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A Comprehensive Review on the Main Honey Authentication Issues: Production and Origin

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Abstract: Honey is a highly consumed natural product, not only for its taste and nutritional value, but also for its health benefits. Owing to characteristics that are essentially or exclusively related to the specific region or particular local environment and flora, honey can be classified as a premium product generally perceived as a high-quality and valued product because of its desirable flavor and taste. Consequently, honey has been a target of adulteration through inappropriate/fraudulent production practices and mislabeling origin. Globally, authentication of honey covers 2 main aspects: the production, with main issues related to sugar syrup addition, filtration, thermal treatment, and water content; and the labeled origin (geographical and/or botanical) and "organic" provenance. This review addresses all those issues, focusing on the approaches to detect the different types of honey adulteration. Due to the complex nature of honey and to the different types of adulteration, its authentication has been challenging and prompted the development of several advanced analytical approaches. Therefore, an updated, critical, and extensive overview on the current and advanced analytical methods targeting markers of adulteration/authenticity, including nontarget fingerprint approaches will be provided. The most recent advances on molecular, chromatographic, and spectroscopic methodologies will be described, emphasizing their pros and cons for the identification of botanical and geographical origins.

Keywords: analysis, authenticity, botanical and geographical origins, honey, production

Introduction

According to the standards of *Codex alimentarius* (FAO 2001), "honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature." The definition of honey under the European Union (EU) legislation is very similar, with the difference that it stipulates the bee species as being *Apis mellifera* (Directive 2014/63/EU).

Honey is a widely consumed natural product, not only desirable for its taste and nutritional value, but also for its health benefits. Since ancient times, honey consumption is generally associated with its medicinal properties, being traditionally used for healing wounds and burns and for the treatment of colds and sore throats. More recently, several studies have associated honey with many other medicinal effects, as it was shown to have antibacterial, hepatoprotective, hypoglycemic, antihypertensive, gastroprotective, antifungal, anti-inflammatory, and antioxidant effects (Frans and others 2001; Akbulut and others 2009; Gomes and others 2010; Erejuwa and others 2012). Regarding its nutritional value, honey is essentially composed of water and sugars (mainly fructose and glucose). However, other different valuable substances, such as vitamins, minerals, enzymes, flavors, free amino acids, and numerous volatile compounds, are present as minor components (Schievano and others 2013). These compounds, which can vary among different honeys due to factors such as the botanical origin, geographic area, season, technology used for honey extraction, and storage conditions, are responsible for conferring specific/individual organoleptic and nutritional properties to honey. Even being present at small amounts, they are reported to represent a fingerprint and, therefore, are used to differentiate honeys by botanical and geographical origins, as well as to define their quality (Schievano and others 2013).

According to Article (2) of Directive 2001/110/EC, the country or countries of origin where the honey is harvested have to be declared on the label and, in the case of more than 1 member state or third country, the origin of the honey has to be declared as a "blend of EC honeys," "blend of non-EC honeys," or "blend of EC and non-EC honeys." Depending on the botanical origin, honey can be classified as monofloral or multifloral, if arising predominantly from a single or from several plant species, respectively. In the last few decades, owing to its more appreciated flavor and aroma, and also to particular pharmacological attributes, there has been higher consumer demand for monofloral honey, thus increasing its commercial value (Pires and others 2009). Honey can also be classified according to its geographical origin, with particular types being from specific areas within the EU, bearing the

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labels of Protected Designation of Origin (PDO) and Protected Geographical Identification (PGI) labels, processed and prepared in given geographical areas using recognized expertise (Regulation (EEC) No. 2081/92). As happens with other products, PDO and PGI honeys generally present characteristics that are essentially or exclusively related to the specific region or particular local environment with inherent natural and human factors (Regulation (EEC) No. 2081/92). Currently, there are 23 PDO and 8 PGI honeys registered in the EU, with Portugal the country with the highest number (9 PDO honeys), followed by Spain (5 PDO and 1 PGI honeys) and France (2 PDO and 3 PGI honeys) (Table 1) (European Commission 2017).

Owing to its refined and unique flavor and taste, monofloral, PDO, and PGI honeys are generally perceived as high-quality products and, consequently, the most susceptible to be adulterated through incorrect labeling and fraudulent admixing with lower-cost and low-quality honeys. Moreover, the increased population concerns with health and well-being and the consequent growing demand for "natural" food products with therapeutic and healing properties has contributed to increase their economic value, making them vulnerable adulteration targets. Therefore, honey has been associated with fraudulent practices for a long time (White 2000). During the last several decades, documents have been developed aiming at proposing tools to assess the quality and authenticity of honey, protecting consumers, and promoting fair competition among producers. The quality of honey is mainly determined by its sensory, chemical, physical, and microbiological characteristics (Khalil and others 2012), with the required composition criteria (sugar content, moisture content, water-insoluble content, electrical conductivity, free acid, diastase activity, and hydroxymethylfurfural [HMF] content) being described in standards and legislation (FAO 2001; Directive 2001/110/EC). On the other hand, the authenticity of honey is more difficult to assess since several types of adulterations have already been described for this matrix.

The main concerns related to honey authenticity have been focused on the geographical and botanical origins, but the addition of other nonallowed substances, such as syrups or sugars, is also an important issue. Adulteration practices, such as overfeeding the bees with sucrose or other sugars, harvesting prior to maturity, and the overuse of veterinary drugs, are still performed by some producers around the globe to answer to the demands of a competitive market (Bogdanov and others 2004; Sahinler and others 2004; Guler and others 2007). In a global way, the authentication of honey encompasses mainly 2 different aspects that should be both considered: production and origin (Figure 1). Regarding honey production, the main authenticity issues, namely processing, addition of sugars/syrups, and water content, will be particularly described. Additionally, the claims of organic, botanical, and geographical origins, mostly associated with high-quality honeys, will be addressed, focusing on the chemical parameters, melissopalynology, and the most advanced DNA-based methods and spectroscopic approaches for honey authentication.

