) the sampling procedure may average the isotopic data over extended periods or seasons.

5.2.4.1 Applications

The use of strontium abundance isotope variation as a geographic "tracer" for determining the provenance of food has been growing over the last decade and a huge number of published research papers and reviews (Kelly *et al.* 2005; Drivelos & Georgiou 2012; Zhao *et al.* 2014) attest its potentiality for this purpose. One of the pioneers on this topic is Peter Horn, who exploited the potential of the $^{87}Sr/^{86}Sr$ method for the assignment of the geographical origin of wines (Horn *et al.* 1993, 1998).

Due to technological innovation and instrumental developments, it has been possible to extend the use of this indicator to several foodstuffs including: wines (Di Paola-Naranjo *et al.* 2011; Dehelean *et al.* 2012; Durante *et al.* 2013; Marchionni *et al.* 2013); cheese (Pillonel *et al.* 2003; Fortunato *et al.* 2004); asparagus (Swoboda *et al.* 2008); butter (Rossmann *et al.* 2000); caviar (Rodushkin *et al.* 2007); coffee (Rodrigues *et al.* 2011; Techer *et al.* 2011; Liu *et al.* 2014); cider (García-Ruiz *et al.* 2007); meat (Franke *et al.* 2007; Rummel *et al.* 2010; Baroni *et al.* 2011); milk (Crittenden *et al.* 2007); potatoes (Zampella *et al.* 2011; Adamo *et al.* 2012); tomato (Trincherini *et al.* 2014); rice (Ariyama *et al.* 2011, 2012); and fish (Kennedy *et al.* 2000; Wolff *et al.* 2012). For all these food products, it has been possible to use the Sr isotope ratio for provenance purposes due to its potential as territorial marker as well as the active participation of the metal in the metabolism of living organisms.

Table 5.2.1 lists the scientific literature published in the last ten years based on the use of $^{87}Sr/^{86}Sr$ for geographical origin purposes. In many applications, the direct indicator has been used alone or in combination with other analytes, such as multi-elemental content or the determination of organic compounds and isotope ratios of light elements. From an analytical point of view, the issues related to the development of geographical traceability models for food can be worked out by two different approaches: (1) based on a discriminating approach which aims to differentiate "homologous" food products but of different geographical origin (Kennedy *et al.* 2000; Rossmann *et al.* 2000; Almeida & Vasconcelos 2001; Barbaste *et al.* 2002; Pillonel *et al.* 2003; Fortunato *et al.* 2004; Crittenden *et al.* 2007; Franke *et al.* 2007; Rodushkin *et al.* 2007, 2011; Rodrigues *et al.* 2010, 2011; Rummel *et al.* 2010; Voerkelius *et al.* 2010; Ariyama *et al.* 2011, 2012; Bontempo *et al.* 2011; Goitom Asfaha *et al.* 2011; Wolff *et al.* 2012; Trincherini *et al.* 2014; Zhu *et al.* 2014); and (2) a more systematic approach, objectively linking the food product to its area of origin and discriminating it from the others on the basis of its provenance.

The first approach relies on the use of the properties related to the food; the second approach also considers those related to its territory. In the latter case, analysis of the

Table 5.2.1 Summary of the relevant literature relating to the use of $^{87}Sr/^{86}Sr$ to determine geographical origin of food.

Commodity	Instrument	Geographical origin	Reference
Alpine cheese	TIMS	Italy	Bontempo *et al.* 2011
Asparagus	MC-ICP-SFMS	Austria, Germany, Hungary, Peru, Slovakia, Netherlands	Swoboda *et al.* 2008
Beef	TIMS/MC-ICP-MS	France, Germany, Greece, Ireland, Italy, Spain, UK	Rummel *et al.* 2012
Butter	TIMS	Alpine region, Germany, USA, Netherlands, Argentina, New Zealand, Australia	Rossmann *et al.* 2000
Cabbages	TIMS	China, Korea	Bong *et al.* 2012
Cane, beet sugar	TIMS, MC-ICP-MS	Moldova, Poland, USA, Netherlands, France, Germany, Hungary, Costa Rica	Rodushkin *et al.* 2011
Caviar	MC-ICP-MS	Sweden, Finland	Rodushkin *et al.* 2007
Cereal	TIMS, MC-ICP-MS	Austria, Germany, Denmark, UK, Italy, France, Poland, Ireland, Iceland	Goitom Asfaha *et al.* 2011
Cheese	MC-ICP-MS	Alpine, pre-Alpine, Bretagne, Finland, Canada, Australia	Fortunato *et al.* 2004
Cheese	TIMS	Germany, Austria, Finland, Switzerland, France	Pillonel *et al.* 2003
Ciders	MC-ICP-MS	England, Switzerland, France, Spain	García-Ruiz *et al.* 2007
Coffee	MC-ICP-SFMS	Hawaii islands	Rodrigues *et al.* 2011
Coffee beans	MC-ICPMS	South America	Rodrigues *et al.* 2010
Coffee beans	MC-ICP-MS	Africa, USA, Asia	Liu *et al.* 2014
Coffee beans	TIMS	Réunion Island	Techer *et al.* 2011
Fish	VG-Sector and TIMS	White and West River	Kennedy *et al.* 2000
Fish	MC-ICP-MS	Upper Colorado River Basin (western USA)	Wolff *et al.* 2012

(Continued)

Table 5.2.1 (Continued)

Commodity	Instrument	Geographical origin	Reference
Grain	HR-ICPMS (Element2; ThermoFisherScientific)	Japan, USA, Australia, Canada	Ariyama *et al.* 2011
Grape juice	MC-ICP-MS	Italy	Durante *et al.* 2013
Meat	TIMS	Argentina	Baroni *et al.* 2011
Milk	TIMS	Australia, New Zealand	Crittenden *et al.* 2007
Orange juice	TIMS	Europe, North/South America, Africa	Rummel *et al.* 2010
Peanut seeds/testa	ICP-Q-MS	Japan	Zhu *et al.* 2014
Potatoes	MC-ICP-MS	Italy	Zampella *et al.* 2011; Adamo *et al.* 2012
Poultry, beef	MC-ICP-MS	Brazil, France, Germany, Hungary, Switzerland	Franke *et al.* 2007
Rice	HR-ICPMS	Japan, USA, China, Thailand	Ariyama *et al.* 2012
Rice, barley, wheat	HR-ICPMS single collector	Japan ??	Shinozaki *et al.* 2010
Szegedi paprika	MC-ICP-SFMS	Hungary, Spain, France, Germany	Brunner *et al.* 2010
Tea	MC-ICP-MS	India	Lagad *et al.* 2013
Tomato (berries, passata, etc.)	TIMS	Italy, China	Trincherini *et al.* 2014

Water	MC-ICP-MS	South Korea	Kim *et al.* 2013
Water	MC-ICP-MS	??	Yang *et al.* 2011
Water	–	Denmark	Frei and Frei *et al.* 2011
Water	TIMS, MC-ICP-MS	European water	Voerkelius *et al.* 2010
Wheat	TIMS	Argentina	Podio *et al.* 2013
Wine	TIMS	Italy	Marchionni *et al.* 2013
Wine	ICP-Q-MS	Romania	Dehelean *et al.* 2012
Wine	q-ICPMS	South Africa	Vorster *et al.* 2010
Wine	ICP-MS	Portugal	Almeida and Vasconcelos 2004
Wine	ICP-MS	Portugal, France	Almeida and Vasconcelos 2001
Wine	TIMS	Argentina	Di Paola-Naranjo *et al.* 2011
Wine	ICP-SF-MC-MS	Chile, California, Madeira, Chablis, St Emillion, Australia, Portugal, South Africa	Barbaste *et al.* 2002

matrices that come from the territory, namely soils, water, plants, etc., is unavoidable (Swoboda *et al.* 2008; Brunner *et al.* 2010; Baroni *et al.* 2011; Di Paola-Naranjo *et al.* 2011; Frei and Frei 2011; Techer *et al.* 2011; Zampella *et al.* 2011; Bong *et al.* 2012; Durante *et al.* 2013; Kim *et al.* 2013; Marchionni *et al.* 2013; Podio *et al.* 2013; Petrini *et al.* 2014). On these bases, it is possible to assume that it is the territory that helps to shape the fingerprint of the product. An *a priori* systematic study of the performance of the Sr isotope ratio within the factors that characterize the "production cycle" of a food, soil–water–plant–fruit–raw materials–food finished, is therefore the starting point in developing a model for the geographical origin of food. In this case, it is possible to establish a unique link between the final product and its territory of origin. On the other hand, more structured considerations have to be made for food of animal origin, such as beef and cheese. For instance, the strontium isotopic composition of animal tissues only reflects to some extent that of the vegetation, animal feed, water consumed, and territory of origin. In fact, variations may occur when animals switch between areas during fattening or in response to the use of supplements in animal feed. In this case, different parameters need to be taken into account in order to consider the association between animal and its growing area.

The reader is referred to the references in Table 5.2.1 for further detailed information about the numbers of samples analyzed, the analytical methods, and chemometrics techniques used in the different applications.

From an analytical point of view, the experimental approach mainly consists of the following steps: (1) develop a suitable analytical method for the samples digestion and/or for the extraction of the bioavailable Sr fraction of soils; (2) perform the separation of Sr from the matrix if Rb is present, to avoid any isobaric interferences; and (3) convert the collected data to the corresponding isotope ratio values by means of the appropriate mathematical corrections for the mass bias and spectroscopic interferences present, especially important when aiming at obtaining very accurate measurements with multi-collector ICP-MS.

For the development of a geographical traceability model, there are some aspects that need to be considered before the implementation of the analytical methodology: the representativeness of the sampling procedure (Totaro *et al.* 2013); the local geochemistry of the soil (Price *et al.* 2002); the fertigation (applying fertilizer through irrigation) practices (Green *et al.* 2004; Hosono *et al.* 2007); and the food-making processes. The obtained model is therefore strictly related to the reality of where the food comes from.

In the following sections two different examples are presented and discussed in order to highlight the powerful properties of a $^{87}Sr/^{86}Sr$-based geographical traceability model. Both the examples present the results of distinct pilot studies aimed at developing geographical traceability models for two typical Italian food products, namely Ligurian PDO extra virgin olive oil and Lambrusco PDO wines. Different aspects will be tackled, in particular the representativeness of sampling, the variability of the investigated indicators along the production chain, and the influence of the making procedure. All these aspects are relevant for the development of isotopic maps able to characterize a food/territory as well as to discriminate a sample as a function of its geographical origin. $^{87}Sr/^{86}Sr$ values in all samples have been determined by using a double-focusing magnetic sector multi-collector ICP mass spectrometry. The straightforward methods, the high sample-throughput, and the high precision and accuracy of the measurements are some of the advantages which allow a huge amount of samples to

be processed; at the same time, the increased analytical production allows the use of chemometric tools of data analysis.

5.2.4.2 Case Study: Extra Virgin Olive Oils

This work summarizes the results of a research project focused on the development of a geographical traceability model for Ligurian extra virgin olive oil, which received the protected designation of origin (PDO) in 1997 (Regulation (EC) No. 123/97; Decree of 23 August 2011).

Extra virgin olive oil (EVOO) belongs to the superior olive oil category; by regulation, it can be solely obtained from the fruits of olive tree *Olea Europaea* by mechanical tools (Commission Regulation (EU) Nos 61/2011, 182/2009, 2568/91). EVOO is one of the main components of the Mediterranean diet, and was declared Intangible Cultural Heritage by UNESCO thanks to its various health benefits. The healthy effects can be mainly found in the unique chemical composition that characterizes the EVOO. Its unique properties are influenced by several parameters such as the type of cultivar, the collection period, agricultural practices employed in the field, the territory of origin, and the technologies used for the processing stages. Chemical and physical parameters of Ligurian EVOO are defined in the specific Commission Regulation and sensory examination is used for grading the virgin olive oils by means of selected trained tasters operating as a panel test (Commission Regulation (EC) No 796/2002, International Olive Oil Council Doc. No 15, 2013).

In the context of food research and control, authentication (Marcos Lorenzo *et al.* 2002; Guimet *et al.* 2005; Peña *et al.* 2005; Cosio *et al.* 2006; Alonso-Salces *et al.* 2010; Dais and Hatzakis 2013) and geographical traceability (Cajka *et al.* 2010; Camin *et al.* 2010; Casale *et al.* 2010; Mannina *et al.* 2010; Bevilacqua *et al.* 2012; Perri *et al.* 2012) of EVOO are of utmost importance considering both the high added value of the product and the consequent issues related to possible adulteration and/or frauds. First and foremost, a great volume of research has been focused on evaluating the inter- and intra-relationship between the sensorial parameters and the concentration of organic compounds (Angerosa *et al.* 2004; Zunin *et al.* 2004; Ollivier *et al.* 2006; Kalua *et al.* 2007; López-Feria *et al.* 2007; Panagou *et al.* 2008; Cajka *et al.* 2010; Gómez-Coca *et al.* 2012) given the importance of the these parameters in the estimation of the quality of an EVOO sample. Nevertheless, it has been proven that the concept of authenticity is rather complex, since it includes many factors ranging from adulteration detection to the monitoring compliance with the traditional protocols and the geographical origin.

Aware of these aspects, many analytical methodologies have been proposed mainly based on: DNA markers (Perri *et al.* 2012); trace elements (Benincasa *et al.* 2007; Cabrera-Vique *et al.* 2012); stable isotopes (Camin *et al.* 2010; Chiavaro *et al.* 2011); and tools for acquiring a "fingerprint" of the product, from blind analysis techniques such as electronic nose, near infrared (NIR) spectroscopy (Christy *et al.* 2004; Bendini *et al.* 2007; Galtier *et al.* 2007; Casale *et al.* 2010; Mannina *et al.* 2010; Haddi *et al.* 2011; Bevilacqua *et al.* 2012). However, as suggested by different authors (Janin *et al.* 2014), the next step toward authenticity/traceability for EVOO is the possibility of linking the product to its territory of origin and consequently to develop an analytical methodology able to identify, instead of discriminate, the geographical origin of EVOO.

In this context, to the authors' knowledge, this work represents the first attempt to systematically study the $^{87}Sr/^{86}Sr$ variability along the soil–branch–olive–olive-oil chain in order to develop a robust geographical traceability model. Indeed, 16 Ligurian olive farms belonging to the *Consortium di Tutela Olio d'oliva PDO Riviera Ligure* were investigated. For each of the different producers, samples of soil, branch, and olives from the plants grown near the sampled soil were collected. In addition, an aliquot of the produced olive oil was also taken. This study has been focused on the determination of ^{87}Sr /^{86}Sr in all the collected matrices in order to: examine any trend between several farms (inter-site) and within the same farm (intra-site); check for the variability of the investigated isotopic ratio along the production chain (soil–plant–fruit); and develop isotopic maps representative of the area of production of Ligurian extra virgin olive.

5.2.4.2.1 Sampling and Sample Treatment

In order to develop a strontium isotopic map of Liguria region, it was necessary to plan a representative sampling in terms of number of farms, extension of the cultivation sites, pedological/geological features of territory, and the production of olive oil. A collaboration with the Settore Servizi alle Imprese (Sector Services to Agricultural Enterprises) and the Consorzio di Tutela Olio d"oliva PDO Riviera Ligure was important in the selection of representative farms.

In the Liguria region, there are 13419 companies involved in olive production covering an area of about 11 000 hectares (about 25% of the regional total utilized agricultural area or UAA). A detailed analysis of these data shows that the percentage of land used for this type of cultivation is about 44% for Imperia district, 20% for Savona, and around 16% for the provinces of Genoa and La Spezia.

In the case of Ligurian EVOO, according to the different environmental conditions and cultivation, the production regulation (Decree of 23 August 2011) identifies three areas of production:

1) Riviera dei Fiori, covering the whole territory of the district of Imperia (68 municipalities), with an olive production area of approximately 2063 hectares.
2) Riviera del Ponente Savonese, including 48 municipalities in the district of Savona, with a production area of approximately 106 hectares.
3) Riviera di Levante, comprising 39 and 24 municipalities of the districts of Genoa and La Spezia, respectively, with a total area of approximately 172 hectares.

For the aims of the project, 16 farms (Fig. 5.2.2) were investigated: 7, 5 and 4 farms in each of the districts of Imperia, Savona, and La Spezia, respectively.

In accordance with the above reported information, the Imperia district plays a relevant role within the utilized agricultural area (UAA) with the largest number of companies. For each of the selected districts, the selection of individual producers was made on the basis of the extension of the respective cultivation area, EVOO production, and geographical location. A variable number of soil samples (from three to four) was collected at each farm, depending on the total extension of the cultivated field. Soils, branches, and olive samples were collected as described in the following sections.

Information about sampling – number of collected samples, geographical position, and GPS coordinates – is reported in Table 5.2.2.

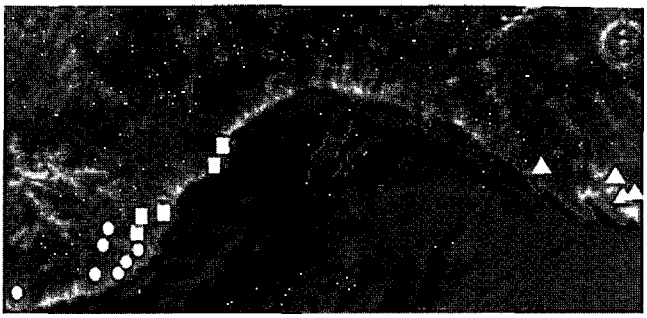


Figure 5.2.2 Farms investigated for the development of a traceability model for Ligurian PDO extra virgin olive oil: Imperia district producer (circle), Savona producer (square), and La Spezia district farms (triangle).

Table 5.2.2 Number of soil, branch, and olive samples collected, location of the farm, and GPS coordinate.

Farm	Soil samples	Branch samples	Olive samples	GPS coordinate
Imperia, Riviera dei Fiori				
A	5	5	5	43° 49.661' N, 007° 36.999' E
B	5	5	5	43° 55.347' N, 008° 02.513' E
C	4	4	4	44° 00.475' N, 007° 57.780' E
D	4	4	4	43° 53.489' N, 007° 55.282'E
E	5	5	5	43° 58.091' N, 008° 05.574' E
F	4	4	4	43° 52.951' N, 008° 00.976' E
G	3	3	3	43° 57.561' N, 007° 59.194' E
Savona, Riviera del Ponente Savonese				
H	4	4	4	44° 03.306' N, 008° 10.892' E
I	5	5	5	44° 02.091' N, 008° 05.620' E
L	4	4	4	44° 10.652' N, 008° 22.098' E
M	4	4	4	44° 13.902' N, 008° 25.034' E
N	3	3	3	44° 00.458' N, 008° 04.462' E
La Spezia, Riviera di Levante				
O	4	4	4	44° 05.740' N, 009° 59.240' E
P	4	4	4	44° 10.356' N, 009° 38.606' E
Q	4	4	4	44° 06.437' N, 010° 00.614' E
R	4	4	4	44° 09.326' N, 009° 56.611' E

5.2.4.2.2 Soil Sampling and Treatment

Soil samples were dug using a single-gauge auger set to avoid disturbing samples at a maximum sampling depth of 100 cm. The first 10 cm of the obtained core sample was discarded. The collected soils were properly pre-processed. Briefly, 100 g of each soil

sample was dried and pre-processed to a final average particle size within the 200–300 μm range using a centrifugal mill. To determine the Sr bioavailable fraction in soil, an extraction procedure with 1 mol L^{-1} NH_4NO_3, DIN 19730 (Hall *et al.* 1998) was used. At the end of the extraction process, each solution was filtered with cellulose acetate membrane (pore size 0.20 μm) into a 30 mL PFA bottle (Durante *et al.* 2013).

For each sample, strontium concentration was determined in order to dilute the solution to achieve a final Sr value as close as possible to 200 μg kg^{-1} with HNO_3 8 mol L^{-1}, before the Sr/Rb separation. On the basis of previous results, the best precision in instrumental Sr isotope ratio measurements is achieved when this Sr concentration is used. On the other hand, according to the chemical/physical properties of the resin (Horwitz *et al.* 1992), the maximum retention capability of the complex Sr-resin is achieved when working with a HNO_3 8 mol L^{-1} solution.

5.2.4.2.3 Branch and Olive Treatment

The sampling of branches and olives was carried out from the olive trees grown in proximity to the collected soils. Olive and branch dissolution was performed on a maximum sample aliquot of 1 g for both the matrices, accurately weighed in the MW (microwave) vessels, before adding 6 mL HNO_3, 1 mL H_2O_2, and 3 mL H_2O. Between each cycle of mineralization, a washing cycle (Durante *et al.* 2013) was always carried out.

At the end of the digestion process, an almost colorless, pale yellow, sample was obtained and each solution was transferred into PFA bottles; the content of strontium was accurately determined and, finally, samples were diluted in order to obtain a final strontium concentration of 200 μg kg^{-1} with HNO_3 8 mol L^{-1}.

5.2.4.2.4 Analytical Determination: Strontium Isotope Ratio

The analytical determination of strontium isotope ratio mainly consists of: (1) mineralization of the organic matrices (branches and olives); (2) extraction of the bioavailable element fraction from soils; (3) determination of strontium content; (4) Rb/Sr separation procedure; (5) Sr isotope ratio measurements; and (6) mass-bias correction and data reduction.

As far as the separation procedure is concerned, the $^{87}Sr/^{86}Sr$ determination could be mainly influenced by the presence of the isobaric interference of ^{87}Rb. This interference is usually minimized through a matrix simplification procedure, which generally involves a SPE (solid phase extraction) separation whose specificity and selectivity for strontium is dependent on the HNO_3 concentration (Horwitz *et al.* 1992). The separation procedure used in this work, also dependent on the matrix to be analyzed (i.e., elemental and strontium content), has previously been optimized (Durante *et al.* 2013). Briefly, the following steps were performed: (1) loading and washing of the resin with high-purity water; (2) resin activation with HNO_3 8 mol L^{-1}; (3) sample loading and elution of interference with HNO_3 8 mol L^{-1}; and (4) recovery of Sr with high-purity water.

Finally, a proper aliquot of HNO_3 65% w/w was added to all the water eluted fractions to obtain a final HNO_3 solution as close as possible to 4% w/w.

Regarding Sr isotope measurements, a [blank/sample/blank/standard/blank] bracketing sequence was adopted in order to check for any instrumental drift. NIST SRM 987, $SrCO_3$, certified for its Sr isotopic composition ($^{87}Sr/^{86}Sr$ certified value of 0.71034 ± 0.00026; Moore *et al.* 1982) and a "generally accepted" value of 0.71026 ± 0.00002

(Stein *et al.* 1997), the uncertainty expressed as twice the standard deviation (2*s*), was used for the bracketing procedure.

The average of the measured intensities of bracketing-blanks (HNO_3 4% w/w) was calculated and subtracted from the measured intensities of the respective standards or samples. The strontium isotope ratios of standards and samples were then calculated according to the mathematical procedures for the isobaric interferences and mass-bias corrections (Durante *et al.* 2013). During the different instrumental sessions, the $^{87}Sr/^{86}Sr$ values obtained for NIST SRM 987 were used for the evaluation of the accuracy and precision of the instrumental measurements.

5.2.4.2.5 Instrumentation

A Pulverisette 14 rotor mill, supplied by Fritsch, ECO Scientifica, Milan, Italy, equipped with a Titan 12 ribs rotor, a 500 μm trapezoidal perforation sieve ring and a Teflon-coated collecting pan, working at 16 000 rpm, was used to pre-process the soil samples.

Olive and branch dissolution was achieved using an Ultrawave digestion system (Milestone, Inc.) equipped with five × 20 mL quartz vessels. The microwave instrumental parameters and conditions used for olives and branches are reported in Tables 5.2.3a and 5.2.3b, respectively. The Ultrawave apparatus consists of a single reaction chamber where samples are placed for microwave digestion under high-temperature and high-pressure conditions.

Strontium concentrations in the soil and branch solutions were determined by using an atomic absorption spectrometer (AAS; Varian, Spectra AA 220FS) equipped with the introduction/dilution system SIPS 10, provided by the same company. A quadrupole inductively coupled plasma mass spectrometer (ICP-qMS; XSeries II, ThermoFisher Scientific) was used for the analysis of olive solutions. For more information about the instrumental parameters and experimental conditions, the reader is referred to a previously published manuscript (Durante *et al.* 2013).

Strontium isotope ratio measurements were acquired via a MC-ICP-MS spectrometer (Neptune, ThermoFinnigan, Bremen, Germany). The instrument consists of a double-focusing, multi-collector mass spectrometer with a forward Nier–Johnson

Table 5.2.3a Microwave digestion program used for the mineralization of olive samples

Step	Power (W)	Temperature (°C)	Ramp (min)	Pressure (bar)	Time at temp (min)
1	1500	100	15:00	160	00:00
2	1500	130	10:00	160	00:00
3	1500	200	10:00	160	10:00

Table 5.2.3b Microwave digestion program used for the mineralization of branch samples.

Step	Power (W)	Temperature (°C)	Ramp (min)	Pressure (bar)	Time at temp (min)
1	1500	100	5:00	160	00:00
2	1500	200	15:00	160	05:00

geometry. The instrument is equipped with nine Faraday collectors (eight movable and a central fixed collector). Data acquisition, performed in low-resolution mode, was simultaneous for all measured ion masses. All the instrumental parameters are reported in a previous study (Durante *et al.* 2013). Due to the low Sr concentration in the olive samples ($< 100\,\mu g\,kg^{-1}$), the measurements of $^{87}Sr/^{86}Sr$ were performed with the aid of a desolvating apparatus (APEX-IR, manufactured by Elemental Scientific Inc.) in order to enhance the instrumental sensitivity.

5.2.4.2.6 Accuracy and Precision Evaluation

The accuracy and precision of the experimental procedure were monitored on the NIST SRM 987 solution at $180\,\mu g\,kg^{-1}$ Sr, analyzed several times in each measurement sequence. All the monitored intensities were subjected to the correction procedures, that is, subtraction of the blank, interference, and mass-bias correction (Durante *et al.* 2013).

The results of the daily measurement sequence yield an indication of short-term stability and repeatability, while the entire dataset gives a measure of the long-term reproducibility. All results – the average of the $^{87}Sr/^{86}Sr$ values of the SRM 987 measured daily, repeatability and reproducibility – are expressed as percent relative standard deviation (RSD%) and reported in Table 5.2.4.

Considering the uncertainty given by the standard deviation, all the average values lie within the range of the NIST certified value (0.71034 ± 0.00026) and are in excellent agreement with the commonly accepted high-precision value of 0.71026 ± 0.00002. Furthermore, optimum results have been achieved in terms of long-term stability (i.e., all working sessions) obtaining an RSD% value of 0.001%.

Moreover, to test the entire analytical procedure (namely pretreatment process, Sr/Rb separation, measurements, etc.) an internal control sample (a branch sample) was repeated for each batch of measured samples. In particular, the control sample was repeated six times, obtaining an average value and a standard deviation (s) of 0.70925 and 0.00001, respectively. The obtained uncertainty, expressed as $2s$ (0.00002), perfectly matches the attainable instrumental precision for this element. Since all samples were generally once prepared and measured, the uncertainties associated with the final results are expressed as that of the control sample: $2s = 0.00002$.

5.2.4.2.7 $^{87}Sr/^{86}Sr$ for Soil, Branch and Olive Samples

Before dealing with the development of Sr isotopic maps, different aspects needed to be tackled: (1) the trend of the investigated isotopic ratio along the production chain (soil–plant–fruit); (2) the differences present among the investigated farms (inter-site variability);

Table 5.2.4 Average $^{87}Sr/^{86}Sr$ values for NIST SRM 987 obtained for each measurement day with respective to relative standard deviation (RSD). Average $^{87}Sr/^{86}Sr$ and corresponding RSD values for all the working sessions are reported in the last column of the table.

SRM 987	1-seq	2-seq	3-seq	4-seq	5-seq	All-seq
Average	0.710257	0.710262	0.710270	0.710270	0.710274	0.710267
RSD%	0.002	0.001	0.0005	0.0007	0.0008	0.0009

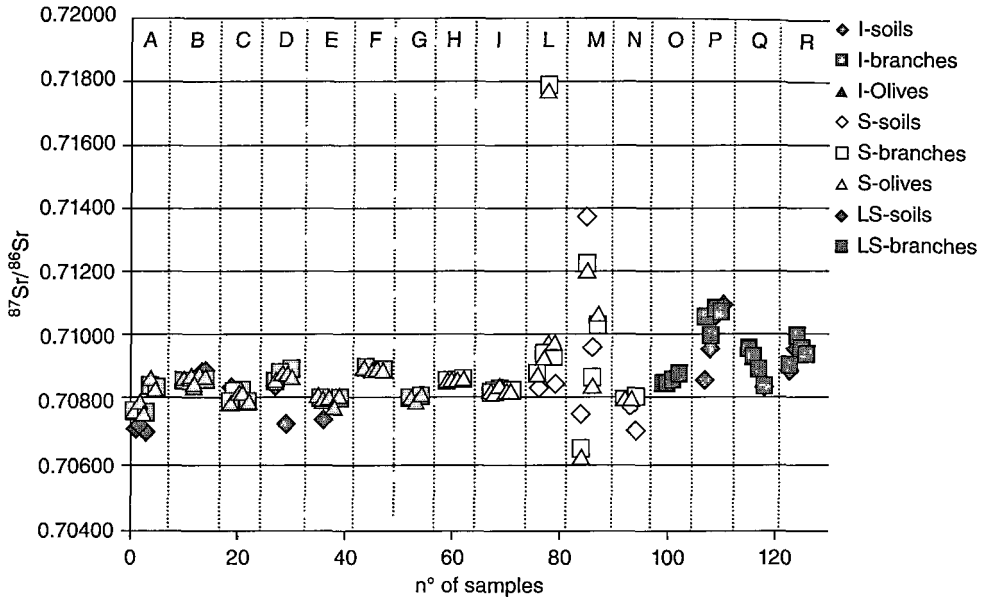


Figure 5.2.3 Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different soil fractions extracted with NH_4NO_3, the different branches, and olive samples from the EVOO farms.

and (3) the differences among sampling sites within the same farm (intra-site variability). The values of ^{87}Sr /^{86}Sr for branches, olives and soils are therefore reported in Figure 5.2.3. Producers within the classes A–G, H–N, and O–R are located within the Riviera dei Fiori (Imperia), Riviera di Ponente (Savona), and Riviera del Ponte di Levante (La Spezia) zones, respectively.

Considering the variability of $^{87}Sr/^{86}Sr$ in the soil–olive–branches system, the dataset indicates a good match between the matrices belonging to the same chain/sampling site for most of the producers (B, C, F, G, H, I, O, Q, R). In fact, in several cases the $^{87}Sr/^{86}Sr$ values of olives perfectly overlap those of the respective soils and branches. However, it is worth noting that there are minor correspondences between the values obtained for the soils and the respective olives samples for some of the sites from producers A, D, E, L, M, N, P. In particular, in most of these cases the values of soil samples appear to be lower than that of the respective branches and olives. These differences could be due to several factors such as: (1) the performed sampling (olives, when present, were sampled from different trees grown as close as possible to the site of respective soils sample); (2) the extracted soil fraction, which could not perfectly mimic the bioavailable fraction; and (3) the practices of fertilization/irrigation used by the producers which, as has been shown (Hosono *et al.* 2007), could affect the final $^{86}Sr/^{87}Sr$ value by as much as 25%. However, for these sites the differences between $^{86}Sr/^{87}Sr$-branches and $^{86}Sr/^{87}Sr$-olives are minor (sometimes not statistically representative) compared with the differences between $^{86}Sr/^{87}Sr$-soil and $^{86}Sr/^{87}Sr$-olive. The former comparison therefore shows a greater and relevant direct link between plants and olive than the latter comparison of soil to olive.

Taking into account $^{86}Sr/^{87}Sr$ data within the soil samples of the same farm (intra-site variability), the obtained values highlight the presence of a reasonable heterogeneity for

producers A, D, E, N, P, Q, and R. For instance, the field belonging to producer A has three sites with a lower isotopic value with respect to the other sites. In particular, considering the uncertainty associated with the obtained isotopic values, the difference between the two groups is statistically significant; it is around 0.001 for both soils and branches and around 0.0001 for olives. Generally speaking, these discrepancies could be attributed to the heterogeneity that characterizes the geological and lithological features of the Liguria region. In fact, the morphology and geology of this region, whose borders are shared with the Ligurian Alps and the Ligurian Apennines in the north and with the Ligurian Sea in the south, is discontinuous due to the orogenic events that led to its formation. In particular, the Liguria region is mostly a mountainous and hilly area; there is no real plain, but rather a modest coastal plain. Furthermore, it is possible to find soils of predominantly carbonate and clay or with the presence of silt or granite, which cause the variation in the strontium isotope values within the investigated areas.

The isotopic values obtained for the different matrices collected in the sampling sites of producers L and M (Savona district) show a peculiar trend, different from the other producers. Concerning producer L, the data appear to highlight the presence of an area characterized by a mixture of carbonate/calcareous rocks and clays, with $^{87}Sr/^{86}Sr$ values within the range 0.70846–0.70979. Furthermore, the observed variation in soil–branch–olives belonging to the first two sites is strongly suggestive of a strict relationship between the investigated food (olive) and the geology of the respective territory. Nevertheless, this correspondence is slightly lower for the last site, with a lower correspondence between the isotopic values of olive and branch samples with respect to that one of the respective soils. A particular trend is present at the third site, where the isotopic values obtained for branch and olive (0.71778 and 0.71773, respectively) practically overlap but are far from the corresponding soils (about 0.008 units higher).

Regarding producer M, there is a pronounced intra-field variability with values of $^{87}Sr/^{86}Sr$ ranging from 0.70760 to 0.71380 for soil samples (typical of carbonate/limestone rocks and clay) and from 0.70636 to 0.71211 for branches and olives. Figure 5.2.3 clearly shows a connection between the values found for these last two matrices. On the other hand, the values obtained for the fraction extracted from the soils are systematically higher than those of the respective olives and branches, confirming the difficulty in obtaining the real bioavailable fraction of element intake from the plants using the DIN 19730 procedure.

Finally, by comparing the data obtained for all monitored producers, it seems that it is not possible to discriminate the three geographical areas of production by means of this indicator, since its variability within the same district is extremely high. Nevertheless, with the exception of producer M, a regular variation of strontium isotope values can be observed proceeding from east to the west (slightly higher values) of the region of Liguria. In addition, the latter shows a higher intra-site and inter-site variability of $^{87}Sr/^{86}$ Sr values with respect to those from Imperia district.

Considering the complexity of the issues that underlie the development of geographical traceability (representativeness of sampling, operational steps, matrices pretreatment, etc.), this work is a pilot study that, starting with the characterization of the production chains of several Ligurian olive farms, set the stage for the development of the geographical traceability model of Ligurian EVOO by using the isotopic ratio of Sr.

Regarding the trend of $^{87}Sr/^{86}Sr$ values, the results have drawn attention to several aspects. On one hand, the excellent tracer potential of the used indicator has been

demonstrated due to the high degree of agreement between the values determined in the whole soil–branch—olive chain. On the other hand, the complexity in developing a traceability model when various factors are involved, including the heterogeneity of the geology of the investigated area, the practices of fertilization/irrigation used, etc., has been highlighted. In any case, it has been shown how a preliminary systematic study of the system under investigation has identified and resolved certain issues. The obtained results highlight that, in the case of a complex soil geology, when the soil fractions are obtained according to the DIN19730 procedure, it is difficult to mimic the plant bioavailable elements assumption. When this is the case, it could be an excellent approach to monitor the geographic marker directly in the plant/tree. In fact, the differences between $^{87}Sr/^{86}Sr$ for branches and olives are often lower and somewhat more representative (the roots of the tree, given their extension, are able to represent larger volumes with respect to a single site of sampled soil) compared to those between olives and soils.

Despite the excellent results obtained in this work, it is worth noting that reliable results can be obtained by considering a larger and statistically representative sampling of the area and of the finished product in addition to other geographical tracers, such as the isotopic ratio of lights elements, which may contain further information.

5.2.4.3 Case Study: Lambruschi PDO

The data reported here are part of a long-term research project (http://www.progettoager.it) focused on the development of geographical traceability models of oenological products, Lambrusco PDO and Trento DOC, by means of primary indicators, such as isotope ratios of radiogenic and light elements and metals content, and secondary indicators, NMR spectra and polyphenols fingerprint. The project is characterized by an innovative analytical methodology which requires a thorough knowledge of all the matrices that characterize the investigated systems, namely soils, branches, grape juices, intermediate products, and wines. For these reasons, a representative sampling for each investigated matrix (soils, branches, and wines) was performed (Totaro *et al.* 2013) and different primary indicators were monitored. The entire dataset was processed using chemometrics tools in order to: (1) obtain useful information about the differences between/similarity among the samples; and (2) build a unique geographical link between the investigated wines and their territory of provenance.

The development of geographical traceability models requires an in-depth knowledge of the entire investigated system and its characteristics. In addition to the phenomena that influence the strontium isotope ratio from the soil to the grapes, the procedures applied in converting the raw materials into final products should be also analyzed.

The winemaking process may encompass several steps which may introduce changes to the element pattern, due to both natural and anthropogenic/extraneous sources. Several studies have been carried out on the variation of some element concentrations during the cellar practices (Eschnauer *et al.* 1989; McKinnon *et al.* 1992; Jakubowski *et al.* 1999; Kristl *et al.* 2002; Almeida & Vasconcelos 2004; Castiñeira Gómez *et al.* 2004; Kment *et al.* 2005; Rossano *et al.* 2007; Catarino *et al.* 2008; Cheng & Liang 2012). In particular, storage tanks, pipes, and other wine cellar equipment (brass and stainless steel based tools) increase the content of Cd, Cr, and Pb (Kristl *et al.* 2002) or Fe, Al, and Cr (Kment *et al.* 2005). Significant contributions to Al (McKinnon *et al.* 1992), rare Earth elements (REEs) (Jakubowski *et al.* 1999; Rossano *et al.* 2007), and exchangeable

cations such as Mg, Ca, and Na (Castiñeira Gómez *et al.* 2004) were found, due to the use of additives for purification or de-acidification processes. Furthermore, variations in the element content occur also as a consequence of the fermentation process (Eschnauer *et al.* 1989; Castiñeira Gómez *et al.* 2004). As a consequence, an accurate study to verify whether the strontium isotope ratio is also influenced is necessary in order to finally achieve reliable results on the basis of this indicator.

If no sources of external strontium are added (i.e., the use of additives to stabilize the process and eventually to clarify the final product), an increase or decrease in the strontium concentration should not directly affect the isotope ratio. In any case, it is worth investigating whether and which strontium contaminations occur during the vinification. Generally, an increase of strontium is found in the first steps of the winemaking process, probably released by seeds and skins, while a slight decrease during the ageing period is a consequence of strontium precipitation with colloidal particles (Almeida & Vasconcelos 2004). These two phenomena are therefore natural, not being due to external sources, and should not affect the $^{87}Sr/^{86}Sr$ isotope ratio. On the contrary, the addition of bentonites used for wine clarification, or calcium carbonate to de-acidify, could be the main extraneous sources of strontium (Horn 1993).

In the literature, it has been demonstrated that the winemaking process generally does not affect the strontium isotope ratio values. Nevertheless, the rationale of these results relies on the fact that additives are not used and no relevant difference (around the fourth figure) is present in the $^{87}Sr/^{86}Sr$ value. In fact, the only attempt to investigate the influence of the winemaking process on the strontium isotope ratio (Almeida & Vasconcelos 2004) reveals no differences in the values measured in the different phases. However, for the former investigation a quadrupole-based ICP-MS was used for the evaluation of the strontium isotope ratio. This instrument is characterized by a low precision, generally not sufficient to discriminate differences among samples in terms of variation of the isotope ratio values.

Starting from the recent observation that mass discrimination may also occur for heavy-element isotopic systems (Johnson *et al.* 2004), and that this experimental evidence is obtained as a result of the high sensitivity of the MC-ICP-MS instruments, we now discuss the isotope ratio data obtained from the monitoring of the winemaking process of several producers of Lambrusco PDO wines in order to assess whether and how the production process can cause variations in the measured values. Samples for each step of the process (grape juice, intermediate products, wine) were tracked for seven Lambrusco winemaking chains, coming from four Modena district wineries. For some of the investigated producers, it has been possible to assess a correspondence in the isotopic values from the respective soil and vine-branch samples.

All analytical determinations of the strontium isotope ratio were made as described in the previous case study (Section 5.2.4.2; EVOO application).

5.2.4.3.1 Sampling and Sample Treatment

Lambrusco wine is one of the main products of the Modena district, classified as a red sparkling product obtained by the fermentation of grapes of Lambrusco varietals from well-defined areas of the district of Modena (Decree of 23 March 2010; Decree of 29 March 2010; Decree of 27 December 2010).

Different grape varietals can be used, namely: Lambrusco di Sorbara, Lambrusco Salamino, Lambrusco Grasparossa, and Lambrusco di Modena. According to their rule

of production, the cultivation of grapes is fixed in three distinct and well-defined areas of the district of Modena. Lambrusco of Modena has recently been added to the list, which can be produced by mixing all the typical Lambrusco grape varieties grown in Modena district, as stated in the rule of production.

During the winemaking process different modifications can occur, namely the addition of organic additives to stabilize the winemaking procedure as well as of selected yeast to support fermentation or bentonite (clay mineral) to eventually clarify the final product.

5.2.4.3.2 Lambrusco Winemaking Sampling

In order to obtain a representative sample set, collaboration with two local Lambrusco wine producers (PB and MO) and two wineries (Winery 1 and Winery 2) was established. Four Lambrusco winemaking processes from the four wineries/producers were monitored in the period from September 2011 to April 2012. For each of the investigated process, representative samples of: (1) grape juices, obtained from two different Lambrusco grape varieties, namely Sorbara and Grasparossa grapes; (2) intermediate products of each winemaking production step; and (3) final commercial Lambrusco wines were obtained. Regarding the winemaking processes, an aliquot of sample was systematically collected after every variation occurred during the process (e.g., filtration, use of additives, mixing of the content of different silos, etc.). The additives used in the Lambrusco productions to make the wine sparkling, namely rectified concentrated must (MCR), red concentrate, etc., were also sampled when available. All the collected samples were stored at 0°C until analysis.

Given the particular characteristics of each wine production, detailed information about the investigated winemaking processes as well as the collected samples are reported in Tables 5.2.5–5.2.8.

Table 5.2.5 Collected samples for the PB winemaking chain.

Sample code	Information
PB1a	Grape juice from the first silo after filtration
PB1b	Grape juice from the second silo after filtration
PB2ab	Mix of products PB1a and PB1b at the end of the fermentation process
PB3ab	Fermented sample after SO_2 addition
PB4ab	Lambrusco Sorbara wine

Table 5.2.6 Collected samples for the MO winemaking chain.

Sample code	Information
MO1	Grape juice
MO2	Fermented grape juice
MO3	Fermented juice, with bentonites, pvpp, and gelatin added
MO4	Lambrusco Grasparossa wine

Table 5.2.7 Collected samples for Winery 1 winemaking chain.

Sample code	Information
CR1	Grape juice
CR2	Grape juice after perlite filtration
CR3	Grape juice waiting for the fermentation process to start
CR4	Fermented juice after low temperature stabilization
CR5	Product after tangential filtration
CR6	Lambrusco Grasparossa wine

Table 5.2.8 Collected samples for Winery 2 winemaking chain.

Sample code	Information
CM1a	First silo of Lambrusco Sorbara grape juice, added with SO_2
CM1b	Second silo of Lambrusco Sorbara grape juice, added with SO_2
CM2ab	*Mixing of CM1a and CM1b samples, after flotation process and* starting fermentation
CM3ab	Fermented juice
CM4ab	Fermented juice after centrifugation
CM5ab	Sample after low temperature stabilization, with addition of PAG_CM1
CM6ab	Lambrusco Sorbara wine
PAG_CM1	*Concentrated must obtained from Sorbara grapes*

Briefly, in the case of PB producer the starting must is obtained from Lambrusco Sorbara grapes and is initially stored in two silos of 300 hL capacity. After the mechanical harvesting, the grape crushing is directly performed in the field. Grapes are put into a destemming machine, conveying must and grape skins in the collecting tank. A small amount of SO_2 is added to delay fermentation processes. In the winery, the must is stored in two silos, enzymes and tannins added for oenological use to facilitate skins separation and clarification, and finally roughly filtered (samples PB1a and PB1b).

The contents of the two silos are then mixed in a fermentation tank, 500 hL capacity, and yeasts are added. After one week, the fermentation process stops due to the complete transformation of the sugar substrate (sample PB2ab). SO_2 is added to the fermented sample and transferred to an autoclave (sample PB3ab). Different products (e.g., MCR or red concentrate) may be added to produce the sparkling wine. Finally, the wine is bottled (sample PB4ab).

The wine production of MO producer begins with Lambrusco Grasparossa grapes. The collected grapes are brought to the winery and treated by a mechanical crusher/destemmer. The grape must is stored with its skins in a concrete tank (sample MO1). Later, skins are removed and yeasts are added to start the fermentation. After about one month, the fermentation process ends (sample MO2) and clarifying substances are added to the wine such as bentonites, polyvinylpolypyrrolidone (pvpp), and gelatin (sample MO3). The wine ages for a period and is then bottled (sample MO4).

Regarding Winery 1, the wine is made from Lambrusco Grasparossa grapes. After the harvest, the grapes are crushed and the must is transferred to a 1500 hL wine tank (sample CR1). The must is filtered with perlites (sample CR2), and the fermentation starts with selected yeasts (sample CR3). At the end of the fermentation process, must is centrifuged and added to rectified concentrated must. The product is stabilized at low temperature (sample CR4), subjected to tangential filtration (sample CR5), and finally bottled (sample CR6).

Finally, in Winery 2, the raw materials are two starting silos filled with Lambrusco Sorbara grape must (samples CM1a and CM1b), mixed together at different steps of the production procedure. The grapes are crushed in the winery and stored in silos, with the addition of SO_2, pectolytic enzymes, gelatins, and N_2 to facilitate the separation of skins and other waste material from the must. The must is then transferred to a fermentation tank (sample CM2ab) for about a week until the end of the fermentation process (sample CM3ab). The fermented liquid is centrifuged to remove yeasts and particulates (sample CM4ab). The product is stabilized at low temperature until the next stage of the wine production (sample CM5ab). The final product is obtained by a passage in autoclave and the addition of concentrated must (sample CM6ab).

5.2.4.3.3 Soil, Branch and Juice Sampling

Since PB and MO are two local wine producers, it was also possible to investigate the relationships between winemaking production and the geological substratum of the vineyards. In particular, strontium isotopic ratios values determined in grape/juice or wine samples were compared to respective ratios measured in the respective soils and vine-branches. Soil and branch sampling procedures have been carefully described in a previous publication (Durante *et al.* 2013). Briefly, the soil holes were dug using a single-gauge auger set to avoid disturbing the samples, at a maximum sampling depth of 60 cm. The obtained core sample was divided into five aliquots, 10 cm length each, after discarding the first 10 cm. The depths are labeled as follows: UP for 10–30 cm and DW for 30–60 cm. For producer PB, two vineyards (situated in the in-plane area of Modena district) V1 and V2 were investigated, collecting three and five samples of soils, respectively.

Producer MO's vineyard is located in a hilly area, and previous studies have shown the heterogeneity of its morphological and geological features (Bertacchini *et al.* 2013). Five soil samples were acquired and the obtained core sample was divided into five aliquots (always discarding the first 10 cm), in order to be as representative as possible of the investigated area. Samples were labeled: (a) 10–20 cm; (b) 20–30 cm; (c) 30–40 cm; (d) 40–50 cm; and (e) 50–60 cm.

From each farm, vine branch and grape juice samples were collected from the grapevines growing in proximity to the location of the soil samples. Vine branches were picked during two different periods of the years (spring and summer) in order to

investigate the influence of the weather conditions and/or the vineyard treatments on the monitored $^{87}Sr/^{86}Sr$ indicator.

5.2.4.3.4 Soil, Branch and Juice Pretreatments

Soils and branch samples were pre-processed as described in Section **5.2.4.2** (EVOO applications). Grape juices and concentrated musts were obtained and wine digestion performed via a microwave (MW) -assisted procedure (Durante *et al.* 2013) on a maximum sample aliquot of 6 g, accurately weighted into quartz vessels before adding 4 mL concentrated (65% w/w) HNO_3. This mixture was left to react for at least 30 minutes before the microwave digestion. Operatively, five vials (40 mL) fitted with Teflon caps were placed on the rack and an appropriate control sample (a vine-branch sample) was repeated once a day to monitor the procedure performances. At the end of the digestion process, all the samples were transferred to PFA bottles and diluted with 65% HNO_3 to obtain a solution with a final HNO_3 concentration equal to 8 mol L^{-1}.

5.2.4.3.5 $^{87}Sr/^{86}Sr$ in Lambrusco Winemaking

$^{87}Sr/^{86}Sr$ was determined for the grape juices, intermediate products, and commercial wines for all the investigated winemaking processes. A measurement uncertainty, expressed as twice the standard deviation of the control sample, of ±0.00003 was associated with each value. The data obtained from the four batches are reported in Figure 5.2.4.

From this graph, some general consideration can be pointed out. First of all, the $^{87}Sr/^{86}Sr$ values are in agreement with the range found in soils of Modena district (Durante *et al.* 2013). The values related to Lambrusco Grasparossa variety (MO and Winery 1 batches) are on average higher than those of the Lambrusco Sorbara (PB and CM batch). Indeed, the cultivation area of Lambrusco Grasparossa grapes is located in

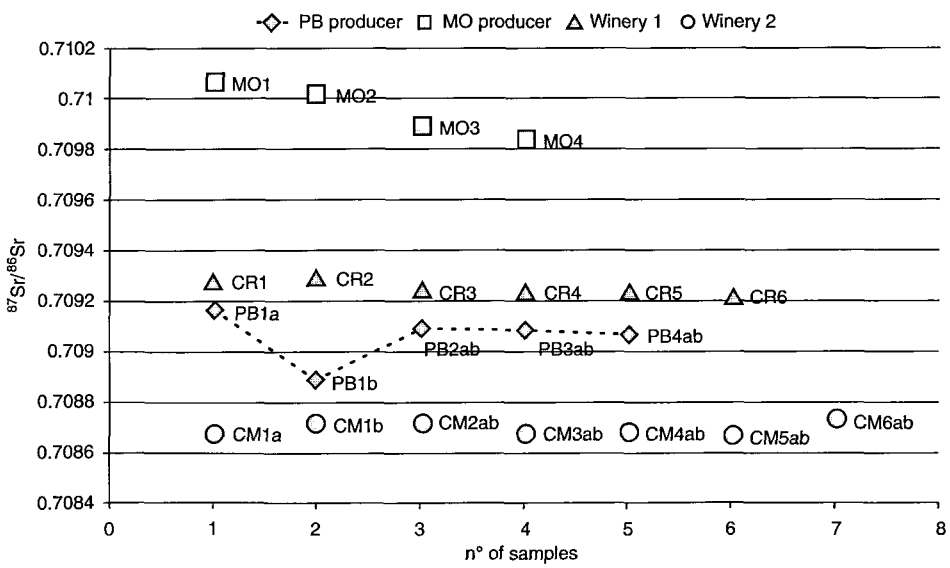


Figure 5.2.4 Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different matrices from the four investigated winemaking procedures.

the southern part of the Modena district which comprises hill and in-plain territory, while Sorbara variety is mainly cultivated in in-plain regions. A rather constant $^{87}Sr/^{86}Sr$ value is generally found, but some exceptions and peculiarities in the winemaking chains require more detailed discussion.

As regards the PB winemaking process, the data interpretation is simpler. The production comprises two starting silos (each of 300 hL) of grape juices (PB1a and PB1b samples), which yield different strontium isotope ratios values since grapes come from various fields (vineyards) of the producer but are located in the PDO zone as stated by the production rule. In the second step, these samples were mixed to obtain a value which is within the range of the two starting juices according to the mixing rule (Hosono *et al.* 2007). Samples acquired from this intermediate stage to the final commercial wine demonstrate identical (differences are below the measurement uncertainty) $^{87}Sr/^{86}Sr$ values.

Different considerations can be highlighted for the strontium isotope ratio values obtained for MO winemaking procedure. In this case, the standard deviation of the four collected samples is slightly higher than the measurement uncertainty. On the one hand, the $^{87}Sr/^{86}Sr$ values measured for the samples from the first two steps, MO1 and MO2, present no significant differences. On the other hand, a decrease in the $^{87}Sr/^{86}Sr$ ratio is found for the fermented must, MO3 sample, probably as a consequence of the addition of clarifying agents (bentonites, pvpp, gelatins) which may introduce an exogenous Sr contribution. Finally, the value obtained for the commercial wine, MO4, is perfectly in agreement with that obtained from the previous step.

As far as the results obtained for Winery 1 are concerned, it is worth noting that the wine production is significant, around 1500 hL for batch. The strontium isotope ratio value is perfectly transferred from grape juice to the intermediate products and then to the final product, demonstrating that the use of perlite (CR2) or the tangential filtration of the last step (CR5) do not affect the value observed in the commercial wine (CR6).

Finally, during the production of commercial wines of Winery 2 (around 1000 hL), the contents of two silos are mixed (CM1a and CM1b, respectively). The two starting grape juices demonstrate similar isotopic ratio values. During the winemaking procedure, no significant variability in the strontium isotope ratio is found (standard deviation is equal to 0.00003). The addition of filtered must of Sorbara grapes (PAG_CM1) does not introduce changes, since the measured $^{87}Sr/^{86}Sr$ is not significantly below the values monitored in the intermediate products. The grapes used to obtain PAG_CM1 probably come from approximately the same area as those used for the produced wine.

5.2.4.3.6 $^{87}Sr/^{86}Sr$ from Soils to Lambrusco Wines

Figure 5.2.5 reports the $^{87}Sr/^{86}Sr$ values of the soils, grape juices, and vine branches of three vineyards, two belonging to PB producer (V1 and V2) and one to MO. An uncertainty equal to ±0.00003 (Durante *et al.* 2013) is associated with each value, calculated as twice the standard deviation of a replicated soil samples. It is evident that samples can be discriminated on the basis of their isotopic values. In particular, vineyards belonging to PB producer (situated in an in-plane area) are characterized by lower values with respect to MO (situated in a hill zone). Such differences are likely related to the different geology of the soils in the two areas; according to the properties of the strontium isotope ratio, it is possible to state that hill soils, which yield higher values, were formed from older rocks.

As far as inter- and intra field-variability is concerned, V1 and V2 soils samples have quite similar isotope ratios; considering the mean value for each producer, V2 field yields slightly higher values than V1. Small differences are found among the sampling points of V1 producer; points 1 and 3 yield higher values than point 2. Vertical variability seems negligible, since the isotope ratio of the UP and DW fractions are nearly the same for all sampling points. V2 soil samples are characterized by a limited variability both among the sampling points and depths (except for at point 2, which presents lower values).

It is possible to determine a greater variability of the hill samples with respect to the in-plain samples, regarding both the sampling sites and depths. In particular, the main differences are found for hole 4 (which yields higher values than the others) and along the vertical profile of hole 5 (there is an increase in the isotope ratio from depth a to e). This latter trend is quite peculiar; some researchers have described these isotope variations as a function of the sampling depth, explaining the experimental evidence as a consequence of the different origin, and thus geology, of rock fragments which can produce stratification of soils (Prohaska *et al.* 2005).

Considering the entire dataset (Fig. 5.2.5), it emerges that the $^{87}Sr/^{86}Sr$ values of the juices have a different range of variability depending on their provenance; they are closer to isotopic values obtained for the soil fractions. Values obtained for juices from V2 field perfectly match those monitored in the respective soil samples; in particular, better correspondence is found with the lower soil depth (UP). Differences are around the fifth decimal figure, hence not statistically significant compared to the uncertainty associated with the measurements. As far as the hill farm (MO) and V1 samples are concerned, the variation between the Sr isotope ratio values of soil extracts (considering

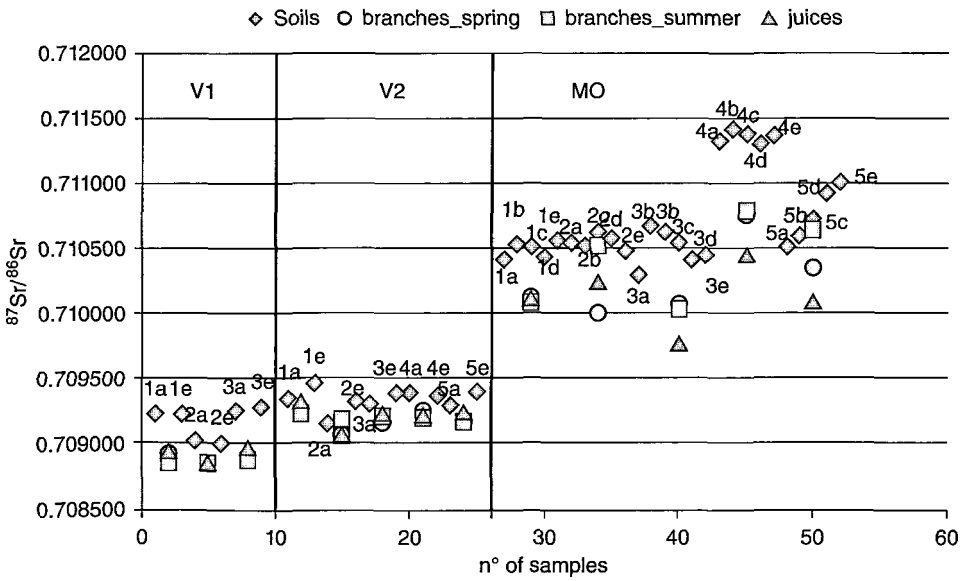


Figure 5.2.5 Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different soil fractions extracted with NH_4NO_3, the different vine branches, and grape juice samples from the three investigated vineyards.

the UP sample) and that of grape juices is around the fourth decimal digit. In detail, $^{87}Sr/^{86}Sr$ obtained for juices of producer MO ranges between 0.70978 and 0.71045 and the worst soil–juice correspondence is found for site 4 with a difference of 0.00088. As concerns V1 samples, isotopic values vary from 0.70886 to 0.70898 and the maximum discrepancy (0.00028) is relative to site 1.

On the basis of the described results, a good correlation exists between soils and juices for the investigated in-plain area. Furthermore, considering the geochemical classification of the investigated soils (i.e., mainly clayey-loam for sites of V1 and V2 fields and calcareous with the presence of some oldest granite debris for site MO; Soils of Emilia Romagna; http://geo.regione.emilia-romagna.it/cartpedo), the obtained isotopic data for the juices are consistent with that determined for the corresponding rocks and soils by other authors (Horn *et al.* 1998).

Regarding the samples from the hill area, the situation is certainly more complex since the discrepancies are higher than the precision of the measurement. Even if traceability models based on $^{87}Sr/^{86}Sr$ values with soil–food differences to the fourth decimal digit could be considered optimal (Swoboda *et al.* 2008; Brunner *et al.* 2010; Voerkelius *et al.* 2010), other aspects which influence the correlation degree could be highlighted. Indeed, the differences found between $^{87}Sr/^{86}Sr$ values of soils and of juices for hill samples are too high to only be due to procedural uncertainties or Sr segregation in the soil. As a consequence, when the geology, structure, and composition of soil is complex, the bioavailable fraction extracted with NH_4NO_3 was not able to perfectly reproduce the plant uptake. In order to assess the accuracy of the previous explanation, the strontium isotope ratio was measured for the branches from the grapevines grown close to the soil sampling points. In this way, it is possible to obtain information about the plant uptake, using the grapevine as a direct sampler. From Figure 5.2.5, the excellent correlation between isotopic values measured in branches and juices is evident. In fact, differences in samples from producer V1 are significantly reduced, passing from the fourth to the fifth decimal digit, and remain at the fifth decimal digit for V2 samples. As far as producer MO is concerned, the branch–juice correlation certainly improves with respect to soil–juice, even if a perfect agreement is found only for points 1 and 2 when considering the mean value of the branches collected in the two periods. The differences for the other sampling points remain over the measurement uncertainty, although limited to a maximum value of 0.0004.

Branch values for the two sampling periods are the same (differences not statistically significant) for almost all the investigated samples. A strange situation occurs for the branches of point 2 of producer MO, which yield a wide range of values but with an average which perfectly matches the juice value.

These results confirm that the Sr uptake from minerals is not uniform, but depends on many parameters such as the solubility, water content, mineral type, temperature, etc., in the root space of the plant. From these considerations, that the greater similarity of values between juices and vine branches with respect to soils was clear, confirming the "sampling capacity" of the plants in traceability studies. The experimental evidence confirms that plants are able to optimize the bioavailable element uptake on a time basis, when needed, and over a spatial scale, where available. The branch values were compared with those obtained for the matrices from the winemaking procedure for PB and MO producers (Figs 5.2.6 and 5.2.7, respectively).

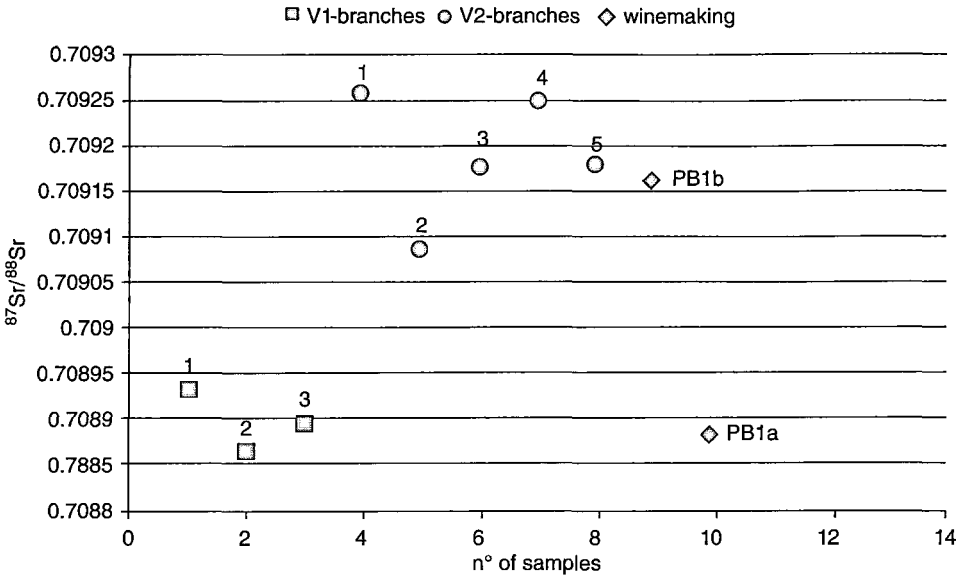


Figure 5.2.6 Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different matrices describing producer PB's procedure and respective vineyards.

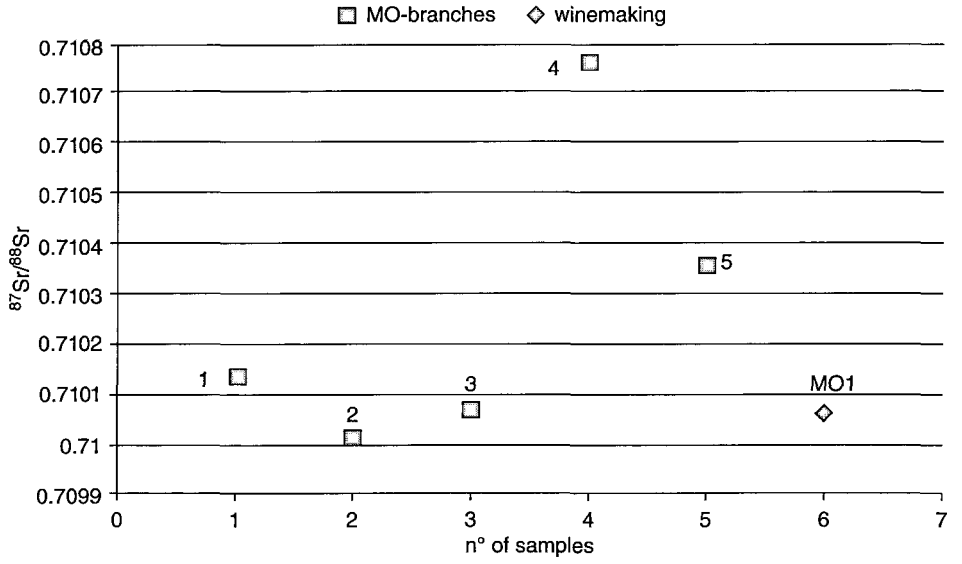


Figure 5.2.7 Strontium isotopic ratio ($^{87}Sr/^{86}Sr$) obtained for the different matrices describing producer MO's procedure and respective vineyards.

From Figure 5.2.6, it emerges that PB producer used juices from both its fields; in particular, there is a good agreement between the used raw materials, PB1a and PB1b, and the strontium isotopic range characteristic of the V1 and V2 vineyards, respectively.

Considering the strontium isotope range of variability of MO's vineyard (Fig. 5.2.7), data obtained for the grape juice used in the winemaking chain of Lambrusco Grasparossa are in perfect agreement with the values of the strontium isotope ratio of the vineyard of origin, that is, it falls within the respective range of variability.

The use of $^{87}Sr/^{86}Sr$ as geographical tracer of food origin is related to the constancy of its value in its transfer from the soil to the plant and then into the final product. Until now, studies of the features of this indicator suggest that the effect of isotope fractionation is not significant. As a consequence, all the transformations and biological processes which occur along the winemaking chain (e.g., fermentations, precipitations, etc.), should not affect the $^{87}Sr/^{86}Sr$ values. However, cellar practices sometimes include the use/addition of different additives such as clarification or deacidification agents as well as concentrated musts, which could release strontium. The $^{87}Sr/^{86}Sr$ ratio found in the final product may therefore be influenced by these winemaking processes.

In this case study, the influence of the winemaking process on the transfer of $^{87}Sr/^{86}Sr$ signature from the grape juices to the final product was investigated considering Lambrusco PDO productions chains. For most of the investigated winemaking chains, the strontium isotope values obtained for all the samples are in excellent agreement, showing no changes during the production; the possibility to directly relate the final product to its raw materials and geographical area therefore exists.

On the other hand, some productions could present variations in the value of the isotope ratio of the final product (e.g., for MO producer). These variations highlight the need to perform a tailored investigation in order to identify the peculiarities of each process. In fact, a more detailed knowledge of the production procedures could allow a better understanding of the data, obtaining reliable information about the influence of different sources on the strontium isotope ratio along the winemaking chain.

All things considered, the $^{87}Sr/^{86}Sr$ is certainly a powerful geographical traceability marker due to its characteristics which allow the discrimination, both horizontally and vertically, of soils samples with different origins and characteristics. However, the great variability of the indicator could also be considered as a drawback, since it contributes to data dispersion, leading to a more difficult distinction and interpretation of samples from different geographical areas.

The possibility of using the plant as a sampling device could reduce the dispersion of the data, allowing a better discrimination of the samples and processes. Further, by considering the branch values (which were not dependent on the period of sampling), it could be possible to overcome problems associated with soil sampling and its structure and composition, as well as those related to the evaluation of the bioavailable fraction of soil, namely the plant uptake. The described approach could be considered very promising in geographical traceability studies.

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6

Nuclear Magnetic Resonance – Metabolomics

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6.1 Introduction

Why are nuclear magnetic resonance (NMR) methodologies used in food analysis? In order to answer this question it is important to consider that food is a complex matrix and different NMR approaches can be useful to investigate its specific aspects.

Food is a complex matrix because it can have a complex physical structure, and because it is a mixture of many primary and secondary metabolites widely ranging in concentration and chemical properties. For instance, milk contains proteins as micelles, fats as emulsion, and sugars, vitamins, and minerals in solution. Food composition can change due to biochemical processes (ripening, aging, etc) and manufacturing processes, etc. As a result, as well as components that are already present in crude products, others can arise during storage and processing or can be added for preservation or specific nutritional reasons.

Depending on the specific problem, different strategies (i.e., target analysis, metabolic profiling, metabolic fingerprinting, and metabolomics) can be used in the study of metabolite composition of foodstuffs. Among these strategies, metabolomics, a non-biased identification and quantification of all metabolites in a biological system, is the most comprehensive approach. Metabolic profiling requires identification and quantification of a number of selected metabolites belonging to various classes of compounds, whereas metabolic fingerprinting is used when a simple classification of food samples without identification of individual specific metabolites is sufficient. Metabolomics (Fiehn 2002) as a discipline, which investigates the metabolites present in biological systems, is closely related to nutrition studies since the major part of nutrients are metabolites produced in biochemical reactions. The metabolome (Oliver *et al.* 1998) is made up of many thousands of metabolites which are the final result of the gene expression. In metabolomics, a wide range of different compounds with different chemical structures, solubility, and concentration therefore have to be analyzed.

NMR is recognized as one of the main analytical methodologies in metabolomics, giving a complete view of the foodstuffs metabolites and, together with suitable statistical

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analysis, providing information regarding foodstuff quality, geographical origin, processing, safety of raw material, digestion processes, and so on (Verpoorte *et al.* 2007; Consonni and Cagliani 2010; Mannina *et al.* 2012; Marchese *et al.* 2014). NMR is one of the most suitable techniques to obtain "high-throughput" spectroscopic/structural information about a wide range of compounds with a high analytical precision (Mannina *et al.* 2012). It can be applied to study complex mixtures without the prerequisite separation of components. NMR is especially helpful for the characterization and identification of molecular structure in a straightforward way.

NMR is a robust quantitative method: the amount of a selected metabolite in a mixture can be directly compared in different samples quite independently from sample composition. Moreover, due to the proportionality of ^{1}H NMR signal integral to the molar concentration of the corresponding metabolite, any biases against certain classes of organic compounds are avoided.

The sensitivity of NMR is considered one of the main limitations, especially compared with higher sensitivity of MS. However, continuous developments in hardware (magnet strength, probe head design, console electronics) have allowed the sensitivity of NMR to be much increased. The required amount of material to be analyzed by NMR in solution may be minimized through the use of the recently developed cryogenically cooled NMR probes (cryoprobes), which take advantage of the fact that the radio frequency electronics generate less noise at low temperature. By reducing the temperature of the NMR detection coil and the preamplifier, large increases in NMR sensitivity have been achieved in the last few decades. Cryoprobes and microprobes (Schlotterbeck *et al.* 2002; Spraul *et al.* 2003) offer the chance to minimize the amount of material needed to perform the NMR analysis down to the microgram scale, making NMR spectroscopy in solution a suitable technique for the analysis of a very low amount of soluble compounds.

Liquid-state high-resolution ^{1}H NMR has been extensively used in foodstuffs analysis (Mannina *et al.* 2012). With this approach liquid foodstuffs can be analyzed directly, whereas solid and semisolid foods require an extraction. The extraction procedure is probably the most critical step aimed at the quantitative transfer of the metabolites from the solid matrix into the solution. Foodstuffs contain a wide variety of compounds with totally different polarities, so no single extraction is able to extract all metabolites in a single experiment.

To avoid any extraction step, another NMR approach can be used in the case of semi-solid food matrix, namely high-resolution magic angle spinning (HR-MAS) NMR. Rapid spinning of the sample, typically at 4–6 kHz, at the magic angle (around 54.7°) provides a reduction of line-broadening effects typical for solids caused by sample heterogeneity and residual anisotropic interactions. Highly resolved NMR spectra are produced; moreover, within the same experiment it is possible to identify polar and non-polar metabolites simultaneously. The metabolic composition of different foodstuffs (Brescia *et al.* 2002; Amato *et al.* 2004; Shintu and Caldarelli 2005; Ritota *et al.* 2010) has been determined by means of ^{1}H HRMAS NMR using common 1D and 2D pulse techniques.

Another NMR approach worth mentioning is ^{2}H site-specific natural isotopic fractionation (SNIF) NMR. A well-known application of ^{2}H SNIF NMR is the determination of the geographical origin of wine (Martin and Martin 1983), approved by the EU in 1990 (EU regulations 2670/90, 2347/91, and 2348/91). This methodology is based on

the correlation between the isotope ratio of deuterium to hydrogen ($^2H/^1H$) of methyl and methylene groups of ethanol and the geographic origin of wines. Relative deuterium concentration and specific deuterium-site locations have also been determined in other alcoholic beverages, fruit juices, aromas and perfumes, fats and oils, milk, and drugs (Rossman Rossmann, 2001).

As it is well known, NMR is a key method for the identification of compounds in foodstuffs (Novoa-Carballal *et al.* 2011). The NMR analysis and structural assignment of foodstuffs is challenging because these samples generally constitute a myriad of distinct compounds such as saccharides, polysaccharides, lipids, peptides, amino acids, etc., which are present in a large range of concentrations. These very complex mixtures inevitably give rise to ^1H NMR spectra that are highly crowded due to the presence of numerous, often scalar-coupled, resonances. The NMR assignment of foodstuff samples is mainly based on two strategies, namely, spectral comparison using databases of NMR spectra and/or application of 1D and 2D/nD NMR editing and/or correlation experiments. In order to confirm assignments it is also useful to spike the investigated sample with an appropriate amount of a pure chemical standard once its presence has been inferred, or at least suspected. Considering the high level of complexity of foodstuff samples, the highest information content can only be obtained by combining all of these approaches.

In this chapter, we report examples of NMR metabolomics application to the analysis of olive oils, fruits (kiwifruits and blueberries), and genetically modified products (tomatoes and lettuce). In some cases, the strategy of metabolites assignment is also reported.

6.2 Olive Oils

Extra virgin olive oil is a natural fruit juice obtained from olive fruits only by mechanical operations. It is considered a fundamental component of Mediterranean diet due to its nutritional and sensory quality. International regulation (EEC 1991) has established analytical criteria to define the genuineness of olive oil (detection of adulterations with seed oils or other solvent extracted oils) and quality grade (extra virgin, virgin, "lampante," "refined," "pure," etc.). This official regulation involves panel test analysis and many different analytical procedures such as chemical analyses (acidity, number of peroxides), chromatographic analyses (fatty acid composition, triacylglycerols, sterols) and UV spectroscopy (ΔK, K232). NMR methodology gives information that can be considered complementary to those obtained using the conventional analyses (Mannina and Segre 2009). It is important to underline that the NMR analyses are still not official, that is, they are not officially recognized by the public institutions. However, due to the significant advantages of NMR methodology we hope it will become an official method for extra virgin olive oil characterization.

The determination of the geographical origin of extra virgin olive oils is a debated issue with many implications. An important act of legislation, the PDO (Protected Designation of Origin; EEC 1992), allows the identification of some Mediterranean extra virgin olive oils with the names of the geographical areas where they are produced. This certification improves the value of the Mediterranean products and assures a product of high quality and defined origin. However, olive oil is a complex matrix and

complementary analytical methodologies are required for its exhaustive characterization. In addition to conventional analyses such as panel test, chemical, and chromatographic analyses, ^{1}H and ^{13}C NMR spectroscopy has therefore been successfully used for chemical characterization of olive oils giving information on major and minor metabolites (Mannina and Segre 2009).

^{1}H NMR spectrum allows the detection of both major components, mainly triglycerides, and minor components such as diglycerides, terpenes, aldehydes, β-sitosterol, squalene, and cycloartenol, whereas ^{13}C NMR spectrum can provide valuable information about the fatty acid composition and the fatty acid positional distribution on the glycerol moiety of different olive oils. An NMR protocol for olive oil characterization has been developed using intensity measurements of selected resonances from major and minor compounds and their statistical elaboration (Mannina and Segre 2009).

Using this protocol it has been possible to obtain olive oil characterization in terms of geographical origin and authenticity providing an important contribution in the debated problem of PDO characterization and confirmation (Mannina *et al.* 2001*a*, *b*, 2005, 2009*a*; 2010; D'Imperio *et al.* 2007).

It is important to underline that the capability of NMR spectroscopy to distinguish between olive oils on the basis of their geographical origin seems unique; no other non-NMR analytical methods suitable for the determination of quality and genuineness are appropriate for this type of discrimination.

The geographical characterization can be on regional, national, and Mediterranean scales. Figure 6.1 depicts some examples of classification on a regional scale. In Figure 6.1a the grouping of extra virgin olive oils from two PDO areas of Veneto (Mannina *et al.* 2005), namely, PDO Garda and PDO Veneto is shown. Selected ^{1}H NMR resonances have been submitted to statistical analysis (ANOVA and PCA).

In Figure 6.1b extra virgin olive oils (D'Imperio *et al.* 2007) obtained from different areas of Lazio (southern area, northern area and the centre) are grouped according to the area of origin. The intensity of some minor components, such as aldehydes, terpenes, and squalene, as well as the content of β-sitosterol turned out to be the most discriminating factors in this geographic classification. NMR-statistical protocols have also been used to investigate the role of some agronomic aspects of the olive oil chemical composition (D'Imperio *et al.* 2007; Mannina *et al.* 2009*a*). An interesting point is the possible effect of altitude. In order to evaluate this effect, we have applied the NMR methodology to olive oils from Lazio region for which the altitude of the olive trees was known. The purpose was to distinguish olive oils originating from olive trees grown on hills that are higher than 400 m with respect to olive oils originating from lower altitudes. The statistical result shows that olive oils are well grouped according to the altitude. High levels of terpenes and wax were observed in the samples from low altitude, whereas a high level of squalene was present in the samples from hill-top cultivations.

In Figure 6.1c the classification of olive oils from three different areas of Tuscany is reported. In two of these districts, Lucca (A samples) and Arezzo (AR samples), the same cultivars typical of these geographical areas are present, whereas in a third district, Seggiano (S samples), only monovarietal olive oils of Seggianese cultivar are present.

A classification of olive oils on a national scale (Mannina *et al.* 2001*b*) is reported in Figure 6.1d. Extra virgin olive oils harvested in three years and from different Italian regions (Tuscany, Lazio, and Lake of Garda) were analyzed and submitted to statistical analysis. Olive oils are grouped according to the geographical areas. The separation

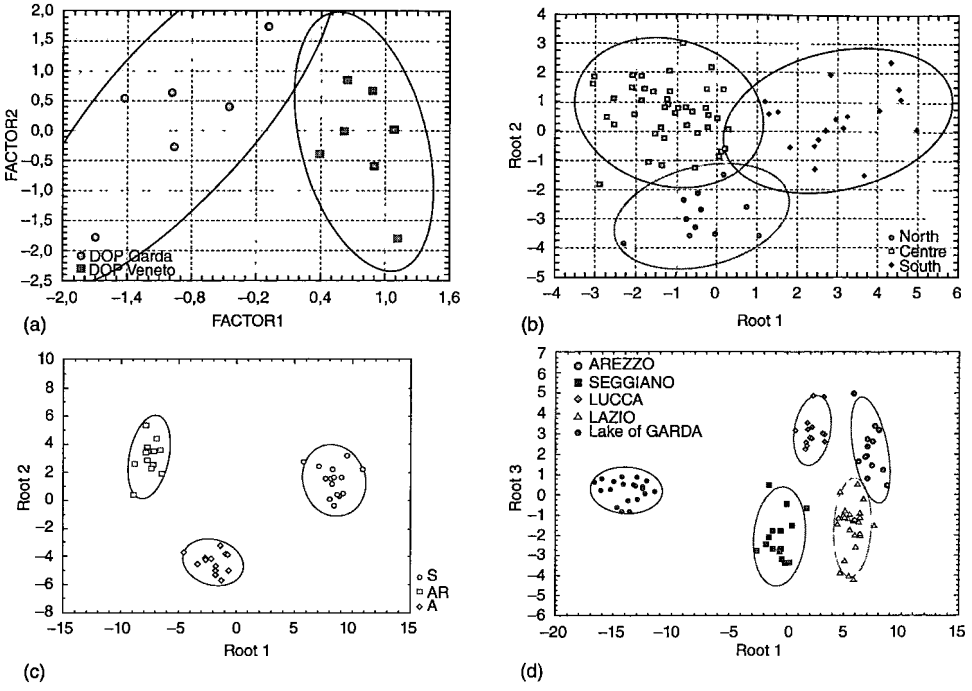


Figure 6.1 Multivariate statistical analyses of olive oils ^{1}H NMR spectra. (a) PCA scores plot of olive oils from two Italian PDO areas: Veneto and Garda. (b) LDA plot of olive oils from Lazio (Italian region). (c) LDA plot of olive oils from three different areas of Tuscany (Italian region). A: Lucca; AR:Arezzo; S: Seggiano. (d) LDA plot of olive oils from different Italian regions (Tuscany, Lazio, Lake of Garda). Adapted from Mannina *et al.* (2005), D'Imperio *et al.* (2007), Mannina *et al.* (2001a), and Mannina *et al.* (2001b).

between olive oils from different regions is more marked with respect to the separation of olive oils coming from different areas of the same region discussed, since on a national scale the differences in the pedoclimatic conditions and cultivar noticeably increase.

A modeling approach using olive oils from Mediterranean areas has also been proposed. In this case, olive oils of three consecutive harvesting years from Mediterranean areas (Italy, Spain, Greece, France, and Turkey) have been analyzed by ^{1}H NMR (Mannina *et al.* 2010). Olive oil samples from Liguria, an Italian region, have been chosen as a case study. Two modeling analyses, PLS-DA and SIMCA, have been used to build up statistical models, to discriminate between Ligurian and non-Ligurian olive oils and to define the Ligurian olive oil class, confirming the declared provenance.

PLS-DA as a discriminant technique is focused on the differences between samples from different classes, whereas SIMCA as a class-modeling technique is more focused on the similarities among samples of the same class. The results of these analyses are given as classification accuracy, sensitivity, and specificity, and are reported considering all samples together (global model with samples of three harvesting years together) and considering every specific harvesting year. The percentages showed that a good classification has been obtained and the "class" olive oils from Liguria have been well defined with respect to the other olive oils. This result suggests that the NMR and chemometric approach extended to a relevant number of samples representative of the Mediterranean

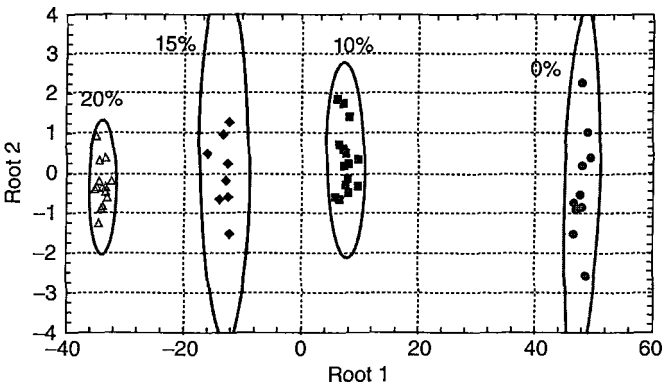


Figure 6.2 Linear discriminant analysis applied to the test samples of olive oil containing different percentages of hazelnut oils analyzed at 600, 500 and 400 MHz proton NMR frequencies. The percentage of hazelnut oil in olive oil is indicated on the figure. The ellipses correspond to a 95% confidence region for each group. Adapted from Mannina *et al.* (2009b).

production could be used to create statistical models specific for geographical areas well known for olive oils production.

Adulteration of food products is a relevant problem since it impacts quality and safety, important to consumers. Olive oil adulteration with hazelnut oils (Mannina *et al.* 1999, 2009*b*; Garcia-González *et al.* 2004) is one very difficult to detect since hazelnut and olive oils have a very similar chemical composition. A simple protocol based on ^{1}H NMR measurements, combined with a suitable statistical analysis which allows detection down to 10% adulteration of olive oil with refined hazelnut oils, has been proposed (Mannina *et al.* 1999, 2009*b*; Garcia-González *et al.* 2004). The protocol has been tested by peer laboratories using spectrometers operating at different magnetic fields (600, 500, and 400 MHz). The LDA applied to the intensity of four selected ^{1}H signals (diallylic protons of linolenic acid, diallylic protons of linoleic acid, methylenic protons of palmitic and stearic fatty chains, and methyl-18 of β-sitosterol) of 10 test oil samples analyzed at 600, 500, and 400 MHz is reported in Figure 6.2: olive oils are grouped according to the percentage of hazelnut oil.

NMR can also be useful to obtain information about the olive oil cultivar. In this case, the ^{13}C NMR methodology was used giving information on the fatty chain distribution on glycerol moiety. As an example, the case of monovarietal Sicilian olive oils (Mannina *et al.* 2003) from the same area of Sicily, an Italian region, is reported. The results of LDA analysis applied to ^{13}C data (see Fig. 6.3) show that olive oils are grouped according to the specific cultivar. This grouping occurs for particular ^{13}C resonances all belonging to fatty chains in the *sn* 1,3 position of the glycerol moiety.

6.3 NMR for Investigating Fruit Metabolomics

Fruits, as for other foodstuffs, are a complex mixture containing many metabolites, widely ranging in concentration and biological activities, which have to be analyzed in order to obtain a comprehensive chemical picture. NMR, as a one of the most suitable

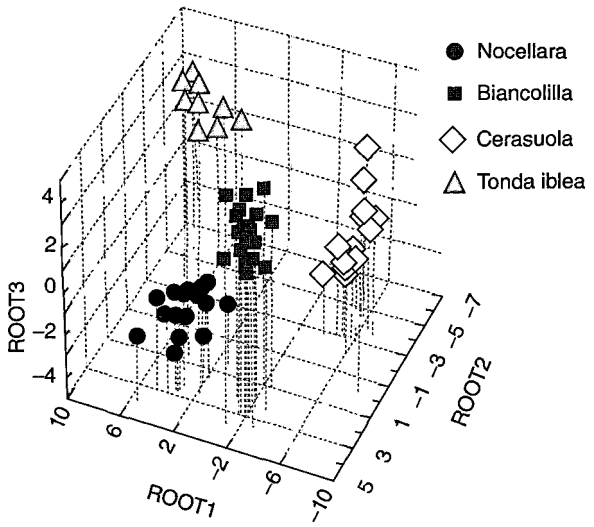


Figure 6.3 Linear discriminant analysis of Sicilian olive oils from four different varieties. Adapted from Mannina *et al.* (2003).

methodologies to obtain "high-throughput" spectroscopic/structural information on metabolite composition, has been applied in fruit metabolomics. Using this approach several fruits such as mango (Gil *et al.* 2000), melon (Biais *et al.* 2009), water melon (Tarachiwin *et al.* 2008), peaches (Capitani *et al.* 2013*a*), kiwifruits (Capitani *et al.* 2010, 2013*b*), and blueberries (Capitani *et al.* 2014) have been investigated, yielding information on fruits ripening, varieties, quality, and adulteration.

For consumers, desirable attributes of fruit are flavor, fragrance, and healthy properties which obviously depend on the fruit chemical composition. In particular, the flavor of the fruit flesh depends on the balance between soluble sugars and non-volatile organic compounds. Different sugars are responsible for different sweetness levels whereas organic acids give the perception of acidity. As a consequence, qualitative and quantitative analysis of metabolites in fruits are both important for commercial success. Furthermore, the knowledge of the metabolic profiling of fruits at different developmental stages may allow the most suitable time of harvesting to detect nutrients at different growth times to be assessed, and to discriminate various cultivars.

We report two examples regarding NMR metabolomics applied to fruit, specifically kiwifruit and blueberry.

6.3.1 NMR Metabolomics Applied to Kiwifruits

We report results obtained by NMR on three cultivar of kiwifruit, namely Hayward, Zespri, and CI.GI. Specifically, a comprehensive metabolic profiling of aqueous extracts of kiwifruit was studied over the season by means of high-field NMR spectroscopy (Capitani *et al.* 2010, 2013*b*).

The assignment of the ^{1}H NMR spectrum of kiwifruit aqueous extract was obtained by 1D and 2D NMR experiments and literature data and, when necessary, using standard compounds (see Fig. 6.4). In the spectrum malic acid (MA), citric acid (CA), quinic acid (QA), ascorbic acid (AA), lactic acid (LA), and shikimic acid (SHA) were identified by

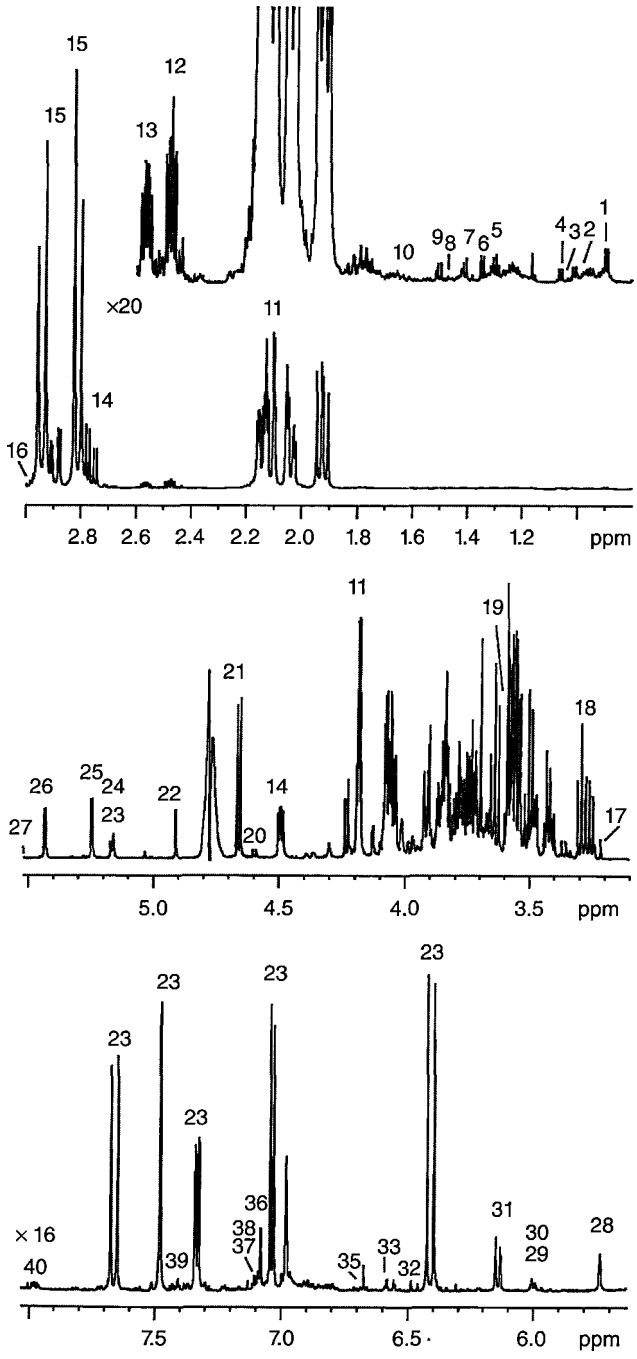


Figure 6.4 600.13 MHz ^{1}H NMR spectrum of a kiwifruit extract in aqueous solution. The assignment of some peaks is reported: 1, U1; 2, LEU; 3, ILE; 4, VAL; 5, U2; 6, THR; 7, LA; 8, U3; 9, ALA; 10, ARG; 11, QA; 12, GLN; 13, GLU; 14, MA; 15, CA; 16, ASN; 17, CHN; 18, MI; 19, FRUpy; 20, β-GAL; 21, βGLC; 22, AA; 23, GLUtCA; 24, GAL-U; 25, αGLC; 26, SUCR; 27, U5; 28, U6; 29, GLUcCA; 30, U7; 31, U10; 32, U11; 33, U12; 35, SHA; 36, U14; 37, U15; 38, U16; 39, U17; 40, URI. Reproduced with permission from Capitani *et al.* (2010).

means of their diagnostic peaks. In all cultivars, QA was found in a higher proportion of the total acids during early fruit development. In the case of Hayward and CI.GI, SHA disappeared at stages of development later than July.

In Hayward and CI.GI the highest concentrations of some amino acids such as arginine (ARG) and glutamine (GLN) were found in the early stages of development, whereas a very different trend was found in Zespri. In the latter case ARG increased over the season and the concentration of GLN was rather constant until September, before decreasing. Concentrations of ARG, GLN, threonine (THR), asparagine (ASP), and alanine (ALA) were found to be higher in Zespri than in CI.GI and Hayward. Triptophan (TRP) was present at a similar concentration in all cultivars, the highest level being found at the early stage of development. The level of histidine (HIS) was found to be higher in Zespri than in CI.GI and Hayward kiwifruits over the season. From July until October, the level of phenylalanine (PHE) was also found to be higher in Zespri than in Hayward and CI.GI. In Hayward and CI.GI the highest level of PHE was found at the early stage of development, whereas in Zespri the highest level was found from August until October.

In all cultivars the level of some sugars increased during fruit growth, reaching the highest level in December. In Hayward galactose (GAL) began to be detectable in August, decreased until October, and then increased again. In CI.GI this compound was detectable as early as in June, reached its highest concentration in September, and then decreased. In Zespri the level of GAL monotonically increased from June to December. In all cultivars the level of myo-inositol (MI) increased between June and August, and decreased at a later stage of development.

In the literature, an increased production of phenolic compounds and flavonoids has been related to reduced susceptibility or increased resistance to fungal or microbial infection in immature fruits. In this frame, a relevant amount of O^3-β-D-glucopyranosyl-trans-caffeic acid (GLUtCA) along with a low amount of O^3-β-D-glucopyranosyl-cis-caffeic acid (GLUcCA) was found in immature Hayward and CI.GI kiwifruits, whereas in the case of Zespri the level was definitely reduced. Another phenolic compound, namely 3-O-α-L-rhamnopyranosyl quercetin (Q-RAM), was detected in immature Hayward and CI.GI kiwifruits but not in Zespri. On the other hand, Zespri kiwifruits displayed the presence of neochlorogenic acid (NCGA), which can be considered a specific marker of this variety. In Hayward and CI.GI kiwifruits a significant amount of epicatechin (EPC) was also detected in August, whereas in Zespri the amount of this compound was found to be low over the season.

To analyze the multivariate structure of the data, a PLS analysis was applied to the intensity of ^{1}H resonances always present during the entire investigated period of development. Figure 6.5a shows a PLS plot of samples scores (PC1 v. PC2) obtained in the case of Hayward kiwifruits. These first two PCs account for 95% of the variability within the data, PC1 providing for 91% and PC2 for 4%. A clear grouping of the samples according to the harvesting month is observable. Samples collected in June, July, and August are well grouped according to the specific campaign of measurement, and separated mainly along PC2. The other samples collected later in the season appear separated according to the specific campaign mainly along PC1. It is worth noting that the effect of the growth is visible as a trend along PC1. In fact, the centroid of each group moves towards high PC1 values according to the kiwifruit growth. The contribution of the variables to this classification is given by the variable loadings reported in Figure 6.5b.