Production

Honeybees are the keystone in honey production to obtain the final product available in commercial display. The intervention of the beekeeper is also of utmost importance to maintain the honeybee colonies, to collect and to transport the honey from the hives, and to proceed with its extraction and packaging. Within all this, there are several potential sources of honey contamination and adulteration that should be considered. Those are originated from the contact of the bees with contaminated water, air, and plants (such as the case of contamination with pesticides, heavy metals, microorganisms, and genetically modified organisms [GMO]), and from the adulterations associated with processing, packaging, and inadequate beekeeping practices, such as overheating, feeding the bees during honey production, the addition of different sugars and/or syrups after production, and mislabeling. In the following subsections, adulteration and quality issues regarding honey production, namely addition of sugars, thermal processing, and water content, will be described, together with the parameters and tools used for their evaluation.

Addition of sugars

Sugars are the main components of honey, comprised of mainly glucose and fructose, but also several minor oligosaccharides. Thus, adulteration by the addition of carbohydrate materials is a type of fraud that should be carefully considered as it would be, by principle, difficult to detect if the added sugars are tailored to mimic those naturally existing in honey. The presence of sugars as adulterants in honeys can be related to the direct addition of syrups, at certain ratios after production, to increase honey sweetness or to overfeed the bees during the main nectar period in order to recover more honey from hives. Inexpensive sugars or industrial syrups are generally used for this purpose, with well-known adulterants being sugar syrups, such as corn syrup (CS) and high-fructose corn syrup (HFCS), glucose syrup (GS), sucrose syrup, inverted syrup (IS), or high-fructose inulin syrup (HFIS), which are produced from sugar cane or sugar beet (Anklam 1998; Guler and others 2007; Tosun 2013).

Honeys adulterated by sugar addition can present, in fact, changes in some chemical and/or biochemical parameters, such as enzymatic activity, electrical conductivity, and contents of specific compounds (HMF, glucose, fructose, sucrose, maltose, isomaltose, proline, ash) when compared to a control. However, when evaluating a blind sample, normal variation of these parameters should be considered when interpreting results. Moreover, some chemical parameters, such as HMF content, formerly suggested as a test to detect the addition of IS, may be ambiguous because HMF and enzymatic activity vary in different honeys and can spontaneously change in honeys when subjected to heat or abusive storage in warm environments (Ajlouni and Sujirapinyokul 2010). Sugar (sucrose, fructose, and glucose) content and other biochemical properties, such as proline content and electrical conductivity, were determined by Guler and others (2007) aiming at discriminating pure blossom honey from those adulterated by overfeeding the bees with sucrose syrup (SS). The results showed that sugar content could not be used to discriminate adulterated honeys because more than 95% of the sucrose given to overfeed the bees was converted to fructose and glucose. On the contrary, the authors reported that proline content and electrical conductivity could be satisfactorily used for that purpose. However, it should be mentioned that, in this study, honey samples were taken from just 5 bee colonies and the results should be further confirmed by data from other studies.

Stable carbon isotope ratio analysis (SCIRA), expressed as $^{13}C/^{12}C = \delta^{13}C$ (‰), has been proposed for a long time to detect this type of adulteration (Martín and others 1998; Elflein and Raezke 2008; Simsek and others 2012; Tosun 2013; Luo and others 2016; Silva and others 2016) and has been used as the official analytical method in many countries (AOAC 2005; Xue and others 2013). The principle of this method is based on the fact that monocotyledonous (C4) and dicotyledonous (C3) plants have distinct carbon isotope ratios that are produced by different

Table 1–Protected designation of origin (PDO) and protected geographical indication (PGI) honeys registered in European Commission, Agriculture and Rural Development DOOR in 2017 (European Commission 2017)

Honey	Classification	Country	Region	Botanical origin	Registration year
Mel de Barroso	PDO	Portugal	Boticas, Chaves, Montalegre, Vila Pouca de Aguiar, Murça	Monofloral from heather	2005
Mel dos Açores	PDO		Azores islands	Multifloral Monofloral from incenso (*Pittosporum ondulattum*)	1996
Mel do Alentejo	PDO		Different regions of Évora, Beja and Portalegre districts	Monofloral from lavender Monofloral from eucalyptus Monofloral from orange blossom Multifloral	1996
Mel do Parque de Montezinho	PDO		Bragança and Vinhais	Multiflower	1996
Mel do Ribatejo Norte	PDO		Alcanena, Ourém, Ferreira do Zêzere, Tomar, Vila Nova de Barquinha, Torres Novas	Multifloral	1996
Mel da Serra da Lousã	PDO		Different regions of Coimbra and Leiria districts	Multifloral Monofloral from heather	1996
Mel da Serra de Monchique	PDO		Monchique, Aljezur, Lagos, Portimão and Silves	Multifloral	1996
Mel das Terras Altas do Minho	PDO		Different regions of Viana do Castelo, Braga, Vila Real, Porto and Aveiro districts	Monofloral from heather Multifloral	1996
Mel da Terra Quente	PDO		Mirandela, Vila Flor, Moncorvo, Freixo de Espada à Cinta, Mogadouro, Alfândega da Fé, Macedo de Cavaleiros, Carrazeda de Anciães, Vila Nova de Foz Côa, Valpaços	Monofloral from lavender Multifloral	1996
Miel Villuercas-Ibores	PDO	Spain	Province of Cáceres	Monofloral from chestnut Monofloral from retama (*Retama sphaerocarpa*) Multifloral Honeydew	2017
Miel de Liébana	PDO		Cabezón de Liébana, Camaleño, Cillorigo de Liébana, Pesaguero, Potes,Tresviso, Vega de Liébana	Honeydew Monofloral from heather	2016
Miel de Tenerife	PDO		Tenerife Island	Monofloral from "Retama del Teide" (*Spartocytisus supranubius*) Monofloral from "Tajinaste" (*Echium* spp.) Monofloral from Avocado (*Persea americana*) Monofloral from Chestnut Monofloral from Heather Monofloral from Shortpod mustard (*Hirstfeldia incana*) Monofloral from Common ice plant (*Mesembryanthemum crystallinum*) Monofloral from Fennel (*Foeniculum vulgare*) Monofloral from "Poleo" (*Bystropogon Origanifolius*) Monofloral from "Pitera" *(Agave americana*) Monofloral from Malpica (*Carlina xeranthemoides*) Monofloral from Arabian Pea (*Aspalthium bituminosum*) Honeydew Multifloral	2014

(*Continued*)

Table 1–Continued.