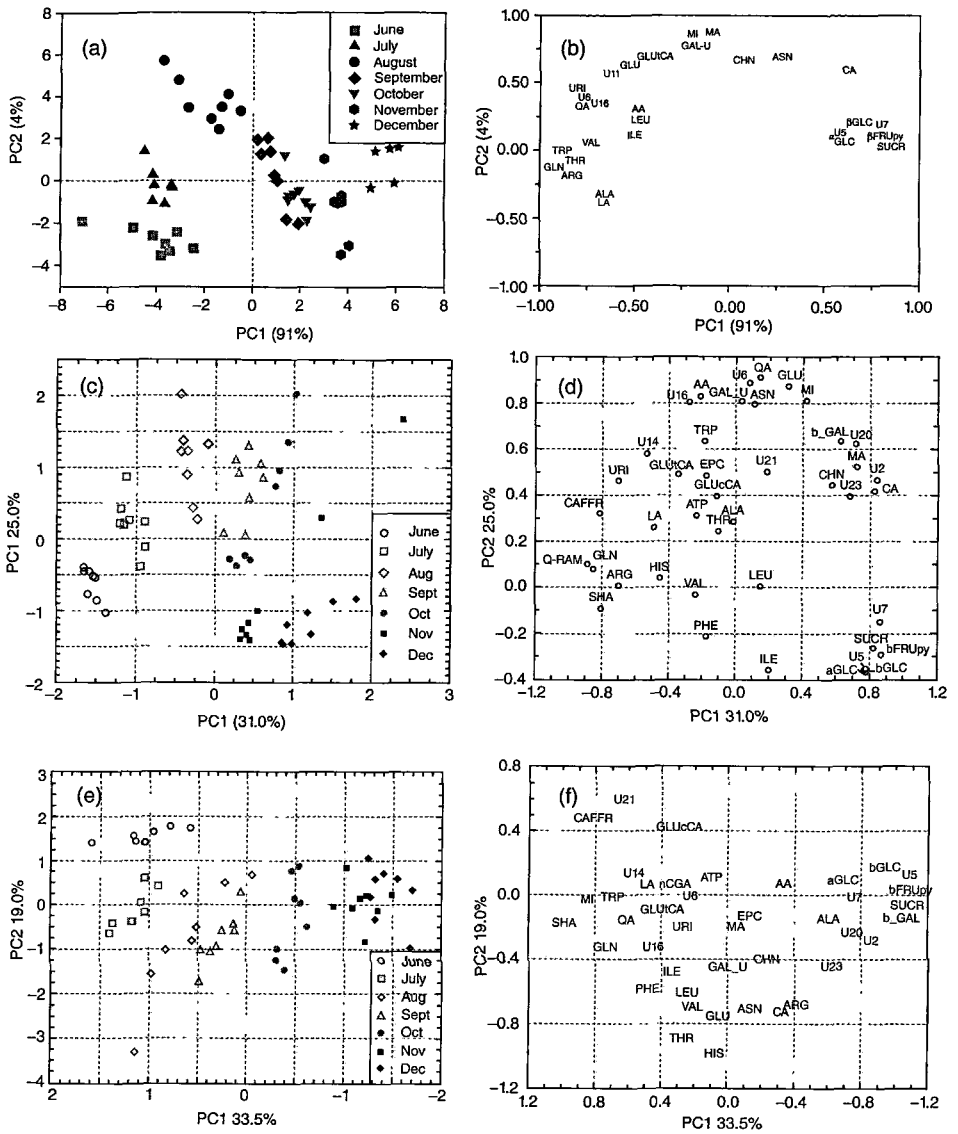


Figure 6.5 (a, b) PLS and (c–f) PCA sample scores and variable loadings plots of NMR metabolic profiles. (a, b) Hayward kiwifruits; (c, d) Cl.GI kiwifruits; (e, f) Zespri kiwifruits. Adapted from Capitani *et al.* (2010, 2013*b*).

Many amino acids (VAL, ILE, THR, TRP, LEU, ALA, ARG, GLN), organic acids (LA, AA, QA), URI (uridine) and U6, U16 unassigned compounds present in the highest concentration in the first periods, and U5, U7, βFRUpy (β-fructopyranose), SUCR (sucrose), αGLC (α-glucose), βGLC (β-glucose), and CA present in the highest concentration in the later months, mostly contribute to PC1 and are responsible for the grouping according to the harvesting period. On the other hand, MI, GAL-U (galactose-U), MA, CHN (choline), ASN, GLUtCA, GLU (glutamate), and U11 are the variables which

make the highest contribution to PC2 and are mostly related to the classification of samples harvested in August from others. These results seem to suggest that August is the key month for kiwifruit development, when a drastic change in the metabolic trend occurs.

Figure 6.5c shows the PCA plot of sample scores (PC1 v. PC2) of CI.GI kiwifruits. These first two PCs account for 56% of the variability within the data, PC1 providing for 31% and PC2 for 25%. In accordance with results reported in the case of Hayward kiwifruits, the effect of the developmental stage is visible as a trend along PC1. Samples collected from June to August are grouped according to the harvesting time along PC1 and PC2. The contribution of the variables to this grouping is given by the variable loadings reported in Figure 6.5d. GLN, ARG, SHA, URI, and Q-RAM (3-O-α-L-rhamnopyranosyl quercetin) present the highest concentration in the first months, and SUCR, GLC, βFRUpy, βGAL (β-galactose), MA, CA, CHN, U7, and U2 present in the highest concentration in the last months, mainly contribute to PC1 and are responsible for the grouping according to the stage of development. Figure 6.5e shows a PCA plot of sample scores (PC1 v. PC2) of Zespri kiwifruits. These first two PCs account for 52.5% of the variability within the data, PC1 providing for 33.5% and PC2 for 19.0%. PC1 mostly classifies kiwifruits according to season. Samples collected in September and October are separated according to harvesting time along PC1, whereas samples collected later over the season partially overlap. The contribution of variables to the grouping is given by the variable loadings reported in Figure 6.5f. SHA, MI, TRP, and GLN, present in the highest concentration in the first period, and GLC, SUCR, βFRUpy, βGAL, and U5, present in the highest concentration in the last months, are mainly responsible for the grouping according to the stage of development.

Figure 6.6a shows the mean values and standard deviations of PCA scores at each stage of development relative to the first two principal components obtained over the

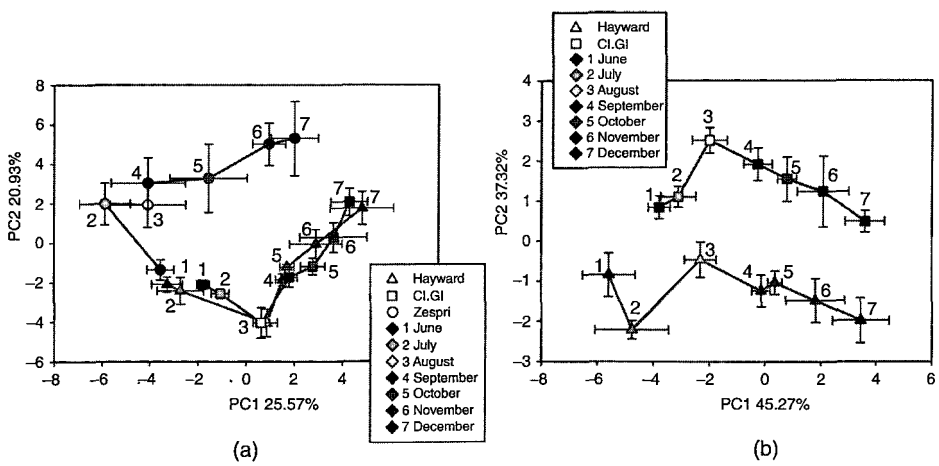


Figure 6.6 (a) PCA applied to the intensity of selected ^{1}H resonances, monitored as a function of the developmental stage in Zespri, CI.GI, and Hayward kiwifruits. Means and standard deviations of the scores are reported. (b) Means and standard deviations of the scores of the first two components of the PLS2 regression model between Hayward and CI.GI kiwifruits. Adapted from Capitani *et al.* (2013*b*).

whole data matrix. The first principal component is related to the developmental stage of the fruit, whereas the second is related to the cultivar. The evolution of the scores along PC1 shows that Zespri metabolic profile always precedes their Hayward and CI.GI counterparts, confirming the Zespri attitude to an earlier ripening. Hayward and CI.GI profiles were substantially coupled along the first and the second component. This observation is in agreement with the common origin of CI.GI and Hayward being both varieties of *Actinidia deliciosa*. To investigate the possibility of different behavior, PLS2 analysis was carried out on Hayward and CI.GI samples. The score plot obtained from the analysis showed that, with the exception of June and July, data relative to CI.GI and Hayward were not separated along PC1, that is, the stage of development, whereas they were well separated along PC2 (Fig. 6.6b). The most relevant metabolites responsible for this separation were found to be ARG, CHN, MI, QA, SUCR, URI, and HIS.

To summarize, the metabolite profile of aqueous extracts of Hayward, Zespri, and CI.GI kiwifruits monitored over the season allowed the development of kiwifruit to be investigated. The application of statistical analysis to NMR data allowed the grouping of samples according to the kiwifruit development and cultivar.

6.3.2 NMR Metabolomics Applied to Blueberries

Another example of the application of NMR metabolomics in fruits regards the case of blueberry. Untargeted NMR metabolite profiling of aqueous and organic extracts from blueberries, as well as targeted NMR analysis focused on anthocyanins and other phenols, has been reported in literature (Capitani *et al.* 2014).

The ^{1}H NMR spectrum of aqueous extracts of blueberry is dominated by the strong signals of sugars in the 3.0–5.5 ppm spectral range, whereas vertical expansions of the spectrum are necessary to observe other metabolites (Fig. 6.7). Glucose (αGLC and βGLC), fructose (α-fructofuranose, β-fructofuranose, and β-fructopyranose), and MI were identified by means of the characteristic spin systems in the TOCSY map. Nine amino acids, namely ALA, ARG, ASN, GAB (γ-aminobutyrate), GLN, LEU, VAL, THR, and GLU, were identified by their characteristic chemical shifts. MA, CA, QA, and SHA were identified by their diagnostic peaks. Furthermore, CGA (chlorogenic acid) was identified by means of the ^{1}H and ^{13}C chemical shifts and the characteristic spin system in the TOCSY experiment.

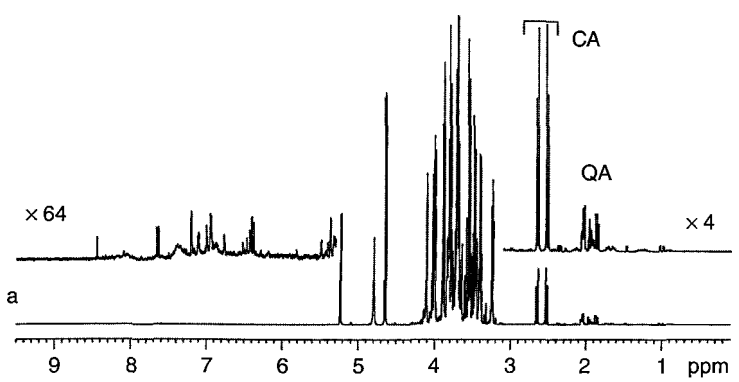


Figure 6.7 The ^{1}H NMR spectrum of an aqueous extract of blueberry. Adapted from Capitani *et al.* (2014).

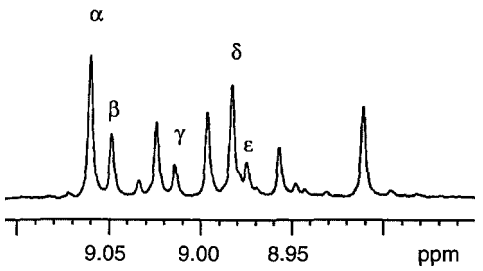


Figure 6.8 The 8.90–9.10 ppm ^{1}H spectral region of the SPE extract of blueberry. α: malvidin-3-galactoside, β: malvidin-3-glucoside, γ: petunidin-3-glucoside, δ: delphinidin-3-galactoside; and ε =delphinidin-3-glucoside. Solvent: the mixture CD_3OD/CF_3COOD (95:5 v/v). Adapted from Capitani *et al.* (2014).

The mixture of deuterated methanol and trifluoroaceitc acid has been found to be the best solvent system (in terms of spectral resolution) for NMR analysis of anthocyanins. However, because the identification of sugar moiety of anthocyanins in the mixture with other more abundant sugar metabolites is very difficult, a C18 SPE extraction of anthocyanins and other phenolic compounds allowed us to overcome this problem. The assignment of anthocyanins and other phenolic compounds was based on 2D experiments and literature data, and was confirmed by the addition of the corresponding standard compounds. The ^{1}H spectrum shows the signals of the glycosidic moiety of anthocyanins in the region between 3 and 5.5 ppm and those of the aglicon mojety between 6 and 9 ppm. The 8.90–9.10 ppm spectral region shows the presence of at least 14 anthocyanins (Fig. 6.8). In this region only signals of H-4 protons of anthocyanidins are present and the spectrum can be read as chromatogram, each signal indicating a specific compound. ^{1}H-^{1}H ROESY confirmed the proximity of H-4 protons and the anomeric protons of the corresponding sugars resonating in the range 5.25–5.35 ppm. Specifically, five anthocyanidins were identified as malvidin-3-glucoside, malvidin-3-galactoside, delfinidin-3-glucoside, delfinidin-3-galactoside, and petunidin-3-glucoside. Furthermore, Q-RAM (3-O-α-L-rhamnopyranosyl quercetin) and CGA were identified. The assignment of Q-RAM aromatic protons and rhamnose signals was based on TOCSY experiment and literature data (Hanamura *et al.* 2005).

The assignment of ^{1}H spectrum of blueberry organic extract dissolved in $CDCl_3$ was obtained using 2D experiments and literature data. The presence of triacylglycerols as one of the major components was indicated by the ^{1}H signals of the glycerol moiety at 4.19, 4.35, and 5.29 ppm. Diacylglycerophospholipids were also present in the organic extract; specifically, phosphatidylcholine and phosphatidylethanolamine were identified by means of the ^{1}H resonance of their hydrophilic head group at 3.29 and 3.25 ppm, respectively. Both esterified and not esterified fatty acids were identified due to the position of CH_2-2 group at 2.36 and 2.31 ppm, respectively. Further, the signal of CH_2-2 group showed a long range correlation with ^{13}C signals of COOR at 175.7–176.0 ppm or COOH at 195.4 ppm. Saturated, mono-, di-, and tri-unsaturated fatty acid chains were assigned by means of 2D spectra and literature data. Digalactosyldiacylglycerol was identified by the diagnostic anomeric signals at 4.93 and 4.24 ppm. The spectrum also showed the presence of β-sitosterol reported as major sterol in blueberry.

6.4 NMR Metabolomics of Transgenic Vegetable Food

The genetic engineering of comestible plants, aimed to improve their traits, productivity, resistance to pathogens and infections, has raised the concern of consumers and regulatory authorities. The principal criterion for assessing the safety of new foodstuff is the substantial equivalence (OECD 1993; FAO/WHO 2000), that is, if the composition of the transgenic foodstuff does not differ in a meaningful way from the unmodified variety, and whether a biotech food shares similar health and nutritional characteristics with its conventional counterpart. The establishment of substantial equivalence is not an easy and straightforward task. First of all, even traditional foodstuffs that have a long story of consumption are not sufficiently characterized and defined in terms of metabolic composition. Taking into account the natural variability of the crops, either associated with variable environmental conditions such as weather, soil, location, rain, etc. or derived from the multitude of naturally different genotypes, it is clear that a better definition of the "reference point," that is, natural metabolic variability of traditional crops, is an obligatory step in a substantial equivalence enquiry.

One of the simplest and most powerful ways to characterize the metabolic composition of any crop is in fact the metabolite profiling analysis by ^{1}H NMR. NMR-based identification and quantification of metabolites requires minimal preparation and handling, and no derivatization. The essential points for the effective application of NMR methodology in the study of transgenic foods consist of appropriate sample choice and treatment, reproducible and simple extraction procedures, acquisition and processing of NMR spectra and, last but not least, a proper multivariate statistical treatment of NMR data.

Ideally, genetically engineered (GE) crops and their conventional counterparts should share an identical genetic background in order to address metabolic differences specifically to the transgene action. In this context, the performance of a given (stable homozygous) GE cultivar is compared to the same non-GE cultivar that provided material for the transformation. A careful control of environmental conditions (light intensity, photoperiod, temperature, humidity, etc.) and cultivation conditions (soil composition, watering, fertilization, etc.) can minimize the exogenous effects on plant metabolite variation. Sampling procedures should consider diurnal cycles of metabolism, particularly in situations where these may alter the metabolite content. The GE and control plant tissue to be analyzed must be sampled from the plants at the same or comparable developmental stage; otherwise, developmentally regulated metabolic changes can mask any other effect. A sufficient number of samples for every combination of factors (genetic, environmental, etc.) should be analyzed to obtain statistically significant results. The larger the variability of metabolic content, the higher the number of samples required for the analysis. The number of GE and control samples should be comparable to avoid bias in the statistical treatment of data.

Simple methods for extraction of water-soluble and lipo-soluble metabolites were usually applied (Bligh and Dyer 1959; Sobolev *et al.* 2005). To avoid any intermediate step (e.g., extraction by H_2O followed by freeze-drying), deuterated water was used for a direct extraction of metabolites from the lyophilized tomato fruits and lettuce leaves tissues (Sobolev *et al.* 2003, 2005). During this extraction some enzymatic activity may take place altering metabolic composition, for example, due to sucrose hydrolysis or polyphenols oxidation. An alternative one-step extraction procedure was used in the

study of transgenic tomatoes (Le Gall *et al.* 2003) and maize (Barros et al. 2010). Instead of deuterated water, a mixture of 70% CD_3OD and 30% D_2O phosphate buffer was applied for extraction of polar metabolites.

The metabolic profiles of control fruits and transgenic tomatoes overexpressing the maize transcription factors LC and C1 have been compared at three ripening stages (Le Gall *et al.* 2003). LC and C1 are known to regulate the flavonoid/ phenylpropanoid biosynthesis and have been used in tomato in order to enhance its antioxidant capacity. In fact, NMR data have confirmed the increased production of six flavonols (kaempferol, dihydrokaempferol, and naringenin glycosides) in the transgenic tomato line as the result of genetic modification. Unexpectedly, the metabolic changes were not restricted to the flavonoid/phenylpropanoid biosynthesis pathway, but the levels of at least 15 other metabolites were found to be different in transgenic tomatoes with respect to conventional tomatoes. Significant changes (2–3-fold or less) were found in the level of primary metabolites such as organic acid (citric and malic acids), amino acids (arginine, asparagine, γ-amino butyric acid, glutamine, isoleucine, phenylalanine, valine) and sugars (sucrose). The division between transgenic and control groups observed by PLS analysis was modulated by ripening; it was most clearly observed at the ripe stage and less clearly at the turning and the green stages.

NMR-based metabolite profiling of transgenic tomato fruit engineered to accumulate spermidine and spermine due to the expression of transgene SAMdc from yeast has revealed distinct metabolic trends in the transgenic and wild-type/azygous fruits at four different ripening stages (Mattoo *et al.* 2006). A fruit-ripening-specific promoter was used to drive the expression of SAMdc; fruits from transgenic plants therefore accumulated higher polyamines in a ripening-specific manner, and the phenotypes showed increased antioxidant capacity and enhanced fruit processing quality. NMR spectroscopy was used to monitor the level of 22 primary metabolites. Glutamine, asparagine, choline, citrate, fumarate, and malate accumulated in the red transgenic fruit, while the levels of valine, aspartic acid, sucrose, and glucose were significantly lower as compared to the control (wild-type and azygous) red fruit. Upon the metabolite profiling analysis the authors suggested that polyamines were perceived as nitrogenous metabolites by the fruit cells, which in turn resulted in the stimulation of carbon sequestration. Further studies of the same transgenic tomatoes were undertaken in order to discover the influence of agricultural practice on their metabolic composition (Neelam *et al.* 2008). Two alternative farming practices have been compared: standard black polyethylene bed and cover-crop-based sustainable system using leguminous hairy vetch (*Vicia villosa*; HV). The analysis of the metabolic composition of the fruits has revealed a surprising synergy between transgene expression and HV cover-crop cultivation system. HV activates the same metabolic pathways and genes as those activated by the introduction of the ySAMdc transgene. HV also modulates transgene expression and seemingly affects the pattern and quantity of metabolites during fruit ripening.

Transgenic tomato fruits with severely reduced methyl jasmonate levels, and as a result a reduced content of polyamines, were obtained by silencing a fruit-ripening-associated lipoxygenase (LOX), SlLoxB, using a truncated LOX gene (Kausch *et al.* 2012). Using NMR spectroscopy the level of 30 metabolites was monitored in tomato fruit during on-shelf ripening. Transgenic fruits contained significantly reduced amount of 19 out of 30 metabolites examined, mostly amino acids, citrate, succinate, myo-inositol, choline, and trigonelline as compared to the wild-type azygous counterparts. PCA

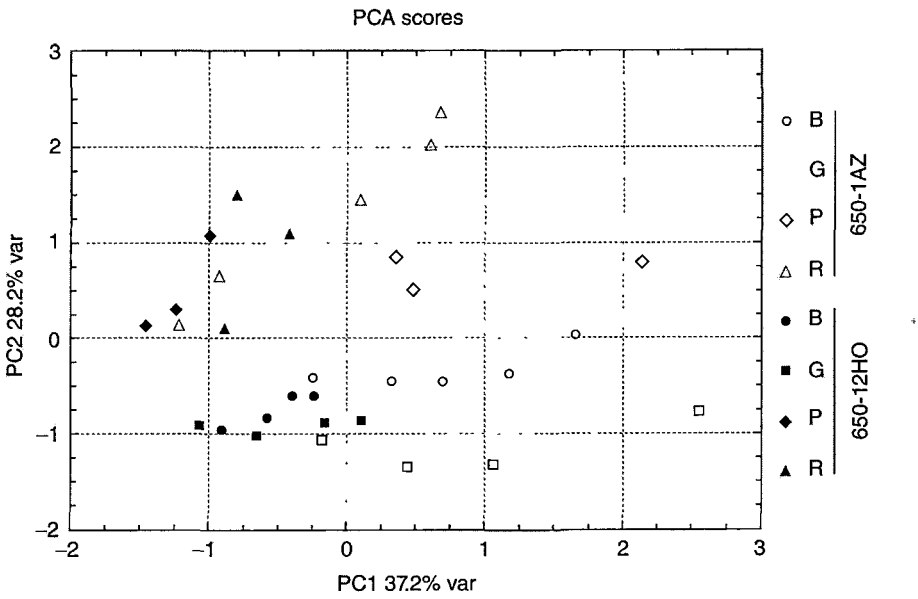


Figure 6.9 Principal component analysis (PCA) map of 31 tomato fruit samples. Stages of fruit ripeness listed are G (green), B (breaker), P (pink), and R (red). Open symbols represent samples from azygous 650-1AZ control line, while the filled symbols represent corresponding fruit samples from the LOX-silenced 650-12HO line. Reproduced with permission from Kausch *et al.* (2012).

analysis of NMR metabolic profiles showed that separation between transgenic and control samples was modulated by ripening; the best separation was between pink fruits (see Fig. 6.9).

Lettuce is a self-fecundating crop with very high homozygosis grade, and all marketed varieties of lettuce are (virtually) highly inbred (Mou 2008). Even a small genetic variation due to a transgene insertion can therefore be easily revealed. Two examples of genetically engineered lettuce studied using NMR metabolite profiling include transgenic lettuce lines (*Lactuca sativa* cultivar "Cortina") expressing the *asparagine synthetase* A (*asn*A) gene from *Escherichia coli* (Sobolev *et al.* 2007), and lettuce (*Lactuca sativa* cultivar "Luxor") over-expressing the Arabidopsis KNAT1 gene (Sobolev *et al.* 2010). Lettuce plants expressing the *asn*A gene are characterized by a higher leaf mass and dry weight and quicker bolting and flowering than controls. Interestingly, a dramatic increase of inulin (1-kestose and higher oligomers) content (up to 30 times) was observed in transgenic lettuce leaves (Sobolev *et al.* 2007, 2010). This appeared as an unexpected effect because the transgene aims to modify the asparagine content, together with the N status, rather than that of carbohydrates. Inulins are plant storage carbohydrates and non-digestible dietary fibers considered probiotics; their presence can therefore be favorable, responding to consumers' demand for low caloric and tasty food with health benefits.

Lettuce over-expresses the A. *thaliana* KNAT1 homeobox gene and exhibits vascular net modifications (mid-vein shortening and hyper-branching), lamina margin modifications (lobbing and fringing), compact curly butter-heads, and flowering anticipation

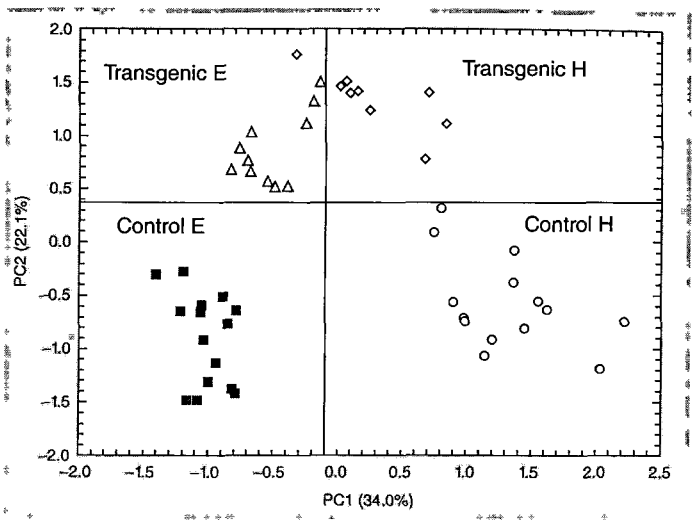


Figure 6.10 PCA scores plot of NMR metabolic profiles from control and transgenic lettuce leaves at precocious (E) and butter-head (H) developmental stages. Adapted from Sobolev *et al.* (2010).

compared to control wild type. The NMR analysis furnished the levels of 22 water-soluble metabolites (mostly amino acids, organic acid, and sugars) in leaves of transgenic and control lettuce at precocious and butter-head developmental stages (Sobolev *et al.* 2010). Explorative statistical analysis such as PCA indicated the development as a main source of metabolic variations (first principal component, 34.0% of variability) followed by transgene expression (second principal component, 22.1% of variability; see Fig. 6.10).

A main effect of the transgene was in altering sugar metabolism. The interaction between the transgene expression and development processes lead to a drop in glucose and fructose levels in butter-heads of transgenic lettuce, suggesting that the simple sugars are major, if not direct, targets of transgene KNAT1 control.

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7

Chromatography

7.1

Introduction to Chromatography – Techniques

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7.1.1 Introduction

Food authenticity is frequently related to a high-quality product. It is normally associated with the use of certain production systems, ingredients, and/or the origin of the product (Di Stefano *et al.* 2012). The concept of "authentic" does not yet have an official definition, but a food item can be perceived as "authentic" if: it complies with the existing regulations; it has the correct composition to assume its legal name; the composition, origin, and production system complies with its label information; and no adulteration by substitution with a cheaper or similar ingredient has been made to reduce the production cost. The current legislation in the European Union only regulates that food labeling, advertising, presentation, and packaging "shall not mislead consumers" (EC 2008). However, as a way to preserve and support the potential to differentiate the quality and authenticity of products from specific regions, the EU has created specific quality schemes (Protected Designation of Origin, PDO; Protected Geographical Indication, PGI; the Traditional Specialty Guaranteed, TSG; and the Organic farming and Outermost regions). These nominations are indicated in the product labels with a specific logo, identifying products and foodstuffs farmed and produced according to exact specifications (EC 2012). These schemes aim to guarantee to the consumers the origin and methods of production, to deliver an effective marketing message that identifies a value-added product, and to reinforce the high-quality production of small rural business across Europe, protecting their products from fraudulent imitations (EC 2012). However, the certification of these labels is only made by the producers and controlled at a documental level; it is therefore urgent to develop objective markers that could provide an analytical control of the authenticity of these added-value products (Di Stefano *et al.* 2012).

These quality indications have huge importance considering the globalization of the food markets which have made available a great variety of food products. The occurrence of several food scandals in the recent years has also raised awareness of authenticity questions. Examples include the case of the addition of melamine to high-protein feed and milk-based products, resulting in the death of six infants and illness of more than 50 000 in 2008 (Ingelfinger 2008); in 2013 the presence of unlabeled horse and

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pork meat was detected in "beef" minced meat products in different samples from several European supermarkets (Premanandh 2013). These events tend to alarm and reduce the confidence of consumers in certain food products. Nowadays, consumers want quality guarantees that enable them to make a conscientious and free choice when buying food (Di Stefano *et al.* 2012).

There are many different fraudulent practices that compromise the "authenticity" of a product. However, they can mainly be grouped into three categories. The first corresponds to the complete or partial replacement of a food ingredient or valuable authentic constituent with a less expensive substitute (or alternative animal species in the case of some meat and fish products, or plant species or varieties in the case of vegetal products). The addition of small amounts of a non-authentic ingredient and the omission of an authentic and valuable constituent or food ingredient in a food product are also common product manipulations that reduce the production cost. Other cases of fraud are the false label claims that tend to add more value to the product (e.g., claiming a determined geographic origin of species, or a specific production process such as organic farming) (Bosque-Sendra *et al.* 2012; Moore *et al.* 2012; Johnson 2014).

These fraudulent practices cause consumers to lose confidence in the products and also cause problems for producers who experience greater difficulty in selling their authentic product due do unfair competition from adulterated products (Reid *et al.* 2006). The detection of an adulteration can be difficult and complex due to the natural variation of food products generated by different growing regions, storage conditions, sample treatment, and processing techniques. The development of suitable, comprehensive analytical techniques that objectively assess the authenticity of a food product are needed. The analytical techniques available have been evolving continually to provide plausible solutions to the fraudulent events that continue to arise (Aparicio and Aparicio-Ruiz 2000). The development of food authentication analytical techniques has to overcome several challenges that are inherent to food matrixes that consist of a great number of nutrients and non-nutrients compounds, such as proteins, peptides, lipids, carbohydrates, amino acids, fatty acids, organic acids, nucleic acids, phytochemicals, minerals, and just about any other small molecules (additives, such as colorants, aromas, preservatives, and other exogenous compounds) (Ibáñez *et al.* 2013). These compounds are chemically very different, ranging from the small organic molecules (usually up to 1000 Da) to macromolecules (biopolymers), and can possess a wide range of polarities; some are apolar (e.g., oils) while others are strongly polar (e.g., amino acids) (Di Stefano *et al.* 2012). The methods have to be able to chemically identify foods or chemical entities that differentiate and authenticate each food and find unique marker compounds or, more often, small analytical differences between patterns. In general terms, current approaches to the authenticity control of food can be either compound-oriented or pattern-oriented. In the first, the analysis focuses on determining specific components with known molecular structures and chemical properties that should be simultaneously responsible for a biological effect or an organoleptic trait characteristic of that food product (Jiang *et al.* 2010; Di Stefano *et al.* 2012). In the second approach, the studies target all detectable components, studying a complete profile of the samples. This is even harder due to the large natural variability between natural products, whose composition can be influenced by external factors such as climatic conditions and production regimes.

The use of highly sophisticated analytical techniques is becoming essential to obtain a definitive authentication of food products and to identify new types of

frauds; such frauds are continually evolving, becoming more complex and difficult to detect (Reid *et al.* 2006). The large amount of data generated in profiling or fingerprinting studies also requires a multivariate data analysis to avoid false conclusions. Through chemometric methods it is possible to qualitatively group or classify unknown samples with similar characteristics, and quantitatively determine the presence of adulterants in the samples (Moore *et al.* 2012). So far, there is no single analytical technique that can overcome the variability inherent to a food product and simultaneously cover all aspects of food analysis. Analyzing different classes of compounds in food requires diverse and specialized methodologies. There is usually a tendency to focus on the analysis of a given class of compounds (Di Stefano *et al.* 2012). Studies based on the presence and quantification alone of organic constituents, minerals, light- or heavy-element isotope ratios, or in conjunction with chemometric analysis, have been presented to prove the authenticity of certain food products (Luykx and van Ruth 2008).

A considerable number of methodologies have been developed for the assessment of the authenticity and traceability of foods based on spectroscopy (UV, NIR, MIR, visible, Raman), isotopic analysis, chromatography, electronic nose, polymerase chain reaction, enzyme-linked immunosorbent assay, and thermal analysis (Cserhati *et al.* 2005; Reid *et al.* 2006). According to the review by Moore *et al.* (2012) on this subject, there are about 16 commonly used instrumental technologies for detecting food adulteration. Between them, the use of liquid chromatography, infrared spectroscopy, gas chromatography, isotope ratio mass spectrometry, and hyphenated mass spectrometry techniques are referred to in the greatest numbers. In this chapter the use of the most common and also the promising new developments in chromatographic methods for food authenticity analyses are addressed. We focus on the advantages of these techniques in the fight against fraudulent procedures, describing the latest analytical developments and applications that were published in the period 2009–2014.

7.1.2 Chromatography

Chromatographic techniques operate on the principle of separation of compounds through the adsorption and/or partition of the molecules between a mobile and a stationary phase. Separation results from the different strengths of adsorption of the molecules between the two phases (Forgács and Cserháti 2003). The IUPAC definition of chromatography states that (Ettre 1993): "Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction."

A chromatographic system normally comprises four components (Ettre 2003; Ardrey 2003*a*):

1) a device to introduce the sample into the system;
2) a mobile phase defined as the fluid that percolates through or along the stationary bed; this may be a liquid (liquid chromatography), a gas (gas chromatography), or a supercritical fluid (supercritical-fluid chromatography);

3) a stationary phase, part of the chromatographic system through which the mobile phase flows and where the distribution of the solutes between the two phase occurs; this could be a solid, a gel, or a liquid; and
4) a detector that corresponds to a device capable of monitoring the change in the composition of the eluent by measuring physical or chemical properties.

The mobile and stationary phases are the only components directly related to the compound separation. These interact in different extents with the mobile and/or stationary phases, taking different times to move across the system until reach the detector (Ardrey 2003*a*). The character of the stationary and mobile phases, the packaging of the stationary phase, and the driving forces of separation allow the distinction between several types of chromatographic techniques. The most frequently used in food analysis are gas chromatography (GC) (solid stationary phase and gas mobile phase), high-performance liquid chromatography (HPLC) (stationary phase filled in column, liquid mobile phase moved by pump), thin-layer chromatography (TLC) (planar solid surface, liquid mobile phase moving by capillary forces or by a pump system), and several electrically driven procedures (capillary zone electrophoresis and micellar electrokinetic chromatography) (Forgács and Cserháti 2003).

Food authenticity and traceability chromatographic analysis were developed to obtain a rapid and reliable separation of chemically similar compounds in complex food matrices. Those methods identify, and if possible quantify, the presence of typical components of foods and the presence of legal or illegal additives, environmental pollutants, and xenobiotics (Cserhati *et al.* 2005). Liquid and gas chromatography are capable of separating and enabling the identification of almost any type of molecule present in a food sample. In this work special attention is paid to the latest innovations of liquid and gas chromatography in authenticity food analysis. The analytical techniques are separated into two major sections: liquid chromatography (LC), in particular HPLC, used to detect compounds such as proteins, amino acids, carbohydrates, vitamins, phenolic compounds, tryglycerides, chiral compounds, pigments, and many others; and gas chromatography (GC), more suited to the analysis of naturally volatile or semi-volatile molecules (Reid *et al.* 2006). Special attention is given to the development of new technologies, new detectors, and improvements in traditional chromatography systems. Techniques such as UPLC (ultra-high performance liquid chromatography), HPLC-MS (high-performance liquid-chromatography mass spectrometry), 2DLC (two-dimensional liquid chromatography), GC-MS (gas chromatography–mass spectrometry) will be discussed; these may be used alone or, more commonly, in combination with different detectors in the latest research on food authenticity issues.

7.1.2.1 HPLC and Hyphenated Techniques

High-performance liquid chromatography (HPLC) is one of the most common forms of liquid chromatography (LC) used to separate, analyze, and quantify compounds that are necessarily dissolved in a solution (Luykx and van Ruth 2008). It was developed in the 1960s as an improvement over column liquid chromatography, and has been applied to the analyses of non-volatile compounds. It is today the most popular food analysis technique, being recognized for its wide suitability and high accuracy in qualitative and quantitative analyses. This straightforward, robust, and reproducible technique is also reasonably priced (Michael 2011).

In HPLC, the liquid mobile phase is delivered under high pressure (up to 400 bar) at a constant flow rate, while the stationary phase is packed into a column capable of withstanding the necessary high pressures (Ardrey 2003*a*). According to the composition of the stationary phase, compounds will be separated based on their charge (weak/strong cation or anion exchange chromatography), molecular mass (size exclusion chromatography), hydrophobicity/polarity (reversed-phase HPLC, hydrophobic interaction chromatography), and specific characteristics (affinity chromatography) (Luykx and van Ruth 2008). The reversed-phase HPLC is the most applied technique, where the mobile phase is more polar than the stationary phase. The use of mixtures of solvents of different polarities to achieve the compound separation is frequent, creating a wide range of possibilities to be employed as mobile phases. However, a mobile phase suitable for separating highly polar compounds will lead to a long retention time for non-polar analytes, and *vice versa*. To overcome this, the separation is often achieved by creating an elution gradient that changes the composition of the mobile phase in a controlled manner during the chromatographic run. The most widely used columns contain a chemically modified silica stationary phase, being the polarity of the column determinate by the nature of that chemical modification (Ardrey 2003*a*).

The great number of detectors that can be coupled to an HPLC system is also one of the main advantages of this technique. The most common detectors used in HPLC are the ultraviolet–visible (UV-Vis) light absorbance detector, diode array detector (DAD), fluorescence detector (FLD), evaporative light scattering detector (ELSD), electrochemical detector, diffractometer, and mass detector (MS). Each detector available to be coupled to an HPLC adds more possibilities for detecting different types of compounds, being selected accordingly to their sensitivity to the compounds of interest (Luykx and van Ruth 2008; Jiang *et al.* 2010). Due to the great variety of compounds that HPLC systems can separate, the detectors available have also evolved. One example is the introduction of a new detector based upon aerosol charging (the charged aerosol detector, CAD), developed to be an alternative to ELSD, chemiluminescent nitrogen detector, and refractive index detector for the detection of non-ultraviolet and weakly ultraviolet active compounds and for UV-absorbing compounds in the absence of standards. CAD demonstrated greater sensitivity than the ELSD system, a wider test range, and better reproducibility (Jiang *et al.* 2010; Vehovec and Obreza 2010). This detector has already been used in some food authenticity studies, for example in the determination of geographical origin of palm oil by chromatographic fingerprinting of triacylglycerols content (Ruiz-Samblas *et al.* 2013) and in the authentication of the composition of traditional Chinese medicinal herbs (Jiang *et al.* 2010).

In the last five years, a great numbers of HPLC applications have been developed for food authenticity analysis. A summary of some of the most significant is presented in Table 7.1.1. The works were grouped by the type of food matrices, highlighting that the authentication of fruit juice and functional food products and supplements are currently the most-studied matrices by HPLC methods. Determination of authenticity of honey and different types of oil by HPLC methods was also described in several recent publications (see Table 7.1.1).

Regarding the compounds of interest in the LC authenticity works compiled in Table 7.1.1, the large number of different chemical compounds used for authenticity assessment is evident. Phenolic compounds are clearly the most studied classes of compounds in food products of vegetal origin in the last years (Table 7.1.1). Nonetheless,

Table 7.1.1 Examples of LC applications in food authenticity analysis between 2009 and 2014.

Matrix	Authenticity issue	Authenticity analysis approach	Compounds of interest	LC systems used	Reference
Cereals	Species/variety identification	Compounds profile	Alkylresorcinols (phenolic lipids)	HPLC-DAD; HPLC-APCI-MS/MS	Knödler *et al.* 2010
Cereals	Species/variety identification	Specific compounds	Two biomarker peptides	HPLC-ESI-MS/MS	Prandi *et al.* 2012
Coffee	Production process	Specific compounds	Carbohydrates	HPLC-HPAEC-PAD; HPLC-UV	Domingues *et al.* 2014
Dairy	Species/variety identification	Compounds profile	Soluble whey proteins: albumins (lactoglobulin, lactoalbumin and serum albumin), immunoglobulin (IgG, IgA and IgM), proteoso-peptones and lactoferrin	HPLC-DAD	Rodriguez *et al.* 2010
Dairy	Species/variety identification	Compounds profile	Trypitc peptides (derived from β-casein)	UPLC-TSQ-MS/MS; HPLC-ESI-QTOF-MS/MS	Russo *et al.* 2012
Fruit	Geographical origin	Finger-printing	Metabolites	UPLC-QTOF-MS	Díaz *et al.* 2014
Fruit juice	Species/variety identification	Compounds profile	Amino acid profiles	HPLC-UV	Pomilio *et al.* 2010
Fruit juice	Production process	Compounds profile	Anthocyanins, betacyanins, synthetic red pigments, hydroxycinnamic acids, hydroxybenzoic acids and catechins	HPLC-DAD-fluorescence	Obón *et al.* 2011
Fruit juice	Species/variety identification	Compounds profile	Phenolic compounds	HPLC-DAD	Abad-García *et al.* 2014
Fruit juice	Species/variety identification	Compounds profile	Profiles of polyphenols	UPLC-DAD-fluorescence; HPLC-MS	Díaz-García *et al.* 2013

(Continued)

Table 7.1.1 (Continued)

Matrix	Authenticity issue	Authenticity analysis approach	Compounds of interest	LC systems used	Reference
Fruit juice	Species/variety identification	Compounds profile	Organic acids (citric, malic, quinic, and tartaric acid)	HPLC-MS/MS	Ehling and Cole 2011
Fruit juice	Species/variety identification	Finger-printing	Metabolites	UPLC-QTOF-MS	Jandrić *et al.* 2014
Fruit juice	Species/variety identification	Compounds profile	Profiles of polyphenols	HPLC-UV; HPLC-ESI-LTQ-Orbitrap	Navarro *et al.* 2014
Fruit juice	Species/variety identification	Compounds profile	Carotenoids profile	LCxLC-DAD-APCI-IT-TOF-MS	Dugo *et al.* 2009
Fruit juice	Species/variety identification	Specific compounds	Carbon 13 isotope ratio ($\delta^{13}C$) of organic acids, glucose and fructose	HPLC-*co*-IRM	Guyon *et al.* 2014
Functional food/ supplements	Production process	Compounds profile	Flavone glycosides and terpene lactones	HPLC-DAD	Chandra *et al.* 2011
Functional food/ supplements	Species/variety identification	Compounds profile	Phenolic compounds (anthocyanin and ellagitaninns)	HPLC-DAD-fluorescence -MS^2	Borges and Crozier 2012
Functional food/ supplements	Geographical origin	Specific compounds	Procyanidins (A-type trimers, procyanidin A2, catechin, epicatechin, and B-type dimers and trimers)	UPLC-MS^2	Jungfer *et al.* 2012
Functional food/ supplements	Species/variety identification	Compounds profile	Flavonoids profiles	HPLC-DAD-ESI/MS^n	Ferreres *et al.* 2012
Functional food/ supplements	Species/variety identification	Compounds profile	Carotenoids profile	HPLC-DAD; UPLC-DAD-ESI-MS	Pop *et al.* 2014
Functional food/ supplements	Species/variety identification	Specific compounds	Gingerols and six other gingerol-related compounds	HPLC-DAD; HPLC-MS/MS	Yudthavorasit *et al.* 2014

Functional food/ supplements	Species/variety identification	Compounds profile	Phenolic compounds	HPLC-DAD-ESI/MS^n	Ferreres *et al.* 2014
Functional food/ supplements	Species/variety identification	Compounds profile	Carotenoids	HPLC-DAD-APCI-MS	Liu *et al.* 2014
Functional food/ supplements	Production process	Specific compounds	Puerins I–VIII	HPLC-DAD-ESI-MS	Wang *et al.* 2014*c*
Functional food/ supplements	Species/variety identification	Specific compounds	Botanical species biomarkers	HPLC-MS; HPLC-ESI-QTRAP-MS/MS	Mathon *et al.* 2013
Functional food/ supplements	Species/variety identification	Specific compounds	Ginsenosides Rb_1, Rb_3, Rd, and F	HPLC-ELSD; UPLC-QTOF-MS	Lu *et al.* 2013
Honey	Production process	Specific compounds	10-HDA, proteins, amino acids	HPLC-DAD; HPLC- fluorescence	Wytrychowski *et al.* 2013
Honey	Species/variety identification	Compounds profile	Phenolic compounds	HPLC-DAD	Cavazza *et al.* 2013
Honey	Production process	Specific compounds	Six maltooligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose)	UPLC-ELSD	Zhou *et al.* 2014
Honey	Species/variety identification	Compounds profile	Phenolic compounds	HPLC-ECD*-DAD (*ECD: electrochemical detection)	Wang *et al.* 2014*a*
Honey	Species/variety identification	Specific compound	2-acetylfuran-3-glucopyranoside	HPLC-DAD; HPLC-QTOF- MS	Xue *et al.* 2013
Honey	Species/variety identification	Compounds profile	Phenolic compounds	HPLC-UV; HPLC-LTQ-OrbiTrap	Campone *et al.* 2014
Meat	Production process	Specific compounds	γ-tocopherol; α-tocopherol	HPLC-DAD-fluorescence	Rey *et al.* 2014

(Continued)

Table 7.1.1 (Continued)

Matrix	Authenticity issue	Authenticity analysis approach	Compounds of interest	LC systems used	Reference
Meat	Species/variety identification	Compounds profile	Fibrinopeptides	HPLC-MS/MS	Grundy *et al.* 2013
Meat	Species/variety identification	Compounds profile	Peptides	HPLC-ESI-LQT-Orbitrap; nano LC-QTOF-MS	Von Bargen *et al.* 2013
Nuts	Species/variety identification	Compounds profile	Triacylglycerols and tocopherols	HPLC-ELSD; HPLC-DAD-fluorescence	Barreira *et al.* 2012*a*
Nuts	Species/variety identification	Compounds profile	Tocopherols	HPLC-DAD-fluorescence	Barreira *et al.* 2012*b*
Oil	Geographical origin	Compounds profile	Triacylglycerols	HPLC-CAD	Ruiz-Samblas *et al.* 2013
Oil	Geographical origin	Compounds profile	Phenolic compounds	HPLC-ESI-TOF-MS	Ouni *et al.* 2011
Organic eggs	Production process	Compounds profile	Carotenoids	HPLC-DAD	van Ruth *et al.* 2011
Organic sweet peppers/lettuce	Production process	Compounds profile	Sugars, organic acids, and vitamin C	HPLC-refractive index detector; HPLC-MS/MS	Flores *et al.* 2013
Traditional food	Species/variety identification	Compounds profile	Organic acids and polyphenols	HPLC-DAD	Willems and Low 2012
Wines/vinegars	Species/variety identification	Compounds profile	Flavonol profiles	HPLC-DAD-ESI-MS^n	Castillo-Muñoz *et al.* 2010
Wines/vinegars	Production process	Compounds profile	Nonanthocyanic phenolic compounds	HPLC-DAD; HPLC-DAD-ESI-MS	Sanz *et al.* 2012

compounds such as peptides, amino acids, sugars, organic acids, carotenoids, tocopherols, and even diverse unidentified metabolites were also used as biomarkers of food authenticity. This high diversity of the compounds shows the huge complexity of certain food matrices and also the great plasticity of the LC systems.

From the different types of detectors found in the authenticity works summarized in Table 7.1.1, there are two that have a clear preference. DAD is still one of the most used detectors in this field, now being (in many works) coupled to a mass detector. The coupling of mass detectors to HPLC systems has shown valuable applications in the food science sector, being among the fastest-developing fields in science (Di Stefano *et al.* 2012). Due to the relevance of mass detectors in the recent development of authenticity studies, this subject is discussed further in the following section.

The quantification of compounds with similar or different structures in some HPLC authenticity studies is of utmost importance in distinguishing between the authentic and the adulterated product (Ouni *et al.* 2011; Díaz-García *et al.* 2013; Rey *et al.* 2014). The information obtained can be used directly from the chromatographic instrument (e.g., peak areas in fingerprinting studies) or can be derived information where an identification and quantification of the obtained signals is attempted (i.e., profiling) (Bosque-Sendra *et al.* 2012). Recent HPLC authenticity studies show a clear tendency to simultaneously identify and quantify a great number of compounds, studying the sample profile instead of one or two compounds (see Table 7.1.1). The use of the metabolic chemical profile of a food will include many unknown components as well as the easily identified components that would usually be used for targeted analysis, potentially providing a more efficient and powerful tool for food authenticity (Jandrić *et al.* 2014). In an untargeted approach the use of high-throughput techniques is required to profile a large number of very different compounds, with diverse chemical and physical properties. These studies generate a large and complex datasets, whose analysis requires the use of appropriate advanced software tools and multivariate statistical analysis to identify and characterize the compounds more relevant to the authenticity of the food product (Bosque-Sendra *et al.* 2012; Jandrić *et al.* 2014). The most employed chemometric methods in the studies summarized in Table 7.1.1 are principal component analysis (PCA) (Flores *et al.* 2013; Xue *et al.* 2013; Ferreres *et al.* 2014), linear discriminant analysis (LDA) (Ouni *et al.* 2011; Barreira *et al.* 2012*b*; Ferreres *et al.* 2014), orthogonal projection to latent structures discriminant analysis (OPLS-DA) (Pomilio *et al.* 2010; Díaz-García *et al.* 2013; Navarro *et al.* 2014), cluster analysis (CA) (van Ruth *et al.* 2011; Xue *et al.* 2013; Ferreres *et al.* 2014), and generalized linear model (GLM) (Grundy *et al.* 2013).

Currently, the evolution of the HPLC analytical systems is tending towards miniaturization, using smaller columns and reduced volume of solvents to achieve a faster separation of compounds. One example is ultra-high-performance liquid chromatography (UPLC) which is capable of increasing the resolution of a mixture of complex compounds, within a fraction of the time, and using a considerably lower quantity of solvent as compared to the traditional HPLC system (Di Stefano *et al.* 2012). UPLC columns are packed with sub-2 μm particles and the system operates at higher pressures (<1000 bar), reaching high linear velocities over the stationary phase that improves the resolution while reducing analysis time (Jing *et al.* 2013). Due to their characteristics the use of UPLC systems has become common in profiling researches, allowing for the analysis of a group of compounds from one or more chemical classes, even when a metabolomic approach is followed in the investigation of food authenticity (Jandrić *et al.* 2014). To

maximize the potential obtained by the UPLC high-separation capacity the use of mass detectors is recommended, being the choice of many food authenticity studies (Russo *et al.* 2012; Díaz *et al.* 2014; Jandrić *et al.* 2014; Pop *et al.* 2014). In many cases the tandem mass spectrometry technique is employed using the multiple reaction monitoring (MRM) scanning method (Di Stefano *et al.* 2012; Jungfer *et al.* 2012; Russo *et al.* 2012; Ibáñez *et al.* 2013; Lu *et al.* 2013; Jandrić *et al.* 2014) (see Table 7.1.1).

However, due to the natural complexity of food matrices, sometimes it is difficult to overcome the overlapping of compounds with new column technologies and even the use of mass detectors (Herrero *et al.* 2009). Multidimensional-liquid-chromatography systems (e.g., LCxLC) were developed to improve the separation of compounds of very complex matrices. Using a comprehensive liquid-chromatography configuration (LC×LC), the whole sample is analyzed in two dimensions. Combining two or more independent, or nearly independent, separation steps (columns) increases the separation power of the corresponding one-dimensional HPLC, achieving a higher sensitivity and resolution. These are ideal characteristics for the analysis of complex matrices such as foods, especially when combined with mass detectors (Herrero *et al.* 2009; Jing *et al.* 2013). These systems can achieve a great enhancement of the peak capacity, which is by far higher than that obtained after improving by any means the one-dimensional separations (Herrero *et al.* 2009). As well as its advantages, there are still some problems related to the connection of the two systems such as: poor orthogonality and small peak capacity in the two dimensions; incompatibility of the eluents in two dimensions and between the mobile phases and the MS detector; and the different separation speed in the two dimensions (usually slower in the second dimension). The system has to guarantee that two constituents separated in the first dimension remain separated in the second dimension (Herrero *et al.* 2009; Jing *et al.* 2013). To reduce the disadvantage created by the solvent immiscibility, new interfaces to couple the two dimensions were developed, also reducing the injection volumes necessary for the second column. The use of new stationary phases and new combinations of different types of liquid chromatography – for example reverse phase liquid and hydrophilic interaction chromatography (RPLC/HILIC); size exclusion chromatography and reverse phase liquid chromatography (SEC/RPLC); ion exchange and size exclusion chromatography (IEC/SEC) – were able to achieve good results in the analysis of very complex food matrices (Jiang *et al.* 2012). Besides the effectiveness in the separation of the compounds, only a few studies have been able to perform the quantification of the compounds (Mondello *et al.* 2008). In the work presented by Herrero *et al.* (2009) the applications of LC×LC in food analysis are reviewed, showing a great number of different groups of compounds of interest and very different food matrices analyzed, proving that the use of this technique might be a valuable tool in the determination of authenticity biomarkers.

7.1.2.1.1 Liquid Chromatography–Mass Spectrometry

Mass spectrometers (MS) are the most selective detectors, being able to detect the presence and facilitate the identification of compounds in a food extract. The great strength of MS is that it can provide the molecular weight of an analyte and information about the structure of the molecule. In a first stage, mass spectrometers promote the ionization and fragmentation of the compounds followed by the physical separation of charged fragments and their detection. The mass spectrum obtained in the analysis indicates the ion intensity *vs* mass/charge ratio (m/z), giving indications of the original structure of

the compound (Kaklamanos *et al.* 2012). Coupling the HPLC system to a MS detector means that even more data are available for the identification of unknown compounds with or without reference standards, such as their retention times, UV shifts, molecular weight, and fragmentation patterns (Jing *et al.* 2013). The on-line combination between HPLC and MS offers the best sensitivity, selectivity, and productivity, improving further by the evolution of both LC and MS technologies (Kaklamanos *et al.* 2012; Ibáñez *et al.* 2013). This technique has already been proven in the determination of a very wide spectrum of classes of molecules with similar structure (e.g., carbohydrates) and even in cases of compounds with a great diversity of structures and polarities (Di Stefano *et al.* 2012). Another advantage attributed to LC-MS methods concerns the sample preparation, which is normally made by simple extraction procedures without the need of derivatization reactions (Kaklamanos *et al.* 2012).

The connection between the two systems can be made in a direct manner, although there are certain aspects that need to be ensured to maximize the capabilities of the hyphenated system. The use of volatile buffers with low ion strength (e.g., 5–50 mM) is recommended; the use of strong acids should be avoided as they could suppress the ionization of the compounds in the mass detector unit. The flow rate should be low; the optimum rate is dependent on the design of the ion source used. Regarding the ionization step, the devices used in LC-MS systems have to be able to promote the evaporation of the solvent along with ionization of molecules. There are several ionization sources usually hyphenated with HPLC systems and whose selection depends mostly on the type of the compounds of interest (Kaklamanos *et al.* 2012). The use of an electrospray ionization source (ESI) is recommended to promote the ionization of polar molecules, while ionization sources such as atmospheric pressure chemical ionization (APCI) or atmospheric pressure photo ionization (APPI) are used for the analysis of low-polarity compounds (Ardrey 2003*b*; Di Stefano *et al.* 2012). There are also different types of mass spectrometers (MS) that can be used in HPLC analyses. They can be generally classified as low resolution (LR-MS) (e.g., single-quadrupole (Q), triple quadrupole (TSQ), ion trap (IT), linear ion trap (LIT), and triple quadrupole-linear ion trap (QTRAP)), or high-resolution (HR-MS) (e.g., Orbitrap, Fourier transform ion cyclotron resonance (FTICR), time-of-flight (TOF), hybrid ion trap-time-of-flight (IT-TOF), hybrid quadrupole-time-of-flight (Q-TOF), hybrid linear ion trap quadrupole Orbitrap (LTQ-Orbitrap)). This classification is based on the mass accuracy and sensitivity that can be achieved by each type of equipment (Jing *et al.* 2013).

The use of LR-MS spectrometers is common due to its ease of operation, relatively low cost (when compared to HR-MS), and small size. Their use in authenticity studies represents one of the biggest advances in this field, offering innumerable possibilities to identify new compounds that could help determine the authenticity of a food product (Castillo-Muñoz *et al.* 2010; Borges and Crozier 2012; Ferreres *et al.* 2012, 2014; Jungfer *et al.* 2012). The ion trap (IT) is one of the most sensitive instruments within the class LR-MS, capable of fast scanning and with a reasonable price. The IT has a three-dimensional geometry, composed of three electrodes with hyperbolic surfaces, the central ring electrode, and two adjacent end-cap electrodes. The ions are trapped inside this structure until a radiofrequency voltage (RF) is applied, causing the instability of ions of a particular m/z that will move in the direction of the detector. The complete mass spectrum is achieved by varying the RF voltage applied (Ardrey 2003*b*). The current IT also allows a tandem mass analysis, performing a multiple stage mass spectrometry

(MS^n) by using additional operations performed sequentially in time. The MS^n spectra obtained in an IT by low-energy collision-induced dissociation (CID) is useful to study the possible fragmentation pathways of target parent compounds, helping to determine the structure of that molecule (Jing *et al.* 2013). The IT tandem mass analysis (MS^n) was recently used to detect different compounds, from the most common flavonoids (Castillo-Muñoz *et al.* 2010) to alkyreocinols (Knödler *et al.* 2010) and carotenoids (Liu *et al.* 2014), whose profile contributes to the authentication of white grape cultivars, durum wheat, and a herb (*Scutellaria barbata*) used in traditional Chinese medicine, respectively (see Table 7.1.1).

The quadrupole analyzer (Q) is a simple and robust LR-MS option that can be used for quantification purposes. It consists of four rods where the opposite pairs are electrically connected. When a specific voltage with a determined radio frequency (RF) and direct current (DC) component is applied, ions of a particular m/z follow a stable trajectory through the rods toward the detector. A mass spectrum is obtained by varying the RF and DC voltages in a systematic way to bring ions of increasing or decreasing m/z ratios to the detector (Cavazza *et al.* 2013). To improve the selectivity of LR-MS, the use of a triple quadrupole detector (TSQ) with a MS/MS configuration was developed, and is today one of the most utilized configurations in LC food authentication studies (see Table 7.1.1). In this tandem mass (MS/MS) configuration, one stage of analysis is used to isolate an ion of interest, while a second stage is used to probe the relationship of this ion with others, from which it may have been generated. The use of tandem mass spectrometry yields structural information about the compounds, allowing an easier identification and quantification of the compounds (Ardrey 2003*b*; Di Stefano *et al.* 2012).

These multistage analyzers perform a scanning technique called multiple reaction monitoring (MRM), capable of monitoring with high sensitivity a large number of compounds in very complex samples. The operational steps of MRM analysis occur in three sequential sets of quadrupole rods. In the first, the information about the molecular ions present is received (also called parent ions). In the second stage (more specifcally, in the second quadrupole), a collision-induced decomposition of each parent ion takes place, the expected resultant fragment ions of which are detected in a second analyzer (third quadrupole). Normally, two different ion transitions from each precursor ion are monitored to unequivocally identify and quantify the target compound. Usually the most intense product ion is used for quantification purposes. To further increase the sensitivity and specificity of this MRM analysis, the selected reactions are monitored within a specific time frame corresponding to the retention time of target compound, and the instrument is tuned to detect a few selected masses (i.e., maximize the signal accordingly to the precursors ions) in the moment of elution, instead of scanning a wide mass range during the analysis. The TSQ can monitor between 10 and 100 MRM reactions in each given scan cycle (1–3 s) (Ardrey 2003*b*; Di Stefano *et al.* 2012).

This technique was successfully applied to the study of organic acids in fruit juices for compositional characterization and authenticity evaluation (Ehling and Cole 2011). These authors developed a HPLC-MS/MS method that detects the presence of low levels of tartaric and quinic acid in pomegranate juice, the levels of which are relevant in the detection of the fraudulent addition of other cheaper juices (e.g., grape juice). Another example of the use of MRM analysis is the UPLC-ESI-MS/MS methodology developed by Russo *et al.* (2012) for the authentication of DOP buffalo mozzarella. The authors were able to detect and quantify the fraudulent addition of bovine milk in the

production of buffalo mozzarella cheese due to the high sensitivity of MRM-based MS and the wide dynamic range of TSQ spectrometers. Another type of fraud that has been raising concerns with consumers and authorities is the use of binding products or food 'glues' from bovine and swine origin, used throughout the food industry to increase the meat use rate and profit margins. The species origin of the binding agent, which has been added to foods at a concentration of 10% (v/w), was successfully assessed in 43 test materials of an inter-laboratory trial using a TSQ and monitoring the presence of two types of fibrinopeptides (A and B) from pig and cow blood plasma (Grundy *et al.* 2013). MRM analysis was also used in the quantification of different botanical biomarkers in different food supplements (Mathon *et al.* 2013). The identification of 98 biomarkers from the 78 plants selected in this study was performed by tandem mass spectrometry using a hybrid triple quadrupole/linear ion trap technology (QTRAP). This detector allowed the identification and quantification of the biomarkers in a single analysis, by combining TSQ scanning and MRM functionality with sensitive linear ion trap scans.

For the identification of unknown compounds to characterize samples profiles and distinguish between species, production processes, or even to identify the presence of unwanted compounds, the most powerful MS detectors are gaining more and more applications (Dugo *et al.* 2009; Ouni *et al.* 2011; Lu *et al.* 2013; Xue *et al.* 2013; Domingues *et al.* 2014; Jandrić *et al.* 2014; Navarro *et al.* 2014). HR-MS offers a more reliable identification of the target compounds, being able to attain a mass error of 1–5 ppm, providing a unique elemental composition assigned directly from the measured mass, further increasing the selectivity of the detection method (Jing *et al.* 2013). Detectors such as TOF, Orbitrap or Fourier transform (FT) mass spectrometers have as their main advantage the determination of elemental formulae in the analysis of compounds with a mass of 3–500 Da. In the TOF system the ions produced in the ion source receive the same kinetic energy, being their velocity inversely proportional to the square root of its mass. The time taken to pass the flight tube of the mass spectrometer is therefore used to determine the m/z of the ion. Higher flight tube distances increase the resolution of the TOF analyzer that will be able to measure very small differences in time required for ions of a similar m/z to reach the detector. The TOF has a fast scanning capability, being recommended for fast analysis (e.g., UPLC) or when high chromatographic resolution is involved (e.g., LCxLC) (Ardrey 2003*b*; Di Stefano *et al.* 2012).

The authentication of olive oil is one of the most studied subjects due to its high value, which also makes it a preferred target for adulteration with cheaper oils (Aparicio *et al.* 2013). A total of 98% of the composition of olive oil corresponds to triglycerides, so the characterization and quantification of their minor constituents by LC (e.g., various polyphenols, phytosterols, chlorophyll, carotenoids, oleuropeine, tocopherols (vitamin E), retinol (vitamin A), and phylloquinone (vitamin K)) could be used for authentication purposes. One example is the work developed by Ouni *et al.* (2011) where a LC-ESI-TOF-MS method was applied to study the phenolic profile of olive oils from different geographical regions. The authors did not find qualitative differences; the differentiation of the geographic origin of the olive oil was possible due to quantitative differences between the several phenolics identified.

In many food authenticity works, a tandem mass configuration corresponds to the combination of a TSQ or IT mass spectrometer with the TOF analyzer (QTOF and IT-TOF, respectively) (Dugo *et al.* 2009; Lu *et al.* 2013; Xue *et al.* 2013; Díaz *et al.* 2014; Jandrić *et al.* 2014) (see Table 7.1.1). In QTOF, the final stage of the triple quadrupole is

replaced by an orthogonal time-of-flight (TOF) mass analyzer (Ardrey 2003*b*). This equipment was proven to be useful in the identification of an adulteration marker, the 2-acetylfuran-3-glucopyranoside, used to detect the fraudulent addition of rice syrup to honeys (Xue *et al.* 2013). The use of an UPLC-QTOF-MS system was also applied in the authentication of different fruit juices in an untargeted metabolite fingerprinting approach (Jandrić *et al.* 2014). In this work, 21 metabolites were selected to differentiate pineapple, orange, and grapefruit juices and their admixtures down to 1% adulteration level (Jandrić *et al.* 2014). A similar analysis system was also used to identify geographical origin biomarkers to differentiate oranges from Valencia (Spain) from Argentinean, Brazilian, and South African oranges. The authors identified a geographic origin marker, the citrusin D, that was seasonal-independent and whose structure was determined by the accurate mass data and MS^E fragmentation spectrum (Díaz *et al.* 2014). The IT-TOF mass spectrometer was used to clarify the native carotenoid profile of different orange juices. For that, the authors developed an LCxLC-DAD-APCI-IT-TOF-MS methodology. Several important epoxycarotenoid esters were first identified in the samples; it was suggested that the relative proportions of 5,6- and 5,8-epoxycarotenoids be used as markers of juice freshness (Dugo *et al.* 2009). Another tandem mass combination between LR-MS and HR-MS spectrometers is the coupling of the IT with the Orbitrap or ion cyclotron resonance analyzers. These tandem instruments have an extreme high resolution power allowing the exact mass of the analyte to be determined from HPLC-full scan MS datasets (Di Stefano *et al.* 2012).

In Fourier transform ion cyclotron resonance (FTICR) mass spectrometers the ions are trapped in a strong magnetic field combined with a weak electric field; the image detected is converted using the Fourier transform to the frequency domain and then to mass spectra. Due to its design, this equipment has great stability and can achieve the highest values of mass resolving power and mass accuracy (ultra-high mass-resolution), being able to discern ions with similar m/z ratios such as isotopic peaks of high-mass ions (Zubarev and Makarov 2013). However, the cost of acquisition and operation of these instruments is still one of the main disadvantages of its use (Kaklamanos *et al.* 2012).

The Orbitrap analyzer is one of the most recent MS detectors from the FTICR family. It was introduced in 2005 as an accurate and compact mass detector, and operates by radially trapping ions around a central spindle electrode. An outer barrel-like electrode is coaxial with the inner spindle electrode and m/z values are measured from the frequency of harmonic ion oscillations, along the axis of the electric field, experienced by the trapped ions. The ion frequencies are measured by acquisition of time-domain image current transients, with subsequent use of fast Fourier transforms (FFTs) to obtain the mass spectra (Hu *et al.* 2005). The first commercial implementation of the Orbitrap was in a hybrid instrument (LTQ Orbitrap) combined with a linear ion trap quadrupole (Zubarev and Makarov 2013). Their use in analytical laboratories is becoming more common, and they have already been applied to food authenticity purposes (von Bargen *et al.* 2013; Campone *et al.* 2014; Navarro *et al.* 2014). One of these studies (von Bargen *et al.* 2013) focused on the detection of trace contaminations of horse meat and pork, identifying 12 specific tryptic peptides against chicken, lamb, and beef. The ability of the LQT-Orbitrap to perform MRM^3 analysis allowed the detection limit to be lowered to 0.13% pork contamination in beef. The low detection levels achieved using MRM and MRM^3 methods were comparable to the most sensitive PCR and ELISA

methods available, showing the great potential of the LC-HR-MS as an alternative analytical technique for meat speciation and authentication. The LQT-Orbitrap capabilities were also used to assess the authenticity of a suspicious cranberry extract (Hu *et al.* 2005) by identifying the presence of A-type proanthocyanidins, characteristic of and more abundant in cranberry-based products, and for the identification of the phenolic profile of natural honeys (Campone *et al.* 2014).

Another promising MS-based detector for HPLC authenticity studies is the isotope ratio mass spectrometer (IRMS), connected to the LC system through an interface assuring the chemical oxidation (*co*) of the compounds entering the detector. IRMS is designed for high-precision isotopic analysis; the carbon isotope ratio ($^{13}C/^{12}C$) is the most commonly studied (Godin and McCullagh 2011). This technique was recently applied to determine carbon 13 isotope ratio of organic acids, glucose and fructose in lime and lemon juices (Guyon *et al.* 2014). In this study, a confidence domain was establish for the average $\delta^{13}C$ values of authentic juices which allowed the detection of any unlabeled sugar added to commercial juice samples. This technology was also used in studies of honey (Cabanero *et al.* 2006), caffeine (Zhang *et al.* 2012), and wine authenticity (Cabanero *et al.* 2010).

LC-MS-based analytical techniques have gained a favorable reputation in the unambiguous detection and accurate quantification of a large variety of compounds in foods. The great success of these techniques results mainly from the combination of the high separation capacity of most recent HPLC systems with the power of mass spectrometry to identify molecular structures; these techniques are also in constant evolution and offer a great diversity of instruments with enhanced detection capabilities. This confirms the importance of this technique for the future of routine food authenticity/adulteration studies, capable of decreasing the detection limits, improving the capability for multicomponent analysis, and allowing the simplification of sample preparation procedures (Di Stefano *et al.* 2012).

7.1.2.2 GC and Hyphenated Techniques

Since its introduction in the early 1950s, gas chromatography (GC) is known by its sensitivity, stability, and high efficiency in the analysis of food samples. GC is one of the most universal separation techniques used for characterizing the food volatile fraction (aroma and flavor), identifying and quantifying several organic contaminants and residues in complex food samples, analyzing and characterizing fat (low-chain fatty acids, fatty acid methyl esters (FAMEs), triglycerides, sterols, etc.), verifying the compliance with quality standards and safety requirements, and authenticating the composition and origin of certain foods (Luykx and van Ruth 2008; Cordero *et al.* 2012). GC is more usually employed in the analysis of non-polar compounds, but polar compounds can be also analyzed if they are first derivatized (Michael 2011). In general terms, the mixture of compounds to be analyzed is initially injected into the GC systems, then vaporized in a heated chamber. The gas mixture is then transported through the GC column by the flow of an inert carrier gas (mobile phase); the compounds are then separated by their interaction with the coating of the column. In comparison with HPLC, the mobile phase in GC has a very limited role in the separation process (Luykx and van Ruth 2008). The use of GC systems in authenticity evaluation is also very common within the methods applied in this field (Moore *et al.* 2012). Following the same procedure used for the LC

section, some of the most relevant authenticity works based on GC methods from the period 2009–2014 are presented in Table 7.1.2.

The same 13 groups of food matrices and also the same authenticity issues are found in these studies. Regarding the compounds of interest of each study, it is possible to note a clear difference between the GC and LC systems (see Table 7.1.2). The exceptions were recorded for the analysis of carbohydrates (Ruiz-Matute *et al.* 2010; Willems and Low 2012; Wytrychowski *et al.* 2013; Yang *et al.* 2014) and organics acids (Jumhawan *et al.* 2013), the detection and quantification of which was also performed by GC.

In GC analysis the introduction of the sample into the chromatographic system determines the resolution attained, dependent upon the specific injection system. The most common injection methods can be divided into hot-injector vaporization methods (split/splitless injection) and on-column injection. In the former, the sample is introduced by a syringe into a hot chamber, which is then promptly vaporized and transferred by the carrier gas flow into the column. The main difference between the split/splitless injection modes is the use of a closed valve for a fixed time in the splitless mode. This forces the entire flow to pass through the inlet into the column, which is more appropriate for a highly diluted sample. In the on-column injection, the sample will be placed inside the GC column without any vaporization. The increase of the column temperature causes the increase of the solute vapor pressure that will then be transported by the carrier gas through the column. This technique is more suitable for analyzing samples with thermally labile and/or high-boiling point compounds (Cordero *et al.* 2012).

Another type of injection in GC systems is the injection of vapor samples through headspace (HS) injection. In this mode the sample vapor is concentrated at the head of the column, injected through a transfer line or a classical split or splitless inlet. Headspace sampling is considered a robust method of sample preparation for the analysis of volatiles compounds. The purge-and-trap technique is one of the most-used HS sampling modes and employs equipment in which the sample is purged with a carrier gas (e.g., helium) for a fixed time and temperature. The sample volatile compounds are concentrated onto a suitable material (the trap) by an adsorption or sorption process, according to the trap material. The cartridge containing the trap is then heated under a flow of carrier gas, causing desorption or vaporization of the previously trapped compounds that will be moved to the head of GC column (Cordero *et al.* 2012). The isolation of the analyte from the sample matrix is particularly important in GC analysis to avoid the occurrence of false responses due to the presence of degradation products from the matrix (Michael 2011).

After the introduction of the sample into the column, the compounds are dynamically equilibrated and separated between the stationary and the mobile phases, depending on the vapor pressure of each compound. The more volatile compounds have more affinity for the mobile phase, and are eluted first. The GC columns (stationary phase), commonly known as "capillary columns," are coated as a thin film on the inner column wall, generally consisting of fused silica tubings of different diameters in the case of food analysis systems. These columns must be maintained at a suitable temperature and continuously swept by the carrier gas. Commonly used gases include nitrogen, helium, and hydrogen (Luykx and van Ruth 2008).

The appearance of the open tubular columns for GC systems greatly improved the separation efficiency of these systems, used today in most of the analytic systems due to

their higher sensitivity, longer lifetimes, low bleeding rates, and shorter analysis times. The separation in these columns is also dependent on their polarity and selectivity. The low-polarity columns (e.g., 100% polydimethylsiloxane or 95% dimethyl-5% diphenyl-polysiloxanes) are the most used, covering 80% of GC applications (Cordero *et al.* 2012).

One special type of GC column that has proved very useful in food authenticity studies is those with the possibility to perform the separation of enantiomer compounds. The chiral flavor and fragrance components of natural origin generally have a characteristic distribution of enantiomers, attributable to stereoselectively controlled biogenetic mechanisms. One example is the stereoselective reactions of enzymes in the biosynthesis of volatiles that normally results in a large amount of one enantiomer, while in an organic synthesis a racemic mixture is produced. Any changes in the enantiomeric ratios could therefore indicate a fraudulent manipulation or the addition of synthetically produced chemicals (Spanik *et al.* 2014). This fact was the basis of the development of several food authentication methods to identify adulterations, monitor fermentation processes, evaluate shelf-life, determine the geographic and botanic origin, and in the control of technological processes (Ravid *et al.* 2010; Cordero *et al.* 2012; Verzera *et al.* 2014).

The addition of "natural" or "synthetic" flavors to food has to be discriminated in the product label by law (EC 2008), where enantioselective GC (Es-GC) analysis provides a decisive tool to monitor the conformity of the label and check the product authenticity. The derivatized cyclodextrins (CDs) are the most widely used chiral selectors for GC chiral recognition of underivatized analytes, in particular the GC enantiomer separation of natural and synthetic flavors and fragrances (Cordero *et al.* 2012). These columns were used in the authentication of the botanical origin of different honeys (Spanik *et al.* 2014; Verzera *et al.* 2014) to identify the addition of artificial flavors to different fruit-flavored foods and beverages (Ravid *et al.* 2010; Cagliero *et al.* 2012) and to characterize the aroma-active compounds of fermented honeybush, a traditional plant from South Africa used for tea (Roux *et al.* 2012).

The GC enantiomeric analysis was also used in the characterization of pure enantiomers of limonene and carvone in mentha essential oils, being identified the (S)-limonene and (R)-carvone as the unique enantiomeric forms existing for both compounds in *Mentha spicata* and *Mentha piperita* species (Barba *et al.* 2013). Besides the chiral separation, this study used on-line coupled reversed-phase liquid chromatography with gas chromatography and mass spectrometry (LC-GC-MS). The coupling of LC and GG has the advantage of fractioning and cleaning up the sample in the LC system, transferring much cleaner fractions to the GC and transferring only the fractions of interest (Cordero *et al.* 2012). This on-line combination of LC-GC has also proved valuable in the analysis of intact phytosteryl/phytostanyl fatty acid esters in enriched yogurt, cheese-based spread, and milk dairy foods (Esche *et al.* 2013). The phytosteryl/phytostanyl fatty acid esters from vegetal origin are added to several daily foods due to their impact on the cholesterol-lowering effect and also for technological reasons. In this work, a LC-GC-MS method was able to analyze the phytosteryl/phytostanyl esters in low-fat and fat-based products, creating a base for authenticity assessment of these enriched novel foods (Esche *et al.* 2013).

Regarding the detection of the compounds separated in the GC systems, there are several detectors that can be coupled to GC systems. This multiplicity of options is one of the key reasons for the great success of these systems. They can be generally classified

as universal (measure nearly all components in a mixture) or selective (detect components with a specific structural feature in their molecules). Examples of universal detectors include the thermal-conductivity detector (TCD) and the flame-ionization detector (FID). The latter is commonly used in authenticity works (see Table 7.1.2) due to its high sensitivity, stability, large linear response range, and low noise capacities. In the former (FID), the organic molecules are degraded by oxidation in a hydrogen-rich flame between two electrodes into smaller charged species; the changes caused in electrical conductivity are monitored, electronically amplified, and recorded (Luykx and van Ruth 2008; Cordero *et al.* 2012). The most common selective detectors include: the electron-capture detector (ECD) (detection of halogenated compounds, nitro-aromatics, highly conjugated molecules and metal chelates); the nitrogen/phosphorus detector (NPD) (detection of analytes containing nitrogen and/or phosphorus); and the flame photometric detector (FPD) (detection of organosulfur and organophosphorus compounds). However, the most-used detector in GC systems in the latest authenticity publications is the MS detector which can provide reliable information for the qualitative analysis of the complex constituents (see Table 7.1.2) (Eiceman *et al.* 1996). The use of MS detection in GC methods has become usual over the past 30 years due to the decrease of the cost and complexity, along with the increase in stability and durability that made them the most common GC detectors (Eiceman *et al.* 1996). Due to the great importance of MS detectors in the evolution of authenticity works, the GC-MS coupling is discussed further in the following section.

Another GC-based method that still has an important function in different food authenticity studies is the GC-olfactometry (GC-O). This is the term used to describe techniques that use human assessors to detect and evaluate volatile compounds eluting from a GC separation (Delahunty *et al.* 2006). This method was recently used in the authentication of the volatile profile of the honeybush (Roux *et al.* 2012) and grilled beef (Resconi *et al.* 2012).

Besides the evolution of the injection systems, columns, and detectors for GC analysis, one of the major trends in this area is also the use of very fast analysis (HSGC) in smaller instruments. A "fast" GC analysis normally takes less than 10 minutes, and can be considered "ultra-fast" if it takes 1 minute or less. The use of short columns with narrow inner diameters (0.1 mm or less) or short capillary columns are most common in high-speed analysis (Cordero *et al.* 2012). However, as speed and resolution in HSGC are inversely correlated, this technique relies upon the optimization of several parameters such as the sample introduction, selection of the stationary phase film thickness, the temperature program, the flow of the carrier gas, and the selection of suitable detectors (Eiceman *et al.* 1996). Another approach to increase the selectivity of HSGC is to use a second column of differing polarity to obtain a multidimensional separation. These latter developments allow for real-time monitoring and on-site analysis (portable GC) (Luykx and van Ruth 2008). A HSGC method was develop by Cagliero *et al.* (2012) to check the authenticity of fruit-based foods and beverages. These authors described a fast Es-GC-MS method that was able to reduce the normal time of analysis by more than 50%.

As occurs in LC systems, there are many compounds found in food matrices that cannot be easily separated in GC systems, showing a similar chromatographic retention and MS fragmentation patterns. The use of multidimensional GC techniques (MDGC) and comprehensive two-dimensional GC (GCxGC) can overcome these limitations,

Table 7.1.2 Examples of GC applications in food authenticity analysis between 2009 and 2014.

Matrix	Authenticity issue	Authenticity analysis approach	Compounds of interest	LC systems used	Reference
Beer	Species/variety identification	Fingerprinting	Volatile compounds	GC-TOF MS	Cajka *et al.* 2010
Cereals	Species/variety identification	Compounds profile	Fatty acids methyl esters, phytosterols and vitamins	GC-MS	Bodroza-Solarov *et al.* 2014
Coffee	Species/variety identification	Compounds profile	Silylated components (TMS) of the unsaponifiable fraction	GC-FID	Pacetti *et al.* 2012
Coffee	Species/variety identification	Compounds profile	Citric acid, malic acid, and the inositol/pyroglutamic acid ratio	GC-Q/MS	Jumhawan *et al.* 2013
Dairy	Production process	Specific compounds	Intact phytosteryl/phytostanyl esters	On-line LC-UV-GC -FID; GC-MS	Esche *et al.* 2013
Dairy	Species/variety identification	Fingerprinting	Lipid fraction	GC × GC-MS/ FID; GC-TOF-MS	Tranchida *et al.* 2013
Fruit	Production process	Fingerprinting	Volatile compounds	HS-GC/MS	Steingass *et al.* 2014
Fruit	Species/variety identification	Fingerprinting	Volatile compounds	GC-MS/MS	Vrhovsek *et al.* 2014
Fruit	Production process	Compounds profile	Methyl butanoate, ethyl butanoate, hex-(2E)-enal, methyl hexanoate, buthyl butanoate, ethyl hexanoate, hexyl acetate, linalool, hexyl butanoate, octyl isovalerate, -decalactone and octyl hexanoate	GC-C-IRMS; GG-MS; Es-GC-FID	Schipilliti *et al.* 2011
Fruit juice	Production process	Compounds profile	γ-lactones, monoterpenes and a C13-norisoprenoid ketone	HS-Es-GC-MS	Ravid *et al.* 2010

(Continued)

Table 7.1.2 (Continued)

Matrix	Authenticity issue	Authenticity analysis approach	Compounds of interest	LC systems used	Reference
Fruit juice	Production process	Specific compounds	Racemic c- and d-lactones: a-ionone, linalool, nerolidol, ethyl 2-methylbutyrate, 2- methylbutyric acid and 2-methylbutanol	Es-GC-MS	Cagliero *et al.* 2009
Fruit juice	Species/variety identification	Compounds profile	Alkylesters and carbonyl compounds (hexanal,trans-2-hexenal), alcohols (1-hexanol, 1-butanol, cis-3-hexenol)	APCI-S; GC-MS	Gan *et al.* 2014
Functional food/ supplements	Species/variety identification	Fingerprinting	Volatile compounds	GC-MS	Socaci *et al.* 2013
Functional food/ supplements	Species/variety identification	Compounds profile	Oligosaccharides	GC-MS	Yang *et al.* 2014
Functional food/ supplements	Species/variety identification	Compounds profile	Fatty acid profile	GC-MS	Pop *et al.* 2014
Functional food/ supplements	Species/variety identification	Fingerprinting	Volatile compounds	GC-MS	Wang *et al.* 2014*b*
Functional food/ supplements	Species/variety identification	Fingerprinting	Volatile organic compounds, mainly terpenoids	GC-TOF-MS; Es-GC-MS; GC-O	Roux *et al.* 2012
Honey	Production process	Specific compounds	Carbohydrates	GC-FID	Wytrychowski *et al.* 2013
Honey	Species/variety identification	Compounds profile	Volatile organic compounds	GC-MS; Es-GCxGC-FID	Spanik *et al.* 2014
Honey	Species/variety identification	Compounds profile	Volatile compounds	HS-Es-GC-MS	Verzera *et al.* 2014
Meat	Production process	Fingerprinting	Volatile compounds	GC-O	Resconi *et al.* 2012

Meat	Production process	Compounds profile	Hydrocarbons	GC-MS	Hwang *et al.* 2014
Nuts	Species/variety identification	Compounds profile	Fatty acids	GC-FID	Barreira *et al.* 2012*a*
Nuts	Species/variety identification	Compounds profile	Fatty acids	GC-FID	Mikkelsen *et al.* 2014
Oil	Species/variety identification	Compounds profile	Fatty acids	GC-FID	Hrastar *et al.* 2009
Oil	Species/variety identification	Compounds profile	Sterols	GC-FID	Al-Ismail *et al.* 2010
Oil	Species/variety identification	Compounds profile	Fatty acids, sterols	GC-FID (FA); GC-MS (sterols)	Li *et al.* 2011
Oil	Production process	Specific compounds	*Trans*- and *cis*-phytol	GC-MS	Vetter *et al.* 2012
Oil	Geographical origin	Compounds profile	Triacylglycerols	HTGC-MS	Ruiz-Samblas *et al.* 2012
Oil	Species/variety identification	Specific compounds	pure enantiomers of limonene and carvone	LC-GC-MS	Barba *et al.* 2013
Oil	Geographical origin	Fingerprinting	Volatile organic compounds	GC-C-IRMS; Es-GC-FID; GC-FID	Schipilliti *et al.* 2010
Organic	Production process	Specific compounds	Phytanic acid and pristanic acid	GC-MS	Vetter *et al.* 2010
Traditional food	Species/variety identification	Specific compounds	Carbohydrates	GC-MS; GC-FID	Ruiz-Matute *et al.* 2010
Traditional food	Species/variety identification	Compounds profile	Oligosaccharide profiles	GC-FID	Willems and Low 2012

(Continued)

Table 7.1.2 *(Continued)*

Matrix	Authenticity issue	Authenticity analysis approach	Compounds of interest	LC systems used	Reference
Wines/vinegars	Production process	Compounds profile	Volatile compounds (furfural, acetic acid, ethyl acetate, 3-hydroxy-2-butanone, 3-methyl-1-butanol, isopentyl acetate, benzaldehyde, phenylethyl alcohol)	HS-GC-MS	Xiao *et al.* 2011
Wines/vinegars	Species/variety identification	Compounds profile	cyclic polyalcohols	GC-MS	Allonon *et al.* 2011
Wines/vinegars	Production process	Specific compounds	Lactones (γ- and δ-)	HS-GC-TSQ-MS	Langen *et al.* 2013
Wines/vinegars	Geographical origin	Compounds profile	Methanol and higher alcohols; monoterpene alcohols; fatty acids and their ethyl esters, Acetates, 1-hexanol, diethyl succinate and 2-phenyl ethanol; volatile phenols; sulfur compounds;	GC-FID; GC- FPD; GC-MS	Gómez-Meire *et al.* 2014
Wines/vinegars	Species/variety identification	Specific compounds	γ-butyrolactone, γ-hexalactone, trans-whiskey lactone, γ-octalactone,cis-whiskey lactone, γ-nonalactone, γ-decalactone, δ-decalactone, and γ-undecalactone	GC-MS	Pérez-Olivero *et al.* 2014

due to increased separation power and the possibility of adopting different separation mechanisms in the two chromatographic dimensions (Herrero *et al.* 2009). In MDGC the sequential transfer of compounds from the first to the second column is usually done using an on-line heartcut that only allows the transportation of some key analytes from the first to the second column. In contrast, in GCxGC all compounds separated in first dimension are transferred to the second column, which must be operated at high speed. In this case, the interface between the two columns is a modulator that increases the amplitude of the signal and facilitates the transfer to the second dimension (Herrero *et al.* 2009). These systems can be coupled to several different detectors that are able of a fast response time (e.g., FID, ECD, NPD, and MS).

Due to their high resolution capacity, mass accuracy, and scanning speed, the TOF-MS is one of the best options for coupling with a GCxGC system. The main advantages of this type of GC chromatography include: the increased peak capacity; improved resolution and enhanced mass sensitivity, which improves accuracy of the classification and identification of unknown compounds; and the significant reduction of sample preparation procedures. The main disadvantages include the high cost of the equipment and its maintenance and the difficulties in optimizing the method. The use of comprehensive GCxGC systems in food analysis is mostly focused on safety issues, such as the determination of pesticides, contaminants, and harmful compounds in different food matrices (Herrero *et al.* 2009). However, both GCxGC and MDGC have shown favorable results in the composition of diverse food matrices (e.g., essential oils composition, flavor compounds of alcoholic drinks, fruits, coffee, cheese, honey, milk) (Herrero *et al.* 2009; Tranchida *et al.* 2013; Spanik *et al.* 2014). In most cases, the results obtained by GCxGC comprise highly complex fingerprinting profiles that could be used to differentiate similar species, food origins, and processing methods (Luykx and van Ruth 2008; Herrero *et al.* 2009; Cordero *et al.* 2012). In these cases and others where fingerprinting methodologies are employed, the use of chemometric methods to evaluate the generated data is necessary to detect the profile patterns and to obtain an analytical authenticity marker for that food product.

7.1.2.2.1 Gas Chromatography–Mass Spectrometry

The unequivocal identification of the composition of food matrices is sometimes one of the most reliable approaches to the authenticity assessment of these products. Coupling the high separation capacity of the GC systems with MS spectrometers is the most effective method to analyze volatile and thermally stable compounds in food matrices. The advances in GC-MS techniques has been dependent on the instrumental improvements to obtain higher mass resolution, accurate mass measurement, higher sensitivity, enhanced fragmentation capabilities, and better linearity (Ibáñez *et al.* 2013).

In GC-MS, the compounds are mainly ionized through electron impact (EI) or chemical ionization (CI) techniques, considered soft ionization methods. Although these provide less structural information, the lack of fragmentation and the high efficiency of ionization allow simple mass spectra with high sensitivity (Eiceman *et al.* 1996). In combination with a chromatographic separation data, such as the compound's linear retention index or its relative retention, the identification or at least characterization of the compound is possible (Cordero *et al.* 2012). The use of a full-scan EI source mass spectrum is one of the most common methods for identification in GC-MS. However, the use of selected ion monitoring (SIM) or tandem techniques with multiple ion monitoring

(MIM) can provide more useful information in the case of more complex mixtures by improving the selectivity and sensitivity of the analysis (Eiceman *et al.* 1996; Cordero *et al.* 2012). The mass detection capabilities will also be enhanced when a high-resolution MS spectrometer is used.

Regarding the low (LR) or high (HR) resolution of the MS instruments usually coupled to GC systems, the more recent GC-MS-based studies have focused on determining the authenticity of different food products, revealing a prevalence of LR-MS detection systems (see Table 7.1.2). As well as their low resolution, the scanning velocity of these instruments is not limiting, being suitable for fast-GC and comprehensive GCxGC methods (Cagliero *et al.* 2012; Cordero *et al.* 2012; Tranchida *et al.* 2013). As described before for LC-MS (Section *7.1.2.1.1*) the use of a tandem mass configuration in GC-MS is also an option to increase the sensitivity and selectivity of the detector, particularly for trace analysis.

The use of TSQ or IT-MS coupled to GC methods allows the study of the fragmentation pattern of selected precursor ions. This technique was employed in the characterization of the volatile profile of different fruits (Vrhovsek *et al.* 2014) and wines (Langen *et al.* 2013). On the other hand, in cases where the determination of a superior mass accuracy is needed for compound identification, coupling with HR-MS spectrometers will greatly enhance the capacity of the analysis. HR-MS has higher acquisition rates and resolving power which, together with accurate mass and data from fragmentation (MS/MS), could facilitate the determination of the molecular structure of unknown compounds (Ibáñez *et al.* 2013).

TOF is one of the most used GC-HR-MS options in authenticity studies (Cajka *et al.* 2010; Roux *et al.* 2012; Tranchida *et al.* 2013) due to the ability to achieve a high volume of data across a narrow peak in full scan operation and to operate over a wide spectral mass range and fast scanning (Cordero *et al.* 2012).

Another GC-MS-based technique that has demonstrated great potential in the authenticity control of foodstuffs is GC-C-IRMS. The on-line combustion (C) of compounds separated by GC allows the exact determination of the isotope distribution. Small differences in stable isotope ratios were correlated to different geographical origin of mandarin oils (Schipilliti *et al.* 2010) and to the authenticity of the aromas of flavored strawberry foods (Schipilliti *et al.* 2011).

MS-based GC analyses have recently become one of the most preferred methods for the evaluation of the authenticity of very different food products (Moore *et al.* 2012). Analysis tools in this field are constantly evolving to respond to the needs of legislators, consumers, and producers and also to avoid fraudulent practices. Complete characterization of certain classes of compounds (profiling) by GC-MS is one of the main tools in the authenticity area, where most studies are focused on the determination of differences between profiles. Profile analysis by different chemometric tools reliably distinguishes between species and production processes by determining relevant compounds that may be selected as authenticity markers.

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7.2

Chromatography – Applications

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7.2.1 Introduction

Nowadays, the new and sophisticated methods used for food frauds necessarily involve the development of robust, efficient, sensitive, and cost-effective analytical methodologies for their detection. The use of these advanced analytical approaches is necessary to guarantee the safety, quality, and traceability of foods in accordance with legislation and demands of consumers (Cifuentes 2012). Due to the chemical complexity of foodstuffs, the results of high-resolution analytical techniques are extremely useful for their analysis. In this sense, enhancements in analytical accuracy, precision, detection limits, and sample throughput have expanded the practical application of chromatographic techniques for the detection of food frauds and to control food authenticity. The coupling of these techniques with mass spectrometry (MS) constitutes a further advancement for identification of quality markers. Moreover, the use of chemometric tools is generally required as a final stage of the authenticity evaluation. Among the different chromatographic techniques, both gas (GC) and liquid chromatography (LC) are the most widely used to detect food frauds. Some examples of chromatographic applications considering the type of analyzed compounds are discussed in this chapter.

7.2.2 Carbohydrates

Carbohydrates are polyhydroxyaldehydes and polyhydroxyketones which are important constituents of foods. They are a widely known source of energy, but also possess different bioactivities (immunostimulatory, prebiotic, antioxidant, etc.) and technological functionalities (emulsifying, gelling, etc.). Carbohydrates can be classified according to their degree of polymerization (DP) into four chemical groups: mono- (DP1); di- (DP2); oligo- (DP3-20); and polysaccharides (>DP20). In this chapter we will not consider polysaccharides, taking into account the limitation of chromatographic techniques for their analysis.