Honey	Classification	Country	Region	Botanical origin	Registration year
Miel de Granada	PDO		Province of Granada	Monofloral from chestnut Monofloral from rosemary Monofloral from thymus Monofloral from avocado Monofloral from orange blossom (*Citrus* sp.) Monofloral from french lavender (*Lavandula stoechas*) Multifloral	2005
Miel de La Alcarria	PDO		Province of Guadalajara and Province of Cuenca.	Monofloral from rosemary Monofloral from spike lavender (*Lavandula latifolia* Medicus) Multifloral	1996
Miel de Galicia; Mel de Galicia	PGI		Autonomous Community of Galicia	Multifloral Monofloral from eucalyptus (*Eucaliptus* sp.) Maonofloral from chestnut (*Castanea* sp.) Multifloral from blackberry (*Rubus* sp.) Monofloral from heather (*Erica* sp.)	2007
Miele della Lunigiana	PDO	Italy	Province of Massa Carrara	Monofloral from acacia Monofloral from chestnut	2004
Miele Varesino	PDO		Province of Varese	Monofloral from acacia (*Robinia pseudoacacia* L.)	2014
Miele delle Dolomiti Bellunesi	PDO		Province of Belluno	Multifloral Monofloral from acacia Monofloral from lime (*Tilia* spp.) Monofloral from chestnut Monofloral from rhododendron (*Rhododendron* spp.) Monofloral from dandelion (*Taraxacum* spp.)	2011
Miel de Corse; Mele di Corsica	PDO	France	Corsica Island	Honeydew from Maquis Monofloral from chestnut Multifloral	2000
Miel de sapin des Vosges	PDO		Departments of Meurthe-et-Moselle, Moselle, Haute-Saône and Belfort Territory	Honeydew	2005
Miel d'Alsace	PGI		Alsace: Alsatian slopes of the Vosges and Jura mountain ranges for silver fir honey; hills south of the Vosges (Lower Rhine and Upper Rhine in the forests of Brumath and Haguenau) for chestnut honey; forests of Hardt (Upper Rhine) for Lime honey.	Silver fir Chestnut Lime Acacia Honeydew Multifloral	2005
Miel de Provence	PGI		Six departments of the Provence – Alpes – Côte-d'Azur region, with the exception of the some communes and cantons from the departments of Alpes-de Haute-Provence, Hautes-Alpes and Alpes-Maritimes. Drôme Provençale area and the eastern part of Gard.	Multifloral Monofloral	2005
Miel des Cevénnes	PGI		Departments of Ardèche, Aveyron, Gard and Lozère (mountain range which borders the Massif Central to the south-east)	Multifloral Monofloral from heather (*Erica arborea, Erica cinerea, Calluna vulgaris*) Monofloral from chestnut Monofloral from raspberry Monofloral from blackberry	2015

(*Continued*)

Table 1–Continued.

Honey	Classification	Country	Region	Botanical origin	Registration year
Kočevski gozdni med	PDO	Slovenia	Kočevje, Kostel, Osilnica, Loški Potok, Sodražica in Ribnica ter delno občine Črnomelj, Semič, Dolenjske Toplice, Žužemberk, Velike Lašče in Dobrepolje	Honeydew Spruce Fir Lime	2011
Kraški med	PDO		Karst area	Honeydew from forest Multifloral from blossom Acacia (*Robinia pseudoacacia*) Lime (*Tilia* sp.) Chestnut (*Castanea sativan*) St Lucie cherry (*Prunus mahaleb*) Cherry (*Prunus avium*) Winter savoury (*Satureja montana*)	2013
Slovenski med	PGI		Slovenia	Acacia (*Robinia pseudoacacia*) Lime (*Tilia* spp.) Chestnut (*Castanea sativan*) Fir (*mainly honeydew*) Spruce (*mainly honeydew*) Multifloral (pollen of fruit trees, *Castanea sativa, Acer* sp., *Trifolium repens and plants of the Asteraceae* family) Honeydew from forest	2013
Miel - Marque nationale du Grand-Duché de Luxembourg	PDO	Luxembourg	Grand Duchy of Luxembourg	Multifloral	2000
Miód z Sejneńszczyny / Łoźd/ Seinų/Lazdijų krašto medus	PDO	Poland Lithuania	Poland: 4 municipalities in Sejny county and 5 municipalities in Suwałki. Lithuania: twelve civil parishes in the Lazdijai District municipality.	Multifloral	2012
Podkarpacki miód spadziowy	PDO	Poland	17 State forest districts (Rymanów, Komańcza, Lesko, Baligród, Cisna, Wetlina, Stuposiany, Lutowiska, Brzegi Dolne, Strzyżów, Bircza, Dukla, Brzozów, Dynów, Kańczuga, Radymno, Krasiczyn) and Bieszczadzki Park Narodowy and Magurski Park Narodowy	Honeydew from European silver fir (*Abies alba*)	2010
Miód wrzosowy z Borów Dolnośląskich	PGI		Boundaries of the area in which nutrient heather stands	Heatther (Ericaceae)	2008
Miód kurpiowski	PGI		Kurpie	Multifloral	2010
Miód drahimski	PGI		Municipalities of Czaplinek, Wierzchowo, Barwice, Borne Sulinowo and in the Borne Sulinowo Forest District, located in the Drawa Lake District.	Buckwheat (*Fagopyrum*) Heather (*Calluna vulgaris*) Colza (*Brassica napus* var. *arvensis* Lime (*Tilia* spp.) Multifloral	2011

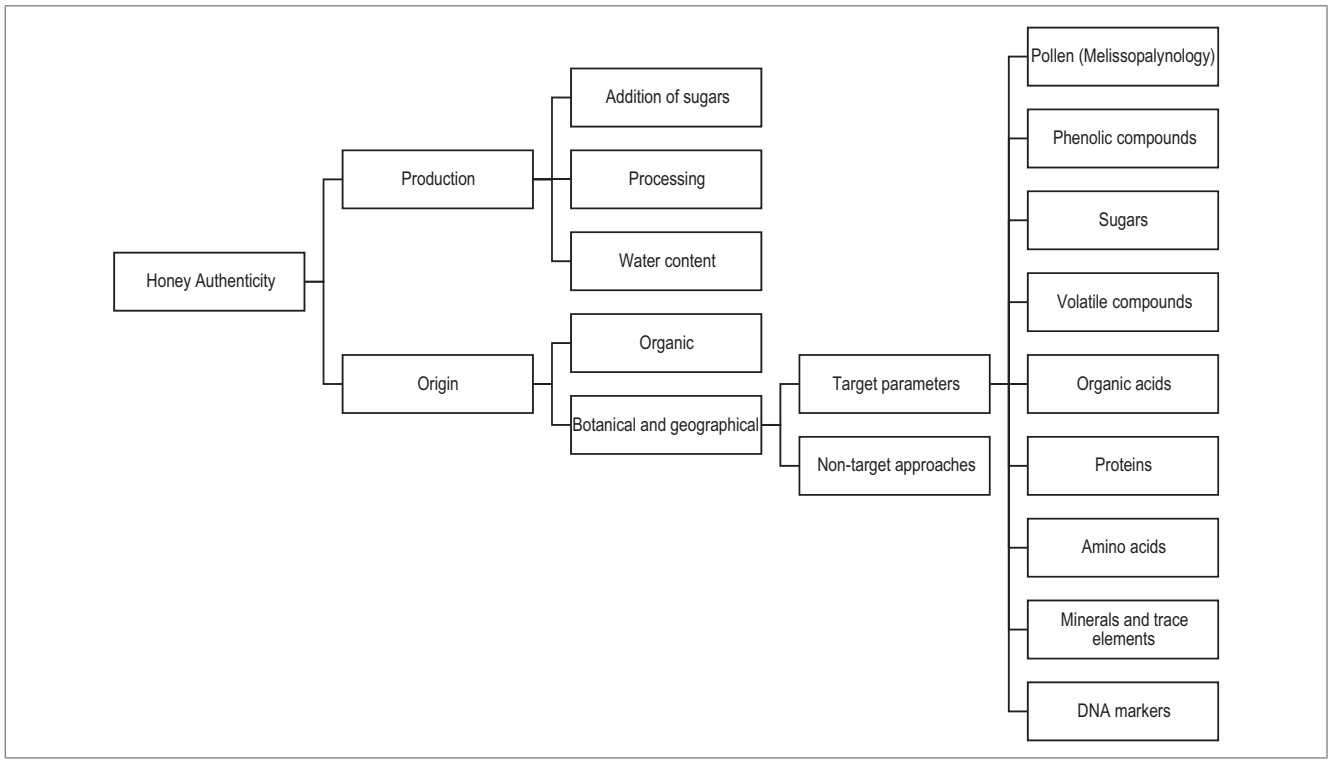

Figure 1–Schematic representation of the main issues related to honey authentication.

photosynthetic cycles (Cengiz and others 2014). C3 plant species use the Calvin and Benson cycle yielding ^{13}C values near −25‰ (generally presenting a $^{13}C/^{12}C$ ratio ranging from 23‰ to 28‰), while C4 plants mainly use the Hatch-Slack cycle, thus yielding ^{13}C values near −10‰ (generally presenting a carbon isotope ratio ranging from 9‰ to 15‰) (Anklam 1998; Simsek and others 2012; Guler and others 2014). Considering that most plants used as nectar sources the honeybees are C3 plants, and both cane and corn, the 2 main sources of industrial sugar syrups are C4 plants, authentic honeys are expected to have the characteristic properties of C3 plants, rather than those of C4 plants. Consequently, honey samples having $\delta^{13}C$ less negative than −23.5‰ could be considered as suspicious (Simsek and others 2012). However, as the $\delta^{13}C$ values for themselves may not be sufficient to detect honey adulteration with C4 sugar syrups, the carbon isotope ratio should also be determined in the honey proteins, which are used as internal standard. For authentic honeys, a mean difference of +0.1‰ (range: +1.1 to −0.9‰) has been measured with more negative differences, indicating the addition of C4 plant sugars (Anklam 1998). Thus, there is general agreement that in pure honeys the difference between the $\delta^{13}C$ values of the honey and its protein should not exceed 1‰, in addition to the $\delta^{13}C$ of honey that should be more negative than −23.5‰ (Simsek and others 2012; Cengiz and others 2014; Guler and others 2014). Nevertheless, although SCIRA has proved to be useful in detecting honey adulteration with sugar cane or corn syrups, the addition of C3 plant sugars, such as sugar beet, cannot be evidenced by this method. Tosun (2013) applied SCIRA in honey samples prepared with different known amounts of HFCS, GS, and SS from sugar beet. The results showed that, while adulteration using HFCS and GS (both C4 sugar syrups) could be detected to a certain extent, the same was not feasible in the case of SS produced from sugar beet (C3 metabolic pathway plant).

The use of SCIRA has also been proposed to evaluate indirect adulteration of honey carried out by excessive supplementary feeding the bee colonies. Guler and others (2014) used SCIRA to detect adulterated honey produced by honeybee colonies fed with different levels of commercial industrial sugar (C3 and C4 plants) syrups. SCIRA showed to be useful in detecting the indirect adulteration of honey by overfeeding bees with C4 (corn or sugar cane) sugar syrups to a certain extent, while failing in detecting adulteration using C3 sugar syrups (beet sugar) due to the similarities in isotopic composition. A new approach to determine $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$ values of honey, and its extracted protein, individual sugar (sucrose, glucose, and fructose) ^{13}C isotope ratios, sucrose, and reducing sugar contents of 800 commercial honey samples was proposed using an elemental analyzer and liquid chromatography coupled to isotope ratio mass spectrometry (IRMS). The approach allowed to reliably detect honey with a C4 sugar content of <0%, providing effective evidence of honey authenticity (Dong and others 2016).