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Carbohydrates are commonly analyzed either by GC or LC; however, both techniques have their advantages and disadvantages. Carbohydrates must be transformed into volatile compounds through appropriate derivatization procedures for GC analysis (Ruiz-Matute *et al.* 2011). Several efficient methods have been proposed; the formation of trimethylsilyl ethers, trifluoroacetates and alditol acetates is the most popular, giving a different compound for each anomeric form of the sugar (up to six). Converting sugars to oximes prior to trimethylsilylation reduces the peaks formed for each sugar to two: *E*- and *Z*- oxime isomers. Regarding GC column selection, methylpolysiloxanes are the most widely used, although other more slightly polar phases have also been proposed such as 50% phenylmethylpolysiloxane (Sanz *et al.* 2004*a*). Recently, the use of several commercial stationary phases based on ionic liquids for the analysis of mono- di- and trisaccharides has been also evaluated (Rodriguez-Sanchez 2015). The best results in terms of resolution and peak symmetry were obtained when a SLB™-IL82 (1,12-di(2,3-dimethylimidazolium)dodecane bis(trifluoromethylsulfonyl)imide) column was used.

GC usually allows a good separation of mono-, di-, and trisaccharides, although coelution of carbohydrates of the same DP is observed when complex mixtures are considered. Coupling of GC with electron impact (EI) MS helps carbohydrate identification considering the typical fragments, even at low abundance, produced for the different glycosidic linkages (Sanz *et al.* 2002; Brokl *et al.* 2009).

Regarding analysis of carbohydrates by LC, derivatization is not mandatory as in GC, but it is in some cases necessary (either pre-column or post-column), mainly to enhance sensitivity in the detection during analysis or to improve separation. Most derivatization methods are based on the condensation of a carbonyl group in carbohydrates with primary amines, which possess a chromophore or fluorophore substituent, to give a Schiff base which is then reduced to a *N*-substituted glycosyl amine. Reductive amination has been carried out with 2-aminopyridine, different trisulphonates, esters of *p*-aminobenzoic acid, 2-aminoacridone, etc. (Sanz and Martínez-Castro 2007). Other reactions are based on acetylation or perbenzoylation of carbohydrates, which overcomes problems of solubility in organic solvents or improves the chromatographic properties on reverse-phase columns, respectively.

Different operational modes for the analysis of carbohydrates are used; the most common for food authentication are high-performance anion exchange chromatography (HPAEC) and hydrophilic liquid chromatography (HILIC). HPAEC is based on the separation of carbohydrates which are negatively charged under eluting conditions (mobile phase: NaOH pH>13 and a gradient of increasing concentration of sodium acetate). Stationary phases are composed by polymeric, non-pourous, MicroBead™ pellicular resins such as polystyrene/divinylbenzene or ethylvinylbenzene/divinylbenzene substrates agglomerated with Microbead™ quaternary amine functionalized latex, which are highly resistant to high pHs (Dionex 2004).

Different structural characteristics of the molecules (charge, molecular size, monomeric composition, and glycosidic linkages) affect the chromatographic separation. Elution of carbohydrates with the same glycosidic linkage composition is principally based on increasing molecular weight; however, this order can change when families of oligosaccharides with different linkage variants are mixed (i.e., isomaltohexaose elutes before maltotriose; Morales *et al.* 2006). On the other hand, considering the extremely basic pH and the high salt concentration of mobile phase, direct coupling with MS is

not allowed in HPAEC. This technique therefore requires the use of standards for the identification of complex mixtures of carbohydrates with different DP and glycosidic linkages. Regarding HILIC, amide-bonded stationary phases are the most commonly used for the analysis of carbohydrates to control food authenticity.

The analysis of carbohydrates by both GC and LC has proven to be especially useful for the authentication of different foods, and several applications have been covered in peer-reviewed journals and books (Prodolliet and Hischenhuber 1998; Soria *et al.* 2008). Some of the most characteristic examples, with special emphasis on those more recently developed, are described.

Honey is probably one of the most complex mixtures of carbohydrates existing in nature. Considering its added value as a natural product, its limited supply, and its relatively high price, honey has been the target of different frauds and the assurance of its quality is of special relevance. Honey fraud can involve the addition of either lower-cost sugar syrups or its sale under a false botanical or geographical origin (Cotte *et al.* 2003). Molasses, corn syrups (CS), invert syrups (IS) obtained by acidic or enzymatic hydrolysis from refined beet or cane sucrose, and high-fructose corn syrups (HFCS) with different degrees of isomerization (20% 20HFCS; 40% 40HFCS; 80% 80HFCS), mainly produced by enzymatic hydrolysis and isomerization of corn starch, are the most common sweeteners that can be added directly to honey after harvest or fed to bees during the harvest to improve yield (Swallow and Low 1994). The variations in honey carbohydrates and the similarities with sugar syrup composition hinder the detection of these adulterations.

Different strategies have been suggested to detect honey adulterations using either GC or LC: (1) study of carbohydrate concentration ratio values; (2) evaluation of chromatographic fingerprints; and (3) detection of an appropriate indicator (a carbohydrate present in adulterated honeys and absent from original honeys).

Taking into account the variable concentration of carbohydrates in honeys and some sugar syrups, characteristic disaccharide concentration ratio values, such as fructose/glucose or maltose/isomaltose, were proposed as markers to determine honey authenticity many decades ago (Doner *et al.* 1979). With the advent of capillary columns GC methods were improved, achieving a better separation of carbohydrates and the detection of a higher number of di- and trisaccharides in honeys. Horvath and Molnar-Perl (1997) developed a GC-MS method for the simultaneous quantitation of major and minor honey constituents. These authors established new concentration ratios such as maltose/turanose, sucrose/turanose, and maltotriose/raffinose+erlose+melezitose to control honey authenticity. However, this strategy is only valid when honeys are adulterated with highly hydrolyzed starch syrups such as CS, with a high concentration of maltose or maltotriose.

Other studies have been based on the evaluation of the GC di- and trisaccharide honey profiles, which can change when some syrups are added. The GC fingerprint of di- and trisaccharides has been used to determine adulterations with IS (Low and South 1995). In 2003, Cotte *et al.* (2003) combined the use of data from HPAEC with a pulse amperometric detector (PAD) and GC with flame ionization detector (FID) with a statistical processing by principal component analysis to demonstrate the addition of IS and CS to honey samples.

Morales *et al.* (2008) proposed a HPAEC-PAD method for the analysis of honey oligosaccharides with DP higher than 3 (mono- and disaccharides were removed using activated charcoal) which enabled the detection of honey adulterations with CS and

HFCS. As shown in Figure 7.2.1, important changes were observed in the oligosaccharide profiles of honey adulterated with 20% CS (Fig. 7.2.1a) and with 20% HFCS20 for which oligosaccharides up to DP16 and DP15 were observed, respectively. Only slight variations were found after adulterations of honey with HFCS40 (Fig. 7.2.1c) and no changes were detected when honey was supplemented with HFCS80, due to the low amount of oligosaccharide present in the syrup. This method allowed the detection of adulterations with up to 5% of CS and HFCS20 and 20% of HFCS40.

More recently, Zhou *et al.* (2014) developed a rapid Ultra-Performance Liquid Chromatography (UPLC)-evaporative light scattering detector (ELSD) method (running time of less than 6 min) to distinguish honey adulterated with maltooligosaccharides. These authors used an amide-based HILIC stationary phase and a mobile phase with pure water and acetonitrile containing 0.2% triethylamine. However, only oligosaccharides up to DP 7 could be detected by this method and detection limits were relatively high ($0.10 - 0.20\,mg\,g^{-1}$).

Although differences in concentrations or chromatographic profiles of carbohydrates present in honey are the most common approaches to detect adulterations, specific markers absent from honeys have been also found. This is the case for difructose anhydrides (DFAs) which are non-fermentable pseudo-disaccharides consisting of two fructose residues with different types of linkage (Defaye *et al.* 2000). These compounds are produced during heating of sugars or sugar-rich food and have been detected in fructose corn syrup and invert syrups by GC-MS (Ruiz-Matute *et al.* 2007). DFAs were found to be good markers of honey adulteration with HFCS and IS. However, a previous removal of monosaccharides from honey samples was required before GC analysis since their high concentrations in honeys make the analysis of minor compounds present in adulterated samples difficult, specifically DFAs (Ruiz-Matute *et al.* 2007). Three different methods – yeast treatment, activated charcoal fractionation, and the combined use of activated charcoal and pressurized liquid extraction (PLE) – were proposed for this purpose (Ruiz-Matute *et al.* 2007, 2008). The optimized procedure allowed the detection of 5% adulteration with HFCS and IS. GC–MS has also been used to detect honey adulteration with high fructose inulin syrups (HFIS); the best marker was inulotriose since it was not detected in any of the 107 honeys analyzed (Ruiz-Matute *et al.* 2010).

On the other hand, honey composition depends on the plants used by the bees, which can produce nectar or honeydew, and also on the climatic and environmental conditions. Floral, vegetable, regional, territorial, or topographical origin should be specified on the label of the product. The development of analytical methods to determine the authenticity of honeys is therefore of great importance.

Several studies have been focused on the classification of honey origins depending on their carbohydrate composition; however, strict classifications cannot be made, considering the high quantitative variability found. Cotte *et al.* (2004) created a comprehensive carbohydrate database of different honeys from seven monofloral varieties analyzed by chromatographic techniques (GC and HPAEC). High fructose and glucose ratios could be characteristic of acacia and chestnut botanical varieties, whereas the high contents of raffinose could discriminate fir honeys. Sanz *et al.* (2004*b*) analyzed honeys from Madrid by GC and proposed the differences in sucrose, α,β-trehalose, and an unknown disaccharide concentrations to discriminate *Echium* honeys. De la Fuente *et al.* (2011) quantified mono-, di-, and trisaccharides from several Spanish honeys (*Citrus*, heather, *Eucalyptus*, rosemary, *Echium*, *Rosaceae*, and multifloral honeys) following a GC method

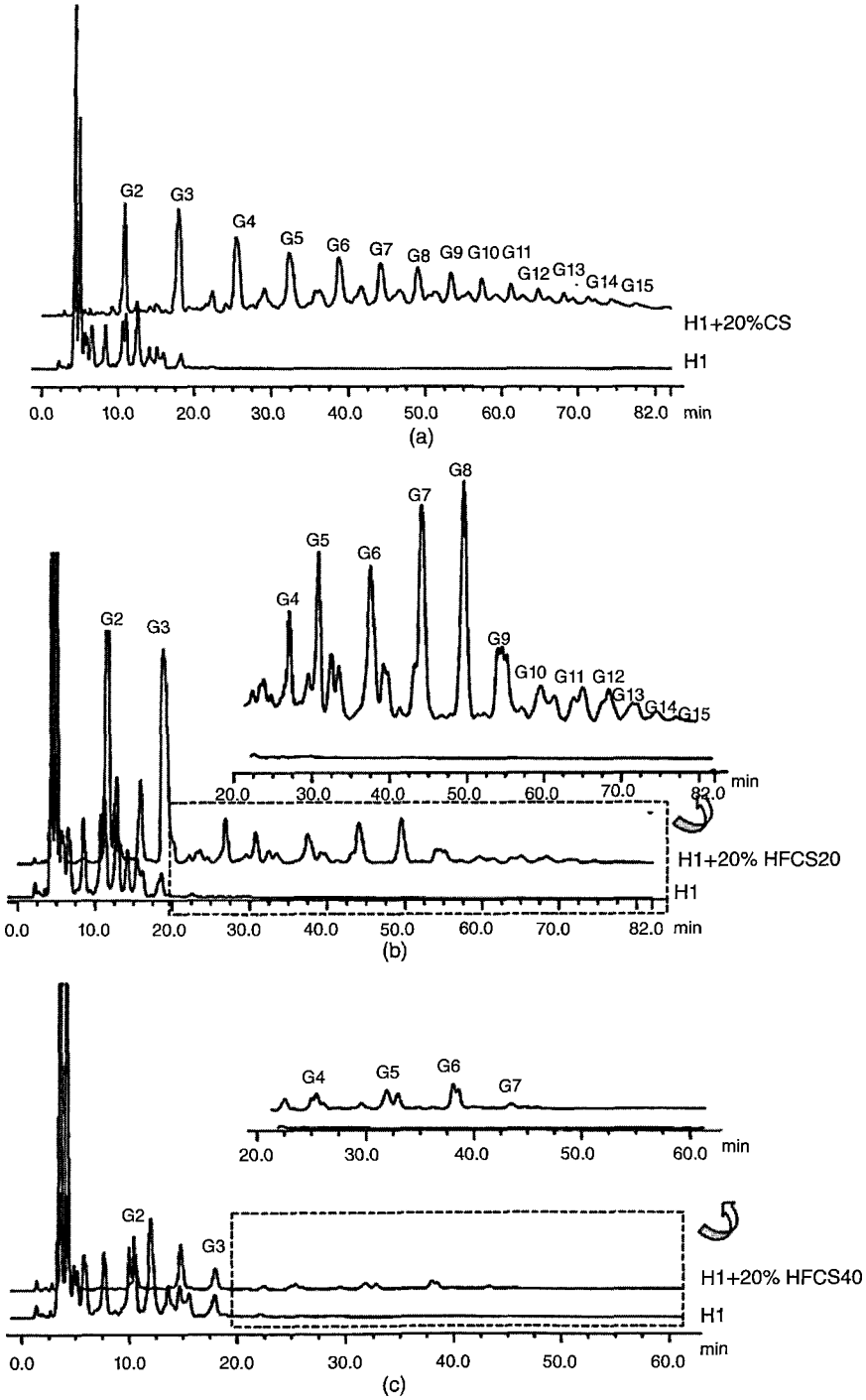


Figure 7.2.1 HPAEC-PAD profiles of honey oligosaccharide fraction (H1) adulterated with (a) 20% CS; (b) 20% HFCS20; and (c) 20% HFCS40. *Source:* Morales *et al.* (2008). Reproduced with permission from Elsevier.

based on the use of two different stationary phases, methyl and 65% phenylmethylsilicone. Although several carbohydrates were found to be characteristic of the most important honey types, the high variability of their concentrations in honey did not allow an unambiguous classification of these main unifloral sources.

Some studies have been focused on the distinction of floral honeys and honeydew honeys, the latter prepared by bees from secretions of living parts of plants or excretions of plant-sucking insects on the living part of plants. Bogdanov and Baumann (1988) found that honeydew honeys showed higher melezitose concentrations (5.3%) than floral honeys (0.5%), whereas raffinose was only detected in honeydew honeys. These authors developed a LC-refractive index (RI) method using an amino column and a mixture of acetonitrile and water as eluent. However, similar to previous approaches, a drawback of this method was the high variability of carbohydrate concentration in honeys. Sanz *et al.* (2005) found that quercitol (1,3,4/2,5-cyclohexane-pentol) could be a good marker to distinguish *Quercus* sp. honeydews from nectar honeys. Quercitol was present in a broad concentration range (0.01–1.50 g/100 g) in honeydews, but never higher than 0.01 g/100 g in samples characterized as nectar honeys.

Some sugar syrups can also be the target of frauds. Agave syrup is produced from the sap of the agave plant, specifically *Agave tequilana* and *Agave salmiana*, and has been gaining great attention in the last years as a table top sweetener and as a food ingredient considering its low glycemic index (Willems and Low 2012). GC-FID has been used to detect adulterations of this syrup with HFCS 55 and HFCS 90 using α- and β-isomaltose as marker (see Fig. 7.2.2) or with sucrose syrup using sucrose as marker. Adulterations of agave syrup with dextrose syrups could also be detected on basis of HPAEC-PAD oligosaccharides profile considering the presence of DP2-25 glucooligosaccharides in adulterated samples.

Maple syrup is produced from the sap of the sugar maple tree (*Acer saccharum*) and can be the target of adulterations with cheaper sweeteners. Stuckel and Low (1995) showed that HPAEC-PAD oligosaccharide profiles can be used to detect up to 5% adulteration of maple syrup with HFCS and beet medium invert sugar (BMIS).

Carbohydrates are also the main constituents of fruit juices which account for more than 98% of the total soluble solids. Considering the relatively high price and large production of juices, they can be the target of adulteration and frauds (partial replacement of fruit juice with water, sugar, fruit-derived products (pulpwash, peel, etc.), or with less expensive fruit varieties not declared in labels; or addition of inexpensive sweeteners with a composition resembling that of the authentic sugars). GC carbohydrate profiles have been used to detect adulteration of orange and apple juices with both IS and HFCS at percentages down to 5% (Low 1996). The addition of IS to juices was detected by the presence of two marker peaks IS1 and IS2 (Stöber *et al.* 1998) using GC and with a pre-equilibration method with dry pyridine at 50°C for 20 min before derivatization of the samples (Low *et al.* 1999). Although IS1 and IS2 may also be present in thermal-processed authentic juices, the ratio IS1/IS2 in heated juices is markedly different from that observed for apple and orange juices adulterated with IS. These markers were further identified as turanose and O-β-D-fructofuranosyl-(2→6)-D-glucose, which is thought to be formed during sucrose inversion (Thavarajah and Low 2006).

The adulteration of pear juices with different sweeteners (HFCS and IS) can also be detected by GC-FID at levels of 0.5–3.0% (v:v) using different markers (e.g., isomaltose for HFCS42, HFCS 55, and HFCS 90; O-β-D-fructofuranosyl-(2→6)-D-glucose for total

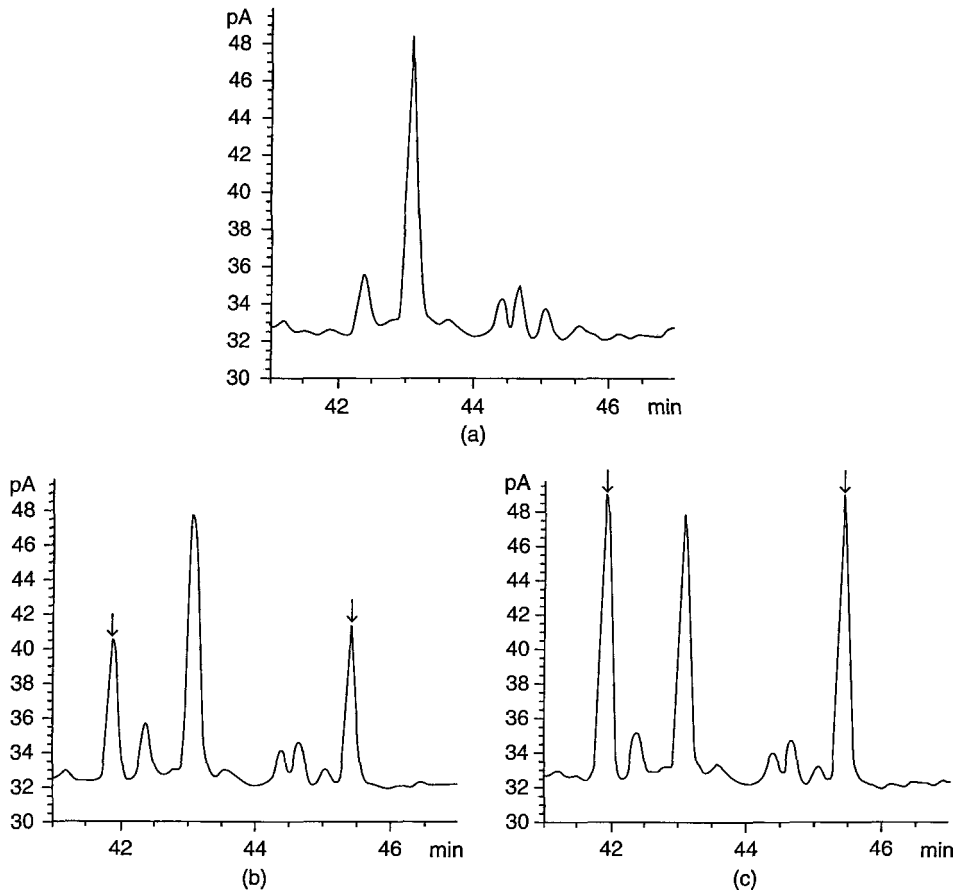


Figure 7.2.2 GC-FID disaccharide profiles of (a) agave syrup and the same syrup intentionally adulterated with (b) 5% and (c) 10% of HFCS 90. *Source:* Willems and Low (2012). Reproduced with permission from the American Chemical Society.

IS; and inulobiose for hydrolyzed IS) (Willems and Low 2014). Moreover, the developed GC-FID method could be used to detect the addition of pear to apple juice via arbutin detection and determine if a pear juice was produced using enzymatic liquefaction via the presence of cellobiose.

A hybrid liquid chromatographic system with conductivity and differential RI detection has been designed for the analysis of cations and carbohydrates (fructose, glucose, sucrose, and maltose) present in orange and apple juices. Data obtained by this method allowed the distinction between natural juices and intentionally adulterated juices with glucose-fructose syrups and sucrose syrups (Muntean 2010). HPAE-PAD profiling can also be used to confirm the presence of HFCS and HIS in pear juice (Willems and Low 2014).

GC-FID has been proven to be a useful tool to determine orange juice authenticity. Analysis of trimethylsilyl carbohydrates of orange juices showed that *myo*-inositol concentration and the *myo*-inositol/fructose ratio were lower in samples made from

concentrates than in freshly squeezed or straight-processed juices and could be used as markers of juice genuinity (Villamiel *et al.* 1998).

Coffee can also be the target of adulterations with different impurities and foreign matters such as husks (or straw), corn, barley, wheat middling, brown sugar, soybean, rye, triticale, acai, etc. (Domingues *et al.* 2014). Carbohydrates have been found to be useful markers for the detection of these fraudulent additions. In general, total xylose content (produced by hydrolysis of xylan present in some vegetable adulterants) has been proposed as a good marker for adulterations with coffee husk and twigs, whereas high levels of total glucose could indicate adulteration with maltodextrin, caramel, or cereals, and high levels of free fructose may be an indicative of adulteration with chicory or soybean (Prodolliet and Hischenhuber 1998; Nogueira and do Lago 2009; Pauli *et al.* 2014). Coffee samples should be previously submitted to a hydrolysis procedure using either sulfuric (Blanc *et al.* 1989) or chloridric acids (Girard *et al.* 2006) at high temperature (100°C) to transform polysaccharides into free monoșaccharides. LC-ultravilolet-visible (UV-Vis) methods using a post-column derivatization for the analysis of mannitol, xylose, glucose, and fructose have been proposed to verify the addition of coffee husk extracts at concentrations above 25% (Blanc *et al.* 1989). However, HPAEC-PAD is the technique most commonly used (Girard *et al.* 2006; Zambrozi Garcia *et al.* 2009; Pauli *et al.* 2014). Girard *et al.* (2006) found that the specification limits, above which a soluble coffee should be considered as adulterated, were 2.46% for total glucose and 0.45% for total xylose. Other authors have proposed the use of other separation techniques such as capillary zone electrophoresis to determine these markers (Nogueira and do Lago 2009).

The analysis of free sugars by HPAEC-PAD has also been suggested to detect coffee adulteration with 5% of extracts of chicory and cereal blend. Higher levels of sucrose, glucose, and fructose were detected in adulterated samples (Bernal *et al.* 1996). Other methods based on the analysis by GC of free monosaccharides, disaccharides, and polyalcohols present in coffee and coffee substituents based on cereals, carob, or chicory have been proposed to detect adulterations with these products (Montilla *et al.* 2006).

7.2.3 Food Proteins and Peptides

Food proteins provide nitrogen primarily in the form of essential amino acids. A low consumption during childhood of protein-based food and food ingredients (as cereal grains, flours, eggs, meat fish, milk, and derived products) leading to severe protein deficiency can cause kwashiorkor and marasmus in infants and young children and, in less severe forms, may hinder physical and mental development (Moore *et al.* 2010). Besides providing nutritional value, proteins also contribute to the functional properties of food by imparting texture (foam, emulsion, or gel) and flavor, which are organoleptic properties that influence human interest in purchasing and consuming foods. Because of both the high nutritional value and functionality, the market value of many protein-based food ingredients is determined, in part, by their total protein content (Barbano and Lynch 1990; Wrigley 1994; Owusu-Apenten 2002). This renders these products highly susceptible to be adulterated for economic profit, usually by the fraudulent substitution of high-priced proteins with another similar but cheaper ingredient.

Analytical methods currently adopted by the food industry to verify the quality and purity of protein-based food products are based on determining the total nitrogen content through traditional methods such as Kjeldahl or combustion (Dumas) with a numerical conversion to apparent protein content (Krotz *et al.* 2008), which do not distinguish protein-based nitrogen from non-protein-based nitrogen. Their lack of protein selectivity leaves consumers and industry vulnerable to food adulterations. This fact highlights the need for the development and establishment of new authenticity and purity analytical methods with higher discrimination power against the presence of non-protein nitrogen sources.

Among the emerging techniques for protein analysis, a number of chromatographic approaches have been tested, accepted, and employed in the authenticity of protein-based foods and food ingredients as a faster, cheaper, and easier-to-handle alternative to other emerging protein-based methodologies (e.g., electrophoretic, immunological, and DNA techniques). This is due to their advantageous separation characteristics allowing the rapid, accurate, and reliable identification and quantification of amino acids, peptides, and proteins in complicated food matrices. Moreover, the recent advances in MS and bioinformatics have favored the emergence of novel proteomics-based chromatographic methods, including LC and GC online coupled to MS (LC-MS and GC-MS) or tandem MS (GC-MS/MS and LC-MS/MS). These techniques have shown great convenience and suitability for food analysis due to their ease of automation and high-throughput capability. They are being increasingly used in authenticity studies: as a powerful tool for the development of simple and fast methodologies for routine use; for the analysis of complex or highly processed food matrices; and for the accurate quantification of trace levels of analytes with a high degree of selectivity (Gallardo *et al.* 2013; Wang *et al.* 2013).

Proteomics-based chromatographic applications are particularly abundant for authentication of milk, meat, fish, and products derived from them; however, some chromatographic methods for authentication of egg products and cereal grains and derived products (pasta) can also be found.

Milk and dairy products have been important trading foods since historical times, and therefore one of the earliest foodstuffs subjected to adulteration. There are several ways in which the authenticity of milk and dairy products may be challenged. One of the most common illegal practices in the dairy industry, especially for cheeses with Protected Designation of Origin (PDO), is the undeclared mixing of high-quality milk such as buffalo's, sheep's and goat's with cheaper cow's milk (Herrero-Martínez *et al.* 2000; De La Fuente *et al.* 2005; Calvano *et al.* 2014). There may be safety issues attached to the type of adulteration, since many people are allergic to cow's milk and therefore choose milk, cheese, and yogurt from other animals, such as sheep and goats. Applications of LC in this field have been reported by a number of research groups. A method to detect bovine milk in a mixture with ovine milk using anion-exchange fast protein liquid chromatography (AE-FPLC) was described by Laezza *et al.* (1991). They found a good correlation between the amount of bovine milk present in the mixture (up to 60%) and the peak area of bovine α-lactalbumin. De Frutos *et al.* (1991, 1992) used Reverse Phase Liquid Chromatography (RPLC) with UV detection at 205 nm to enable the separation of whey proteins (α-lactalbumin (α-la), β-lactoglobulin (β-lg), and bovine serum albumin (BSA)) from bovine, ovine, and caprine species and the detection of milk mixtures from different species. They succeeded in identifying cow's milk in ewe's and goat's milk, detecting

ewe's milk in milk mixtures, and detecting the incorporation of bovine milk (>10%) during the production of cheese made from ewe's milk. Separation time was about 8 min. Very similar results with RPLC-UV were obtained by Romero *et al.* (1996), Pellegrino *et al.* (1992), De Noni *et al.* (1996) and Torre *et al.* (1996), a further reduction of analysis time to less than 2 min being achieved in the latter work by using a perfusion chromatography column. A more sensitive detection of the addition of cow's milk to goat's and ewe's milk and cheeses by the analysis of whey proteins and their genetic variants has been described using RPLC with UV detection at 215 nm (Ferreira and Cacote 2003) and diode array detector (DAD) (Rodríguez *et al.* 2010), using β-lg peak as molecular marker. These methods allowed for the quantification of milk species with a detection limit of 2–4%. Ferreira's method was successfully applied for authenticity evaluation of commercial PDO ewe's and goat's cheeses. Fraudulent addition of bovine milk in water buffalo's milk and Mozzarella cheese may be also detected by RPLC-UV (205 nm) (Enne *et al.* 2005) at a level down to the EU law limit of 1%. The use of casein fractions to detect cow's, ewe's, and goat's milk occurs to a lesser extent. Haasnoot *et al.* (1986) evaluated the use of a method based on HPAEC with UV at 280 nm for the separation of caseins of different species. Adulterations of goat's and ewe's milk can be detected quantitatively with bovine α_{S1}-caseine as a marker and a detection limit of about 2% and 4%, respectively. The presence of cow's milk in sheep's and goat's milk cheeses can be estimated with the cow's milk α_{S1}-casein degradation product, α_{S1}-I-caseine, as an indicator. HILC with UV detection at 280 nm has been also used for the separation and determination of α_{s1}-, α_{s2}-, β- and κ-caseins in raw cow's, ewe's, and goat's milk, their blends and cheeses (Bramanti *et al.* 2003). By means of these model relations it was possible to estimate the addition of 10% and more of cow's milk into ewe's and goat's milk. Veloso *et al.* (2002) used a method of RPLC-UV (280 nm) for the detection of different milk types, through which they succeeded in detecting and quantifying the addition of more than 5% of cow's milk in goat's milk. This detection was based on the presence of α-casein peak. Mayer *et al.* (1997) reported the determination of the percentages of cow's, ewe's, and goat's milk in cheeses by HPCEC-UV based on the quantification of para-κ-casein from the different species within 45 min. Authors of all these works concluded that LC-UV is a very sensitive and reproducible technique for studying the percentage of milk from different species in milk and dairy products and highlighted the importance of using a proper calibration curve to achieve accurate results.

LC-MS(/MS) methodology, usually with an electrospray ionization (ESI) interface and different mass analyzers (quadrupole (Q) or ion trap (IT)), has also been applied for the identification and quantification of species in milk and dairy products, allowing more accurate results. β-lactoglobulin whey protein has therefore also been used for the detection and quantification of undeclared cow's milk addition in goat's and water buffalo's milk (Chen *et al.* 2004; Czerwenka *et al.* 2010) by means of RPLC-ESI-IT MS, using retention times and accurate molecular masses to detect the presence of cow's milk at levels as low as 5%. Guarino *et al.* (2010) developed a RPLC-ESI-IT MS/MS method based on the detection of a sheep-specific peptide from the digestion of casein with plasmin, a method that is able to detect up to 2% of sheep's milk in goat's and cow's milk cheeses. Species-specific peptides from the tryptic digestion of casein have also been used for the detection and quantification of cow's, ewe's, buffalo's, and goat's milks in milk mixtures (Cuollo *et al.* 2010), by using (RP)-LC-ESI-Q MS methods that are able to detect extraneous milk concentrations as low as 0.5%. Likewise, peptides naturally arising in the cheese from the spontaneous proteolytic

process have been analyzed by LC-ESI-MS to identify marker peptides suitable to detect cow's milk in sheep's milk cheeses at a level of 1% (Sforza *et al.* 2008).

Another common fraud carried out in the dairy industry to reduce the production cost and increase the protein content is the undeclared addition of cheaper vegetable proteins (such as those from soybeans), milk powder, waste from the milk-processing industry (such as whey rennet solids from the cheese production), or non-protein nitrogen sources (such as melamine and its metabolites, including ammeline, ammelide, and cyanuric acid), which can be potentially harmful (allergenic or toxic). Several LC approaches, including RPLC, ion pair RPLC, size exclusion chromatography (SEC), and UPLC coupled to UV, photodiode array detector (PDA), and MS detectors, as well as GC-MS methodologies, have been applied for the detection of this kind of adulteration. Table 7.2.1 summarizes some of these studies.

Similarly to dairy products, undeclared mixing of animal species and inappropriate labeling of the origin is also one of the most common frauds in the trade of raw meat, fish, and shellfish products aiming to sell low-priced species in a fraudulent substitution of more valuable higher-priced species. In addition to economic consequences, potential human health hazards are intrinsic to these fraudulent practices due to the possible introduction of common fish allergens that may aggravate symptoms of sensitive patients to specific animal species (Carrera *et al.* 2013). Likewise, mislabeling of "halal" foods by mixing meat from specific animal species such as dog or horse, as demonstrated by the recent scandal of the presence of undeclared horsemeat in foods containing raw beef meat (Brown 2013), is a serious issue in many religions including Judaism, Islam, and Buddhism (Mahanarongchai and Marranca 2007; Khattak *et al.* 2011; Satter 2013; Rahman *et al.* 2014; Di Giuseppe *et al.* 2015). When testing the authenticity of meat and fishery products, species identification is the most important issue in the raw material trade for consumer protection.

In this sense, LC and LC-MS(/MS) applications developed with this purpose have shown to be effective, fast, and relatively inexpensive. Among them, RPLC has been used by Piñeiro *et al.* (1997) to identify gadoid fish species. Chromatograms of phylogenetically related fish species were different, enabling species identification based on the presence or absence of one or more peaks. RPLC of water-extractable proteins from fish white muscle tissue has also been used for the identification of seven fish species (salmon, cod, monk, trout, rockfish, catfish, and shark) (Ashoor and Knox 1985). The use of BSA was necessary for the qualitative identification of fish species, since only the expression of retention times relative to BSA gave good results. This compound was also used as an internal standard for quantification. However, response factors of the different peaks were not known, which makes quantification of unknown samples difficult. Recently, Carrera *et al.* (2010) developed a new strategy for the extensive characterization and *de novo* sequencing of all parvalbumins (PRVBs) isoforms for all commercial species from the Merlucciidae family. For each PRVB, mass spectra obtained by LC-ESI-IT MS/MS from two digests (trypsin, Glu-C) were followed by database searching and *de novo* sequenced manually with the help of several bioinformatics tools. PRVBs peptides with the highest interspecific variability were selected as species-specific peptide biomarkers to effectively identify all the species from Merlucciidae family, even in cooked fish products (Carrera *et al.* 2011). Likewise, López *et al.* (2002) used a LC-ESI-IT MS method to validate the tropomyosin peptides specific to three European marine mussel species (*Mytilus edulis*, *M. galloprovincialis*, and

Table 7.2.1 Chromatographic methods used in authenticity studies for detection of undeclared addition of foreign ingredients to milk and meat products.

Product	Method	Result	Reference
Bovine milk	LC-ESI-MS	Detection of fraudulent addition of milk powder	Czerwenka *et al.* 2006
	FPLC-UV	Detection of undeclared addition of foreign proteins, such as soybean, pea, brown rice and hydrolyzed wheat proteins	Hewedy and Smith 1989
	RPLC-UV		García *et al.* 1997; Kruså *et al.* 2000; Wu *et al.* 2014
Skim milk powder	RPLC-UV		Espejas *et al.* 2001
	UPLC-UV		Jablonski *et al.* 2014
	LC-MALDI-Q-TOF MS		Cordewener *et al.* 2009
	LC-MS/MS		Luykx *et al.* 2007
Skim milk powder and sweet buttermilk powder	SEC-UV	Detection of undeclared addition of rennet whey total solids based on glycomacropeptide peak	Olieman and van den Bedem 1983; Olieman and van Riel 1989
	Ion-pair RPLC-PDA		Bordin *et al.* 2001
Cow's milk and milk-based infant formula	LC-ESI-MS/MS	Detection of melamine and cyanuric acid	Desmarchelier *et al.* 2009
	GC-MS		Squadrone *et al.* 2010
	HR-GC-isotope dilution MS		Koh *et al.* 2011
Heat-processed meat products	Perfusion RPLC-UV	Detection of undeclared addition of soybean proteins	Criado *et al.* 2005 Castro-Rubio *et al.* 2005*a*, *b*, 2006; Castro *et al.* 2005
	RPLC-UV	Detection of undeclared addition of soybean proteins, caseinate, and whey proteins	Ashoor and Stilles 1987

M. trossulus), previously detected by matrix-assisted laser desorption/ionization (MALDI)-TOF PMF (peptide mass fingerprinting) and sequenced by ESI-IT MS/MS.

With respect to the trade of raw meat products, Ashoor and Osman (1988) described a RPLC-UV method for the identification of raw beef, pork, veal, lamb, chicken, turkey, and duck based on water-soluble proteins. Meat cuts or parts from different species resulted in different chromatographic profiles, which enabled the detection and identification of meat blends. The same method was applied to determine the amount of chicken and turkey in unheated chicken–turkey mixtures (Ashoor and Osman 1988). Mixtures of 5–100% of raw chicken in turkey could be detected. Giarretta *et al.* (2013) recently proposed the myoglobin (Mb) as a powerful molecular marker to evaluate the presence of

undeclared meat addition in raw beef burgers by using anion exchange (AE)-UPLC with visible detection at 409 nm (maximum absorbance of Mb) for the separation and identification of edible animal species (beef, chicken, horse, ostrich, pig, and water buffalo). The method was validated to verify the undeclared presence of pork meat in minced beef meat with a sensitivity of 5%. Very recently, the same research group presented a fast AE-UPLC-Vis method to selectively detect the presence of horse meat in raw beefs with a sensitivity of *c.* 0.1%, also using Mb as molecular marker (Di Giuseppe *et al.* 2015). Proteomics-based LC methods have also been applied for species identification and accurate quantification in meat products. Sentandreu *et al.* (2010) developed a sensitive LC-MS/MS methodology by using myosin light chain-3 (MLC-3) as biomarker for detecting and quantifying chicken meat in different meat mixtures, either raw or cooked, at a level of 0.5%.

In the case of processed meat products, the fraudulent addition of meat substitutes based on non-meat proteins (such as soybean, wheat and milk proteins, egg white, yeast extract, gelatin, and bovine blood plasma) is also a frequent practice in the meat industry due to their low cost and relevant nutritional and technological characteristics. However, they are also considered potentially allergenic ingredients, so their undeclared addition is forbidden or limited in some countries where recommendations and/or regulations concerning the content of added non-meat proteins to specific meat products exist (Soares *et al.* 2014). As far as processed meat products are concerned, reliable detection of this kind of adulteration should also be studied for authentication. There are only a limited number of chromatographic methods available for the (semi)quantitative determination of non-meat proteins, especially soybean proteins, in processed meat products (Leitner *et al.* 2006) (see Table 7.2.1). Another common and forbidden or limited practice is the addition of mechanically recovered meat (MRM), with high content in cholesterol, to processed meat products. A nano-LC-MS/MS method has been applied to identify two potential markers for chicken MRM, hemoglobin, and a protein similar to myosin-binding protein C (Surowiec *et al.* 2011). Likewise, due to the Bovine Spongiform Encephalopathy (BSE) epidemic, the European Union prohibited feeding ruminants with proteins processed from animal tissues such as fish meal, meat, and bone meal (MBM), and banned feeding all livestocks with proteins from the same species. Several LC and LC-MS methods, based on the detection of peptide markers specific to the animal tissue, have been developed to detect animal protein additives in feeds (Ocaña *et al.* 2004; Buckley *et al.* 2009).

Since proteins present in whole egg, yolk, and albumen (egg white) are excellent sources of nutrients and possess valuable functional properties, egg products, including refrigerated-liquid, frozen, dried, and specialty products, are considered high-risk materials for economically motivated adulteration with undeclared non-protein nitrogen sources aiming to increase the protein content, such as melamine and cyanuric acid. To detect this type of adulteration, Mondal *et al.* (2010) demonstrated the ability of a clean-up-free LC-MS/MS method, originally developed for cow's milk and milk-powdered infant formula, to simultaneously quantify melamine and cyanuric acid in egg powder with a detection limit of 0.02 and 1.0 ppm, respectively. Very few publications report on the use of LC for detection and quantification of egg proteins in food products (e.g., poultry products), probably due to the complexity of the protein fraction (especially the lipoprotein fraction present in yolk) compared to milk proteins (Dierckx and Huyghebaert 2000). Existing analytical methodologies are based on SEC-Fast Protein Liquid Chromatography (FPLC) (Sajdok *et al.* 1989) and RPLC-UV (Takeuchi *et al.* 1992).

When cereal-based products are concerned, the most common illegal practice is the sale of pasta made from common wheat (*Triticum aestivum* L.) or a mixture of common and durum wheat (*Triticum durum* Desf.). EU legislation states that pasta must be prepared only with durum wheat. Chromatographic methods applied to detect the wheat varieties used for pasta production and authenticity verification are based on the analysis of the gliadin fraction (McCarthy *et al.* 1990) and water-soluble proteins (albumins) (De Noni *et al.* 1994) by RPLC.

7.2.4 Fatty Acids and Triacylglicerols

Food lipids, including oils (liquid) and fats (solid), are represented by several components, fatty acids (FA) being the major constituents. FA are generally esterified on the glycerol backbone as triacylglycerols (TAG) (also referred to as triglycerides). TAG represent the major dietary form of FA; however, they are also part of the phospholipid molecule. Phospholipids, which generally represent less than 1% of total dietary lipids, are found in cell membranes, milk fat globules, and eggs. A major phospholipid is phosphatidylcholine (EFSA 2010).

Triacylglycerols (TAG) and fatty acids (FA) facilitate the absorption of other fat-soluble components such as vitamins. In the body, fatty acids are not only a major source of energy, but also serve many vital functions (e.g., structural components of cell membranes, precursors for bioactive molecules, regulators of enzyme activities such as protein myristoylation, and regulation of gene expression), so are needed for metabolic integrity and human health (EFSA 2010).

Dietary fat is an important source of fatty acids, since many of them cannot be synthesized by the body (these are called essential FA or EFA) or are synthetized to a very low extent. This is mainly provided by edible oils and butter, but also by dairy, meat, and fishery products. Among them, some plant oils, such as extra virgin olive oil, and butter, are considered as high-quality products due to their nutritional and physiological beneficial effects to humans since they are potential sources of antioxidants, fat-soluble vitamins, and heart-healthy fats. Because of this, they command a high market value which renders these products highly susceptible to economic fraud; this is commonly achieved by mixing with cheaper plant oils or animal fats to achieve the highest profit. Verification of quality and purity of high-quality oils and butter is therefore focused on the reliable determination of geographical origin and the identification of botanical and animal species. Generally, this has been carried out for many years by determining the FA and TAG composition (García *et al.* 2013; Purcaro *et al.* 2013).

Since free FA and TAG of plant oils and animal fats are semi-volatile, both LC and GC techniques can be employed for their measurement. A number of GC and LC approaches, including hyphenated methodologies (GC/LC-MS), have been used for authentication of high-quality oils and butter by means of the reliable, selective, and accurate separation, identification, and quantification of FA and TAG.

Stefanoudaki *et al.* (1997) studied the potential of the TAG profile obtained by LC with RI detection for authentication of Cretan olive oils. Data concluded that TAG composition can be successfully employed for the classification of olive oil samples. RPLC-RID has also been applied for the detection of adulteration of sesame oils with perilla oil at a level of 5% (Lee *et al.* 2001). LC combined with various MS detection

procedures have also been frequently applied for the analysis of edible oils. Almond, avocado, corn germ, grape seed, linseed, mustard seed, olive, peanut, pumpkin seed, sesame seed, soybean, sunflower, walnut, and wheat germ oils have been analyzed by LC-Atmospheric pressure chemical ionization (APCI) MS and MALDI-TOF MS. Both techniques classified 67 oil samples correctly according their TAG profile and only 6 samples were classified incorrectly (Jakab *et al.* 2002*a*, *b*; Jakab and Forgács 2002). Likewise, the TAG and diacylglycerol (DAG) composition of several plant oils (including hazelnut, pistachio, poppy-seed, almond, palm, Brazilnut, rapeseed, macadama, soybean, sunflower, linseed, *Dracocephalum moldavica*, evening primrose, corn, amaranth, and *Silybum arianum*) has been characterized by LC combined with APCI-MS and UV detection. The high separation capacity of the technique makes it suitable for the determination of the composition of different plant oils, and the comparison of the TAG and DAG profile of oils may help their identification (Holcapek *et al.* 2003).

Unlike LC, sample preparation for GC analysis of FA and TAG usually involves their derivatization by esterification (leading to fatty acids methyl esters, FAME) or transesterification, respectively. GC methods of TAG were developed later than the FAME technique due to the need for thermally stable stationary phases, as well as the thermal stability of TAG themselves. Because of this, LC became a more widely used analytical technique for TAG (Purcaro *et al.* 2013). However, analyzing the TAG composition is usually more suitable for authentication purposes as compared to FA profiling. The reason is that the genetically controlled stereo-specific distribution of FA moieties on the glycerol backbone is preserved and the information content of intact TAG is therefore usually higher. The method adopted within the UE Regulation 213 for detection of butter adulteration by cheaper non-milk fats (such as mutton fat and edible oils from soybean, sunflower, cocoa, rapeseed, linseed, wheat, corn, cotton, palm, and fish) is based on the analysis of TAG by GC-FID (Precht 1991*a*, *b*, 1992). TAG composition of a considerable number of oils, including argania, corn, cotton, grape, olive, soy, cocoa, palm, sunflower, and peanut oils has been also analyzed by GC-FID with the aim of identifying potential species-specific markers to detect possible adulterations by other oils or the adulteration of butter by oils (Rezanka and Rezanková 1999). High-resolution (HR) GC has been applied for the determination of the TAG profile for the detection and quantification of the addition of 5% cocoa butter equivalents to cocoa butter for chocolate elaboration (Simoneau *et al.* 1999). Regarding olive oil (*Olea europaea* L.), current guarantees of the botanical and geographical origin of extra virgin olive oil (EVOO) is of huge importance world-wide due to the recent introduction to the market of high-quality monovarietal EVOO (Angerosa *et al.* 2006; Frankel 2010; García *et al.* 2013). Monovarietal EVOO could therefore be more susceptible of economic fraud; the most common practice is the addition of seed oils, such as sunflower, soybean, hazelnut oil, and refined olive oil. In this sense, TAG and some minor components of the EVOO (1,3-diacylglycerols (DGs) and fatty acid alkyl esters (FAAEs), promoted by unsuitable practices related with olive oil extraction and scarce quality of olive fruits), have been analyzed by different GC methodologies as potential markers of changes in the EVOO composition due to forbidden blending (Marsilio *et al.* 2001; Pérez-Camino *et al.* 2001, 2008; Garrido-Delgado *et al.* 2011*a*, *b*, 2012; Bosque-Sendra *et al.* 2012).

FA can also be reliable markers for authentication of seed oils, in this case a previous amendment is needed in order to make them conform to the modification in composition induced by genetic improvement or environmental factors of new cultivation areas,

for instance: palmitic acid has been used as a marker to detect the contamination of cottonseed oil by palm oil (Aparicio and Aparicio-Ruiz 2000); linolenic acid (LA) to identify the fraudulent addition of the cheaper soybean oil to groundnut and sunflowers oils (with less than 0.1% of LA) or the mixture of soybean oil with rapeseed oil (with a lower content of LA); and erucic acid and stearidonic acid has been used for the detection of the mixture of evening primrose oil with borage and blackcurrent oils, respectively (Lercker 1988). Likewise, isotopic analysis of individual FA, carried out by GC coupled to a stable isotope ratio mass spectrometer (IRMS) via a combustion interface, has been successfully applied to test the authenticity of olive oils (Spangenberg *et al.* 1998; Spangenberg and Ogrinc 2001).

Moreover, FA and TAG composition, determined by GC or LC, can also be used as molecular markers in authenticity studies with coffee and dairy, meat, and fishery products (see Table 7.2.2) for reliable identification and quantification of the undeclared mixing of high-quality species with cheaper products (Cserháti *et al.* 2005; García *et al.* 2013; Purcaro *et al.* 2013).

Table 7.2.2 Chromatographic methods used for authentication of coffee and dairy, meat, and fishery products by using fatty acids (FA) and triacylglycerols (TAG) as molecular markers.

Product	Method	Result	Reference
Cheese	HR-GC-FID	Identification of the cream origin and the protected designated origin of Swiss cheeses	Collomb *et al.* 2002
	GC-MS	Detection of 68 volatile flavor compounds characteristic of Roncal cheese	Izco and Torre 2000
Milk	GC-FID	Detection of fat from non-dairy origin	Villanueva *et al.* 1988
Mold- and long-ripened cheeses			Molkentin 2013
Infant formula		Analysis of *trans* fatty acids to evaluate the quality of vegetable oils used in infant formula	Dionisi *et al.* 2002
Organic milk		Use of delta 13C and the α-linolenic acid content of milk fat as indicators of organic milk	Molkentin 2009
Cow milk and Parmigiano/ Reggiano cheese	GC-MS	Use of ω-cyclohexyl fatty acids as quality markers	Marseglia *et al.* 2013
Cured meat products	GC-MS	Use of FA and TAG profiles to identify the origin of Iberian pork products	Horcada *et al.* 2013
Halal meat	GC-FID	Differentiation of lard, chicken, mutton, and beef fat for halal authentication purposes	Nizar *et al.* 2013
Fish	GC-FID	Detection of fraudulent substitution of wild sea bass by farmed sea bass	Eder 1995
Coffee	GC-FID	Assessment of the relative amounts of *Arabica* and *Robusta* coffee varieties in a mixture to prevent possible fraud in commercial coffee blends	Romano *et al.* 2014

7.2.5 Volatile Compounds

Volatile compounds comprise a wide range of low-molecular-weight organic compounds with diverse chemical groups. This type of compounds contributes to the typical odor or taste of foods and has been used as markers for authentication in a varied number of foodstuffs. In unifloral honeys, the aroma profile can be considered a "fingerprint" due to the fact that it is directly related to the plant nectar extracted by the bees (Overton and Manura 1994; Amtmann 2010; Escriche *et al.* 2011). Consequently, volatile fraction assessment could be a useful tool for the characterization of geographical or botanical origins (Verzera *et al.* 2001; Cuevas-Glory *et al.* 2007; Castro-Vázquez *et al.* 2010). Furthermore, and given the high price of certain honey types, adulteration with low cost and nutritional value substances or mislabeling regarding the botanical origin may occur; consequently, the development of reliable, rapid, and cost-effective methodologies for their discrimination and classification based on their botanical origin is of high importance (Aliferis *et al.* 2010). Additionally, considering that volatile compounds in honey are an extremely complex mixture of different compounds with a great variety in functional groups that are present at low concentration levels, their extraction requires the use of effective sample treatment methods (Spánik *et al.* 2014).

The predominant chromatographic technique employed for the analysis of volatile compounds is GC and its combination with solid-phase microextraction (SPME), which was firstly introduced by Arthur and Pawliszyn (1990), has shown to be a powerful approach in food authentication. Concretely, headspace (HS) -SPME may represent an optimal method for the isolation of honey volatile compounds since it is a solvent-free and inexpensive technique, together with a reliable detection of low concentrations of analytes (Cuevas-Glory *et al.* 2012). In this sense, HS-SPME-GC/MS fingerprinting of honey volatiles combined with state-of-the-art chemometric analyses has shown to be a robust, reliable, non-time-consuming, and cost-effective methodology for the routine discrimination and classification of honey samples based on their botanical origin (Soria *et al.* 2003; Aliferis *et al.* 2010; Moniruzzaman *et al.* 2014). As an example, Figure 7.2.3 shows GC-MS profiles of heather and rosemary honey volatiles fractionated by SPME using polyacrylate (PA) fiber, illustrating the great variability of well-resolved compounds obtained by combining SPME with GC (Soria *et al.* 2003). Nevertheless, other isolation techniques have largely been applied, such as the traditional liquid–liquid extraction into organic solvent, Likens–Nickerson methodology, purge-and-trap systems or ultrasound-assisted extraction (Anklam 1998; Cuevas-Glory *et al.* 2007; Soria *et al.* 2009) as is shown in Table 7.2.3.

The GC pattern of volatile compounds of honeys has been used profusely as a useful marker for fingerprinting their botanical origin and/or their geographical discrimination. However, due to the complexity of volatile honey compounds (which can be classified in seven major groups: aldehydes, ketones, acids, alcohols, esters, hydrocarbons, and cyclic compounds; Kaskonienė and Venskutonis 2010), and given that more than 600 organic compounds have been identified in the extract of volatiles or headspace isolates of honeys up to date (Jerkovic *et al.* 2009*b*), most studies consider groups of compounds rather than specific compounds as indicators of honey floral origin (Guyot *et al.* 1998*a*, *b*; Castro-Vázquez *et al.* 2007, 2014; Aliferis *et al.* 2010). Kaskonienė and Venskutonis (2010) summarized the most important volatile compounds proposed as possible markers in unifloral honeys, and these are listed in Table 7.2.3.

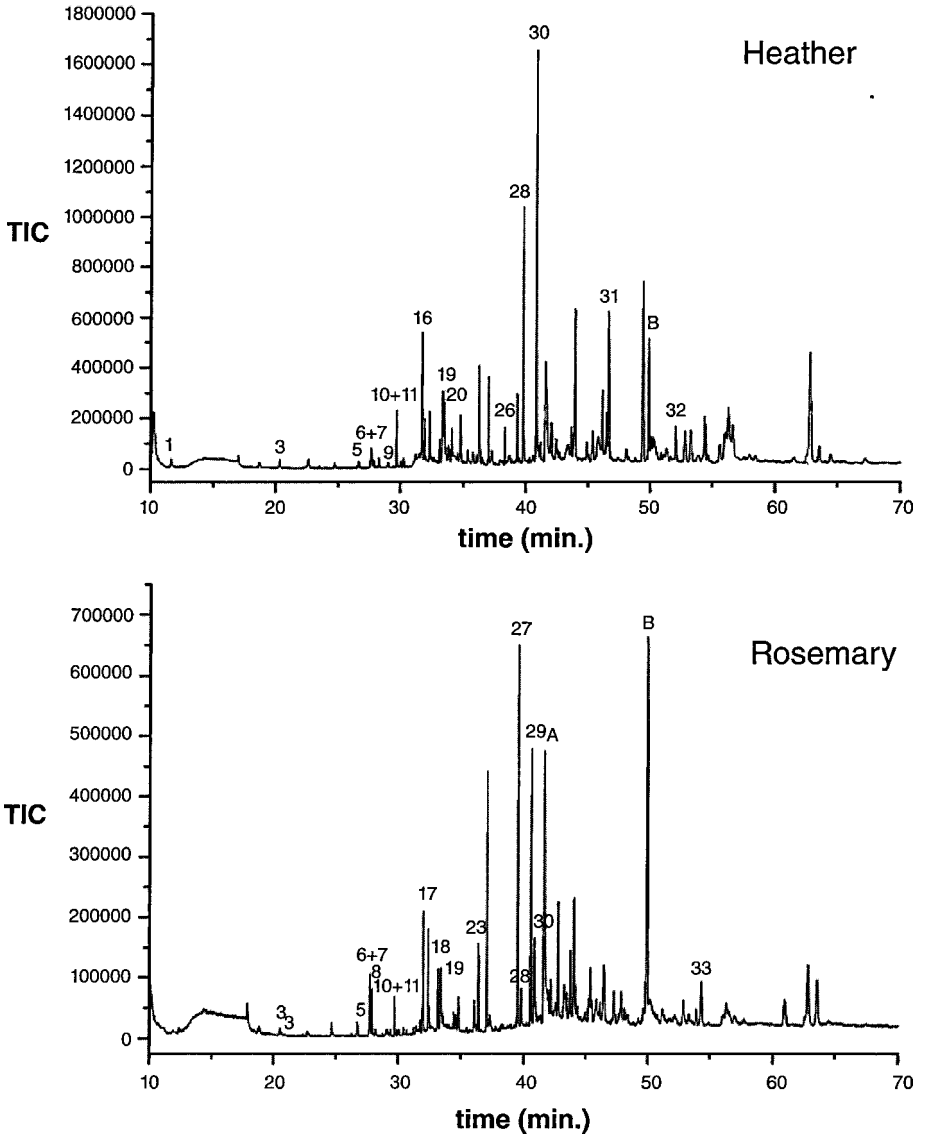


Figure 7.2.3 TIC profiles obtained by GC-MS from heather and rosemary honey volatiles fractionated by SPME using PA fiber. For identification of numbered peaks, see table 4 of original article. *Source:* Soria et al. (2003). Reproduced with permission from Wiley.

More recently, the study of enantiomer distribution of some chiral volatile organic compounds in different honeys has been explored for the determination of their botanical origin either using GC-MS (Verzera *et al.* 2014) or two-dimensional GC (Spánik *et al.* 2014), using SPME for the previous extraction of volatile compounds. These studies have reported significant differences in enantiomer ratio of different terpene alcohols, such as linalool and its oxides, 4-terpineol, or lilac aldehydes, which might potentially constitute a new and reliable analytical approach for honey authenticity.

Table 7.2.3 Most important volatile compounds in unifloral honeys. Source: Kaskonienė and Venskutonis (2010). Reproduced with permission from John Wiley & Sons.

Honey source	Compound	Extraction method	GO[a]	Reference
Rhododendron	n-Decane, lilac aldehyde, 2-aminoaceto phenone, benzenedicarboxylic, nonanal, isobutylphthalate, damascenone	SPME	TR	Senyuva *et al.* 2009
Christ's thorn	Nonanal, 4 isomers of lilac aldehyde, decanal, methyl nonanoate, hexanoic and 2-ethylhexanoic acids	SPME	HR	Jerkovic *et al.* 2009a
Sage	Tetrahydro-2,2,5,5-tetramethylfuran, 3-hexenyl ester of butanoic acid, 2-methylbenzene, maltol, methyl ester of 3-furanocarboxylic acid, benzeneacetic acid	SPME	HR	Lusic *et al.* 2007
	Benzoic acid, phenylacetic acid, p-anisaldehyde, a-isophorone, 4-ketoisophorone, dehydrovomifoliol, 2,6,6-trimethyl-4-oxocyclohex-2-ene-1-carbaldehyde, 2,2,6-trimethylcyclohexane-1,4-dione, and coumaran	Solvent	HR	Jerković *et al.* 2006
Chestnut	High percentage of phenylacetic acid, low percentage of benzoic acid, also 4-aminoacetophenone, 2-aminoacetophenone	Solvent	HR	Jerković *et al.* 2007
	n-Decane, phenylacetaldehyde, phthalic acid, α- α-dimethylphenyl acetate, p-anisaldehyde	SPME	TR	Senyuva *et al.* 2009
	2-Methyldihydrofuranone or α-methylbenzyl alcohol or 3-hexen-1-ol and dimethylstyrene	Purge-and-trap[b] (Tenax™ TA trap)	EU	Radovic *et al.* 2001
	2-Methylcyclopentanol, diethylphenol	SPME	IT	Guidotti and Vitali 1998
	Acetophenone, 1-phenylethanol, 2-aminoacetophenone	Likens–Nickerson	FR and IT	Guyot *et al.* 1998
	Acetophenone, 2-aminoacetophenone	Likens–Nickerson	Catalonia and Basque area (ES)	Bonvehí and Coll 2003

(Continued)

Table 7.2.3 (Continued)

Honey source	Compound	Extraction method	GO[a]	Reference
Erica arborea	Benzoic acid, decanoic acid, high levels of cinnamic acid, isophorone, 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohexen-2-en-1-one	Likens–Nickerson	EU	Guyot *et al.* 1999
Calluna vulgaris	Phenylacetic acid, dehydrovomifoliol, (4-(3-oxo-1-butynyl)-3,5,5-trimethylcyclohexen-2-en-1-one)	Likens–Nickerson	EU	Guyot *et al.* 1999
Heather	4-Methylbicyclo[2,2,2]octan-1-ol or 4-ethylphenyl acetate	Purge-and-trap[a] (Tenax™ TA trap)	EU	Radovic *et al.* 2001
	High contents of benzene and guaiacol, *p*-anisaldehyde, propylanisole and *p*-cresol	Likens–Nickerson	ES	Castro-Vázquez *et al.* 2009
	α-Isophorone, 2-hydroxy-3,5,5-trimethylcyclohexanone, furfuryl alcohol, benzyl alcohol, 2-phenylethanol	SPME	ES	de la Fuente *et al.* 2005
Buckwheat	3-Methylbutanal, 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon), β-damascenone	Solvent	U.S.A. and ENG	Zhou *et al.* 2002
Blue gum	8,9-Dehydrotheaspirone and 3-oxo-α-ionone	Solvent	AU	D'Arcy *et al.* 1997
Eucalyptus	1-Octene or 2,3-pentanedione	Purge-and-trap* (Tenax™ TA trap)	EU	Radovic *et al.* 2001
	Nonanal, ethylphenyl acetate, phenethyl alcohol	SPME	TR	Senyuva *et al.* 2009
	Acetoin, 5-hydroxy-2,7-dimethyl-4-octanone, p-cymene derivatives, 3-caren-2-ol and spathulenol	Likens–Nickerson	ES	Castro-Vázquez *et al.* 2009
	Acetoin, diacetyl, 2,3-pentanedione, dimethyldisulfide	SPME	ES	de la Fuente *et al.* 2005
	2-Hydroxy-5-methyl-3-hexanone, 3-hydroxy-5-methyl-2-hexanone	SPME	ES	de la Fuente *et al.* 2007*a*
	2,3-Pentanedione, acetoin, 1-hexyl alcohol, 2-acetyl-5-methylfuran, furfuryl propionate, 2-phenylacetaldehyde, nerolidol	Likens–Nickerson	Catalonia and Basque area (ES)	Bonvehí and Coll 2003

Lime tree	2-Methylfuran or α-terpinene or α-pinene oxide or α-terpene or methyl isopropyl benzene or 4-methylacetophenone	Purge-and-trap[a] (Tenax™ TA trap)	EU	Radovic *et al.* 2001
	Ethylmethylphenol, carvacrol, estragole	Likens–Nickerson	FR	Guyot *et al.* 1998*a*
Citrus	Lilac aldehydes, dill ether, methyl anthranilate, 1-*p*-menthen-9-al isomers	SPME	GR	Alissandrakis *et al.* 2007
	Methyl anthranilate	Likens–Nickerson	Florida (USA)	White and Bryant 1996
	(*E*)-Linalool oxide, lilac aldehydes, lilac alcohols, limonyl alcohol, sinensal isomers, α-4-dimethyl-3-cyclohexene-1-acetaldehyde	Likens–Nickerson	ES	Castro-Vázquez *et al.* 2009
	Limonene diol, methyl anthranilate	SPME	IT	Piasenzotto *et al.* 2003
	Lilac aldehydes, methyl anthranilate	SPME	ES	de la Fuente *et al.* 2005
	α and β sinensal	Likens–Nickerson	ES	Castro-Vázquez *et al.* 2007
Lavender	Heptanal	Purge-and-trap[a] (Tenax™ TA trap)	EU	Radovic *et al.* 2001
	Hexanal, nerolidol oxide, coumarin, high concentrations of hexanol, and hotrienol	Likens–Nickerson	ES	Castro-Vázquez *et al.* 2009
	n-Hexanal, n-heptanal, phenylacetaldehyde, coumarin	Dynamic headspace	FR	Bouseta *et al.* 1992
	n-Hexanal, n-heptanal, hexanol, phenylacetaldehyde	Likens–Nickerson	PT and FR	Guyot-Declerck *et al.* 2002
Acacia	*cis*-Linalool oxide and heptanal	Purge-and-trap[a] (Tenax™ TA trap)	EU	Radovic *et al.* 2001
Sunflower	α-Pinene or 3-methyl-2-butanol	Purge-and-trap[a] (Tenax™ TA trap)	EU	Radovic *et al.* 2001

(Continued)

Table 7.2.3 (Continued)

Honey source	Compound	Extraction method	GO[a]	Reference
Cotton	Cinnamaldehyde, cinnamyl alcohol, cinnamic acid, neryl and geranyl nitrile, benzenepropanol, homovanillyl alcohol, (*E*)- and (*Z*)-*p*-methoxy-cinnamic acid, 2-methyl-*p*-phthalaldehyde, coniferaldehyde, *p*-coumaric acid, ferulic acid, scopoletin, scoparone	Solvent	GR	Alissandrakis *et al.* 2005
Rape	Dimethyl disulfide	Purge-and-trap[a] (Tenax™ TA trap)	EU	Radovic *et al.* 2001
Willow	Methyl salycilate	SPME	ES	de la Fuente *et al.* 2007*b*
Rosemary	Lilac aldehyde isomers	Likens–Nickerson	ES	Castro-Vázquez *et al.* 2009
Haze	Methyl-*p*-anisaldehyde, *p*-anisaldehyde, *p*-cresol, trimethoxybenzene, 5-hydroxy-2-methyl-4H-pyran-4-one, lilac aldehyde isomers (A, B, C, D), phenethyl alcohol, benzenacetaldehyde	Purge-and-trap[a] (Tenax™ TA trap)	JP	Shimoda *et al.* 1996
Cashew	Furfuryl mercaptan, benzyl alcohol, δ-decalactone, eugenol, benzoic acid, isovaleric acid, phenylethyl alcohol, 2-methoxyphenol	Purge-and-trap[a] (Tenax™ TA trap)	BR	Moreira *et al.* 2002
Thyme	3,4,5-Trimethoxybenzaldehyde	SPME	TR	Mannas and Altug 2007
	1-Phenyl-2,3-butanedione, 3-hydroxy-4-phenyl-2-butanone, 3-hydroxy-1-phenyl-2-butanone, phenylacetonitrile, and carvacrol	SPME	GR	Alissandrakis *et al.* 2007
	3-Hydroxy-4-phenyl-2-butanone, 3-hydroxy-1-phenyl-2-butanone, 2-methylpropionic acid, 4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0] hept-1-yl)-3-buten-2-one	Solvent	GR	Alissandrakis *et al.* 2009
Thymus capitatus	1,3-Diphenyl-2-propanone, (3-methylbutyl)benzene, 3,4,5-trimethoxy benzaldehyde, 3,4-dimethoxy benzaldehyde, vanilline, thymol	SPME	PS	Odeh *et al.* 2007
Thymelaea hirsute	Benzene propanol, benzyl alcohol, nonanol, hexanol, 4-methoxyphenol	SPME	PS	Odeh *et al.* 2007
Tolpis virgata	3,5-Dihydroxytoluene, tridecane	SPME	PS	Odeh *et al.* 2007

Marmeleiro	Isovaleric acid, γ-decalactone, benzoic acid, vanillin	Purge-and-trap[a] (Tenax™ TA trap)	BR	Moreira *et al.* 2002
Cambara	Benzaldehyde, 2,3-methylbutanoic acid, benzonitrile, 2-phenylethanol	Solvent	BR	Moreira and de Maria 2005
Strawberry-tree	α-Isophorone, β-isophorone, 4-oxoisophorone	Purge-and-trap[a] (Carbopack™ trap)	Sardinia (IT)	Bianchi *et al.* 2005
Fir honeydew	Acetonitrile, methyl-2-buten-1-ol, *n*-hexanol, 3-hexanol, 1-propyne, 2-furanmethanol, 5-methyl-2(5H)-furanone, 4-methylphenol, hexadecanoic acid, methylheptanoate	SPME	HR	Lusic *et al.* 2007
Honeydew	n-Decane, nonanal, α-α-dimethylphenyl acetate, nonanol, 2-methyl heptanoic acid	SPME	TR	Senyuva *et al.* 2009
Nodding thistle	(E)-2,6-dimethyl-6-hydroxy-2,7-octadienoic acid, (E)-2,6-dimethyl-3,7-octadiene-2,6-diol, (Z)-2,6-dimethyl-2,7-octadiene-1,6-diol, (E)-2,6-dimethyl-6-hydroxy-2,7-octadienal, lilac aldehydes, lilac alcohols	Solvent	NZ	Wilkins *et al.* 1993
Turkish pine	3-Carene and unidentified compound (*m/z* 55, 79, 91, 107, 123, 162)	Purge-and-trap[a] (Tenax™ TA trap)	TR	Tananaki *et al.* 2007
Oak honeydew	*trans*-Oak lactone, aminoacetophenone, propylanisol	Solvent	ES	Castro-Vázquez *et al.* 2006

a) Geographic origin: EU: Europe (honey from three and more Europe countries); AU: Australia; BR: Brazil; CN: China; ENG: England; ES: Spain; GR: Greece; HU: Hungary; HR: Croatia; IR: Iran; IT: Italy; FR: France; JP: Japan; NZ: New Zealand; PS: Palestine; PT: Portugal; RO: Romania; SK: Slovakia; TR: Turkey; USA: United States of America.

b) Dynamic headspace extraction with desorption tube, filled with corresponding sorbent.

HS-SPME-GC analysis of volatile compounds using MS and/or FID in combination with multivariate analysis (Angerosa *et al.* 1999; Berlioz *et al.* 2006; Tena *et al.* 2007; Manai *et al.* 2008; Tura *et al.* 2008; Pouliarekou *et al.* 2011), as well as HS-SPME-GC × GC-MS (Torres Vaz-Freire *et al.* 2009) have also been successfully used for the fingerprint pattern recognition in olive oils from different varieties. Concerning the detection of adulteration in oils, volatile analysis has scarcely been used due to the composition of major components as fatty acids and sterols is more useful for that purpose (Soria *et al.* 2008). Nevertheless, Krist *et al.* (2006) were able to detect the adulteration of poppy seed oils with sunflower oils at different levels (5–40%, w/w) by using HS-SPME-GC-MS on the basis of detection of α-pinene as the most characteristic volatile component in sunflower seed oils. In fact, α-pinene was predominantly present in the headspace of all analyzed sunflower oil varieties in a manner independent of their triacylglycerol profiles. Filbertone (E-5-methylhept-2-en-4-one), a chiral compound which is found in hazelnuts, has been proposed as a marker to detect the fraudulent addition of this oil to olive oil. An initial sample extraction with either simultaneous distillation (SDE) or supercritical fluid technology (SFE) followed by GC or directly by LC-GC analysis, using a chiral column in both cases, was optimized for the determination of the enantiomeric composition of filbertone and, consequently, for the detection of the adulteration of olive oil with hazelnut oil (Blanch *et al.* 1998, 1999; Ruiz del Castillo *et al.* 1998; Flores *et al.* 2006). Later, Pérez-Pavón *et al.* (2009) developed a methodology for the determination of filbertone with a limit of detection of 0.27 $\mu g\, L^{-1}$ in different types of olive oils, based on HS-programed temperature vaporization (PTV)-GC-MS. Mildner-Szkudlarz and Jelen (2008) investigated the effectiveness of three different methods – SPME-GC-FID, electronic nose based on metal oxide (MOS) sensors, and SPME-MS – for the analysis of volatile compounds with subsequent principal component analysis (PCA) treatment of data. In all cases, these methods allowed the detection of olive oil adulteration with different contents of hazelnut oil ranging from 5 to 50% (v/v). However, the SPME-GC-FID approach was described as the slowest method.

Finally, volatile compounds determined by GC have been occasionally proposed for authentication of other foodstuffs such as cheese (Mariaca *et al.* 1997; Cornu *et al.* 2005), milk (Contarini *et al.* 1997), balsamic vinegars (Cocchi *et al.* 2004), mushrooms (Malheiro *et al.* 2013), hams (Narváez-Rivas *et al.* 2010), coffee (Risticevic *et al.* 2008), or spices (Alonso *et al.* 1998; Dimandja *et al.* 2000).

7.2.6 Phenolic Compounds

Phenolic compounds are a large class of well-known phytochemicals, ubiquitous in plants as well as in foods and beverages from vegetal origin. They show a large diversity of structures, including rather simple molecules such as phenolic acids through polyphenols such as stilbenes or flavonoids, to polymers derived from these various groups (Cheynier 2012). Although they have been analyzed by GC (Daher and Gülaçar 2008), some drawbacks, such as the non-volatility which implies the introduction of a derivatization step as well as the fact that the use of high temperature could damage some compounds due to their thermolability, means LC is the preferred technique for both separation and quantification of phenolic compounds. Although different LC operation modes can be applied for the analysis of phenolic compounds, the use of reverse-phase

C18 columns is the most predominant, combined with UV, fluorometric, and/or MS detection (Khoddami *et al.* 2013)

In honeys, the main reported phenolic compounds are flavonoids, including pinobanksin, pinocembrin, quercetin, myricetin, chrysin, galangin, luteolin, and kaempferol, and phenolic acids such as ferulic, caffeic, homogentisic, syringic, kynurenic, rosmarinic, and abscisic acids. Since geographic and botanical origins can have an important influence on the composition of phenolic constituents, some of these compounds can be promising markers for honey authentication as reviewed by Kaskonienè and Venskutonis (2010). In this context, there is an important number of studies addressing the characterization of the phenolic fraction in honeys by LC and its application to honey botanical or geographic characterization (Andrade *et al.* 1997; Yao *et al.* 2004*a*, *b*; Michalkiewicz *et al.* 2008; Escriche *et al.* 2011, 2014; Oelschlaegel *et al.* 2012; Campone *et al.* 2014; Karabagias *et al.* 2014). Nevertheless, there are other aspects which might have an effect on the composition of phenolic constituents in honey such as seasonal and environmental factors, thermal processing, or their intrinsic structural variety following biosynthesis by the floral sources of honey, which makes it difficult to use the presence of phenolic compounds for the precise determination of the botanical origin (Kaskonienè and Venskutonis 2010).

Phenolic compounds are part of the polar fraction of virgin olive oils and play a key role on the organoleptic characteristics of this natural food product (Ryan and Robards 1998), as well as on the stability of the oils to autoxidation (García *et al.* 2003). Although the phenolic composition of oils is strongly affected by the oil extraction procedure (Giovacchino *et al.* 2002) and agronomic factors, such as olive variety, location, altitude, or maturation degree (Uceda and Hermoso 1999), the lignan 1-acetoxypinoresinol has been used for the authentication of Picual olive oils following its analysis by reversed-phase LC and UV-absorption, fluorescence, and electrochemical detection, as well as by GC-MS analysis (Brenes *et al.* 2002). Additionally, phenolic compounds have also been used in chemometric studies together with chemical composition data (fatty acids, sterols, triterpenes, and triglycerides) and other quality characteristics (acidity, peroxide value, extinction coefficients at 232 and 270 nm) for the characterization and authentication of virgin olive oils from different varieties and/or geographical areas (Stefanoudaki *et al.* 2000; Boggia *et al.* 2002).

Díaz-García *et al.* (2013) developed an UPLC method with the simultaneous use of PDA and fluorescence detection to identify and quantify in a single analysis up to 70 polyphenols (23 anthocyanins, 15 flavonols, 6 hydroxybenzoic acids, 14 hydroxycinnamic acids, 4 flavanones, 2 dihydrochalcones, 4 flavan-3-ols, and 2 stilbenes) present in seven fruit juices (strawberry, American cranberry, bilberry, sour cherry, black grape, orange, and apple), allowing the verification of the authenticity of these fruit juices. More recently, Navarro *et al.* (2014) also applied capillary zone electrophoresis (CZE) and RPLC-DAD methods combined with PCA analysis to the authentication of fruit products (fruits such as cranberry, blueberry, grapes, and raisins, fruit-based products such as grape-juice and cranberry-juice, and commercial cranberry-based products) based on the compositional profiles of polyphenols. In a similar way, Primetta *et al.* (2013) determined the anthocyanidin profile by RPLC-DAD of bilberry and concluded that it could be a consistent feature to be used for the authentication and quality control of bilberry raw material and its extracts. Finally, Seeram *et al.* (2006), who characterized the phenolic fraction in strawberries by RPLC-ESI-MS, suggested that the phenolic

fingerprinting of strawberries could also be useful for the authentication of strawberry-based food products.

7.2.7 Organic Acids

Adulterations in fruit juices are normally performed by the addition of exogenous sugar, readily available less-expensive nutritive sweeteners, and/or organic acids. In consequence, organic acids in combination with major sugars (i.e., sucrose, glucose, and/or fructose) have been proposed as useful markers for authentication of different fruit juices such as pineapple (malic and citric acids; Jamin *et al.* 1997), orange (gluconic, lactic, malic, tartaric, oxalic, fumaric, citric, and isocitric acids; Guyon *et al.* 2013), and lemon (citric acid; Guyon *et al.* 2014) by determining their $^{13}C/^{12}C$ isotope ratios. This approach is based on the fact that naturally occurring elements exist in different isotopic forms and the distribution of these isotopes in the chemical compound profile of a fruit juice depends upon a variety factors such as growing region, temperature, and photosynthetic pathway (Perez *et al.* 2006; Willems and Low 2014). The articles mentioned above combined the separation of organic acids by LC (using RPLC or ion exchange chromatography (IEC)) and their ^{13}C isotope ratio determination by IRMS. In fact, the coupling of IEC) together with IRMS has been achieved through a liquid interface, ensuring a chemical oxidation (co) of organic matter and allowing the $\delta^{13}C$ determination of single compounds from a complex mixture without any purification steps (Godin and McCullagh 2011).

Nuncio-Jáuregui *et al.* (2014) reported adulteration of pomegranate juice by the addition of grape or peach juices. In the case of the addition of the grape juice, these authors observed an important increase in tartaric acid ($> 1.0\,mg\,L^{-1}$) which was detected in addition to citric and malic acids by ion-exchange LC-DAD, together with a decrease in potassium, an increase in proline, and the presence of some volatile compounds such as linalool and linalool oxide.

Regarding honeys, Daniele *et al.* (2012) quantified the presence of up to 14 organic acids by analyzing 140 samples originating from 7 different botanical origins by IEC. While gluconic acid was the major organic acid in honeys belonging to 6 botanical origins (i.e., acacia, chestnut, rapeseed, lavender, linden, and sunflower), galacturonic acid was the predominant organic acid in fir honey. In combination with the $^{13}C/^{12}C$ isotope ratios determined by IRMS and with data correlation by PCA, these authors concluded that organic acids can also be used as internal standards to detect honey adulteration and/or to differentiate honey samples as a function of their botanical origin.

7.2.8 Conclusions

Food adulteration leads to mislabeling, which affects public health in terms of food quality and safety. Global incidences of food adulteration are increasing; an important task of modern analytical chemistry is therefore to provide robust, efficient, and sensitive analytical methodologies for the assurance of food authenticity. The high resolution provided by chromatographic techniques in the separation of complex food matrices makes these techniques a valuable tool for genuineness assignment. Moreover, their coupling with mass spectrometry affords additional structural information, which

is crucial for compound identification as well as providing more accurate, more precise, and faster analysis of food components at very low concentrations. As illustrated in this chapter, LC, GC, and coupled MS techniques are suitable analytical techniques for the detection, identification, and quantification of analytes in complex food samples, and are at present the most widely used techniques for food-quality control in authenticity studies.

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8

Vibrational and Fluorescence Spectroscopy

8.1

Vibrational Spectroscopy

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8.1.1 Introduction

The authentication of foods and agricultural products is of primary importance for both consumers as well for the modern food industry. From a legislative point of view, quality standards were established through the requirement for quality labels that specify the chemical composition of each product. From an economic point of view, product authentication is essential to avoid unfair competition that can eventually create a destabilised market and disrupt regional or national economies (Arvanitoyannis *et al.* 1999, 2005; Cozzolino 2009, 2012; Arvanitoyannis 2010; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011). The authenticity of foods has two different aspects: authenticity with respect to production; and authenticity with respect to description. For example, geographical and botanical origin, 'natural', 'organic', 'raw', 'unheated' and 'fresh v frozen', and has been analysed using different methods and techniques (e.g. volatile compounds, minerals, carbohydrates and protein identification) (Arvanitoyannis *et al.* 1999, 2005; Cozzolino 2009, 2012; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011).

Adulteration can also take many forms, including the addition of sugars, acids, volatile oils, over-dilution of a concentrate, addition of juices of other fruits to a premium juice, the use of concentrates in a 'fresh' product and the use of low-quality products recovered from what are normally waste products of manufacture (Cozzolino 2009, 2012; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011). Food adulteration has been practised since ancient times but has become more sophisticated in the recent years. Foods or ingredients most likely to be targets for adulteration include those which are of high value or are subject to the vagaries of weather during their growth or harvesting. The practice of adulteration commonly arises for two main reasons: firstly it can be profitable; and secondly adulterants can be easily mixed and are subsequently difficult to detect (Cozzolino 2009, 2012; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011).

To counter these issues, manufacturers subject their raw material and by-products to a series of quality controls which include high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), thin-layer chromatography (TLC), enzymatic tests and physical tests to establish their authenticity and hence

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guarantee the quality of the products manufactured for the consumers (Arvanitoyannis *et al.* 1999, 2005; Cozzolino 2009, 2012; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011).

In recent years, the combination of novel instrumental techniques and chemometric methods has resulted in the development of rapid methods combining multivariate (MVA) data analysis with techniques such as near-infrared (NIR) and mid-infrared (MIR) in order to qualitatively and quantitatively analyse several food matrices (Arvanitoyannis *et al.* 1999, 2005; Cozzolino 2009, 2012; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011). Infrared radiation (IR) is the region between the visible (Vis) and the microwave wavelengths regions of the electromagnetic spectrum. The nominal range of wavelengths for NIR range is between 750 and 2500 nm (4000–13 400 cm^{-1}), while for the MIR the spectral range is defined from 2500 to 25 000 nm (400–4000 cm^{-1}). In this region, solid, liquid or gaseous samples can absorb some of the incoming infrared radiation at specific wavelengths or frequencies, resulting in a 'fingerprint' or spectrum of the sample (Cozzolino 2011; Smyth and Cozzolino 2011, 2013; Subramanian and Rodrigez-Saona 2009; Woodcock *et al.* 2008; Karoui *et al.* 2010).

Spectral 'signatures' in the MIR result from the fundamental stretching, bending, and rotating vibrations of the sample molecules, whilst NIR spectra result from complex overtone and high frequency combinations at the shorter wavelengths (Woodcock *et al.* 2008; Subramanian and Rodrigez-Saona 2009; Karoui *et al.* 2010; Smyth and Cozzolino 2011, 2013). Although NIR intensities are 10–1000 times lower than for the MIR range, and the peaks concomitantly smaller, highly sensitive spectrometers can be built through several means including the use of efficient detectors and brighter light sources (Cozzolino 2009, 2011; Subramanian and Rodrigez-Saona 2009). This allows concentrated bulk or even aqueous materials to be scanned and analysed quickly and easily. Spectral absorbances or peaks in the MIR frequencies are often sharper and better resolved than in the NIR. All the higher overtones derived from the O-H, N-H, C-H and S-H bands from the MIR region are still observed in the NIR region, although much weaker than the fundamental frequencies in the MIR (Subramanian and Rodrigez-Saona 2009). In addition, the existence of combination bands (e.g. C-O stretch and N-H bend in protein) gives rise to a crowded NIR spectrum with strongly overlapping bands. A major disadvantage of this characteristic overlap and complexity feature of the NIR spectra has been the difficulty of quantification and interpretation of the spectra without the use of MVA methods. On the other hand, the broad overlapping bands can reduce the need for a large number of wavelengths in calibration and analysis routines (Subramanian and Rodrigez-Saona 2009; Cozzolino 2011; Smyth and Cozzolino 2011, 2013).

The combination of MVA with either NIR or MIR spectroscopy is needed in order to enhance the information generated in order to increase their use for wide applications including composition measurement, traceability and authenticity. The combinations of MVA with analytical instruments therefore have the ability to determine more than one component at a time and can act as a support to establish links to other sample characteristics (McClure 2003; Brereton 2007; Cozzolino 2009, 2012; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011; Cozzolino and Murray 2012). The use of MVA in combination with modern instrumental methods also provides the ability to detect patterns in a dataset and to develop mathematical models to predict or monitor composition and other characteristics such as authenticity and traceability. Applications of both NIR and

MIR spectroscopy combined with multivariate data methods such as principal component analysis (PCA), linear discriminant analysis (LDA) and partial least-squares discriminant analysis (PLS-DA) are the most commonly used to investigate the authentication and traceability of foods (McClure 2003; Cozzolino 2009, 2012; Karoui *et al.* 2010; Weeranantanaphan *et al.* 2011; Cozzolino and Murray 2012).

This chapter review the applications and uses of NIR and MIR spectroscopy to authenticate and trace several food products. Examples of these applications to authenticate and trace fish, seafood, fish by-products, wine, honey, cereals and meat products are provided.

8.1.2 Instrumentation and Software

Many reports and reviews have examined and discussed the vast amount of studies related to IR instrumentation. The purpose of this section is to give an overall and brief introduction to the different available instruments used to target authenticity and traceability issues in foods. Generally speaking, an IR spectrophotometer (both MIR and NIR) consists of a light source (e.g. a tungsten halogen lamp), sample presentation accessories, monochromator and detector and optical components such as lenses, collimators, beam splitters, integrating spheres and optical fibres (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Huang *et al.* 2008; Roggo *et al.* 2007; Cozzolino 2009).

Basically, two main methods are used to collect the NIR spectra of a given sample: reflectance and transmittance. In reflectance (e.g. generally used to analyse solids or powder samples) the light source and detector are mounted under a specific angle (e.g. 45° in order to avoid specular reflection; McClure 2003; Nicolai *et al.* 2007; Huang *et al.* 2008). In transmittance (e.g. generally used to analyse liquid samples) the light source is positioned opposite to the detector, while in interactance mode the light source and detector are positioned parallel to each other in such a way that light due to specular reflection cannot directly enter the detector. In both reflectance and transmittance methods, integrated spheres may also be used to collect light and increase the signal to noise ratio. Transmission measurements, on the other hand, need very high light intensities which can easily burn the sample surface and alter its spectral properties (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009).

Generally speaking, NIR spectrophotometers are conveniently classified according to the type of monochromator. For example, in a filter instrument, the monochromator is a wheel holding a number of absorption or interference filters, while its spectral resolution is limited. On the other hand, in a scanning monochromator instrument a grating or a prism is used to separate the individual frequencies of the radiation either entering or leaving the sample. The wavelength divider rotates, allowing radiation of the individual wavelengths to subsequently reach the detector (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009).

In the case of Fourier transform (FT) spectrophotometers, interferometers are used to generate modulated light; while the time domain signal of the light reflected or transmitted by the sample onto the sample can be converted into a spectrum via a fast Fourier

transform (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009). Often a Michelson interferometer is used, but polarisation interferometers can also be mounted in some spectrophotometers.

Recently, photodiode array (PDA) spectrophotometers have been introduced. They consist of a fixed grating that focuses the dispersed radiation onto an array of silicon (Si, 350–1100 nm) or indium gallium arsenide (InGaAs, 1100–2500 nm) photodiode detectors. There is definitely a shift towards PDA systems because of their high acquisition speed (the integration time is typically 50 ms, but can be as low as a few milliseconds) and the absence of moving parts which enables them to be mounted on online fruit grading lines (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009).

Laser-based systems do not contain a monochromator, but have different laser light sources or a tunable laser. Acousto-optic tunable filter (AOTF) instruments use a diffraction-based optical-band-pass filter that can be rapidly tuned to pass various wavelengths of light by varying the frequency of an acoustic wave propagating through an anisotropic crystal medium (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009).

Liquid crystal tunable filter (LCTF) instruments have been introduced. They use a bi-refringent filter to create constructive and destructive interference based on the retardation in phase between the ordinary and extraordinary light rays passing through a liquid crystal. In this way they act as an interference filter to pass a single wavelength of light (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009). By combining several electronically tunable stages in series, high spectral resolution can be achieved.

Micro-electro-mechanical systems (MEMS) combine mechanical parts, sensors, actuators and electronics on a common substrate through the use of micro-fabrication technology. This technology represents a paradigm shift for industrial spectroscopy, and enables a variety of new industrial applications (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009).

In recent years multi- and hyperspectral imaging systems have been incorporated. In this case multispectral (a few wavelengths) or hyperspectral (a continuous range of wavelengths) imaging systems are required. Such systems produce a spectral data cube, a spectrum at every 2D spatial position.

In early systems a sequence of spatial images was acquired by means of an NIR camera and a set of band-pass filters. While the acquisition can be fast, the disadvantage of this approach is that only a limited number of wavelengths can be analysed, and these need to be known *a priori*. In a more recent approach, a line of spatial information with a full spectral range per spatial pixel is captured sequentially to complete a volume of spatial-spectral data. This is usually achieved by means of a spectrograph which disperses an incoming line of radiation into a spectral and spatial matrix which is captured by the camera. The horizontal and vertical pixels on the camera capture spatial and spectral information, respectively, providing full spectral information at every spatial position (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009). The object must be moved stepwise under the camera by means of an actuator while at each step a line is scanned,

but this is not necessarily a disadvantage when the system is mounted on a grading line on which the fruit is physically transported anyway. Novel developments include focal plane array cameras in combination with LCTF, AOTF or other monochromator principles which allow for much faster acquisition spectra.

As shown above, a variety of IR spectrophotometers from several manufacturers exists today, providing a number of different solutions for light dispersion, detectors and sample presentation configurations. What instrument to choose is largely dependent on the application; there is basically a trade-off between price and performance. The application of FT-MIR for food authenticity is of special interest due to the presence of sharp and specific absorption bands derived from the sample. In conventional laboratory FT-MIR spectrophotometers commonly used in food routine analysis (liquids), the sample is analysed through a short-path-length transmission cell. The advantage of the transmission cell is that it provides very accurate and reproducible spectroscopic measurements as well as allowing an easily control of the temperature of the sample. However, the use of transmission cells has several drawbacks, for example: care is needed for filling and cleaning the cell; the variations in sample path length due to window wear; and sample turbidity and viscosity. Sample thickness is very important for both these measurements: if it is too thick, the IR beam will be attenuated beyond the range where absorption is proportional to chemical concentration; if it is too thin, the absorption will be too low and the acquired spectral signal will be flooded in noise (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009).

Most of the current instruments used for the routine analysis of liquids are still based on measurements employing transmission cells using either CaF_2 or glass cuvettes. The strong absorption in the MIR region is of analytical advantage, generating several characteristic and rich sharp peaks in the MIR spectrum. Samples containing high amounts of water, ethanol and organic compounds such as wine or juice possess highly absorptive properties in the MIR region (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009). It is therefore not possible to use long path lengths when FT-MIR is used during analysis of clear liquid samples, determining the need for centrifugation or sonication of the sample prior to analysis.

In recent years a potential alternative to transmission measurements in liquids and solids is the use of attenuated total reflectance (ATR) cells (Dambergs *et al.* 2015). This technique is based on the principle of evanescence, and is usually used for the analysis of highly absorptive liquid samples or surfaces. In ATR spectroscopy, penetration depth into the medium is dependent on the wavelength and the refractive index of the sample, but is much lower than in transmission-type-spectrometers (10–50 μm). Even the regions which show total absorption by water in transmission spectra are therefore accessible for analysis using ATR. In the last two decades, different FT-MIR instruments have incorporated different sample presentation methods such as single bounce-attenuated total reflection (SBATR) and transmission with variable and defined path length (Dambergs *et al.* 2015).

Raman spectroscopy is based on fundamental vibration modes that can be assigned to specific chemical functional groups within a sample molecule, and can therefore potentially provide useful information for fingerprinting or qualitative identification (Shultz *et al.* 2005; Schulz and Baranska 2007; Li-Chan 2010). Raman spectroscopy has

also been explored as an emerging methodology in food authenticity and traceability (Shultz *et al.* 2005; Schulz and Baranska 2007; Li-Chan 2010). Raman spectroscopy, and similarly NIR and MIR, is a constantly developing technique that allows for the rapid, non-destructive, reagent-less and high-throughput processing of a diverse range of sample types. Raman spectroscopy coupled with microscopy has recently been used with great success, allowing the identification and quantification of photochemical distribution directly from the sample (Shultz *et al.* 2005; Schulz and Baranska 2007; Li-Chan 2010). Despite the advantages of Raman spectroscopy, when compared with MIR or NIR the development of this technique has been less extensive and slower due to complex instrumentation and high cost (Shultz *et al.* 2005; Schulz and Baranska 2007; Li-Chan 2010).

Many instrument manufacturers provide their spectrophotometers with specific software or have close collaborations with commercial software developers to facilitate exporting and importing the measured spectra, allowing for real-time predictions of composition. Alternatively, most of the analyses and calibration tools can also be found in commercial, shareware and freeware software and in open-source environments such as the R-project (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009). Some companies supply software packages that are fully integrated and can only calibrate using scans from their instruments. Others have their software broken into separate components and the calibration software can be used with scans from several platforms. Manufacturers typically include the software required to control the instrument as well as to create calibrations as part of the basic package that goes with the instrument. Access to pre-existing calibration equations developed by the manufacturer is usually a negotiable item.

In addition to specific software, there are various stand-alone software packages used by chemometric experts performing advanced algorithms as well as new multivariate methods and techniques development (Blanco and Villaroya 2002; McClure 2003; Cozzolino *et al.* 2006; Nicolai *et al.* 2007; Roggo *et al.* 2007; Huang *et al.* 2008; Cozzolino 2009). Modern software assesses whether unknowns fit within an equation as well as whether the equation would be improved by adding reference method values for a particular sample. Some programs also allow easy export of spectral and analytical data to laboratory information management systems (LIMS) so that the spectral data generated for a sample can be combined with other information and analytical data for a sample. All the manufacturers use advanced chemometrics in their calibration equations. Calibrations are developed using multivariate techniques such as principal component (PCR) or partial least-squares (PLS) regression.

Although commercially available IR instruments in the marketplace (e.g. Bruker, Foss, Unity, Thermo, etc.) have or include standalone software (e.g. OPUS, WinISI) that both controls the instrument operation and facilitates the development of new calibrations, other commercial software can be used. Examples of commercially available software include the Unscrambler package (http://www.camo.com) which is menu-driven and user-friendly, offers a range of pre-processing techniques, and handles spectra from several instruments. The Grams group, distributed by the Thermo Scientific company (http://www.thermoscientific.com), is a general-purpose software package for handling spectroscopic data including chemometrics. The PLS toolbox from Eigenvector Research (http:/www.eigenvector.com) offers the flexibility of different add-on tools that can be used in MatLab, in which a minimal level of programming is required. Many

other statistical packages are also available, for example SIMCA (http://www.umetrics.com), SAS (http://www.sas.com), Statistica (http://www.statsoft.com) and R (http://r-project.com) also provide multivariate calibration and data analysis tools.

8.1.3 Applications of Vibrational Spectroscopy in Food Authenticity

8.1.3.1 Fish and Seafood

The use of a VIS and NIR fibre-optic probe combined with LDA was reported as a method to classify correctly (up to 79%) salmon samples into three categories according to their Kramer shear force (Isaksson *et al.* 1989, 1995). These authors concluded that non-destructive VIS-NIR fibre-optic probe measurement gives good predictions of Kramer shear force. The same authors also suggested that most useful application of VIS-NIR might be in salmon production plants to classify fillets into broad classes according to texture before further processing or sale (Isaksson *et al.* 1989, 1995).