Recently, alternative chromatographic approaches have been used to analyze sugars in honey, namely liquid chromatography coupled to a refractometry detector, anion exchange chromatography coupled to pulse amperometric detection (HPAEC-PAD), and gas chromatography coupled to a flame-ionization detector (GC-FID) or mass spectrometry (GC-MS). Even though major sugars are considered not suitable as authentication markers of honey, some of the studies showed the presence of disaccharides and small amounts of trisaccharides and tetrasaccharides in honey, but also demonstrated the nonexistence of oligosaccharides with high a degree of polymerization (DP). Considering that several sugar syrups are obtained by enzymatic hydrolysis of starch, they can contain a large amount of oligosaccharides with high DP (Megherbi and others 2009; Wang and others 2015). Therefore, oligosaccharides with high DP have been suggested as indicators to

detect honey adulteration with starch syrups. Morales and others (2008) evaluated the profile of high-molecular-weight oligosaccharides in genuine honeys and in 9 sugar syrups, including CS and HFCS with different degrees of isomerization, and they found it possible to detect honey adulteration with CS down to 5%. Megherbi and others (2009) used HPAEC-PAD to establish trace polysaccharide fingerprints in order to detect adulterated honeys with CS. The combination of reversed-phase solid-phase extraction and HPAEC-PAD revealed the presence of polysaccharides (DP11 to DP17) coming from corn syrup that can be used as markers to detect adulteration down to the 1% level (Megherbi and others 2009). However, this method does not allow to produce accurate quantitative results due to the unavailability of pure standard oligosaccharides with DP >7. Recently, Wang and others (2015) evaluated a series of artificially adulterated honeys with high-fructose syrups, including those made from corn and rice (this last, a C3 plant species) using HPAEC-PAD and reported the presence of a marker peak of starch syrups on the chromatograms. The authors claimed that the proposed method could detect the addition of both C4 and C3 starch syrups to honey in a simple and low-cost way. HPAEC-PAD combined with chemometrics was proposed for detecting and quantifying the presence of industrial sugar syrup added to honey samples from 10% to 40% (Cordella and others 2005). The method allowed the characterization of authentic and adulterated honey samples at a 96.5% level of correct classification using linear discriminant analysis (LDA) followed by a canonical analysis. Additionally, the same authors conducted a field experiment and showed that adulteration was also possible via bee-feeding syrups. This practice could cause chemical modifications to honey quality similar to direct insertion of sugar syrup into honey.

The carbohydrate composition of high-fructose inulin syrups (HFIS) has been studied by Ruiz-Matute and others (2010) using a GC-MS method to provide better resolution for honey oligosaccharide analysis, though requiring a derivatization step that might lead to very complex chromatograms (Wang and others 2015). Ruiz-Matute and others (2010) analyzed 107 floral honeys (heather, rosemary, eucalyptus, and citrus) and suggested to use inulotriose as a marker for honey adulteration with HFIS. This compound was not detected in any of the analyzed honey samples, while high concentrations were found in all the ones containing HFIS. The addition of rice syrup has recently emerged as an increasing adulteration in the honey market due to its very difficult detection. Being a C3 plant, the use of common SCIRA is not feasible to detect it in this syrup. According to Xue and others (2013), rice syrup is produced by the hydrolysis of polysaccharides and oligosaccharides, thus containing low levels of these compounds. As an alternative, they developed a simple, fast, and effective methodology using liquid chromatography with diode array detection (HPLC-DAD) to analyze 160 samples of different honeys (acacia, jujube, rape, linden, litchi, clover, and multifloral honeys from Chinese beekeepers) and also 32 representative rice syrups purchased in China. The obtained results allowed selecting a suitable marker present only in rice syrup, namely 2-acetylfuran-3-glucopyranoside, which proved to be accurate and effective for its detection as an adulterant in honey. Du and others (2015) developed a method to assess adulteration of honey with multi-class sugar syrups based on ultra-high-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS). The method allowed the simultaneous detection of polysaccharides, difructose anhydrides, and 2-acetylfuran-3-glucopyranoside as adulteration markers, enabling identifying the presence of 10% of sugar syrup in honey in less than 30 min (Figure 2).

According to several authors, the use of spectroscopic techniques based on infrared (IR), Raman spectroscopy, and NMR are considered useful alternatives to detect sugar adulteration in honey. Compared to other analytical approaches, in general, spectroscopic methods have the advantages of not being destructive, providing fast, simple, and low-cost screening analysis. However, these techniques generally need to be combined with multivariate data analysis, require a large set of diversified samples to allow establishing a suitable database, and the availability of special equipment, often of high cost. Moreover, data obtained by the use of classification models are not always very accurate in differentiating authentic from adulterated honeys. Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) and multivariate analysis was successfully used to detect and quantify different sugar adulterants (corn syrup, high-fructose corn syrup, and invert sugar) in honeys (Irudayaraj and others 2003; Gallardo-Velázquez and others 2009; Rios-Corripio and others 2012). Similarly, near-infrared (NIR) spectroscopy in combination with chemometric methods was investigated for the qualitative and quantitative detection of beet syrup adulteration of honey (Li and others 2013), adulteration of honey with fructose and glucose mixtures (Zhu and others 2010), and adulteration of honey with HFLS (Chen and others 2011).

Recently, Li and others (2012) used Raman spectroscopy to detect honey adulterated with HFCS and maltose syrups (from 10% to 40% w/w adulteration level). Adaptive iteratively reweighted penalized least squares (airPLS) was used to remove the background of spectral data, and partial least-squares-linear discriminant analysis (PLS-LDA) was used to develop a binary classification model. Spectra data were collected from a total of 74 authentic honeys of 10 botanical origins, as well as from 132 HFCS-adulterated honeys (corresponding to laboratory admixing of 44 authentic honeys with different amounts of syrup) and 150 maltose syrup-adulterated honeys (corresponding to laboratory admixing of 50 authentic honeys with different amounts of syrup). Classification of authentic honey samples showed a total accuracy of 91.1% (authentic honey vs. adulterated honey with HFCS), 97.8% (authentic honey vs. adulterated honey with MS), and 75.6% (authentic honey vs. adulterated honey with HFCS and MS), while classification of honey adulterants (HFCS or maltose syrup) gave a total accuracy of 84.4%.