Seafood processing often removes morphological properties of the seafood species that enable the consumer to distinguish one type of organism from another. For this reason, species substitution is the most common form of economic adulteration in the seafood industry. Both VIS and NIR spectroscopy were evaluated to detect and quantify species authenticity and adulteration in crabmeat samples. To test the ability of NIR spectroscopy to trace seafood, Atlantic blue crabmeat samples were adulterated with blue swimmer crabmeat in 10% increments (Gayo and Hale 2007). These authors reported the use of several data pre-treatments to the NIR spectra such as moving average, combination of first and second derivatives, and the use of multiplicative scatter correction (MSC), in addition to the raw data, before PLS regression models were developed (Gayo and Hale 2007). The results obtained in this study suggested that VIS and NIR spectroscopy can be applied to detect and quantify species authenticity and adulteration in crabmeat. The same authors explored the use of both VIS and NIR spectroscopy to define the economic adulteration of crab meat samples. Atlantic blue and blue swimmer crab meat samples were adulterated with surimi-based imitation crab meat in 10% increments. These results suggested that VIS and NIR spectroscopy can be successfully used to detect adulteration in crab meat samples adulterated with surimi-based imitation crab meat (Gayo *et al.* 2006).

Shrimp (*Pandalus borealis*) samples from a commercial freezer trawler were thawed and subjected to different treatments such as in ice, water and brine of various salt concentrations before being steam-cooked and peeled (Brodersen and Bremner 2001). The NIR spectra were obtained for samples of whole shrimp and of minced fresh from the various treatments. The use of PCA allowed the differentiation between frozen and thawed material, different salt content, pH in the flesh, cooking period (temperature) of either in whole or minced shrimp, or both (Brodersen and Bremner 2001).

8.1.3.2 Fish and Meat By-Products

Since the bovine spongiform encephalopathy (BSE) crisis, the use of animal proteins in animal feed has been prohibited by the European Union (EU) (Cozzolino and Murray 2004, 2012). NIR microscopy was used to identify different classes of animal

by-products (De la Haba *et al.* 2007). Samples of fish meals ($n = 10$) and meals of land-animal origin ($n = 50$) were ground, sedimented and analysed using an Auto Image Microscope connected to a FTNIR. Sediment fraction particles were spread on a Spectralon plate, presented to the NIR microscope and scanned over the 1100–2500 nm region (De la Haba *et al.* 2007). The support vector machine (SVM) algorithm was used to construct models to identify class origin. Models correctly classified 100% of the samples in the calibration set and 95–95.5% in the validation set. The results demonstrated the potential of FT-NIR microscopy as a rapid method for distinguishing between fish and land-animal particles (De la Haba *et al.* 2007).

The use of NIR spectroscopy combined with chemometrics was used to identify and authenticate fishmeal batches made with different fish species. Samples from a commercial fishmeal factory ($n = 60$) were scanned in the NIR region (1100–2500 nm) in reflectance. PCA, PLS-DA, and LDA based on PCA scores were used to identify the origin of fishmeal produced using different fish species. The PLS-DA modes correctly classified 80 and 82% of the fishmeal samples while LDA models correctly classified >80% of fishmeal samples according to fish species (Cozzolino *et al.* 2002, 2005; Cozzolino and Murray 2012).

8.1.3.3 Wine

Verification of the authenticity of wine and other alcoholic beverages has become a potential application of both NIR and MIR spectroscopy, and several reports can be found in the literature about this topic (Picque *et al.* 2001, 2005; Cozzolino *et al.* 2009; Edelman *et al.* 2011; Dambergs *et al.* 2015). In order to illustrate this important application, a few examples are provided.

The use of VIS-NIR was investigated to discriminate Riesling and unwooded Chardonnay grown in Australia, with an accuracy of up to 95% (Cozzolino *et al.* 2003). Both VIS and NIR spectroscopy combined with multivariate analysis were also used to classify commercial Tempranillo wines from Australia and Spain (Liu *et al.* 2006; Liu 2007). In this study the authors used different multivariate data methods such as PCA, PLS-DA and LDA to classify Tempranillo wines according to their geographical origin. Results indicated that PLS-DA models correctly classified 100% and 84.7% of the Australian and Spanish Tempranillo wine samples, respectively. Similar results were obtained when Riesling wines samples from Australia, New Zealand, France and Germany were analysed using NIR spectroscopy (Liu *et al.* 2008). The concatenation of NIR and MIR spectroscopy combined with MVA methods was used to authenticate and discriminate Shiraz wine samples grown in different regions of Australia (Riovanto *et al.* 2011). Wines belonging to the controlled designation of origin (DO) Rías Baixas (Spain) were classified according to their denomination of origin using UV, VIS and NIR spectroscopy alone or in combination. Samples were classified using PCA, soft independent modelling of class analogy (SIMCA), LDA and support vector machine (SVM). These results show that combinations of UV/VIS/NIR spectra and chemometric methods can be used as a rapid technique to classify subzones into a DO such as Rías Baixas (Martelo-Vidal *et al.* 2013).

A similarity index (SI) based on MIR spectra of wines was developed in order to identify grape varieties (Bevin *et al.* 2006, 2008). The classification of red wines made from different grape varieties using FT-MIR was reported by several authors as well as the

use of ATR-FT-MIR as a tool to differentiate Greek red wines on the basis of grape variety (Tarantilis *et al.* 2008). The use of UV-VIS spectroscopy combined with SVM as a reliable analytical tool in order to discriminate the wines produced in the different specific regions of Spain was also reported (Acevedo *et al.* 2007). These authors highlighted that it is important to take into account that each brand in the same DO can present significant differences and, in order to prevent fraud, it is necessary to include samples in the training set as well as a large number of different brands. The results obtained recommend the suppression of non-relevant wavelengths in search of better accuracy. The importance of these conclusions relies on the design of real-time systems, where we can focus on simpler devices (Acevedo *et al.* 2007).

In the Italian oenological industry, the regular practice used to naturally increase the colour of red wines consists of blending them with a wine very rich in anthocyanins, namely Rossissimo (Ferrari *et al.* 2011). In the Asian market, on the other hand, anthocyanins extracted from black rice are frequently used as correctors for wine colour. This practice does not produce negative effects on health; however, in many countries, it is considered as a food adulteration. In order to discriminate wines containing anthocyanins originating from black rice and from grapevine, using a reliable spectroscopic technique requiring minimum sample preparation, two series of samples were prepared from five original wines that were doctored with different amounts of Rossissimo or of black rice anthocyanins solution, until the desired colour index was reached (Ferrari *et al.* 2011). The samples were analysed by both FT-NIR and NMR and the resulting spectra matrices were subjected to multivariate classification with variable selection. The NIR spectra allowed the classification of the test set samples with an efficiency of about 70%, while the classification models based on wavelet variables selection yielded a classification rate in validation greater than 95%. The use of 2D correlation analysis was also evaluated and applied to both the FT-NIR and NMR matrices in order to recognise the spectral zones bringing the same chemical information (Ferrari *et al.* 2011).

Four rapid and low-cost analytical systems based on methods such as NIR and UV-Vis spectroscopy, headspace-mass-based artificial nose (E-Nose) and a voltametric artificial tongue, combined with chemometric pattern recognition techniques, were used to target wine authenticity issues such as the distinction between wine samples from the same Italian oenological region, according to the grape variety (Casale *et al.* 2010). Certified ($n = 59$) samples belonging to the Barbera d'Alba and Dolcetto d'Alba appellations collected from the same vintage (2007) were analysed. The instrumental responses were analysed after proper data pre-processing, and were used as fingerprints of the characteristics of the samples (Casale *et al.* 2010).

The volatile composition and MIR spectroscopic profiles of South African young cultivar wines combined with chemometrics was proposed as a method to identify compositional trends as well as to distinguish between different grape cultivars (Louw *et al.* 2009). Data were generated by GC and FTMIR spectroscopy and investigated by using analysis of variance (ANOVA), PCA and LDA. Significant differences were found in the volatile composition of the cultivar wines, with marked similarities in the composition of Pinotage wines and white wines. Both the volatile composition and the FT-MIR spectra contributed to the differentiation between the cultivar wines. The best discrimination model between the white wines was based on FT-MIR spectra, correctly classifying 98.3% of the wine samples, while the combination of spectra and volatile compounds achieved 86.8% of correct classification (Louw *et al.* 2009).

The FTMIR spectra of 65 sweet wines produced in Cyprus and other countries were collected in order to study the authenticity and uniqueness of the Cypriot traditional wine Commandaria that is produced from sun-dried grapes (Ioannou-Papayianni *et al.* 2011). FTMIR spectroscopy and chemometrics were able to differentiate all the types of Commandaria (non-fortified, fortified and commercial) from various sweet wines from other countries (Ioannou-Papayianni *et al.* 2011). Differentiation between different Greek wines was also reported using ATR and MIR spectroscopy (Tarantilis *et al.* 2008).

8.1.3.4 Cereal grains

Cereal grains are among the most important of the foods we grow, where a large proportion of cereals are milled and marketed as flour in order to produce bread, cakes, biscuits and many other commodities (Pojić *et al.* 2008; Jespersen and Munck 2009; Himmelsbasch 2010; Pojić and Martilović 2013). A number of reports on the use of NIR spectroscopy as an analytical tool in wheat quality determination are available in the literature, see recent reviews by other authors. However, few publications report on the use of vibrational spectroscopy to target authenticity and classification issues in cereals. Recent reports stated that the ultimate goal of the application of the NIR spectroscopy is not solely the best estimates of the reference values, but a discrimination of the samples according to their spectral properties reflecting their functionality (Miralbés 2008; Pojić *et al.* 2008; Pojić and Martilović 2013).

The application of NIR spectroscopy for classification purposes is based on the fact that samples with different spectral responses have different physical and/or chemical properties (Pojić *et al.* 2008; Jespersen and Munck 2009; Himmelsbasch 2010; Pojić and Martilović 2013). The first application of NIR spectroscopy for classification purposes was reported by Bertrand *et al.* (1985) for the identification of wheat varieties suitable for bread baking. Delwiche and Norris (1993) reported the successful classification of ground samples of hard red winter (HRW) and hard red spring (HRS) wheat samples. The use of algorithms such as artificial neural network (ANN) classification of wheat classes was also reported (Chen *et al.* 1995). Delwiche and Massie (1996) reported successful classification of individual kernels of wheat classes using PLS or multiple linear regression (MLR). Dowell (1998, 2000) reported the successful application of NIR spectroscopy for the classification of vitreous and non-vitreous wheat kernels, suggesting that the scattering effects, grain hardness, and differences in starch and protein content between vitreous and non-vitreous kernels contributed more to the classification of vitreous and non-vitreous kernels. Cocchi *et al.* (2004, 2005) and Foca *et al.* (2009, 2011) demonstrated the successful application of NIR spectroscopy to differentiate between bread wheats and flours sourced from different baking quality characteristics. In Europe, Miralbés (2008) demonstrated the discrimination of wheat varieties originating from Spain and France.

Another application of NIR spectroscopy for classification purposes was reported by Dowell *et al.* (2009), related to the selection and sorting of waxy wheat kernels using an automated single kernel NIR sorting system. Delwiche *et al.* (2011) also reported the utilisation of the NIR spectroscopy for the differentiation of the starch waxy genotypes with accuracies of 90–100%. The combination of NIR spectroscopy and genetic markers were used to classify five wheat varieties sourced from Syria. The author of this study concluded that the combination of NIR with other genetic techniques could be a powerful tool to detect and classify wheat varieties (Saleh 2012).

Delwiche *et al.* (1995) used NIR and PCA to classify amylose content in rice while Campbell *et al.* (2000) combined NIR with PCA analysis to identify and classify maize endosperm mutants. In barley, the main application of NIR in relation to classification was related to the discrimination between grains from normal and mutant barley samples (Munck *et al.* 2004). The use of derivatives of NIR spectra to remove linear trends associated with the changing particle size distribution from one sample to the next of the same type of biological material was used to classify barley varieties grown in Australia (Wiley *et al.* 2009).

The feasibility of using NIR spectroscopy combined with chemometrics was investigated to discriminate wheat from different geographical origins. A total of 240 wheat samples from two growing years from four major wheat-producing regions in China were analysed. After spectral data pre-treatment to wheat kernel and whole wheat flour, LDA based on PCA scores and DPLS were applied to classify samples according to their origin. The results showed that there were significant differences among the NIR spectra of wheat samples from different regions. The authors concluded that wheat could be classified based according to their geographical origins with a combination of chemometrics and NIR (Zhao *et al.* 2013). The effects of origin, genotype, harvest year, and their interactions on wheat NIR spectra were also evaluated in order to determine the differences in NIR fingerprints of wheat from different geographical origins in three regions of China from two harvests. The results showed that origin, genotype, year and their interactions all had significant effects on wheat NIR fingerprints. The second overtones of N–H and C–H stretching vibrations and a combination of stretch and deformation of C–H group in wheat were mainly influenced by the geographical origin. The wavelength ranges 975–990 nm, 1200 nm and 1355–1380 nm contained enough information to develop robust discriminant models (Zhao *et al.* 2014). The use of attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was also reported to discriminate flours of 26 maize landraces from southern Brazil (Kuhnen *et al.* 2010).

8.1.3.5 Honey

The Council Directive 74/409/EEC of the European Communities gives a general definition of honey and indicates the main varieties that can be marketed in the European Union (Arvantoyannis *et al.* 2005). Several reports can be found on the use of vibrational spectroscopy to authenticate honey samples (Ruoff *et al.* 2006*a*, *b*; Woodcock *et al.* 2007, 2009). Figure 8.1.1 shows the second-derivative NIR spectra of honey samples sourced from different origins (pollen source).

Authentic honey samples from two seasons (2004/2005 and 2005/2006) were collected from Ireland ($n = 2$), Italy ($n = 30$), Austria ($n = 40$), Germany ($n = 36$), mainland France ($n = 46$) and Corsica ($n = 219$). Overall correct classifications of 82% (FDA) and 87% (PLS) were obtained for a separate validation set comprising samples from both harvests (Etzold and Lichtenberg-Kraag 2008). Classification models based on MIR spectra were developed using PCA and validated with samples from various botanical origins (Hennessy *et al.* 2010). Honey samples sourced from Brassica spp., Robinia pseudoacacia, Calluna vulgaris, Trifolium spp., Tilia spp., Helianthus annuus, Centaurea cyanus and honeydew honey were used for calibration. The results demonstrated that honey samples from rape, false acacia, heather and honeydew can be classified correctly

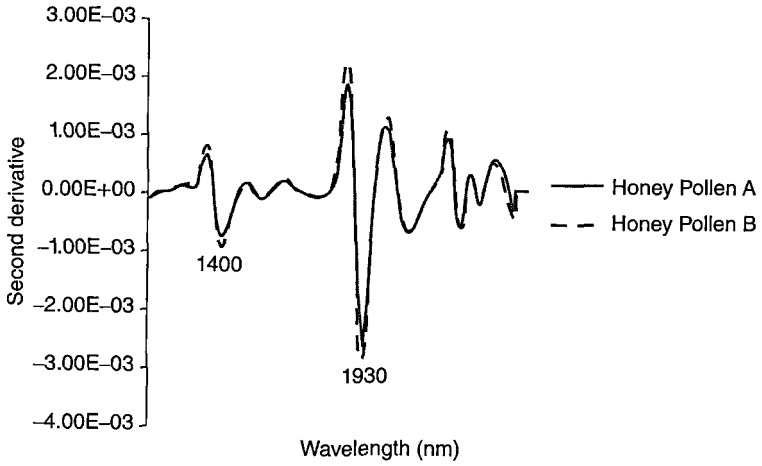


Figure 8.1.1 Second-derivative NIR spectra of honey samples sourced from different origins.

by FT-MIR and were comparable to those obtained using chemical and sensory analysis (Hennessy *et al.* 2010).

The potential of FT-MIR-ATR was evaluated for the authentication of 10 unifloral (acacia, alpine rose, chestnut, dandelion, heather, lime, rape, fir honeydew, metcalfa honeydew, oak honeydew) and one polyfloral honey types ($n = 411$ samples) previously classified with traditional methods based on chemical, pollen count and sensory analysis (Ruoff *et al.* 2006*a*, *b*). Chemometric evaluation of the spectra was carried out by applying PCA and LDA, and the error rates of the discriminant models were calculated using Bayes' theorem. Honey samples ($n = 48$) from a wide variety of geographical and botanical sources were analysed at five temperatures using NIR spectroscopy. These results demonstrate that the successful characterisation of the botanical source of a honey may be obtained by NIR spectroscopy (Ruoff *et al.* 2006*a*, *b*).

FT-NIR was evaluated for the authentication of eight unifloral and polyfloral honey types ($n = 364$) previously classified using traditional methods such as chemical, pollen and sensory analysis. Chemometric evaluation of the spectra was carried out by applying PCA and LDA. Authentic, unfiltered honeys ($n = 373$; 219 Corsican and 154 non-Corsican) were collected over two production seasons (Hennessy *et al.* 2008, 2010). The goal was to create a specific spectral fingerprint for Corsican honey followed by preliminary data examination using PCA (Hennessy *et al.* 2008, 2010). The best PLS discriminant models developed using full cross-validation, a variable selection algorithm and second-derivative data pre-treatment provided correct classification results of 90.0 and 90.3% for the Corsican and non-Corsican honey samples, respectively. Using separate calibration and validation samples from this same honey collection, the highest correct classification values of 90.4 and 86.3% for Corsican and non-Corsican honey samples, respectively, were obtained using a variable selection procedure (Hennessy *et al.* 2010).

The potential of NIR spectroscopy to determine the geographical origin in 167 unfiltered (88 Irish, 54 Mexican and 25 Spanish) and 125 filtered honey samples (25 Irish, 25 Argentinean, 50 Czech and 25 Hungarian) was evaluated using transflectance mode (Woodcock *et al.* 2009). Following preliminary examination using PCA, modelling methods applied to the spectral dataset were PLS regression and SIMCA; various

pre-treatments were also evaluated by the authors. For unfiltered honey, the best SIMCA models provided correct classification rates of 95.5, 94.4 and 96% for the Irish, Mexican, and Spanish samples, respectively. Classification rates obtained were around 96, 100, 100 and 100% correct classifications for the Argentinean, Czech, Hungarian and Irish honey samples, respectively, using a second derivative (Woodcock *et al.* 2009). Overall, although both SIMCA and PLS provided encouraging results, better classification rates were found using PLS regression.

Diffuse reflectance mid-infrared Fourier transform spectroscopy (DRIFTS) and multivariate statistical analysis methods were used for the identification and classification of honey from different floral sources. In total, 82 honey samples (robinia, chestnut, citrus, polyfloral) were scanned by DRIFTS in the region 4000–600 cm^{-1} and the spectra was also transformed using first and second derivatives (Bertelli *et al.* 2007). Spectral data were analysed using PCA, DA and classification tree analysis. Classification rates obtained were near 100% using discriminant and classification tree analyses. Classification models were successfully validated with a one-third leave-out method, and a classification of about 100% was achieved (Bertelli *et al.* 2007).

The adulteration of honey with sugars is the most crucial quality assurance concern in the honey industry. The use of FT-MIR-ATR was evaluated to determine the level (1–25%, wt/wt) of invert cane sugar adulteration in honey. On the basis of spectral data compression using PCA, PLS, LDA and CVA, models were developed and validated (Bertelli *et al.* 2007). FT-MIR-ATR was used to quantify three different adulterants (corn syrup, high fructose corn syrup and inverted sugar) in honeys of four different locations in Mexico (Chiapas, Oaxaca, Estado de Mexico and Morelos) (Gallardo-Velazquez *et al.* 2009). Classification of the Mexican honeys from the four different states carried out using SIMCA provided a 100% correct classification rate and no false-positive results were obtained (Gallardo-Velazquez *et al.* 2009).

The application of FT-MIR-ATR spectroscopy as a screening tool for the determination of the type of sugar adulterant in honey was investigated. Adulterants considered were sugars (glucose, fructose and sucrose) and invert sugars (cane invert and beet invert). Predictive models were developed to classify the adulterated honey samples using DA analysis. LDA was used to discriminate the type of adulterant in three different honey varieties. An optimum classification of 100% was achieved for honey samples adulterated with glucose, fructose and sucrose and beet and cane invert sugars. Results demonstrated that discriminant analysis of the spectra of adulterated honey samples could be used for rapid detection of adulteration in honey (Irudayaraj and Sivakesava 2001; Irudayaraj *et al.* 2003; Kelly *et al.* 2004, 2006).

A collection of authentic artisanal Irish honeys ($n = 580$), some of which had been adulterated with fully inverted beet syrup ($n = 280$), high-fructose corn syrup ($n = 160$), partial inverted cane syrup ($n = 120$), dextrose syrup ($n = 160$) and beet sucrose ($n = 120$), were evaluated. Both SIMCA and PLS classification methods allowed discrimination between authentic honey and honey adulterated by beet sucrose, dextrose syrups and partial inverted corn syrup with correct classification rates of 96.2, 97.5, 95.8 and 91.7%, respectively (Irudayaraj and Sivakesava 2001; Irudayaraj *et al.* 2003; Kelly *et al.* 2004).

NIR spectroscopy combined with chemometrics methods has been used to detect adulteration of honey samples. Spectral data of samples containing authentic ($n = 68$) and adulterated ($n = 67$) honey samples were compressed using wavelet transformation (WT) and PCA (Kelly *et al.* 2004, 2006). Five classification modelling methods,

including least-squares support vector machine (LS-SVM), SVM, BP-ANN, LDA and K-nearest neighbours (KNN), were tested to correctly classify pure and adulterated honey samples. According to the authors the use of WT proved to be more effective than PCA as a means of variable selection, while the best classification models were achieved with LS-SVM. A total accuracy of 95.1% was obtained by LS-SVM. The results showed that WT-LS-SVM can be used as a rapid screening technique for the detection of this type of honey adulteration with good accuracy and better generalisation (Irudayaraj and Sivakesava 2001; Irudayaraj *et al.* 2003; Kelly *et al.* 2004, 2006).

8.1.3.6 Meat and Meat Products

Many consumers place particular emphasis on non-compositional aspects of meat that are not related to composition (e.g. protein, fat, colour) such as geographical origin, production methods and rearing or feeding systems. Examples of these applications are summarised in a very comprehensive and recent review by Weeranantanaphan *et al.* (2011). Figure 8.1.2 shows the score plot of the first two principal components derived from the NIR reflectance spectra of homogenised pork and chicken samples. The figure shows the separation between samples according to their origin (species) based on their NIR spectra.

8.1.4 Concluding Remarks and Future Perspectives

Both NIR and MIR spectroscopy combined with MVA have been extensively used to measure chemical composition in many foods (e.g. protein, moisture and oil). However, the most important development of these technologies has been as tools or methods for authenticity, discrimination or traceability of foods. Adapting and using these technologies

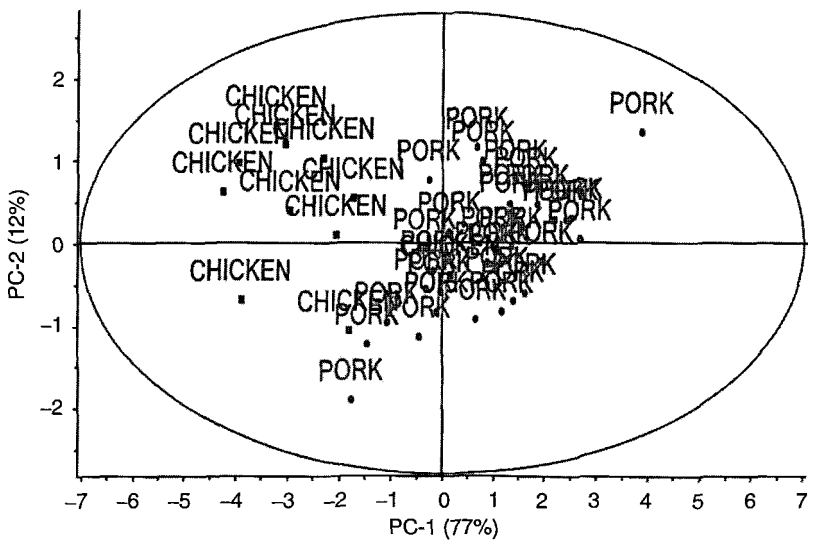


Figure 8.1.2 Score plot of chicken breast meat and pork loin meat analysed using near-infrared reflectance spectroscopy.

is a promising method to efficiently and reliably monitor and control important characteristics in the food process, as well as to improve our understanding about chemical and biochemical characteristics associated with origin, in order to maintain a sustainable food production as well as to guarantee to the consumers the origin of the food.

Infrared and other vibrational spectroscopy technologies have been introduced as fingerprinting techniques to analyse agricultural products and foods, and in recent years have been rediscovered by several research groups in broad applications. The main advantages of these techniques over the traditional chemical and chromatographic methods are the rapidity and ease of use in routine operations. Vibrational-spectroscopy-based methods are well known to be non-destructive techniques which require minimal or zero sample preparation.

From the literature published it is clear that the breadth of these applications, either in routine use or under development, is showing no sign of diminishing. The combination of different vibrational spectroscopy analytical techniques with multivariate methods could be used as a sample fingerprinting tool on a large scale. However, the chemical basis of this separation is not addressed using this type of methodology and other analytical techniques (i.e. GC-MS) need to be used or combined in order to reveal the fundamental causes of the separation/classification.

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8.2

Fluorescence Spectroscopy

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8.2.1 Fluorescence

8.2.1.1 Basic Principles

Fluorescence spectroscopy (also known as fluorometry or spectrofluorometry) is a type of electromagnetic spectroscopy which analyzes the fluorescence of a sample. It is an analytical technique of moderate selectivity and extremely high sensitivity, and can be applied to the detection of a very wide range of analytes in environmental and biological samples. It is applied for analysis of concentrations in the nanogram per milliliter range (and sometimes in the picogram per milliliter range) in complex matrices.

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation of higher energy. The fluorescent substance is called fluorophore. The general principles can be illustrated by a Jabłoński diagram (Jabłoński 1933), as shown in Figure 8.2.1, and consists of 3 steps:

1) An electron of the fluorophore absorbs a photon ($h\nu_{ex}$) and is excited from its ground electronic state to one of the various vibrational states in the excited electronic state.
2) The excited molecule interacts with the molecular environment in a number of different ways such as vibrational relaxation, quenching, and energy transfer, moving to the lower vibrational state of the first excited state.
3) The electron returns to one of the various vibrational levels of the ground electronic state, emitting a photon ($h\nu_{em}$) in the process. As molecules may drop down into any of the several vibrational levels in the ground state, the emitted photons will have different energies and therefore different frequencies.

Based on the fact that the excitation can occur at any of the vibrational states of the excited states and the emission to any of the vibrational states of the ground state, excitation and emission do not occur only at one single wavelength but rather over a distribution of wavelengths.

Usually, the emission spectrum of a molecule is a mirror image of its excitation spectrum moved to lower energies. This symmetry is a result of the similarities of the

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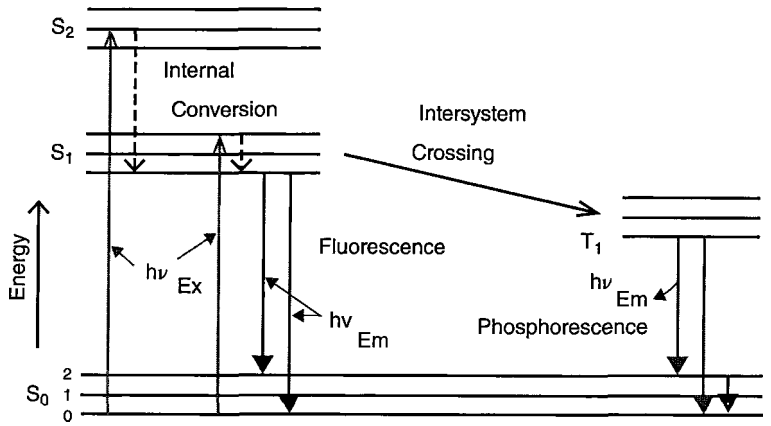


Figure 8.2.1 Jabłoński diagram illustrating the fluorescence and phosphorescence process.

vibrational levels of S_0 and S_1 involved in both excitation and emission. However, in some cases there are more bands, for example in the case of excitation from the transition S_0 to S_3.

The difference in wavelengths or energy units between the absorbed and the emitted photon is called the Stoke's shift. The process of emitting light runs within nanoseconds and is temperature independent. In some cases the electron does not go to an energetically lower vibrational state of the excited state, but to an intermediate excited triplet state; in this case the decay lasts longer and is temperature dependent, and the phenomenon is known as phosphorescence (Fig. 8.2.1.)

Due to the fact that fluorescence is characterized by two independent wavelengths, the specificity of the method is significantly improved in comparison to absorption spectroscopy. Fluorescence spectroscopy is also a very sensitive technique, permitting in some cases measurements down to levels of parts per billion. That is due to the fact that the photons emitted are measured against a low background and there is a multitude of high-efficiency detectors for the visual part of the spectra range.

Fluorescence is not a property of all molecules. Among molecules presenting fluorescence are polyaromatic hydrocarbons and heterocycles with rigid molecular skeletons. The most food important fluorophores are presented in Table 8.2.1 (http://www.models.kvl.dk/foodfluor).

The fluorescence ability of a fluorophor can be measured by the quantum yield or quantum efficiency Q, defined:

$$Q = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}}.$$

8.2.1.2 Instrumentation

Figure 8.2.2 shows a schematic diagram of a spectrofluorometer. It includes a Xenon lamp or laser as a source of exciting light. The light beam passes through a monochromator (in the case of the Xenon lamp) and the outgoing monochromatic beam is focused

Table 8.2.1 List of food-fluorophores.

Fluorophore	Concentration (M)	Excitation λ_{max} (nm)	Emission λ_{max} (nm)	I_{max} [a]	Solvent
Phenyl alanine	10^{-3}	258	284	32	Water
Tyrosine	10^{-5}	276	302	28	Water
Tryptophan	10^{-5}	280	357	100	Water
Vitamin A Retinol)	10^{-5}	346	480	16	Iso-octane
Vitamin B_2 (Riboflavin)	10^{-5}	270 (382,448)	518	167	Water
Vitamin B_6 (Pyridoxin)	10^{-5}	328	393	51	Water
Vitamin E (α-Tocopherol)	10^{-4}	298	326	167	Iso-octane
Nicotinamide adenine dinucleotide (NADH)	10^{-4}	344	465	16	Water
Adenosine triphosphate (ATP)	10^{-3}	292	388	33	Water
Chlorophyll A	10^{-6}	428	663	56	Iso-octane
Hematoporphyrin	10^{-5}	396	614	27	Iso-octane

a) The intensities are normalized according to Tryptophan (100)

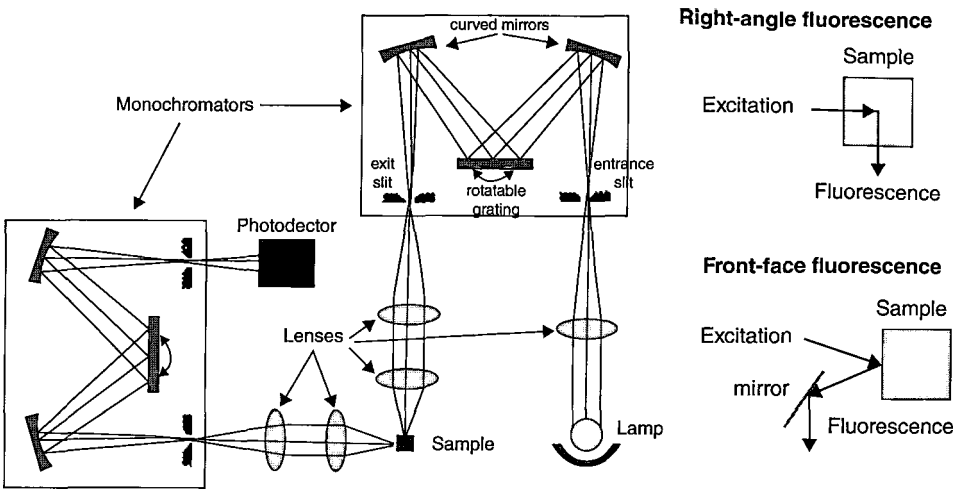


Figure 8.2.2 Schematic diagram of a spectrofluorometer (left) and of front face and right angle fluorescence set-up (right).

onto the sample. The fluorescence is emitted from the sample in all directions. The recording can be made at a 90° angle relative to the excitation radiation, or at a 180° angle (Fig. 8.2.2). In the first case there is a minimization of parasite radiation (due to the excitation beam). In the second case, films or solid samples can be measured and there is no inner filter effect. The 180° method is known as front-face fluorescence spectroscopy (FFFS). The emitted radiation reaches the photomultiplier after passage through a second monochromator, to record the intensity with regards to wavelength.

8.2.1.3 Types of Fluorescence Spectra

There are three main types of spectra in fluorescence spectroscopy.

1) The emission spectra when a sample is excited at a fixed wavelength λ_{ex}: an emission spectrum is produced by recording the emission intensity as a function of the emission wavelength λ_{em}. By recording the emission spectra for a range of excitation wavelengths and combining them, a contour plot (a three-dimensional surface data with emission intensity as a function of excitation and emission wavelengths) is created that is the sample's total luminescence (Fig. 8.2.3a).
2) The excitation spectra when λ_{ex} is scanned while the observation is conducted at the fixed λ_{em}: from the excitation and emission spectra maximum peaks ($\lambda_{max,ex}$, $\lambda_{max,em}$, respectively) the Stoke's shift can be calculated. Stoke's shift (nm) $= \lambda_{max,em} - \lambda_{max,ex}$.
3) The synchronous scanning fluorescence spectra (SSFS): the broad nature and spectral overlap of conventional fluorescence spectra (especially in the cases of multicomponent samples as in the case of foods) can be overcome, and enhanced selectivity can be obtained using SSFS (Lloyd 1971). In SSFS, the λ_{ex} and λ_{em} are scanned simultaneously (Ndou and Warner 1991). Depending on the scan rate, three basic types of the SSFS technique are possible. The most popular SSFS technique is the constant-wavelength, according to which the scan rate is constant for both monochromators and, therefore, a constant wavelength interval $\Delta\lambda$ is kept between

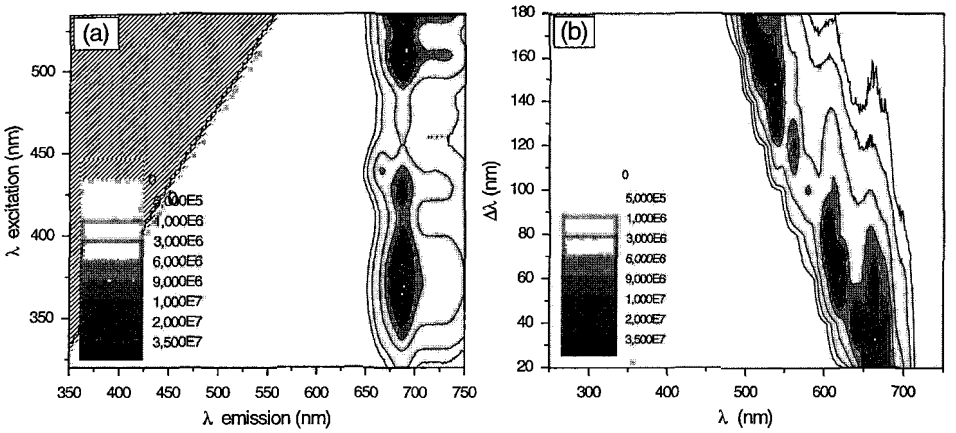


Figure 8.2.3 Contour plots of (a) total luminescence and (b) total SSFS of edible olive oil.

λ_{em} and λ_{ex}. By recording the synchronous spectra for a range of $\Delta\lambda$ and combining them all together a contour plot can be created; that is the sample's total SSFS (Fig. 8.2.3b). The other two SSFS techniques are the variable-angle SSFS when the excitation and emission wavelengths may be varied simultaneously but at different rates and the constant-energy SSFS.

8.2.1.4 Factors Affecting Fluorescence Intensity

There are several factors influencing the fluorescence intensity of biological samples. The most important of them are described in the following sections.

8.2.1.4.1 Concentration and Inner Filter Effect

The fluorescence intensity (I_f) of a fluorophore excited with a light of intensity I_o, according to the quantum efficiency Q, is defined $I_f = QI_o$, but in many cases there is an error due to the absorption of the light from the sample and the equation has to be modified to:

$$I_f = QI_o\left(1-10^{\varepsilon lc}\right)$$

or, in the case of samples with optical density <0.05,

$$I_f = 2.3QI_o\varepsilon lc$$

where c is the morality of the fluorophore, ε the molar absorptivity, and l the optical depth. We can see that there is a linear relation between the concentration and fluorescence intensity at low concentrations (absorbance <0.05). However, at higher concentrations a part of the excitation light intensity or/and a part of the emitted light intensity is absorbed and the signal decreases. This phenomenon is called the inner filter effect and can be a source of errors. A method to avoid the inner filter effect is the use of the front-face fluorescence technique.

8.2.1.4.2 Quenching

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample due to deactivation of the excited molecule by either intramolecular interaction (collision) or intermolecular activity (interaction with other molecules). One of the best-known quenchers is oxygen. Quenching can also be caused by high temperatures or donor acceptor energy transfer.

8.2.1.4.3 Scatter

Scattering and reflection of the incident light is a problem for the fluorescence measurements, especially in the case of FFFS, in turbid solutions, and solid opaque samples. There are two main mechanisms of scattering: the elastic Rayleigh scatter and the inelastic Raman scatter. In the Rayleigh scatter the scattering light has the same wavelength as the incident light and its interference can be avoided if there is a difference of some wavelengths between the excited and emitted light. Due to the construction of the monochromators, some light at the double (triple, etc.) wavelength of the excitation light will be present in the signal.

Raman scattered light reflects an energy loss or gain in comparison to the incident light due to coupling with the vibrational states of the molecule.

8.2.1.4.4 Molecular Environment

The polarity of the local environment of a fluorophore affects the intensity and wavelength of the emitted light. In addition the pH affects the fluorescence, especially in the case that it can evoke different structures or conformations (e.g., proteins).

8.2.2 Chemometrics

Foods generally contain several intrinsic fluorophores and other phenomena which influence the recorded fluorescence signals. Since fluorescence spectra of foods even in the case of SSF are typically composed of broad overlapping fluorescence bands, containing information of all sample components, the analytical information contained in spectra is multivariate in nature and therefore non-selective. In addition, differences between the samples may cause very slight spectral differences that are difficult to distinguish. For these reasons the use of chemometric methods to extract the analytical chemical information contained in the fluorescence spectra is necessary. By using various multivariate analysis techniques, several analytical variables can be related to the properties of the analyte(s). Furthermore, these techniques allow the grouping of the samples with similar characteristics.

Chemometrics is the science of extracting information from chemical systems by data-driven means. The chemometric methods that are used are in the form of multivariate and multiway data analysis.

Methods such as principal component analysis (PCA), common component and specific weights analysis (CCSWA), partial least squares regression (PLS), principal component regression (PCR), parallel factor analysis (PARAFAC), etc. have proven to be powerful techniques for the extraction of valuable information from fluorescence spectra of food systems (Christensen *et al.* 2006; Sádecká and Tóthová 2007).

PCA is often the first stage in fluorescence data exploration and the most common data analytical approach used for exploring, visualizing, and mining the data. PCA is a variable reduction procedure. It uses an orthogonal transformation to convert a set of possibly correlated (because they are measuring the same property) variables into a set of values of linearly uncorrelated principal components. The first step is the standardization of the variables and then the calculation of the principal components as linear combination of input variables. For example, for the first principal component (PC1):

$$t_1 = a_{11}X_1 + a_{12}X_2 + a_{13}X_3 + \cdots a_{1j}X_j$$

where t_1 is the vector coordinates of PC1, X_j is fluorescence intensities at wavelength j, and a is a coefficient. The first principal component corresponds to the maximum variability in the data, while the other components correspond to the residual variability (Miller and Miller 2010).

The PLS method is based on the linear transition from a large number of original descriptors (e.g., peak intensities) to a new variable space based on a small number of orthogonal factors (latent variables). The factors are mutually independent linear combinations of original descriptors.

PLS regression is used for building quantitative models, for example predicting specific concentrations from the measured spectra. In PLS, the average performance of the calibration is usually estimated through cross-validation. The method is evaluated based on the cross-validation parameter R^2 and root mean square error (RMSE), defined:

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}\left(X_{\mathrm{obs},i} - X_{\mathrm{model},i}\right)^2}{n}}$$

where X_{obs} are the observed values and X_{model} are modeled values (Brereton 2007; Esposito Vinzi *et al.* 2010).

PCR is a regression analysis technique that uses the principal components provided by PCA. According to this technique, the outcome on a set of covariates is subject to regression based on a standard linear regression model. It uses PCA for estimating the unknown regression coefficients in the model.

The objective of CCSWA is to describe several data tables observed on the same n samples by recovering the maximum inertia (total variance) of each of them. It is used to investigate the relationships among various data tables and to extract latent variables from information of different nature (Mazerolles *et al.* 2006).

Multiway methods are heavily used in chemometric applications, and especially in the area of food analysis. Multiway data analysis refers to multivariate data analysis performed on data arrays which are three-way or even higher as opposed to two-way data. In our case, the fluorescence intensity and the wavelengths of excitation and emission constitute a classical three-way data array. The most popular multiway mathematical method applied is the PARAFAC (Christensen *et al.* 2006). More specifically, PARAFAC decomposes the fluorescence intensity and excitation and emission matrices presented in a three-way array into trilinear components according to the number of

fluorophores present in the samples. PARAFAC is a generalization of PCA to higher-order arrays, but some of the characteristics of the method are quite different from the ordinary two-way case (Bro 1997).

Moreover, various other methods can be used for the chemometric analysis of fluorescence spectra such as linear discriminant analysis (LDA; Sikorska *et al.* 2005), factorial discriminant analysis (FDA; Hammami *et al.* 2010), or K-nearest neighbors (KNN; Sikorska *et al.* 2005).

8.2.3 Applications in Foods and Drinks

The first use of fluorescence in food applications was more than 70 years ago (Thistle *et al.* 1943). However, the recent development of simple, accurate, and low-cost fluorometers, combined with computers, has provided the opportunity for fast, reliable, repeatable measurements and elaboration of the spectra with complicated chemometric techniques. Many fluorometric methods are therefore developed to check the authenticity, adulteration, quality, and composition of foods (Christensen *et al.* 2006; Karoui and Dufour 2008; Karoui and Blecker 2011). The most important studies for the basic categories of foods are described in the following sections.

8.2.3.1 Edible Oils

Edible oils are a category of components that are used by almost all the consumers. Olive oil in particular is an important ingredient in the diet of the Mediterranean and Balkan people and also constitutes a large part of their agricultural production with a big economical value. Virgin olive oil especially is highly appreciated all over the world because of its fine aroma, pleasant taste, and health benefits.

The storage of edible oils for a long time or under inappropriate conditions results in their deterioration, mainly through oxidation. Oxidation of edible oils reduces shelf life and nutritional value, while at the same time producing toxic compounds. Moreover, olive oil and especially virgin olive oil are expensive, so unscrupulous sellers adulterate them. Various analytical methods are used to detect adulteration of oils and especially olive oil. Most of them are based on the quantification of fatty acids, triglycerols, sterols, and hydrocarbons by gas chromatography (Andrikopoulos *et al.* 2001) or high-performance liquid chromatography (Tsimidou and Macrae 1987; Aparicio and Aparicio-Ruiz 2000).

Although analytical methodologies used in commercial and government laboratories cover almost all edible oil characterizations and adulteration detection, they are time-consuming and expensive. Fluorescence spectroscopy, in combination with multivariate statistical analyses, has been proven to be a promising screening method for predicting quality parameters and adulteration in edible oil samples (Wolfbeis and Leiner 1984; Sikorska *et al.* 2005; Poulli *et al.* 2006, 2009*a*). The fluorescence spectra of oils and especially of olive oil have been measured, and the different emission regions attributed to its components (Zandomeneghi *et al.* 2005). The emission spectra of edible oils excited at 350 nm have a region associated with pigments and tocopherols (342–357 nm); there is a region related to conjugated dienes (value of K232) and trienes (value of K270) as well as hydrolysis products (426–463 nm); a region around 501 nm

correlates with vitamin E; and a region exists related to chlorophylls and pheophytins (665–685 nm) which is very intense at virgin olive oil (Sayago *et al.* 2004). Similar results were obtained by total luminescence spectra (Poulli 2009).

There is a very popular test (but not secure) to discriminate virgin olive oil from other oils based on the fluorescence of chlorophylls and pheophytins. By shining a green pen laser through a clear glass bottle containing the oil, if the laser light appears orange or red as it passes through the oil it is extra virgin olive oil. If the laser light remains green as it passes through the oil, it is has been adulterated.

Emission spectra with a laser excitation at 370 nm (Mbesse Kongbonga *et al.* 2011), but also at 532 nm (Mu *et al.* 2013), have been used to detect some refined and unrefined oils and also to detect in some cases the deterioration of oils under heating. Kyriakidis and Skarkalis (2000) used an excitation wavelength of 360 nm to monitor and differentiate edible vegetable oils (olive oil, olive residue oil, refined olive oil, corn oil, soybean oil, sunflower oil, and cotton oil). Furthermore, using excitation/emission fluorescence matrices and two multiway methods – unfold PCA and PARAFAC– Guimet *et al.* (2004) managed to distinguish between virgin olive oils and pure olive oils.

As an alternative to the Folin–Ciocalteu assay (F-C) Papoti and Tsimidou (2009) proposed a fluorimetric determination with excitation/emission wavelengths set at 280/320 nm of the total phenol content in virgin olive oil, olive fruit, and leaf polar extracts.

During the last decade, instrumental improvements have allowed simple and fast recording of SSFS and total luminescence spectra, and therefore the ability to use them in analytical applications. Poulli *et al.* (2005) used SSFS combined with multi-dimensional chemometric techniques for classification of virgin olive oils according to their quality. In addition, based also on SSFS and using a non-parametrical KNN method and LDA, Sikorska *et al.* (2005) managed to differentiate edible oils including soybean, sunflower, rapeseed, peanut, olive, grape seed, linseed, and corn oils.

Single-emission spectra can be used for the detection of olive oil adulteration with hazelnut oil. Sayago *et al.* (2004, 2007) and Poulli *et al.* (2007) demonstrated the ability of SSFS in combination with a partial least squares regression model for quantitative determination of adulteration of virgin olive oil with olive-pomace, corn, sunflower, rapeseed, soybean, and walnut oil at levels as low as 2.6%, 3.8%, 4.3%, 4.2%, 3.6%, and 13.8% (w/w), respectively. In a more detailed study, the same group (Poulli *et al.* 2006) used 136 mixtures of 15 different virgin olive oil samples adulterated with sunflower oil at varying levels (0.5–95%) and detected adulteration down to 3.4% (w/v). They used SSFS with 80 nm wavelength interval and PLSR analysis. Dankowska and Małecka (2009) applied a similar methodology for quantitative determination of adulteration of extra virgin olive oil with olive oil at levels down to 8.9% and 8.4%. The measurements were acquired at 60 and 80 nm wavelength interval at the excitation wavelength 350 and 302 nm, respectively. Ge *et al.* (2014) applied the same methodology for the quantification of adulteration of walnut oil with sunflower oil. By using SSFS with an 80 nm wavelength interval and (PLSR) analysis, adulterations at levels down to 0.3% (v/v) were detected.

The potential of fluorescence spectroscopy to monitor olive oil oxidation under thermal and UV stress has been demonstrated by Poulli *et al.* (2009*a*). In this study spectra acquired in the range 300–500 nm during 12 h accelerated oxidation stress reveal five different classes after PCA. Linear correlations between parameter values versus exposure time were observed during extra virgin olive oil deterioration under UV at different

temperatures. The same group (Poulli *et al.* 2009*b*) studied the deterioration under heating at 100, 150, and 190°C of edible vegetable oils (extra virgin olive, olive, pomace, sesame, corn, sunflower, soybean oils, and a commercial blend of oils). They used SSFS obtained at an 80 nm wavelength interval, combined with PCA. Engelsen (1997) monitored the physicochemical changes that occur in oil during an industrial frying operation, using several spectroscopic techniques including total luminescence. Tena *et al.* (2012) studied the thermoxidation process of virgin olive oils using total luminescence spectra. The emission profile obtained from three-factor PARAFAC model allowed the extraction of specific information about phenols, pigments, and oxidation products, respectively. Similar results were obtained by Cheikhousman *et al.* (2005). The variation of the fluorescent characteristics of rapeseed oil during thermal oxidation was studied by Sun *et al.* (2013) using the PARAFAC method and two-dimensional correlation technique.

In a study of the efficiency of Nigella seed extract as natural antioxidant (Faten *et al.* 2012), the degradation of sunflower oil during accelerated oxidation at 120 and 140°C was monitored by FFFS. Contour diagrams of time-resolved fluorescence intensities were recently used as unique fingerprints for the characterization of peanut oils (Mu *et al.* 2014).

8.2.3.2 Meat and Meat Products

The use of fluorescent spectroscopy for the evaluation of meat products is based on the measurements of fluorescence from collagen, in connective and adipose tissues, as well as protein.

Fluorescence for quality control of meat and fish products was first proposed in 1986; this patented method was based on excitation at 340 nm. Under this excitation, pure meat gives only weak fluorescence signals whereas bone, cartilage, connective tissues, and fat give a strong signal with a maximum at 390 nm and a shoulder at 475 nm for the fat or 455 nm for the bone, cartilage, and connective tissue (Jensen *et al.* 1986).

Swatland and his group were the pioneers in the use of fluorescence for meat quality control, with a series of papers starting in 1987 (Swatland 1987). Based on the fluorescence spectra of tissues, they managed to predict taste (Swatland *et al.* 1995*a*), beef chewiness (Swatland *et al.* 1995*b*), juiciness, hardness, and palatability (Swatland 2006). These predictions are based on the amount and distribution of connective and adipose tissue in the meat. Following this methodology, numerous works used the fluorescence spectroscopy to correlate autofluorescence signals of various meat samples to several characteristics, for example collagen content in meat batter (Egelandsdal *et al.* 2005) or sensory-related quality parameters such as tenderness (Frencia *et al.* 2003).

Møller *et al.* (2003) used fluorescence spectra of raw and salted (3 months), matured (11 and 12 months), plus aged (15 and 18 months) Parma hams produced from two muscles (semi-membranosus and biceps femoris) to monitor major chemical/physical changes during processing and to predict their age. By employing a five-component PARAFAC model of a three-dimensional data array (sample × emission × excitation) a good prediction of the age was achieved, with a relative error of approximately 1 month. Moisture content, sensory and instrumental color, and proteolysis value were also predicted.

Veberg *et al.* (2006) used FFFS to detect early lipid oxidation in freeze-stored minced turkey meat. The fluorescence data were highly correlated to specific markers of lipid

oxidation such as 1-penten-3-ol and hexanal, as well as 2-thiobarbituric acid reactive substances and sensory analysis of rancidity. Gatellier *et al.* (2007) confirmed the previous work using FFFS as a valuable index of lipid oxidation in freeze-stored chicken meat in air.

Constant-energy SSF was used to determine the quantity of benzo(a)pyrene, one of the most carcinogenic polycyclic aromatic hydrocarbons, in roasted, smoked, and charcoal-grilled foods. Using the second derivative of constant-energy SSF, meat, fish, and some other food samples were analyzed and the quantity of benzo(a)pyrene was determined with a recovery of 97.7% (Li *et al.* 2011). In another work, the use of SSFS coupled with chemometrics was investigated for the determination of heterocyclic aromatic amines in cooked meat samples. The three-dimensional SSFS dataset was analyzed using PARAFAC analysis and N-PLS regression method. The results obtained showed good correlation between the spectral and analytical data (Sahar *et al.* 2010).

The SSFS combined with chemometric methods was examined for the determination of microbial load on aerobically stored chicken breast fillets. SSFS spectra were recorded in an excitation wavelength range of 250–500 nm with $\Delta\lambda$ of 20–180 nm and analyzed with the PARAFAC and PLSDA (partial least square discriminant analysis) methods (Sahar *et al.* 2011). Oto *et al.* (2013) investigated the potential of fluorescence spectroscopy for the non-destructive evaluation of ATP content and plate count on pork meat surface stored aerobically at 15°C for 3 days. Based in the change of tryptophan and NADPH fluorescence with the growth of microorganisms, microbial spoilage on meat could be detected. Using PLSR analysis, ATP content and plate count were predicted with good determination coefficient. Aït-Kaddour *et al.* (2011) investigated the potential of portable spectrofluorimeters for the determination of the microbial spoilage of minced beef. Fluorescence spectra obtained for different excitation (280, 320, and 380 nm) yielded intensity variations that could relate to biochemical changes occurring during meat spoilage. A chemometric method (PLS-R) was used to correlate microbiological growth with fluorescence spectra and predict the spoilage level of meat product.

8.2.3.3 Fish and Fish Products

Fluorescence spectroscopy has also been exploited for providing spectral signatures in a short time that can be used as fingerprints of fishes and fish products.

Davis and Reece (1982) found that two fluorescent emissions develop in fish muscle during frozen storage, and suggested the use of fluorescence for evaluation of the frozen fish.

Andersen and Wold (2003) studied the fluorescence of salmon and cod muscle in comparison with the fluorescence of collagen type I and type V. The excitation wavelength was 332 nm and emission peaks obtained around 390, 430, and 480 nm for both fish muscle and collagen. They failed to predict texture and gaping score, but demonstrated that fluorescence of fish muscle could be related to the storage time.

In a more detailed study, the research team of Dufour *et al.* (2003) investigated the use of fish muscle fluorescence for the monitoring of the fish freshness. They recorded the aromatic-amino- and nucleic acids, as well as its tryptophan and NADH emission spectra, on cod, mackerel, salmon, and whiting fillets. For all three spectra, some differences according to storage time appeared, allowing evaluation of fish freshness. The best results were obtained for the first spectra. Karoui *et al.* (2006*b*) later used the same

methodology to differentiate frozen–thawed from fresh fish. They recorded the emission spectra of tryptophan and NADH and multicomponent analysis was also applied. The best results were obtained from the NADH emission spectra, since 100% correct classifications for the calibration and validation spectra were achieved.

In the study of Olsen *et al.* (2006), FFFS was used to detect early lipid oxidation in salmon pâté. The pâté was enriched with cod liver oil to increase the n-3 content, and metal chelators (citric acid or calcium disodium ethylenediamine tetraacetate) were also added. The results showed that fluorescence spectroscopy can be used for monitoring lipid oxidation in salmon pâté from an early stage.

In a preliminary investigation performed by Pedersen *et al.* (2002) concerning fish oils, total fluorescence spectroscopy combined with three-way chemometric methods was used in an attempt to determine the level of dioxins and polychlorinated biphenyls (PCBs) contamination. The results showed that by using appropriate chemometric models a dioxin determination with prediction errors below 1 ng kg^{-1} can be achieved.

Liu *et al.* (2012) used the SSFS for the determination of pyrene in the gills of exposed fish. They used a $\Delta\lambda$ of 50 nm and a peak due to pyrene was observed at an excitation around 334 nm, allowing the determination of pyrene in the gills of fish. Li *et al.* (2011) also used the second-derivative of constant-energy SSFS for the determination of the quantity of benzo(*a*)pyrene in roasted, smoked, and charcoal-grilled fishes.

8.2.3.4 Eggs

The first use of fluorescence for the quality-control testing of dried egg powder was developed During World War II, to check the egg powder shipped to Britain (Thistle *et al.* 1943). Posudin (1998) recorded the emission spectra of different eggs after excitation at 405 nm. The spectra display two maxima at 635 and 672 nm. These preliminary results lead to the conclusion that fluorescence spectroscopy could be a promising approach for quantitative estimation of porphyrin in eggs, and thus determine egg freshness.

The group of Karoui *et al.* (2006*c*, *d*) showed the ability to detect the egg freshness by FFFS by recording fluorescent Maillard reaction products on thick and thin albumens (excitation, 360 nm; emission, 380–580 nm) and vitamin A scanned on egg yolk (emission, 410 nm; excitation, 270–350 nm). FDA was used to classify the eggs according to their date after being laid.

8.2.3.5 Caviar

Airado-Rodríguez *et al.* (2010) used FFFS to measure the quality of cod caviar paste. Multivariate curve resolution was employed to decompose the overall fluorescence spectra into pure fluorescent components and calculate the relative concentrations of protoporphyrin IX, photoprotoporphyrin, and fluorescent oxidation products in the different samples.

8.2.3.6 Milk and Cheese Products

Milk and dairy products contain several intrinsic fluorophores such as aromatic amino and nucleic acids, vitamins, NADH, and chlorophyll. A series of information about milk can be extracted from chemometric analysis of the emission spectra of Tryptophan, aromatic amino and nucleic acid, and riboflavin (excitation at 290 nm,

250 nm, and 380 nm, respectively), as well as the excitation spectra of vitamin A (emission at 410 nm).

Karoui *et al.* (2005*a*) determined the geographic origin (lowland, mid-mountain, and mountain) of milks from the Haute-Loire department (France). Hammami *et al.* (2010) also used these spectra coupled with FDA technique to identify sheep milk originating from different feeding systems (corn, soy bean, and scotch bean). Similar results were also obtained by Rouissi *et al.* (2008). In another work, a good discrimination between milk samples collected from Comisana and those produced from Sicilo-Sarde ewes was achieved by Zaïdi *et al.* (2008).

Fluorescence spectroscopy can also be used for measuring chemical and physical changes in dairy products caused by processing and storage (Andersen and Mortensen, 2008). Dufour and Riaublanc (1997) applied PCA to fluorescence spectra of raw, heated (70°C for 20 minutes), homogenized, and homogenized and heated milks. A good discrimination between samples as a function of homogenization and heat treatment applied to milk samples was observed. Similar results were obtained by the same group in subsequent work by Kulmyrzaev *et al.* (2005). Birlouez-Aragon *et al.* (1998, 2002) proposed and patented (Birlouez-Aragon 1998) a rapid and sensitive fluorimetric method to evaluate the heat treatment of commercial milk. They named it FAST (fluorescence of advanced Maillard products and soluble tryptophan). This method is based on the fluorimetric measurement of the transparent soluble fraction of milk at pH 4.6, first at 290 nm/340 nm and then at 350 nm/440 nm as maxima excitation/emission wavelengths of Tryptophan and the advanced Maillard products, respectively. Guan *et al.* (2005) used the FAST method for the determination of skim milk powder adulteration in fresh milk. The FAST method was used in many cases for the detection of Maillard products (e.g. Damjanovic Desic and Birlouez-Aragon 2011). In another work, Schamberger and Labuza (2006) heated milks at higher temperatures (up to 140°C) to assess the Maillard browning by FFFS and showed that this technique has the potential to be of use as an online instrument for monitoring and control of thermal processing of milk. Similar results were found by Liu and Metzger (2007) who used PCA to analyze the spectra of nine samples of non-fat dry milk from three producers and stored at different temperatures (4, 22, 35, and 50°C) for 8 weeks. A novel rapid and non-destructive FFFS method to quantify furosine and lactulose in heat-treated milk was developed by Kulmyrzaev and Dufour (2002). They applied PCA to recorded excitation and emission spectra of ultra-high-temperature processed (UHT) milks, overheated UHT, pasteurized milks, and mixtures of them. Furosine and lactulose can be used as indicators of heat damage of milk and for distinction of UHT milk, pasteurized milk, and in-container sterilized milk. Lately, using total luminescence spectra and by modeling the data with PARAFAC, it was possible to separate the samples according to the variations in heat treatment (Hougaard *et al.* 2013).

Boubellouta and Dufour (2008) showed the potential of SSFS in the characterization at the molecular level of milk changes during mild heating from 4 to 50°C and acidification in the pH range of 6.8 to 5.1. Similarly, they used SSFS to examine the structure and rheology characteristics of Comté (hard) and Raclette (semi-hard) cheeses as a function of temperature (Boubellouta and Dufour 2012).

Karoui *et al.* (2005*b*) showed that FFFS is efficient for the geographic determination of Gruyère cheeses. They used a similar technique for the detection of geographic origin of milks (Karoui *et al.* 2005*a*). In a preliminary study, Abbas *et al.* (2012)

demonstrated the potential of SSFS to predict some physicochemical parameters (ash and protein) of heterogeneous and anisotropic matrix such as Blue and Fourme cheeses. Similarly, Karoui *et al.* (2008) evaluated the usefulness of FFFS to predict some chemical parameters and the melting point of semi-hard and hard cheeses. A similar methodology was used by Purna *et al.* (2005) to predict processed cheese meltability. Karoui *et al.* (2007*a*) used the 400–640 nm emission fluorescence spectra to monitor the changes in spectra and oxidation of 24 semi-hard cheeses, confirming previous works by Dufour *et al.* (2000) and Mazerolles *et al.* (2001). In another study, Andersen *et al.* (2005) used total fluorescence spectroscopy to evaluate the light-induced oxidation of semi-hard cheese. Herbert *et al.* (2000) by applying PCA and FDA to the emission spectra of tryptophan and the excitation spectra of vitamin A managed to detect the identity and the structure of soft cheeses.

Christensen *et al.* (2005*a*) used PARAFAC analysis and total fluorescence spectroscopy (excitation wavelengths 270–550 nm and emission wavelengths 310–590 nm) in the evaluation of yogurt during storage over a period of 5 weeks at 4°C. The same group used a similar method to monitor the stability of processed cheese (Christensen *et al.* 2003).

8.2.3.7 Cereals

The principal contributions to cereal flour fluorescence are from amino acids, ferulic acid, riboflavin, vitamins, and carotenoids. The first study concerning fluorescence study in cereals was published in 1982. Jensen *et al.* (1982) described a method for quantifying pericarp, aleurone, and endosperm in wheat milling fractions by their autofluorescence characteristics. Fluorescence data from wheat fractions were evaluated by the PLS method, initially calibrated against manually dissected botanical parts and synthesized mixtures with known compositions. In a similar study the same laboratory team (Kissmeyer-Nielsen *et al.* 1985) used the same method to quantify the botanical composition of rye and rye milling fractions. The classification was based on aromatic amino acids (excitation 275 nm, emission maxima at 335 nm), ferulic acid (excitation 350 nm, emission maxima at 420 nm), and a compound (excitation 420 nm, emission maxima at 520 nm), the latter being attributed to rivoflavin (Zandomeneghi *et al.* 2003).

Symons and Dexter (1994) showed that aleurone and pericarp fluorescence measurement can be useful for the determination of flour refinement and diverse intermediate. These results can be used for monitoring wheat flour refinement and milling efficiency.

Zandomeneghi (1999) demonstrated the possibility of recognizing the different species of cereals, even of different cultivars, by FFFS. In another study (Ram *et al.* 2004), emission spectra (370–670 nm) were recorded after excitation at 350 nm to identify red and white wheat cultivars. In a later work, Karoui *et al.* (2006*a*) confirmed the potential of intrinsic fluorescence spectroscopy to differentiate between processed grains (flours, pasta, and semolinas) of different wheat cereal products.

Recently, Zeković *et al.* (2012) classified intact cereal flours (wheat, corn, rye, buckwheat, rice, and barley) by SSFS combined with PCA and PLS analysis. Discrimination and classification were possible at low intervals of 20 nm or less, and the best results were achieved with a combination of 7 and 20 nm intervals.

8.2.3.8 Honey, Sugar, and Syrups

Honey composition is closely associated with its botanical origin, which is closely related to the geographical area of its origin; soil and climate characteristics determine melliferous flora as well as the presence of different minerals arising from soil, dust, etc.

The group of Ruoff and Karoui (Ruoff *et al.* 2005, 2006; Karoui *et al.* 2007*b*) used the front-face fluorescence spectroscopy as a tool for the classification of honey samples according to their botanical origins. In their last work honey (62 samples from Switzerland), fluorescence spectra from seven floral origins, namely acacia, alpine rose, chestnut, rape, honeydew, alpine polyfloral, and lowland polyfloral, were recorded. They used three sets of excitation spectra (excitation 250 nm and emission 280–480 nm; excitation 290 nm and emission 305–500 nm; and excitation 373 nm and emission 380–600 nm) and a set of emission spectra (emission 450 nm, excitation 290–440 nm). By using subsequent PCA and FDA, a correct classification of 100% for the calibration and 90% for the validation samples was achieved. Sergiel *et al.* (2014) recently distinguished honey samples due to their different botanical origin by the simple and fast analysis of their phenolic fraction separated by means of solid phase extraction and detected by using total SSFS. SSFS was also applied to differentiate between pure honey and sugar syrup samples. The ratio of the intensity of SSFS (with a $\Delta\lambda$ 40 nm) at 365 nm to that of 435 nm could be used to monitor adulteration of honey with cane sugar syrup (Ghosh *et al.* 2005). In another study, the adulteration of honey with rice syrup was detected using total fluorescence spectra combined with chemometric methods (Zhao *et al.* 2013).

Concerning sugar, it is important to note that approximately 99.999% of the sugar is sucrose, which is not fluorescent. It is the very small fraction of impurities such as amino acids, phenols, and their reaction products that are detected by the fluorometer. It is therefore the complex mixture of a very small amount of natural and process-related components that are being measured and provide information concerning the origin and process of the sugar.

The first report concerning the sugar fluorescence spectra was published in 1972 (Carpenter and Wall 1972). In 1995, Nørgaard (1995) performed multivariate chemometric analysis of beet sugars fluorescence spectra and showed that it is possible to classify white sugar samples according to factory and to predict quality parameters such as ash, amino-nitrogen, and color. In a following study (Baunsgaard *et al.* 2000*a*), the same group confirmed these results. Bro (1999) applied the multiway chemometric model PARAFAC to the total fluorescence spectra of 268 beet sugar samples from a sugar plant in Scandinavia to resolve the profiles of the fluorophores.

In a detailed investigation of impurities in various sugar juices, chemometrics was applied for the evaluation of their fluorescence spectra. PARAFAC revealed four fluorophores that were responsible for the main fluorescence of beet sugar solutions. Two of the components were assigned to tyrosine and tryptophan originating from the beet (Munck *et al.* 1998). PARAFAC was also applied in a fluorescence study of 47 raw cane sugar samples representing a varied selection. Three individual fluorophores with 275/350 nm, 340/420 nm, and 390/460 nm as their approximate excitation/emission maxima were found. The 275/350 nm fluorophore is considered as a color precursor to the color developed during storage and the 340/420 nm and 390/460 nm fluorophores show colorant polymer characteristics (Baunsgaard *et al.* 2000*b*).

The quality class of sugar is determined according to European standards in which the degree of coloration is a main parameter; there is therefore a strong economical motivation to minimize the formation of color during the process. For this reason, Andersson *et al.* (1997) used total luminescence spectra from 47 samples of intermediary sugar products (thick juices) prior to the final heating stages and combined with them with PCA to obtain information concerning the process. Orzel *et al.* (2012) recently developed a robust calibration technique for the fluorescence spectra to be used for monitoring sugar production.

Recently, Clement *et al.* (2010) used fluorescence spectroscopy to assess the physicochemistry and typicity of maple syrup. They found two major regions of fluorescence, which were mostly the same for sap and syrup. The first was at 320 nm, excited at 275 nm, and the second one at 460 nm, excited at 360 (syrup) or 370 nm (sap). The first peak diminishes as harvesting season progresses while the second peak increases, making it possible to predict the harvesting period of syrup from its spectra. The color of syrup and bacterial counts in sap were also predicted from syrup spectra. Results show that sap spectra are related to syrup spectra and could potentially be used as a predictor of quality prior to transformation. The same group (Panneton *et al.* 2013) investigated the potential of fluorescence spectroscopy for the characterization of maple syrup flavors. Their preliminary results showed that the fluorescence spectra can be used to describe the four main flavors (maple, confectionery, empyreumatic, and woody) as well as off-flavor of maple syrup.

8.2.3.9 Fruits

One of the first uses of fluorescence for checking the quality of fruits was in 1988 by Steiner *et al.* (1988) when it was discovered that figs contaminated with afalatoxins appear a bright greenish-yellow fluorescence if they illuminated with UV light (365 nm).

Chlorophyll fluorescence has been used as a measure of chloroplast function and indirectly the physiological status of whole plants and plant organs. Chlorophyll fluorescence declines with ripening, probably due to chlorophyll loss and a loss in chloroplast competence. These assumptions allow the use of chlorophyll fluorescence to detect differences in maturity, ripeness, or senescence of fruits (DeEll *et al.* 1995; Song *et al.* 1997). Similar results were also obtained for golden papaya fruit (Bron *et al.* 2004).

Fluorescence spectra of apple juices were recorded with excitations at 265 and 315 nm. The data were analyzed by chemometric methods (PCA, PLS, SIMCA), and classification of the juices according to the variety and maturity of the apples was achieved. Based on this result, a possible correlation between picking date and the traditional harvest indices was achieved (Seiden *et al.* 1996).

The chlorophyll fluorescence kinetics of apples stored under different conditions to induce mealiness were recently measured. Three levels of mealiness were assigned to three different storage conditions according to a protocol established in an EU project (Moshou *et al.* 2005).

8.2.3.10 Alcoholic Beverages

Wines with origin identification are generally higher priced and bring in a higher profit to the producers than other wines. Concerning consumers, drink authenticity is a guarantee of safety and quality.

The fluorescence contribution of both monomeric and polymeric anthocyanins was studied by comparing their emission spectra to monitor the evolution of anthocyanin derivatives and to distinguish them from their anthocyanin precursors. These results allowed the evolution of anthocyanin pigments during wine aging to be monitored, but may also be useful to determine age markers or even geographical markers (Agati *et al.* 2013). Wan *et al.* (2012) used PCA to differentiate and evaluate the character parameters of the total fluorescence spectra of Jiangxi wines. Their results showed that there was definite different information among the wine samples from different manufacturers. In another study Airado-Rodríguez *et al.* (2009) recorded the total fluorescence spectra of 57 red wine samples from different origins. When the PARAFAC score values were plotted against each other, they discriminated the wines according to origin (country) and grape variety. Similarly, in a preliminary study concerning 42 wines produced in the geographic origin of Changli country, Yin *et al.* (2009) showed that the PCA performed on their total luminescence spectra allowed a good discrimination of wine samples. Dufour *et al.* (2006) demonstrated the implementation of FFFS along with chemometric methods for the discrimination of different wines according to variety, typicality, and vintage. A number of 120 wines of French and German origin were examined. Emission (275–450 nm) and excitation (250–350 nm) fluorescence spectra of phenolic compounds were recorded straight on the wine samples. The evaluation of wine spectra was performed using PCA and FDA. A good discrimination among French and German wines was accomplished by performing PCA on the excitation spectra and, by performing FDA of the emission spectra, correct classification of typical and non-typical Beaujolais at a rate of 95% was observed (Dufour *et al.* 2006).

In an effort to evaluate wine samples according to their appellation and ageing condition, Airado-Rodríguez *et al.* (2011) used FFFS combined with PARAFAC. Total luminescence spectra of untreated wine samples were obtained at 245–345 and 300–500 nm for excitation and emission, respectively. First of all, PARAFAC was applied for exploratory analysis and the four obtained components were assigned to different fluorophores present in the wine samples according to bibliographic data. It was proved that further interpretation of PARAFAC-score values allows the discrimination and clustering of wine samples that belong to different appellations. When plotting the scores of the second PARAFAC component against the third, a good separation between Rioja and Ribera del Guadiana samples was therefore accomplished. Furthermore, the effect of ageing was tested using the same technique. Consequently, an improved discrimination between Rioja and non-Rioja samples for crianza and reserva wines compared to young wines was established (Airado-Rodríguez *et al.* 2011).

Sádecká *et al.* (2009) used total luminescence and SSFS recording followed by PCA and HCA to differentiate brandies from wine distillates. Both PCA and HCA carried out on the emission spectra (360–650 nm) recorded at excitation wavelength 350 nm and SSFS (200–700 nm) collected at $\Delta\lambda = 90$ nm. The results indicate that fluorescence spectroscopy offers an efficient approach for the authentication of brandies. Using a similar methodology, Uríčková *et al.* (2013) differentiated brandies from mixed wine spirits. The studies were performed on 16 brandies from 3 different producers and 30 mixed wine spirits from 5 different producers. The same technique can be used to discriminate brandy, whisky, slivovice, and juniper drink (Tóthová *et al.* 2008).

The study of Sikorska *et al.* (2004) demonstrates the ability of SSFS to characterize and differentiate beers. The total luminescence and SSFS exhibit a relatively intense

short-wavelength emission ascribed to aromatic amino acids, and a less intense emission in the long-wavelength region which may originate from B vitamins. Classification of beers based on their SSFS was performed using non-parametrical KNN method and LDA. Very good discrimination was obtained by both methods with a low classification error. The same group (Sikorska *et al.* 2006) used total luminescence and SSFS in combination with multivariate data analysis to monitor changes in beer during storage. PCA of SSFS showed clear clustering of samples according to storage conditions. Successful classification of differently stored samples was accomplished using both the nearest-neighbor method and LDA. Additionally, the same group used PLS to develop calibration models between SSFS and excitation/emission matrices of beers on one hand, and analytical concentrations of riboflavin and aromatic amino acids on the other (Sikorska *et al.* 2008). They used the same technique to quantify vitamin B_2.

In a preliminary study Christensen *et al.* (2005*b*) documented that both autofluorescence spectra and europium induced delayed fluorescence spectra by the aid of multivariate modeling, demonstrating the potential to predict bitterness in beers.

Callejon *et al.* (2012) recorded the total fluorescence spectra of 19 sherry vinegars from 14 different producers. By applying the PARAFAC analysis to their data, adequate classification according to the ageing time in wood barrels was achieved.

8.2.4 Conclusions and Perspectives

This chapter has described applications of fluorescence spectroscopy for the authentication of food. During the past 30 years, various applications of fluorescence spectroscopy for food authentication have been developed. The fluorophore contents of foods show characteristic spectra that can be evaluated with chemometric methods to provide information about the origin, quality, processing, and even bacterial counts or presence of toxic contaminants. The forthcoming challenge is the construction of large data banks in order to be used in qualitative and quantitative analysis as well as the implementation in routine control. Additionally, the development of reliable and low-cost instruments will allow the fluorescence spectroscopy to be used as an *in situ* test, in industry and in commerce.

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9

Molecular Techniques – Genomics and Proteomics

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9.1 Introduction

The need for complete and truthful information about the food products we eat is a primary request from consumers. In recent years many regulations have been introduced in order to avoid food adulteration, mislabeling, and fraud (see Chapter 1). For these reasons, the authentication of food products must be guaranteed; accurate and reliable analytical methods are therefore needed in order to verify that the components used in a food product are of the nature or quality declared by the seller. Many different instrumental techniques have been proposed for food authentication, and even though traditional methods are being extensively used, the use of molecular tools for the definition of molecular targets such as DNA-based and proteomics techniques is complementing existing methodologies. Today, most of the DNA- and protein-based methods for food authentication are related to species detection and identification. The either inadvertent or deliberate substitution of an appreciated high-quality species for another of lower quality and with a lower price is especially frequent (Pascoal *et al.* 2008*c*). In these cases, this adulteration leads to mislabeling and commercial fraud. Another main focus of food authenticity is the determination of the origin of the food, tracing back the primary product to a certain region, with special interest in the protection of products of certified origin (PDO and PGI) and the determination of plant cultivars. Furthermore, the monitoring of the geographical origin and populations of animal species used for food production is important to avoid overexploitation of nature and protect endangered species. In addition, food authenticity also includes the detection of genetically modified organisms (GMO) and allergens, both requiring quantification to fulfill regulations established by the European Union.

The limitations of classical protein-based methods, such as electrophoretic and immunological assays, have been resolved with the introduction of methods based on DNA amplification and hybridization (Pascoal *et al.* 2008*a*). Since DNA is identical in

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all somatic cells of an organism, it is invariant whether the DNA is extracted from blood, muscle, liver, or any other tissue. Furthermore, the high stability of DNA allows the analysis in highly processed food products. Most DNA-based methods for food authentication consists of the highly specific amplification of DNA fragments by PCR. This technique presents a high potential due to its fastness, sensitivity, and specificity. Recently, due to recent advances in mass spectrometry (MS), proteomic tools have been proposed as fast, sensitive, and high-throughput means for authenticity assessment and traceability of food products (Piñeiro *et al.* 2003). Proteins can act as markers for many properties of the food products all along the food chain from farm to fork, and therefore proteomics can be applied for a systematic search of new marker proteins or peptides. After this first discovery step using reference samples, reliable analytical methodologies can be developed for the targeted detection and quantification of those previously identified markers in real unknown samples (Gallardo *et al.* 2013).

In this chapter we provide a comprehensive and updated overview of the molecular techniques that have been applied for the authentication of food components. In Section 9.2 DNA-based methodologies are described, and in Section 9.3 we focus on proteomics applications.

9.2 DNA-Based Methods

The authentication of food products by DNA-based techniques is mainly focused on the analysis of the composition of a certain food product and the detection of species substitution, as well as the mislabeling of the food products. Due to the high specificity of DNA, complex mixtures can be analyzed and closely related specimens differentiated.

Species identification in food of plant origin is mainly focused on the detection of allergens (Mafra *et al.* 2008). With the aim of detecting an allergenic protein in a food product, DNA-based methods have been developed, targeting any specific DNA gene of the corresponding plant species. Special effort has been made in the detection and quantification of low levels of the high allergenic hazelnut, peanut, and celery by real-time PCR-based methods (Stephan and Vieths 2004; Hupfer *et al.* 2007; Piknová *et al.* 2008). Recently, the simultaneous detection of 12 allergens by a multiplex real-time PCR assay has been developed (Köppel *et al.* 2012).

Quantification is also required in food authenticity when the food or feed product contains ingredients originating from a plant that may be genetically modified, such as cotton, maize, rapeseed, potato, soybean, etc. Genetically modified ingredients have to be labeled if the content exceeds 0.9% in the case of authorized GMOs and 0.5% in the case of non-authorized GMOs. Real-time PCR is the only valid method that is applied for the maintenance of GMO regulation in the EU and the general requirements and definitions, as well as validation of the analytical protocols, are established by the EU. Quantification of a GMO is based on the quantification of the GM-specific event sequence and the quantification of the endogenous reference sequence of the corresponding ingredient (Marmiroli *et al.* 2008). Besides event-specific assays, screening for GMO in food products is carried out by PCR targeting common promotor sequences, such as P-35S and T-NOS. Likewise, multiplex PCR assays have been described to detect various GMO events simultaneously and a commercial kit has been developed by Eppendorf Array Technologies, combining multiplex PCR and microarray hybridization

(DualChip®GMO). The application of DNA-based methods for GMO detection in food and feed products has been reviewed by Marmiroli *et al.* (2008).

Plant species identification by DNA-based methods has also been carried out for authentication of cereals, legumes, and olive oil (Mafra *et al.* 2008). Olive oil is a frequently adulterated food product and has been the focus of many studies aiming to determine the authenticity and absence of low-quality oils (Costa *et al.* 2012). The differentiation of several oil-producing plants that may be possible adulterants of olive oil has been successfully achieved by PCR single-strand conformation polymorphism (PCR-SSCP) targeting the plastid DNA genes *rbc*L and *trn*L (Spaniolas *et al.* 2010; Wu *et al.* 2011). Besides species identification, the varietal composition is of special interest for the olive oil producer and consumer and has been the focus of many studies, including a huge international project (OLIV-TRACK EU project) in which different olive cultivars were distinguished and identified by DNA-based methods (Agrimonti *et al.* 2011; Costa *et al.* 2012).

The study of genetic diversity at the intra-species level is of interest for food authenticity purposes where the determination of the geographic origin or a certain population is required. On one hand this is the case in food products of certified origin (POD, PGI; Nicoloso *et al.* 2013). The determination of the geographical origin by specific plant DNA-profiles has been applied to PDO and PGI honey (Laube *et al.* 2010). Likewise, the presence of the four required durum wheat cultivars in PDO Altamura bread has been verified by a microsatellite-based PCR approach (Pasqualone *et al.* 2010). On the other hand, food authentication aims at the registration of the location of seafood capture to control overfishing and monitor the illegal trade of protected and endangered stocks. For example, geographical populations of the black tiger shrimp have been extensively studied by DNA-based methods (Li *et al.* 2007; Khamnamtong *et al.* 2009; Mandal *et al.* 2012). Likewise, genetic stock identification of chum salmon has been carried out (Moriya *et al.* 2007).

Nevertheless, the application of DNA-based methods to food authenticity is mainly focused on the discrimination of animal species of commercial interest, to avoid the fraudulent addition of low-price species. The identification of animal species in the food sector by DNA-based methods has been extensively reviewed for meat products originating from domestic animals (Ballin 2010; Montowska and Pospiech 2010), meat of game animals (Fajardo *et al.* 2010), seafood (Rasmussen Hellberg *et al.* 2010), and dairy products (De La Fuente and Juárez 2005).

For authenticity purposes of food products, nuclear, mitochondrial, and plastid DNA have been targeted; the genes 12S rRNA and 16S rRNA of ribosomal DNA, the cytochrome B gene (cytb) and cytochrome c oxidase I gene (COI) are used most often for the discrimination of animal species. Ribosomal and mitochondrial DNA is present in high copy numbers that increase the sensitivity and lower the detection limit of the analysis. Furthermore, these genes exhibit a high inter-specific variability, but also conserved regions at intra-specific level, enabling the amplification of regions that are common to a large group of specimens. In contrast, nuclear genes exhibit a higher inter- and intra-specific variability due to biological factors. However, they are the preferred target genes for quantification assays, since they are normally encoded in a low and stable copy number across the population of a species.

PCR-based methods can be divided into two groups: those that target many loci and those that amplify a single loci. In the first case, techniques such as randomly amplified

polymorphic DNA (RAPD) and simple sequence repeats (SSR) are included and fingerprint-like amplification patterns are obtained on the base of which discrimination can be carried out by comparison to reference samples. These methods have the advantage that they do not require *a priori* knowledge of the DNA sequences, but they cannot be applied to mixtures of different species and highly degraded DNA. In the second group, a specific biomarker DNA, the DNA sequence of which is known, is selected and amplified by the application of specific or universal primers. Afterwards, the amplification products are differentiated due to the variability in their DNA sequences and the presence of single-nucleotide polymorphisms (SNPs) by methods such as single-strand conformation polymorphism (SSCP), restriction fragment-length polymorphism (RFLP), or DNA hybridization to specific probes. The advantages of working with biomarker DNA are that an analysis of mixtures can be carried out, as well as real-time PCR that allows a quantitative assessment of adulteration in a food product. Table 9.1 gives an overview of DNA-based methods and some related studies carried out in food authentication. The different DNA-based approaches are discussed in more detail in the following sections.

9.2.1 Randomly Amplified Polymorphic DNA (RAPD)

The technique of RAPD is based on PCR amplification of random sequences in the corresponding specimen by using a set of arbitrary primers. As a result, every primer generates a large number of amplification fragments that are visualized by electrophoresis and exhibit a specific fingerprint. Species and strains are than distinguished by the presence or absence of characteristic bands. The methodology is rapid and simple; no previous knowledge of the DNA sequences is necessary and no further analytical steps are required. The methodology has been applied to seafood to differentiate groupers and wreck fish from fraudulent nile perch filets (Asensio *et al.* 2002) and four mussel species (Rego *et al.* 2002), as well as to discriminate 27 legume species obtained from 63 seed meal samples (Weder 2002). Likewise, the technique has been used to determine the origin of fresh meats (cattle, goat, sheep, camel, pork, wild swine, donkey, cat, dog, rabbit, and bear). The results showed high efficacy in identifying meat products of a single species, but is not as successful when analyzing mixtures of raw minced meat from sheep–pork, horse–beef or beef–sheep (Arslan *et al.* 2005), its main disadvantage. Furthermore, the reproducibility of the method requires a strict protocol with standardized conditions to carry out a comparison with reference fingerprints and high-quality DNA that is difficult to obtain from highly processed food products.

Consequently, the use of RAPD for species identification in food authenticity is less common, but the technique has been frequently applied to genetic diversity studies, such as the analysis of geographic populations of shrimp (Rajakumaran *et al.* 2013) and varietal tracing of olive oil (Martins-Lopes *et al.* 2008).

9.2.2 Simple Sequence Repeats (SSR)

Simple sequence repeats (SSR), also called short tandem repeats (STR) or microsatellites, are polymorphic loci in nuclear DNA that consist of tandem repeats of a few nucleotides and are amplified by PCR resulting in specific fingerprints. The methodology setup is labor-intensive and requires statistical analysis. However, it is one of the most suitable DNA-based tools for the authenticity of food products with a special

Table 9.1 Summary of the DNA-based methods applied to food authentication.

Technique	Reference
RAPD	
Meat	Saez *et al.* 2004; Arslan *et al.* 2005
Paté	Calvo *et al.* 2001
Marine mammals	Martinez and Daníelsdóttir 2000
Legumes	Weder 2002
Fish	Asensio *et al.* 2002
Mussels	Rego *et al.* 2002
Olive oil	Martins-Lopes *et al.* 2008
SSR	
Meat	Orrú *et al.* 2006; Conyers *et al.* 2012
Bread	Pasqualone *et al.* 2010
Rice	Minh and Rakshit 2009
Olive Oil	Vietina *et al.* 2011; Pérez-Jiménez *et al.* 2013
FINS	
Meat	Girish *et al.* 2004; Karlsson and Holmlund 2007; Kitano *et al.* 2007; Lago *et al.* 2011
Fish	Sotelo *et al.* 2001; Jérôme *et al.* 2003; Pérez *et al.* 2005; Blanco *et al.* 2008; Pedrosa-Gerasmio *et al.* 2012
Other seafood	Chapela *et al.* 2002; Chapela and Sotela 2003; Santaclara *et al.* 2006
Species specific primers	
Meat	Rodríguez *et al.* 2004
Fish	Asensio 2008; Rasmussen Hellberg *et al.* 2010
Other seafood	Bendezu *et al.* 2005; Pascoal *et al.* 2011
Dairy products	Mafra *et al.* 2007; Gonçalves *et al.* 2012
Rice	Minh and Rakshit 2009
Real time PCR	
Meat of domestic animals	Laube *et al.* 2007; Jonker *et al.* 2008; Cammà *et al.* 2012; Kesmen *et al.* 2012; Ulca *et al.* 2013
Meat of game animals	Fajardo *et al.* 2008; Santos *et al.* 2012
Fish	Hird *et al.* 2005; Itoi *et al.* 2005; Lopez and Pardo 2005; Herrero *et al.* 2010; Rasmussen Hellberg *et al.* 2010; Chuang *et al.* 2012; Velasco *et al.* 2013
Milk	López-Calleja *et al.* 2007
Cheese	Lopparelli *et al.* 2007; Dalmasso *et al.* 2011
Honey	Laube *et al.* 2010
Basmati rice	Lopez 2008

(*Continued*)

Table 9.1 (Continued)

Technique	Reference
SSCP	
Fish	Céspedes *et al.* 1999; Rehbein *et al.* 2002; Rehbein 2005; Schiefenhövel and Rehbein 2013
Other seafood	Fernández *et al.* 2002; Khamnamtong *et al.* 2005
Olive oil	Spaniolas *et al.* 2010; Wu *et al.* 2011
PCR-RFLP	
Meat	Partis *et al.* 2000; Girish *et al.* 2007
Meat of game animals	Fajardo *et al.* 2006
Fish	Rehbein *et al.* 2002; Calo-Mata *et al.* 2003; Perez *et al.* 2004; Dooley *et al.* 2005
Other seafood	Chapela *et al.* 2003; Khamnamtong *et al.* 2005; Santaclara *et al.* 2006, 2007; Pascoal *et al.* 2008*c*, 2012
Coffee	Spaniolas *et al.* 2006
Microarray	
Meat	Peter *et al.* 2004; Teletchea *et al.* 2008
Seafood	Kochzius *et al.* 2010
Olive oil	Consolandi *et al.* 2008

focus on breed, population, and geographical origin assurance. In this sense, sets of microsatellites have been developed with authentication purposes in meat (Orrú *et al.* 2006; Conyers *et al.* 2012), seafood (Li *et al.* 2007), rice (Minh and Rakshit 2009), and olive oil varieties (Vietina *et al.* 2011; Pérez-Jiménez *et al.* 2013).

9.2.3 DNA Sequencing

Forensically informative nucleotide sequencing (FINS) is by far the most reliable analyzing technique with which a precise and unequivocal identification can be obtained. Universal primers are selected for PCR amplification that may react with any species, and sequencing of the obtained DNA-fragments is carried out. For the identification of an unknown target DNA-sequence, comparison with reference sequences deposited in public databases is carried out by bioinformatics tools that determine the highest percentage of DNA-sequence similarity, or by the creation of a phylogenetic cluster that groups together the unknown DNA target with the corresponding reference species. The challenge of this approach is that it can only be applied to isolated specimens and not to mixtures of food products. That is why DNA-sequencing and FINS have frequently been applied for species discrimination in pure meat and seafood products, as shown in Table 9.1.

Lago *et al.* (2011) studied 42 different meat animal species, including those that are commonly consumed and also exotic meats that are part of the diet in other parts of the world. FINS was then applied to analyze 20 commercial products of fresh and processed food, revealing 15% of mislabeling. Pascoal *et al.* (2008*a*, *b*) carried out various studies

aiming to discriminate prawn and shrimp species by the construction of phylogenetic relations of the DNA-sequences of the 16S rRNA/tRNA^{Val} (Pascoal *et al.* 2008*a*) and cytb (Pascoal *et al.* 2008*b*) genes. Figure 9.1 shows the phylogenetic tree of the 16S rRNA gene sequences of a number of crustacean species.

DNA-barcoding represents important progress in DNA-sequencing as a tool for species identification and food traceability (Galimberti *et al.* 2013). The Consortium for the Barcode of Life (CBOL) directs a global effort to assemble a sequence reference library for every animal and plant species on Earth, with the aim of identification and delineation. Small marker genes act as a "barcode," and the cytochrome c oxidase I (COI) gene has been selected for animal species. For plant species the genes *rbc*L and *mat*K are discussed. DNA-barcoding has been especially focused on seafood authentication; the Fish Barcode of Life (FISH-BOL; http://www.fishbol.org) was launched (Hanner *et al.* 2011), aiming to detect frauds and mislabeling in this sector. The method has been applied to analyze 37 commercially processed seafood products from markets and groceries (Nicolè *et al.* 2012), 68 samples of tuna sushi from restaurants (Lowenstein *et al.* 2009) and 28 smoked fish products (Smith *et al.* 2008).

9.2.4 Multiplex PCR with Species-Specific Primers

Once the DNA-sequences are known, the search for variability can be carried out and specific primers can be designed for the identification of certain species with importance in food authenticity. The detection is carried out by electrophoresis and the presence of a specific band corresponding to the amplified DNA sequence. For the determination of more than one species in mixtures, multiplex PCR is carried out and has been successfully applied to the simultaneous detection of meat (Rodríguez *et al.* 2004) and seafood species (Asensio 2008; Rasmussen Hellberg *et al.* 2010). In a further study, the authentication of commercial dairy products manufactured from the milk of cow, sheep, goat, and buffalo has been tested, revealing that 12.5% of the products failed to conform to their description of contents (Gonçalves *et al.* 2012). Likewise, the detection of cows' milk in goat cheese was possible by a duplex PCR with species-specific primers (Mafra *et al.* 2007).

9.2.5 Real-Time PCR

The PCR amplification of a species-specific DNA region can be measured in real time by detection of a fluorescence signal that increase with the concentration of the amplified product, allowing the quantification of the initial concentration of the target DNA in the food product. Real-time PCR is rapid and sensitive and has been successfully applied to the detection and quantification of a certain animal species relative to the total amount of meat or seafood in a food product. Although this approach is expensive and the generation of false-positive signals has frequently been reported, it is the only technique with which a quantitative assessment can be carried out; an estimation of concentration of the fraudulent ingredient can therefore be determined.

Real-time PCR has been applied to detect frauds in meat products, such as the identification and quantification of turkey, chicken, beef, goat, sheep, and pork meat in complex food products (Laube *et al.* 2007; Cammà *et al.* 2012). Likewise, real-time PCR was used to quantify the fraudulent addition of horse and donkey meat in commercial meat products (Chisholm *et al.* 2005) and to detect pork meat down to a concentration of 0.1% in

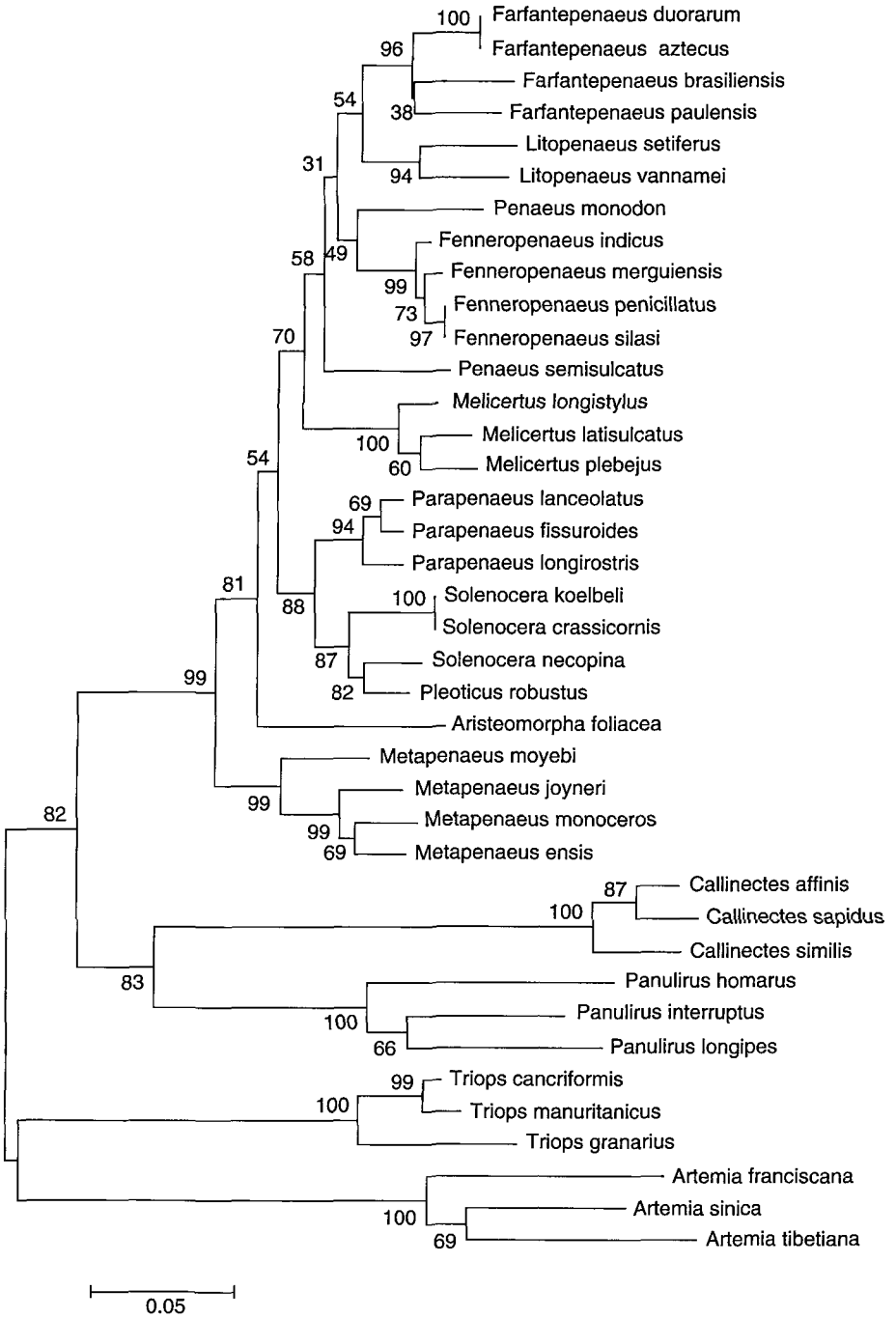


Figure 9.1 Phylogenetic tree of the 16S rRNA gene sequences of crustacean species.

Turkish processed Halal meat products (Ulca *et al.* 2013). In another study, test mixtures containing beef, pork, horse, mutton, chicken, and turkey have been studied, resulting in the possible identification of these species down to a level of 0.01%. This real-time PCR approach has been tested in more than 150 meat samples and compared to DNA hybridization. With real-time PCR more frauds and mislabeled products could be detected (Jonker *et al.* 2008). Furthermore, cervid meat species (red deer, fallow deer, roe deer) and hare have been quantified in meat mixtures of game animals (Fajardo *et al.* 2008; Santos *et al.* 2012). In products of marine origin, real-time PCR was used to quantify thunnus in canned products (Chuang *et al.* 2012), to determine haddock (Hird *et al.* 2005), mackerel (Velasco *et al.* 2013), and to differentiate two eel species (Itoi *et al.* 2005). In dairy products, the fraudulent addition of cows' milk to products manufactured of milk from different ruminants needs to be detected and quantified. In several studies, cows' milk has been quantified by real-time PCR approaches in raw and heat-treated cow/sheep milk mixtures (López-Calleja *et al.* 2007) and buffalo cheese (Dalmasso *et al.* 2011).

The detection of animal species is also important in feeding products, since the commercialization of animal feeds infected by prions proved to be the main cause of transmission of bovine spongiform encephalopathy (BSE). In an inter-laboratory comparison, four different developed real-time PCR procedures have been tested in three different laboratories to detect cattle or ruminant species in typical compound feeds, fortified with meat and bone meals from different animal species at different concentration levels. As a result, all laboratories were able to detect cattle meat and bone meals down to 0.1% in the feed products (Prado *et al.* 2007).

9.2.6 PCR-SSCP

Similar to the search for specific regions, the comparison of DNA-sequences has the objective of determining single-nucleotide polymorphisms (SNPs). SNPs play an important role as molecular markers in genomics and are used to study the genetic diversity of breeds, as well as to differentiate species.

The simplest approach to detect SNPs is the single-strand conformation polymorphism (SSCP) that is based on the differences in the electrophoretic mobility of single strands of a PCR amplicon, which differ in their DNA-sequences. After realizing PCR with primers common to a certain group of interest, the fragments are denatured by heat or organic solvents and separated by conventional electrophoresis in polyacrylamide gels (PAGE) or by capillary electrophoresis (CE). This technique is inexpensive, convenient, and sensitive and has been successfully applied to the identification of closely related fish species such as salmon fish (Rehbein 2005) and eel (Rehbein *et al.* 2002), as well as shrimp species (Khamnamtong *et al.* 2005) and clams (Fernández *et al.* 2002).

The amplification of the plastid DNA genes trnL and rbcL(UAA) with universal primers allowed the discrimination of eight plant species commonly used for oil extraction (Spaniolas *et al.* 2008; Wu *et al.* 2011). In this way, the adulteration of olive oil could be detected; however, the approach was problematic for mixtures due to small differences in the electrophoretic behavior of some plant species and the relatively low resolution.

9.2.7 PCR-RFLP

PCR combined with restriction fragment-length polymorphism (PCR-RFLP) has been frequently applied in food authentication. This DNA-based approach is based

on the specific fragment patterns that result after cutting PCR products with restriction endonucleases. If the sequences are known, the best restriction enzyme can be chosen by determining the cutting sites and calculating the fragment sizes theoretically. For an unequivocal identification of an elevated number of species, usually more than one restriction enzyme is required. Figure 9.2 shows the theoretically determined fragment sizes when cutting a 359 bp fragment of the mitochondrial cytb gene of beef, pork, chicken, and turkey meat with the restriction enzymes *Hae*III, *Mbo*I, and *Hinf*I. This approach has been applied to meat species authentication of 50 commercial raw, cured, or highly processed meat products, resulting in 30% of incorrect labeling. The complete absence of a declared species of high commercial value, such as beef or roe-deer, was observed in four cases (Pascoal *et al.* 2004). The same authors also successfully applied PCR-RFLP for species identification of cod (Calo-Mata *et al.* 2003) and shrimp species (Pascoal *et al.* 2008*c*). A methodology has been patented by Pascoal *et al.* (2008*a*) for the identification of 20 prawn and shrimp species and is based on the amplification of a 515–535 bp region of the 16S rRNA and part of the $tRNA^{Val}$ gene (Spanish patent number: PCT/ES2007/070212). An improvement of the usually applied gel electrophoresis (PAGE) to visualize the specific fragment patterns has been the use of Lab-on-a-chip Capillary Electrophoresis (CE) (Dooley *et al.* 2005).

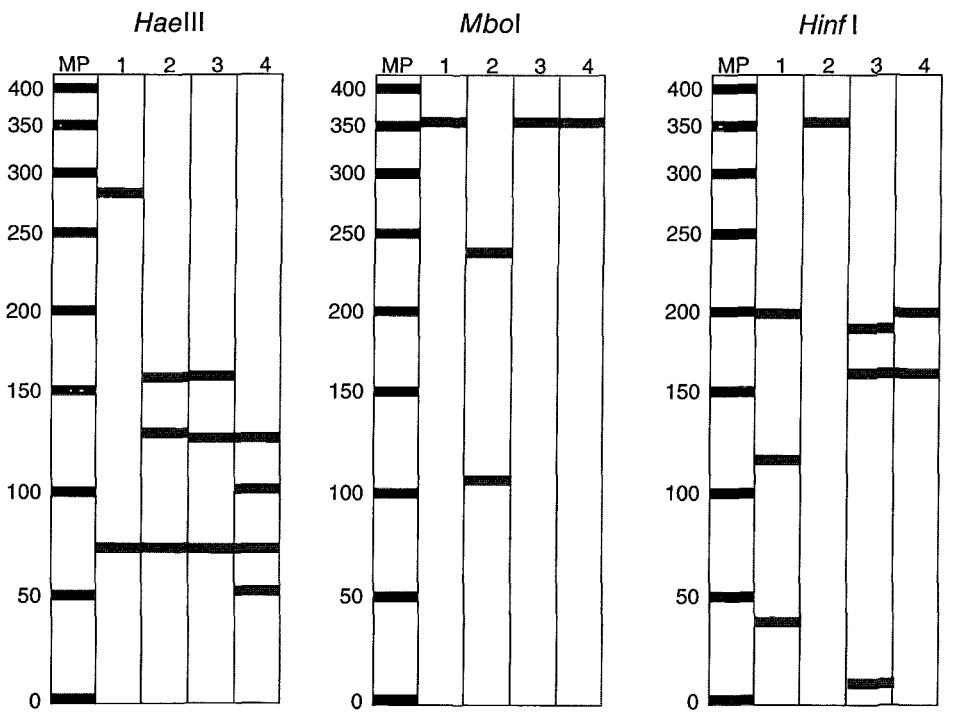


Figure 9.2 Theoretically determined fragment sizes obtained when cutting a 359 bp fragment of the mitochondrial cytb gene of beef, pork, chicken, and turkey meats with the restriction enzymes *Hae*III, *Mbo*I, and *Hinf*I. MP: molecular weight marker; 1. beef; 2. pork; 3. chicken; 4. turkey.

9.2.8 DNA Hybridization and Microarrays

A further, more advanced approach to detect SNPs is the hybridization to specific probes. DNA microarrays are miniature high-density arrays that can have from a few hundred to several hundred thousand oligonucleotides (probes) capable of taking part in hybridization reactions. The sample to be analyzed is usually labeled with a fluorophore and hybridized to the array. In this way, a high number of different species can be analyzed simultaneously and in a short time; this technique is therefore of special interest for food authenticity purposes. Furthermore, subtle differences in the DNA sequences can be distinguished. In this sense, the microarray technique is commonly applied to study the genetic diversity of breeds of ruminants (Bongiorni *et al.* 2009) and populations of marine origin (Moriya *et al.* 2007). Similarly, the microarray technique has been applied to study olive cultivars to assure the authenticity of olive oil (Consolandi *et al.* 2008). The methodology was based on the highly specific ligase detection reaction (LDR), coupled to a universal microarray, and allowed the differentiation of 8 olive cultivars by detecting 17 SNPs in 9 different genes that were amplified in a multiplex PCR.

DNA microarray approaches have also been applied for the species identification in food authentication. An ample study allowed the detection of 71 vertebrate species in food samples of a single species of mixtures up to five different species by an oligonucleotide microarray consisting of species-specific probes (Teletchea *et al.* 2008). Kochzius *et al.* (2010) developed a microarray platform that is able to identify 30 commercially important fish species. For a clear discrimination, 64 species-specific probes are necessary (Kochzius *et al.* 2010).

A number of further studies (Table 9.1) have demonstrated the high potential of DNA microchips for food authenticity applications. As a result, today there are commercial DNA-chips available for species identification in meat products (Chipron LCD array kit, Chipron, Germany; Carno check detection system, Greiner BioOne). These platforms are able to detect up to 14 animal species and have been tested in more than 70 commercial meat samples, confirming the successful performance and the potential to be implemented for routine use in any food control agency (Iwobi *et al.* 2011).

9.2.9 Peptide Nucleic Acid (PNA) -Based Approaches

Recently, peptide nucleic acids (PNAs) have been used in hybridization and microarray assays, replacing the specific probes constituted from DNA. PNAs are DNA mimics in that the negatively charged sugar-phosphate backbone is replaced by a neutral polyamide backbone composed of N-(2-aminoethyl) glycine units. PNAs can bind complementary DNA or RNA in hybridization processes. PNAs have several advantages compared to DNA when used for hybridization: the improved thermal stability of PNA/DNA complexes; the high affinity to the corresponding DNA target that lowers the detection limits; the selectivity that allows the detection of a single base mismatch; and the relative independence from environmental conditions that allows the use in complex matrices such as foods (Sforza *et al.* 2011).

PNAs have been applied to GMO detection of five transgenic events (Roundup Ready soybean and Bt11, Bt176, Mon810, GA21 maize) after amplification by multiplex PCR with event-specific primers (Germini *et al.* 2005).

9.3 Proteomics for Species and Geographical Origin Authentication

Proteomics has been defined as the large-scale analysis of proteins in a specific biological system at a particular time (Pandey and Mann 2000), and includes the study of protein characterization, functions, interactions, localizations, and quantification. The goal of proteomics is the investigation of a proteome, usually based on mass spectrometry (MS) techniques such as matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS and electrospray ionization–ion trap (ESI-IT) MS. The different proteomic workflows can be divided in two general approaches: bottom-up and top-down proteomics.

In the bottom-up approach, proteins are digested with a protease and the resulting peptidic fragments are analyzed in a mass spectrometer. Trypsin is the protease most often used, due to the fact that the peptides that are produced are usually optimal for MS analysis. This approach has two different variations: gel-based, where proteins are separated on a polyacrylamide electrophoresis gel by means of molecular weight or pI or both (in this case, named two dimensional electrophoresis, 2-DE) previous to the protein digestion; or the so-called shotgun approach, where all the proteins in the sample are digested with no previous separation. Peptide mass fingerprinting (PMF), where the masses from the peptides from the digestion of a protein isolated from a 2-DE gel are obtained by MALDI-TOF MS, is the most used gel-based methodology (Pappin *et al.* 1993). The PMF spectrum of the isolated protein is then assigned to a protein from a database. On the other hand, in the shotgun approach, the resulting high-complexity peptide mixture is separated by one- or two-dimensional liquid chromatography and analyzed by MS. The tandem MS (MS/MS) spectrum of each peptide is obtained and searched against a peptide database in order to obtain the sequence of the peptide and the name of the protein which contains that peptide (peptide fragmentation fingerprinting; Eng *et al.* 1994). If the protein is not present in the databases, the sequence of the protein has to be obtained from those MS/MS spectra by *de novo* sequencing (Shevchenko *et al.* 1997). In addition to structural information of the protein, such as sequence or post-translational modifications (PTMs), quantitative information, such as the total abundance of a protein in a sample or the relative amount of a protein between different samples, can be obtained from the MS or MS/MS data using different approaches (Panchaud *et al.* 2008).

Top-down proteomics represents a different approach. In this case, the step of digesting the proteins is skipped, and the whole protein is fragmented directly inside the mass spectrometer (McLafferty *et al.* 2007). Although this approach avoids the variability introduced by the digestion step, is not used very often due to the currently limited performance of the instruments.

Since proteins can act as markers for many food features along the food chain, proteomic tools represent promising approaches for studying food quality, safety, and traceability. For instance, MS of proteins and peptides can be used for both the characterization of markers in reference samples and for the subsequent monitoring of those diagnostic peptides in real foodstuffs. Figure 9.3 shows the proteomics approaches that are being used for the discovery, characterization, and monitoring of food authentication markers. The combination of different proteomic tools, taking advantage of the high-throughput MS-based techniques, represents a robust and powerful strategy for protein detection, identification, and characterization.

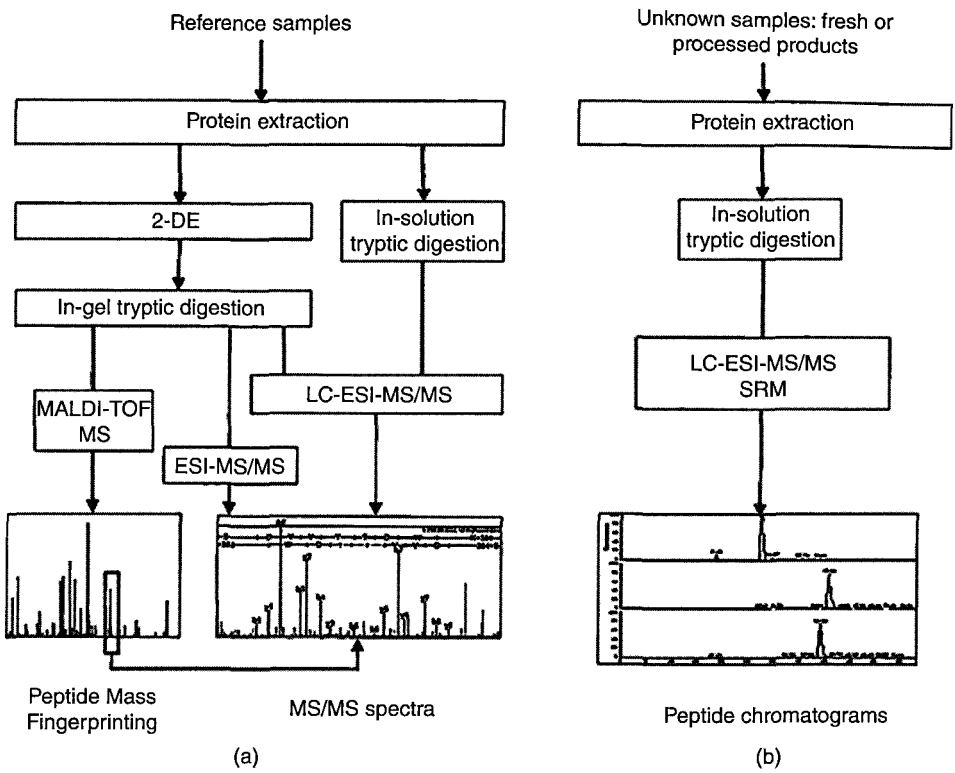


Figure 9.3 Proteomic approaches considered for the (a) identification and characterization and (b) detection and quantification of species-specific diagnostic peptides for authentication purposes. Source: Gallardo *et al.* (2013).

According to how the molecules are analyzed in the mass spectrometer, we could divide MS-based methods into those involving only one stage of mass spectrometry analysis (MS^1 or MS), and those involving two steps of mass spectrometry selection/analysis, with a fragmentation stage occurring in between the two stages (tandem mass spectrometry, MS/MS or MS^2). In addition, when previous information about the target protein or peptide is known, the use of the MS acquisition modes named directed analysis, such as selected ion monitoring (SIM) and selected reaction monitoring (SRM), is recommended. However, protein-based methods based mainly on electrophoresis are still frequently used and reported. Applications of proteomics to food authenticity are listed in Table 9.2.

9.3.1 Gel-Based Methods

Protein electrophoretic patterns have been extensively used for the development of methods aimed at the detection and authentication of the species present in food products (Rehbein 1990; Lockley and Bardsley 2000), mainly using SDS-PAGE, IEF, and 2DE. In SDS-PAGE, the proteins are separated according to molecular weight; the protein profiles from different samples can therefore be compared. SDS-PAGE has been used for the differentiation of crab and shrimp meat (Civera and Parisi 1991), and even

Table 9.2 Summary of the proteomics-based methods applied to food authentication.

Main technique	Species/food products	Target	Reference
SDS-PAGE	Ginseng varieties	–	Yeh *et al.* 2006
IEF	14 shrimp and prawn species	SCPs	Ortea *et al.* 2010
	Ewe, goat and cow milk in cheeses	Whey proteins	Mayer 2005
	17 flatfish species	–	Bossier and Cooreman 2000
	Dry bean cultivars	–	Ruiz Guerrero *et al.* 2001
2-DE	8 gadoid fishes	Parvalbumins	Piñeiro *et al.* 1998
	9 flat fishes	Sarcoplasmic proteins	Piñeiro *et al.* 1999
	5 fish species	Myosin light chains	Martinez and Friis 2004
	3 *Thunnus* species	Triose phosphate isomerase	Pepe *et al.* 2010
	Cattle, pig, chicken, turkey, duck, and goose	Myosin light chains	Montowska and Pospiech 2012, 2013
	2 cattle breeds	–	Timperio *et al.* 2009
ESI MS	Pig, beef, sheep, and horse meats	Hemoglobin/myoglobin	Taylor *et al.* 1993
HPLC-ESI MS	Cow and goat milk	β-lactoglobulin	Chen *et al.* 2004*b*
	Cow, sheep, goat, and buffalo milks	caseins	Cuollo *et al.* 2010
CE-MS	Cow, goat, and sheep milk	β-lactoglobulin	Müller *et al.* 2008
MALDI-TOF protein profiling	Cow, ewe, and buffalo milk	α-lactalbumin and β-lactoglobulin	Cozzolino *et al.* 2001
	Mozzarella cheese	α-lactalbumin and β-lactoglobulin	Cozzolino *et al.* 2002
	25 commercial fish species	Sarcoplasmic proteins	Mazzeo *et al.* 2008
	Honey	–	Wang *et al.* 2009
	Cow, sheep, goat, and buffalo milks	Caseins	Cuollo *et al.* 2010
MALDI-TOF PMF	5 hake species	Parvalbumins and NDKA	Piñeiro *et al.* 2001
	3 European mussels	TM	López *et al.* 2002*b*
	10 Merlucciidae hake and grenadier species	Parvalbumins	Carrera *et al.* 2006
	11 Merlucciidae hake and grenadier species	NDK B	Carrera *et al.* 2007
	Penaeus monodon, *Fenneropenaeus indicus*	AK	Ortea *et al.* 2009*a*

Table 9.2 (Continued)

Main technique	Species/food products	Target	Reference
	6 shrimp and prawn species	AK	Ortea *et al.* 2009*b*
	Pandalus borealis (Northern shrimp)	AK	Pascoal *et al.* 2012
	2 Sperata species	Triosephosphate isomerase	Barik *et al.* 2013
MALDI-TOF peptide profiling	Sheep, goat, and cow milk	–	Calvano *et al.* 2012
	Campania white wines	–	Chambery *et al.* 2009
MS/MS	5 hake species	Parvalbumins and NDKA	Piñeiro *et al.* 2001
	Soybean in meat products	Glycinin, β-conglycinin	Leitner *et al.* 2006
	11 Merlucciidae commercial hakes and grenadiers	NDKB	Carrera *et al.* 2007
	Bovine gelling agent in meat	Fibrinopeptides	Grundy *et al.* 2007
	7 shrimp and prawn species	AK	Ortea *et al.* 2009a
	Soy and pea in skimmed-milk powder	Glycinin, β-conglycin, legumin, vicilin	Cordawener *et al.* 2009
	Sheep milk in goat and cow cheeses	Casein	Guarino *et al.* 2010
	Pandalus borealis (Northern shrimp)	AK	Pascoal *et al.* 2012
	22 fish species	–	Wulff *et al.* 2013
SIM	3 European mussels	TM	López *et al.* 2002*b*
	Chicken in meat preparations	Myosin light chain 3	Sentandreu *et al.* 2010
pSRM	11 Merlucciidae hake and grenadier species	NDK B	Carrera *et al.* 2007
	7 shrimp and prawn species	AK peptides	Ortea *et al.* 2011
	11 Merlucciidae hake and grenadier species	Parvalbumins	Carrera *et al.* 2011

2-DE: two-dimensional electrophoresis; AK: arginine kinase; CE: capillary electrophoresis; ESI: electrospray ionization; HPLC: high-performance liquid chromatography; IEF: isoelectric focusing; MALDI-TOF: matrix-assisted laser desorption/ionization-time-of-flight; MS: mass spectrometry; MS/MS: tandem mass spectrometry; NDKA: nucleoside diphosphate kinase A; NDKB: nucleoside diphosphate kinase B; PMF: peptide mass fingerprinting; pSRM: pseudo-selected reaction monitoring; SCPs: sarcoplasmic calcium-binding proteins; SIM: selected ion monitoring; TM: tropomyosin.

for the differentiation of shrimps at species level (An *et al.* 1988). In an inter-laboratory study, SDS-PAGE also enabled the identification of several commercial fish species after cooking by comparison with reference samples (Etienne *et al.* 2000). Yeh *et al.* (2006) proposed SDS-PAGE as a technique for the authentication of ginseng proteins, since they were able to differentiate between two major ginseng varieties: Oriental and American.

IEF, a technique consisting of the separation of proteins in a polyacrylamide gel according to their isoelectric points (pI), was adopted by the Association of Official Analytical Chemists as the only official validated method for species identification (AOAC 1984). IEF methods for the authentication of different kind of foods have been reported. An *et al.* (1989) and Wei *et al.* (1990) report the use of pH 4.0–6.5 IEF in urea gels for the differentiation of shrimp species even when samples had been heated. IEF urea gels also allowed the differentiation of shrimp from fish and cephalopod meat (Rehbein 1995) and the identification of 10 commercial fish species after cooking (Etienne *et al.* 2000). Bossier and Cooreman (2000) used a computerized databank of IEF protein profiles to identify 17 flatfish species. Renon *et al.* (2005) used the IEF sarcoplasmic protein pattern to distinguish between swordfish, blue marlin, and Mediterranean spearfish in fresh, frozen, and cold-smoked fillets. The IEF profiles of the sarcoplasmic calcium-binding proteins (SCPs), a kind of heat-stable proteins found in fish and crustacean muscle, have provided species-specific patterns for cooked and raw samples from shrimp, fish, and lobster (Rehbein 1995). SCPs also produced species-specific protein bands for the unambiguous identification of 14 commercial shrimp species in a recent work using native IEF of water-soluble sarcoplasmic proteins (Ortea *et al.* 2010). Apart from seafood, IEF has been used for the authentication of some other food commodities such as legume seeds, being able of differentiate between several dry bean cultivars (Ruiz Guerrero *et al.* 2001), and cheeses, where IEF of whey proteins was useful for the detection of ewe, goat, and cow milk (Mayer 2005).

2-DE, where proteins are successively separated according to pI and molecular weight, has also been revealed as a valuable tool for the discrimination of species, being able to differentiate between very closely related species that have shown identical IEF protein profiles. Differences in the 2-DE profiles of the parvalbumin protein fraction have been used for the discrimination between fish species such as gadoid fishes (Piñeiro *et al.* 1998), flat fishes (Piñeiro *et al.* 1999), and hake species (Piñeiro *et al.* 2001). Since parvalbumins are heat-resistant proteins, it was claimed that the methodologies described could be used for the identification of those fish species in heat-treated products. Apart from parvalbumins, some other proteins have demonstrated the potential for discriminating fish species by 2-DE, such as triose phosphate isomerase, proposed as a marker for the tuna species *Thunnus thynnus* (Pepe *et al.* 2010), and the myosin light chains (MLCs) for differentiation of cod, saithe, haddock, mackerel, and capelin (Martínez and Friis 2004). Interestingly, MLCs also showed as markers for the breeding stock, since the differentiation of two stocks was achieved. 2-DE also achieved the differentiation of puffer fish species using the urea soluble protein extracts (Chen *et al.* 2004*a*). Quantitative differences between two marine mussels species were detected in some proteins by 2-DE, although these changes were not only caused by genetic differences but also by environmental variations (López *et al.* 2002*a*). In decapoda, Ortea *et al.* (2009*a*) proposed the protein arginine kinase as biomarker for shrimp species identification, due to its variability in the 2-DE profile. Montowska and Pospiech (2012, 2013) found inter-species differences in 2-DE protein patterns between cattle, pig, chicken, turkey, duck, and goose, in both raw meat and processed products such as sausages. The differences were mainly due to species-specific patterns of the muscle myosin light chain (MLC) isoforms; they were therefore proposed as markers for authentication of meat products. 2-DE has even been used for the differentiation of cattle breeds, and differences between Holstein and Chianina breeds were found (Timperio *et al.* 2009)

Since electrophoretic methodologies show some limitations, such as changes and degradation of proteins due to food processing, low resolving power for closely related species, misidentification when analyzing samples with a mixture of several species, or a need for reference samples, authentication methods using MS have gained much attention recently.

9.3.2 MS-Based Methods

Several proteomic workflows involve only one stage of mass spectrometry analysis (MS^1 or MS), meaning that only the mass of the analyte is obtained; since the peptide or protein is not being fragmented and no MS/MS spectra are being obtained, no structural information is derived.

The most representative approach including this kind of analysis is PMF. Here, proteins isolated by 2-DE are digested with a protease, usually trypsin, and the mass of the resulting peptides are obtained by one-stage MS analysis, generally using MALDI-TOF mass spectrometer. The mass list obtained for that particular protein acts as its fingerprint, and can be searched against a protein database in order to identify the protein. Since inter-species differences in the sequence of a specific protein can be detected comparing the PMF for that protein between species, MALDI-TOF PMF has been extensively used for species authentication. PMF of the parvalbumin fraction allowed the classification of several hake species (Piñeiro *et al.* 2001), and even the differentiation of hakes in two groups depending on the geographical origin, namely Euro-African and American (Carrera *et al.* 2006). Barik *et al.* (2013) found differences in the PMF profiles of the protein triosephosphate isomerase between two commercially important river fish species from the genus *Sperata*. López *et al.* (2002*b*) reported species-specific peptides for three species of commercial marine mussels, detected by PMF analysis of the protein tropomyosin. Fingerprints of the sarcoplasmic protein arginine kinase were used as markers for six shrimp species of commercial relevance (Ortea *et al.* 2009*b*). In this work, a mathematical method for drawing a PMF spectra dendrogram, which can help in the classification of unknown samples by comparing with reference samples, was reported.

In addition to PMF, another type of analysis comprising one-stage MS has been widely applied to food authentication: in protein profiling, the mass of the whole proteins are obtained and compared between samples. The great advantage of this method is its rapidity, due to the fast sample preparation step that avoids protein digestion. Mazzeo *et al.* (2008) obtained specific MALDI-TOF MS profiles for 25 different fish species, representing the highest number of species included in a fish authentication study. MALDI-TOF protein profiling has been used for the determination of the geographical origin of honey from Hawaian bees (Wang *et al.* 2009). It has also been used for the detection of fraudulent practices in the dairy industry, such as the addition of bovine milk to ewe and buffalo milk (Cozzolino *et al.* 2001) and adulteration of mozzarella cheese (Cozzolino *et al.* 2002), using whey proteins as biomarkers. The protein β-lactoglobulin has been used for the detection and quantification of cow milk adulteration in caprine milk (Chen *et al.* 2004*b*) or in either caprine or ovine milk (Müller *et al.* 2008) by means of HPLC-ESI-MS or capillary electrophoresis-MS, respectively, detecting the presence of cow milk at levels as low as 5%. Casein has also being used to detect milk adulteration in MALDI MS and LC-ESI MS methods, which were able to

detect concentrations as low as 0.5% of bovine, ovine, buffalo, and caprine milks in milk mixtures (Cuollo *et al.* 2010). Calvano *et al.* (2012) described a method using MALDI-TOF analysis of milk tryptic digest able to detect up to 5% level of sheep and goat milk adulteration. A similar approach, but using the tryptic digests of wine proteins, provided a signature for Campania white wines (Chambery *et al.* 2009). Regarding meat authentication, Taylor *et al.* (1993) were able to differentiate between several sources (pig, beef, sheep, and horse) using ESI MS of purified hemoglobin and myoglobin.

9.3.3 MS/MS-Based Methods

MS/MS involves two steps of mass spectrometry analysis with a fragmentation stage occurring in between the two stages: the selection of the precursor ion to fragment in the first step; and the analysis of the ion fragments that result from that fragmentation. From the analysis of the MS/MS spectrum, the sequence of the corresponding peptide can be obtained. MS/MS is being used for both the discovery and characterization of species-specific peptidic markers in reference samples and for the subsequent detection of those diagnostic peptides in real samples. Once identified and characterized, differential peptides could be used in immunoassay kits for the cheap detection of the species, or in the development of fast and highly specific directed MS assays.

MS/MS has been used for the identification of species in seafood products. Interspecies differences in the sequence of parvalbumins and the protein nucleoside diphosphate kinase B (NDK B) were used for the differentiation of hake species (Piñeiro *et al.* 2001; Carrera *et al.* 2007). Several species-specific peptides from the sarcoplasmic protein arginine kinase were identified than can serve as diagnostic peptides for the identification of the seven most commercial shrimp and prawn species, by means of *de novo* sequencing of the corresponding MS/MS spectra (Ortea *et al.* 2009*a*; Pascoal *et al.* 2012). Wulff *et al.* (2013) used a library of complete MS/MS spectra datasets for identifying 22 different fish species, showing that the method was able to identify heavily processed samples in addition to fresh samples.

Adulteration of meat has also been investigated by means of MS/MS. Leitner *et al.* (2006) reported a method for detecting soybean proteins added to processed meat products. Grundy *et al.* (2007) were able to detect concentrations of bovine gelling agents as low as 5% in different food matrices by the MS/MS detection of the species-specific fibrinopeptides.

Regarding adulteration in dairy products, MS/MS analysis was applied to the detection of soy and pea proteins in skimmed milk powder (Cordawener *et al.* 2009), and Guarino *et al.* (2010) reported a method combining liquid chromatography and MS/MS for the detection of as low as 2% of sheep's milk in goat's and cow's cheeses using a sheep-specific casein peptide.

9.3.4 Directed Approaches

When peptidic biomarkers have been already characterized, it is much faster to directly search for the specific known peptides in the protein extract using the MS acquisition modes included under the term "directed analysis," such as: selected ion monitoring (SIM) where only the peptides or proteins with selected m/z values are detected; selected reaction monitoring (SRM), where only some of the specific MS/MS fragment

from a specific peptide in monitored; and pseudo-SRM (pSRM), sometimes called selected MS/MS ion monitoring (SMIM) or parallel reaction monitoring (PRM), where only those peptides of interest are fragmented and recorded (Jorge *et al.* 2007; Peterson *et al.* 2012; Sherrod *et al.* 2012).

SIM acquisition of species-specific tropomyosin-derived peptides was applied to the differentiation of three commercial mussel species by LC-MS analysis (López *et al.* 2002*b*). The mass spectrometer was programmed to continuously monitor species-specific tropomyosin peptides that had been previously identified and characterized. In pSRM the MS/MS spectra of the target peptides are registered, yielding a higher specificity than SIM. SMIM-based methods have been used for the identification of seafood products. Ortea *et al.* (2011) developed a methodology able to identify the most commercial shrimp and prawn species in no longer than 90 minutes, combining ultrasound assisted sample preparation and LC-SMIM MS analysis of species-specific peptides from the protein arginine kinase. Carrera *et al.* (2011) reported a method for the fast monitoring of parvalbumin species-specific peptides for 11 hake species. Since parvalbumins are heat-resistant proteins, the methodology allowed the identification of the species in processed and cooked products.

Sentandreu *et al.* (2010) developed a sensitive LC-MS/MS methodology for detecting chicken meat in meat mixes. The extracted ion chromatograms for two chicken-specific peptides from myosin light chain 3 were used to calculate the amount of the peptides after normalizing with synthetic internal standard peptides, allowing the detection of as low as 0.5% chicken meat in pork meat.

9.4 Future Trends

Major challenges regarding food authentication are: (1) the development of fast, easy, and cheap methodologies for routine use; (2) detecting smaller fragments, which could be analyzed in both raw and processed products; and (3) achieving the multiplexed identification and quantification of several authenticity markers in a food product. The targeted MS/MS acquisition method termed selected reaction monitoring (SRM) can help to meet these challenges as they allow high-throughput, selective, and sensitive multiplexed detection, identification, and quantification of marker peptides in complex matrices (Gallien et at. 2011). Since sensitivities reach low ppm levels, it is a real alternative to ELISA and rtPCR methods. SRM is the reference quantitative MS-based technique to analyze small molecules, and is currently being commonly used to verify candidate biomarker proteins in a hypothesis-driven approach following discovery studies, although its application to the food research area has been scarce to date. In addition to SRM, some other recently developed MS acquisition methods promise much for the future, since they can help to decrease the LOD levels and to increase selectivity. For instance, data-independent analysis acquisition (DIA) modes, such as the sequential window acquisition of all theoretic mass spectra (SWATH) (Gillet *et al.* 2012) and the elevated-energy MS (MS^e) (Geromanos *et al.* 2009), allow the quantitative analysis of low-abundance peptides in the same run that is used for protein identification. Furthermore, future improvements in high-resolution MS instruments will enable the application of top-down proteomics to the high-throughput analysis of food products at the intact protein level.

Regarding DNA-based methods, real-time PCR and DNA-microarrays are the most promising techniques for future applications in food authenticity, due to the ability of multiplexing and quantification. Furthermore, the potential to be integrated in miniaturized systems, such as microfluidic systems and novel nanobiosensors with enhanced adaptability, versatility, and sensitivity, makes them especially attractive for the food industry and control laboratories.

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10

Immunological Techniques

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10.1 Introduction

As the food supply chain becomes increasingly global and intricate, it continues to present new risks and challenges. One of these risks that has gained a great deal of attention recently has been food fraud, a collective term that embraces the conscious substitution, addition, tampering, or misrepresentation of food and food ingredients, or dishonest statements made about a product solely for economic gain (Moore *et al.* 2012). Although food fraud is nothing new and was even reported in ancient times (Hutt and Hutt 1984), the current global nature of the food supply not only creates an atmosphere that is conducive to such acts but also increases the impact of any disastrous consequences, making it essential for the various national authorities to work together in a concerted effort to curb such illegal practices. According to the US Congressional Research Service (CRC), it is not known how widespread food fraud is either in the USA or worldwide; most cases go undetected because they do not result in health problems or the quality is not noticeably degraded (Johnson 2014).

Although any food is technically a target for misrepresentation, some tend to be more frequently misrepresented than others. A recent news report identified 15 foods that are frequently counterfeited: olive oil was top of the list, closely followed by honey, fish, scallops, balsamic vinegar, saffron, vanilla, coffee, cinnamon, black pepper, caviar, milk, juices, meat, and wine, in that order (Fox News 2014). Compared to other industries, the profit margins on food production are relatively small (Schieber 2008) and hence such fraudulent acts are not unexpected and are likely to always be an issue.

Many methods based on different techniques have therefore been developed for the quality control of these food items to enforce the applicable national and international regulations and also to address consumers' economic, health, and ethical concerns associated with such fraudulent acts. These methods include spectroscopy, chromatography, polymerase chain reactions (PCR), immunoassays, electrophoresis, differential scanning calorimetry, and chemometrics (Schieber 2008). Among these, immunoassays have become widely recognized among manufacturers and regulators alike as highly desirable, rapid, and efficient methods for the quality control of food and agricultural products. This chapter focuses on the latest immunochemical techniques for ensuring food authenticity and integrity.

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10.2 Immunoassays

Immunoassays are analytical techniques that rely on the basic immunology concept of the specific interaction between an antibody and its antigen. Antibodies are glycoproteins secreted by higher vertebrate animals in response to exposure to an antigen, which is a foreign substance that has the potential to induce the formation of antibodies under appropriate conditions. Immunoassays were originally developed to facilitate the study of immunology; they are now finding widespread applications outside the clinical field as they can be used to detect a host of molecules, ranging from proteins to small organic molecules in a complex sample matrix (Hsieh 2010). An immunoassay can work in one of two ways: either the antibody can be used to capture its specific antigen in a matrix; or the antigen can be used to trap its specific antibody in a matrix. The former is most often used in the food industry, where antibodies are developed against specific antigens (allergens, toxins, pathogens, etc.) and then used as capture molecules to trap their target antigens. The production of specific antibodies is therefore the first crucial step in the development of an immunoassay.

The antibodies that are used as probes for the target antigen in a food matrix can be either polyclonal antibodies (pAbs) or monoclonal antibodies (mAbs). pAbs are antibody reagents that contain many different antibodies in the anti-serum, each of which recognizes a different epitope (the specific region on the antigen that the antibody binds to) on the same antigen and is produced by a different antibody-producing cell. In contrast, mAbs are produced by a single hybridoma cell (produced in the laboratory by fusing an antibody-producing cell with a non-antibody producing myeloma cell) that binds to a single epitope (Grothaus *et al.* 2006). Although pAbs take less time (about 3–4 months) to produce and are also less expensive to prepare, the nature and the quality of the antibodies varies from animal to animal and from batch to batch. The Nobel Prize-winning technique for producing mAbs developed by Köhler and Milstein (1975) is more expensive to produce, requires greater skill, and takes more time to develop, but once the desired mAb has been isolated and selected it can be cultured perpetually, providing a virtually unlimited source of invariable and highly specific reagent. These advantages outweigh the initial development costs for many immunoassay manufacturers, and are therefore becoming more desirable for commercialized immunoassay kits (Hsieh 2010).

Every immunoassay needs a way to measure the primary antibody–antigen interaction and the various techniques developed all depend on the selection of an appropriate amplification method to improve the assay sensitivity. Early techniques involved visualizing the precipitation that occurred following the binding of multiple antibody molecules to large numbers of antigen molecules, with relatively slow processes such as immunodiffusion techniques requiring overnight incubation and large quantities of immunoreagents. The subsequent development of radioimmunoassays that use radio isotopes as labels made this process much faster, allowing early quantitative measurement of the antibody–antigen complex and needing only small quantities of radioactive material. However, this technique is confined to specially equipped laboratories because it requires highly trained technicians and the use of hazardous radio isotopes and sophisticated instruments to measure the radioactivity.

A major step forward that opened the door for the more general use of immunoassays was the development of enzyme labels. Enzyme immunoassays use enzymes as labels by

converting a colorless substrate to a colored product in the solution to generate a measurable signal that reveals the amount of antibody–antigen binding for the assay. Engvall and Perlmann (1971), who pioneered this approach, developed a type of enzyme immunoassay that they named enzyme-linked immunosorbent assay (ELISA). ELISA involves reactants that are bound to a solid support, typically in the form of a 96-well plastic microtiter plate, to be separated from unbound materials through a simple washing step. This has become the most commonly used immunoassay for commercial immunoassay kits for food applications, as the 96-well plastic microtiter plates permit a high throughput, allowing 48 samples to be analyzed in duplicate simultaneously. Further developments have created alternative labeling and amplification techniques such as the use of nanogold particles in immunohistochemical staining assays and lateral flow immunochromatographic strip assays (LFS), magnetic spheres for concentrating target molecules, and various immunosensors.

There are now many variants of immunoassay formats. Examples include indirect v. direct and competitive v. noncompetitive immunoassays that depend on how and which reactant (antibody or antigen) is immobilized on a solid support (which can be a nitrocellulose membrane, a 96-well plastic microtiter plate or immunotubes). LFS, which is the same principle upon which the home pregnancy test is based, is easy to use (requiring no training), low in cost, fast (results can be visualized in 10–20 min), and can be used in the field as it requires no equipment. More detailed information about these immunoassays is provided in Hsieh (2010). The user-friendly, high throughput, economical, highly specific (distinguishing between isomeric forms of an antigen), and very sensitive (detecting targets at concentrations of parts per million or lower) features and fast analysis time of immunoassays have made them popular tools for analyzing various types of food and food ingredients.

10.3 Meat Speciation

Because of the high prices commanded by meat, meat products are prone to species substitution or adulteration and are commonly misrepresented. The perpetrators easily get away with such fraudulent practice because the flesh of meat from various species cannot be readily distinguished visually by nonspecialists. The difficulty is compounded when meat is ground and incorporated into value-added products. A number of cases of misrepresented meat have been reported in the US and in other parts of the world (Hsieh *et al.* 1996; Cawthorn *et al.* 2013; Lawrence 2013). The economic, ethical, health, and food safety issues associated with such fraudulent practices have led to efforts to develop better methods for meat speciation that can be used to enforce the existing labeling regulations promulgated to protect the consumer and to ensure fair trade. Methods such as electrophoresis, chromatography, and polymerase chain reaction (PCR) are currently available for meat speciation, but these methods are relatively expensive, slow, and require experienced analysts. Hsieh (2005) provides a comprehensive review of the methods available for meat species identification, including immunoassays. Immunoassays are gaining widespread acceptance as user-friendly, rapid, and economical alternative methods for meat speciation based on mAbs or pAbs that target either a single species (e.g. pork, horse, sheep, or chicken) or multiple species (e.g. poultry, mammalian, ruminant, or all land animals) as needed to address particular issues, as summarized in Table 10.1.

Table 10.1 Immunoassays for meat species identification.

Target species	Target antigen	Antibody used	Assay type	LOD	Sample tested	Cross-reactivity	Reference
A. Single species identification							
Chicken	Chicken muscle protein	mAb BC9 as capture Ab and goat anti-rabbit IgG conjugated to peroxidase as detection Ab	sELISA	1%	Raw	NR	Martin *et al.* 1991
Chicken	Chicken muscle protein	mAbs 9C6 and 6F7	iELISA	1–5%	Raw and cooked	None	Sheu and Hsieh 1998
Horse	Horse muscle protein	Affinity purified anti-sera as capture Ab and peroxidase-conjugated affinity purified anti-sera as detection Ab	sELISA	1%	Raw	NR	Martin *et al.* 1988
Pork	1. Porcine IgG	1. Antiserum	1. iELISA	1.1%	Raw	NR	Martin *et al.* 1998
	2. Porcine serum albumin	2. Antiserum	2. Immuno-diffusion	2.3–5%			
Pork	Porcine serum albumin	Affinity-purified anti-serum	cELISA	10 mg/g	Raw and mildly cooked	NR	Ayob *et al.* 1989
Pork	Porcine muscle protein	mAb DD9	iELISA	1%	Raw	Did not react with bovine caseins, soy proteins, gelatin, and bovine serum albumin	Morales *et al.* 1994
Horse	Horse muscle protein	mAb DD3	iELISA	10 g kg^{-1}	Raw	Did not react with soy proteins, bovine caseins, gelatin, and bovine serum albumin	Garcia *et al.* 1994

Pork	Troponin I	mAb 5H9	iELISA	0.5%	Raw and cooked	NR	Chen and Hsieh 2000
Pork	Porcine muscle protein	Anti-serum	cELISA	0.5–1%	Raw and cooked	NR	Sawaya *et al.* 1990
Pork	Troponin I	mAb 8F10 as capture Ab and mAb 5H9 as detection Ab	sELISA	0.05–1%	Raw and cooked	Did not react with egg albumin, soy proteins, gelatin, milk proteins, and blood	Liu *et al.* 2006
Horse	Troponin I	mAbs 4E7 and 3E3	cELISA	1%	Raw and cooked	None	Hsieh and Ofori 2014
Sheep	Troponin I	mAbs 7F6 and 6F11	sELISA	0.25–0.5%	Raw and cooked	Slight cross-reaction with soy but no reaction with milk, egg albumin, and gelatin	Liu 2006
B. Multiple-species identification							
Poultry	Chicken muscle protein	mAbs 3E12 and 1A5	iELISA	1–5%	Raw and cooked	None	Sheu and Hsieh 1998
Turkey and chicken	Chicken muscle protein	mAbs 5D2 and 6G8	iELISA	1–5%	Raw and cooked	None	Sheu and Hsieh 1998
Beef, pork, sheep, horse, and deer	Troponin I	mAb 2F8	iELISA	0.5%	Raw and cooked	None	Hsieh *et al.* 1998
Chicken and turkey	Chicken muscle protein	mAbs AH4, BC9, and CF2	iELISA	ND	Raw	Did not react with casein, gelatin and soy	Martin *et al.* 1989
Chicken and turkey	Chicken muscle protein	pAbs	iELISA	126ppm	Raw and cooked	NR	Berger *et al.* 1988
Beef, sheep, horse, pig, camel, and kangaroo	Serum proteins	Affinity purified anti-serum	iELISA	10%	Raw	NR	Whittaker *et al.* 1983

(Continued)

Table 10.1 (Continued)

Target species	Target antigen	Antibody used	Assay type	LOD	Sample tested	Cross-reactivity	Reference
Beef, chicken, pig, and horse	Albumin	Anti-sera	Dot-blot	0.6%	Raw	NR	Macedo-Silva *et al.* 2000
Beef and sheep	Troponin I	mAbs	LFS	0.5%	Raw and cooked	None	Rao and Hsieh 2007

sELISA: sandwich ELISA; iELISA: indirect ELISA; cELISA: competitive ELISA; Ab: antibody; mAb: monoclonal antibody; pAb: polyclonal antibody; NR: not reported; LFS: lateral flow strip assay

Using antisera or pAbs as the probe in an immunoassay system for meat species identification requires laborious affinity purification and affinity depletion procedures to remove cross-reactive antibodies with other non-target species, which often results in low reactivity against the target species after complete depletion or problems with cross-reactivity with non-target species because of incomplete depletion. To avoid false positives, it is important that newly developed assays are tested for cross-reactivity against common food additive proteins such as soy, gelatin, casein, and so on, particularly for assays that employ pAbs as these tend to be more cross-reactive.

A number of factors need to be considered during the development of a new assay. For successful commercialization of an assay, it is imperative that it is able to detect cooked species, as most meat products are consumed after cooking or have been processed with methods that may involve severe heat treatment. Methods originally developed for cooked products can easily be applied to raw meat samples simply by heating the raw samples prior to analysis. In order to analyze a cooked meat product effectively, the target antigen must either be able to withstand cooking or maintain its solubility and antigenicity after heating. The use of anti-sera raised against serum albumin as a probe in several immunoassays renders them only useful for raw meat analysis because of the general heat-labile nature of the antigen. Additional concerns may be raised as serum albumin is obtained from blood and is also found in milk due to transcytosis (proteins crossing the capillary endothelia into the mammary gland) (Tuma and Hubbard 2003). As a result, false positives caused by cross-reaction with dairy protein ingredients, especially in cases involving anti-sera to beef and goat meat, can be a problem; it is therefore desirable that the marker protein for immunoassays developed for meat speciation be unique to muscle. In our laboratory troponin I, a subunit of troponin that is a contractile protein in the muscle thin-filament has been identified as a suitable muscle marker protein for meat speciation in raw, cooked, and pressure-cooked meats (Chen and Hsieh 2001, 2002).

Most of the immunoassays shown in Table 10.1 are based on the format of antigen immobilized indirect ELISA (iELISA). iELISA is unsuitable as an analytical tool for routine analysis when a large number of routine samples are involved, because of the inconvenience of coating unknown sample extracts onto microplates. Sandwich immunoassay is the preferred type of immunoassay and the most frequently used immunoassay variant for commercial test kits because of its higher sensitivity and user-friendliness (Liu *et al.* 2006). In a sandwich type of immunoassay system, the target antigen (the analyte) is trapped between the capture antibody and the detecting antibody, and therefore requires either a pair of different mAbs with complimentary antigen binding properties, or pAbs. Based on a highly thermal-stable marker antigen, the mAb-based sandwich ELISA (sELISA) reported by Liu *et al.* (2006) was shown to detect levels as low as 0.05% of pork in both raw and heat-processed (132°C for 2 h) laboratory-prepared chicken and beef spiked with pork. This detection limit was ten times lower than the previously reported detection limit of 0.5% (w/w) when the same porcine-specific mAb 5H9 was used alone in iELISA (Chen and Hsieh 2000). This immunoassay (Liu *et al.* 2006) has now been commercialized as the first mAb-based sELISA for porcine meat detection (MELISA® for Pork by ELISA Technologies, Inc.). All other commercial immunoassay test kits employ pAbs as species probes.

10.4 Fish and Shellfish Authentication

Fish is popular with consumers worldwide and, as is the case with meat, commands premium prices for certain species. Over 90% of the seafood consumed in the US is imported (NOAA 2012). This situation creates the right environment for substituting lower-valued fish species for high-valued fish species at different trading levels, as these fish products are usually imported in the processed form and are therefore devoid of the morphological characteristics that enable end-users to easily identify the species. The literature is rife with examples of malpractice, generally involving the misrepresentation of high-valued fish such as snapper and grouper (Hsieh *et al.* 1997; Jacquet and Pauly 2008).The illegal practices of fish substitution are a concern, particularly at the restaurant level where the fish has been heat-processed before it arrives at the table and labeling is not required (USA Today 2013). Such practices not only hurt the consumer's pocket but also have safety implications as the consumer may be exposed to heavy metals, antimicrobial agents, and other contaminants associated with particular groups of fish. The methods currently used for species identification by the regulatory agencies are protein-based isoelectric focusing (IEF) (Hsieh *et al.* 1997; Renon *et al.* 2001) and DNA-based polymerase chain reaction techniques (Quinteiro *et al.* 2001; Aranishi *et al.* 2005), both of which are laboratory-based techniques that require expensive equipment, highly trained personnel, and authentic fish standards that can be analyzed side by side with the sample being tested. As yet, immunoassays developed for the identification of single fish species have not been very successful or seen common use due to the difficulties associated with identifying species-specific marker antigens among the huge number of highly diverse food fish species that would be suitable for developing antibodies with the desired species specificity. At present, the only commercial rapid immunoassay available is for Pangasius catfish, mainly basa and tra, species identification (EZ Pangasius® by ELISA Technologies, FL USA).

Several immunochemical methods have been tried for fish species identification to combat fish fraud and mAbs and pAbs have been used as probes in immunoassays for speciation in both raw and cooked fish products. However, most of the assays developed have either suffered from cross-reactions with other non-target fish species or have only been tested against a limited number of species. iELISAs based on either mAbs or pAbs have been developed to target the substitution issues affecting grouper (*Epinephelus guaza*) and wreck fish (*Polyprion americanus*) (Asensio *et al.* 2003*b*, *c*), and sole (*Solea solea*) (Cespedes *et al.* 1999), which are frequently adulterated or replaced with less desirable and less expensive fish species in their raw state. The species-specificity of the developed antibodies was determined by testing against a total of 12, 20, and 10 fish species, respectively. An iELISA employing mAb (Asensio *et al.* 2003*a*) was developed to distinguish grouper and wreck fish from 18 other species, either in their native state or when heat-treated (heated for 85°C for 10 min or 121°C for 20 min). Unfortunately, the anti-grouper mAb developed cross-reacted with wreck fish. This is a common problem; a mAb-iELISA developed for the authentication of raw and cooked red snapper (*Lutjanus campechanus*) (Huang *et al.* 1995) was unable to differentiate red snapper from other snapper samples and also weakly cross-reacted with virtually every sample tested (fish and meat).

As mentioned earlier in connection with meat species identification, iELISA is not a user-friendly assay. It is primarily used in the early stages of developing a new assay to reveal the reactivity between the antigen and the antibody during antibody characterization and has

never been the choice of final assay for unknown sample analysis or a commercial assay. Other immunoassays have also been developed for the speciation of fish that has undergone other processes, for example in the substitution of whole sardines (*Sardina pilchardus Walbaum*) with other fish in the canned product (Taylor and Jones 1992), the substitution of Alaska pollock with cheaper fishes in surimi (Reed and Park 2010), and the substitution of smoked salmon or trout with stained smoked bream (Carrera *et al.* 1997). A study by Reed and Park (2010) found that while a newly developed pAb-based competitive ELISA (cELISA) was capable of detecting Alaska pollock in crabsticks that had been cooked at 90°C for 3 min, there was also a cross-reaction with Pacific whiting and threadfin bream. However, there was no cross-reaction with dried egg white, whey protein concentrate, or soy protein isolate, all of which are common ingredients in crabstick formulations.

More recently, an sELISA employing a pair of mAbs (F7B8 and T7E10) (Hsieh *et al.* 2009) was developed for identification of imported raw and cooked farm-raised and Asia-imported Pangasius catfish, primarily tra or swai (*Pangasius hypophthalamus*) and basa (*Pangasius bocourti*), which are commonly used as substitute for domestic catfish, grouper, and other such high-priced fish in dishes served at American restaurants. This sELISA is based on the detection of a thermal-stable 36 kDa muscle protein and could sensitively detect 0.5% or 0.1% of basa or tra adulteration in mixed crab meat products, respectively, without any cross-reactivity among a total of 70 common food fish, shellfish, meat species, and five common food proteins tested. The same study reported the development of an additional mAb capable of differentiating between two Pangasius species, basa and tra. pAbs being raised against this same 36 kDa antigenic protein and directed to bind to the conserved region of all fish species have recently been used to develop an sELISA that positively and strongly reacts with all the fish species tested without cross-reactivity with non-fish substances, and is able to detect fish that has undergone a wide range of processing such as cooking, smoking, salting, drying, and canning (Chen and Hsieh 2014). This type of assay is clearly of value for those responsible for enforcing the Food Allergen Labeling and Consumer Protection Act (FALCPA) (FDA 2004) to protect fish-allergic individuals, as fish is one of the "big eight" allergenic foods.

A number of researchers have looked at the problem of the species identification of shellfish. Fernandez *et al.* (2002) utilized pAbs raised against clam soluble proteins as iELISA probes for the identification of three clam species: *Ruditapes decussatus* (grooved carpet shell, a culinary delicacy that sells for twice the price of other clams); *Venerupis rhomboides* (yellow carpet shell); and *Venerupis pullastra* (pullet carpet shell). Besides the fact that test samples had to be analyzed separately with these three antibodies, the assay was tested only on raw clams. An *et al.* (1990) employed mAb 4H2-10D3, which recognizes a 17.7 kDa component of rock-shrimp-specific protein M, in iELISA to distinguish between raw and cooked pink and white rock shrimp, reporting that the assay was able to distinguish rock shrimp from 23 other commonly consumed seafood and meat samples.

Although as the above discussion shows, a number of assays have been developed for the species identification of both raw and processed fish and shellfish, the species-specificity of the antibodies utilized as probes in most of these immunoassays has been determined by testing against only a few species. This is largely due to the huge number of food fish species that are available worldwide, which makes it highly challenging to identify species-specific markers for single fish species identification with any certainty

because it is simply not feasible to test the assay against every single species. However, once an assay has been developed, testing as many of the commonly used species available in a particular region as possible is critically important to ensure the species specificity of the assay and avoid the issue of false positives. This can be a major problem: Reed and Park (2010) noted that when they tested the anti-Alaska pollock antibody that was isolated in their study against only 3 species (Pacific whiting, Alaska pollock, and threadfin bream), it cross-reacted with all three. Certainly, if these assays are to be commercialized, the importance of testing large numbers of species to determine the species-specificity of these developed assays cannot be overemphasized. Although they are not without their own drawbacks, DNA-based assays and IEF have therefore had to be used rather than immunoassays for fish species identification because of the unavailability of effective specific immunoassays.

10.5 Fruit Juices

Due to the rampant falsification of fruit juices (Fry *et al.* 1995), the quality control of these products has become a critical concern for many consumers. In the past, falsification of fruit juices involved simple dilution with water or the addition of cheaper artificial ingredients such as sugars, acids, and colorants, but as more information about the chemical composition of fruit juices became known, falsification methods have become more sophisticated and now often involve the substitution of fruit juices with pulp and peel extracts or with cheaper fruit juices (Mears and Shenton 1973). Routine methods for fruit juice authentication are not specific enough to detect and quantify the presence of fruit juice components originating from waste materials (peel and pulp) or other fruit juices (Sass-Kiss and Sass 2000). Instead, analytical methods such as protein-based immunoassays that have the capacity to be not only species-specific (distinguishing one juice from another) but also tissue-specific (distinguishing between exocarp, mesocarp, and endocarp), are promising for fruit juice authentication. Differences in the protein composition of the exo-, meso-, and endocarp of citrus fruits (Clements 1966) and in the species-specific immunogenicity of orange and lemon juice (Cantagalli *et al.* 1972) have already been reported in the literature, leading researchers to investigate the possibility of authenticating fruit juices using immunoassays, among other instrumental analytical methods.

From as early as the 1970s, the literature has contained reports of immunoassays in the form of immunodiffusion being developed for fruit juice authentication (Cantagalli *et al.* 1972; Firon *et al.* 1979). More recently, the western blot technique has begun to be utilized for the quality control of fruit juices using species-specific and tissue-specific pAbs as probes to detect peel contamination in fruit juices (Sass-Kiss and Sass 2000, 2002). The use of western blot, which is a relatively complex and slow procedure, makes these assays unsuitable for the routine quality control of fruit juices, so the authors suggested that the development of an ELISA using pAbs as probes for orange juice authentication might be a useful avenue for future research. This would not be straightforward, however, as commercial fruit juices are often subjected to processes such as ultra-pasteurization that destroy the antigenic protein, so the intensity of the antigenic proteins that form the basis of peel contamination may be reduced to such a low level that they do not show a clear result.

As western blot and immunodiffusion are both unsuitable for the routine quality control of fruit juices, no effective immunoassay has yet been developed for verifying juice authenticity. The very few immunoassays available that have focused on the quality control of citrus fruits, with a particular emphasis on orange juice. However, non-citrus fruit juices are equally prone to misrepresentation and therefore also deserve attention. Future efforts should therefore be directed not only at developing faster and more user-friendly immunoassays for citrus fruit authentication, but also for the authentication of non-citrus fruit juices.

10.6 Botanical Origin of Honey

Many of the properties of honey that give it its distinctive character, such as sweetness, color, and geographical location, are associated with its botanical origin (Perezarquillue *et al.* 1995; Schmidt 1996) and honey from particular geographical locations and/or unifloral honey (honey produced predominantly from the nectar of a single plant species) command premium prices. Mislabeling of honey through mixing honey originating from different floral or geographical locations is a common fraudulent practice (Cajka *et al.* 2009).

Efforts to authenticate the botanical origin of honey are important for consumers because of the economic and health issues associated with misrepresentations. Traditionally, the botanical origin is determined through melissopalynology, a process that involves the microscopic analysis of the pollen that remains in the honey after filtration or centrifugation (Lutier and Vaissiere 1993). However, this requires an experienced analyst with prior knowledge of pollen morphology if it is to achieve dependable results. In addition, the effectiveness of melissopalynology is contingent on the amount of pollen present, which may be a problem for processed honey as filtration depletes the pollen population. A number of methods involving the multivariate analysis of the sensory and physico-chemical parameters of honey have been developed in the quest to provide an alternative method to melissopalynology for determining the floral source of honey, described in a review article by Kaskoniene and Venskutonis (2010). However, these methods not only involve the use of expensive equipment that requires an experienced analyst, but must usually be combined with chemometrics for more statistically reliable results. The parameters that are being used to establish authenticity also change with time and storage conditions (Wunderlin *et al.* 1998; Sancho *et al.* 1992; Castro-Vazquez *et al.* 2008; Saric *et al.* 2012), so must be treated with caution in the interpretation of results when honey samples are not fresh.

Although the proteins that originate from the hypopharyngeal gland of bees or from the plant nectar are present only as minor components of honey (0.05–0.79%; Azeredo *et al.* 2003), they have been found useful as a marker of the botanical origin of honey using immunoassays (Baroni *et al.* 2002, 2004; Won *et al.* 2009) and other analytical methods such as fast peak liquid chromatography (Iglesias *et al.* 2006), gel filtration chromatography (Mohammed and Azim 2012), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Wang *et al.* 2009), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-AGE) (Marshall and Williams 1987; Lee *et al.* 1998). Anti-pollen pAbs developed against sunflower (*Helianthus annuus*) and eucalyptus (*Eucalyptus sp.*) pollen have been utilized in an

immunoblot to differentiate sunflower honey from eucalyptus honey and other unifloral honeys (Baroni *et al.* 2002). Due to the laborious nature of immunoblot, which makes it unsuitable for routine analysis, these researchers went on to utilize anti-sunflower pollen antibodies as a probe in cLEISA to detect and quantify sunflower pollen in honey samples (Baroni *et al.* 2004). Although their results showed a significant correlation ($R > 0.975$; $p < 0.001$) with the results obtained by melissopalynology, the high detection limit (10%) restricts its usefulness. Won *et al.* (2009) used pAbs raised against major proteins in honey produced by *A cerana* (used in the production of Korean Native Honey) and *A mellifera* (used in the production of imported European Honey) in iELISA to discriminate between the two and also to detect the adulteration of highly prized Korean honey with the much cheaper European variety. However, as with the previous method, the anti-European honey and anti-Korean honey iELISAs both suffered from a high detection limit of 20%. Although various formats of immunoassay, namely immunoblot, iELISA, and cELISA, have all proven useful in determining the floral origin of honey, these assays can identify the type of pollen present but cannot provide any information regarding whether other pollens that do not bind with the particular antibody used may also be present in the product. In order for immunoassays to play a useful role in the authentication of the floral origin of honey, a myriad of antibodies targeting pollens from different plants must therefore also be developed, which may not be feasible. Perhaps for now the emphasis should instead be on developing antibodies against those pollens that are used in the making of premium honey. Also, given the high detection limits observed for these assays, it might be helpful if future work was directed towards the development of sELISAs that improve the assay sensitivity.

10.7 Irradiated and Genetically Modified Foods

The link between diet and physical well-being is now understood by many and the demand for foods produced naturally has increased considerably as a result. Consumers are increasingly demanding organic foods and shying away from foods that have been produced by genetic manipulation, produced using pesticides, or subjected to irradiation, as they believe such foods could cause them harm. To protect consumers, who have a right to information and clear labeling to help them make informed choices, both the European Union (EU) (Directive 1999/2/EC) and the US Food and Drugs Administration (FDA) (21 CFR 179) require that irradiated foods be labeled as such. However, whereas the EU requires every irradiated ingredient (e.g., spices) in a product to be clearly indicated on the label, this is not the case in the US. The EU has also made it mandatory to label genetically modified (GM) foods (Directive 2000/13/EC), while the US does not require special labeling for GM foods unless the GM food is significantly different from its conventional counterpart (57 FR 22984). The relatively lax and ambiguous regulations in the US have created an environment that is conducive to the misrepresentation of irradiated or GM foods, so there is an urgent need for reliable methods for detecting such foods. Although methods relying on various techniques for the detection of irradiated or GM foods have been developed and adopted by different regulatory bodies, the discussion below focuses specifically on the use of immunoassays as analytical tools to detect irradiated or GM foods.

10.7.1 Detection of Irradiated Foods

When foods are subjected to irradiation, the ionizing radiation induces changes in the DNA content of the food. In itself this is not unusual; many changes in the DNA may similarly be induced by traditional food treatments such as heating or freezing. This notwithstanding, there are some compounds that are unique to irradiated foods and have therefore been suggested as markers for their identification. Dihydrothymidine (DiHT, $C_{10}H_{16}N_2O_5$), which is produced as a result of the reaction between thymidine and free radicals due to the ionization of molecules surrounding the DNA under anoxic conditions (Deeble *et al.* 1990), has been suggested as a potential marker for irradiated foods. Accordingly, antibodies raised against DiHT have been used as probes in cELISA to detect irradiated wheat (Deeble *et al.* 1994) and prawns (Tyreman *et al.* 1998, 2004). A competitive ELISA was developed for these studies because, unlike protein antigens, the molecular size of DiHT (MW 244) is too small to construct a sELISA. Deeble *et al.* (1994) reported that when calf thymus and wheat samples were irradiated under anoxic conditions, the cELISA could detect DNA extracts in samples irradiated at doses as low as 0.04 kGy for calf thymus and 2 kGy in the case of wheat. However, when samples were irradiated in the presence of oxygen, which is likely to be the case in real-world samples, the assay showed no response because DiHT is only formed under anoxic conditions, limiting the potential utility of immunoassays targeting DiHT.

2-alkylcyclobutanones (2-CBs) have also been identified as specific markers for the identification of lipid-containing foods such as pork, liquid whole egg, and chicken that have been subjected to irradiation (Boyd *et al.* 1991; Crone *et al.* 1993; Stevenson 1994). Elliott *et al.* (1995) were the first to develop an iELISA to detect irradiated chicken meat using pAbs raised against conjugated 2-CB. In their study, pAbs raised against 2-CBs as probes in cELISA were able to detect chicken irradiated at doses ranging from 2.5 to 10 kGy with a detection limit of 0.064 μg of 2-CB per gram of extracted chicken fat. The cELISA developed also showed a good correlation ($r^2 = 0.93$) with the results obtained from gas chromatography mass spectrometry (GC-MS), which at the time was the methodology of choice for the detection of 2-CBs. In a later study, Nolan *et al.* (1998) also raised pAbs against 2-CB to detect irradiated liquid whole egg (LWE) using ELISA (unspecified format). Although initially the ELISA could detect 2-CBs in LWE samples irradiated with doses ranging from 0.5 to 10 kGy, the results were not reproducible; this was ascribed to the possibility that the 2-CBs present in LWE were not able to bind to the pAbs due to the formation of protein aggregates during irradiation, which entrapped the 2-CBs. Compared to the previous method (Elliott *et al.* 1995) involving lipid extracts, this method had the advantage of allowing samples to be analyzed directly, thereby shortening the analysis time.

In summary, antibodies raised against two radiolytic products, DiHT and 2-CB, have been found useful in distinguishing irradiated foods from non-irradiated foods. Given that most foodstuffs contain DNA, these methods could potentially be applied to other foods. However, since the amount of DiHT or 2-CB formed is proportional to the dose of irradiation, these assays need to be validated against products such as white potatoes that are required to be subjected to low irradiation doses (0.05–0.15 kGy) and also products containing irradiated ingredients such as spices that are used in relatively small amounts. Lastly, as novel processing technologies such as pulsed electric fields, high-pressure processing, and ohmic heating become a routine part of food processing,

these radiolytic products that are considered markers of food irradiation may also be found in food subjected to these novel processing technologies. More studies are therefore needed to confirm that DiHT and 2-CBs are indeed unique to irradiated foods. Identifying other radiolytic indicators for future immunoassay development for irradiated food detection would be both helpful and feasible.

10.7.2 Detection of GM Foods

According to data published by the International Service for the Acquisition of Agri-Biotech Applications (ISAAA), the world crop acreage planted with GM crops has been increasing steadily since the commercial cultivation of transgenic crops began in the early 1990s, reaching 174 million hectares as of 2013, 5 million hectares more than just a year earlier (ISAAA 2013). Given this growth in the rate at which GM crops are being cultivated, methods for monitoring their presence in food are needed more than ever to meet consumer demand for information and protection. A great deal of progress has been achieved in the development of methods such as polymerase chain reaction (PCR), mass spectrometry, chromatography, near-infrared spectroscopy, herbicide assays, and immunoassays (Tripathi 2005) for the detection of GM foods. Commercial immunoassay kits are particularly desired because they are convenient, fast, and can easily be performed in the field (Kumar *et al.* 2010).

Multiple problems associated with the use of chemical insecticides have led to the development of pest-resistant transgenic crops carrying insecticidal proteins. The soil bacterium *Bacillus thuringiensis* (Bt) has proven to be an outstanding source of insecticidal protein genes and has therefore been widely used for transgenic expression in a variety of crops (Kumar 2003). In order to detect GM plants, the insecticidal protein Cry1Ab, an endotoxin produced by Bt, and the Vip-S protein (a recombinant Vip type protein which is an exotoxin produced by Bt) have been targeted as potential markers in Bt gene modified plants for immunoassay development. A competitive capillary electrophoresis immunoassay technique that uses a mAb raised against Cry1Ab with laser-induced fluorescence (Giovannoli *et al.* 2008) and a chemiluminescent sELISA employing two commercial Cry1Ab-specific antibodies (a mAb paired with pAb) (Roda *et al.* 2006) have been developed to detect transgenic maize that expresses the insecticidal protein Cry1Ab. Kumar *et al.* (2010) have developed a rapid LFS assay using pAbs for the detection of transgenic plants (in this case brinjal or eggplants) carrying the Vip-S protein. All of the methods mentioned so far have focused on the detection of non-processed GM foods, but researchers are now beginning to extend this to include foods that have been processed in some way. For example, Diaz *et al.* (2002) and de Luis *et al.* (2009) utilized a commercial ELISA kit (pAb-based Cry9C ELISA Plate Kit by EnviroLogix Inc., Portland, ME) and pAb-based sELISA to detect the insecticidal proteins Cry9C and Cry1Ab in processed transgenic maize, respectively. Both groups reported that processing drastically reduced the levels of these proteins to the point that they became undetectable.

To date, the emphasis has clearly been on the detection of GM foods carrying the gene for the Bt insecticidal proteins. However, given that there are now a number of other GM foods carrying other traits that are becoming commercially available, or are about to be commercialized, future research should be directed towards the development of immunoassays targeting these other GM foods. In addition, since the target

proteins for these immunoassays are expressed by the inserted gene, they may be affected by the same problems noted above due to food processes such as heating. Efforts should therefore also be directed towards the generation of antibodies against denatured proteins or antibodies that bind to heat-stable regions on the peptide to ensure they are effective against the target protein in processed GM samples. Using more sensitive labels to further amplify the reaction signals could also help overcome the problem of the low quantities of target antigens present in processed GM foods.

10.8 Conclusions

The globalization of our food supply has created an ideal environment for food fraud. The situation has been worsened by the worldwide recession, which not only tempts manufacturers to indulge in fraudulent practices but the resulting budget constraint also prevent regulators from implementing effective control strategies. Rapid, fast, economical, high-throughput, easy-to-use, and sensitive immunoassays have therefore become the diagnostic tool of choice for food authentication, as evidenced by the myriad of commercial immunoassay kits now available. In particular, single-step self-performing assays such as LFS have the added advantage of allowing samples to be analyzed without the need for additional expensive equipment, which not only allows field-testing of samples but also provides valuable diagnostic tools in developing countries where resources in terms of equipment and trained analysts tend to be limited. However, as with any technology, immunoassays suffer from limitations such as cross-reactivity, the fact that they are often ineffective in processed foods, and the unavailability of commercialized test kits for many types of food products. As identified marker antigens and specific antibodies in a wide range of processed and unprocessed food materials are not always available, so far immunoassays for the detection of food authenticity have been restricted to a few types of foods (mainly food of animal origin). Future assay development would be wise to place a stronger emphasis on exploring thermally and chemically stable markers and developing high affinity antibodies, preferably mAbs. Effective immunoassays have an enormous market potential and will eventually serve as potent weapons in the battle to ensure food authenticity and safety for consumer protection.

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11

Sensory Analysis

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11.1 Introduction

The life-long learning of any human being starts when it instinctively differentiates the pleasant sensations from the unpleasant ones.

The dependence on our five senses has been decisive for our survival since ancient times. Nowadays, this dependence is still determinant because our senses direct our brain towards a specific product, most times in an unconscious way. Moreover, today's consumers are more knowledgeable and demand products not only safe and nourishing but also with a high organoleptic quality to satisfy their senses; sensory analysis has therefore become a necessity in many industrial sectors.

Sensory science studies both the physical and the psychological responses of our senses to the characteristics of a product, providing valuable information. Indeed, despite major advances in the field of electronic noses, tongues or eyes, no one can yet supply the traditional sensory analysis. However, to get reliable results in sensory analysis, a well-trained panel of assessors and suitable statistical tools to interpret their evaluations are needed, as well as precise analysis protocols, common sensory vocabulary and standardised test rooms.

There are multiple applications of sensory analysis, but it is essential in the foodstuff field. Undoubtedly, the ability to provide a scientific evaluation and a quantitative measurement of the appearance, odour, taste and texture of any food is critical to set performance standards and to evaluate changes or improvements in today's competitive business environment (Iannario *et al.* 2012).

11.2 Organoleptic Evaluation and Food Quality

The physiology of our senses has been widely studied as well as the perception process, the adaptation to different stimuli and the interactions among them. In all cases, when the receptor cells of any of the five senses are stimulated by a chemical or physical agent, they establish a synaptic connection with the nerves and send an electrical signal to the processing centre of the brain, where this signal is translated into a sensation (Sancho *et al.* 1999).

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This sensation can be measured as a response by applying one of the four sensory analysis categories: sensitivity, quantity, quality or hedonic value (Pangborn 1984).

Food quality is a complex concept because it depends on the consumer's expectations and how the product consumption matches these expectations. This consumer-based process is related not only to who is performing the evaluation but also to a wide range of situational and contextual factors (place, time, temperature, social setting); this evaluation can therefore be considered as an abstract construct of a multi-dimensional nature (Verdú Jover *et al.* 2004). Sensory analysis should therefore be considered from a physical but also from a psychological point of view.

11.3 Human Sensory Panels: Response and Subjectivity

Sensory analysis allows the description of profiles, the determination and quantification of the organoleptic characteristics of a product, and the monitoring of quality control. All these applications indicate that a human sensory panel is necessary to check the specific properties of the standard samples against the properties of real products (from different batches, runs, treatments) to detect differences. In all cases, the response obtained has to be properly evaluated because the sensory evaluation varies both among panellists – they are individuals with different sensitivities, preferences and product knowledge – and within a given panellist across time, depending on his fatigue, stress and health (Sancho *et al.* 1999). Moreover, the detection of any sensory stimuli does not follow a step function in which detection jumps from 0 to 100% at some special value. Indeed, the probability of detection gradually increases with the intensity of a stimulus, so the sensory responses should be treated as a statistical concept. Panellists are therefore required to have a reasonable level of sensory perception, commitment and motivation, but they should also be trained in the use of standardised and systematic sensory methods in order to obtain reliable results.

Apart from these inherent working problems related to panellists' subjectivity and to the variability of the responses over time, there are other important weaknesses to be considered when applying sensory analysis. It is expensive and time-consuming (both when training the sensory panel and when performing the sensory analyses), too cumbersome to be introduced as a routine procedure and not suitable for on-line monitoring (Ghasemi-Varnamkhasti *et al.* 2010).

Since sensory analysis provides very valuable and unique information, the development of techniques that imitate the human sensory response but avoid the mentioned drawbacks is therefore the objective of many current studies.

11.4 Instrumental Sensory Analysis

11.4.1 Looking for Objectivity

Many efforts are being made to develop instrumental techniques that could recognise objectively and quickly specific sensory perceptions in the same way as an expert tasting panel does. The following sections describe the state of art and the new trends that are emerging in this area.

11.4.2 Gas Chromatography-Olfactometry (GCO)

The aroma is one of the most decisive organoleptic properties in food evaluation. It is due to several different chemical volatile compounds whose origin can be the raw material, the different reactions taking place during the production or the processing, or the evolution of the product itself during its aging. Moreover, this large number of volatile compounds presents different physical and chemical properties and a wide range of concentration levels. One of the major challenges is therefore obtaining a representative aroma extract that ensures the aroma characterisation success. This is why several different pre-treatment techniques have been applied to isolate the foodstuff aroma, such as static headspace, dynamic headspace, liquid–liquid extraction or solid phase microextraction, among others (Delahunty *et al.* 2006; d'Acampora Zellner *et al.* 2008; Plutowska and Wardencki 2008; Brattoli *et al.* 2013).

However, the real odour-active compounds among the entire volatile fraction have to be identified and quantified to characterise the aroma of a food. This implies the use of an analytical tool able to correlate sensory and instrumental measurements: the gas chromatography-olfactometry (GCO) technique.

11.4.2.1 GCO Device: How it Works

Gas chromatography-olfactometry (GCO) is based on the sensory evaluation of the different compounds eluting separately from a chromatographic column to determine which are odour-active, providing instrumental and sensory results. For this purpose, the flow coming out from the chromatographic column is split to allow a simultaneous detection of the eluted analytes by the nose of a properly trained person through a special plug called 'odour port' and by a conventional chemical detector such as a flame ionisation detector or a mass spectrometer (Fig. 11.1). By these means it is possible to perceive, describe and measure the duration and intensity of the eluted odorants in parallel with the drawing of a chromatogram (Acree 1997).

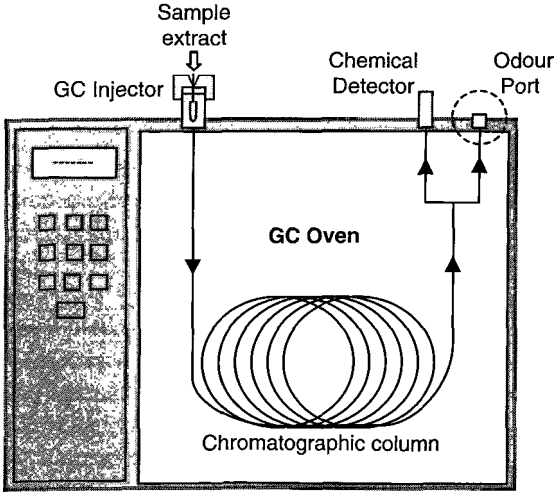


Figure 11.1 GCO device.

11.4.2.2 Olfactometric Techniques

The first sensory evaluation of the contribution of a compound to the whole aroma of a food was carried out in 1963, when Rothe and Thomas defined the 'odour aroma value' (OAV) as the ratio between the concentration of the compound in the sample and its threshold sensory perception (Rothe and Thomas 1963). Those compounds with an OAV >1 will therefore have an influence on the aroma perception. Lately, with the introduction of the GCO technique (Fuller *et al.* 1964), this concept was also considered when developing different olfactometric techniques to collect and process the GCO data. These techniques can be classified into four categories according to the measurement carried out: dilution analysis techniques; time-intensity techniques; posterior intensity techniques; and detection frequency techniques. Their advantages and disadvantages have been widely studied in different matrices (van Ruth 2001; Delahunty *et al.* 2006; d'Acampora Zellner *et al.* 2008; Plutowska and Wardencki 2008).

11.4.2.3 Applications

Although the GCO could be considered as a mere screening technique to differentiate volatile compounds into odour-active and non-odour-active compounds, the large number of studies that can be found in the literature shows the huge potential of this technique (Grosch 1993, 2001; van Ruth 2001; Delahunty *et al.* 2006; d'Acampora Zellner *et al.* 2008; Plutowska and Wardencki 2008; Ebeler and Thorngate 2009; Rouseff *et al.* 2009; Brattoli *et al.* 2011; Croissant *et al.* 2011; Sefton *et al.* 2011).

The most common application in food analysis is the characterisation of aroma profiles to describe the different aromatic qualities by identifying the compounds responsible for the whole aroma. This process provides odour profiles of any foodstuff. Moreover, some products have aromatic specificities that give them an added value. This is the case for those products from a certain region, produced using singular raw materials or produced using specific technologies.

Other GCO applications are related to the continuous and increasing demand for those compounds (natural or synthetic) reproducing the aromatic sensations produced by specific foods. In these cases it is necessary to determine the so-called 'key compounds' or 'character impact aroma compounds', those odorants that provide the aroma essence of a particular product due to its higher odour aroma value. These studies are rather complex because food aroma is usually due to the interaction of several substances with character impact aroma.

Another group of aromatic substances that can only be determined by GCO are the so-called 'off-flavours'. Their identification becomes very important because they modify the original foodstuff aroma by introducing undesirable aromatic notes due to changes in the concentration of some odorants, the presence of compounds that are not normally present or the loss of key compounds.

All these applications make the GCO a good quality control tool allowing the aroma evolution to be traced all along the foodstuff production process. However, the challenge of detecting trace amounts of highly odour-active compounds in complex food matrices has promoted the development of more sensitive methods. Recent studies in the sensory field are focused on the use of two-dimensional gas chromatography (GCxGC) to enhance the results obtained with the single GC-O systems (Chin and Marriott 2015; Cordero *et al.* 2015).

11.4.3 Electronic Nose

The human sense of smell acts as a chemodetector that continuously monitors changes in the aroma composition (Swiegers *et al.* 2005). This sensory system is the most complex because of its great sensitivity and wide range of different perceptions, as it enables the detection and discrimination of hundreds of low-molecular-mass organic compounds almost instantly. Nowadays, one of the most important challenges in food sensory science is the development of electronic noses (e-noses) with the aim of emulating the human sense of smell.

11.4.3.1 Emulating the Sense of Smell

The different e-noses developed have been designed on the basis of how the human olfactory system operates. The volatile compounds interact with a detection system that generates a set of signals containing the information related to the aromatic characteristics of the sample. These responses are then processed with chemometric techniques to compare and/or classify as fast as possible the samples according to their volatile composition, in the same way as our sense of smell.

11.4.3.2 Types of Electronic Noses: Instrumental Aspects

The e-nose comprises three elements: the sample handling system; the detection system; and the data analysis system. The most common sampling technique is the static headspace (SHS), because of its simplicity and speed. If its sensitivity is not high enough, some concentration techniques can be added, although they increase the time of analysis.

The two more usual detection systems are those based on gas sensors and those based on mass spectrometry.

Gas sensors operate on the basis of physical and/or chemical interactions of the volatile compounds with an array of solid-state gas sensors; each one of them has a partial specificity and gives a fast response to the interaction. The most used sensors are metal-oxide semiconductors (MOS) that operate at high temperatures and those called conducting polymer sensors (CP) that operate at room temperature. The CP sensors present a better selectivity than MOS sensors and they are less susceptible to poisoning, although the polymer response is sensitive to humidity. This is an important problem when analysing foodstuffs where water is one of the major components of the sample headspace. Since the use of an array of the same type of gas sensors does not give a suitable signal when dealing with complex matrices, the studies are increasingly focused on the combination of different types of gas sensors to produce hybrid systems. This implies a normalisation of the different sensor outputs (Strike *et al.* 1999).

The e-noses based on mass spectrometry (MS) were developed later (Dittmann *et al.* 1998). They are based on the simultaneous ionisation and fragmentation of all the volatile compounds in the ionisation chamber of a mass spectrometer. Each fragment ion (m/z ratio) abundance resembles the response of different sensors, so the number of sensors is much larger than in the gas-sensor-based e-noses. The sensitivity and even the selectivity of this instrument can be easily improved using the selected ion monitoring (SIM) mode. However, one of the most important problems of this type of e-nose is its signal instability over time due to different causes such as gradual fouling of the ion

source, vacuum instability, aging of the ion multiplier or change of a filament. To solve these problems, different strategies such as the internal standardisation (Marsili 2000), the standard gas addition technique (Pérès *et al.* 2002) or the calibration transfer (Vera *et al.* 2010*a*) can be used.

Other techniques that have also been used as e-nose detection systems are the quartz crystal microbalances (QMB), the surface acoustic wave transducers (SAW) or the fibre-optic chemical sensors.

11.4.3.3 Applications of E-Noses in Food Analysis

As volatile compounds can provide an accurate food fingerprint, the main area where e-noses have been used is food control, not only in the analysis of raw materials but also testing them throughout their corresponding chains of production and conservation.

Complete reviews about e-nose applications in food analysis have been published during the last decade (Deisingh *et al.* 2004; Martí *et al.* 2005; Baldwin *et al.* 2011; Wardencki *et al.* 2013; Loutfi *et al.* 2015). Authenticity assessment and fraud control, both closely related to the monitoring of food volatile profiles, are undoubtedly the areas where e-noses have been most widely used.

In recent years however research has been directed towards the emulation of human noses, trying to wholly calibrate e-noses from a sensory point of view as a consumer or an expert routinely does with the food he/she tastes. In this way, e-noses can provide objective information about measures otherwise subjective, helping food industries and regulators to establish instrumental organoleptic profiles of the tested products (Lerma-García *et al.* 2010; Santos *et al.* 2010; Apetrei *et al.* 2012; Ouyang *et al.* 2014).

11.4.4 Electronic Tongue

Sense of taste involves examining a particular product by means of touch and taste. Although the tactile perception is widely distributed throughout the body, it only converges with the sense of taste into our mouth, so this perception is almost exclusive for food and drink. The need for robust and fast instruments to evaluate the perceived tastes (sweet, sour, bitter, salty and umami) and other mouth-feel perceptions (temperature, astringency, viscosity, burning, touch, body, prickling or pain) when consuming any foodstuff is therefore evident.

11.4.4.1 Emulating the Sense of Taste

An electronic tongue (e-tongue) tries to emulate our biological tasting model, so it is necessary to design a sensor array system able to provide different signals after the interaction with all the different chemicals related to the sense of taste. Since there are several interactions, this system provides a huge amount of data that requires the use of chemometric techniques for multivariate analysis, as will be discussed in Section 11.5.

11.4.4.2 Types of Electronic Tongues

The e-tongue consists of a system of non-selective (or poorly selective) chemical sensors with a complementary program for the pre-treatment and the processing of the signals (data). As in the e-nose, a sampling system is also needed but, in this case, adapted to non-volatile compounds.

The main types of commercially available e-tongues are based on (1) potentiometric and (2) voltammetric techniques, and also on (3) optical sensors, mainly the Fourier transform infrared spectroscopy (FT-IR).

1) *Potentiometric systems*: The first studies on e-tongues used ion-selective electrodes. However, the first commercialised instrument was based on a system of non-selective sensors composed of different lipid/polymer membranes. Later, the e-tongue term was introduced by using a system of chalcogenide glass sensors (Legin *et al.* 1996). Since then, the systems have been improved to implement these instruments in food quality control (Legin *et al.* 1997) and use them for quantitative measurements of chemical species in complex solutions (Gallardo *et al.* 2005). The challenges to improve the results (sensitivity, robustness, etc.) and to reduce the sample consumption led to the development of miniaturised potentiometric sensors based on silicon technology and membranes of polyvinyl chloride (PVC) that can be doped with Co and Pt-porphyrinates (Verrelli *et al.* 2007). Moreover, the use of some surfaces as a set of non-specific potentiometric sensors allowed the development of an array containing different electrodes (Gil *et al.* 2008).
2) *Voltammetric systems*: When working with voltammetric e-tongues a set of voltage pulses are applied over two electrodes (one of them is the reference electrode). Those electro-active compounds of the sample reacting to that potential are reduced or oxidised and the current aroused can be measured. These systems show high selectivities, high signal-to-noise ratios and low detection limits, and can be operated in different measurement modes. However, they do have some drawbacks, including their temperature dependence, the drifts in the sensor response due to the surface alteration, and their applicability, which is limited to redox-active substances (Escuder-Gilabert and Peris 2010).
3) *Other types of electrochemical electronic tongues*: Many other techniques have also been used as sensors in the e-tongue detector systems, including: the impedance, the amperometry, the resonance frequency, the surface acoustic wave (SAW), the quartz crystal microbalance (QMB) or the Fourier transform infrared spectroscopy (FT-IR). This latter technique deserves a special mention because of its applicability to a large number of compounds, but also because of the quality of information it provides. FT-IR is based on the stretching, contracting and bending of the chemical bonds that the infrared light promotes on most organic chemicals. Together with this wide applicability, the e-tongues based on these systems provide information about the real composition of the sample because the infrared energy emitted or absorbed depends on the structure of the molecules present in the sample. Moreover, the ATR-FT-IR (attenuated total reflection Fourier transform infrared) increases the range of applications because it allows the analysis of both solid and liquid samples; it has therefore become a very useful tool in food analysis.

Although e-tongues were developed after e-noses, the increasing use of these devices is evident in the recent literature (Deisingh *et al.* 2004; Escuder-Gilabert and Peris 2010; Jain *et al.* 2010; Baldwin *et al.* 2011; Cosio *et al.* 2012; Tahara and Toko 2013; Wardencki *et al.* 2013; Bagnasco *et al.* 2014; Ha *et al.* 2015; Simoes Costa *et al.* 2015).

Because e-tongues can measure every dissolved substance (even volatiles), most of these studies are related to successful applications for monitoring taste compounds, assessment of food shelf-life, prediction of freshness or spoilage, or authentication of different foodstuffs such as waters, olive oils, wines, juices, meats, fish, fruits or dairy products. There exist both qualitative and quantitative applications, with quantitative analyses being especially significant since e-tongues achieve better sensitivities than humans.

As with e-noses, most of the applications developed allow taste-related properties to be measured. There is a trend in recent years to calibrate the devices against human panels, in order to reproduce the overall response they provide for a specific flavour (Apetrei *et al.* 2012; Ouyang *et al.* 2014).

11.4.5 Multivariate Data Analysis

Data generated in sensory analysis are multivariate by nature; either they come from panel tests or from instrumental techniques such as e-noses or e-tongues. Those multivariate data need to be processed with suitable multivariate data analysis methods. Multivariate data analysis in sensory science is a very broad topic, and excellent books and software have been written (Le and Husson 2008; Næs *et al.* 2011). However, in this section we will focus on multivariate analysis of sensory data for food authentication purposes.

First, it should be noted that the only food product whose quality is determined from sensory analysis (apart from other chemical parameters) is olive oil. Differentiation among the three quality categories – extra virgin, virgin and lampante – is performed on the basis of the scores for the fruity attribute (positive) and the scores for the negative attributes (defects), provided by an official test panel. Analysis of such data, however, is performed in a univariate way, using the median of all the panellists and comparing it with regulatory limits (European Commission 2013).

However, sensory data in food authentication have been used alone or in combination with other chemical, physical or instrumental measurements for characterisation or discrimination purposes. Instrumental measurements related to sensory characteristics of foodstuffs, such the those provided by e-noses or e-tongues, have also been used to assess authenticity. Finally, measurements from different instrumental techniques have been used to correlate them to sensory attributes.

Analytical instrumental specifications can therefore be defined to characterise the quality of a sample, in the same way as the tasting panel. Detailed analysis of instrumental variables, with the help of sensory information and, in some cases, GCO provides chemical information about the compounds responsible for the behaviour of a given attribute.

Different multivariate analysis techniques have been used depending on the purpose of the study. Principal component analysis (PCA) and cluster analysis are the two techniques used most often when the aim is to perform a preliminary exploratory analysis in order to assess the repeatability of the measurements, detect clear outliers and/or visualise the natural sample grouping.

When the purpose is to classify a sample into a predefined category, then two groups of techniques can be used: class-discriminating or class-modelling techniques. Class-discriminating techniques define delimiters between established classes so that problem samples are always assigned to one of the classes. Class-discriminating

techniques include linear discriminant analysis (LDA), k-nearest neighbours (kNN), partial least squares–discriminant analysis (PLS-DA), support vector machines (SVM) and artificial neural networks (ANNs), the latter specially suited when data show a non-linear behaviour.

Class-modelling techniques calculate a separate model for each established class, so that an unknown sample can be either assigned to that class or rejected. The most popular class-modelling technique is soft independent modelling of class analogy (SIMCA).

Finally, when the purpose is to predict sensory attributes from instrumental data, partial least squares regression (PLSR) is probably the most popular regression method. PLSR is especially suited for datasets with more variables than samples.

Three important issues have to be taken into account when developing multivariate models involving sensorial data, described as follows.

1) Quality and reliability of attribute sensory scores: for that, data from a trained test panel should be used whenever possible.
2) Number of samples: statistical inferences and conclusions from results obtained using datasets with limited number of samples should be taken carefully.
3) Model validation: the multivariate classification or prediction models developed should always be validated against an independent set of samples.

The application of multivariate statistical techniques for authentication, including sensory data, has been reviewed for agricultural products (Tzouros and Arvanitoyannis 2001) and for specific food commodities such as meat (Arvanitoyannis and van Houwelingen-Koukaliaroglou 2003), dairy products (Arvanitoyannis and Tzouros 2005), olive oil (Arvanitoyannis and Vlachos 2007), maize (Arvanitoyannis and Vlachos 2009), rice (Vlachos and Arvanitoyannis 2008) or coffee (Barbin *et al.* 2014).