Higher accuracy was reported by Bertelli and others (2010) who studied the feasibility to detect honey adulteration using 1D and 2D NMR spectra and multivariate statistical analysis. The authors claimed several advantages of this technique, such as the fact of not needing a calibration with internal standards or component extraction prior to the analysis, and having a remarkable selectivity and the capacity of identifying unknown compounds at a molecular level, providing both structural and quantitative information on a wide range of chemical species. When applying the technique to the analysis of 63 authentic and 63 adulterated honeys with 10% to 40% of commercial sugar syrups, the best discriminant model, obtained by 1D-spectra, showed a predictive capacity of 95.2%, with some samples being incorrectly classified. Nevertheless, none of the adulterated samples was classified as being authentic. Although the obtained results seem to be promising, even considering that honeys from different botanical origins and different commercial syrups were used, the number of samples was still regarded as being low. This remark can be further extended to several other studies using spectroscopic methods, as frequently the works include

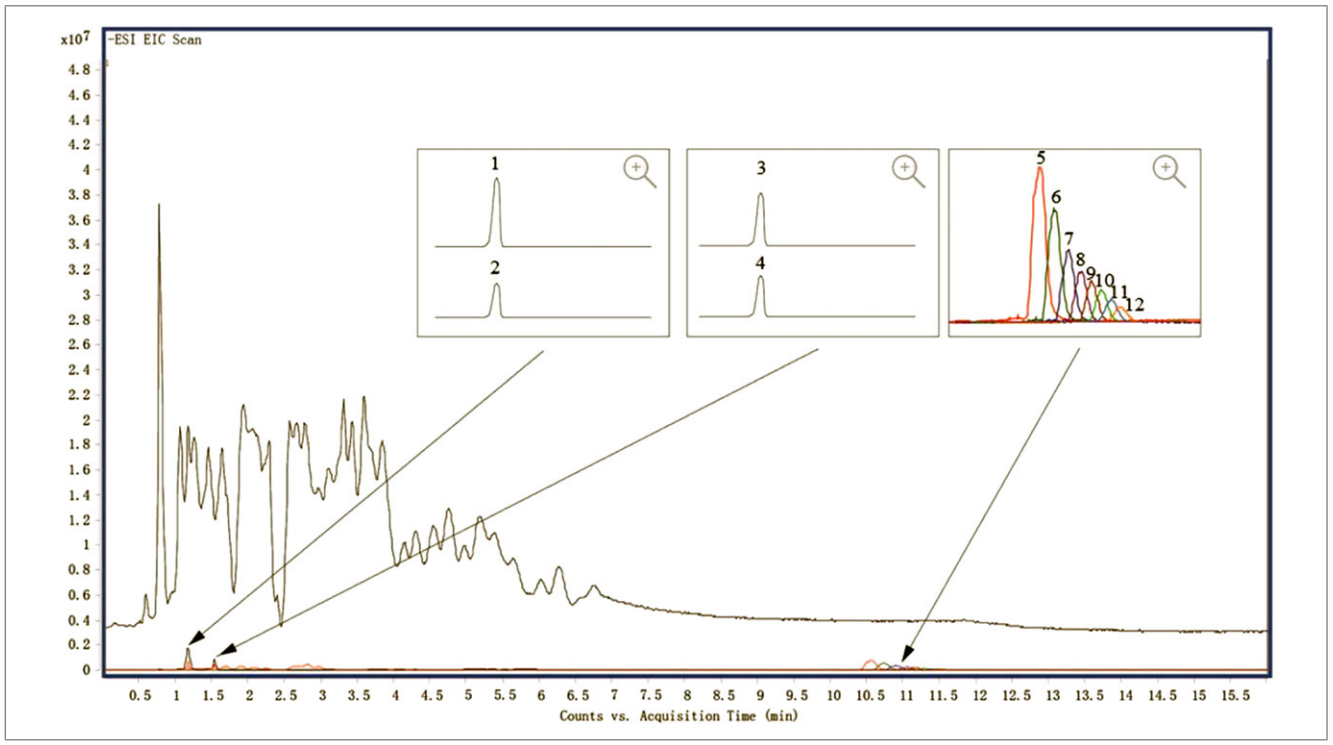

Figure 2–Chromatogram obtained by using UHPLC/Q-TOF-MS of rape honey from China with 10% mixed syrups. Peaks 1 and 2 correspond to $[M - H]^-$ and $[M + COOH]^-$ of 2-acetylfuran-3-glucopyranoside (AFGP), respectively; peaks 3 and 4 correspond to $[M - H]^-$ and $[M + COOH]^-$ of difructose anhydrides (DFAs), respectively; and peaks 5 to 12 correspond to $[M - 2H]^{2-}$ of polysaccharides (DP 12–19). Reprinted from Du and others (2015) with permission from American Chemical Society.

sets with a low number of authentic and adulterated samples, with the latter generally being limited to a few laboratory-model mixtures.

Processing

During the industrial processing of honey, some treatments, including heat-assisted filtration and centrifugation, can be used. In the case of filtration, a process commonly used by producers to remove some foreign matter that might appear in honey, legislation has specific rules for the use of filters, defining also that filtered honey must be labeled as such (Directive 2001/110/EC; FAO 2001).