Sensory data have been used alone or in combination with other chemical, physical or instrumental measurements for characterisation or discrimination purposes. Some examples include differentiation of potato cultivars from different geographical Greek regions using PCA, cluster analysis and LDA (Arvanitoyannis *et al.* 2008), discrimination between the five registered designations of origin (RDOs) of French virgin olive oils using LDA (Ollivier *et al.* 2006), species discrimination among three kinds of puffer fish using discriminant factor analysis (DFA) (Zhang *et al.* 2012), discrimination of beer samples of the same brand but produced in four different factories using LDA and data fusion strategies (Vera *et al.* 2011) or discrimination between sweet and bitter almonds using PLS-DA (Borràs *et al.* 2014).

Sensory attributes can also be predicted from instrumental measurements (Smyth and Cozzolino 2013). Some examples include the prediction of different sensory attributes and age from the near-infrared reflectance spectra of Cheddar cheeses stored for up to 9 months using PLS regression (Downey *et al.* 2005), the identification and prediction of sensory attributes of virgin olive oils (lampante and extra) from HS-MS data using SIMCA and PLS regression (López-Feria *et al.* 2008) or the prediction of the sensory grades of butter and butter oil from their proton transfer reaction mass spectrometry (PTR-MS) data using PLS-DA (van Ruth *et al.* 2007).

Finally, multivariate classification techniques have been increasingly applied for external (colour, texture, surface defects, etc.) food quality evaluation using computer vision systems, such as hyperspectral imaging (Huang *et al.* 2014).

11.5 Future Trends

Given the complexity of food there is no single instrumental technique capable of properly correlating the instrumental measurements with all the sensory attributes. Different instrumental techniques are therefore necessary to properly mimic the panel response. Independently of the techniques used, the term that better matches this field is 'instrumental sensometry': a tool that combines the results of the instrumental analytical techniques with the sensory evaluation made by a panel of tasters and with the chemometric analysis techniques in order to define the sensory specifications of a product quickly and objectively (Fig. 11.2).

The analytical responses of these instruments allow key attributes (positive or negative) of the organoleptic quality of foodstuffs to be evaluated based on the presence of different chemicals. By applying the suitable chemometric techniques, the chemical composition of the samples can be compared to find similarities or differences between them that will provide the correlations with the sensory properties (aroma, colour, flavour) defined by an expert taste panel. This will allow a purely instrumental and objective organoleptic profile to be defined.

Important efforts are already being made to characterise different parameters of the sample by multivariate analysis using different rapid analytical techniques. In this field, data fusion should be considered because of its potential and the quality of information provided. Some hopeful results have been obtained up to now by means of an electronic nose system based on mass spectrometry (HS-MS), an electronic tongue system based on mid-infrared spectroscopy with Fourier transform (FT-IR) and near-infrared (NIR) spectroscopy, and an electronic eye based on UV-Vis spectrophotometry (Vera *et al.* 2010*a*, *b*, 2011; Fragoso *et al.* 2011*a*, *b*; Borràs *et al.* 2015).

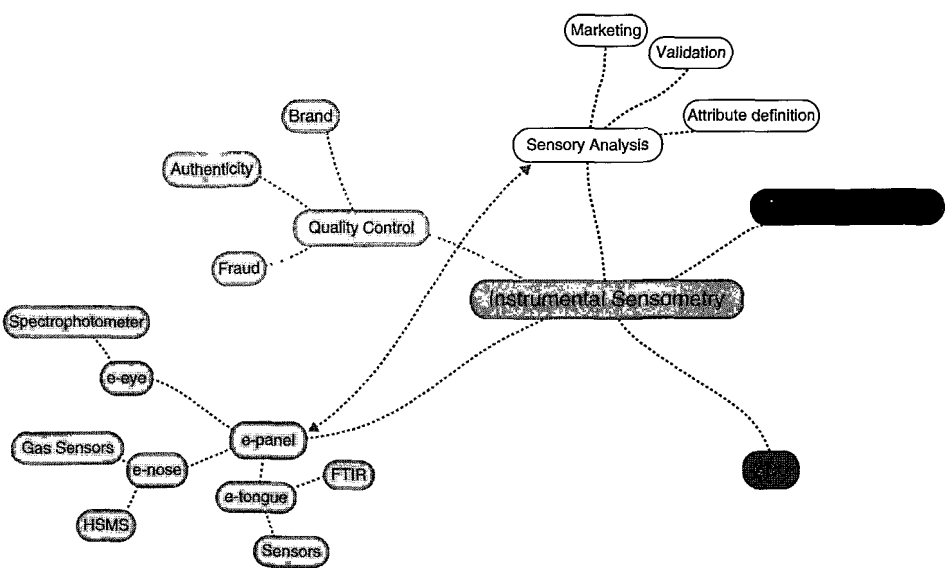


Figure 11.2 Mindmap showing the relations between the different agents involved in instrumental sensometry.

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12

MALDI Mass Spectrometry: A Promising Non-Chromatographic Technique

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12.1 Introduction

Over the last decades, safety and quality control in all food production processes have progressively attracted a great deal of attention. Indeed, the assessment of standard quality criteria has become a crucial step for authorities, companies and institutions involved in guaranteeing high-quality and safe products for end-user consumers (Chen 2014). To ensure food safety, the whole food production chain from raw materials to the operations of food processing, transport, manufacturing, storage and consumption, should be controlled.

To help consumers and agencies in this very tricky subject, many tools have been developed and disseminated. For example, the US Pharmacopeia Convention (USP), a non-profit scientific organisation that provides standards for quality assessment of medicines, food ingredients and dietary supplements, has created an online database (www.foodfraud.org) containing a collection of reports about food frauds and analytical methods, in an attempt to prevent new illegal practices (Moore *et al.* 2012). The data collected (from 1980 to 2010) in scientific papers (scholarly records) showed that the food commodities mainly subjected to adulteration are olive oil (Woodbury *et al.* 1998; Blanch *et al.* 2000; Ruiz-Mendez *et al.* 2001; Gamazo-Vazquez *et al.* 2003; Pavon *et al.* 2009; Fasciotti and Pereira-Netto 2010; Mildner-Szkudlarz and Jele 2010; Torrecilla *et al.* 2010), milk (Barron *et al.* 1990; Precht 1991; Veloso *et al.* 2002; Goudjil *et al.* 2003; Hurley *et al.* 2004; De la Fuente and Juarez 2005; Goswami and Gupta 2008; Muller *et al.* 2008; Calvano *et al.* 2009*a*), honey (Cajka *et al.* 2009; Wang *et al.* 2009; Chudzinska and Baralkiewicz 2010; Gomez-Perez *et al.* 2012), saffron (Sabatino *et al.* 2011), fruit juice (Burnett and Beuchat 2001; Rosales-Conrado *et al.* 2005), coffee (Gil-Agusti *et al.* 2005; Zambonin *et al.* 2005; Risticevic *et al.* 2008) and fish (Masseo *et al.* 2008; Fernandez-No 2010; Kalachova *et al.* 2011). Demand for the advance and application of efficient and powerful tools to quickly evaluate indefinite food components or chemical and microbial food contaminants has therefore increased considerably.

In this background, the application of 'omic' sciences to food has recently developed in foodomics, a comprehensive discipline that combines food and nutrition with analytical

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techniques and bioinformatics tools (Cifuentes 2009; Herrero *et al.* 2010*a*). The analytical techniques typically used in food analysis are nuclear magnetic resonance (NMR; Fardet *et al.* 2007; Shepherd *et al.* 2011; Longobardi *et al.* 2013), atomic spectroscopy (AS; Fodor 1992; Jorhem 2000; Baker *et al.* 2012), infrared (IR) spectroscopy (Ismail *et al.* 1999; Milosevic *et al.* 2004; Teye *et al.* 2013), polymerase chain reaction (PCR; Postollec *et al.* 2011; Talkington 2013) and mass spectrometry (MS), mostly coupled to separation techniques such as high-performance liquid chromatography (HPLC; Di Stefano *et al.* 2012; Holčapek *et al.* 2012; O'Mahony *et al.* 2013), gas chromatography (GC; Zambonin *et al.* 2004; Alder *et al.* 2006) and capillary electrophoresis (CE; Piñero *et al.* 2011; Herrero *et al.* 2010*b*).

Recent MS applications also include the use of stand-alone techniques for profiling and imaging such as ambient-ionisation MS and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF), which offer fast and easy sample preparation without the need for analyte derivatisation or chromatographic separation.

The aim of this chapter is to describe the principles and some applications of MALDI as a useful tool for food quality and safety.

12.2 MALDI MS Principles

In the late 1980s, MALDI was introduced in mass spectrometry by Tanaka *et al.* (1988) as a new soft-ionisation technique. For the first MALDI experiment, Tanaka *et al.* used a cobalt powder as a matrix for absorption of a laser beam, while the introduction of small organic compounds is due to Karas and Hillenkamp (1988). Actually, for sample preparation, a matrix constituted by a weak organic acid, is simply mixed with the sample in a large molar excess and the resultant solution is deposited on a microtiter plate and allowed to crystallise. The plate is therefore loaded in the mass spectrometer and a laser beam, typically originated by a UV-laser, hits the spot where co-crystals of matrix and analyte are present. Due to the laser energy, a part of the matrix which has a strong absorption to the laser wavelength is vaporised together with the analyte in a 'plume' expanding at high velocity. The sample ions formed in the ion source are extracted and accelerated in an electric field with high voltages (usually 20 kV). After passing the charged grid, the ions fly into the TOF mass analyser (at field free space), at a time directly proportional to the square root of their m/z. Finally, the ions reach the detector where the conversion and the amplification of ion current in an electrical signal are accomplished. The total process is depicted in Figure 12.1. In this process, the length of the field-free path influences the mass resolution, since the ion separation is achieved over this space. The traditional tube was characterised by a linear configuration with a poor resolution; an improvement was reached using strategies to extend the flight path as the 'electrostatic mirrors', where ions are reflected (reflector ion mode) at the end of the flight tube towards another detector (Schiller and Arnold 2000). Moreover, in order to minimise the initial different distributions in energy and velocity, and to further improve the mass resolution, a special implementation of 'time-lag focusing' or 'delayed-ion extraction' was realised.

The matrix aids the ionisation process in several ways. First, matrix molecules absorb laser light, and then excite helping the transfer of analyte molecules into the gas phase (sublimation). Second, matrix promotes analyte ionisation by chemical reactions: the

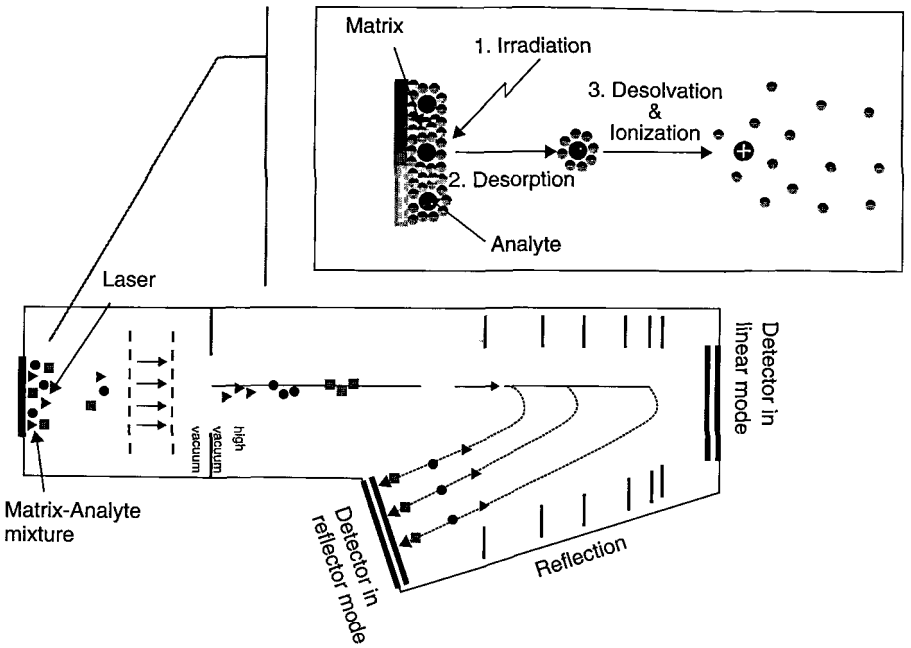


Figure 12.1 Scheme of a MALDI-TOF mass spectrometer.

matrix functions as a proton donor (in positive ion mode) and a proton acceptor (in negative ion mode). Third, the matrix prevents analyte cluster formation because its molecules, added in large molecular excess (100–1000-fold molar excess), separate the analyte molecules from each other. Fourth, the matrix limits the analyte fragmentation, allowing the desorbtion biomolecules of several hundred kDa as intact ions. To achieve best results it is important that matrices have a low molecular weight for easy sublimation, are vacuum stable, promote analyte ionisation and are soluble in the same solvent of the analyte. In Table 12.1 provides a list of the most commonly used MALDI matrices together with their application.

Different ways to prepare the analyte-matrix mixtures exist, although the most widespread still remains the dried droplet method where a saturated matrix solution is mixed with the analyte solution and an aliquot (0.5–2.0 μL) of the resulting solution is applied to the target and allowed to dry. Some of the qbvious advantages using MALDI-MS are the rapid sample preparation and analysis. The sample-matrix mixture is applied directly on the sample probe tip, enabling the simultaneous investigation of numerous samples. It is a very sensitive method and requires a low amount of sample, ranging from pico-moles to atto-moles.

MALDI-MS also has a high tolerance towards salts, buffers and detergent in comparison to other ionisation techniques (Xu *et al.* 2006). However, high concentration of salts and other contaminants can negatively affect the desorption and ionisation of the analytes. In these cases, a previous purification of the sample is necessary.

MALDI-MS also presents some drawbacks such as low shot-to-shot reproducibility (due to spot inhomogeneities) and matrix peaks interference at low m/z ratios. To solve these problems, several efforts have been made. For instance, different matrix-free

Table 12.1 List of some common used UV-MALDI matrices.

Analyte	Matrix
Proteins/peptides	α-cyano-4-hydroxycinnamic acid (CHCA) α-cyano-4-chlorocinnamic acid (CClCA) Sinapinic acid (SA)
Oligonucleotides	3-Hydroxypicolinic acid (HPA) 6-aza-2-thiothymine (ATT)
Carbohydrates	2,5-Dihydroxybenzoic acid (DHB) 2,4,6-Trihydroxyacetophenone (THAP) 3-Aminoquinoline
Lipids, metabolites	2,5-Dihydroxybenzoic acid (DHB) 9-Aminoacridine (9-AA) 2,6- Dihydroxyacetophenone (DHAP)
Synthetic polymers	Dithranol (DIT) Trans-3-indoleacrylic acid (IAA) 2-Mercaptobenzothiazole

approaches involving laser desorption/ionisation (LDI) of analytes have been explored (Calvano *et al.* 2005, 2011; Zambonin *et al.* 2006; Urban *et al.* 2009); alternatively, several new matrices were studied as 1,8-bis(dimethylamino)naphthalene (Schroff *et al.* 2009; Calvano *et al.* 2011), acridine derivatives (Cerruti *et al.* 2012; Monopoli *et al.* 2012), ionic liquids (Calvano *et al.* 2009*b*, 2012*a*; Przybylski *et al.* 2010), binary matrices (Shanta *et al.* 2011; Calvano *et al.* 2013*a*; Fukuyama *et al.* 2014) or reactive matrices (Monopoli *et al.* 2014; Shigeri *et al.* 2014). In many cases, some of these new matrices have been demonstrated to outperform the classical matrices in terms of sensitivity, tolerance to salts, vacuum stability, homogeneity of crystallisation, mass resolution and lack of interfering peaks.

12.3 MALDI-TOF-MS for Food Proteins and Peptides Analysis

In the last few years, several proteomic strategies have been developed for food analysis for different purposes such as authentication, identification of hidden allergens, quality and safety control. Although a separation (by electrophoresis or chromatography) is needed before MS analysis, in the case of conventional proteomics analysis of complex samples this step can usually be omitted when profiling and comparative studies are devoted to address food quality and safety issues. In particular, intact proteins (Cozzolino *et al.* 2012) can be analysed or, alternatively, peptides derived from their enzymatic digestion to identify and localise minor modifications in the protein sequence (Carulli *et al.* 2011). The enzymes typically used for protein digestion are trypsin, LysC, AspN or Glu-C although trypsin represents the most used since it generates peptides within the mass range of 500–5000 Da, which are suitable for mass mapping. After protein digestion a characteristic dataset called peptide mass fingerprint (PMF) is generated;

the experimental *m/z* data are compared with *in silico* protein digests to find the best match that could identify the examined unknown protein.

The most frequently used search programs for PMF are Mascot, MS-Fit and Profound, all available from www.expasy.org freeware site.

12.4 MALDI-TOF-MS for Lipids Analysis

Lipid analysis is traditionally based on chromatographic techniques such as TLC (thin layer chromatography) (Touchstone 1995), HPLC (Nikolova-Damyanova 1997; Graeve and Janseen 2009) or GC-MS (Christie 1998; Bohin *et al.* 2005), although these methods are tedious and time-consuming since they usually include complex sample pre-treatments and, often, derivatisation steps. Recently, lipids determination by MALDI-TOF-MS has been progressively increasing, even if rather limited compared to proteomics studies. This is quite surprising considering that MALDI could be a fast and convenient method to solve this kind of analytical problem: one sample can be analysed in less than one minute; sample plates allow the simultaneous (even automated) analysis of many samples in a single run; and lipid amounts in the range of a few nanograms are required (a very significant improvement compared to the amount required by other techniques, such as nuclear magnetic resonance orNMR). The main problem for the analysis of lipids, which have quite low molecular weights, has been the search for a matrix which should originate few interfering ions. So far, among conventional matrices DHB seems to be the most appropriate for lipid analysis (Schiller *et al.* 1999) since it tends to give a low yield of matrix ions even if it produces inhomogeneous crystals. New approaches and new matrices have recently been proposed such as *para*-Nitro-aniline (PNA) (Steven *et al.* 2013), lumazine (Calvano *et al.* 2010*a*) and ionic liquids (ILs) (Li *et al.* 2005; Calvano *et al.* 2009*b*; Zhou *et al.* 2010), which produce even less interfering matrix peaks and/or improve spot homogeneity.

12.5 MALDI-TOF-MS for Illegal Mixture Detection

12.5.1 Hazelnut Oil in Olive Oil: Lipid Analysis

Due to the several above-mentioned advantages, MALDI-MS can be a very useful tool in the detection of food adulteration. Here we describe simple, rapid and sensitive procedures for proteomic and lipidomic MALDI-MS analysis of vegetable oils and milk, which are the most frequently adulterated foods. Extra-virgin olive oil (EVOO) is considered as a nutraceutical food since it is rich in essential nutrients such as fatty acids, phytosterols, vitamins and natural antioxidants. Adulteration of extra-virgin olive oil (EVOO) with hazelnut oil (HO) or other cheap vegetable oils such as soy, sunflower or nut oils is an illegal practice that could also have health consequences for consumers due to the potential exposure to hidden allergens.

In particular, the use of HO as adulterating agent is a serious concern since it cannot be easily detected by well-established techniques because of its similarity in triacylglycerol, sterol and fatty acid compositions with respect to olive oil. The simplest methods which can easily be implemented routinely in laboratories are those based upon the analysis of particular marker compounds.

For instance, in the case of EVOO adulteration, polar compounds may have a great potential for the characterisation of vegetable oils since phospholipids (PLs) are usually present in seed oils at a concentration range of 10–20 g kg^{-1} (Bernardini 1983), while their amount in EVOOs is 300–400 times lower (Koidis and Boskou 2006). In view of these considerations, Calvano *et al.* (2010*b*) developed a hydrophilic liquid chromatography (HILIC) micro-solid phase extraction to enrich the polar fraction of olive and hazelnut oils before MALDI-TOF-MS analysis. It was found that lysophosphatidylcholines can be a potential marker of HO in EVOO since they are absent from oilve oil. More recently, phospholipids (PLs) were confirmed to be promising markers of EVOO adulteration with HO by using an ionic liquid composed of tributylamine (TBA) and CHCA as a matrix (Calvano *et al.* 2012). In fact, sample pre-treatment focused on the selective PLs extraction (modified Bligh–Dyer with TBA-CHCA instead of methanol), together with the use of a MALDI matrix more suitable for PLs analysis, revealed to be very useful to detect adulteration down to 5%.

In this approach, 1.87 mL of (1:2) $CHCl_3$/TBA-CHCA dissolved in MeOH (at the concentration of 0.035 M) were added to 0.5 mL of oil followed by vigorous vortex-mixing. Then, 0.62 mL of $CHCl_3$ were added and the solution vortexed again. Afterwards, 0.62 mL of H_2O were added and the solution vortexed again. Finally, the solution was centrifuged (15 min at 2000 g) and the organic layer was directly collected, diluted 1:10 in a $CHCl_3$/MeOH (1:1) solution and spotted on the target plate.

Figure 12.2 reports the MALDI-TOF-MS spectra relevant to the Bligh–Dyer extracts of (a) a 100% EVOO sample; (b) an EVOO adulterated at 20% with HO; and (c) a 100% hazelnut oil sample. While the MS spectra of EVOO and HO obtained with the DHB matrix were almost equal (data not shown), very different profiles were obtained for HO and EVOO when TBA-CHCA was used. As can be seen in Figure 12.2c, a remarkable increase of phospholipids signals with a simultaneous decrease of those relevant to

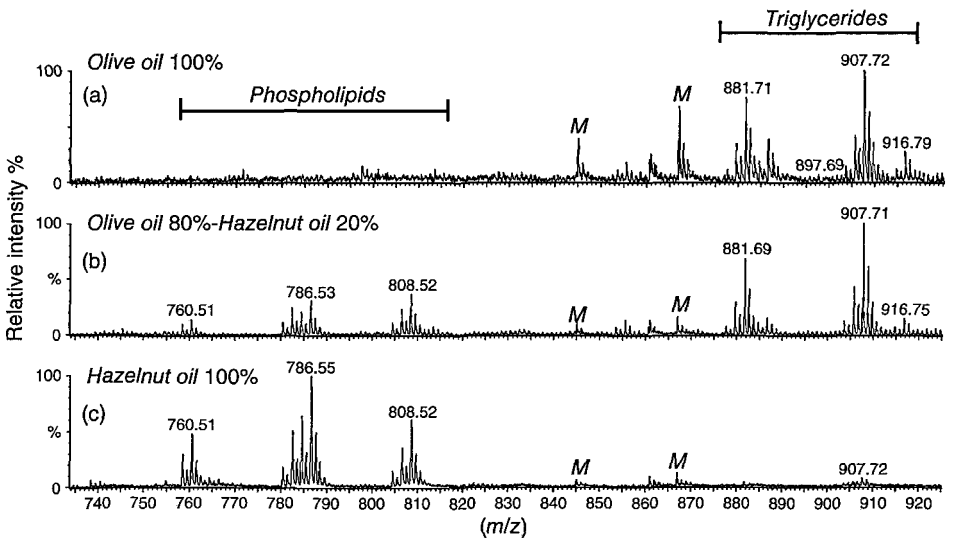


Figure 12.2 MALDI-TOF mass spectra relevant to the Bligh–Dyer extracts of (a) a 100% EVOO sample; (b) an EVOO adulterated at 20% with HO; and (c) a 100% hazelnut oil sample.

triglycerides and diglycerides is observed for HO contrary to EVOO where PLs are absent and the main detected ions are relevant to triglycerides. When the mixture composed of 80% of EVOO and 20% of HO was analysed (Fig. 12.2b), the peaks attributable to marker PLs were still observable, demonstrating the applicability of the method.

12.5.2 Hazelnut Oil in Olive Oil: Protein Analysis

A big issue relevant to the adulteration of EVOO with HO is related to the hidden presence of hazelnut residual proteins in EVOO that could induce severe reactions in allergic subjects. The main allergens known in hazelnut are Cor a 1 (17 kDa), Cor a 2 (14 kDa), Cor a 8 (12 kDa), a lipid transfer protein (LTP), Cor a 9 (globulin 11S) and Cor a 11 (globulin 7S), two oleosin isoforms (Cor a 12, Cor a 13), an isoflavone reductase homologue (Cor a 6), a luminal binding protein (Cor a 10), a 2S albumin (Cor a 14) and a Cor a 9 isoform.

Hazelnut oil may be considered a protein-free food, although the presence of residual proteins depends on its processing method and on the nature of employed kernels. For residual protein detection, different extraction protocols based on cold-acetone, hexane/acetone, diethyl ether/trichloroacetic acid (TCA), ammonium hydrogen carbonate, tris-buffered saline (TBS), Bligh–Dyer can be tested. Among them, cold-acetone was one of the most efficient procedures for hazelnut protein precipitation in oil samples (De Ceglie *et al.* 2014).

To 5 g of pure hazelnut oil, 12.5 mL of cold acetone (4°C) were added, shaken vigorously, kept for 1 h at 4°C and shaken every 10 min, then centrifuged at 5000 rpm for 1 h. The obtained precipitate was washed twice with 1 mL of cold acetone and then dried at 40°C for 5 min in a vacuum concentrator.

For proteomics studies, the pellets were digested in solution following reported protocols. Dried pellets were resuspended in 40 μL of 400 mM NH_4HCO_3 in 8 M urea. 5 μL of DTT (50 mM) were added and the mixture was incubated for 40 min at 60°C and chilled at room temperature. 5 μL of iodoacetamide (150 mM) were added, followed by 45 min of dark incubation at room temperature. After addition of water to dilute urea at 2 M, porcine trypsin was added and the solution was incubated overnight at 37°C. The digestion was stopped with acidification and tryptic digests analysed by MALDI-TOF-MS. In some cases, due to the low concentration of hazelnut-derived peptides in the samples, a procedure of desalting and pre-concentration using C_{18} Zip Tips can be employed.

Figure 12.3 shows the MALDI spectra relevant to the in-solution tryptic digests of EVOO extracts adulterated with (a) 20% and (b) 50% HO obtained from roasted hazelnuts. The marked *m/z* ions belong to *Cor a 9* (1394.70 *m/z*, 1440.79 *m/z*, 1555.74 *m/z*, 1571.80 *m/z*, 1629.83 *m/z*), *Cor a 1* (1528.72 *m/z*) and *Cor a 14* (1453.85 *m/z*) and represent hazelnut peptide markers. This approach can therefore allow the direct detection for routine analysis of specific hazelnut allergens in EVOO at low concentration levels on a small amount of oil without time-consuming pre-treatments.

12.5.3 Cow Milk in Goat and Sheep Milk: Protein Analysis

After vegetable oil, dairy products are the second-most likely food to be subjected to a high number of frauds.

In particular, milk and cheese are expensive foodstuffs of high nutritional value and consumption is spread across the entire population. According to recent legislation in different countries, the most common illegal practices are the selling of skimmed milk

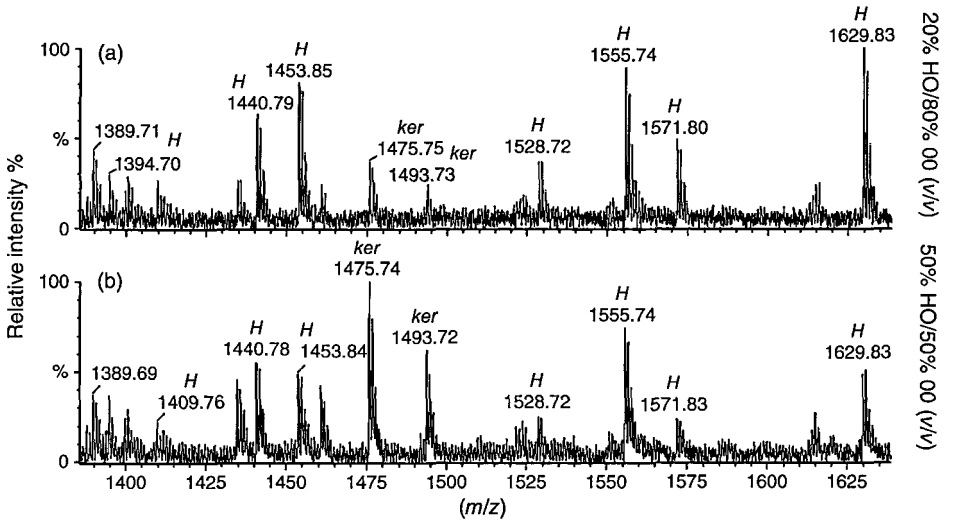


Figure 12.3 MALDI-TOF mass spectra relevant to the in-solution tryptic digests of EVOO extracts adulterated with (a) 20% and (b) 50% of HO obtained from roasted hazelnuts.

and semi-skimmed milk instead of whole milk, milk dilution by water, addition of milk anhydrous products (caseins and caseinate, milk protein concentrate, whey proteins) to liquid milk and the mixing of high-quality milk such as sheep and goat with cow milk.

Adulteration of the commodity, aimed at obtaining economical profits, could also have serious sanitary consequences due to the exposure of consumers to hidden allergens such as cow milk proteins α_{s1}-casein and β-lactoglobulin.

To assess the authenticity of dairy products and milk and to protect consumers' health, many analytical methods have been developed and improved in the last years. For cheese samples, the EU official method (EC 2008) to detect cow's caseins in sheep or goat cheese consists of the isolation of caseins from both cheese samples and reference standards, in their digestion with plasmin and in the isoelectric focusing (IEF) of the resultant γ-caseins. The comparison of γ3- and γ2- caseins with reference samples (containing 0% or 1% of cow milk) allowed any potential illegal presence to be determined. For milk samples, the official method (Gazzetta Ufficiale 2003) consists of the analysis of whey protein profiles by reverse-phase HPLC. In fact, bovine β-lactoglobulin and α-lactalbumin have characteristic retention times different from goat and sheep milk.

The most employed research strategies use liquid chromatography coupled with electrospray (ESI) MS (Chen *et al.* 2004; Guarino *et al.* 2010). However, MALDI-TOF-MS has also proven to be a powerful tool for rapid and sensitive milk adulteration detection in dairy products.

Cozzolino *et al.* (2002) analysed intact proteins of different milk or cheese species, proving that α-lactalbumin and β-lactoglobulin can be used as molecular markers for the detection of bovine milk in ewe and buffalo milk or cheese. Earlier, Angeletti *et al.* (1998) found the presence of bovine milk in water buffalo milk and mozzarella, while Fanton *et al.* (1993) determined the addition of bovine milk to 'Pecorino' cheese samples.

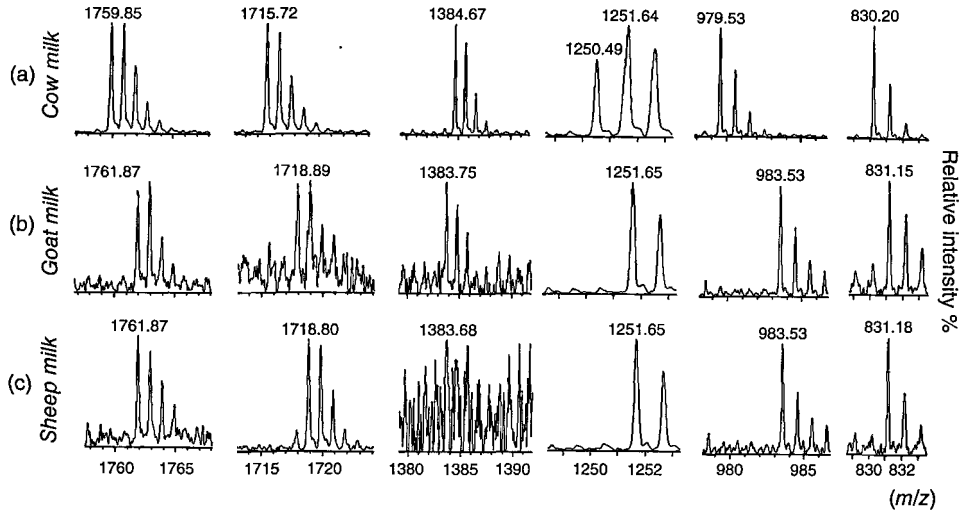


Figure 12.4 Important species-specific peptides for (a) cow; (b) goat; and (c) sheep milk.

The alternative choice of investigating peptides instead of intact proteins could be crucial, since it could determine the location of minor differences in protein sequence, allowing authenticity studies. In order to obtain peptide profiles, milk samples can be simply diluted with water (1:10) and subjected to tryptic digestion (Calvano *et al.* 2012*b*). The resulting digests are analysed by MALDI, obtaining a specific fingerprint for each milk species. As an example, Figure 12.4 reports the main species-specific peptides useful to distinguish between cow, sheep and goat milk samples. The useful goat-specific peptides to be monitored belong to k-casein and some of them can be at *m/z* 1219.74, 1276.74 and 1978.26. It is anticipated that this approach will be applicable to processed dairy products such as cheese, where marker peptides are still present.

12.5.4 Powder Milk in Liquid Milk: Protein and Lipid Analysis

MALDI can also be suitable for detecting the addition of powdered milk to fresh milk. In fact, to obtain milk powder, fresh milk is submitted to heat and pressure treatments, causing chemical changes that affect different components including proteins, phospholipids (PLs) and carbohydrates. The relatively high concentration of reducing sugars (lactose) and lysine-rich proteins or amino-lipids make thermally induced non-enzymatic reactions (Maillard reaction) favourable. The analysis of intact proteins in an ultra-high-temperature (UHT) heated milk sample reveals the presence of lactosylated forms of α-lactalbumin and β-lactoglobulin in both variants, due to the thermal stressing (Carulli *et al.* 2011). As observed in Figure 12.5, both proteins can be detected in their native form together with up to three lactose, suggesting that proteins have been modified under thermal stress. In addition, performing the analysis of tryptic digests allows the localisation of the glycation sites on powder milk proteins and the detection of their illegal presence in fresh milk (Calvano *et al.* 2013*b*).

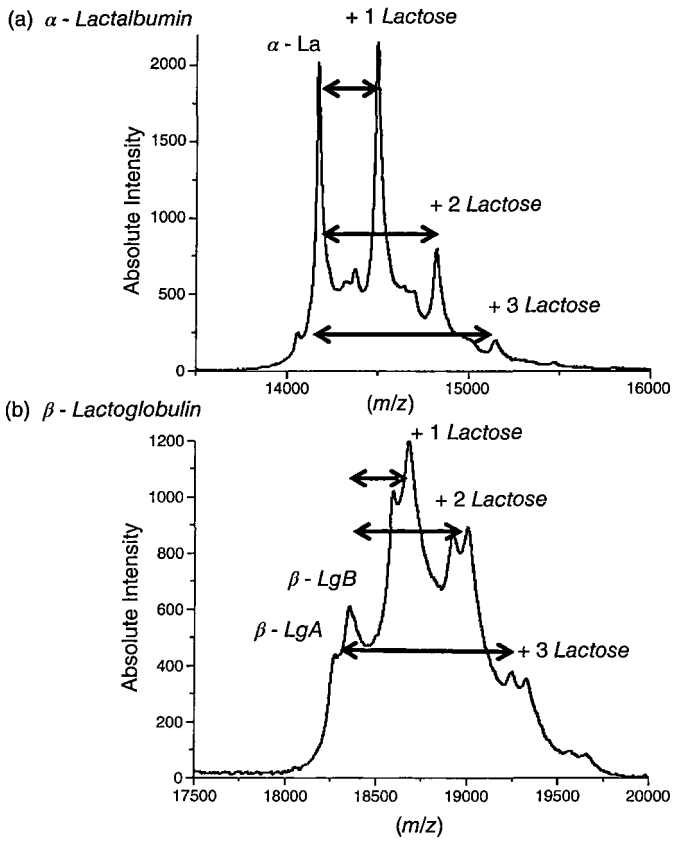


Figure 12.5 MALDI-TOF mass spectra relevant to (a) α-lactalbumin and (b) β-lactoglobulin in a ultra-high-temperature (UHT) milk sample.

On the other hand, PLs also possess a free amino group (such as phosphatidylethanolamines (PEs) and phosphatidylserines (PSs)) and can consequently give rise to different Amadori by-products in food. To this aim, model PLs and sugars (glucose or lactose) were heated, extracted by using traditional Bligh–Dyer or direct in-matrix extraction (DIME) and subjected to MALDI analysis (Calvano *et al.* 2014). Figure 12.6 shows the MALDI mass spectra, acquired in negative ion mode, relevant to the model 1,2-dioleoyl-3-phosphoethanolamine (DOPE) heated in presence of (a) lactose and (b) glucose. As noticed, native DOPE was observed as deprotonated ion at m/z 742.54 together with the lactosylated adduct (mass shift +324) at (a) m/z 1066.75 or (b) the glycated (Δm = 162 Da) adduct at m/z 904.76. The application of the same protocol to milk powder sample (Fig. 12.6c) allowed the detection of various modified phospholipids in both glycated and lactosylated forms.

12.6 MALDI-TOF-MS for Microbial Contamination Detection

The detection of pathogens in food spoiled by microorganisms is a relevant aspect of food safety. A frequently used method is based on the GC-MS characterisation of

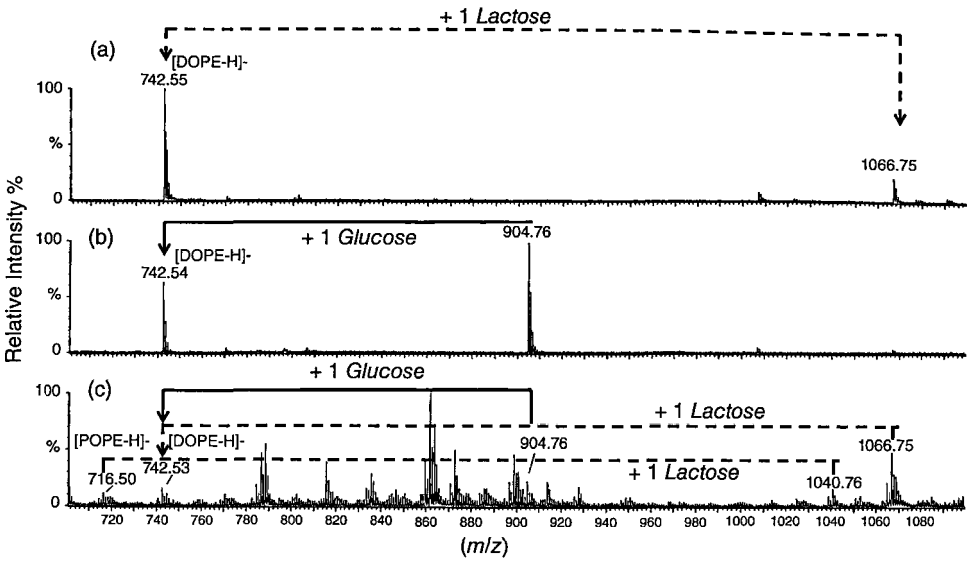


Figure 12.6 Negative MALDI-TOF mass spectra relevant to DOPE heated with (a) β-lactose and (b) glucose, and (c) to a powdered milk sample.

volatile compounds or metabolites arising from food products to identify a particular microbial contamination (Ercolini *et al.* 1997). Thanks to the combination of metabolites profiling and chemometrics, an identification of microbial contaminants is often possible (Xu *et al.* 2010). An alternative approach could be the use of MALDI to directly classify microorganisms or strains contaminating food with the help of proteomics tools. The screening of bacterial samples for quick identification can be achieved with direct acquisition of a subset of the protein profile by MALDI-TOF-MS analysis. Mass spectra are collected from unknown bacterial samples and compared with reference spectra to provide information for a correct identification with a calculated degree of confidence. It is crucial to follow well-defined sample preparation procedures and calibration routines during the collection of replicates to build reference spectra. Afterward, statistical analysis based on algorithms can be used to insert the replicates into a reference spectrum and then into a database (Jarman *et al.* 1999).

For instance, MALDI-TOF-MS has been applied to characterise intact bacterial cells (Pavlovic *et al.* 2013) and ribosomal proteins (Barbuddhee *et al.* 2008), differentiate among bacterial species and genera, or identify unknown bacterial strains (Bohme *et al.* 2010). Examples of the used reference profiles are given in Figure 12.7, where the protein profiles obtained from the direct analysis of intact (a) *Straphylococcus aureus* and (b) *Escherichia coli* are reported. MALDI was also demonstrated to be a rapid screening tool for identification of microorganisms without individual protein identification.

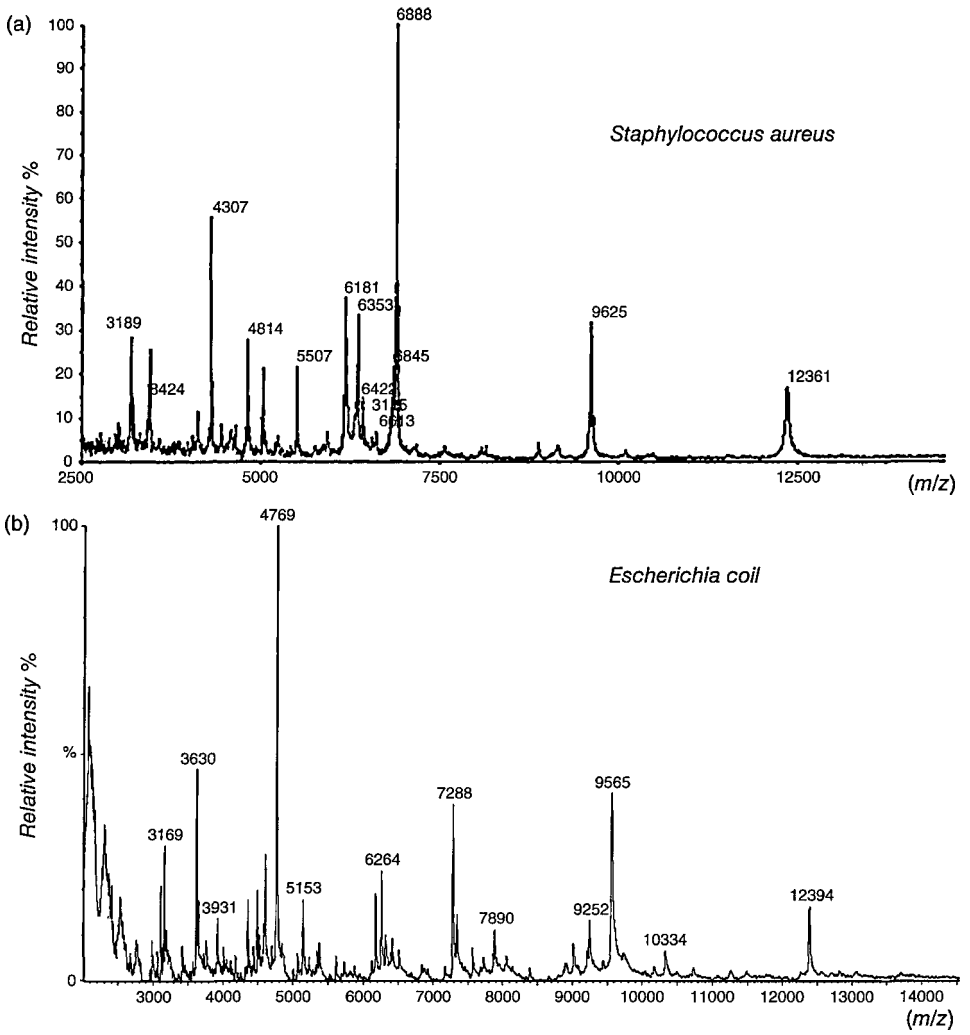


Figure 12.7 MALDI-TOF mass spectra relevant to protein fingerprint of intact (a) *Straphyloccus aureus* and (b) *Escherichia coli*.

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13

Detection of Food Processing Techniques

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13.1 Introduction

Food processing is a powerful weapon of food science and technology. The main goal of food processing is to improve the microbial safety of food by destroying pathogenic and spoilage organisms. Food processing can also improve food safety by destroying or eliminating naturally occurring toxins, chemical contaminants, and antinutritive factors. Except safety, food processing also plays a dramatic role in quality. Food processing techniques are important indicators of quality for religious, ethical, and environmental reasons. Religion may dictate food quality without prior experience through beliefs and traditions. People who follow the Muslim religion can only consume meat products which are produced under "Halal" specifications. Halal is referred to a specific slaughter technique. Consequently, products which do not meet the criteria are immediately excluded (Nakyinsige *et al.* 2012). We may also focus on quality because food safety in the western world is non negotiable. In fact, they are willing to spend high amounts of money for quality goods. According to studies, consumers in the EU, where quality schemes are defined (PDO, PGI, TSG), prefer certificated products even though their prices are higher (Velčovská and Sadílek 2014). Therefore, fraudsters are motivated by profit to use processing in order to adulterate foodstuffs. In this way, overrated products, which does not meet their standards, are purchased. A typical example of this practice is frozen/thawed meat sold as fresh. Retailers take advantage of the fact that it is nearly impossible to visually distinguish fresh meat from thawed meat. Another example of food fraud is the replacement of ingredients by others of lower commercial value.

On this chapter, we review and discuss the possible non-acceptable and/or undeclared processes. These are in detail freezing–thawing, irradiation heating and microwave heating. Additionally, various analytical methods have been developed in order to protect consumers and detect undeclared processing that affects quality. These methods are comprehensively presented in the following sections.

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13.2 Freezing–Thawing

Freezing of meat and fish products is perhaps the best method of increasing the shelf life of these foodstuffs. In contrast to other preservation methods that decrease the nutritive value of foodstuffs, freezing retains ingredients of the products unchanged. The necessary temperature for freezing should be achieved rapidly in order to avoid any spoilage and decrease in quality. The minimization of time for transition from one aggregate state into another (e.g., from liquid to solid) contributes to improved quality of products (Giddings and Hill 1978).

Freezing results to an effective dehydration as water transmits natural state and produces ice crystals. During defrosting, this water cannot be reabsorbed by the tissue microstructure. The factors that influence this process are the texture of the tissue, the thawing rate, and the physiological and biochemical status of the tissue prior to freezing (Pham and Mawson 1997).

Bacterial cells are usually sensitive to freezing which resulting in decrease of their population on the foodstuff. In this way, cell membranes are damaged and DNA molecules are denaturated during freezing and thawing. It is also believed that there is damage to some proteins because of the increase of salt concentration in the cells. Freezing does not make foods sterile, but it is generally accepted that the majority of the pathogenic microorganisms could not grow at low temperatures. In this case, the main concern is that pathogens may survive freezing and begin growing in the thawed product (Arthey 1993).

In conclusion, freezing and storage in a frozen state are very important for the quality of meat and meat products. The proper application of processes is essential to obtain a high-quality product (Nollet and Toldrá 2006). Freezing of meat is also important regarding food safety and has two major challenges to fulfill. On the one hand, the minimization of the possibility that any microorganism may grow. On the other hand, the decrease of meat's microflora population. This is of essential importance in the case of in fish, where a thorough veterinary inspection after fishing is lacking.

13.2.1 Methods of Detection

Selling frozen meat as fresh is a common practice by the retailers. They let meat or fish to thaw, and then they offer it to the consumers as fresh meat. However, the price of fresh or chilled meat is higher than that of frozen–thawed meat. This treatment to meat and fishery products hides safety risks. Thawed meat is a nutritive substrate for microorganisms that can be pathogenic. Therefore, it is an urgent need for analytical chemistry to provide capable techniques to detect frozen–thawed meat that is sold as fresh.

A method able to distinguish fresh meat between thawed one is the enzymatic β-hydroxyacyl-CoA-dehydrogenase (HADH). The HADH method has been widely used and discussed (Chen *et al.* 1988; Toldrá *et al.* 1991; Hoz *et al.* 1993; Fernández *et al.* 1999; Ramos *et al.* 2004; Cheung *et al.* 2015). Originally, one spectrophotometric and one color test method based on the following reaction were cited (Gottesmann and Hamm 1983):

$$\text{Acetoacetyl}-\text{CoA}+\text{NADH}+\text{H}^{+} \underset{}{\overset{\text{HADH}}{\rightleftharpoons}} \beta-\text{hydroxybutyryl}-\text{CoA}+\text{NAD}^{+}$$

The spectrophotometric method measures the conversion rate of NADH to NAD^+ by monitoring the decrease in absorption at 340 nm. Gottesmann and Hamm (1983) reported threshold HADH enzyme activity values for beef, veal, pork, mutton/lamb, game, and poultry; surpassing the threshold values indicates a freeze–thaw treatment. The HADH method is applicable to pressed juice from whole meat, and measures β-hydroxyacyl-CoA-dehydrogenase released from the freezing-induced disruption of mitochondria. A grinding procedure of whole meat also induces disruption of mitochondria, whereby the HADH content is released to the press juice. In this case, a significant HADH difference between fresh ground meat and thawed meat is absent and discrimination is therefore impossible. It is also important to note that differentiation between fresh and thawed meat is only possible with the HADH method if the freezing temperature has been −12°C or below (Gottesmann & Hamm 1983).

Enzymatic methods have not been restricted to the HADH method. Another research group tried to differentiate fresh and thawed chicken meat by the measurement of aconitase activity (Škorpilová *et al.* 2014). The goal of this study was to compare the activity of mitochondrial enzyme aconitase in meat under different storage conditions. The detected enzyme was also found in the exudate of fresh meat. Comparing the enzyme activity measured in fresh meat with the one in thawed meat, indicates higher enzyme activity in the case of frozen-thawed meat. Moreover, another study used the API-ZYM system, which is based on a semi-quantitative method for simultaneous measurement of 19 different enzymes (Ellerbroek *et al.* 1995).

There is a variety of spectroscopic techniques that can detect these changes. Infrared spectroscopy (Al-Jowder *et al.* 1997; Reddy Gangidi and Proctor 2009) was used to study ground meat from pork, chicken, and turkey after storage at −30°C. Subsequently, a principal component analysis was successfully performed on spectroscopic data in the region 1000–1800 cm^{-1}. Nuclear magnetic resonance (NMR) (Foucat *et al.* 2001; Li *et al.* 2012), magnetic resonance imaging (MRI) (Evans *et al.* 1998; Hall *et al.* 1998; Nott *et al.* 1999), and electron microscopy (Sen and Sharma 2004) have also shown a potential in fresh v. thawed discrimination. The use of sensory methods for detection should not be forgotten.

13.3 Irradiation

The purpose of food irradiation is the decrease of microorganisms causing food spoilage like other food processing procedures like freezing and high-temperature treatment. The aim is to extend the shelf life of foods that kept under various conditions such as in shops and households, and to eliminate pathogenic organisms that cause diseases as a result of food consumption. Radiation doses usually employed for food irradiation destroy the majority of microorganisms, but not all of them. The irradiated food is not sterilized. The use of irradiation as a preservation technique can play an important role in cutting losses and reducing the dependence on chemical pesticides. Each year a few hundred thousand tons of food products and ingredients are irradiated worldwide. Furthermore, food irradiation may be used to reduce losses caused by premature ripening of fruits, sprouting of vegetables, and pests. A typical example of that is to prevent germination and sprouting of potatoes, onions, and garlic. In such cases,

irradiation can replace the chemical treatment of foodstuffs (fruits, vegetables, spices, and crops).

International foodstuffs trade, especially seafood, is expanding, increasing the risk of transfer of some diseases; this fact has led the World Health Organization to legalize radiation treatment of food. International health and safety authorities have set the safety of irradiation for all foods up to a dose level of 10 kGy. In fact, an international expert study group appointed by FAO, IAEA, and WHO showed that food treated according to good manufacturing practices (GMPs) at any dose above 10 kGy is also safe for consumption (Diehl 2002). Irradiated products sold in retail packages must be labeled with the logo that is depicted in Figure 13.1.

Why should food irradiation be used rather than other more conventional food processing practices? Ionizing radiation is frequently used for the sterilization of special case diets. These diets are mainly necessary for patients suffering from different disorders of the immune system like tumors and AIDS. Ionizing radiation may also be employed to treat diets for allergic patients (Byun *et al.* 2002). For example, by the irradiation of milk proteins the structure of their epitops can be destroyed in order to reduce their effects as allergens (Lee *et al.* 2001). Sterilized foods for the army and space missions are also increasingly important (Grolichova *et al.* 2004). Additionally, irradiation may be used in several types of raw or minimally processed foods such as poultry, meat and meat products, fish, seafood, fruits, and vegetables.

Analytical methods for the detection of irradiated foods are standardized by the European Committee for Standardization (CEN). CEN has standardized the methods upon a commission mandate. The European standards have been adopted by the Codex Alimentarius Commission as General Methods. Table 13.1 lists the standards of CEN according to the European Commission.

Although properly irradiated food is safe and nutritious, consumers should be able to make their own free choice between irradiated and non-irradiated food. For this purpose, labeling is indispensable. Detection of radiation treatment is highly desirable in order to check compliance to regulations. Significant progress has been made recently in analytical methods utilizing changes in food originating from the radiation treatment. These detection methods are separated in physical, chemical, and biological methods.

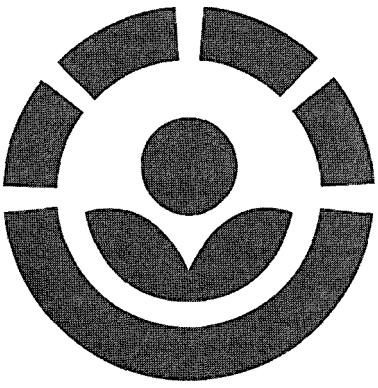


Figure 13.1 Irradiation logo for foodstuffs treated with radiation.

Table 13.1 Standardized EU analytical methods for the detection of irradiated foodstuffs.

Standard	Analytical method
EN 1784:2003	Detection of irradiated food containing fat by gas chromatographic analysis of hydrocarbons
EN 1785:2003	Detection of irradiated food containing fat by gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones
EN 1786:2003	Detection of irradiated food containing bone by ESR spectroscopy
EN 1787:2003	Detection of irradiated food containing cellulose by ESR spectroscopy
EN 1788:2003	Thermoluminescence detection of irradiated food from which silicate minerals can be isolated
EN 13708:2001	Detection of irradiated food containing crystalline sugar by ESR spectroscopy
EN 13751:2002	Detection of irradiated food using photostimulated luminescence
EN 13783:2001	Detection of irradiated food using direct epifluorescent filter technique/ aerobic plate count (DEFT/APC) (screening method)
EN 13784:2001	DNA comet assay for the detection of irradiated foodstuffs (screening method)
EN 14569:2004	Microbiological screening for irradiated food using LAL/GNB procedures

13.3.1 Physical Methods for Irradiation Detection

Electron spin resonance (ESR) spectroscopy is a promising technique for many foodstuffs. This technique detects radiation-specific radicals produced during irradiation of food which can be quite stable in solid and dry components of the food (e.g., bones). The technique is non-destructive, specific and combines simplicity with rapid measurement. The availability of desktop ESR spectrometers reduces the cost of equipment, but this is still substantial and may hamper investigation. Moreover, ESR can detect radiation-induced radicals in packaging materials, offering an indirect control for products irradiated in their package (Dodd *et al.* 1989; Delincée and Soika 2002).

Thermoluminescence (TL) and photostimulated luminescence (PSL) are radiation-specific phenomena resulting from energy storage by trapped charge carriers in dielectric materials following irradiation. Releasing such stored energy by thermal or optical stimulation can result in detectable luminescence emission during the relaxation processes which follow. These approaches can be applied to inorganic components, present either as inherent parts of foods or as adhering contaminants and to bio-inorganic systems. The strengths of these techniques lie in their radiation-specificity and the wide range of sample types which may by analyzed (Sanderson *et al.* 1996). These techniques are commonly used for the detection of irradiation of herbs, spices and their mixtures and for shrimps. Thermoluminescence analysis is used to detect radiation processing of foods which are contaminated with sand or dust. Silicate minerals are isolated and their radiation-induced luminescence is measured and compared to the thermoluminescence from a second measurement after exposure to a defined radiation dose (Soika and Delincée 2004).

13.3.2 Chemical Methods for Irradiation Detection

Irradiation of foodstuffs rich in lipids leads to the formation of various volatile compounds that may be used as potential markers of irradiation processing. The amount of radiolytic products from lipids, such as volatile hydrocarbons, aldehydes, or ketones, is directly linked to the chemical composition of the lipids. Consequently, fatty acids were undergone preferential cleavages in the ester carbonyl region. It should be noted that 2-dodecylcyclobutanone has never been detected in un-irradiated chicken meat and does not seem to be generated by cooking (Boyd *et al.* 1991). In fact, it seems to be a specific marker for irradiation treatment. However, the quantities induced are very small. It is possible that by using novel analytical methods featuring lower detection limits down to the pM level, the specificity to irradiation of this product could disappear, as has already happened for other specific radiolytic products. In the literature, several other radio-induced chemical products are cited as markers. However, none have been found to be characteristic of irradiation.

13.3.3 Biological Methods for Irradiation Detection

There are various biological and microbiological approaches to detect irradiated food. Ionizing treatment of food induces changes in nucleic acids. As a consequence, the methods of identification could be based on changes in DNA. Detection of DNA strand breaks in mitochondrial DNA from beef liver was achieved by analysis using agarose gel electrophoresis (Rydberg 1996). More recent publications deal with the adoption of the limulus amoebocyte lysate (LAL) test combined with a Gram-negative bacterial count for the detection of irradiated poultry meat as an official method in the UK. Additionally, the direct epifluorescent filter technique/aerobic plate count (DEFT/APC) procedure is also used for poultry meat (Scotter *et al.* 1990). Both techniques are based on the difference between dead and live microorganisms, indicating the number of organisms deactivated by irradiation or other decontaminating treatments. Moreover, they may be valuable to determine the hygienic status of the food prior to radiation treatment or/and after processing has taken place. Following irradiation, the amount of supercoiled DNA is reduced, and its analysis could be used as an identification test. Another promising method for detecting DNA fragments in irradiated food uses the microelectrophoresis of single cells (Ostling and Johanson 1984). Among the advantages of the method are its simplicity and the speed with which it can be performed as the electrophoretic separation lasting only 2.5–5 min. Fruit, treated with ionizing radiation for quarantine purposes, may also be checked for insects. An interesting approach is the simple spot test which detects polyphenol oxidase activity in Caribbean fruit flies. It is noticed that the enzyme activity has greatly reduced in irradiated larvae.

13.4 Heating Techniques

Food preparation based on heating techniques aims to extend the shelf life of the foodstuff and to prevent microbiological deterioration. Microwave heating processes are under investigation in order to substitute more conventional heating techniques such as cooking, blanching, drying etc. Fast heating methods based on microwave energy

application to food products can provide high sterilization efficiency compared to conventional methods. Over 60% of homes in the UK and over 90% of homes in the US and Canada feature a microwave oven and an increasing range of processed foods are produced with microwave reheating instructions (Schubert and Regier 2005). Microwaves are electromagnetic waves within a frequency band of 300 MHz to 300 GHz that pass through material and interact with its polar molecules and charged ions. This interaction makes molecules vibrate quickly as they try to align themselves to the rapidly shifting electromagnetic field. This intense vibration generates heat within the material. Microwave in food technology is a target of research and development. Applications are increasingly used in tempering, vacuum drying, freeze drying, dehydration, cooking, blanching, baking, roasting, rendering, pasteurization, sterilization, and extraction.

However, extensive use of microwave heating in food processing has been delayed due to the increased cost of the instrumentation and safety affairs. Microwave energy use is feared for safety reasons associated with radiation leakage and contaminants transferring to foods from susceptors and containers. There are many reports and articles blaming microwave heating for the formulation of undesirable components. As a result, many consumers and microwave oven users were confused. However, these alarming reports were not based on proper scientific investigation. There have been periods of doubt in the public regarding the safety of microwave heating and microwave heated foods. It should be noted that food nutritionists agree on the nutritional advantages of microwave like the improved retention of vitamins and minerals in vegetables. However, as in the case of irradiation, consumers have the right to know if foodstuffs are treated with microwaves.

13.4.1 Methods of Detection

According to our knowledge, there is not any analytical method for the detection of microwave heating (Ballin 2010). The possible explanation for the limited scientific discussion on this issue is that economic benefit from fraudulent behavior could be low. However, as microwave heating is used instead of other traditional techniques, there are some compounds that can be used as markers of thermal processing. The production of Maillard compounds during heating can potentially differentiate the various thermal procedures like boiling, frying and grill. Maillard products are formed above certain temperature which permits the discrimination between long-term cooking at a low temperature from short-term cooking at a high temperature (Belitz *et al.* 2009). For instance, acrylamide is formed above 120°C and could be used as an indicator of products exposed to temperature above 120°C (Schubert and Regier *et al.* 2004; Zhang *et al.* 2005). HPLC and LC-MS/MS can be used to quantify acrylamide. Furthermore, it can be determined whether a foodstuff has been treated with microwaves through its physical properties and appearance. For example, in milk processing, residual sulfhydryl oxidase activity was detected in microwave processed samples and decreased during the storage. No residual activity was detected in the UHT-treated samples. Small differences were observed between the viscosity of the UHT- and microwave-treated white milks. According to color, significant differences were observed between white UHT and microwave-processed samples. Sensory analyses indicated that UHT-treated milks were visibly darker and showed higher caramelization and fatty flavors with higher

astringency compared to the samples treated with microwaves (Salazar-González *et al.* 2012).

13.5 Conclusion

Food processing and preservation technologies must keep fresh-like characteristics while providing an acceptable and convenient shelf life. Additionally, it is of great importance the assurance of safety and nutritional value. Processing technologies include a wide range of methodologies to inactivate microorganisms, improve quality and stability and preserve and minimize changes of foodstuffs characteristics. However, these techniques are also used in a fraudulent way for economical profit. Although analytical chemistry provides many tools for food authentication, there is a gap concerning techniques capable of detecting undeclared processes. This is the challenge that future Food Scientists have to overcome.

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14

Adulteration Stories

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14.1 Introduction

Food frauds have been around from antiquity. However, it is only in the past few years that consumers, regulators, and companies have started to "wrestle" seriously with the problem. Food fraud is the deception of consumers using food products, ingredients, additives, and packaging for economic gain. There are several types of food fraud such as substitution, unapproved enhancements, misbranding, counterfeiting, and others. Food adulteration can be more dangerous than traditional food hazards, because the adulterant could be an unconventional substance that has not been assessed concerning safety for human consumption. Fraudsters adulterate foodstuffs so they can evade existing quality assurance (QA) and quality control (QC) systems that are applied. Unfortunately, the fraudulent foodstuffs satisfy the safety standards that are posed by good manufacturing practice (GMP) and hazard analysis and critical control points (HACCP) plans. On this fate, only the fraudsters know how the food ingredients have been treated. There is an emerging concern that food fraud emerges greater risks than traditional food safety risks. The adulterants used in these counterfeits are often unconventional. A typical example of that is melamine. It was considered neither a potential contaminant nor an adulterant in the food supply before 2007, and was therefore not included in routine QA/QC analyses. Therefore, food safety systems are not designed to protect from the uncountable potential adulterants that may show up in the food supply. Additionally, food fraud recently came to the fore with the horse meat scandal in the EU.

The spread of food fraud is global. In fact, even multinational food companies are involved in food fraud scandals motivated by profit. The global food import bills have been recently estimated at US$ 1.29 trillion by the Food and Agriculture Organization of the United Nations. It is the fifth year in succession that the world bill has surpassed the 1 trillion US dollars. Taking into consideration the great amount of money that is calculated, the high rate of fraud should not be a surprise.

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Another issue of great importance that has to be considered is the potential health effect on consumers. This has led to the introduction of food forensics, a field that is comprehensively described by Aceto (2015) who highlights the most reliable markers that arise from mass spectrometry techniques. In this chapter, we present current and classic adulteration cases in order to provide an update to the scientific community.

14.2 A Flashback

Food fraud is not a new topic (http://www.history.com/news/hungry-history/food-fraud-a-brief-history-of-the-adulteration-of-food, last accessed 11/2015). Criminal fraud has been around for thousands of years whenever the opportunity for profit has arisen. Early food laws and codes had a major focus on adulterants for economic gain. In ancient Rome there was legislation on food adulteration which provided penalties, for example, servitude or exile, for the fraudsters. During the Middle Ages, imported spices were quite valuable. Due to their high prices and limited supply, merchants sometimes combined spices with numerous cheap substitutes such as ground nutshells, pits, seeds, juniper berries, stones, or dust. In the thirteenth century, guilds in France and Germany tried to control adulteration with food control statutes. In addition, King John in England made a proclamation regarding penalties for the adulteration of bread. However, Reformation marked the beginning of a new era where the influence of guilds waned and, along with them, their laws.

During the eighteenth and nineteenth centuries, food adulteration for economical gain became extensive (Carlyle 1993). The reason was the Industrial Revolution, which forced people to disconnect from agriculture and consequently from food production. Not only had they lack of knowledge on food production and processing, but also they did not have any quality standards. Common food frauds that were observed were the extension of milk with water and the mixing of tea, coffee, and spices with dirt and sand, etc.

Unfortunately, the problem for the established legislation, that it prohibited the use of unsafe additives, was the lack of dependable tests and techniques for the determination of the adulterants. Around 1820, the evolution of science had as a result the use of microscopes that could detect differences between ingredients. However, the rise of analytical chemistry did not benefit only the audit authorities but also the fraudsters. By the end of the nineteenth century, manufacturers could "cover" food deterioration in ways that were tough to detect.

Common food preservatives and additives were not studied concerning their toxicological effect, resulting in the use of harmful substances. On this topic, in 1902 Dr Harvey W. Wiley created a group of volunteers known as the "Poison Squad" that were willing to ingest preservatives such as formaldehyde, copper sulfate, and borax. The purpose of this study was to determine the impact of the additives on health of the volunteers. During the twentieth century, society was becoming more and more aware of the food safety. Today, safety standards are high and consumers demand qualitive products. To sum up, tracing food adulteration is a significant challenge due to the complexity of food processing.

14.3 Food Fraud Incidents

Over the last decades, there have been many notable adulteration incidents that had a huge impact on societies and the food industry, resulting in serious health incidents in some cases. In the following sections some of the better-known adulteration incidents, and their methods of detection, are described.

14.3.1 Bootleg Liquor, India, 2015

Bootleg liquor containing methanol killed 84 people and sickened 21 more who drank the cheap, illicit hooch which was bought at small shops in Mumbai. Illegal liquor operations flourish in the slums of urban India and among the rural poor who cannot afford the alcohol that is sold at cellars and liquor stores. The brew is often mixed with cheap chemicals to increase potency and profit, and causes several types of illnesses and death. The most common adulterant is methanol, because of its price. Methanol is normally used as fuel, solvent, or antifreeze agent. Furthermore, is a highly toxic compound that can induce comas, cause blindness, and is deadly in high doses. Finally, the tainted liquor can lead to fits, vomiting, and death.

The production of illicit liquor is a hugely profitable industry across India, where fraudsters pay no taxes and sell enormous quantities of their product. Toxic alcohol deaths are a regular occurrence in India. The bootleggers operate secretly, working in homes, hidden warehouses, and even in forests. They are capable of turning 1 liter of genuine alcohol into 1000 liters of bootlegged swill with chemicals and additives that usually cause no harm, but on occasion can lead to tragedy. The liquor is packed in cans and delivered all over the district and other parts of the state in buses and on trains. The illegal alcohol – commonly called desi daroo or country-made liquor in India – usually costs little and the majority of consumers are poor, daily-wage workers. India has witnessed many incidents of toxic alcohol deaths, for example in July 2009, February 2010, and February 2011 (http://news.yahoo.com/bootleg-liquor-kills-143-people-eastern-india-140530822.html, http://www.dw.com/en/dozens-die-after-drinking-illegally-brewed-alcohol-in-mumbai/a-18528250, last accessed 11/2015).

14.3.2 Horse Meat Scandal, EU, 2013

During January and February 2013, a scandal broke throughout Europe. This scandal started around 15 January when the Food Safety Authority of Ireland (FSAI) was running routine tests on meat products, for example salami, beef meal, and beef burger. After running a routine set of DNA-based tests on those products, a shocking amount of 10 beef burger products out of the total 27 were found to contain equine DNA, meaning that the products contained horse meat although they were not labeled as such. In particular, in one sample the results demonstrated 29% equine DNA, a fact that set alarms ringing for the FSAI. Afterwards, two reports in the UK came to the conclusion that not only there were beef products containing horse meat, but it was a result of "a complex network of companies trading in and mislabeling beef or beef products" (Chair of the Committee on Environment, Food and Rural Affairs, Anne McIntosh MP). In the following months, the scandal gained a tremendous amount of attention as more and more food fraud incidents came up. The timeline as depicted in the popular press is as follows.

- The Food Safety Authority of Ireland (FSAI) announced that horse meat had been found in frozen beef burgers at several Irish and British supermarkets, including Tesco, Asda, Dunnes Stores, Lidl, Aldi, and Iceland. The FSAI conducted tests on a selection of beef and salami products with "best before" dates between June 2012 and March 2014 (15 January 2013).
- It was revealed that the Findus beef lasagne range in the UK, France, and Sweden and the shepherd's pie and moussaka ranges in France contained horse meat without proper declaration. Out of 18 products, 11 tested positive for horse meat ranging over 60% to even 100%. The supplier of those products was a company named Comigel (7 February 2013).
- A study by Stellenbosch University found traces of water buffalo, goat, and donkey meat in various products on sale in South Africa (25 February 2013).
- Gæðakokkar 30% meat pie sold in Iceland is found to not contain any meat at all (27 February 2013).
- One of two Russian suppliers of sausage to IKEA in Russia is found to contain horse meat despite its labeling (7 March 2013).

There are many meat-supplying companies that were involved in this scandal. To begin with, the horse meat that was found in Comigel products originated at Doly Com, a Romanian-based slaughterhouse. An inquiry by the French government showed that "the meat had left Romania clearly and correctly labeled as horse. It was afterwards that it was relabeled as beef." Doly Com supplied the horse meat under a contract to Cyprus-based Draap Trading Ltd, a meat trader which operates in the Netherlands. It is owned by a British Virgin Islands holding company, and Jan Fasen is a director. Draap spelt backwards is paard, the Dutch word for horse. After having the horse meat delivered to a cold storage company in Breda, Draap then sold the frozen meat to Spanghero, who insist that it arrived at their Castelnaudary plant labeled as "Beef – originating in EU." After some processing, Spanghero then sent it to Comigel, where the final products were made. According to French media reports, Spanghero falsified documents regarding the meat. Moving on, the horse meat found in Silvercrest products, another major meat supplier and part of the ABP Food Group, is thought to have originated in Poland.

While horse meat is consumed in various countries, the main reason that this scandal called for attention was the fact that horse meat is considered a taboo food in many English-speaking countries such as the UK, Australia, Ireland, the USA, and English Canada. According to a small survey by a team from University College Dublin and Brunel University, consumers' main concern was that the claims made on labels did not match the contents of food products. However, it should be mentioned that additional concerns included allegations that a "French plant which handled horse meat sold in Britain as beef has previously been at the centre of a major E.coli discovery." Finally, inquiry into horse meat sources in the UK also revealed that Aintree racecourse had a contract with a licensed slaughterhouse in West Yorkshire to remove dead Grand National race horses.

Since the outbreak of this scandal, it spread to 13 other European countries and European authorities decided to find an EU-wide solution. Although labeling with the country of origin in the EU has been compulsory for meat since April 2015, it is still optional for processed meat (Premanandh 2013).

14.3.3 Adulteration with Melamine, China, 2008 and 2007

During the years of 2007 and 2008 in China, many protein-based food products or ingredients were adulterated with melamine, which is a lot cheaper than protein. The main problem is that, although melamine may have a positive role as an ingredient in various materials (e.g., plastic or even concrete), if consumed it can lead to toxicity problems, especially chronic toxicity, resulting in cancer or kidney health issues such as kidney failure. As a consequence, in 2007 a massive number of pets throughout the world died mysteriously from kidney failure. After a while the FDA discovered the root of this problem was wheat gluten, rice protein, or corn gluten, which proved to be wheat flour adulterated with melamine, cyanuric acid, and other contaminants. Those vegetable proteins originated from Chinese companies. The Chinese government originally refused to allow relevant investigations, but as public awareness was raised (along with the famous pet food recall in 2007) many food products consumed by pet or human alike were recalled, and in 27 April 2007 the FDA subjected every vegetable protein-based product imported from China to investigation. In addition, in 2008 there was a huge outbreak with over 300,000 babies in China falling ill and at least 3 dying from kidney stones or kidney failure. The source of this problem was baby milk adulterated with melamine instead of protein. What should be noted, however, is that in this scenario, the adulterers did not use industrial-grade melamine, but an even cheaper and more toxic version of it known as "scrap melamine." What is more surprising is that the Chinese government did not react for a year; effects of this adulteration event were being felt in 2010. Similar events have been spotted in other Chinese food commodities, such as eggs. Finally, a fact which is certainly troublesome, is that Jiang Weisuo, the man who first alerted authorities to what would become the melamine-tainted milk scandal, was murdered in Xi'an city. Authorities are currently using spectroscopy and other methods in order to detect melamine in food products (Kuehn 2009; Xiu and Klein 2010; Zhou and Wang 2011).

14.3.4 Food Extension in the ConAgra Incident, USA, 1997

COnAgraFoods, Inc., an American packaged foods company, was found guilty of illegally spraying water on stored grain to increase its weight and value at its Peavey Grain unit, while also bribing Federal inspectors. The United States Justice Department began investigating practices at Peavey Grain elevators around Terre Haute, Ind., and elsewhere 5 years ago until finally charges were made against ConAgra. Although the fine that the corporation was forced to pay was not large enough (US$ 8.3 million), it served as an opportunity to repay farmers who were financially damaged by this adulteration (http://money.cnn.com/1997/03/19/companies/conagra/, last accesed 11/2015).

14.3.5 Low-Cost Mixture Marketed as 100% Pure Apple Juice in the Beech-nut Incident, USA, 1987

On 13 November 1987, Beech-Nut, a corporation and a subsidiary of Nestle S.A. of Switzerland selling baby food products, pleaded guilty to Federal charges for selling apple juice which was actually flavored sugared water. Further, the product that Beech-Nut had been marketing as 100% apple juice was actually made from beet sugar, cane sugar syrup, corn syrup, and other ingredients, with little if any apple juice in the

mixture. Prosecutors said at the time of the indictment that the bogus apple juice cost about 20% less to make than real apple juice. The suspicions of this adulteration first started in 1977 and were later reinforced during 1982 by Andrew Rosenzweig, who had been hired by the Apple Processors Trade Association to investigate claims of adulterated concentrate. In 1986 FDA investigators initiated an investigation into Beech-Nut's role in the sale of the phony juice and the cover-up, leading to a federal grand jury finding in November 1986 that there was sufficient evidence to indict both Beech-Nut as a corporate entity as well as its two top executives, John Lavery and Neils Hoyvald, on 470 counts of violating the Federal Food, Drug, and Cosmetic Act for selling adulterated apple products. Consequently, Beech-Nut's sales plummeted until 1989 when sales started to increase again. However, the economic losses of the corporation were tremendous. Nowadays, in order to detect adulterations in apple juice products, the main method used is near-infrared spectroscopy along with the use of chemometrics (http://www.nytimes.com/1987/11/14/business/beech-nut-is-fined-2-million-for-sale-of-fake-apple-juice.html).

14.3.6 Arsenic in Beer, UK, 1880 and 1900

This is one of the first and best-known adulteration stories that drew the attention of Frederick Accum and is mentioned in his book *"A Treatise of Food Adulteration and Culinary Poisons."* Most importantly, at the very end of the nineteenth century (1900), a similar incident with the addition of sulfuric acid that was naturally contaminated with arsenic in the sugar of the beer, was the cause of a disease which appeared in Manchester, Salford, and also affected Liverpool and many other places. The source of the epidemic was only discovered from the research of Dr ES Reynolds, who realized that the beer was poisoned with arsenic. The disease was characterized by paralysis of muscles and loss of function in sensory nerves and was similar to peripheral neuritis, which typically resulted from chronic alcohol poisoning. Since many of the patients were clearly only moderate beer drinkers and their symptoms included unfamiliar features such as pigmentation of the skin, Dr Reynolds concluded that they were not suffering from alcohol poisoning but from arsenic poisoning. Until then, 6070 cases were occurred in London, including 70 deaths (Dyer 2009).

14.3.7 Bright Poisonous Inorganic Colors for Sweets, UK, 1858

This incident was first reported in Bradford, England where sweets were adulterated with arsenic. This resulted in the arsenic poisoning of more than 200 people and the death of 20 people (with the first victims being two children). The adulteration remained undetected until Dr John Bell identified arsenic as the cause, which was later confirmed by Felix Rimmington, a prominent chemist, druggist, and analytical chemist. Although this specific incident was accidental, many others were reported around that time. Thomas Wakley detected poisons contained in various items of colored sweets already condemned by Frederick Accum. They included gamboge, a yellow gum that acts as a violent purge and irritant, and brightly colored lead, copper, and mercury compounds. The trade in highly colored sweets had grown extensively in the decade since Accum first mentioned the problem. Even the sweet wrappers were often colored with the same poisonous salts to make the sweets look more attractive. Further incidents raised public awareness, leading to the Pharmacy Act of 1868 in the United Kingdom and to the initial

campaign of Wakley against food adulteration (http://www.historic-uk.com/HistoryUK/HistoryofBritain/Dying-for-Humbug-the-Bradford-Sweets-Poisoning-1858/).

14.3.8 Adulteration of Bread with Alum in London, UK, 1839

During the year of 1839, a considerable number of the poor of London and the children of the middle classes were suffering from rickets. The cause of this phenomenon was unknown, until Liebig found out that the alum which bakers were adulterating the bread with was the source of this problem. He said that since phosphoric acid formed with alumina is a compound hardly decomposable by alkalines or acids, it may perhaps explain the indigestibility of the London bakers' bread, which stroked all foreigners. Arthur Hassall, along with other scientists, pointed out that the only safe way to detect alum in the bread is to incinerate it and examine the ashes. Much effort was required to wipe out the rickets epidemic (Dunnigan 2003).

14.4 Conclusions

To summarize, food fraud is still a very serious problem in food science while consumers are becoming more aware of it. Unknown and unpredicted risks of foodstuffs are emerging. The economic motivation leads fraudsters to pioneer adulteration schemes. In this chapter, we have focused on adulteration stories in order to provide an update to the scientific community.

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15

Organic Foods

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15.1 Introduction

Organic food is food produced in a way that complies with organic standards set by national governments and international organisations (FAO/WHO 2008; Patil *et al.* 2014; Todorova and Ikova 2014; Wachter and Reganold 2014; Wollni and Andersson 2014). In the European Union, organic farming is a way of producing food that respects natural life cycles (European Community 2007). It minimises the human impact on the environment and operates as naturally as possible, in accordance with objectives and principles including the following:

- crops are rotated so that on-site resources are used efficiently;
- chemical pesticides, synthetic fertilisers, antibiotics and other substances are severely restricted;
- genetically modified organisms (GMOs) are banned;
- on-site resources are put to good use, such as manure for fertiliser or feed produced on the farm;
- disease-resistant plant and animal species adapted to the local environment are used;
- livestock are raised in a free-range, open-air environment and are fed on organic fodder; and
- animal husbandry practices are tailored to the various livestock species.

Organic farming is part of an extensive supply chain, which also includes food processing, distribution and retailing, and the EU require producers to obtain special certification in order to market food as organic within their borders. Since 1 July 2010, producers of packaged organic food have been required under EU law to use the EU organic logo (Fig. 15.1; European Commission 2014). The logo and the labelling rules are an important part of organic regulations.

Although several studies have been published comparing the quality of food derived from organic and non-organic origin, it is still not clear if food from organic production *per se* can guarantee product-related added value to consumers (Chen Guo 2008; Zalecka *et al.* 2014). Organic food products are considered healthier, safer and more

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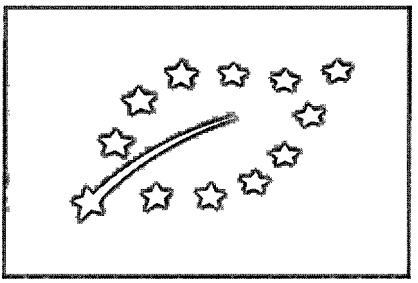


Figure 15.1 Organic logo of the EU.

environmentally friendly than their conventional counterparts, and are also sold at higher prices. Consequently, adulteration of organic plants and fraudulent activities for economic profit are increasing (Zalecka *et al.* 2014).

The main focus of analytical control is on authentication as an analytical verification of the certification process. The use of multi-element and stable-isotope analysis based on atomic spectroscopy for discriminating between organic and conventional plants has been recently reviewed (Laursen *et al.* 2014). Inductively coupled plasma mass spectrometry, stable-isotope analysis of bulk plant tissue and compound-specific isotope analysis based on isotope ratio mass spectrometry are promising tools for documenting the fertilisation history of organic plants. However, these techniques are challenged by the potential diversity of fertilisation practices and the needs of chemometrics to develop statistical models that can classify the agricultural origin of plant products.

New fingerprinting approaches such as ultra-performance liquid-chromatography mass spectrometry, gas-chromatography mass spectrometry and direct analysis in real-time high-resolution mass spectrometry as well as crystallisation with and without the presence of additives seem to be promising methods in terms of time of analysis and detecting organic system-related parameters (Kahl *et al.* 2014).

This chapter presents a comprehensive overview of the different properties measurable, the analytical technique applicable and the chemometrics required to establish the authenticity of organic food. Stable isotope, major trace elements, proteins and metabolites can be used as biomarkers and a wide range of analytical and chemometrics techniques can be applied in this field. The development and validation of analytical protocols to test organic products for their authenticity will contribute to restoring trust in producers of organic food, fairness of the marketplace and the confidence of consumers being offered produce that has been grown according to organic standards.

15.2 Biochemical Markers and Analytical Platforms

The main focus of effort to establish organic food quality is on the authentication as an analytical verification of the certification process. The importance of this issue and its pivotal role is inevitably covered in any review on authentication of food products, such as meat and meat products (Ballin 2010; Kamruzzaman *et al.* 2015), dairy products (Deelstra *et al.* 2014) and food and beverages (Van Leeuwen *et al.* 2014). Furthermore,

Kahl *et al.* (2014) give a status quo report on technologies and analytical methods used to find answers to the various questions related to the field of organic food. Recently, Capuano *et al.* (2013) also compiled the most promising biomarkers/analytical approaches available for the authentication of organic produce. Both reviews conclude that is unlikely that the authentication of organic food products can be attained by the measurement of a single marker. Instead, it requires the determination of multiple markers and/or complex chemical or physical profiles/fingerprints supported by multivariate statistical analysis. In the analytical authentication of organic products, procedures, including sampling and sample preparation, instrumental separation analysis, data analysis, identification of potential marker candidates and biological interpretation, are often performed sequentially to complete the whole approach (Fig. 15.2).

The most important markers useful for organic food authentication are stable isotopes, major trace elements and several organic food components (food metabolites, macronutrients and contaminants). Selected examples of authentication of organic food are summarised in Table 15.1. The application of advanced -omics technologies (including non-target strategies to identify unknown markers or to obtain a characteristic fingerprint able to distinguish organic and conventional food) to acquire knowledge about issues related to authenticity of organic foods is increasing.

15.3 Sampling

The identification of biomarkers capable of distinguishing organic and conventional products involves some already well-known target markers or non-target metabolomics. As the first step, representativeness and random samples need to be taken into account. The main problem with representativeness is that to achieve technical representativeness of a community, the sample size would have to be far too large to

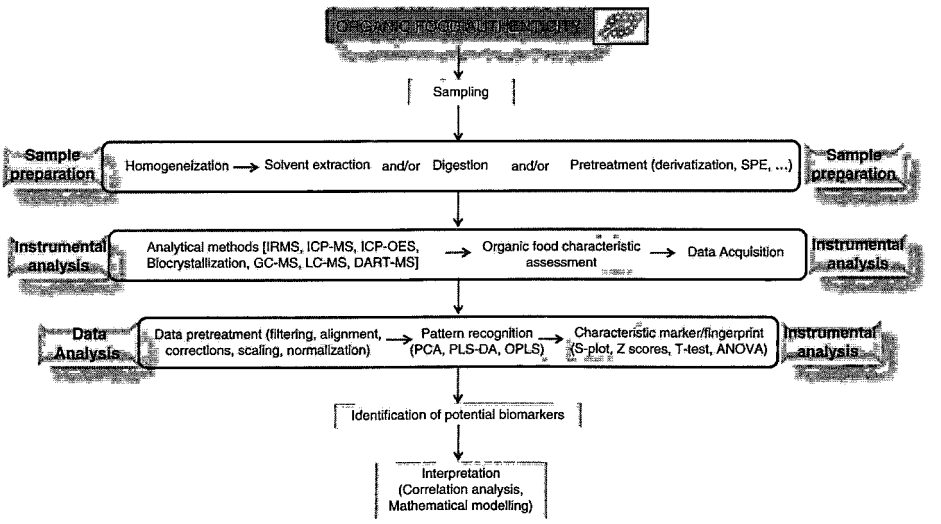


Figure 15.2 Scheme of the authentication of organic food.

Table 15.1 Selected examples of analytical platform discriminating between organic and conventional.

Food	Biomarker	Extraction	Determination	Chemometric	Comment	Reference
Elemental composition						
Wheat, spring barley, faba bean and potato	Multi-elemental fingerprinting	Digested with H_2O_2 in vessels by microwave.	ICP-MS and ICP-OES	Multivariate data analysis PCA	No systematic differences between organic and conventional crops in the content of essential plant nutrients when statistically analysed	Laursen et al. 2011
Isotope ratio mass spectrometry (IRMS)						
Vegetables	Carbon and nitrogen contents and isotopic composition: $\delta^{15}N$ and $\delta^{13}C$	Ground into a fine, homogeneous powder and transferred into tin capsules	EA-IRMS CO_2 was resolved from N_2 by GC	–	Nitrogen isotopes are a way of identifying faster-growing organic vegetables (maturity time to harvest <80 days), as these vegetables tend to be significantly more enriched in ^{15}N than conventionally grown vegetables and natural soil N	Rogers 2008
Eggs	$\delta^{15}N$ and $\delta^{13}C$	Yolk delipidised by extraction with Cl_3CH and methanol, dried overnight and placed on a tin capsule	EA-IRMS Interfering gases were separated by GC	–	Stable nitrogen isotope analysis of egg components is useful to unravel dietary differences between caged or barn hens and free-range hens (both conventional and organic)	Rogers 2009
Dairy products	$\delta^{13}C$ and α-linolenic acid	0.45 g fat milk introduced in a tin capsule and wrapped	EA-IRMS (bulk material) sample was oxidised and interfering gases separated by GC	–	The carbon stable isotope ratio ($\delta^{13}C$) and the R-linolenic acid (C18:3ω3) content of milk fat were examined as general markers	Molkentin 2009

Dairy products	$\delta^{15}N$, $\delta^{13}C$ and α-linolenic acid	Normal and defatted samples were combusted in tin capsules	EA-IRMS (Bulk material)	–	Non-compliant results were obtained for whey as well as both Italian ice creams and cheeses; fatty acids were determined using GC-FID	Molkentin 2013
Vegetables	NO_3^-	Water at 90°C and centrifugation	IC (non-isotopic)	Mann–Whitney U test	Nitrogen and oxygen isotope analysis of nitrate has a potential for differentiation between organic and conventional crops	Mihailova *et al.* 2014
	$\delta^{15}N$ and	N bulk	IRMS (Nbulk)	One-way ANOVA		
	$\delta^{15}N$ and $\delta^{18}O$ from NO_2	$NO_3^- \rightarrow N_2O$ denitrifying *Pseudomonas aureofaciens*	CSIAL: HS-GC-IRMS (different sorbent traps to eliminate interferences)	CDA		
Plants (wheat, barley, faba bean and potato)	δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ or $\delta^{34}S$	Bulk ground materials over dried	IRMS (bulk material)	–	Stable isotopes of hydrogen, carbon, nitrogen, oxygen, magnesium and sulphur as well as compound-specific nitrogen and oxygen isotope analysis of nitrate for discrimination of organically and conventionally grown plants	Laursen *et al.* 2013
	$\delta^{25}Mg$ and $\delta^{26}Mg$	Wet digestion ($HNO_3^- + H_2O_2 + HCl$) clean-up using cationic and anionic exchange resins	MC-ICP-MS (bulk material)			
	NO_3^-	Water at 90°C and centrifugation	IC (non-isotopic)			
	$\delta^{15}N$ and $\delta^{18}O$ from NO_2	$NO_3^- \rightarrow N_2O$ denitrifying pseudomonas aureofaciens	CSIA: IR-MS			
Strawberries	$\delta\ ^{13}C_{VPDB}$ of the volatile components of strawberries	HS-SPME DVB-PDMS-Carboxen 50/30 μm; 15 min at 50°C and 3000 rpm	CSIA: GC-C-IRMS GC-MS (to identify characteristic volatile compounds)	–	An authenticity range was achieved, investigating the carbon isotope ratio of numerous selected aroma active volatile components in organic strawberries	Schipilliti *et al.* 2011

(Continued)

Table 15.1 (Continued)

Food	Biomarker	Extraction	Determination	Chemometric	Comment	Reference
Dairy products	$\delta^{13}C$ and phytanic acid	Fat extraction by PLE with 10 mL cyclohexane/ethyl acetate (46:54); analysis of dry fat: solvent evaporation; analysis of whole milk: overnight evaporation at room temperature	EA-IRMS (bulk material) GC column to removal undesirable gases CSIA: GC-IRMS of the $\delta^{13}C$ of phytanic acid	–	Compare the efficiency of the markers stable carbon isotope ratio ($\delta^{13}C$ value) with the SRR/RRR phytanic acid diastereomer ratio (SRR/RRR) of milk fat for distinguishing different feeding systems	Kaffarnik *et al.* 2014
Salmon	$\delta^{15}N$ and $\delta^{13}C$, fatty acids and carotenoids	Defatted samples combusted in tin capsules	EA-IRMS (bulk material) interfering gases were separated by GC; CSIA: IRMS of FAMEs	Student-*t*	FAMEs were determined by GC-FID and carotenoids by HPLC-UV	Molkentin *et al.* 2015
Biocrystallisation						
Carrots	Assessment of crystallisation pattern	Grind plant material	Glass dish with plant material added with $CuCl_2{\cdot}2H_2O$	The reproducibility between three laboratories in the EU was investigated	To be applied in routine analysis for authentication of organic products, the biocrystallisation method has to be standardised	Busscher *et al.* 2010
Infrared spectroscopy						
Feed	NIR-spectra	–	NIRS, in the spectral range of 1100–2498 nm	PCA, PLS-DA	Data pre-processing: none, autoscaling, first and second derivative, SNV, DOSC; comparison with fatty acid fingerprinting	Tres *et al.* 2012

Proton transfer reaction mass spectrometry (PTR-MS)						
Coffee	Volatile compounds	Headspace air of the ground coffee	PTR-TOF-MS in the mass scan mode, in the range 26–200 amu	PCA, PLS-DA	Coffees were characterised by HS PTR-MS and volatiles were identified by PTR-TOF-MS	Ozdestan *et al.* 2013
GC-MS						
Basil	Non-target metabolomics	0.1 g of basil leaf powder was mixed with 2 mL of a pentane/acetone (90:10, v/v), agitated and centrifuged	GC-qMS splitless injection, EI ionisation, full scan *m/z* 50–500	FOAM, FuRES, PLS-DA and SIMCA	Characteristic components in basil extracts corresponding to highest-ranked useful features were putatively identified	Wang and Harrington 2013
Wheat grain (Triticum aestivum L.)	Non-target metabolomics	15 mg of groundwheat extracted with methanol	GC-IT-MS splitless injection, EI ionisation, full scan *m/z* 50–500	Student's t-test, PCA	Altogether 48 metabolites and 245 non-identified metabolites (TAGs) were detected in the cultivar Runal	Bonte *et al.* 2014
Dairy products	Phytanic acid, SRR/RRR ratio and $\delta^{13}C$	Fat extraction by PLE with 10 mL cyclohexane/ethyl acetate (46:54); conversion to methyl esters by means of BF_3-methanol complex; FAMES were extracted with hexane	GC-qMS splitless injection, EI ionisation, SIM internal standards	–	Compare the efficiency of the markers stable carbon isotope ratio ($\delta^{13}C$ value) with the SRR/RRR phytanic acid diastereomer ratio of milk fat for distinguishing different feeding systems	Kaffarnik *et al.* 2014
Dairy products	Phytanic and pristanic acid	Fat extraction by PLE with 10 mL cyclohexane/ethyl acetate (46:54); conversion to methyl esters by means of BF_3-methanol complex; FAMES were extracted with hexane	GC-qMS splitless injection, EI ionisation, SIM internal standards	ANOVA	Phytanic and pristanic acid cannot be *de novo* synthesised by mammals, and the predominant source for uptake is chlorophyll in food	Vetter and Schroeder 2010

(Continued)

Table 15.1 (Continued)

Food	Biomarker	Extraction	Determination	Chemometric	Comment	Reference
Dairy products	Phytanic acid SRR/RRR ratio	Fat extraction by PLE with 10 mL cyclohexane/ethyl acetate (46:54); conversion to methyl esters by means of BF_3-methanol complex; FAMES were extracted with hexane	GC-qMS splitless injection, EI ionisation, SIM internal standards	ANOVA	The phytanic acid content in milk correlates with the amount of green items in the cows' feed	Schroeder *et al.* 2012
Dairy products	Diastereomer SRR/RRR ratio	Fat extraction by PLE with 10 mL cyclohexane/ethyl acetate (46:54); conversion to methyl esters by means of BF_3-methanol complex; FAMES were extracted with hexane	GC-qMS splitless injection, EI ionisation, SIM internal standards	ANOVA	Milk from an organic cow collected over a period of 30 days showed lower proportions of 3S,7R,11R,15-phytanic acid than milk from a cow raised with conventional feed	Schroeder and Vetter 2011
Dairy products	Potential marker fatty acids: phytanic acid, pristanic acid, 18:3n-3, and 20:5n-3	Fat extraction by PLE with 10 mL cyclohexane/ethyl acetate (46:54); conversion to methyl esters by means of BF_3-methanol complex; FAMES were extracted with hexane	GC-qMS splitless injection, EI ionisation, SIM internal standards	ANOVA	The day-to-day fluctuations were small, and the lowest value in organic milk was generally higher than the highest value in conventional milk	Schroeder *et al.* 2011
LC-MS						
Cabbage	Non-target metabolomics (*c.* 1600 compounds)	2 extractions with 1 mL MeOH/H_2O 50/50 2 with 1 mL acetone/H_2O 70/30	UHPLC-QqTOF-MS ESI 100–1000m/z (PI and NI)	ANOVA-PCA OPLS-DA	Systematic influence of the production system on the chemical composition of the crop was found	Mie *et al.* 2014

Beetroot (*Beta vulgaris* L.) and naturally fermented beetroot juices	Non-target metabolomics	2 g of beetroot or beetroot juiced extracted with methanol, agitated and centrifuged	UHPLC-QqTOF-MS ESI 100–1000m/z (PI and NI)	PCA, LDA	Level of antioxidants and metabolomic fingerprinting in raw beetroots and naturally fermented beetroot juices from organic versus conventional production	Kazimierczak *et al.* 2014
DART-MS						
Tomatoes and peppers	Non-target metabolomics	A methanol–water mixture (1/1.2 mL) was added to 0.2 g of the freeze-dried sample, shaken and centrifuged	DART-TOF-MS (PI and NI) 6000 FWHM m/z 50-950 12 Dip-It tip scanner autosampler	PCA, LDA	The results suggest that the year of production had a stronger influence on the metabolomic fingerprints compared with the type of farming (organic versus conventional)	Novotna *et al.* 2012
Dairy products	Triacylglycerol (TAG) profiles (mass spectra)	Two procedures: (1) dilution of milk (6 mL) with 6 mL of methanol; (2) 4 mL of milk shaken with 4 mL of toluene	DART-Orbitrap 50,000 FWHM *m/z* 50–950 12 Dip-It tip scanner autosampler	Data pre-treatment: constant row sum transformation PCA, LDA	The capability to determine milk from conventional and organic farming was rather low; poor classification rates of the LDA model were obtained	Hrbek *et al.* 2014
Biological methods						
Nectarines	Microbial ecology analysis	DNA extracted after peptone water clean-up, resuspension in breaking buffer and SDS	PCR-DGGE	Cluster analysis and PLS-DA	Results show that yeast and bacterial communities were specific to the farming type, allowing organic fruits to be discriminated from conventionally grown fruits	Bigot *et al.* 2015

CDA: canonic discriminant analysis; ESI: Electrospray; IC: ion chromatography; GC-qMS: gas chromatography single quadrupole mass spectrometry; MC-ICP-MS: multi-collector inductively coupled plasma mass spectrometry; NI: negative ionization mode; PCA: PI: positive ionization mode; UHPLC-QqTOF-MS: ultra-high pressure liquid chromatography-quadrupole time-of-flight-mass sectrometry

analyse. To maintain the representativeness of the research material in an LC-MS non-target metabolomic study, Mie *et al.* (2014) collected 40 kg of beetroots from each farm each year; the samples were collected as four subsamples of 10 kg each from different parts of the field (Kazimierczak *et al.* 2014). Collectively, the time and the method of sampling are of great importance, as they can greatly affect the quality of the analytical sample.

15.4 Sample Preparation and Extraction

Sample extraction can vary greatly depending on the type of analysis performed to establish the authenticity of an organic food. In any case, for solid samples (e.g. grain, bean, rice and fruit kernel, fish or meat), homogenisation of the samples is needed prior to sample extraction. To achieve this, samples are often ground into powders under liquid nitrogen using a mortar and pestle, using a ball-mill with pre-cooling holders or after freezing and lyophilising (Tres *et al.* 2012). In some cases, particularly in dairy products, defatted or delipidised matrices are required. This is achieved by eliminating fat with a non-polar organic solvent, such as CH_2Cl_3 (Molkentin 2013). Instead, in others the fat is the selected sample, which is commonly isolated by pressurised liquid extraction (PLE) (Schroeder *et al.* 2012) using cyclohexane/ethyl acetate. Liquid samples, such as whole milk, are commonly frozen and lyophilised (Schroeder *et al.* 2012), and then processed as solid samples.

Prior to multi-elemental analysis, samples are digested to eliminate the organic matter (Laursen *et al.* 2011). The most frequent treatment is acid digestion or H_2O_2 treatment, many times, using vessels and a microwave oven. Stable isotope analysis requires sample combustion in a tin capsule, and when compound-specific stable isotope analysis is carried out a previous isolation of the compound is needed. For example, nitrate was extracted with water (Mihailova *et al.* 2014), and for the simultaneous determination of $\delta^{15}N$ and $\delta^{18}O$ the denitrifier method was commonly used. The method is based on the reduction of nitrate (NO_3^-) to nitrous oxide (N_2O) using a denitrifying bacterial strain *Pseudomonas aureofaciens* (ATCC No. 13985, recently reclassified as a strain of *Pseudomonas chlororaphis*) that lacks in nitrous oxide reductase, thereby prevents further reduction to N_2. Organic acids, such as phytanic acid, were transformed into their methyl esters by means of 1% of sulphuric acid in methanol and were separated by urea complexation. The filtrate of urea complexation was fractionated by high-speed counter-current chromatography (HSCCC). The fraction containing phytanic acid methyl ester was then analysed (Kaffarnik *et al.* 2014).

Taking the non-targeted metabolomics approach as an example, sample extraction is usually carried out by non-selective methods in order to maximise the number of metabolites determined. A good sample extraction should be conducted knowing the general characteristics of potential metabolites (e.g. polar or non-polar, soluble or insoluble, stable or labile, volatile or non-volatile). In practice, the optimal sample-extraction protocol is often developed on a case-by-case basis depending upon the type of samples and the purpose of the experiment. Volatile components can be separated by headspace alone or in combination with solid-phase microextraction depending on the concentration (Schipilliti *et al.* 2011; Ozdestan *et al.* 2013). In a non-targeted experiment for non-volatile components, the optimisation of solvents and the extraction

method is often required to meet the goal of covering as broad a scope of metabolites as possible. In most cases, the solvent combination of methanol–water with different ratios is used most for foods due to its powerful extraction capacity for polar compounds (Novotna *et al.* 2012; Kazimierczak *et al.* 2014; Mie *et al.* 2014).

Another special case is when biological methods that require DNA extraction are used (Bigot *et al.* 2015). Many different methods and technologies are available for the isolation of DNA. In general, all methods involving disruption and lysis of the starting material are followed by removal of proteins and other contaminants and finally recovery of the DNA. Removal of proteins is typically achieved with proteinase K, followed by salting out and extraction with an organic solvent.

15.5 Instrumental Analysis

15.5.1 Multi-Elemental Composition

The analysis of the multi-element composition of plants mostly relies in atomic spectroscopy including atomic absorption spectroscopy (AAS), graphite-furnace AAS, X-ray fluorescence spectroscopy, inductively coupled plasma optical emission spectroscopy (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS). The application of these techniques for comparing organic and conventional plants was reviewed recently (Laursen *et al.* 2014), demonstrating that ICP-MS was superior to the others.

This is confirmed by a recent example in which the multi-elemental composition of organic and conventional winter wheat, spring barley, faba bean and potato was analysed with ICP-OES and ICP-MS. The crops were cultivated over two years at three geographically different field locations, each accommodating one conventional and two organic cropping systems. The conventional system produced the highest harvest yields for all crops except the nitrogen-fixing faba bean, whereas the dry matter content of each crop was similar across systems. No systematic differences between organic and conventional crops were found in the content of essential plant nutrients when statistically analysed individually. However, multi-elemental fingerprinting with semi-quantitative ICP-MS and chemometrics has the potential to enable authentication of organic crops (Laursen *et al.* 2011).

15.5.2 Stable Isotope Analysis

Isotopes that are not subject to radioactive decay but persist in their same elemental form are stable isotopes (Laursen *et al.* 2014); those most commonly analysed include O, C, N, H and S. The isotopic composition is routinely reported relative to an internationally accepted standard and expressed in parts per thousand:

$$\delta = \left(\frac{R_{\text{sample}} - R_{\text{standards}}}{R_{\text{standards}}} \right) \times 1000$$

where R_{sample} is the isotopic ratio measured for the sample and $R_{\text{standards}}$ that of the international standards.

Stable isotope ratios are typically measured via isotope ratio mass spectrometry (IRMS) and some of its developments, including gas chromatography combustion IRMS (GC-C-IRMS) and derivatives, such as pyrolysis GC-IRMS as well as via multi-collector (MC)-ICP-MS.

Stable isotope ratio analysis of nitrogen has been evaluated for authentication of organic plant products in an increasing number of studies during the past years. Synthetic nitrogen fertilisers are produced from atmospheric nitrogen (δ^{15}Nbulk = 0‰). In contrast, the bulk nitrogen composition of animal manures and composts, which are widely used in organic production systems, is a combination of δ^{15}Nbulk values of a wide range of organic and inorganic compounds and is typically in a range between +4 and +16‰. This could be further used as the basis of a simple authentication tool to detect non-compliant organic farming practices and false labelling of organic produce. However, the discriminative power of the bulk nitrogen isotope method varies greatly with the growing conditions as well as the plant species (Rogers 2008). The application of N stable isotope ratio to differentiate between different egg-laying regimens has also been investigated (Rogers 2009). On the basis of this assumption, free-range eggs would be expected to have more positive δ^{15}N values (due to trophic or denitrification processes) than eggs from caged or barn raised hens.

Stable carbon isotope ratio analysis by elemental analysis isotope ratio mass spectrometry (EA-IRMS) also helps to determine organic milk. A key parameter in this context is the feed supplied to the cows, which depends largely on the farming system. For instance, pasture and hay are essential principal food items supplied in organic milk production. By contrast, conventional farmers take advantage of higher proportions of concentrate and maize silage. Grass-based feed consists of C3-plant, which are more depleted in ^{13}C (δ^{13}C values ranging from −23 to −30‰) than the C4-plant maize (δ^{13}C values of maize silage ranging from −12 to −13‰; Molkentin 2009, 2013, Rogers 2009; Kaffarnik *et al.* 2014; Molkentin *et al.* 2015). The stable isotope ratio of carbon (δ^{13}C) in milk fat is an interesting variable for organic milk authentication, particularly in combination with the α-linoleic acid content, demonstrating that a combination of several markers and different techniques can render optimal results.

In contrast to the stable-isotope analysis of bulk tissues, additional isotopic information can be obtained by compound-specific isotope analysis (CSIA), which focuses on recording isotopic signatures of specific compounds and can be based on both on-line and off-line compound isolation (Laursen *et al.* 2013) encompassing stable isotopes of hydrogen, carbon, nitrogen, oxygen, magnesium and sulphur as well as compound-specific nitrogen and oxygen isotope analysis of nitrate for discrimination of organically and conventionally grown plants. It is recommended that stable isotope databases covering δ^{2}H, δ^{15}N, $\delta^{18}ONO_3^-$ and δ^{15}N NO_3^- from different plant species, geographical origins and fertilisation strategies are generated in order to allow future classifications of organic plant products of unknown agricultural origin. Mihailova *et al.* (2014) investigated compound-specific nitrogen and oxygen isotope analysis of plant-derived nitrate alongside bulk nitrogen isotope analysis for discrimination between organically and conventionally greenhouse-grown lettuce and retail potatoes and tomatoes. The method revealed significant differences between conventional and organic fertilisation.

Authenticity assessment of flavoured strawberry foods was performed using headspace-solid phase microextraction (HS-SPME) coupled to gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) (Schipilliti *et al.* 2011). The use

of GC allows a wider range of components to be investigated, compared to similar studies available in literature (12 compounds were analysed by GC-C-IRMS to build the authenticity range of $\delta^{13}C$ values).

Combined stable isotope analysis of $\delta^{15}N$ and $\delta^{13}C$ in defatted dry matter allowed the differentiation of organically farmed from conventionally farmed salmon and brown trout, whether raw, smoked or graved (Molkentin *et al.* 2015). For the additional distinction of organic and wild salmon, a second analysis of $\delta^{13}C$ in fish lipids was required. Fatty acid analysis completely differentiated the three production types of salmon just by the linoleic acid content in the fish lipids, which was lowest in wild and highest in conventional salmon. Moreover, the elevated myristic acid content allowed organic to be distinguished from wild and conventional salmon. Furthermore, organic and conventional brown trout could be distinguished by combining the oleic acid and gondoic acid contents. Analysis of the free astaxanthin isomeric pattern allowed a clear distinction of conventional and wild salmon, but organic salmon showed variable patterns that did not consistently allow the authentication of their origin. This study also notes the importance of combining different markers.

15.5.3 Biocrystallisation

Copper chloride crystallisation is an analytical method based on the evaluation of the crystallisation patterns that occur when an aqueous cupric chloride solution ($CuCl_2{\cdot}2H_2O$) is crystallised on a glass dish in the presence of a plant extract. The patterns are characteristic of sample material. However, this method has to be standardised to be useful for authentication of organic products. Busscher *et al.* (2010) documented the laboratory process and developed and applied a computerised pattern evaluation tool for selected carrot samples. For standardisation, several factors of influence were tested and the reproducibility between three different laboratories in the EU was investigated. The method is able to differentiate patterns from samples from different farming treatments and processing steps as statistically significant.

However, while this method establishes a footprint characteristic of plant material, and has the advantage of being simple, cheap and does not require sophisticated instrumentation, it is not very specific and cannot compete with the amount of molecular information provided by other techniques. That is probably the reason why there are few applications of this technique.

15.5.4 Infrared Spectroscopy

The ultimate approach in organic product verification would be the development of methods based on rapid techniques that could be applied even during inspections on site. Near infrared spectroscopy (NIRS) is a non-destructive, easily applicable and fast technique that requires minimal or no sample preparation and permits the measurement of several components at once. By recording the response of certain molecular bonds (such as O–H, N–H or C–H) to NIR radiation, NIR spectroscopy generates a spectrum that may be characteristic of a sample and may be considered its 'fingerprint'. Tres *et al.* (2012) evaluated the application of NIRS as a rapid, cost-effective method to verify the organic identity of feed for laying hens. A binary classification model (organic v. conventional feed) was developed using partial least squares discriminant analysis. Spectral regions related to the protein and fat content were among the most important for the classification model.

15.5.5 Proton Transfer Reaction Mass Spectrometry (PTR-MS)

PTR-MS is a very sensitive instrument for the detection and measurement of trace levels of volatile organic compounds (VOCs). It incorporates a soft ionisation process by producing H_3O^+ ions from a discharge ion source which are then combined with the sample gas that enters through a separate inlet. The charge from the H_3O^+ ions is transferred to the VOCs within the sample that have a higher proton affinity. Normal air gases N_2, O_2, Ar, CO_2, etc. have a lower proton affinity and do not become ionised. After passing through a drift tube to remove cluster ions, the VOCs are then analysed and detected by the mass spectrometer. In the coffee sector a diversity of certifications is available, with the best-known being organic and fair trade. Intrinsic markers of products may help to ensure the authenticity of food products and complement administrative controls. Ozdestan *et al.* (2013) studied 110 market coffees with special production traits, characterised by high-sensitivity PTR-MS (HS-PTR-MS), and volatiles were tentatively identified by PTR-time-of-flight (TOF)-MS. Espresso coffees, Kopi Luwak coffee and organic coffees could be distinguished by their profiles of VOCs with the help of chemometrics. A Partial least squares Discriminant Analysis (PLS-DA) classification model was estimated to classify the organic and regular coffees by their HS-PTR-MS mass spectra. The strategy therefore represents a promising approach to rapid organic coffee authentication (Ozdestan *et al.* 2013).

15.5.6 Gas Chromatography–Mass Spectrometry (GC-MS)

GC-MS has been applied to authenticate organic food by target and non-target analysis. Analytical approaches targeted to specific compounds are well established in GC-MS. The most important application to authenticity of organic foods is through characteristic fatty acids such as phytanic acid for the case of daily products. Primary dietary sources of this acid are milk and dairy products as well as seafood. Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid, produced by bacteria by oxidation and bio-hydrogenation of the chlorophyll side chain in the rumen. Two diastereomers (3S,7R,11R,15- and 3R,7R,11R,15-phytanic acid) are naturally produced. The analysis of phytanic acid and its diastereomers is based on formation of its methyl ester and their determination by GC-MS in the selected ion monitoring mode (SIM). Phytanic acid and its degradation product, pristanic acid, have been tested as markers for differentiation of organic and conventional dairy products (Vetter and Schroeder 2010; Schroeder *et al.* 2011). Due to the unique source of grass-based feedstuffs in organic farming, it was tested whether organic cheeses and other organic dairy products are higher in phytanic and pristanic acid concentrations than conventional products. A target value of at least 200 mg/100 g phytanic acid was suggested for the verification of grass-fed, organic dairy products.

The diastereomer composition of phytanic acid has been examined in terrestrial and marine food samples (Schroeder and Vetter 2011). The marine samples were clearly dominated by 3S,7R,11R,15-phytanic acid. Results of this and other studies also indicated that the diastereomer composition in milk depends, at least to a certain degree, on diet, which in turn will have an influence on the composition of rumen bacteria composition. The rumen bacteria are responsible for the production of the phytanic acid and its diastereomeric composition (Schipilliti *et al.* 2011; Schroeder and Vetter 2011).

Kaffarnik *et al.* (2014) compared the efficiency of the markers stable carbon isotope ratio ($\delta^{13}C$ value) with the SRR/RRR phytanic acid diastereomer ratio (SRR/RRR) of milk fat for distinguishing different feeding systems. The low SRR/RRR in the samples indicated limitations in the unequivocal authentication of organic milk.

Non-target identification of biomarkers capable of distinguishing organic and conventional products would be highly welcome to improve the strength of food quality assurance. Metabolite profiling was used for a biomarker search in organic and conventional wheat grain (*Triticum aestivum L.*) of several bread wheat cultivars. Metabolites were extracted using methanol and analysed by GC-MS. Altogether, 48 metabolites and 245 non-identified metabolites were identified. Principal component analysis (PCA) showed a sample clustering according to farming systems and significant differences in peak areas between the farming systems for 10 metabolites. A t-test on data of all cultivars still detected 5 metabolites and 11 non-identified metabolites with significant differences between the farming systems. Based on individual cultivars, metabolite profiling showed promising results for the categorisation of organic and conventional wheat. Further investigations are necessary with wheat from more growing seasons and locations before definite conclusions can be drawn concerning the feasibility of evolving a combined set of biomarkers for organically grown wheat using metabolite profiles (Bonte *et al.* 2014).

Authentication of organic basils using GC-MS chemical profiles has also been demonstrated (Wang and Harrington 2013). Total ion chromatograms of the conventional class and the organic class have been provided in Figure 15.3. The major compositions of conventional and organic basils are similar to a large extent while different feature subsets have been selected by two different feature selection approaches to construct four economic classifiers that are effective in differentiating two classes of basils. Importantly, modelling reduced sets of features proved to be a rapid and viable approach for organic basil authentication that could lead to rapid analytical methods by targeting specific masses and chromatographic peaks.

15.5.7 Liquid Chromatography–Mass Spectrometry (LC-MS)

There are only a few studies that make use of large-scale non-targeted analysis techniques in analysing differences between organic and conventional crops using LC-MS. The level of antioxidants and metabolomic fingerprinting in both raw beetroots and naturally fermented beetroot juices from organic versus conventional production was determined (Kazimierczak *et al.* 2014). In addition, the anticancer properties of the fermented beetroot juices were evaluated. The obtained results showed that organic fresh beetroots contained significantly more dry matter and vitamin C than conventional beetroots, as well as some individual phenolic compounds. The content of total phenolic acids was significantly higher in conventional beetroots. The level of flavonoids was similar in both types. Metabolomic analysis provided the possibility to distinguish clearly between organic and conventional fermented beetroot juices. However, this method was less useful in the case of fresh whole beetroots. It is necessary to continue research on this topic in order to confirm and understand the achieved results (Kazimierczak *et al.* 2014).

Large-scale untargeted LC-MS-based metabolomics was used to compare the composition of white cabbage from organic and conventional agriculture, measuring 1600

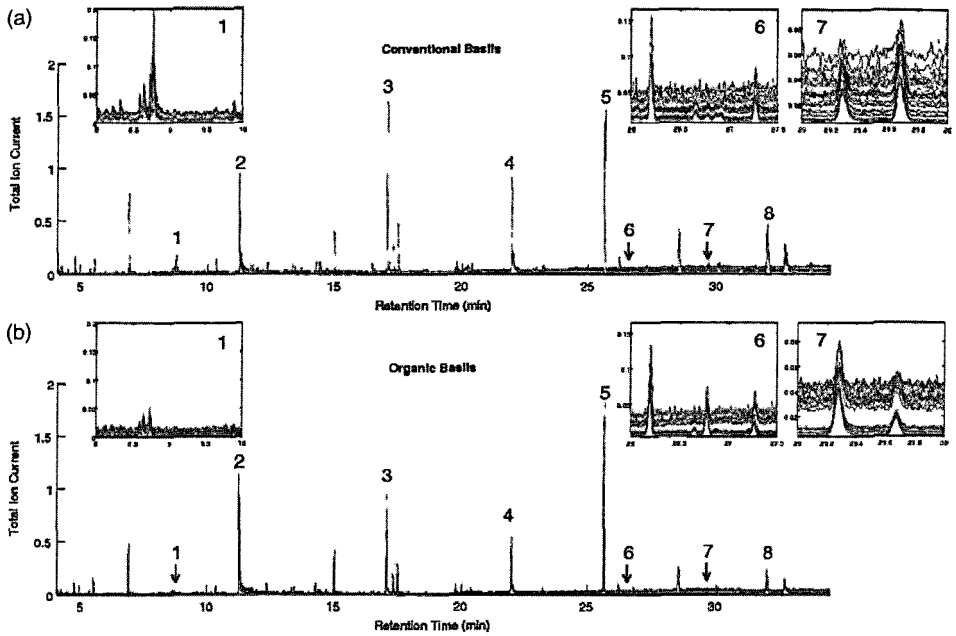


Figure 15.3 Total ion chromatograms of (a) conventional basils and (b) organic basils. Highest-ranked features selected by both feature selection approaches for constructing economic classifiers are found and putatively identified as: (1) unidentified compound(s), 8.8 min; (2) 3-alyl-6-methoxyphenol, 11.3 min; (3) 3,7,11,15-tetramethyl-2-bicyclo[4.4.0]dec-1-ene hexadecen-1-ol 17.5 min; (4) (z)-9-octadecenamide, 22.0 min; (5) 7,7',8,8',11,11',12,12',15,15'-ecahydro-Ψ,Ψ-carotene, 25.6 min; (6) 2,2,4-trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol, 26.8 min; (7) vitamin E acetate, 29.3 min; and (8) γ-sitosterol, 32.0 min. Source: Wang and Harrington (2013). Reproduced with permission of Springer-Verlag.

compounds (Mie *et al.* 2014). Cabbage was sampled over two years from one conventional and two organic farming systems in a rigidly controlled long-term field trial. Results demonstrated that the production system leaves a significant imprint in the white cabbage metabolome that is retained between production years. It was therefore concluded that the investigated conventional and organic management practices have a systematic impact on the metabolome of white cabbage, emphasising the potential of untargeted metabolomics for authenticity testing of organic plant products (Mie *et al.* 2014).

15.5.8 Direct Analysis in Real-Time High-Resolution Mass Spectrometry (DART-HRMS)

The potential of direct analysis in real-time (DART) ambient ionisation technique coupled with high-resolution mass spectrometry (HRMS) in authentication of milk and dairy products was critically assessed. In particular, DART-HRMS was used for several scenarios: (1) discrimination between milks obtained from various farm animal species (cow, goat and sheep); (2) discrimination between cows' milk produced in conventional and organic farming; and (3) detection of vegetable oil added to a milk-based product (soft cheese). Reliable differentiation of cows' milk samples and goats'/sheeps' milk was

possible. The DART-HRMS procedure also allowed milk mixtures prepared at adulteration level of 50% (v/v) to be distinguished. The capability to recognise milk from conventional and organic farming was rather low and poor classification rates of the model were obtained (Hrbek *et al.* 2014).

Other interesting application was reported in a two-year pilot study concerned with the classification of organic versus conventional crops; a DART ion source and a TOF-MS was employed. This novel methodology was tested on 40 tomato and 24 pepper samples grown under specified conditions. The results from the positive ionisation mode enabled better differentiation between organic and conventional samples than the results from the negative mode. The results suggest that the year of production had a stronger influence on the metabolomic fingerprints compared with the type of farming (organic versus conventional). In any case, DART-TOFMS is a promising tool for rapid screening of samples. Establishing comprehensive (multi-sample) long-term databases may further help to improve the quality of statistical classification models (Novotna *et al.* 2012).

15.5.9 Biological Methods

The skin of fresh foods (vegetables, fruits) is not sterile and carries microorganisms or their fragments. The presence of various microorganisms depends on the external environment of the food matrices (soil ecology, spoilage, insects, diseases), but also microorganisms brought by human activity. Bigot *et al.* (2015) discuss the possibility to apply, for the first time, a molecular microbial ecology approach to discriminate organic from conventional food using rDNA fingerprinting of microorganisms. This was performed using a molecular biology method based on the extraction, polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis separation of microbial DNA (PCR-DGGE). Indeed, the analysis of yeast and bacterial genetic profiles of nectarines and peaches showed that it was possible to differentiate fruits grown with different farming methods.

15.6 Data Analysis

As non-target studies usually generate large datasets, dedicated software is needed to handle the data in terms of filtering, peak picking and non-linear retention-time alignment. The current MS-based software available includes XCMS, MZmine and MET-IDEA, which are freely available on-line and allow users to analyse their own data (Novotna *et al.* 2012; Ozdestan *et al.* 2013; Wang and Harrington 2013; Bonte *et al.* 2014; Hrbek *et al.* 2014; Kazimierczak *et al.* 2014; Mie *et al.* 2014). In addition, many instrument companies provide the customers with their own software. Such a data-processing step is critical in non-target studies because, if incongruous, it compromises the whole method, leading to biased or even erroneous outcomes. Prior to statistical data analysis, processed data need to be converted into different scales that include centre scaling, pareto scaling and auto scaling. The scaling varies depending on the properties of the data. The NIRS different data pre-processing techniques have also been evaluated (Tres *et al.* 2012). The models based on data pre-processed using direct orthogonal signal correction (DOSC), standard normal variate (SNV) and first and second

derivatives provided the most successful results in terms of median sensitivity (0.91 in external validation) and median specificity (1.00 for external validation of SNV models and 0.94 for DOSC and first and second derivative models)

With the advances in modern bioinformatics tools, statistical data analysis has become semi-automatic. Currently, two methods (unsupervised principal component analysis or PCA and supervised partial least squares discriminant analysis or PLS-DA) are most commonly used in both target and non-target studies (Novotna *et al.* 2012; Laursen *et al.* 2011; Tres *et al.* 2012; Ozdestan *et al.* 2013; Bonte *et al.* 2014; Hrbek *et al.* 2014; Kazimierczak *et al.* 2014; Mie *et al.* 2014). PCA is often used to show the most important factors of variation defining the dataset of study. A supervised method aims to extract useful information from the dataset with a presupposed hypothesis, enabling prediction of the relationship of the analytical samples in different study groups.

A bootstrapped fuzzy-rule-building expert system (FuRES) and a bootstrapped t-statistical weight feature selection method were individually used to select informative features from GC-MS chemical profiles of basil plants cultivated by organic and conventional farming practices. Feature subsets were selected from two-way GC-MS data objects, total ion chromatograms and total mass spectra, separately. Four economic classifiers based on the bootstrapped FuRES approach – fuzzy optimal associative memory (e-FOAM), e-FuRES, partial least squares discriminant analysis (e-PLS-DA) and soft independent modelling by class analogy (e-SIMCA) – and four economic classifiers based on the bootstrapped t-weight approach – e-PLS-DA-t, e-FOAM-t, e-FuRES-t and e-SIMCA-t – were constructed thereafter to be compared with full-size classifiers obtained from the entire GC/MS data objects (FOAM, FuRES, PLS-DA and SIMCA). By using three features selected from two-way data objects, the average classification rates with e-FOAM, e-FuRES, e-PLS-DA and e-SIMCA were 95.3±0.5%, 100%, 100% and 91.8±0.2%, respectively. The established economic classifiers were used to classify a new validation set collected 2.5 months later with no parametric change to experimental procedure. Classification rates with e-FOAM, e-FuRES, e-PLS-DA and e-SIMCA were 96.7%, 100%, 100% and 96.7%, respectively. Characteristic components in basil extracts corresponding to highest-ranked useful features were putatively identified. The feature subset may prove valuable as a rapid approach for organic basil authentication (Wang and Harrington 2013).

15.7 Conclusions and Future Trends

Certifying the authenticity of organically farmed food is a complicated task. Although important progress has been made in recent years, it is not yet a fully resolved issue. Multivariate elemental fingerprints and stable isotope ratios generated by several complementary atomic spectroscopy techniques, analysed by chemometrics, can be appropriate tools for verifying if fertilisation was conducted according to the organic regulations. However, when used individually, these techniques are challenged by the potential diversity in fertiliser-management practises of organic and conventional plant-production systems.

There are a number of marked analytes that are of interest when establishing the authenticity of organic food such as fatty acids and volatile compounds. Metabolomic

fingerprinting/profiling has recently been demonstrated as a challenging option for a comprehensive characterisation of small molecules occurring in plants, since their pattern may reflect the impact of various external factors.

One of the main problems and most urgent questions relates to the validation of chemometric tools used to perform laboratory techniques. Standard protocols for all multivariate methods regarding the authentication of raw materials must be developed and verified *in situ*.

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16

Screening and High-Throughput Multi-Contaminants Methods

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16.1 Introduction

Over the past years a series of food safety crises have occurred, resulting in risk to human lives and great losses of capital. For this reason, food safety and quality have become of greater importance and attract public concern. Food safety can usefully be defined as the practice of ensuring that foods cause no harm to the consumer (Lawley *et al.* 2008). This covers a broad range of topics, from basic domestic and personal hygiene, to highly complex technical procedures designed to remove contaminants from food and feeds (Luning and Marcelis 2009). The practice of food service comprises the protection of food supply from harmful contamination, the prevention of the development and spread of harmful contamination, and the effective removal of contamination and contaminants (Lawley *et al.* 2008). Food safety practice seeks to protect against and eliminate foods hazards. A food safety hazard can be defined as any factor present in food that has the potential to cause harm to the consumer (Lawley *et al.* 2008).

Hazards are classified in two categories: biological hazards such as bacteria, viruses, parasites, and prions; and chemical hazards such as pesticides, mycotoxins, veterinary drugs and antibiotics, allergens, polycyclic aromatic hydrocarbons (PAHs), persistent organochlorine pollutants (POPs), and many more. Their occurrence in food and their physical and chemical properties, along with health effects, have already been discussed in previous works (Lawley *et al.* 2008; Picó 2008; Tadéo 2008).

The possible presence of the abovementioned hazards in food and feed necessitates the employment of rapid, high-throughput, effective, and reliable multi-residue methods (MRMs) with the aim of verifying the safety and authenticity of the products (Jansson and Kreuger 2010). For instance, MRMs allow the determination of a large number of pesticides in a single analysis, thus reducing its time and cost. However, these methods require fulfillment of certain criteria, such as common sample preparation methods and making compromises on the different physicochemical properties of the target compounds (Petrovic *et al.* 2010). In this respect, robust and sensitive analytical techniques have been developed with the aim of determining a large number of contaminants and their by-products, in a single run if possible. However, it is understandable that a single analytical method may not be applicable for the analysis of such

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a diverse group of compounds. In recent years, considerable improvements have been made in the determination of a large number of contaminants in a single analytical run, with analytical methods shifting from gas chromatography (GC) to liquid chromatography (LC) -based techniques (Alder *et al.* 2006). The ability to interface LC with atmospheric ionization methods and mass spectrometry has offered significant developments in pesticide residue analysis. The progress in ionization techniques, together with advancements in high-resolution mass spectrometry (HRMS) and faster LC methods, have increased the number and the classes of pesticides analyzed simultaneously. Multi-residue analysis in food matrices consists of four steps: sampling; extraction; clean-up; and finally identification and quantitative determination. Most recent and advanced MRMs methods that have been described in the literature are summarized in Table 16.1.

16.2 Sample Preparation

16.2.1 Sample Extraction

An important step in the preparation of food samples prior to final analysis is sample extraction. The procedure consists of transferring the analytes from the primary matrix into the secondary with a concurrent purging of interfering substances (isolation), and increasing the analytes concentrations to a level above the detection limit for a given analytical technique (Martins *et al.* 2013). A number of instrumental-based extraction procedures have been developed to isolate pesticides residues from food of animal origin, including solid-phase micro-extraction (SPME), matrix solid-phase dispersion extraction systems (MSPD), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), liquid–liquid extraction (LLE), quick easy cheap effective rugged and safe (QuEChERS), and finally microwave-assisted extraction (MAE).

16.2.1.1 Solid-Phase Micro-Extraction

Solid-phase micro-extraction (SPME) is a sample preparation technique using a fused-silica fiber that is coated on the outside with an appropriate stationary phase; the analyte in the sample is directly extracted and concentrated onto the fiber coating (Pawliszyn 1997). SPME, especially in combination with GC, is able to extract and detect volatile residues in an easy and relatively fast and cheap approach (Hercegová *et al.* 2007). The stationary phase can vary in film thickness and coating types, targeting different classes of compounds. The syringe device can be inserted in a gas (headspace) or liquid (direct immersion) sample, and the compounds (i.e., pesticides) can be extracted and concentrated in the stationary phase of the fiber (Lehotay *et al.* 2005). A variation of the SPME technique is the use of a stir bar covered with poly(dimethylsiloxane) (PDMS) (Picó 2008). Another extraction technique based on the same principle as SPME is stir-bar sorptive extraction (SBSE) which is performed by stirring the sample with a stir bar for a given time; this technique is suitable for the isolation of less polar pesticide residues (Hercegová *et al.* 2007). The main advantages of SPME extraction compared to solvent extraction are the reduction in solvent use, the combination of sampling and extraction into one step, and the ability to examine smaller sample sizes (Tan and Chai 2011). It can also have high sensitivity and can be used for polar and non-polar analytes in a wide

Table 16.1 Recent advances in MRMs methods (NA: not available)

Compounds	Matrix	Sample preparation	Detection	Sensitivity	Recovery (%)	Reference
10 triazole fungicides	Grapes and strawberries pulp	DI-SPME	GC-TOFMS	LOQs: 0.25–5 ng g^{-1}	91–117	Souza-Silva *et al.* 2013
14 OCPs, 7PCBs	Vegetable oils	MSPD	GC-ECD	LODs: 0.04–0.74 ng g^{-1}	69.6–105.3	Li *et al.* 2014
16 OCPs, 5PCBs	Fish muscle	MSPD	GC-MS	LOD: 0.5 ng g^{-1}	>69	Hela and Papadopoulos 2013
9 pesticides	Eggs	MSPD	GC-MS	LODs: 0.003–0.015 μg g^{-1}; LOQs: 0.010–0.050 μg g^{-1}	62–95	Rubens dos Reis-Souza *et al.* 2013
10 steroid hormones	Chicken, beef, pork, sausage	MSPD	UPLC-MS/MS	LODs: up to 0.001 μg kg^{-1}	76.8–98.7	Fan *et al.* 2014
8 fungicides	Grapes	MAE, MSPD, SLE, QuEChERS	GC-MS	LODs: 0.7–1.7, 1.0–2.6, 1.2–4.3, 1.5–4.1 μg kg^{-1} LOQs: 2.2–5.8, 3.0–8.7, 4.1–14.3, 6.1–13.6 μg kg^{-1}	78–100 66–102 58–88 68–96	Lagunas-Allué *et al.* 2012
32 mycotoxins	Barley	MAE, MSPD, SLE, QuEChERS	UHPLC-Orbitrap MS	NA	72–101 for QuEChERS <67% for 2 mycotoxins	Rubert *et al.* 2012
9 mycotoxins	Flours	MSPD	LC-ESI-MS/MS QqQ	LODs: 0.5–75 ng g^{-1} LOQs: 1–85.24 ng g^{-1}	71.1–89.3	Rubert *et al.* 2011
16 mycotoxins	Dried fruits	QuEChERS	LC-ESI-MS/MS	LODs: 0.08–15 μg kg^{-1} LOQs: 0.2–45 μg kg^{-1}	60–135	Azaiez *et al.* 2014

(*Continued*)

Table 16.1 (*Continued*)

Compounds	Matrix	Sample preparation	Detection	Sensitivity	Recovery (%)	Reference
OCs pesticides	Fish feed	SPE LLE	GC-MS/MS, QqQ	LODs: 0.01–0.11 μg l^{-1} LOQs: 0.02–0.35 μg l^{-1}	92–116	Nardelli *et al.* 2010
OCs pesticides	Fruits	QuEChERS	GC-MS	LOQs: 0.001–0.013 μg kg^{-1}	70–120	Ciéslik *et al.* 2011
7 pesticides	Sugarcane juice	QuEChERS	GC-ECD	LODs: 0.003–0.004 mg l^{-1}	62.9–107.5	Furlani *et al.* 2011
33 pesticides	Colombian fruit	QuEChERS	GC-MS	LOQs: 0.01 mg kg^{-1}	>80	España Amórtegui and Guerrero Dallos *et al.* 2014
OCs pesticides	Food of animal origin	QuEChERS	GC-MS	LOQs: 0.0015–0.0071 mg kg^{-1}	75–112	Surma *et al.* 2014
32 pesticides	Grape juice	QuEChERS	GC-MS-SIM	LOQs: 3.75–9.47 mg L^{-1}	84–99.7	Alves *et al.* 2014
7 pesticides	Palm oil	MSPD	LC-TOF-MS	LODs: 1.5–5.0 μg kg^{-1} LOQs: 2.5–9.0 μg kg^{-1}	72.6–91.3	Sobhanzadeh*et al.* 2011
10 OPPs pesticides	Vegetables fruits	MAE μ-SPE	GC-MS	LODs: 0.07–0.23 μg kg^{-1} LOQs: 0.21–0.69 μg kg^{-1}	93.5–104.6	Wang *et al.* 2013
6 mycotoxins	Breast milk	LLE	HPLC-FLD	LOQs: 0.005–0.03 ng mL^{-1}	73–99.5	Andrade *et al.* 2013
Veterinary drugs	Chicken tissue	QuEChERS	LC-MS/MS	NA	NA	Stubbings and Bigwood 2009
18 veterinary drugs	Milk	QuEChERS	UHPLC-MS/MS	LODs: 1–4 μg kg^{-1}	70–110	Aguilera-Luiz *et al.* 2008
carbamate pesticides	Vegetable oils	QuEChERS	UHPLC-MS/MS	LOQs: 0.09–2.0 μg kg^{-1}	74–101	Moreno-González *et al.* 2014

85 pesticides	Tomatoes, tomarillos, golden berries	QuEChERS	GC-MS	LOQs: 0.02–0.20 mg kg^{-1}	70–120 in most cases	Ramírez-Restrepo *et al.* 2014
14 mycotoxins	Rice	QuEChERS	UHPLC-MS/MS	LODs: 0.5–15 μg kg^{-1}	70–98	Koesukwiwat*et al.* 2014
15 mycotoxins	Cereals, cocoa oil, spice, coffee, nuts, infant formula	QuEChERS	LC-MS/MS	LOQs: 0.05–0.25 μg kg^{-1}	71–118	Desmarchelier *et al.* 2014
31 OPPs	Dried fruits	Extraction with solvents	GC-FPD GC-MS/MS	LODs: 1–10 ng mL^{-1} LOQs: 4–30 ng mL^{-1}	57.6–104.7	Zhao *et al.* 2014
35 pesticides	Edible oils	d-SPE	GC-NCI-MS/MS	LODs: 0.01–1 ng g^{-1} LOQs: 0.05–2 ng g^{-1}	62–110	Deme *et al.* 2014
22 insecticides	Honey	QuEChERS	GC-MS/MS HPLC-MS/MS	LODs: 0.07– 0.2 ng g^{-1} LOQs: 0.2–0.5 ng g^{-1}	63–139	Paradis *et al.* 2014
7 pesticides	Tea leaves, made tea	Extraction with solvents	HPLC-MS/MS	LODs: 0.03–1.00 ng mL^{-1}	90.4–103, 90.3–102	Chen *et al.* 2014
Pesticides	Honey bee pollen	Extraction with solvents	LC-ESI-MS/MS	LODs: 0.03–23.3 ng g^{-1} LOQs: 0.1–50.4 ng g^{-1}	59–117	Kasiotis *et al.* 2014
Neonicotinoids organophosphorus pesticides	Food matrices	Extraction with solvents	ELISA	LODs: 0.51–3.16 μg L^{-1}	82.54–116.29	Yan *et al.* 2014

(*Continued*)

Table 16.1 *(Continued)*

Compounds	Matrix	Sample preparation	Detection	Sensitivity	Recovery (%)	Reference
Neonicotinoids insecticides	Green pepper, eggplant, spinach	Extraction with solvents	ELISA	NA	97.9–109.1	Watanabe *et al.* 2013
Insecticides	Tangerine juices	Extraction with solvents	ELISA	LODs: 0.20±0.04 μg L^{-1} LOQs: 0.50±0.06 μg L^{-1}	95–106	Navarro *et al.* 2013
150 veterinary drugs	Milk	extraction with solvents	LC-QTOF-MS	LODs: 10 ng mL^{-1}	NA	Turnipseed *et al.* 2014
Mycotoxins	Human breast milk	QuEChERS	UHPLC-HRMS	LODs: 2.1–14.3 ng mL^{-1}	64–93	Rubert *et al.* 2014
Polybrominated diphenyl ethers and hydroxylated and methoxylated metabolites	Sediment, fish tissue milk	PLE, GPC	GC-HRMS	LODs: 40 pg g^{-1} dw, 113% pg g^{-1} lw, 12.5 –197 pg g^{-1} lw LODs: 3.28 pg g^{-1} dw, 41.4 pg g^{-1} lw, 20.5 pg g^{-1} lw	83–112	Lacorte *et al.* 2010
Partially hydrolyzed fumonisins, hydrolyzed fumonisins	Maize, maize-based products	Extraction with solvents	LC-HRMS	LOD: 5 μg kg^{-1}, LOQ: 10 μg kg^{-1},	FB1: 90–97 FB2: 87–89 PHFB1: 98–99 PHFB2: 90–93 HFB1: 88–92; HFB2: 81–82	De Girolamo *et al.* 2014

Veterinary drugs	Distillers grains	Extraction with solvents	LC-Orbitrap HRMS	LOQs: 29.1–179.2 μg kg^{-1}	95% of compounds: 71–124; 4% of compounds: 60–89; 1% of compounds: 50	Kaklamanos *et al.* 2013
Antibiotics, pesticides, mycotoxins	Fish feed, fish fillets	Extraction with solvents	UHPLC-QTOF MS	NA	NA	Nácher-Mestre *et al.* 2013
Pesticides	Fruits vegetables	QuEChERS	LC-HRMS	NA	56 for FHX; 94 for FHX–glycoside	Polgár *et al.* 2012
Veterinary drugs	Fish, pork, kidney, pork liver, honey	Extraction with solvents	UPLC-MS/MS UPLC-HRMS	Up to 1 μg kg^{-1}	NA	Kaufmann *et al.* 2010

range of matrices, linking to both GC and LC in an easy, fast and low-cost way (Souza-Silva *et al.* 2013). Some disadvantages of SPME include batch-to-batch variation and robustness of fiber coatings (Ridgway *et al.* 2007).

16.2.1.2 Matrix Solid-Phase Dispersion Extraction

Matrix solid-phase dispersion (MSPD), firstly reported by Barker *et al.* (1989), is based on the adsorption of the sample on an adsorbent such as Florisil, alumina, silica, or diatomaceous earth. It allows the extraction and clean-up in a single step. The extraction of pesticides from homogeneously dispersed food samples in a solid support has been demonstrated in several works (Hela and Papadopoulos 2013; Souza-Silva *et al.* 2013; Li *et al.* 2014). The sample matrix is placed in a column and the polar compounds, such as pigments, are held on the support. Various pesticides can therefore be eluted rapidly with organic solvents and the extract can be analyzed. MSPD manages to eliminate the traditional processes of extraction and clean-up, it reduces processing times and costs, and avoids emulsification (Li *et al.* 2014). It has been applied to the analysis of pesticides in fruit (Radišić *et al.* 2013) and vegetables (Łozowicka *et al.* 2013), as well as in liquid samples such as milk and juices (Lehotay *et al.* 2005). It is also used for pesticide extraction in matrices with high fat content, such as oils (Sobhanzadeh *et al.* 2011). In addition, it has been used for multi-mycotoxins extraction in flours (Rubert *et al.* 2011), tiger-nuts (Rubert *et al.* 2012), and wines (Zhu *et al.* 2007), as well as for POPs extraction (Abhilash *et al.* 2008) and PAHs (Pensado *et al.* 2005).

16.2.1.3 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a process by which a solvent is obtained at its supercritical point, thus possessing the properties of a gas and a liquid (Tan and Chai 2011). The unique properties exhibited by supercritical fluids have been applied for the analysis of pesticide residues in solid samples. SFE is selective and less-solvent consuming, therefore environmentally friendly (Hercegová *et al.* 2007).

In the SFE commercially available equipment, the fluid is pumped at a pressure above its critical point with the sample placed in an inert extraction cell, and the temperature of the cell is increased to overcome the critical point of the fluid (Bogialli and Di Corcia 2009). After depressurization, the analytes are collected in a small volume of organic solvent or on a solid-phase-filled cartridge (Bogialli and Di Corcia 2009). Extraction can be performed in the static, dynamic, or recirculating mode: in the static extraction mode, the cell containing the sample is filled with the supercritical fluid, pressurized, and allowed to equilibrate; using the dynamic mode, the supercritical fluid is passed through the extraction cell continuously; and finally, in the recirculating mode, the same fluid is repeatedly pumped through the sample and, after the required number of cycles, is pumped out to the collection system (Tan and Chai 2011).

SFE is usually an efficient extraction method, primarily applicable to solid samples, and has numerous advantages such as efficacy, selectivity, short extraction times, and low solvent volumes; it also has serious drawbacks such as difficult optimization, high apparatus and maintenance cost, and high blank and noise levels (Zougagh and Ríos 2008). Most of the works that discuss the supercritical fluid extraction of pesticides in food have been previously summarized by Lehotay (1997). Recently, SFE has been used for the extraction of insecticides in sugar beet (Pucarević *et al.* 2013) and the extraction of mycotoxins in maize flour samples (Zougagh and Ríos 2008).

16.2.1.4 Accelerated Solvent Extraction

Accelerated solvent extraction (ASE) is a technique used in sample preparation that combines elevated temperatures and pressures with liquid solvents (Richter *et al.* 1996). ASE has received different names such as pressurized liquid extraction (PLE), pressurized solvent extraction (PSE), high-pressure solvent extraction (HPSE), pressurized hot solvent extraction (PHSE), high-pressure high-temperature solvent extraction (HPHTSE), pressurized hot water extraction (PHWE), and subcritical solvent extraction (SSE) (Sun *et al.* 2012). It has become a popular green extraction technology for different classes of organic contaminants present in numerous kinds of food and feed for food safety (Sun *et al.* 2012).

The analytes diffuse from the core of the matrix to the surface, are then transferred from the surface into the extraction fluid, and are finally eluted. ASE can be carried out with conventional solvents, such as dichloromethane-hexane (Bester *et al.* 2001) or EtAc (Blasco *et al.* 2005). The technique requires dried samples or previously homogenized with a drying or dispersive agent (Blasco *et al.* 2005). The technique is fully automated but requires high-cost equipment (Sun *et al.* 2012). ASE has been used for the extraction of pesticides in fruits (Bester *et al.* 2001; Blasco *et al.* 2005) and PCBs in fish (Ottonello *et al.* 2014), as well as for the extraction of pesticides in tea coupled to gas-chromatography high-resolution isotope dilution mass spectrometry (Feng *et al.* 2013).

16.2.1.5 Liquid–Liquid Extraction

According to a recent review for pesticides analysis in foods of animal origin, the most widely used pesticide extraction techniques from these samples in the last two decades was solid–liquid extraction (SLE) and liquid–liquid extraction (LLE) (Nollet and Toldrá 2013). The SLE procedure consists of grinding chopped samples or extracted fats several times at high speed into selected organic solvents. This technical procedure has been applied to meat and meat products, animal fat, offal, eggs, and fish for extracting different kinds of pesticides (Da Costa Morais *et al.* 2014). Similarly, the LLE procedure consists of shaking liquid samples several times in selected organic solvents to extract pesticide residues (Andrade *et al.* 2013). Moreover, LLE has been used for the extraction of mycotoxins, employing a mixture of aqueous organic solvents depending on the targeted mycotoxins and performed by stirring or by homogenization (Fabiania *et al.* 2010; Andrade *et al.* 2013; Jia *et al.* 2014). The procedure is effective for several toxins and works well in small-scale preparations (Da Costa Morais *et al.* 2014).

16.2.1.6 QuEChERS

Anastassiades *et al.* (2003) developed a variation of LLE called QuEChERS sample preparation procedure (quick, easy, cheap, effective, rugged, and safe), which has been successfully applied to the analysis of hundreds of pesticide residues. QuEChERS approach is very flexible and it serves as a template for modification depending on the analyte properties, matrix composition, equipment, and analytical technique available in the laboratory (Lehotay *et al.* 2010). The template is very rugged and high recoveries will be achieved for many pesticides in many matrices even if different ratios and types of sample size, solvent, salts, and sorbents are used in modifications (Stubbings and Bigwood 2009). The rugged characteristics of the QuEChERS approach have been thoroughly evaluated in the original (Anastassiades *et al.* 2003) and subsequent publications

(Lehotay *et al.* 2005; Díez *et al.* 2006; Cunha *et al.* 2007; Aguilera-Luiz *et al.* 2008; Stubbings and Bigwood 2009; Lehotay *et al.* 2010).

In multi-class, multi-residue pesticide analysis, the sample preparation method inherently necessitates broad analytical scope which makes it impossible to obtain a high degree of clean-up without reducing recoveries for some pesticides (Anastassiades *et al.* 2003). However, greater clean-up can be achieved by using different sorbents in dispersive SPE (d-SPE) if the application has reduced analytical scope (Li *et al.* 2011). QuEChERS method has been successfully applied for the determination of mycotoxins by several authors (Azaiez *et al.* 2014; Desmarchelier *et al.* 2014; Koesukwiwat *et al.* 2014).

Comparing the discussed methodologies, the QuEChERS approach can be used for a variety of matrices with adequate recoveries and precision values. SPME also offers similar recoveries but requires special apparatus (Picó 2008). Moreover, the number of the reported pesticides analyzed is higher with QuEChERS than any other extraction techniques (Koesukwiwat *et al.* 2014). SLE and MSPD are very specific and would depend on the matrix that is being analyzed (Picó 2008). Finally, SPE does not offer adequate recoveries, although the precision is similar to the other methods (Ridgway *et al.* 2007). In general, the use of clean-up steps improves the recoveries and precision values of the applied extraction techniques (Tan and Chai 2011).

16.2.1.7 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) uses microwaves to heat the solvent in a closed and pressurized vessel in order to extract the target compounds from the sample matrix (Tadéo 2008). Using microwave energy allows the solvent to be heated rapidly: an average extraction takes 15–30 min and offers high sample throughput with low solvent consumption (Wang *et al.* 2013). This technique is only applicable to thermally stable compounds due to the increase in temperature during extraction (Lagunas-Allué *et al.* 2012). The main advantages of microwave pretreatment are the low temperature requirement, high extraction rate, automation, and the possibility of simultaneously extracting different samples at the same time without interferences (Camel 2001).

Lagunas-Allué *et al.* (2012) compared four extraction methods – MAE, MSPD, SLE, and QueChERS – by determining various pesticides in grapes. MAE proved more sensitive and faster than others due to the simultaneous extractions that can be performed in the microwave oven rather than the sequential extractions that can be conducted with the other three methods (Picó 2008). However, low-cost and non-specific instrumentation demands are the advantages of the three methods in sample preparation, while MAE requires a special commercial microwave oven which is not available in all laboratories (Picó 2008).

16.2.2 Sample Clean-Up

Sample extracts contain a large number of sample matrix co-extractants. A thorough clean-up minimizes matrix issues, improves sensitivity, permits more consistent and repeatable results, and extends the (capillary) column lifetime (Li *et al.* 2014). The amount of clean-up required will depend on the type of the chromatographic system used, the specificity of the detector used, and the desirable limit of detection (Tadéo 2008). Several approaches have been attempted to eliminate co-extracted interference

from extracts, including gel permeation chromatography (GPC), solid-phase extraction (SPE), and dispersive solid-phase extraction (d-SPE).

16.2.2.1 Gel Permeation Chromatography

Gel permeation chromatography (GPC) separates particles on the basis of size (Rimkus *et al.* 1996). It is best suited for removing high-molecular-weight pigments and lipid materials such as waxes or fats (Rimkus *et al.* 1996; Diaz *et al.* 1997; Buldini *et al.* 2002). GPC systems comprise a LC pump, a fraction collector, and a detector (Buldini *et al.* 2002). The columns are made from polymeric porous microspheres, which enable the separation of compounds according to their molecular weights (Buldini *et al.* 2002). Pesticide fraction is separated from the high-molecular-weight lipid fractions (Gilbert-López *et al.* 2009). In order to reach a higher sample throughput, high-capacity disposable silica (HCDS) columns containing 28 g of acidic, 16 g of basic, and 6 g of neutral silica were employed (Beyer and Biziuk, 2008). Such a column system has found application in purifying extracts from samples characterized by high lipid content, such as poultry, fish and eggs (Beyer and Biziuk 2008).

16.2.2.2 Solid-Phase Extraction

Solid-phase extraction (SPE) is used to separate a variety of analytes from liquid matrices (Simpson 2000). It has been widely employed in the determination of pesticides and contaminants in liquid samples, such as juices, wines, oils, and honeys, as well as for clean-up of organic solvent extracts after analyte isolation by means of liquid chromatography (Picó 2008). It is also used as a purification step to remove interfering compounds co-extracted with all classes of pesticides from a large selection of foods of animal origin, as reviewed by Le Doux (2011).

A multistep purification based on the use of various adsorbents such as florisil, C18, alumina, and silica gel is frequently employed in order to eliminate the interfering substances and matrix fatty materials, which can rapidly decrease the column efficiency and severely affect the accurate identification and quantification of the analytes (Nardelli *et al.* 2010). For food analysis, a widely used strategy is the combination of SPE-QuEChERS with the advantage of relatively low solvent consumption, at least as compared to previous liquid-partitioning-based methods (Lee *et al.* 2011). In the case of fatty matrices, the clean-up was performed using a combination of primary secondary amine (PSA), graphitized carbon black (GCB), and C18 (Beyer and Biziuk 2010). Moreover, various types of co-polymeric sorbents are employed in order to make SPE a more robust purification technique (Peruga *et al.* 2013). Common polymeric sorbents are a hydrophilic-lipophilic balanced (HLB) copolymer of N-vinylpirrolidone and divinybenzene (Peruga *et al.* 2013). An alternative procedure which is similar to QuEChERS but performs the clean-up step using SPE in cartridges has recently been reported; the procedure is characterized by low solvent consumption and increased automation (Gilbert-López *et al.* 2009; Beyer and Biziuk 2010).

16.2.2.3 Dispersive Solid-Phase Extraction

Dispersive solid-phase extraction (d-SPE) is based on a clean-up with PSA (Tan and Chai 2011) and shows great potential in the detection of pesticide residues in foodstuffs (Ciéslik *et al.* 2011; Furlani *et al.* 2011; Lee *et al.* 2011). d-SPE is a clean-up technique that involves mixing the sorbent with a sample that has been pre-extracted with

acetonitrile solvent (Picó 2008). The appropriate sorbent adsorbs matrix co-extractives onto its surface, leaving analytes of interest in the solvent. $MgSO_4$ is added to provide additional clean-up by removing residual H_2O and some other compounds via chelation. The mixture is then centrifuged and the resulting supernatant can be analyzed directly or else subjected to a concentration and/or solvent exchange step if necessary (Kinsella *et al.* 2009).

16.3 Separation and Detection

16.3.1 Gas Chromatography Coupled to Conventional Detectors and Mass Spectrometry

Gas chromatography (GC) using fused-silica capillary columns is the preferred method for the determination of volatile and thermally stable compounds, especially for organochlorine (OC) and organophosphorus (OP) pesticides (Nielsen 2010). Most GC methods employ element selective detectors. Most of the modern conventional GC detectors (FID, NPD, FPD, μ-TCD, μ-PID, μ-ECD) usually have maximum sampling frequencies in the range 20–200 Hz, which is adequate for fast GC analysis (Picó 2008). Typical acquisition rates of modern scanning mass spectrometers, such as quadrupole, ion traps, and magnetic sector instruments, are in the range of 5–15 spectra per second, hence capable of offering the speed for MS detection in the case of fast GC separation. Alternatively, time-of-flight analyzers allowing acquisition rates of up to 500 spectra per second can be used for the detection of eluted peaks under the conditions of fast GC (Picó 2008).

Electron capture detector (ECD) has been used in the analysis of multi-residues (Taylor *et al.* 2004; Zhu *et al.* 2007) because of the extraordinary sensitivity it presents for organic halogen compounds. Nitrogen and phosphorus detector (NPD) is a very sensitive and specific detector that responds to nitrogen and phosphorous compounds (Ravelo-Pérez *et al.* 2008). It is based on FID but differs in that it contains a rubidium or cesium silicate (glass) bead situated in a heater coil, a short distance from the hydrogen flame; it has been successfully applied in the determination of pesticides in fruit juices (Ravelo-Pérez *et al.* 2008). The determination of sulfur- or phosphorus-containing compounds is the job of the flame photometry detector (FPD) (Cai *et al.* 2006; Xiao *et al.* 2006).

The limiting disadvantage of conventional GC analysis is that only a limited number of compounds can be detected in a single determination (Picó 2008). Multi-dimensional chromatography can play a vital role in fast chromatographic separations, with GC×GC already making the transition from a research tool to a routine analytical technique (Adahchour *et al.* 2008). The GC×GC system is set up by a modulator connecting the first-dimension conventional-size column with a short micro-bore column in the second dimension; a large series of high-speed chromatograms is the outcome of the transfer of chromatographic band from the first to the second dimension during the GC×GC run (Dallüge *et al.* 2003). A number of characteristics of GC×GC, such as high peak capacity, enhanced sensitivity, structured chromatograms, and improved identification of the unknown, have been previously reported (Zrostlíková *et al.* 2003).

Gas chromatography mass spectrometry (GC-MS) also shows high selectivity and sensitivity in pesticide analysis; it has been used in various determinations of volatile and semi-volatile compounds in pesticide analysis (Chandra *et al.* 2014; España

Amórtegui and Guerrero Dallos 2014; Ramírez Restrepo *et al.* 2014; Surma *et al.* 2014) Nowadays, GC-MS is the primary analytical technique used for confirmation of results obtained with classical detectors. EU requirements indicate that all confirmatory methods for pesticide residues must use MS detection (European Commission 2013), so GC-MS has become in recent years the preferred primary tool for determination of a large number of food contaminants. Advantages of GC-MS over traditional approaches include: simultaneous quantification and confirmation of target analytes, detection and identification of non-target sample components; and the possibility to resolve co-eluting peaks by different mass spectra (Picó 2008).

There are three available ionization modes of GC-MS: electron impact (EI); positive and negative chemical ionization; and atmospheric chemical ionization. GC-MS in selected ion monitoring (SIM) is commonly used in the determination of different classes of pesticides (Tadéo 2008). The selectivity of GC-MS can be adjusted by the selection of appropriate molecular and fragment ions (SIM mode) to avoid interferences from co-extracted sample materials and improve sensitivity (Alves *et al.* 2014). Single-quadrupole GC-MS under SIM mode has been used for the identification and quantification of organophosphorus and carbamate pesticides in ginseng (Zhou *et al.* 2012). Nevertheless, GC coupled to a triple quadrupole provides more specific and accurate determination of trace components in complex mixtures with lower LODs than GC-MS (Picó 2008). The main limitation of GC-MS is the relatively low sensitivity obtained for many pesticides in the full scan mode when EI is selected (Agüera *et al.* 2002).

GC coupled to tandem mass spectrometry (MS/MS) provides higher sensitivity and selectivity, since it involves at least two stages of mass analysis separated by a fragmentation step (Picó 2008). The most common tandem mass spectrometers for GC are ion trap (IT) and triple quadropole (QqQ). The use of MS/MS improves the selectivity of the technique, sometimes with a drastic reduction in the background and without loss of the identification capability. It enables the analysis of pesticides at trace levels in the presence of many interfering compounds (Deme *et al.* 2014). According to literature, the LODs for most pesticides in GC-MS-MS are 5–20 times lower than those in GC-MS with the same instrument (Martínez-Domínguez *et al.* 2014). It has been widely used for multi-residue determinations in food matrices (Deme *et al.* 2014; Paradis *et al.* 2014; Zhao *et al.* 2014).

High-resolution MS (HRMS) technology is today a reliable complementary alternative, allowing the analysis of a wide range of contaminants in food, providing screening and tentative identification for both non-target and target compounds. In particular, time-of-flight mass spectrometry (TOF-MS) coupled to GC has proven to perform excellently. Augusti *et al.* (2014) described a method using GC-HRMS for the quantification of 17 polychlorinated dioxins (PCDDs) and furans (PCDFs) in fish. Lacorte *et al.* (2010) reported an efficient and comprehensive methodology based on GC-HRMS to simultaneously determine polybrominated diphenyl ethers (PBDEs) from mono- to deca-brominated and hydroxyl (OH-) and methoxy (MeO-) PBDE metabolites in sediment, fish tissue, and milk.

16.3.2 Liquid Chromatography–Mass Spectrometry

LC separation is the technique of choice for the polar and thermally labile compounds and their degradation products. With the availability of diverse LC separation

techniques, such as reverse phase (RP), hydrophilic interaction (HILIC), or ion chromatography (IC), the polarity range of the target analytes can be considerably increased (Nollet and Toldrá 2013). There are several reports on the determination of pesticides in foodstuff by LC-MS (Sharma *et al.* 2010; Chung and Chen 2011; Le Doux 2011; Jin *et al.* 2012). LC-MS methods use short, narrow bore columns and high mobile phase flow rates resulting in ultra-high pressures. The analytical run shortens, while the chromatographic resolution is maintained; it is an important step towards high sample throughput (Farré and Barceló 2013). From the different LC-MS methodologies – the atmospheric pressure interfacing system using electrospray (ESI) or atmospheric pressure chemical ionization (APCI) in either positive or negative ionization modes – the most widely used is the ionization technique (Tadéo 2008). LC-MS using ESI has been applied for the determination of pesticides in honey and bee pollen (Kasiotis *et al.* 2014), in milk (Ehling and Reddy 2014), in fruits and vegetables (Lénárt *et al.* 2013; Zhao *et al.* 2013) and many more (Table 16.1).

Recently, LC-MS/MS with electrospray (ESI) ionization has been gaining importance as a consequence of its very high multi-class pesticide detection capability along with an increase in LC-amenable compounds which are new to the market compared with those amenable to GC (Songsermsakul and Razzazi-Fazeli 2008). This technique provides high selectivity, sensitivity, and reproducibility operating in selective reaction monitoring mode. Nevertheless, LC-MS/MS uses a target analysis workflow where the set-up acquisition method development is extensive and time consuming in order to analyze hundreds of pesticide residues in a single run. Another drawback is that in targeted screening, the compounds cannot be detected if they have not been previously optimized or in performing retrospective data analysis (Goméz-Ramos *et al.* 2013).

HILIC involves polar stationary phase and aqueous-organic mobile phase rich in organic solvents in which water is introduced as a stronger eluting solvent (Nollet and Toldrá 2013). Kittlaus *et al.* (2013) developed a HILIC×RPLC method for the determination of 300 pesticides simultaneously. Tian *et al.* (2012) described the chiral separation of racemic pesticides, including isocarbophos, fenpropathrin, imazalil, ethofumesate, myclobutanil, paclobutrazol, fipronil, and hexythiazox, which was carried out using cellulose (tris-3,5-dimethyl-phenylcarbamate) (CDMPC) and amylose (tris-3,5-dimethyl-phenyl-carbamate) (ADMPC) -coated silica as chiral stationary phases by liquid chromatography (LC) under reversed-phase (RP) conditions. Applications of multidimensional LC×LC in pesticide analysis have been described by Dugo *et al.* (2008).

The most exploited ion sources in LC-MS analysis are electrospray ionization (ESI) and atmospheric-pressure chemical ionization source (APCI). The development of atmospheric-pressure photoionization technology (APPI) has expanded the range of compounds amenable to LC-MS to include non-polar compounds (Hanold *et al.* 2004). APPI has demonstrated capability in the determination of pesticides (Soler *et al.* 2008; Botitsi *et al.* 2011). APCI obtains superior sensitivity at high flow rates compared to ESI. Moreover, APCI is less susceptible to matrix effects than ESI since ionization of the target analytes is performed in the vapor phase and not in solvent (Covey *et al.* 2009; Maragou *et al.* 2011).

High-pressure liquid chromatography (HPLC) typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. Analyte

retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase (Nollet and Toldrá 2013). With HPLC, a pump provides the higher pressure required to propel the mobile phase and analyte through the densely packed column, allowing better separation on columns of shorter length when compared to ordinary column chromatography (Jin *et al.* 2012). HPLC methods could employ reversed-phase with C18 or C8 columns and aqueous mobile phase, followed by UV adsorption, UV diode array, and fluorescence or mass spectrometric detection.

MRMs are most frequently carried out using liquid chromatography (LC) or GC coupled to triple-quadrupole mass spectrometry (QqQ-MS) (Alder *et al.* 2006; Picó 2008). HPLC with MS/MS is frequently used for pesticide determinations (Zhou *et al.* 2013; Chen *et al.* 2014). Peng *et al.* (2012) developed a rapid determination method that could be performed without any clean-up or determination step with HPLC-ESI-MS. Nowadays, UHPLC is the separation technique of choice in pesticide analysis with several examples of improved speed and sensitivity (Petrovic *et al.* 2010; Masiá *et al.* 2013). Kaufmann *et al.* (2010) compared liquid chromatography selectivity as provided by two types of liquid chromatography detectors, HRMS and MS/MS. The data indicated that the selectivity of LC-HRMS exceeded if high-resolution mass spectrometry (HRMS) data is recorded with a resolution of 50 000 full width at half maximum (FWHM) and a corresponding mass window. UHPLC coupled to TOF-MS enables the effective screening and tentative identification of a large number of species even without the need for standards, as shown by Grimalt *et al.* (2010).

Turnipseed *et al.* (2014) identified over 200 veterinary drug residues using LC-QTOF-MS. The screening levels of detection for over 150 veterinary drug residues in milk were determined, and over half of those tested can be detected at concentrations of 10 ng mL^{-1} or less. Rubert *et al.* (2014) described a method that allowed the detection of target mycotoxins in human breast milk based on QuEChERS extraction and UHPLC-HRMS detection. The method was fully validated, with recoveries ranging from 64% to 93% and relative standard deviations being lower than 20%.

There are many reports on the use of screening methods by LC-TOF-MS (Fernández-Alba and García-Reyes 2008; Ferrer *et al.* 2012) and Orbitrap for the high-throughput screening of pesticide and veterinary drug residues in various matrices (Wang *et al.* 2012; Jović *et al.* 2013; Gómez-Pérez *et al.* 2014; Jia *et al.* 2014). A methodology for the systematic screening of pesticides and their main metabolites in fruit and vegetable samples using LC-HRMS and accurate-mass database search of parent compounds and their diagnostic fragment ions was described by Polgár *et al.* (2012).

Nácher-Mestre (2013) described the development of a rapid screening method for the detection and quantification of undesirable organic compounds in aquaculture products using UHPLC-QTOF-MS. A qualitative validation was carried out for over 70 representative compounds including pesticides, antibiotics, and antibiotics in fish feed and fish fillets spiked at 20 and 100 μg kg^{-1}.

Kaklamanos *et al.* (2013) proposed a multi-residue method for the detection of veterinary drugs in distiller grains by LC-Orbitrap HRMS and proved that LC coupled to an Orbitrap mass spectrometer is a promising analytical technique, suitable for official residue control of a variety of veterinary drugs. The isolation and characterization of partially hydrolyzed fumonisins (PHFB1 and PHFB2) and hydrolyzed fumonisins (HFB1 and HFB2) with LC-HRMS was reported by De Girolamo *et al.*

(2014). According to the results, mycotoxins level ranged over 60–5700 μg kg^{-1} for fumonisins, 10–210 μg kg^{-1} for partially hydrolyzed fumonisins, and 30–200 μg kg^{-1} for hydrolyzed fumonisins.

16.3.3 Ambient Mass Spectrometry

Ambient ionization interfaces applied for pesticides analysis are desorption electrospray ionization (DESI), paper spray ionization, low-temperature plasma ambient ionization, direct analysis in real time (DART), and atmospheric-pressure glow-discharge ionization (APGDI) (Harper *et al.* 2008). A DESI-MS/MS method was developed for the determination of atrazine on cabbage leaf, and 60 pg mm^{-2} was the lower concentration level of the linear range (Zhang *et al.* 2009).The determination of strobilurin fungicides (azoxystrobin and pyraclostrobin) in wheat samples by DESI-MS^n was described by Schurek *et al.* (2008). Moreover, an important multi-pesticide DESI-MS/MS method was developed by García-Reyes *et al.* (2009). Sixteen pesticides were measured on fruit peels and extracts (lemon peel, green pepper, acetonitrile extracts of pear, lemon, apple, tomato, persimmon, grapes, and orange). In that study, the limits of detection for tomato and orange samples ranged between 1 and 90 μg kg^{-1}. DART is primarily applied for small target analytes in contrast to DESI (Hajslova *et al.* 2011), and its application in pesticide screening has to be further exploited.

Recently, DART interface has been applied for the analysis of strobilurine fungicides in wheat grains (Schurek *et al.* 2008). In this study, a quantitative analysis of samples was performed by DART-TOF-MS, and results were found in accordance to those obtained by LC-MS/MS; however, the limits of detection were, in general, four- to five-fold lower with LC-MS/MS (Schurek *et al.* 2008) than with DART. Strobilurine residues were further confirmed by DESI instrumentation (Schurek *et al.* 2008). APGDI has only been applied for rapid screening in foodstuffs (Jecklin *et al.* 2008). A rapid screening method APGDI-QqTOF-MS of 10 pesticides was developed in fruit juices and on the skin of whole fruits and vegetables, and sample pre-treatment was not required (Jecklin *et al.* 2008). Overall, some of the recent developments in the MS detection of pesticides are useful for rapid and real-time analysis of products for surveillance programs (Jecklin *et al.* 2008).

16.3.4 Immunoassays

Immunoassays (IA) provide rapid, sensitive, and cost-effective analysis for a variety of pesticide residues and mycotoxins. The usefulness of these techniques is appreciated during screening analyses when a large number of samples have to be analyzed in parallel for a single analyte within a short time (Mercader *et al.* 2014). Direct competitive enzyme linked immunosorbent assay (ELISA) has been described in multi-enzyme tracer formats for the simultaneous analysis of insecticides in fruit juices (Watanabe *et al.* 2007), for the analysis of carbaryl and metolcarb (Sun *et al.* 2010), as well as for neonicotinoids and organophosphorus pesticides (Yan *et al.* 2014) and for the detection of florfenicol antibiotic in food of animal origin (Sheu *et al.* 2013). Mycotoxins, such as aflatoxin M1, have also been detected with ELISA in dairy products (Tosun and Ayyildiz 2013) and other residues in different agricultural products and juices (Liu *et al.* 2013*b*; Navarro *et al.* 2013; Watanabe *et al.* 2013). The main disadvantage is that the immune reactivity of the primary antibody might be adversely affected by labeling with enzymes

or tags. Moreover, there is no flexibility in the choice of the primary antibody label from one experiment to another (Shrivastav *et al.* 2002).

16.3.5 Biosensors

A biosensor is an analytical device which converts a biological response into an electrical signal (Liu *et al.* 2013*b*). Biosensors are potentially useful as pre-screening methods for direct field use, and the general requirements for a routinely used biosensor include low cost, short assay time, and limited sample pre-treatment (Liu *et al.* 2013*a*).

March *et al.* (2009) developed a quartz crystal microbalance (QCM) immunosensor for the determination of the insecticide carbaryl and 3,5,6-trichloro-2-pyridinol. The detection was based on a competitive conjugate immobilized immunoassay format using monoclonal antibodies. Hapten conjugates were covalently immobilized, via thioctic acid self-assembled monolayer, onto the gold electrode sensitive surface of the quartz crystal. This covalent immobilization allowed the reusability of the modified electrode surface for at least 150 assays without significant loss of sensitivity (March *et al.* 2009).

Other techniques have also been proposed to determine pesticide residue contents in juice and wine, such as kinetic spectrophotometric method (Abbasi *et al.* 2010), fluorescence, and differential pulse polarography (Mercan *et al.* 2007). Thermometric biosensors (dcTELISA) based on an enzyme thermistor have been developed for the determination of atrazine in corn (Qie *et al.* 2013). Biosensor technology has been used for the sensitive and reliable monitoring of veterinary drug residues in food (Huet *et al.* 2010; McGrath *et al.* 2012). The technique relies on contacting the food sample or extract with a residue-specific antibody to form a drug-antibody binding pair. It is easy to use, fast, and offers excellent sensitivity for high-throughput screening (March *et al.* 2009). Most methods developed used optical biosensor instrumentation are based on surface plasmon resonance (Picó 2008).

16.4 Conclusions

The field of food analysis for contaminants is complicated due to the large number of possible residues. In addition, their determination in food samples is even more complex, since in most cases the concentration of the matrix components is much higher than the concentration of the target residues. MRMs are therefore preferred due to their efficiency in checking a large number of compounds in one single analysis. Simplifying complicated and time-consuming sample pre-treatment is a pending problem. Following the current trends towards simplification of the methods as well as solvent reduction, several extraction procedures have been introduced such as SPME or MSPD, with QuEChERS proving to be the most competitive of all. In terms of detection, GC coupled with EI-MS and LC ESI-MS/MS (triple quadrupoles) and today HRMS (QTOF-MS and Oritrap-based technologies) are the most commonly used techniques for multi-residue screening. Such systems allow target residue identification and quantification with an adequate concentration range and reproducibility in complex food matrices at extremely low levels (ng kg^{-1}). HRMS systems have demonstrated their excellent analytical performance, allowing the analysis of a wide range of compounds in food, providing screening and tentative identification for both non-target and target

compounds, overcoming the limitations of low-resolution systems. They provide high resolution, accurate mass and high full-scan sensitivity and selectivity. They can be applied in full-scan mode or MS/MS mode, and in sequential or simultaneous mode. Retrospective analysis is always possible when a new food chemical hazard appears. This translates into a rapid, effective option for routine laboratories.

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17

Chemometrics – Bioinformatics

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17.1 The Role of Chemometrics in Food Authentication

Quality and authenticity are integrated measures of several characteristics either deliberately produced (sophistication, adulteration, counterfeit, color, appearance, etc.) or not (purity, contamination, degradation, etc.), as well as of the ensemble of features capable of linking the product to its origin, provenance, and typicality. In order to overcome the subjectivity which is inherent in some of the definitions reported above and in the procedures adopted to control them, mainly based on paper documentation, there is a need to introduce chemical, physico-chemical, and organoleptic determinations which can be related to the different aspects of quality, in other words, an exhaustive characterization of foodstuff. The analytical methodology actually evolved from the analysis of a subsets of properties on the basis of some *a priori* knowledge of possible constituents/contaminants, to broad sample characterization by using instrumental techniques, going from chromatographic-spectrometric series, through hyper-spectral imaging and higher dimensional spectroscopic techniques, to batteries of sensors/microarray devices.

In terms of data analysis, this implies a change of paradigm from the analysis of mainly univariate data (i.e., a limited number of determined analytes) to multivariate data analysis of the collected instrumental responses in order to capture the required information. This approach relies on data-driven discovery, that is, information is achieved from an understanding of the underlying inner relationships among variables as highlighted by data analysis *a posteriori* (Munck *et al.* 1998; Perrot *et al.* 2011; Li Vigni *et al.* 2013).

With this new perspective in mind, is evident that the role of chemometrics is of the utmost importance. Regardless of the fact that new chemometrics methods are continuously being updated and improved upon (Brown *et al.* 2009), two main distinctive characteristics are required: (1) data exploration and graphical representation; and (2) deep model validation through all steps of data processing. This also explains why chemometrics tools based on latent variables, for example, PCA, SIMCA, PLS, and PLS-DA, are still so popular and powerful. In fact, these methods are based on projecting the data to a low-dimensional, easily interpretable space and providing an effective data display both for exploration and interpretation.

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The presentation of these methods will be the core of this chapter as well as discussing the importance of tackling several other aspects such as the nature, the type, and the scale of the variables, the possible presence of outliers, and the choice of the most appropriate pre-processing method.

17.1.1 Authenticity Issues

According to ISO definitions (ISO IEC 27000 2014, section 2, Terms and definitions): "Authenticity is a property or characteristic of an entity. An entity is authentic if it is what it claims to be" and "Authentication is a process that is used to confirm that a claimed characteristic of an entity is actually correct. To authenticate is to verify that a characteristic or attribute that appears to be true is in fact true."

The compliance to label is therefore one of the main requisites to be verified in the authentication process. This seems particularly challenging especially for typical/traditional foods, for example PDO or PGI labeled, since these certifications require that the product must be produced and/or processed and prepared in a particular geographical area. The consumer must therefore be protected from counterfeiting and/or marketing of 'sounding like products' from other regions or countries.

The main issue that analytical methods have to face is that geographical origin, raw materials, and transformation processes have to be taken into account; this requires a more holistic approach, that is, switching from the situation where "I know what to look for" (separation, identification, quantification of definite analytes/attributes) to "I want to know" (looking for a chemical signature by acquiring a food fingerprint).

Furthermore, from the point of view of data analysis all these aspects introduce distinct sources of variability leading to rather complex systems.

17.1.2 Nature and Challenge of Datasets Arising from Fingerprinting Techniques

At variance with targeted analytical approaches that focus on detecting predetermined compounds of interest, fingerprinting techniques allow the adoption of a hypothesis-free approach to food authentication (Munck *et al.* 1998; Ellis *et al.* 2012; Li Vigni *et al.* 2013*a*; Grauwet *et al.* 2014). In fact, chemical fingerprinting implicitly considers all compounds present in the investigated food products/processes, and may also reveal unexpected compounds, food quality changes, and contrasting differences between products/processes. This makes it a comprehensive, unbiased methodology.

Fingerprinting techniques range from chromatography or spectroscopic-based (Li Vigni *et al.* 2009; Ritota *et al.* 2010; Bevilacqua *et al.* 2012; Cozzolino 2012; Checa and Saurina 2013; Tomassini *et al.* 2013) measurements to landscapes obtained by any hyphenated combination of the previous techniques (Christensen *et al.* 2006; Cocchi *et al.* 2008; Pereira *et al.* 2011), or from signals obtained by means of sensor arrays such as electronic noses or tongues (Baldwin *et al.* 2011; Śliwińska *et al.* 2014), microarrays, etc. to imaging and hyperspectral imaging techniques (Pereira *et al.* 2009; Elmasry *et al.* 2012; Amigo *et al.* 2013).

A common trait of fingerprinting data is the huge number of variables obtained for each sample and, in the case of hyphenated techniques, the presence of more than two sources of variability (e.g., retention times and mass to charge ratio). Moreover, shape information as well as intensities of peaks/bands is also of relevance. Information

retrieval is therefore not trivial when considering that many sources of unwanted variability affect the data such as baseline, background contributions, or peaks shift. The need to consider further variability due to raw materials, seasonality, agricultural practices, and food processing, that are aspects which all play a role in authenticity tasks, justifies the need for chemometrics methods. In particular, multivariate screening tools are needed to give a graphical representation of multivariate data, highlighting the data patterns and the relationships among objects and variables. The relevance of this step in food authenticity must be stressed. In fact, the multivariate exploratory tools through the synergy of data reduction and graphical display make it feasible to recover the distinctive traits of a specific food product and to contrast it with respect to all the substitute products without having to formulate an *a priori* hypothesis on which the salient constituents capable of conferring this specificity might be.

In this case, prior knowledge is used after data reduction to interpret the highlighted patterns and to validate possible conclusions that will lead to a new hypothesis generation. When using prior knowledge in advance, interactions among the different constituents, subsystems, or processing steps are lost and this strongly limits the ability to assess an authenticity model. Guided by exploratory multivariate data analysis results, a modeling step can then be undertaken, for example by discriminant analysis or other supervised classification tools.

17.2 Methodology

To illustrate all available chemometrics tools is beyond the scope of this chapter; instead, at each step of what can be considered a data analysis pipeline (Fig. 17.1), a few basic chemometrics methods are presented.

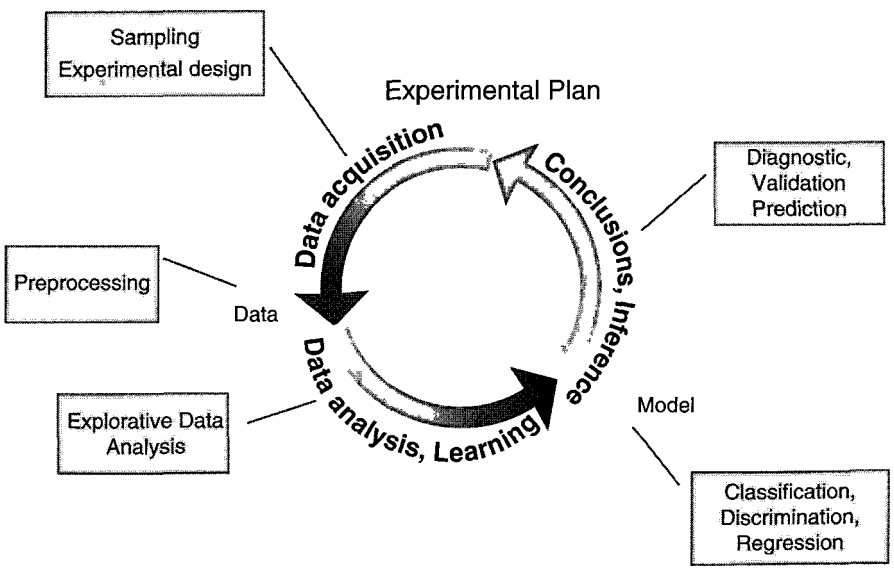


Figure 17.1 A schematic view of data analysis pipeline. Rectangular boxes highlight the specific data analysis tasks that chemometrics tools accomplish.

17.2.1 Exploratory Data Analysis

Exploratory data analysis (Tuckey 1977; Li Vigni *et al.* 2013*a*) should always be the starting step allowing for data inspection through graphical representation. When dealing with a few variables the main graphical tools for descriptive statistics, that is, information about variable distribution features, are frequency histograms and box-whisker graphs (Massart *et al.* 2005). The latter are very useful to signal the presence of outliers and to highlight location and variation changes between different groups of data or among several variables. An example is shown in Figure 17.2.

To visualize multivariate data, a projection step is needed in order to enable a graphical representation in terms of a few 2D or 3D plots. Multivariate analysis methods based on latent variables projection, such as principal component analysis (PCA), provide the best tool to highlight variable correlations and sample similarities/differences, to reduce the variable space to lower dimensions and to allow graphical outputs that are easy to read and interpret. It is worth highlighting that, in the frame of exploratory analysis, data reduction (compression of all the information to a small set of parameters) has to be accomplished without introducing distortion of the data structure or at least strongly limiting it.

A description of the salient feature of PCA is given here, while a detailed illustration of the method can be found elsewhere (e.g., Joliffe 2002; Esbensen *et al.* 2009; Li Vigni *et al.* 2013*a*). Considering a data table $\mathbf{X}_{N,J}$, (N samples by J variables), PCA projects the data from its J-variable space to A-latent factors/components space. This is accomplished by bilinear decomposition of the original data:

$$\mathbf{X}_{N,J} = \mathbf{T}_{N,A} \cdot \mathbf{V}^{\mathrm{T}}{}_{J,A} + \mathbf{E}_{N,J} \tag{17.1}$$

where $\mathbf{T}$ is the scores matrix and holds the new latent variables, that is, the coordinates of data points (samples) in the PCA space; $\mathbf{V}$ is the loadings matrix and contains the weights with which each original variable contributes to the principal components (PCs); and $\mathbf{E}$ is the residual matrix, that is, the unmodeled part of $\mathbf{X}$.

The scores scatter plots give a picture of the dominant object patterns; analogously, the loadings plots show the complementary variables patterns, as well as the correlation structure among the variables. The residual matrix $\mathbf{E}$ can be used as a diagnostic tool for the identification of outlying samples and/or variables, and of anomalous trends in residuals plot.

From an algebraic point of view, a PCA calculation is a maximization problem (retaining maximum data variance) under constraints (orthogonality and normalization of scores and loadings vectors). It can be solved by eigenvectors/eigenvalues decomposition (Golub and Reinsch 1970; Wu *et al.* 1997).

For practitioners there are two critical issues in PCA analysis: (1) to assess the appropriate number of principal components, A; and (2) to choose the pretreatment to be applied to the data, since PCA is sensitive to the relative scaling of the original variables.

The first issue is to assess the appropriate number of principal components, A. The maximum number of PCs that can be extracted is equal to the number of original variables J (for full rank data matrix, in that case PCA is an invariant orthogonal transformation), but in general the aim is to retain only a few significant components. PCs are nested; the first principal component has the largest possible variance (i.e., it accounts for as much of the variability in the data as possible), and each succeeding component

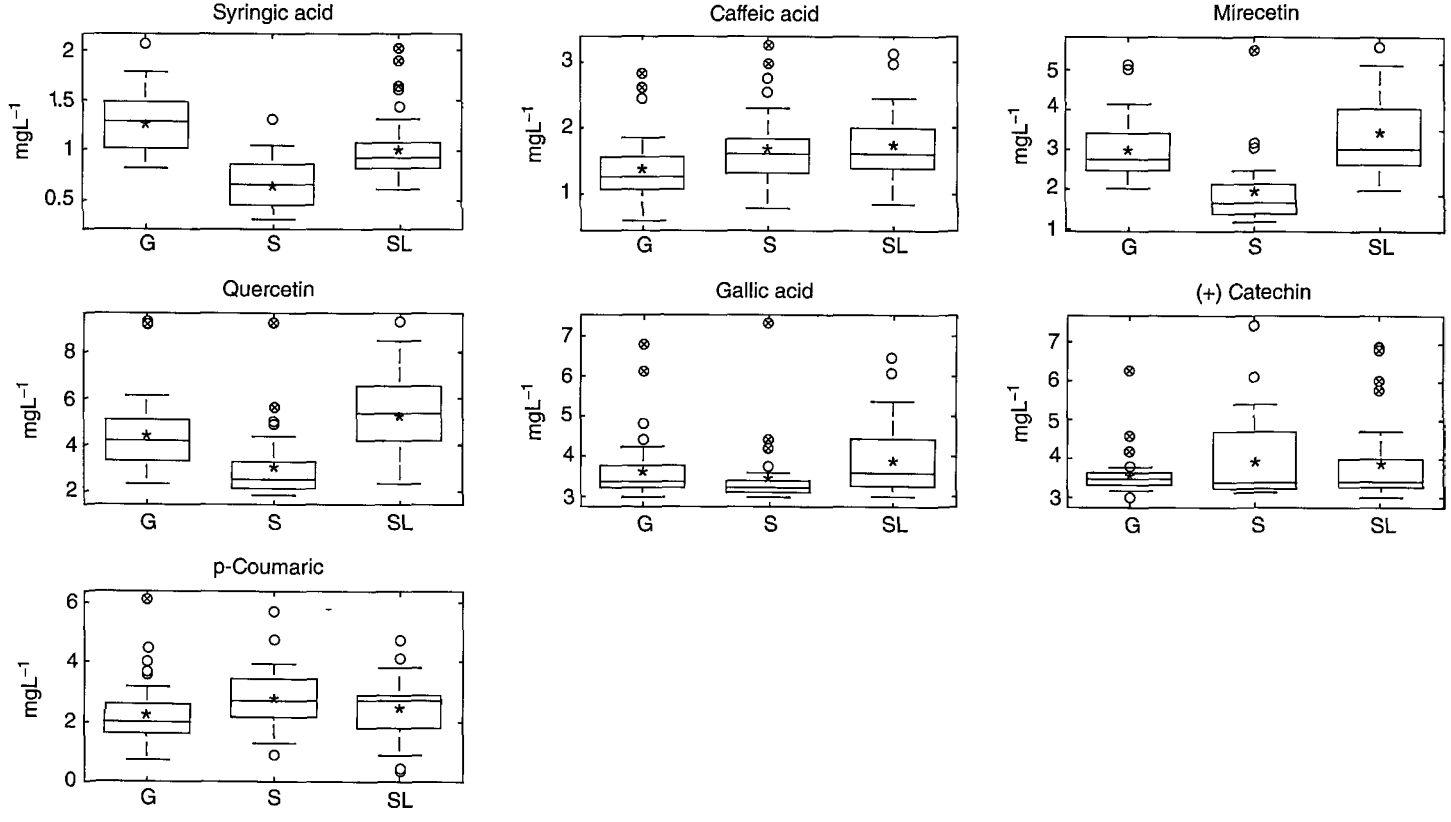


Figure 17.2 Box and whiskers plots for a dataset consisting of three Lambrusco PDO wine varieties – Grasparossa (G), Sorbara (S), and Salamino (SL) – characterized by some phenolic compounds. The boxes enclose the interquartile range (IQR = 75° (UQ) – 25° (LQ) percentiles) and whiskers extend to the minimum and maximum values not considering extreme values (values higher than the UQ + 1.5×IQR or smaller than LQ – 1.5×IQR). The solid line is the median and the asterisk (*) the mean. This representation offers a quick view of compounds potentially useful to distinguish the three varieties such as Mirecetin, potential outliers (crossed circles), and asymmetry of distribution.

in turn has the highest residual variance. Criteria to select the number of PCs can be based on explained variance, for example plotting the cumulative variance versus the number of PCs (scree plot) and retaining PCs until the increment of explained variance between two consecutive components is not significant. It is assumed that since noise is unstructured, PCs accounting for noise explain the same small fraction of data variance and a plateau in scree plot is reached.

Another or complementary criterion can be the sequential exploration of scores plots to keep components until scores plots show interesting trends, and stop when they start to be unstructured (Fig. 17.3).

Cross-validation (CV) (Bro *et al.* 2008; Camacho and Ferrer 2012) or other methods that rely on validation, such permutation tests and bootstrapping (Efron 1981), are recommended when the PCA model will be used for further aims such as building a reference category model. In this case the capability of the PCA model to reproduce data not considered in the model calculation has to be considered for the selection of the appropriate number of components.

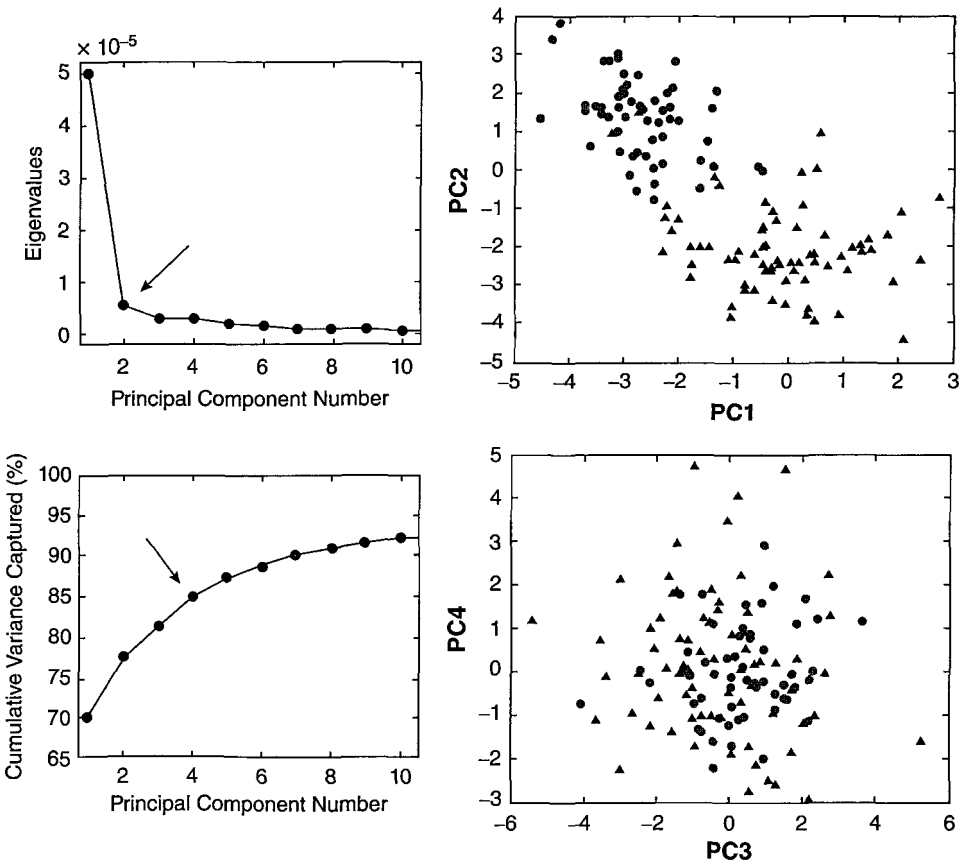


Figure 17.3 An example of a dataset with two categories of samples (triangles and squares) of a Scree plot (left side) either considering eigenvalues v. number of PC (top left) or cumulative explained variance v. number of PC (bottom left). The corresponding scores plots are shown on the right. Scree plots may suggest the choice of 2–4 PCs; score plots point to two PCs.

The second issue is to choose the pretreatment to be applied to the data, since PCA is sensitive to the relative scaling of the original variables. In order to prevent variables with much larger scales or higher variance dominating the PCA model, scaling can be applied. Most frequently used procedures include scaling each variable to unit variance (autoscaling) or scaling blocks of variables to the same variance (Wold *et al.* 1983; Bro and Smilde 2003; Cocchi *et al.* 2006*a*), as shown in Figure 17.4. Block scaling is particularly useful to balance the contribution of peaks/bands in signal with very different intensity without up-weighting the baseline contribution.