According to the *Codex* standards, honey shall not be heated or processed to such an extent that can cause changes to its essential composition or that impairs its quality (FAO 2001). Therefore, treatments such as the use of high temperatures are not allowed. However, it is general knowledge that some producers use high/uncontrolled heating for pasteurization purposes and/or for liquefaction. Pasteurization is used to kill osmophilic yeasts, being carried out for 7.5 min at 63 °C or for 1 min at 69 °C, involving rapid heating and cooling (Bogdanov and Martin 2002). Since consumers, generally (and often incorrectly) associate crystallized honey to be of lower quality or sugar-adulterated honey, some producers apply heat treatments (flash-heating at 80 °C with vacuum for 1 to 2 min, followed by flash-cooling to 55 °C) to melt glucose microcrystals and remove air bubbles because both can act as crystallization nuclei, thus delaying the onset of crystallization at least for 12 mo postprocessing. Both processes affect the quality of honey, causing the loss of volatile compounds and the reduction of enzymatic activity (Bogdanov and Martin 2002). Enzymes have an important role in honey because they are responsible for the transformation of nectar, or honeydew, into honey and can be used as a sensitive indicator of honey exposure to heat during processing and storage. However, it should be noticed that enzyme content can be variable in different honeys owing to its provenance from bees' saliva, which can vary depending on different conditions. Enzymes such as amylases can also be added artificially by producers in order to mask their reduction caused by honey adulteration with syrups (Voldřich and others 2009).

The standard methods used for the evaluation of honey freshness and overheating rely on the determination of diastase activity and HMF content. The effects of temperature and time of treatment on the modification of diastase activity, measured as diastase number, were evaluated by Tosi and others (2008). The results showed a highly variable behavior that makes diastase activity an uncertain parameter to determine if honey has been submitted to heating. Due to this variability, some authors have suggested that enzymatic activity is not a very accurate parameter if analyzed separately from other quality parameters (Anklam 1998). HMF is a furanic compound usually absent or present only at low levels in fresh and unprocessed foods. In honey, HMF can be formed as a result of overheating or during long-term storage, thus being generally used as an indicator for honey freshness and quality. In the EU, legislation establishes that its concentration should not exceed 40 mg/kg, except for honey that originated from regions with tropical temperatures (Directive 2001/110/EC). Zappalà and others (2005) tested and compared 3 different methods for the determination of HMF recommended by the International Honey Commission, namely 2 spectrophotometric methods, according to Winkler (1955) and White (1979), and a HPLC method. The methods were applied to monofloral honeys from acacia, citrus, eucalyptus, chestnut, and

wildflower. Data showed that similar values were generally obtained using both the HPLC and the White methods, while for all types of honey the Winkler method gave higher values compared to the other 2 methods. The HPLC method was proposed as being the most adequate for HMF determination in honey because the substances derived by heat or storage damage can interfere with the spectrophotometric methods. Due to the low precision of the Winkler method, the authors also suggested to avoid its use, as previously recommended by the International Commission of Honey. More recently, a micellar electrokinetic capillary chromatography (MECK-UV) method was proposed as an alternative, with potential for routine and automated analysis of HMF in honeys based on the analytical performance of the method, namely its very short time of analysis (less than 1 min), low cost, and simple sample pretreatment (Rizelio and others 2012). In addition to HMF, Morales and others (2009) suggested to use a combination of HMF and furosine, a chemical marker of the progress of Maillard reaction that has also been used as an indicator of freshness in foods, being a useful parameter for evaluating the quality of fresh honey.

Water content

The water content in honey depends on the climatic, storage, and harvesting conditions (including season of production and human manipulation) and it affects the physical properties of honey, such as viscosity and crystallization, and consequently its quality (Gallina and others 2010). Generally, the water content in honey is less than 20%, with the exception of heather honey, for which the limit is 23% (Directive 2001/110/EC; FAO 2001).

Any excess of water can be removed by centrifugation or vacuum-evaporation during processing. On the other hand, if water is artificially added into honeys or if harvesting is made under high humidity conditions, it can cause fermentation and spoilage. Even though this type of honey adulteration is not considered as being a realistic practice (Bogdanov and Martin 2002), several parameters such as microscopic yeast count and analysis of fermentation products (Martins and others 2008) are used to test honey spoilage, and, thus, indirectly detect this type of honey adulteration.

The determination of honey moisture is an important and a very frequent analysis to establish the quality and marketability of honey. Moisture can be determined gravimetrically, but the refractive index (RI) determination with an evaluation of moisture percentage, using an empirical formula or a relative conversion table, is the most used method. However, it requires heat pre-treatment in crystallized honeys and the empirical formula or relative conversion table used for moisture content evaluation is not correct for every type of honey (Gallina and others 2010). Thus, alternative methods have been suggested, such as the Karl Fischer titration (KFT) (Isengard and others 2001). Gallina and others (2010) compared the values attained with the KFT method of more than 100 samples of honey with the RI values determined for the same samples. Results allowed verifying that KFT was the most reliable method, although displaying slightly higher values in general. Sanchez and others (2010) compared RI and KFT methods using 2 different solvents (methanol and methanol:formamide [1:1]). Similar results were obtained, mainly when the solvent mixture was used. The KFT method with solvent mixture also allowed a reduction in titration time.

Origin

Organic

Nowadays, consumers are increasingly aware of the dangers associated with the possibility of toxic substances in food, preferring those labeled as being "organic." According to legislation, honey is a natural substance and shall not have added any food ingredient to it, including food additives or other nonhoney substances. The production of organic honey should be in good agreement with the rules established by the European legislation on the production and labeling of organic products (Regulation (EC) No 834/2007). Thus, organic honey must be produced following an ecological-based system, using natural resources, and promoting environmental quality, animal welfare, and human health (Gomes and others 2011). Nevertheless, honey contamination can arise from different sources, such as beekeeping practices or the environment surrounding hives. The last one is a complex issue in the classification of organic honey, since nowadays it is almost impossible to produce honey in a totally pollution-free environment (Bogdanov and Gallmann 2008). Environmental contaminants include heavy metals, such as lead, cadmium, and mercury, radioactive isotopes, organic pollutants, pesticides (insecticides, fungicides, herbicides, and bactericides), pathogenic bacteria, and GMO. The contaminants introduced from beekeeping are mainly acaricides, used for *Varroa destructor*, and antibiotics used for the control of bee brood diseases (Bogdanov 2006). Accordingly, beekeepers have to meet strict production standards and conditions to certify honey as "organic," including the absence of contaminants such as pesticides, antibiotics, and organic pollutants. An evaluation of the presence of veterinary drug residues and pesticides is important to recognize honey as organic. The presence of such residues reveals that producers did not use organic production methods, and it might be considered a commercial fraud if labeled as organic (Bogdanov and Martin 2002).