Once a PCA model is obtained, geometrically this model is a hyper plane of A dimensions. Two distances can be defined for each sample (Fig. 17.5): the distance from the hyper plane (from the PC model) that represents a measure of the variation of the data outside the PCA model, expressed by the sum of squared residuals for that objects; and the distance from the origin of the hyper plane (distance within the model space), defined by the sum of squares of scores and referred to as T^2 (this is the Mahalanobis distance (De Maesschalck *et al.* 2000) if scores are scaled to unit variance, which is usually done). T^2, Q, and associate statistics, namely Hotelling-T^2 for T^2, and χ-statistics for Q, can be used to depict possible outliers and extreme behavior samples. Moreover, if the modeled objects belong to the same category, as in classification tasks, statistics limits at a given confidence level for T^2 and Q are used to define the space of that category.

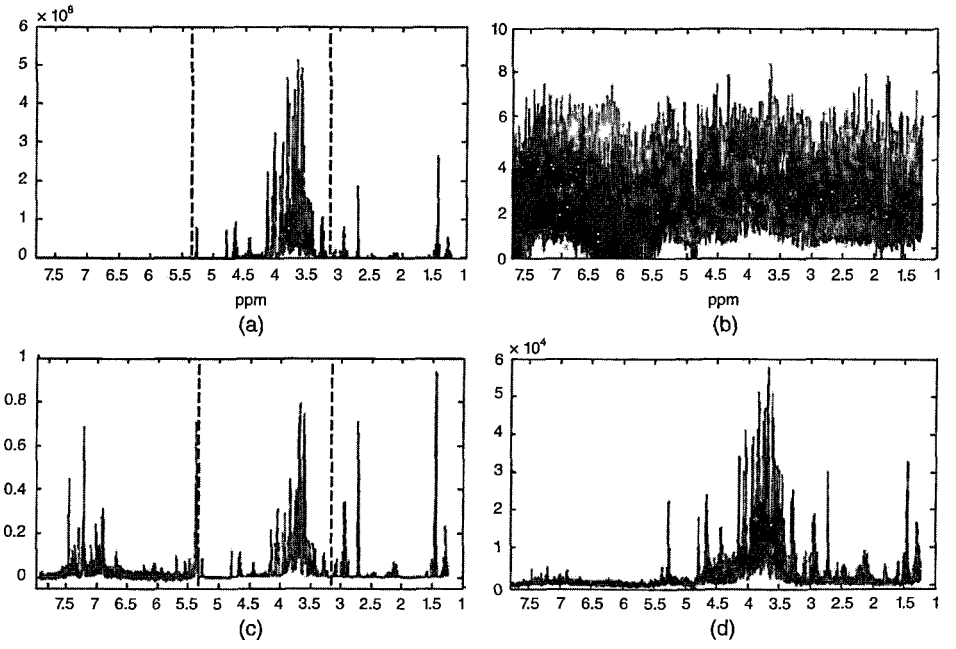


Figure 17.4 Results of different scaling procedures. (a) Raw ^{1}H-NMR spectra collected on *Lambrusco* wines samples; (b) the same spectra after applying autoscaling; (c) the same spectra after applying block scaling to same block variance (the dashed vertical lines highlight the subdivision in three blocks/regions); and (d) the same spectra after applying Pareto scaling.

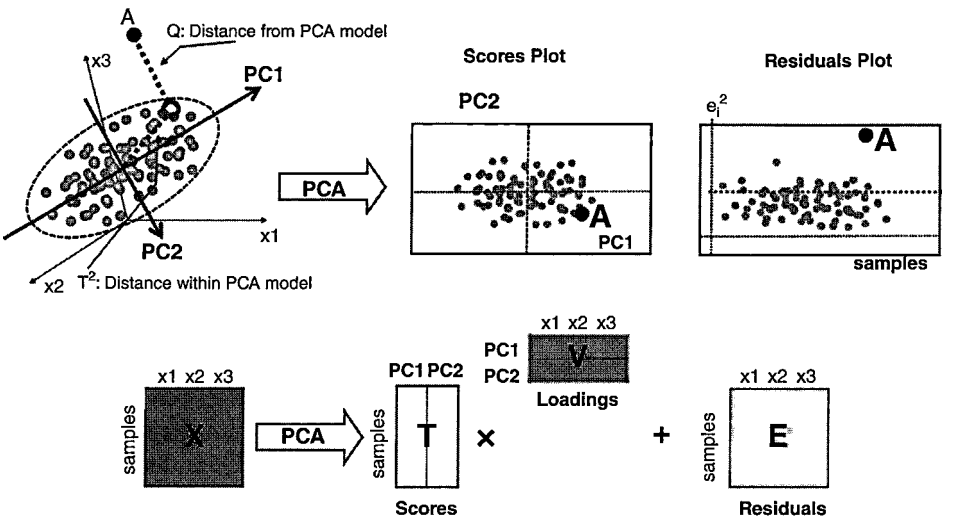


Figure 17.5 Schematic representation of a PCA model, showing PCA decomposition (bottom) and distances of a projected sample in PCA space and residuals space (top).

17.2.1.1 PCA in Practice

As an example, the analysis of the dataset "rheo" is discussed. The data consist of several batches of flour destined for industrial bread production, corresponding to two harvesting campaigns for which the protein content in terms of glutenins and glyadins sub-fractions, chemical, and rheological properties, have been determined (for more details see Li Vigni *et al.* 2013*b*).

The objective of the study is twofold: on the one hand, data exploration in order to depict the main correlation pattern among protein profile and rheological properties, and the extent of variability due to both batch and harvesting year; and on the other hand, to have a reference PCA model capable of distinguishing flour deriving from a wheat mixture mainly comprising Italian wheat (higher than 70%), referred to as "Italian," from flour derived from not-Italian wheat variety (authentication with respect to origin), referred to as "foreign." The PCA model was built by considering only "Italian" flour samples, and a three-PC model was chosen on the basis of the scree plot (Fig. 17.6, top-left). The PC1 v. PC2 scores plot (Fig. 17.6, top right) shows that the main source of variability, as to be expected, is the harvesting year. The loadings values on PC1 (Fig. 17.6, bottom right) indicate which feature determines this separation, for example higher glyadins content, stability, and strength (W) and lower total protein and gluten contents for 2009 samples. Intra- and inter-batch variability are comparable and no clustering of batches is observed (Fig. 17.6, bottom left). In order to use this PCA model as reference for all "Italian" flour, this is a desired feature.

Figure 17.7 shows the T^2 v. Q plot, in which only three samples show peculiar behavior (this is perfectly acceptable considering the reference limits are at 95% confidence level). Sample 14 is not well modeled; sample 48 has extreme behavior in PC space; and sample 21 is a potential outlier. In order to highlight the features responsible for this behavior, the corresponding contribution plots can be inspected (Fig. 17.7). For example, sample 21 has a higher glutenins content, especially for the low-molecular-weight

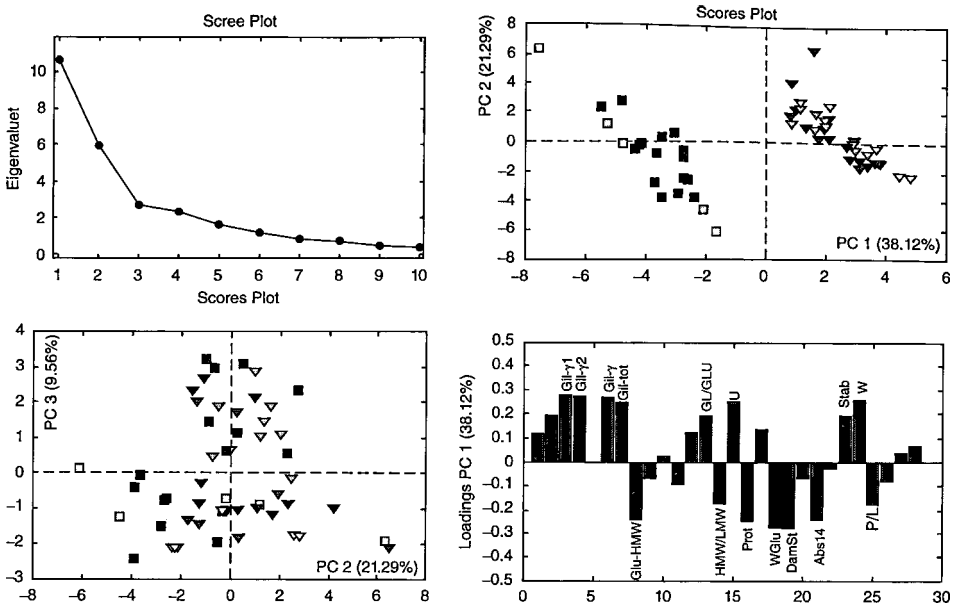


Figure 17.6 PCA of dataset "rheo." In the scores plots the shape of symbols indicates harvesting year (square 2008 and triangles 2009). The different shades of the symbols represent different batches.

fraction (LMW1 and LMW2), hence a lower HMW/LMW ratio (T^2 contributions); at the same time, the gliadyns/glutenin correlation pattern is altered (Q contribution). When "foreign" flour samples from the same two harvesting years are projected on the model, almost all are rejected (Fig. 17.7) for being both not modeled (above Q limit) and extreme in PC space (above T^2 limit).

17.2.2 Classification and Discrimination

Authentication tasks mostly require assessing membership to one or more categories. Typical questions are: "does sample X, labeled as A, really belong to class A?" or "does sample X follow specifications?" and/or "is product X contaminated or not?"

In data analysis terms, these questions can be formulated in the general framework of classification, which means a methodology where mathematical/statistical rules are defined to assign a sample to one or more categories on the basis of the values of a set of variables acquired to characterize the sample. The main difference with respect to exploratory data analysis, which may reveal natural grouping in the data (eventually corresponding to defined categories), is that in classification category information is given *a priori* and used to build the model (i.e., classification is a supervised method), while in exploratory data analysis category information is not used; at most, *a posteriori* is taken into account to color sample points in graphs.

Very many multivariate classification methods have been developed (Vandeginste *et al.* 1998; Bevilacqua *et al.* 2013; Oliveri and Downey 2013; Salvatore *et al.* 2013) and can be distinguished on different bases. Here, the focus is on the fundamental differentiation between two main groups, aimed at discrimination, for example PLS

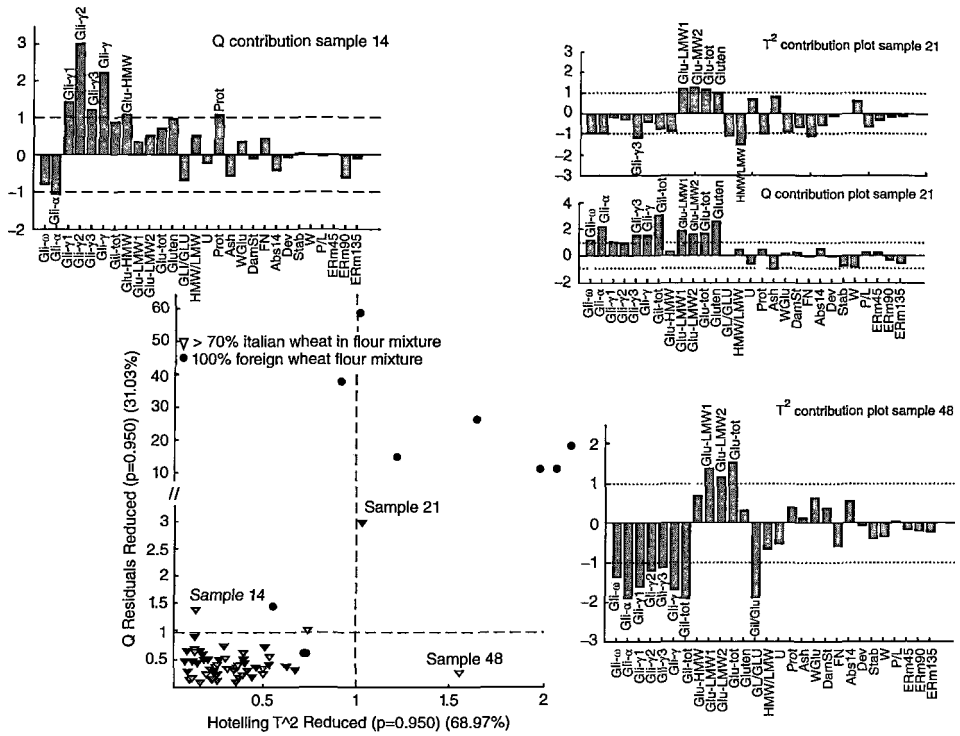


Figure 17.7 PCA of dataset "rheo." Bottom left: T^2 v. Q plot. Shaded symbols are as in Figure 17.6. Grey-filled circles refer to not-Italian flour wheat mixtures. Top right: T^2 and Q contribution plots for outlying sample 21; top left: Q contribution plot for the unfit to model sample 14; and bottom right: T^2 contribution plot for extreme sample 48.

discriminant analysis (Wold *et al.* 2001; Barker and Rayens 2003; Indahl *et al.* 2007), or at class modeling, for example SIMCA (Wold 1976; Wold and Sjostrom 1977; De Maesschalck *et al.* 1999; Forina *et al.* 2008). In discriminant classification, the classification rules are defined in order to find the differences among the classes. From a geometrical point of view this means finding directions (in general terms, hyper surfaces) in variables space capable of segregating the samples belonging to a class in a specific region; there will be as many regions defined as classes. In class modeling the focus is in modeling the similarity between the samples belonging to the same class, not to differentiate the classes. From a geometrical point of view, the class-modeling rules allow a space of the class (in general terms, a hyper volume) to be defined, regardless if there are other classes, and independently for each of them.

This basic difference implies that in discriminant classification a sample is always assigned to one of the defined categories/classes, while in class modeling a sample can either be assigned to just one category/class, to more than one, or to none of them. This is because for the latter each category is modeled independently and classification rules establish whether a sample has to be accepted or refused by a given class model, considering one class model at time. Figure 17.8 shows a schematic illustration of the two approaches.

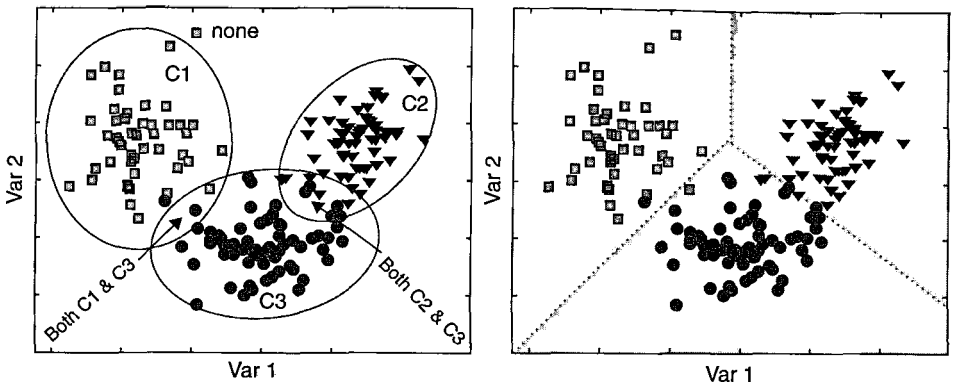


Figure 17.8 Illustration of class-modeling (left) and discriminant (right) approaches to classification.

The common trait in discriminant classification methods is that the decision rules are based on probability criterion (Bayes' rule: a sample is assigned to the class to which it has the maximum probability of belonging), while they differ in the method of defining and estimating probabilities. There are methods that assume a particular probability distribution either from statistical distribution (parametric methods, such as linear discriminant analysis) or estimated from the data itself (non-parametric, such as potential functions), and other methods that directly provide a non-parametric estimation of the probability values by automatic learning from training data such as artificial neural networks (see Section 17.2.6).

Among the discriminant classification methods, the most popular are discriminant analysis, linear (LDA), and quadratic (QDA) (Fisher 1936; McLachlan 1992), extended canonical variate analysis (ECVA; Nørgaard *et al.* 2006) and partial least squares discriminant analysis (PLS-DA; Barker and Rayens 2003).

LDA and QDA assume that the data follow a multivariate normal distribution. LDA also assumes that each class has the same dispersion, that is, a common variance/covariance matrix, resulting in linear boundaries among classes, while QDA relaxes this last assumption allowing for quadratic boundaries. As for multilinear regression, the main limitation of these methods is that they are based on full-rank nonsingular data; they can therefore only operate on data matrices where the number of samples is larger than the number of variables and where the variables themselves have as low a correlation as possible. To overcome this limitation, data reduction can be operated before applying discriminant analysis, for example by using principal component analysis prior to LDA or reformulating the discriminant classification problem in regression terms such as in ECVA or in PLS-DA.

In class-modeling techniques, first a model for each class is built. The normal variation for samples belonging to the class should therefore be estimated, and then for each future/unknown sample it is checked, for each modeled class, whether it falls within the identified normal range of variation or not.

Class-modeling methods include soft independent modeling of class analogies (SIMCA), unequal class-modeling (UNEQ; Derde and Massart 1986), discriminant analysis with shrunken covariances (DASCO; Frank 1988), and a modeling version of the potential-function-based technique (Forina *et al.* 1991).

For the aim of this chapter and for the sake of brevity, as a representative of each type of method PLS-DA and SIMCA are discussed in the following sections.

17.2.2.1 PLS-DA

In partial least squares discriminant analysis (PLS-DA) the discriminant classification problem is reformulated as a regression problem in which the responses matrix Y contains the class membership information in a binary coded form, that is, each Y column refers to a given category and each sample will have a value of 1 in the Y column corresponding to its true class, and 0 (or −1) elsewhere. Accordingly, the classification problem can be reformulated as finding the best regression model by using the PLS algorithm (Wold *et al.* 2001) linking the experimental data measured on the samples X to the binary-coded dummy matrix Y (Fig. 17.9).

$$\mathbf{Y} = \mathbf{XB}. \tag{17.2}$$

In PLS, both **X** and **Y** are decomposed in a PCA-like way:

$$\mathbf{X} = \mathbf{TV}^{\mathrm{T}} + \mathbf{E} \tag{17.3}$$

$$\mathbf{Y} = \mathbf{UQ}^{\mathrm{T}} + \mathbf{F}, \tag{17.4}$$

under the constraint that the corresponding scores have the maximum covariance, that is:

$$\mathbf{U} = \mathbf{TC}. \tag{17.5}$$

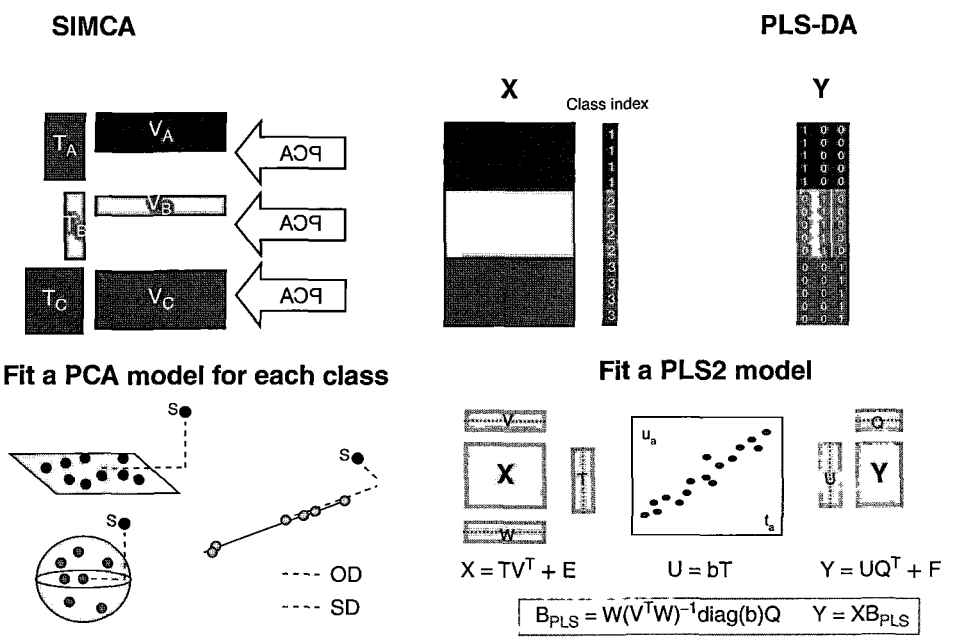


Figure 17.9 Schematic illustration of SIMCA (left) and PLS-DA principles (right).

Additionally, for each latent variable a weights vector **w** is calculated which contains the contribution of each variable in **X** to model the responses **Y** for that dimension; the weights matrix **W** therefore contains the structure in **X** which maximizes the covariance between **T** and **U** in every dimension.

The regression coefficients **B** in Equation (17.2) which relate the predictors **X** with the response **Y** are calculated as:

$$\mathbf{B} = \mathbf{W}\left(\mathbf{V}^{\mathrm{T}}\mathbf{W}\right)^{-1}\mathbf{CQT}. \tag{17.6}$$

PLS-DA has been demonstrated to converge to linear discriminant analysis if the number of PLS latent variables is equal to the number of variables in **X** (Barker and Rayens 2003). The regression coefficients matrix allows prediction of the **Y** values for unknown samples $\mathbf{X}_{\mathrm{new}}$, and as the predicted values ($\hat{\mathbf{Y}}_{\mathrm{new}}$)can assume real values and not only 1 and 0, a classification rule to assign the samples to a given category has to be defined. In general, there are two approaches. First is the "true" discriminant approach where classification is accomplished by assigning the samples to the category corresponding to the highest value of the predicted dummy response. For example, if the classification problem regards three classes, a sample whose predicted Y values are (0.98 0.5 0.1) will be assigned to class 1. This approach when modeling more than two categories may be suboptimal and it is suggested to apply LDA (or QDA) on the X scores or on the Y predicted values (Indahl *et al.* 2007), instead.

A second approach is based on the choice of a class threshold for each category (Wise *et al.* 2009), that is, a value for each dummy **y** above which the sample is assigned to the class and *vice versa* if it is under. The threshold is usually chosen on the basis of classification performance estimated in cross-validation.

17.2.2.2 SIMCA

SIMCA (soft independent modeling of class analogies) is a supervised class-modeling method, first developed by S. Wold in 1976. The basic assumption of SIMCA is that the similarity between samples from a particular class can be captured by a principal component model of opportune dimensionality so that the verification of whether a sample is accepted or rejected by the class model reduces to outlier detection according to some kind of distance to the latent variable model. PCA is separately performed (as well as data pretreatment, such as centering and scaling if needed) on the objects belonging to each class/category, thus providing a scores-**T** and loadings-**V** matrices for each class. The model dimensionality, that is, number of PCs, can differ for the various classes. It is therefore possible to obtain a classification model and at the same time obtain information about the properties of each analyzed class.

Once the PC decomposition is computed:

$$\mathbf{X}_{\mathrm{A}} = \mathbf{T}_{\mathrm{A}}\mathbf{V}_{\mathrm{A}}^{\mathrm{T}} + \mathbf{E}_{\mathrm{A}} \tag{17.7}$$

it is possible to define the distance of the **k**th sample to the model of the class, $d_{k,C}$ as:

$$d_{k,C} = \sqrt{\left(OD_{k,C}\right)^2 + \left(SD_{k,C}\right)^2} \tag{17.8}$$

where $OD_{k,C}$ and $SD_{k,C}$ represent the orthogonal and the scores distances of the sample k to the model of class C, respectively. Orthogonal distance is a measure of the

distance of a sample to the PC subspace and is given by the sum of squared residuals, while score distance indicates how far the individual is from the training objects of the class in the PC space (Fig. 17.9, left side).

Different implementations of SIMCA exist (De Maesschalck *et al.* 1999; Durante *et al.* 2011; Pomerantsev 2008) depending on how *OD* and *SD* are defined and how the classification rule is defined. The two most frequently used implementations are as follows.

1) Original SIMCA (Wold 1976), where for the sample in the calibration set (those on which the class model is built) only the orthogonal distance is considered. Then, for each unknown sample, the distance to the class model is defined according to Equation (17.8) where the scores distance (*SD*) can be evaluated component-wise as the distance from the class boundaries plus a confidence range, or as the Mahalonobis distance from the center of the PCA space of the class. Sample acceptance is evaluated by means of an F-test where the distance to the class of an unknown sample is compared with the reference class distance of calibration samples (by using appropriate degree of freedoms, these distances can be seen as variances). If the test is passed, the object is assigned to that class.
2) Alternative SIMCA (i.e., the version implemented in the PLS Toolbox; Wise *et al.* 2009) where orthogonal distance (in this case called Q) is given by squared residuals and scores distance (in this case defined as T^2) calculated as Mahalanobis distance in PCA space. The classification rule is then based on the reduced distance from the class model:

$$d(red)_{k,C} = \sqrt{\left(Q^2_{k,C}/Q^2_{\text{lim},C}\right)^2 + \left(T^2_{k,C}/T^2_{\text{lim},C}\right)^2} \tag{17.9}$$

3) where the critical values for both distances are obtained from χ-distribution and T^2-Hotelling distributions respectively, considering the calibration sample of the given class *C*. By definition, since each of the two terms in Equation (17.9) are divided by the corresponding critical limits, an acceptance threshold of $\sqrt{2}$ is chosen to decide whether a sample is accepted by the class or not.

17.2.2.3 Assessing Classification Performance

The classification performance in a class-modeling context is estimated by considering the correct acceptance and rejection rate for each category. To this aim, Sensitivity, Specificity, and Efficiency are defined as follows:

$$\text{Sensitivity} = \text{TP}/\left(\text{TP} + \text{FN}\right) = \text{TP}/\text{N}_\text{C} \tag{17.10}$$

$$\text{Specificity} = \text{TN}/\left(\text{FP} + \text{TN}\right) \tag{17.11}$$

$$\text{Efficiency} = \sqrt{\text{Sensitivity} \times \text{Specificity}} \tag{17.12}$$

where TP (true positive) and FN (false negative) refer to the samples belonging to the class being modeled and are the number of samples located inside class boundaries (correctly accepted) and the number of samples falling outside the class model (wrongly

rejected), respectively. The sum of TP and FN equals the number of samples in the modeled class (N_C). On the other hand, TN (true negative) and FP (false positive) refer to the samples not belonging to the class being modeled (extraneous or belonging to other classes), and are the number of samples falling outside class boundaries (correctly rejected) and the number of samples located inside the class space (wrongly accepted), respectively. Sensitivity and Specificity range over 0–1 (or 0–100, if expressed in percentage). Efficiency is the geometric mean of Sensitivity and Specificity, also ranging over 0–1, and resumes the classification performance.

Sensitivity can be estimated in fit, in CV, and for external test set, while Specificity by definition always refers to an estimation of samples not used in building the class model.

Since a sample is always assigned to one of the modeled classes in discriminant classification, the Sensitivity and Specificity are not independent parameters and classification performance is usually expressed as a number of correctly classified samples (correct classification rate):

$$\text{Classification rate} = \left(TN + TP\right) / \left(TN + TP + FN + FP\right) = \left(TN + TP\right) / N_{samples}$$

Correct classification rate can be evaluated in fit, in CV, and in prediction (external/test set); in the latter case it is sometimes called Accuracy.

17.2.2.4 Assessing Model Dimensionality

Classification methods are supervised techniques so require careful validation of the obtained models. In particular, the applicability of a classification model depends on reliability of its predictions. Validation is usually done by testing the model on a "new" set of samples which have not been used in building the model. It has to be pointed out that in model development some parameters have to be set, for example number of components in SIMCA or PLS-DA models, and evaluation of the predictive performance of the model has to be made independently from optimization/choice of model parameters. To this aim several strategies can be adopted, such as using cross-validation or resampling procedures and/or permutation test in order to assess model parameters. An external evaluation set (test set) is then needed to assess predictive capability.

One common mistake is to assess the model dimensionality in SIMCA and PLS-DA models by using the same criteria as in PCA and PLS models, respectively. This is suboptimal since we are interested in evaluating the classification capability of the models. In fact, in SIMCA it cannot be assumed that best reproduction of the class data matrix $\left(\mathbf{X}_C\right)$ as estimated by criteria based on PCs explained variance also leads to the best classification performance. Analogously, considering that the Y-responses hold dummy coded values in PLS-DA, it is not sensible to rely on root mean square error in cross-validation (RMSECV) which is based the squared differences between Y coded and predicted values (which can assume any value).

It is therefore appropriate to evaluate as a function of the number components Sensitivity, Specificity, and/or Efficiency (SIMCA) and correct classification rate (PLS-DA) in cross-validation and to choose the number of components corresponding to a maximum as the best model dimensionality. An external set (test set) will then be used to assess the predictive capability of the chosen model.

17.2.2.5 Case Study

The data presented here were collected and analyzed (Bertacchini *et al.* 2013) in the context of developing an analytical methodology able to assess the quality and authenticity of extra virgin olive oil (EVOO) samples (Casale *et al.* 2010). The study was focused on the characterization of the volatile fraction by using HS-SPME coupled with GC-MS with the main aim of distinguishing EVOO samples from Riviera Ligure-Riviera dei fiori from other Mediterranean samples. In particular, 22 samples were from Apulia, 21 from Liguria (Taggiasca cultivar, PDO certified), and 29 from other Mediterranean countries including Greece, Spain, and Tunisia (referred to here as "not-Italian"). For the illustrative aim of this chapter, that is, highlighting differences between class-modeling and discriminant classification, the PLS-DA model was built with the two classes of Liguria and Apulia. In order to mimic real situations that can be encountered in authentication tasks when the goal is to discriminate the valuable PDO product from the "rest of the world": (1) not all possible counterfeiting products are known or available to get a representative sampling; and (2) there is a main (more frequent) imitating class of products so the problem is formulated as a two-classes issue, but then a fraud is committed with a different counterfeiting product.

The dataset was split into calibration and validation sets, while "not Italian" EVOO were projected on the classification models to assess their Specificity. Figure 17.10 (left side) shows the SIMCA classification results for Liguria class, which is the relevant one from a commercial point of view. The model (three PCs according to minimum CV Efficiency) has 100% Sensitivity (both training and test) and 98% Specificity with only one "not Italian" sample wrongly accepted. The corresponding PLS-DA model (right

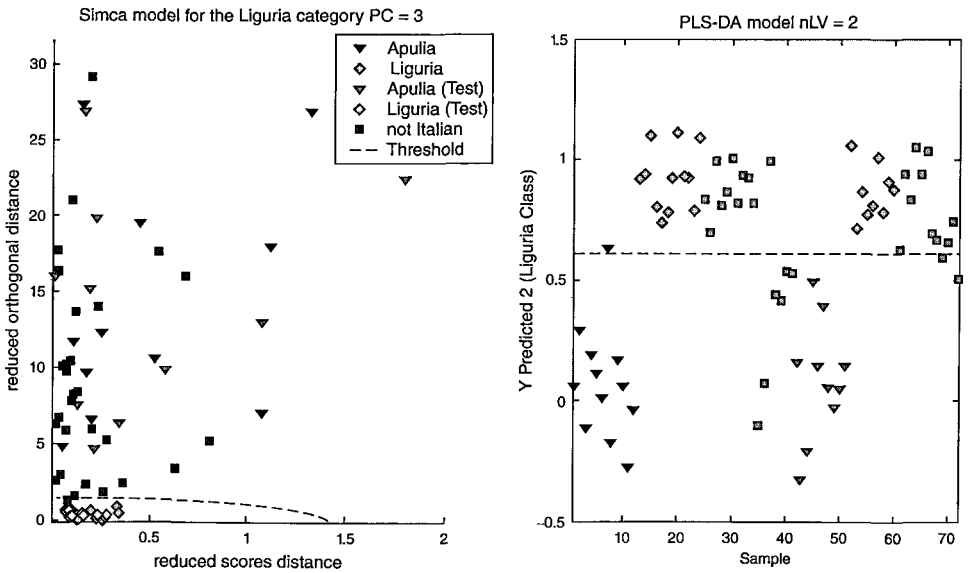


Figure 17.10 SIMCA and PLS-DA classification results for dataset EVOO. Left: the reduced scores distance v. orthogonal reduced distance plot, referred to as the Liguria class. The threshold is calculated according to Equation (17.12) (alternative SIMCA). Right: the dummy Y predicted v. number of sample for the PLS-DA model. The threshold value has been calculated according to lower classification error in cross-validation (venetian blind four split).

side of Fig. 17.10), two LVs according to minimum CV classification error, shows almost 100% correct classification rate (both training and test), but most of the "not Italian" EVOO are wrongly accepted. This is actually the limit of a discriminant approach. More insight can be gained by looking at the information used by the SIMCA and PLS-DA models in terms of the chromatographic profiles of the different classes as shown in Figure 17.11, which reports the SIMCA and PLS-DA parameters useful for modeling interpretation and assessing feature relevance such as discriminant power for SIMCA, variable importance in prediction (VIP) and selectivity ratio (SR) for PLS-DA as described in detail in Section 17.2.4.

Figure 17.11 (left) reports the standard deviation spectrum for each class where common (e.g. Rt region around 30 min for all and around 10 min for Apulia and "not Italian" EVOO) and specific high variance regions can be observed. The SIMCA loadings for Liguria and Apulia classes are reported in the middle part of Figure 17.11; their profile closely follows their respective variance profiles. Finally, the discriminant power plot points to the peaks which are either specific or with varying intensity for the two classes. The PLS-DA regression coefficients and VIP scores (Fig. 17.11, right) point to the regions where Liguria and Apulia differ. Several of these are also characteristic of "not Italian" EVOO however, so that when these are projected on the PLS-DA model, and due to the discriminant standpoint (a sample always has to be assigned to a class), they will also be accepted by Liguria class. Finally, the SR plot focuses on the peaks which are characteristic of Liguria (only important for the target-predictive component).

17.2.3 Multiway Techniques

Multiway data analysis (Smilde *et al.* 2004) is gaining more and more interest in food authenticity applications (Christensen *et al.* 2006; Durante *et al.* 2006; Marini *et al.* 2011; Vosough and Salemi 2012; Rinnan *et al.* 2014) due to the diffusion of second-order analytical techniques, such as chromatography–mass spectrometry (GC-MS, LC-MS), HPLC-DAD, fluorescence emission/excitation spectroscopy, 2D NMR spectroscopy, and so on. Multiway data arrays also arise when a set of samples has been characterized by two distinct sets of variables such as chemical profiles and sensory analysis, or when a set of samples has been characterized by a set of variables taken at different occasions such as at a different times or different sampling sites (Cocchi *et al.* 2006*b*; Li Vigni *et al.* 2013). In the food area multiway problems arise in a number of ways, ranging from storage/ageing (samples, variables, time) to sensory analysis (samples, attributes, judges), batch data (batches, time, variables), and, of course, handling data from hyphenated analytical technique systems. These data have long been treated by unfolding methods, that is, rearranged in a two-way data matrix and analyzed by bilinear models. However, multiway methods are much more efficient (more parsimonious and robust) to analyze multiway data. Further, multiway methods potentially simplify model interpretation.

Nowadays, there is an established set of chemometric multiway methods and algorithms (Smilde *et al.* 2004). Here we mention those that can serve the purposes of exploratory data analysis and classification, the tasks most frequently encountered in food authentication. In particular, the most used multiway decomposition methods are parallel factor analysis (PARAFAC) (Bro 1997) and Tucker3 (Kroonenberg and Deleeuw 1980); both methods accomplish data compression and allow explorative analysis of

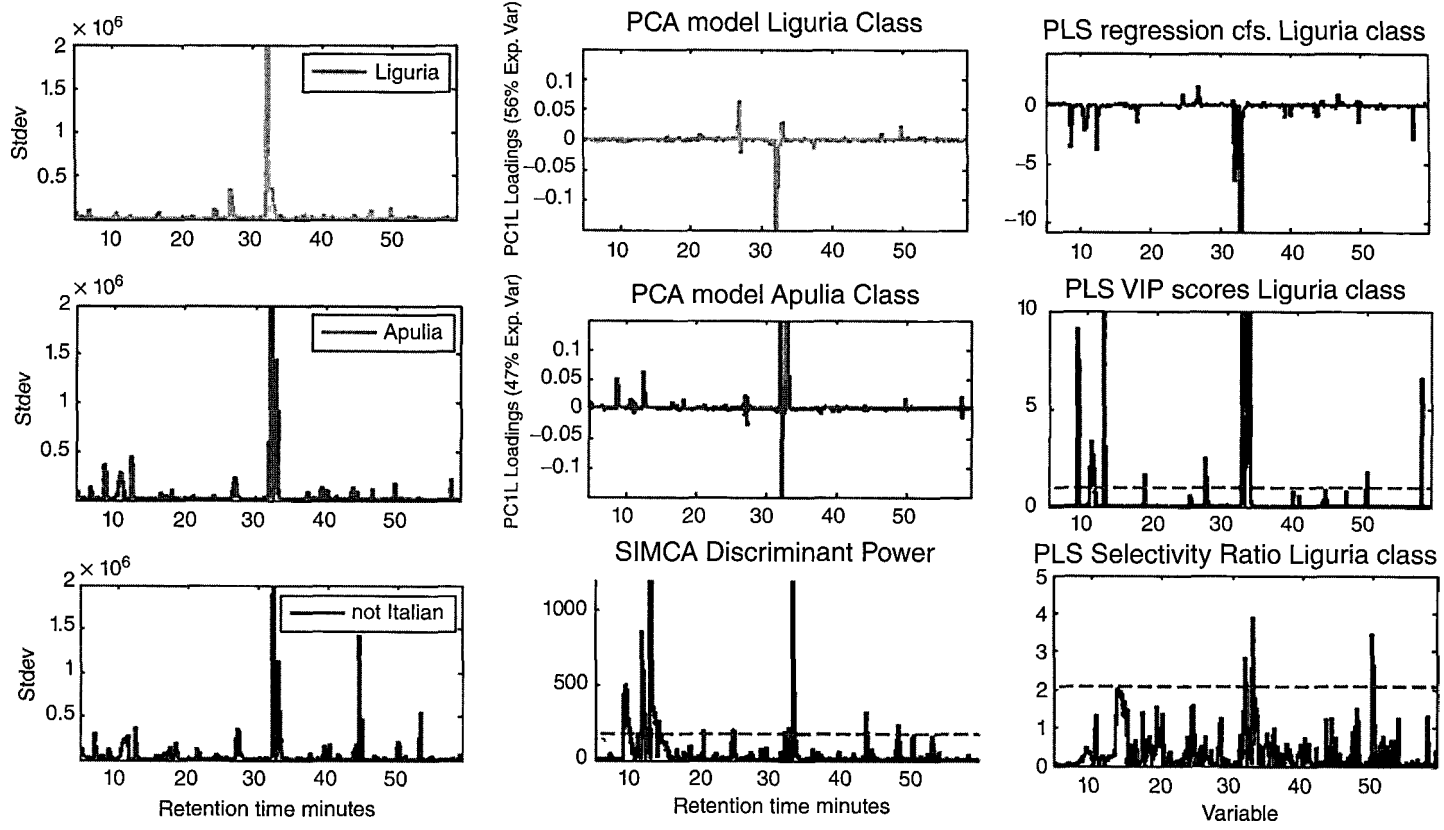


Figure 17.11 SIMCA and PLS-DA model interpretation results for the dataset EVOO. Left: standard deviation profile for each category. Middle: the SIMCA loadings plot for PC1 for the disjoint models of Liguria (top) and Apulia (middle) and classes; SIMCA discriminant power plot (bottom). Right: the regression coefficients plot for the PLS-DA model (top), the VIP score (middle), and the SR values (bottom).

multiway data arrays. Moreover, they serve as a basis for multiway classification tools as NSIMCA (Durante *et al.* 2011) and NPLS-DA (Bro *et al.* 2001), which are an extension to multiway data of SIMCA and PLS-DA.

The decomposition of a three-way data array by PARAFAC and Tucker3 is illustrated in Figure 17.12. Both methods decompose the array in three sets of vectors called loadings, which capture the source of variances pertaining to each mode of the data array and allow exploration of correlation structure and sample trends. However, they differ in their theory and formulation. PARAFAC assumes that the data array can be approximated by a low-rank tri-linear structure, that is, it can be decomposed as a sum of triple outer product of vectors (loadings). The PARAFAC components are not orthogonal or nested, but it also allows for unique models. In other words, PARAFAC is able to uniquely uncover the underlying components, for example, if data follow Beers' law, then a PARAFAC model will estimate the pure spectra even from a mixture. This explains why PARAFAC is especially suited for hyphenated techniques such as HPLC-DAD, or for excitation/emission fluorescence data. In general, PARAFAC recovers the profile corresponding to each unique phenomenon that is generating a variance source in the analyzed data, for example, a time profile if for instance the same variables are measured for the same sample at different harvesting seasons, or in food processing if food samples are measured over maturation time, ageing and so on.

When the dataset does not have a low-rank tri-linear structure, the Tucker3 model may be a suitable choice. The Tucker3 method can be considered an extension of principal component analysis to higher-order arrays. The salient feature of Tucker3 model is that it does not provide a unique solution, as in PCA, to obtain an identified solution, so orthogonality of loading components is imposed. Moreover, the number of extracted components can be different for each mode and to reproduce the original data the outer products between components is weighted by the corresponding core array elements (see Fig. 17.12).

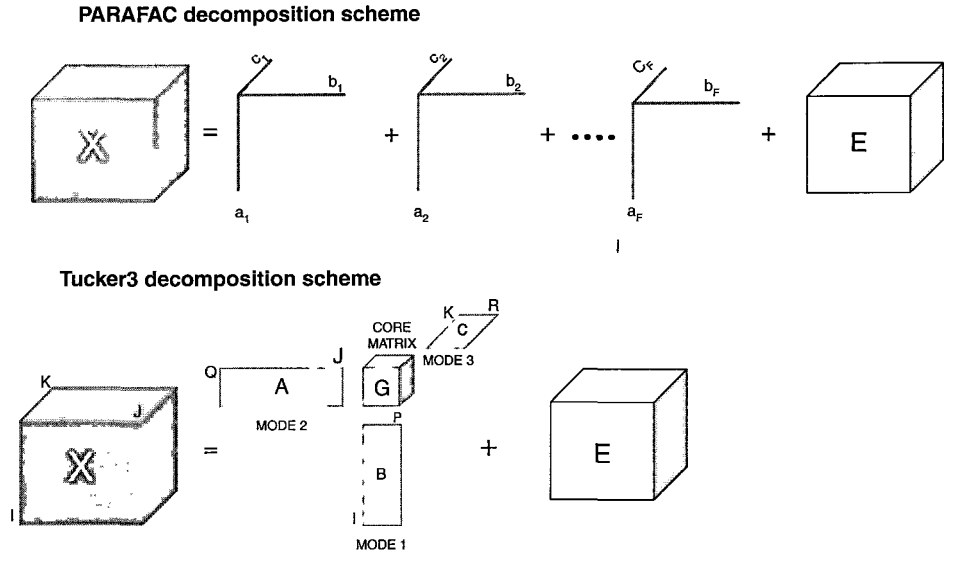


Figure 17.12 Schematic representation of PARAFAC and Tucker3 decomposition methods.

NSIMCA and NPLS-DA allow class modeling and discriminant classification, respectively, and are an extension of SIMCA and PLS-DA; a detailed description is provided Salvatore *et al.* (2013).

17.2.3.1 An Illustrative Example

Figure 17.13 illustrates the three-way nature of the dataset "dough," presented here as a case study, using a mixture of different wheat mixtures prepared in order to assess the best formulation to obtain flour for bread making (Li Vigni and Cocchi 2013). In particular, the leavening process of the dough obtained by each flour mixture was followed by near-infrared spectroscopy (NIR). PARAFAC was used as an explorative data analysis tool to assess similarities/differences among the mixtures and the leavening trends, and to highlight the most influent NIR spectral regions. A two-factor PARAFAC model shows that a common leavening trend along Factor 1 can be depicted (Fig. 17.14, Mode 3 loadings plot, top left). In fact, all samples show the same loadings sign on Mode 1 on Factor 1 (Fig. 17.14, top right), albeit separated in two groups. This trend is associated with a progressive change in the NIR signal from the initial to the final times (Fig. 17.14, Mode 2 loadings plot Factor 1, bottom left), which is in accordance with a gradual transformation of the dough. Factor 2 allows mixtures to be differentiated on the basis of the earliest leavening times (Fig. 17.14, Mode 2 loadings plot Factor 2, bottom left), which make S4 particularly different (Fig. 17.14 top right, Mode 1 loadings plot Factor 2). The two groups of mixtures, highlighted on Factor 1 Mode 1 loadings plot, show that the common leavening profile appears less pronounced for some of the samples (i.e., S1, S2, and S8) with respect to S3, S5, S7, S6, S10, and S9 mixtures. The nested-mode biplot representation (Fig. 17.14, bottom right) helps in the interpretation: each trajectory represents the development of single-mixture dough over leavening time. S2 and S1 therefore have a smaller variability of the NIR signal with the leavening time, while S5, S7, S6, S10, and S9 have a longer leavening trajectory, with S3 and S8 having a slightly different trajectory shape; finally, S4 clearly shows a different evolution.

The Mode 2 loadings plot (Fig. 17.14, bottom left) indicate that the most relevant spectral contributions can be assigned to water (1900–1950 nm region, O–H bending second overtone) and its redistribution across the macro-polymeric components of the dough, such as gluten and starch (region above 2100 nm, overtone, and combination modes contributions from the starch, protein, and lipid fractions).

17.2.4 Assessing Feature Relevance

One of the main advantages when using component models such as PCA and PLS is that both sample space (scores plot) and variables space (loadings plot) can be explored. The aim of this section is to discuss the parameters that can help model interpretation and to highlight relevant features.

17.2.4.1 PCA Models

The basic tools to assess the relevance of variables in PCA are loadings plot and congruence loadings (Lorho *et al.* 2006), which express the correlation among the original variables and the latent variables resulting from the model. To make interpretation more effective, in the graphical representation of loadings both scatter plots and line plots may be considered. In Figure 17.15 an illustrative example is presented: the results of

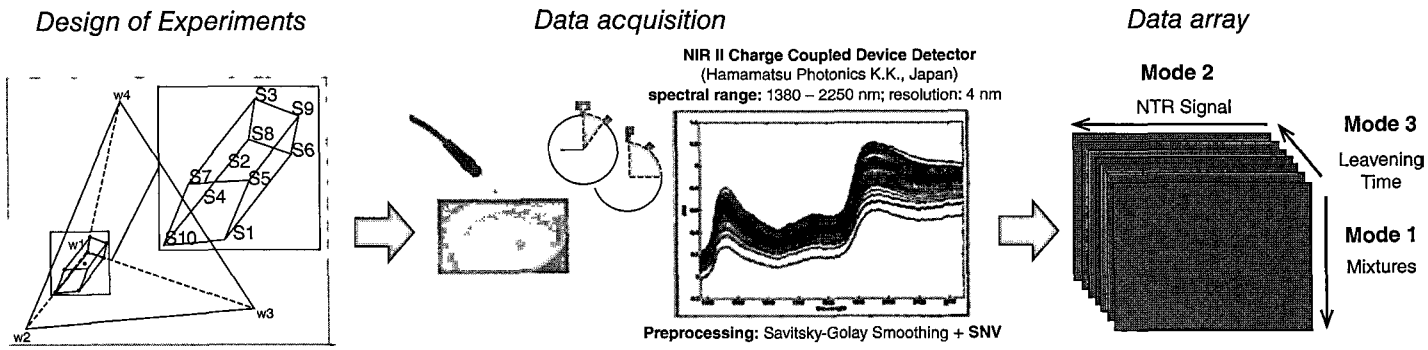


Figure 17.13 Schematic view of the data acquisition steps and data arrangement for the dataset "dough." Left: the experimental domain explored by the ten mixtures according to experiment methodology; since there are constraints on the four wheat varieties the domains is irregular (see zoom). The mixtures labels are also reported.

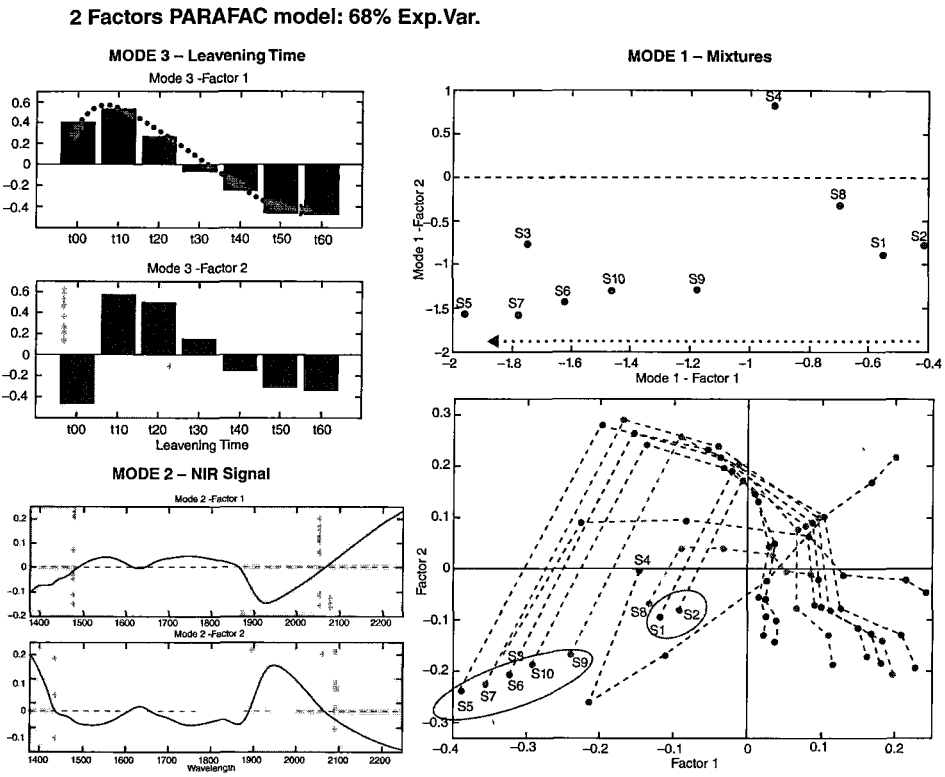


Figure 17.14 PARAFAC results for the dataset "dough." Top-left: MODE 3 loadings bar plots for Factors 1 and 2, respectively. The dotted curve on leavening time v. Factor 1 loadings plot highlights the leavening trend. The lighter shaded area, Factor 2 loadings bar plot, highlights the most relevant leavening times for this factor. Bottom-left: MODE 2 (NIR spectra) line loadings plot for Factor 1 (top) and Factor 2 (bottom), respectively. Top-right: MODE 1 scatter loadings plot, Factor 1 v. Factor 2; the labels refer to the different wheat mixtures (as in Fig. 17.13). Bottom-right: the nested modes 1 and 3 biplot Factor 1 v. Factor 2 is shown. The leavening trajectory of each mixture is represented by a dashed line and the mixtures labels are located at the initial leavening time (T0).

explorative PCA for a set consisting of two (differently aging) specialty balsamic vinegars (Silvestri 2013), namely: (1) Aceto Balsamico di Modena, ABM (industrial made, PGI denomination), with its commercial categories Fascia Alta and Fascia Bassa; and (1) Aceto Balsamico Tradizionale di Modena, ABTM (artisanal made, PDO denomination), with its two commercial categories Affinato and Extravecchio. The scores plot show that PC1 distinguishes ABM from ABTM while the different commercial categories of both products are separated along PC2, with inverse trends. The corresponding loadings profiles (shown as line plot in Fig. 17.15, bottom part) show that PC1 is inversely correlated with water content. In particular, O-H bands, such as the overtone band around 7000 cm^{-1} in the NIR region and O-H stretching above 3000 cm^{-1} in the MIR region, present negative contributions, while the spectral regions attributable to dissolved compounds (sugar, poly-alcohols, etc.), which are more concentrated when a smaller amount of water is present, show positive values (fingerprint region of MIR around 1000 cm^{-1} and combination region in NIR around 4500 cm^{-1}).

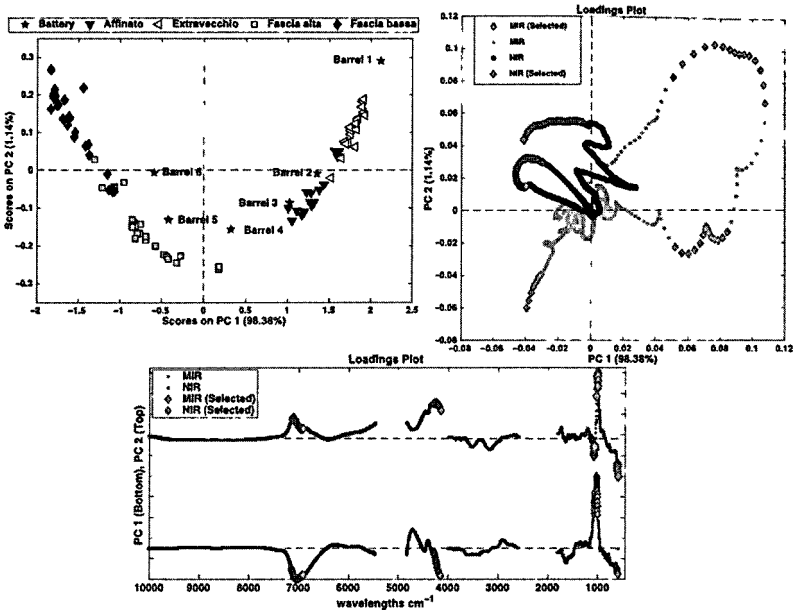


Figure 17.15 Results of PCA for the vinegars dataset. Top left: PC1 v. PC2 scores plot. Samples have different symbols according to specialty and commercial category as reported in the legend; stars are used for the projected vinegar samples from a battery of six barrels. Top right: scatter loadings plot PC1 v. PC2. NIR wavelengths: black dots; MIR wavelengths: grey dots. Grey diamonds indicate the most relevant wavelengths for categories separation. Bottom: line loadings plot for PC1 and PC2.

The behavior shown by PC2, able to distinguish with opposite trends the most valuable products within each of the two classes of vinegars, is quite difficult to interpret and in this case the scatter loadings plot (Fig. 17.15, right side) is more effective. The selected points (grey diamonds) highlight the most influential wavelengths to separate the different categories. These are also reported on PC1 and PC2 line loadings plots for ease of interpretation. Each product occupies a specific quadrant: ABTM Extravecchio (positive scores on both PCs) is mainly characterized by the MIR fingerprint region around $1000\,cm^{-1}$ where sugars and several hydroxylated compounds absorb; in contrast, ABM Fascia Alta is characterized by a lower content of these species and some species which absorb at around $600\,cm^{-1}$; the ABM Fascia Bassa (negative PC1 and positive PC2 scores) are characterized by the higher water content (region around $7000\,cm^{-1}$); and finally ABTM Affinato is characterized mainly by MIR regions around $1050\,cm^{-1}$, attributable to the fermentation product glycerol. When six ABTM samples from a "batteria" (Cocchi *et al.* 2007) were projected on this PCA model (star symbols in Fig. 17.15, left side), it emerges that the parabolic trajectory depicted by ABM and ABTM samples is followed by "batteria" samples according to their ageing; in fact, Barrel 6 indicates the less-aged sample (not marketable) while Barrel 1 indicates the most-aged, ready for market.

17.2.4.2 PLS, PLS-DA Models

In regression modeling it is important to assess the most relevant variables to predict the studied responses either for interpretative purposes (e.g., to gain a better

understanding of the underlying process that generated the data) or to operate feature selection in order to reduce noise or data redundancy. Here, we will not consider proper feature selection methods (Mehmood *et al.* 2012), that is, methods aimed at deleting a set of features and re-assessing the performance of the reduced models (these generally require extensive model validation), but we will focus on PLS/PLS-DA outputs which allow the role of descriptor variables to be interpreted and the features to be ranked according to their relevance, such as congruence loadings, variable importance in projection (VIP; Wold *et al.* 1983; Favilla *et al.* 2013), and selectivity ratio (SR; Rajalahti *et al.* 2009).

PLS (PLS-DA) models furnish the following outputs concerning variables and responses (these are also available for multiway NPLS/NPLS-DA models, that is, a loadings (weights) matrix for each variables mode and a regression coefficients array (of order equal to the number of variable modes in **X** data array for each **Y**-variable):

- **X**-loadings ($\mathbf{V}_{\mathrm{NvarX,A}}$) reflect the importance of x-variables in each component to give a low-rank approximation of the **X** data. Analogously to PCA, this may be represented as 2D or 3D scatter plots or as line/bar plots, and are useful to inspect main source of variance in **X**-block (Fig. 17.15).
- **Y**-loadings ($\mathbf{Q}_{\mathrm{NvarY,A}}$) express the importance of the individual Y-variables in each component; 2D or 3D scatter plot are used to inspect the degree of association of the y-variables. In the case of PLS-DA, **Y**-loadings help to establish which **Y**-variables are modeled by the different PLS components.
- **X**-weights ($\mathbf{W}_{\mathrm{Nvarx,A}}$) express how the information in each x-variable relate to the variation in **Y** for each component. The weights are usually normalized to 1.0, so variables with large loading weight values are important for the prediction of **Y**; the sign of the weights value reflects the type of correlation direct or inverse. As loadings, weights may be represented and inspected as scatter or line plots.
- **X**-regression coefficients ($\mathbf{B}_{\mathrm{NvarX,NvarY}}$) directly link **X**-variables to **Y**-responses, the sign reflecting the type of correlation. However, they are only independent of each other when the **X**-variables are strictly orthogonal (uncorrelated); this is not usually the case, especially with fingerprint techniques, so care should be taken in their interpretation. Moreover, if they are expressed in the original scale of x-variables a direct comparison in terms of their magnitude is not possible.

The most-used ranking parameters that can be derived by PLS models are as follows.

- VIP scores are obtained as the overall sum of all component of **X**-weights weighted by explained Y-variance for each component (SSY_a/SSY_{tot}):

$$VIP_k = \sqrt{\sum_{a=1}^{A} w_{k,a} SSY_a N_{varX} / (SSY_{tot} A)} \tag{17.13}$$

Depending on whether the explained variance for a single **y** or total **Y** variance is considered in the above equation, VIP scores are obtained for each **y** or by referring to all of them (Favilla *et al.* 2013). The VIP value is summed over all **X**-variables close to the number of **X**-variables, hence a threshold of 1 is generally accepted to assess influent variable in **Y** modeling. These are also available for NPLS/NLS-DA models (Favilla *et al.* 2013).

- SR is defined as the ratio for each x-variable of the portion of its variance explained by the predictive PLS component (or target component, calculated as a post preprocessing step), and its corresponding residual variance:

$$SR_k = \frac{\sum_{i=1}^{Nsamples} \hat{x}_{ik}^2}{\sum_{i=1}^{Nsamples} e_{ik}^2} \tag{17.14}$$

The definition of a significance threshold for SR is less straightforward; the most-used values are 3 or 5. Both parameters are reported as line plot.

Figure 17.16 depicts an example showing **X**-loadings, PLS weights, regression coefficients, VIP, and SR for a PLS model (4 LVs, 97% explained Y variance), calibrating the extract concentration in beer samples on the basis of the near-infrared/visible spectra recorded on 60 beer samples in the wavelength range 400–2250 nm (Nørgaard *et al.* 2000). By looking at the mean centered spectra (Fig. 17.16, top left) represented on a greyscale based on extract concentration, a great variability in the 400–800 nm region can be observed which is not related to extract concentration; there also exist three regions where variability is much lower (at around 1000 nm, 1200 nm, and 1300–1400 nm), but is correlated to extract concentration. As expected, **X**-loadings on the first PLS component reflect the variance spectrum profile; PLS weights for the same component, maximizing covariance, depict both this region but also the extract concentration-correlated regions. Regression coefficients are highly negative in correlated regions according to the trend observed in mean centered spectra, and also show smaller contributions

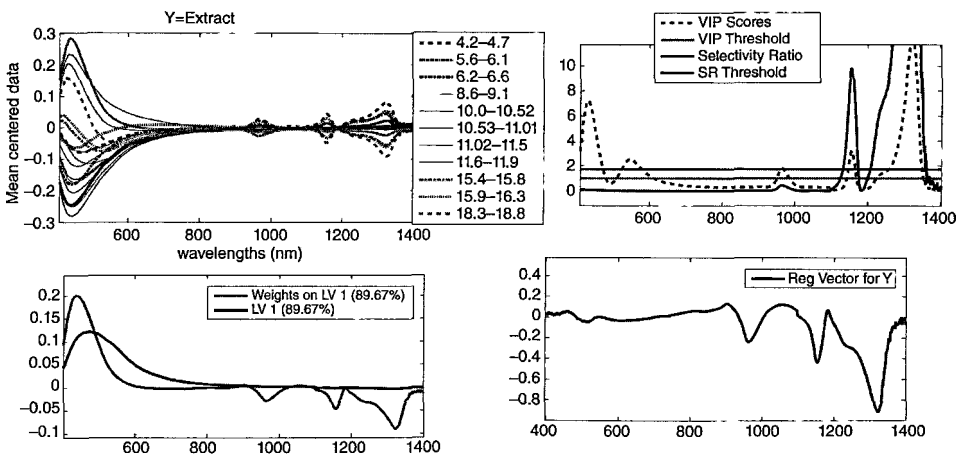


Figure 17.16 Results of a PLS regression model for predicting extract concentration in beer samples on the basis of NIR spectra. Top left: mean centered spectra shaded according to extract concentration. Bottom-left: loadings (black line) and PLS-weights (gray line) plot for first PLS component. Top right: VIP scores (dotted line) and SR values (black line) v. wavelengths with their respective threshold lines. Bottom right: PLS regression coefficients plot.

in the 400–600 nm region with alternating signs probably needed for corrections. VIP scores indicate the influential regions are those highlighted by the regression coefficients, while SR ratio focuses only on the most predictive regions (i.e., around 1200 nm and 1400 nm).

17.2.4.3 SIMCA Model

In SIMCA X-loadings are obtained for each modeled class; as there is a PCA model of each class, they carry the same information as in PCA. However, being obtained separately for each category, X-loadings are not usually so informative about the relevance of X-variables in the classification model. For this purpose, parameters have been defined that are specific to SIMCA models.

- *Modeling power* expresses how much of the original data variance, for a given variable, is described by the class models. It is defined (Wold 1976) as 1 minus the ratio of the residual variance (considering all modeled classes) to original data variance for a given variable, so that it varies between 1 (a variable which is completely relevant to the model) and 0 (a variable which has no role in modeling any of the classes).
- *Discriminant power* (Wold and Sjostrom 1977; Wold *et al.* 1983) expresses the capability of a variable to account for class separation. It is defined for each pair of classes and averaged over all pairs. It is calculated for each variable as the ratio of residual variance for all objects fitted into all classes except for the class to which they belong, and residuals variance for the same objects fitted to classes to which they belong. A value of discriminant power close to 0 indicates a poor capability of class discrimination and a value much above 1 indicates a good capability. However, the discriminant power is not upper-bounded and some criteria to define a threshold have to be adopted (Gosselin *et al.* 2010). In the example reported in Section 17.2.2.5 (Fig. 17.11), the 95° percentile was used as a threshold.

17.2.5 Data Fusion

In very general terms, data fusion is related to data integration and information retrieval from different sources of data in a synergic manner. In recent years, the interest of the scientific community in data fusion has increased (Forshed *et al.* 2007; Mechelen and Smilde 2010; Vera *et al.* 2011; Fernández *et al.* 2013; Silvestri 2013; Silvestri *et al.* 2013, 2014). Dealing with chemical information, the simultaneous investigation of a given set of samples based on a multi-platforms approach allows the complementary information to be evaluated and both sample description and correlation between the variables of different sources to be enhanced.

In food analysis (Di Anibal *et al.* 2001; Vera *et al.* 2011; Silvestri *et al.* 2013, 2014; Drivelos *et al.* 2014) the presence of interferences, uncontrolled variability due to biological, chemical, and physical transformations, and the necessity to unveil and understand the salient features of the samples able to characterize quality, authenticity, etc., renders the adoption of a data fusion (DF) strategy particularly effective. In fact, DF allows the assumptions obtained from the analysis of single techniques to be strengthened or new assumptions to be made which would otherwise remain hidden when looking at the outcome of a single analytical method.

17.2.5.1 DF Application Example

Figure 17.17 shows the analyzed data describing three varieties of PDO Lambrusco wines extensively characterized (Silvestri *et al.* 2014) by using different analytical platforms, in particular ^{1}H-NMR spectroscopy and emission-excitation fluorescence spectroscopy (EEM) as fast fingerprinting techniques and HPLC-DAD for the characterization of the phenolic compounds. Since the analytical outputs are characterized by different dimensionalities (two-dimensional ^{1}H-NMR, and three-way arrays EEM and HPLC-DAD), several multivariate analyses were applied (PCA, PARAFAC, MCR-ALS; De Juan and Tauler 2006) in order to extract and merge, in a hierarchical way, the information present in each dataset. Regarding the HPLC-DAD the information extracted consists of the integrated area of 39 compounds, resolved by multivariate curve resolution methodology, even if only six were identified. The discriminant classification results obtained by the PLS-DA data fused model are reported in Figure 17.18; according to minimum classification error in cross-validation, a five-components model gave only one miss classified in the test set (improved results with respect to using a single technique). The PLS weights plot (Fig. 17.18, bottom) allows the identification of the variables which are the most responsible for class discrimination and synergic information. These are discussed together with the loadings profiles corresponding to the variables input to the data fused model. Considering Sorbara samples (SOR), the first and second PARAFAC factors from EEM are relevant, whose profiles are reported on Figure 17.18 and are compatible with vanillic acid (EEM 1) and trans-resveratrol and *p*-coumaric acid (EEM2). Several compounds of the 39 resolved by MCR on HPLC-DAD data, such as *p*-coumaric and caffeic acids, and two unassigned compounds, belong to the w2 cluster and myrecitin elution window. The first and third components extracted from the ^{1}H-NMR data mainly yields information about the anomeric and the phenolic regions.

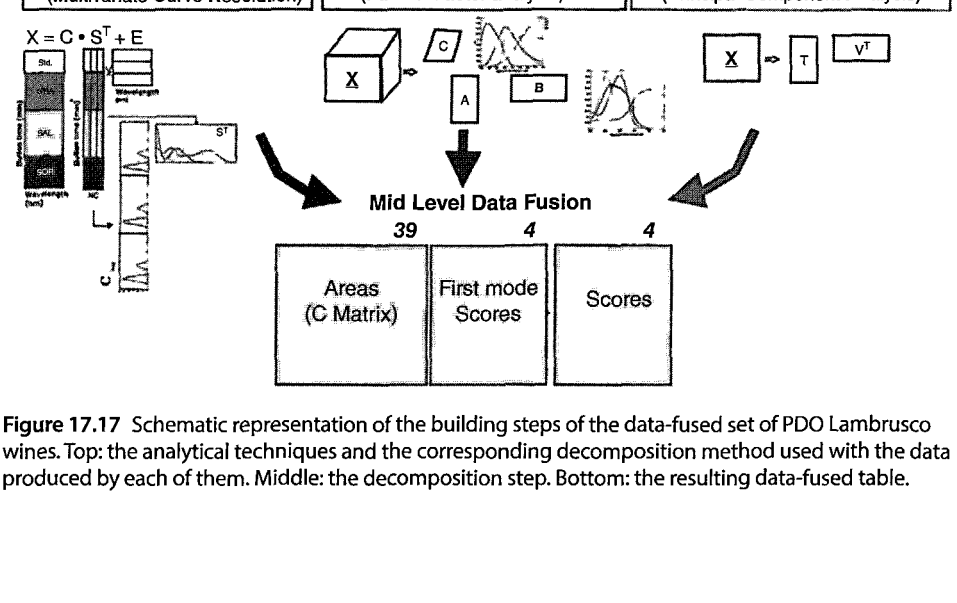


Figure 17.17 Schematic representation of the building steps of the data-fused set of PDO Lambrusco wines. Top: the analytical techniques and the corresponding decomposition method used with the data produced by each of them. Middle: the decomposition step. Bottom: the resulting data-fused table.

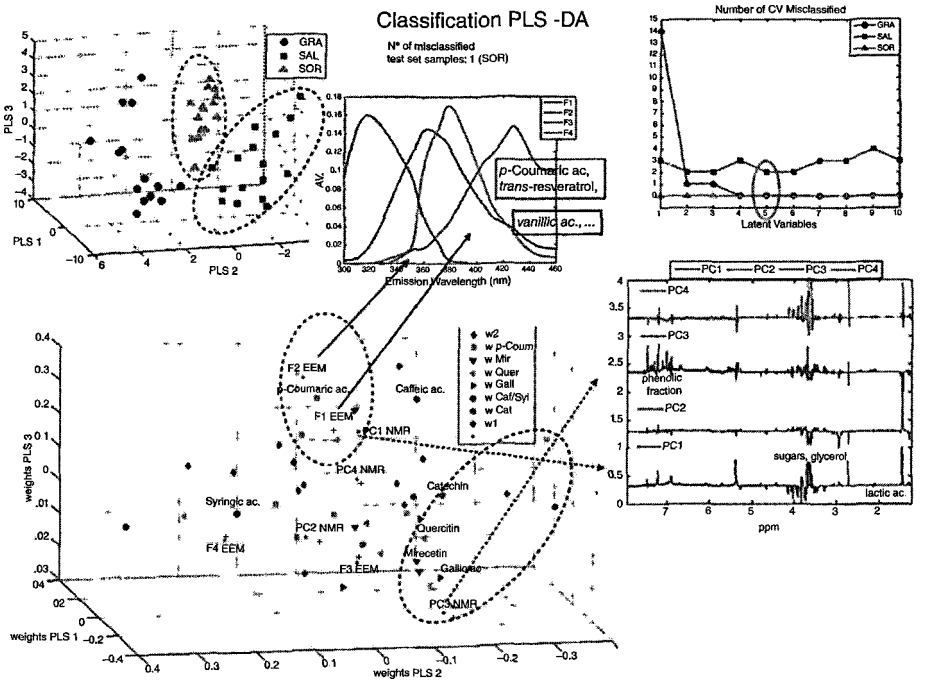


Figure 17.18 PLS-DA results on the data-fused set of PDO *Lambrusco* wines. Top left: Y scores 3D scatter plot. Top right: the classification error in cross-validation (CV) for each class v. number of PLS components. Bottom: PLS-DA X weights 3D scatter plot. The 39 areas from MCR analysis of HPLC-DAD dataset are shaded according to retention time windows, the resolved compounds are labeled by name. Black crosses refer to PARAFAC factors from the EEM dataset (each is labeled by EMM followed by the factor number, e.g., EEM1, EEM2, etc.) and PCs from PCA of NMR dataset (each is labeled by NMR and PCs number, e.g., NMR1, NMR2, etc.). The dotted ellipses highlight variables that are important for discriminating the classes whose samples lie in the corresponding position in scores plot. To recover the information that each PARAFAC factor and PC carries, the mode 3 PARAFAC loadings (EEM model) and PCA loadings (NMR model) are also reported.

A wide group of variables obtained via HPLC-DAD analysis, such as gallic acid, myrecitin, and quercytin, are relevant for the classification of Salamino samples (SAL). Grasparossa samples (GRA), on the other hand, are described mostly by the fourth PARAFAC factor from EEM data and syringic acid and other molecules obtained from the analysis of HPLC-DAD data.

17.2.6 ANN-Based Methodology

Artificial neural networks (ANN) encompass a set of mathematical methods that were initially developed as biological models mimicking the neural activity of the human brain. Framed in this context, there were practical difficulties and theoretical limitations that were overcome by the work of Hopfield (1982). Since then, ANN developed as problem-solving tools based on automatic learning. ANN are sophisticated computational tools capable of handling non-linearity in explorative analysis, discriminant classification, and regression tasks. The use of ANNs for data processing has constantly been increasing (Zupan 2003; Marini *et al.* 2008); regarding food analysis, ANN are

often employed in modeling data obtained by devices known as electronic senses, that is, instrumental techniques or sensors imitating the human senses such as electronic noses, electronic tongues, and computer vision, widely applied today in food analysis for process monitoring and determining the quality and authenticity of foods (Marini 2009; Śliwińska *et al.* 2014).

Here we only give a brief introduction to the methodology; the interested reader is referred to literature for the basics of ANN in chemometrics context (Zupan 2003) and a review of ANN applications in food science (Marini 2009). We do not describe the related methodology, support vector machine (SVM), which has also become very popular recently, but we refer to a tutorial review on this subject (Brereton and Lloyd 2010).

A final remark is that while ANN are very powerful in modeling nonlinear trends when very complex relationships are present in the analyzed data, they are less straightforward with respect to model interpretation and suboptimal in modeling less complex and close to linear phenomena; ANNs are also more prone to over-fitting and more demanding in terms of number of samples to number of variables ratio. For this reason, a good and recommended practice would be to start with simple models, linear or mildly nonlinear, and to resort to ANNs only when the performance of these models is inadequate.

17.2.6.1 A Few Hints About ANN Methodology

Mathematically, ANN are computational tools modeling functional relationships among a set of input and a corresponding set of output variables, operating through a number of connected arithmetic units called neurons (from the analogy with the human brain). Each neuron produces an output value combining the input according to a nonlinear parameterized function. The parameters can be associated with the input as a set of weights:

$$\mathbf{y} = f\left(w_o + \Sigma_i w_i x_i\right) \tag{17.15}$$

or can take part in the definition of the function itself. For example, considering a Gaussian radial basis function:

$$\mathbf{y} = \exp\left[-\sum_i \left(x_i - w_i\right)^2 / 2w_0^2\right]. \tag{17.16}$$

A network connecting each neuron then has to be designed (the network architecture). It is generally represented in a graph where nodes represent single operating units, that is, input parameters (input layer), neurons (hidden layers), and output parameters (output layer), and the weights that modulate the combination of the nonlinear functions are represented as connecting lines. A schematic representation of ANN architecture for two of the most frequently used types of ANN – multilayer feed-forward ANN (MLF) and radial basis function ANN (RBF) – is shown in Figure 17.19. Once the network has been designed, it has to be trained (learning of the network). This is done differently for MLF and RBF, but the general goal is to find the best set of weights (and the parameters defining each RBF) to reproduce the outputs. The weights are adjusted iteratively and the learning is stopped when a minimum error is reached. These ANN can perform supervised tasks such as discriminant classification; in this case the responses are dummy coded variables expressing class memberships and regression.

The main advantage over linear modeling is the increased flexibility of ANNs to handle any kind of complex nonlinear relationship in regression or nonlinear separable

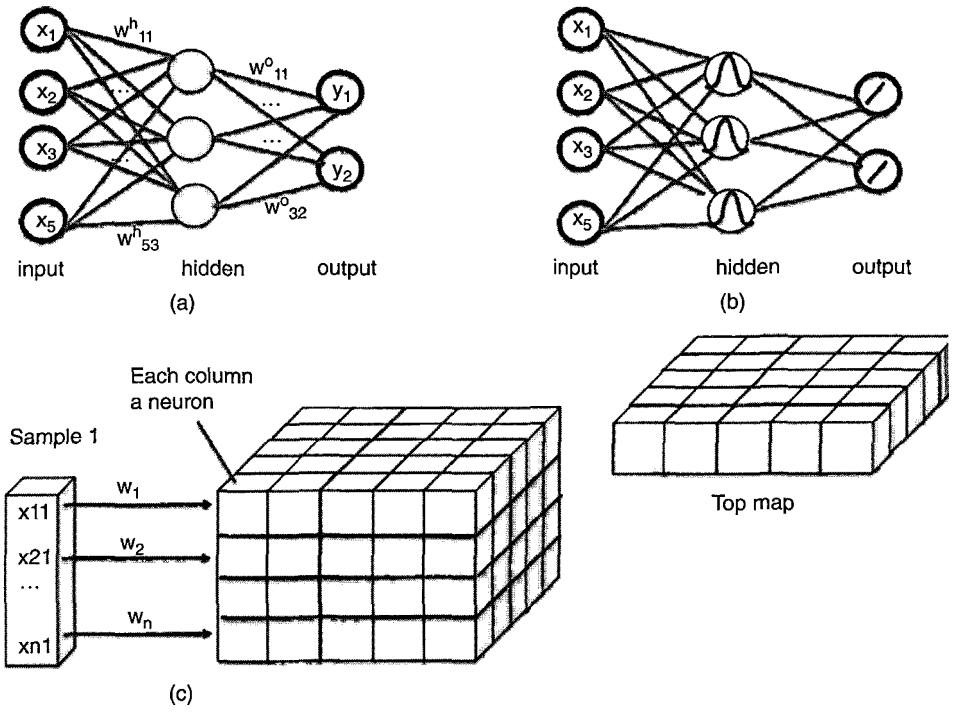


Figure 17.19 Schematic view of (a) MLF-ANN, (b) RBF-ANN, and (c) Khonen SOM.

situations in classification. On the other hand, this flexibility is achieved through the use of a huge number of adjustable parameters which render the methodology prone to over-fitting, implying that a sufficient number of samples (at least as many as the number of adjustable parameters) should be available. Moreover, the numbers of layers of nodes (or neurons) and the learning settings have to be optimized. This generally requires the use of an independent (with respect to the data used for learning of the ANN) validation set to select the best ANN model among the many tried. As a consequence, a second independent external set to evaluate the prediction capability of the chosen model is required; alternatively, a resampling procedure can be used to decide which model to select if only one independent external set is available. In both cases, extreme care has to be taken over dataset splitting and resampling scheme (Marini 2009). Most of the misuses of ANNs are due to both these critical issues, that is, too few samples with respect to network complexity and improper choice of the validation scheme.

For unsupervised tasks, such as explorative data analysis, a self-organizing Kohonen map (SOM) can be used (Kohonen 1982). This is basically a one-layer ANN trained to match the topology of the samples. A SOM map is a regular grid of neurons, generally a 2D hexagonal or rectangular grid, of a given size (the total number of nodes). Each node is associated with a vector of weights of the same dimensions as input variables. In the training phase, the high-dimensional original input is mapped to the low-dimensional SOM map by finding the node with the smallest distance between the weights vector and the sample variables vector. In practice, each node is an "archetype" sample to which all the training samples are compared. The most similar node ("winning unit")

is rotated towards the object by updating its weight vector with an average of the old values and those of the sample, weighted by a learning rate α which decreases as the training iterations progress. The update is also extended to all the other neighboring nodes of the winning unit, making neighboring units more similar than units further away. Kohonen SOM can be turned into a supervised tool by giving class membership information (discriminant classification) and using a second map of identical geometry and size where the winning unit is mapped in the same position; the weights of this map are then adjusted to minimizes the membership errors.

17.2.6.2 A Working Example

The analyzed dataset consists of 252 samples of Italian extra virgin olive oils of different cultivar and geographical provenance: north Apulia (class 1); south Apulia (class 2); Umbria (class 3); east Liguria (class 4); and west Liguria (class 5), the highest-valued product. The oils have been characterized by their fatty acids composition, quantifying seven of them. Here the advantage of a SOM approach with respect to linear methods is shown both at explorative and classification levels when determining which methods allow a better separation of the different categories. In the unsupervised context, the test samples (another 130 samples) are not intended as a validation set but to determine how future samples would project on PCA space and SOM map, respectively.

By looking at PCA scores plot for the first two components (Fig. 17.20a), it is evident that while classes 2 and 5 are linearly separable, classes 1, 3, and 4 are not. The separation did not improve when considering a higher number of PCs. A 12×12 rectangular

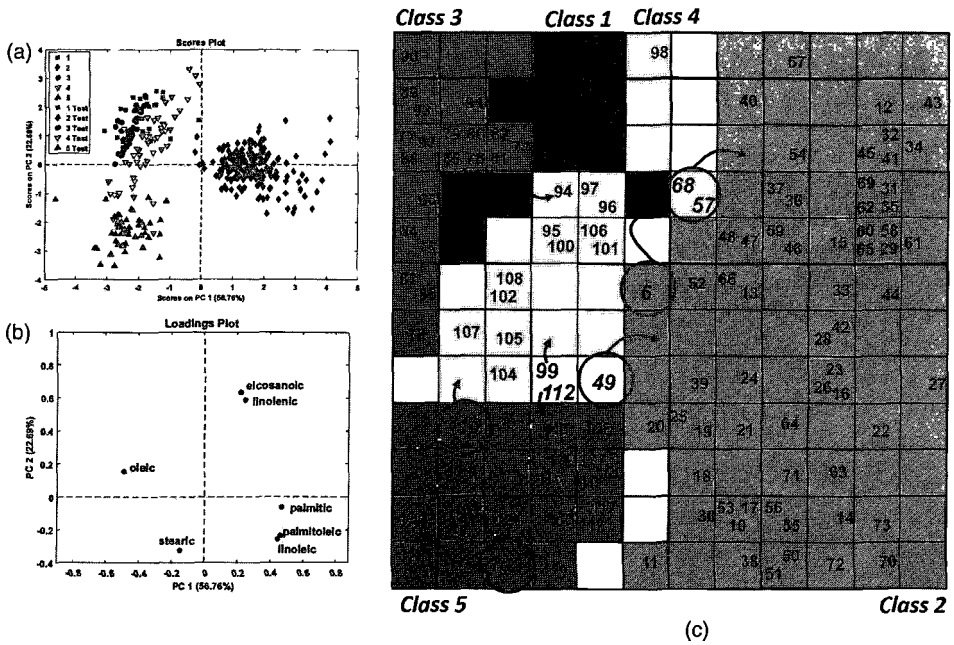


Figure 17.20 PCA and unsupervised SOM results for olive oil dataset: (a) PC1 v. PC2 scores plot; (b) PC1 v. PC2 loadings plot; and (c) SOM top map. Highlighted samples are assigned to a node belonging to no class or to a class different from their own: arrows show the category to which they actually belong.

SOM was trained for 500 iteration (epochs) with a learning rate of 0.5 (for SOM calculation, a MatLab-based routine courtesy of F. Marini, University of Roma La Sapienza, was used). The resulting map is shown in Figure 17.20b; ellipses are shaded according to the class membership of the samples assigned to this node; if there is any sample assigned to a node it is left blank. Numbers labels the test samples, shaded according to class only when mis-assigned. Classes 1, 3, and 4 still overlap but to a lesser extent; mis-assigned samples are mostly from closest-related categories such as east (class 4) and west Liguria (class 5). When considering a supervised linear discriminant classification approach by using PLS-DA (results not shown for brevity), class 2 model is good and classes 5 (it only accepted a few samples of class 4) and 3 (which accepted some samples from class 1 and 4) are satisfactory. Class 1 also accepts almost all samples of class 3, and class 4 is totally confused. In contrast, when using supervised SOM the EVOO belonging to the different classes are well discriminated, with very few faulty assignments (Fig. 17.21a). Moreover, contrary to PCA, there is proximity among south and north Apulia as well as east and west Liguria. The SOM weights can be used to evaluate the importance of each variable in class separation.

Figure 17.21b shows on a shaded intensity scale for each variable the weight values corresponding to each node of the map. It can be seen that palmitic, palmitoleic, and linoleic acids characterize mainly class 1, south Apulia (high positive weights in the nodes of the SOM corresponding to the position where samples belonging to class 1 are mapped). Class 4, west Liguria, is differentiated for a higher content of stearic and oleic acids and lower palmitic, eicosanoic, and linolenic acid content. Classes 2 and 3 show a less definite pattern and their distinction seems mainly due to the different proportion of stearic acids and different modulation of palmitic and linolenic acids. The variable role in class separation deduced from Figure 17.21b is in agreement with the PCA loadings pattern (Figure 17.20b).

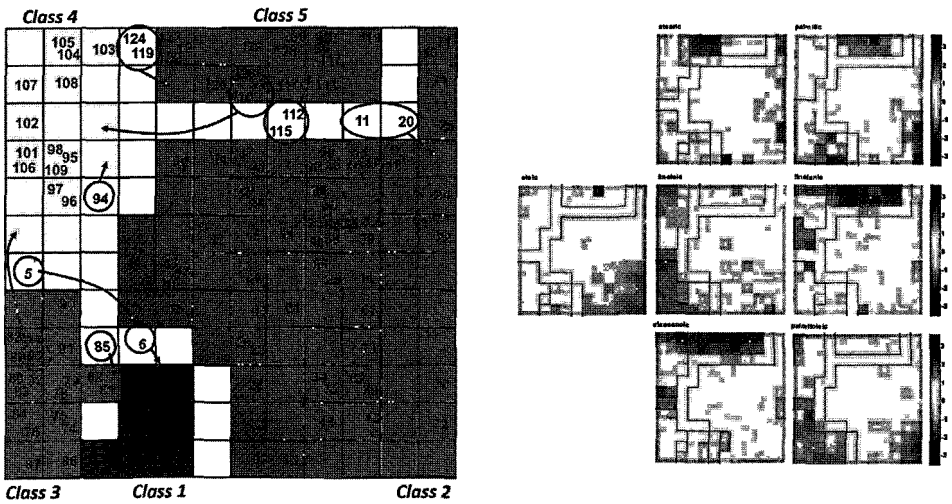


Figure 17.21 Supervised SOM results for olive oil dataset: (a) SOM top map; and (b) SOM variables weights top map. Highlighted samples are assigned to a node belonging to no class or to a class different from their own: arrows show the category to which they actually belong.

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Conclusions and Prospects

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Food authentication is the process by which a food is verified as complying with its label description, for example for its origin, geographical and genetic production method (e.g., organic farming, traditional procedures), and processing technologies (e.g., irradiation, freezing, microwave heating). Of special concern is the declaration of specific quality attributes in high-value products. Proof of provenance has become an important topic in the context of food safety, food quality, and consumer protection in accordance with national legislation and international standards and guidelines. It is of outmost importance to identify and ensure the origin of a commodity and thereby the geographical region of its production. Due to the globalization of food markets and the increased importing of products from other countries, customers over the years seem to be more and more interested in the geographical origin and the concomitant assumed quality of the products they eat and drink. From an economical and legal point of view, the quality of food and drinks and the methods which are used to control and assure it are also of great concern (Georgiou and Danezis 2015).

Authenticity has probably always been a major concern of many consumers, and it is still gaining even more importance. Since the beginning of the twentieth century, organizations that set standards for and control the origin of ingredients and the production process have therefore been established all over the world, for example the French Institut National des Appellations d'Origine (INAO), Italy's Denominazione di Origine Controllata, Spain's Denominación de Origen, South Africa's Wine of Origin or the USA's American Viticultural Areas. As the production of consumer goods according to these standardized procedures can often be rewarded with higher purchase prices, the current production of counterfeit food and illegal food trades is unfortunately a common fact. There is therefore a growing need for control organs to have access to reliable analytical methods that can give a decisive answer about the authenticity of foodstuffs (Balcaen *et al.* 2010).

In Europe, origin is one of the main authenticity issues regarding food. European Union legislation reserving specific names for foods and beverages of a particular quality or reputation has been abundant since the dawn of the European integration process. This eventually culminated in the introduction of a regulatory framework for wines and spirits and quality schemes for food products: protected denominations of origin

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(PDO); protected geographical indication (PGI); traditional specialities guaranteed (TSG); and recently optional quality terms (OQT, for either mountain products or products of island farming) (1151/2012 EU regulation). The purpose of these EU schemes is to protect the reputation of the regional foods, promote rural and agricultural activity, help producers obtain a premium price for authentic products, and eliminate the unfair competition and misleading of consumers by non-genuine products which may be of inferior quality or of different flavor.

Food authenticity evolved after the growing concern of consumers about the quality and origin of the food they eat. Scientific research programs such as Food Integrity MoniQa and Trace enlightened this subject, providing valuable new information. The European Union declared new research projects in the area of food authenticity on Horizon 2020, which is important for people working in the field.

The determination of food authenticity is an important issue in quality control and food safety. In recent years, many serious diseases have appeared related to foodstuff which have motivated the scientific community to work intensely in this area. Authenticity is a quality criterion for food and food ingredients, increasingly referred to worldwide, as a result of legislative protection of regional foods. There is therefore an imperative requirement for food authentication techniques in order to allow consumers to be aware of the food they eat, enhancing safety and quality in the food market. Industries are also seeking the opportunity to assure their food products for their authenticity and gain profit from such assurances. Finally, regulatory bodies are seeking an extended and updated list of analytical methods by which they can confirm the authenticity of products.

The book covers this very important issue – food authentication – which is interesting not only for the scientific community but also the consumers. This area is in a phase of exponential growth, attracting a lot of attention from authorities and media around the world. Food quality and safety is a hot topic and this book covers the most advanced techniques used for food authentication and also a vast number of products around the world. The reader is informed of all analytical breakthroughs in this area during the last years. The book also covers all novel techniques that have recently emerged, including all aspects of food authentication as currently recognized by the scientific community such as: undeclared processing technologies; organic food authentication; multi-contaminant screening methods; consumer perception; and authentic food market analysis.

During the last years, publications concerning analytical methods for food authentication have experienced an exponential growth, as described in Chapter 2. This growth was stimulated by the interest of consumers, regulatory bodies, and industries. Advanced chromatographic and spectroscopic techniques such as liquid-chromatography time-of-flight mass spectrometry (LC-TOF-MS) (Gilbert-López *et al.* 2014; Zhang *et al.* 2014), gas-chromatography time-of-flight mass spectrometry (GC-TOF-MS) (Indrasti *et al.* 2010; Xu *et al.* 2014), and specific natural isotope fractionation nuclear magnetic resonance (SNIF-NMR) (Thomas and Jamin 2009; Pîrnău *et al.* 2013; Bejjani *et al.* 2014; Grégrová *et al.* 2014*a*; Monakhova *et al.* 2014) have seen huge growth. Elemental (D'Archivio *et al.* 2014; Drivelos *et al.* 2014; Hopfer *et al.* 2014; Šelih *et al.* 2014; Baroni *et al.* 2015; Borges *et al.* 2016) and isotope ratio (Durante *et al.* 2014; Grégrová *et al.* 2014*b*; Hansen *et al.* 2014; Rossier *et al.* 2014; Erich *et al.* 2015; Longobardi *et al.* 2015) analysis and molecular techniques (Ortea *et al.* 2009, 2010, 2012; Pascoal *et al.* 2011, 2012; Guerreiro *et al.* 2013; Marín *et al.* 2013; Abdullah and

Rehbein 2014; Lin *et al.* 2014; Druml *et al.* 2015; Di Pinto *et al.* 2015; Ramón-Sierra *et al.* 2015; Shabani *et al.* 2015), such as genomics (Martínez *et al.* 2004; Fontanesi 2009; Coton *et al.* 2010; Primrose *et al.* 2010; Marques *et al.* 2011; Vietina *et al.* 2011; Ferguson *et al.* 2012; Ortea *et al.* 2012; Nicoloso *et al.* 2013), proteomics (Martinez and Friis 2004; Vaidyanathan *et al.* 2007; Barik *et al.* 2013; Colgrave *et al.* 2013; Gallardo *et al.* 2013; Carrera *et al.* 2014; Fraige *et al.* 2015), immuno-techniques (Asensio *et al.* 2008*a*, *b*; Asensio and Samaniego 2009; Puchades and Maquieira 2013; Santaclara *et al.* 2015), sensory analysis (Sivertsen *et al.* 1999; Kallithraka *et al.* 2001; Arvanitoyannis and Vlachos 2007; Sipos *et al.* 2012; Vallverdú-Queralt *et al.* 2013; Wei *et al.* 2014), and food microbial fingerprinting (Dufossé *et al.* 2013; Bigot *et al.* 2015) are increasingly applied for food authentication.

Non-chromatographic mass spectrometry techniques such as proton transfer reaction mass spectrometry (PTR-MS) (Maçatelli *et al.* 2009; Van Ruth *et al.* 2009; Özdestan *et al.* 2013; Tres *et al.* 2014) and matrix-assisted laser desorption/ionization (MALDI-TOF-MS) (Mazzeo *et al.* 2008; Nasi *et al.* 2008; Chambery *et al.* 2009; Wang *et al.* 2009; Cunsolo *et al.* 2013; Bonatto and Silva 2015; Petri *et al.* 2015) are plausible methods of authenticity verification.

Mass spectrometry (MS) techniques provide the base for most of the publications that deal with authentication. This is due to unsurpassed advantages such as high sensitivity, selectivity, throughput, and multi-analyte capabilities of MS techniques. Almost all publications describe how an increase in descriptors, for example elements, δ isotope ratios, and organic compounds by non-target analysis, increase the chances of success and enhance classification models. Finally, the indispensable role of chemometrics and bioinformatics in the utilization of data obtained through analytical techniques should be noted (Borràs *et al.* 2015).

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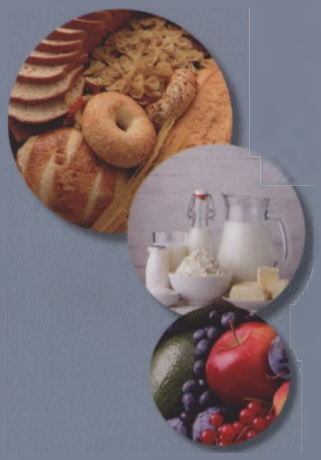
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FOOD AUTHENTICATION

Management, Analysis and Regulation



Edited by CONSTANTINOS A. GEORGIOU
and GEORGIOS P. DANEZIS

The determination of food authenticity is a vital component of quality control. Its importance has been highlighted in recent years by high-profile cases in the global supply chain such as the European horsemeat and the Chinese melamine scandals, the latter of which led to six fatalities and the hospitalization of thousands of infants. As well as being a safety concern, authenticity is also a quality criterion for food and food ingredients. Consumers and retailers demand that the products they purchase and sell are what they purport to be.

This book covers the most advanced techniques used for the authentication of a vast number of products around the world. The reader will be informed about the latest pertinent analytical techniques. Chapters focus on the novel techniques and markers that have emerged in recent years. An introductory section presents the concepts of food authentication, while the second section examines in detail the analytical techniques for the detection of fraud relating to geographical, botanical, species, and processing origin and production methods of food materials and ingredients. Finally, the third section looks at consumer attitudes towards food authenticity, the application of bioinformatics to this field, and the Editor's conclusions and future outlook.

Beyond being a reference for researchers working in food authentication, this book will serve as an essential resource for analytical scientists interested in the field and food scientists aiming to appreciate analytical approaches. This book will be a companion to under- and postgraduate students in their studies in food authentication, and will be useful to researchers in universities and research institutions.

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