Directive 2014/63/EU relating to honey is written according to the specific labeling requirements of Regulation (EC) No 1829/2003, stating that there is no obligation to indicate the presence of genetically modified (GM) pollen in honey on labels for honey if the following conditions are met: such pollen does not exceed 0.9% of the honey, and its presence in honey is adventitious or technically unavoidable. Additionally, according to Council Regulation (EC) No. 834/2007 on organic production and labeling of organic, GMO and products produced from or by GMO are incompatible with the concept of organic production and consumers' perception of organic products. Therefore, their use is prohibited in organic farming or in the processing of organic products such as honey. Nevertheless, as honeybees forage across a long distance to collect nectar and pollen, without being able to distinguish between conventional and GM plants, honey production is at high risk of containing unintended traces of pollen from GMO, if those plants are grown in the vicinity of the hives. This can happen in some European honey-producing countries, such as Spain, Portugal, Czech Republic, and Romania, which cultivate GM maize with the event MON810. Considering that honey production is not sufficient to address the European consumption and industrial needs, approximately 40% of honey consumed in Europe is imported from third-party countries, mostly from Latin American (Argentina, Mexico, Chile, Cuba, Brazil, and El Salvador, accounting for 45% of imports) and Asian countries (China and Thailand, accounting for 33% of imports), where, in some cases, large areas of GM crops (maize, soybean, rapeseed) are cultivated. Thus, honey labeled as organic should ideally be tested for the presence of GMO, especially if imported from non-EU

countries where nonauthorized GM crops are also cultivated and not allowed to be market in EU.

Botanical and geographical

Honey can result from a broad variety of plant species because bees collect nectar from different surrounding flowers. Usually, honey is classified as being monofloral when at least 45% of pollen grains arise from a single species. However, there are some exceptions, namely: honeys that have underrepresented pollen grains, such as in the case of lavender honey, for which only 15% of pollen grain is required for botanical classification as monofloral honey; and honeys having overrepresented pollen grains, such as those from eucalyptus and chestnut that can show a pollen frequency of 70% to 90% (Pires and others 2009). A multifloral honey is composed of pollen grains from several plant species, none of which being considered predominant. Due to their particular flavor, taste, and biological properties, monofloral ones are the most demanded, as previously stated. For that reason, the botanical source of honey is highly connected with its price, and, consequently, producers may be tempted to describe different nectar sources to increase profit. Similarly, the geographical origin is an important parameter with respect to honey differentiation and valorization. Depending on its geographical origin, the region where the hives are located and the surrounding environment, honey can acquire different characteristics and properties.

Considering the increasing global trade and owing to the higher economic value of monofloral, PDO, and PGI honeys, associated with particular origins, those products are particularly prone to adulteration through incorrect labeling and fraudulent admixing with honey of lower value and quality. Thus, in order to protect consumers and promote fair competition among producers, there is a growing need to assess the authenticity of honey, particularly with regard to geographical and botanical origins.

Currently, organoleptic properties, physicochemical analysis, and pollen spectrum are the main criteria for honey classification. Considering the difficulties associated with the traditional melissopalynology, new analytical methodologies for botanical origin discrimination are emerging as alternatives, including those based on chromatography, spectroscopy, and molecular biology. Analytical methods applied to honey characterization and authentication have recently been reviewed with particular emphasis on the spectroscopic techniques (Consonni and Cagliari 2015; Siddiqui and others 2017). The chemometric tools have appeared as analytical complements for honey authentication, which were particularly reviewed by Camiña and others (2012) focusing on the geographical and botanical origins of honeys from numerous countries. So far, several advanced approaches have been proposed aiming at accurately assessing the botanical and geographical origins of honey, by targeting certain minor compounds in honey, including the use of GC-MS, liquid chromatography with mass spectrometry (LC-MS), capillary electrophoresis-time-of-flight mass spectrometry (CE-TOF-MS), matrix-assisted laser desorption/ionization MS (MALDI-TOF MS), and NMR spectroscopy (Schievano and others 2013). However, the assessment of chemical markers, such as volatiles, phenolic acids, sugars, and other constituents of honey can be influenced by beekeeping techniques, environmental conditions, and climate changes, thus leading frequently to unreliable determination of its floral or geographical origin (Madesis and others 2014). Besides, honey is a very complex matrix in terms of pollen origin because bees never visit a single plant species to produce honey, with consequent variability of honey composition. To overcome these problems, the use of DNA markers for pollen identification can offer new alternative tools independent of external conditions of honey production. In the following sections, several analytical methodologies are described aiming at the analysis of particular target parameters for botanical and/or geographical origin discrimination, critically emphasizing their advantages and drawbacks. Similarly, recent advances in nontarget methods will be discussed.

Target parameters.

Pollen. The traditional method used for ascertaining the botanical origin of honey is melissopalynology, which consists of pollen identification by microscopic analysis to determinate the plants visited by the bees during honey production. The identification and quantification of pollen grains in honey sediment is still used as a reference method to establish the botanical origin of honey samples (Pires and others 2009). Nevertheless, this method has several shortcomings, such as the long time of analysis, the availability of a comprehensive collection of pollen grains, and the need of experts with adequate skills and experience to identify different pollen morphologies (Soares and others 2015). Besides, there is the risk of considerable discrepancy of the pollen content in honeys because of several factors, such as seasonal variations, or the sort of flora visited by bees for certain types of honey with "underrepresented" pollen (Escriche and others 2011). Moreover, this methodology is not suited for cases of inadequate honey filtration performed by beekeepers or adulteration by pollen addition. Consequently, melissopalynology is commonly complemented by physico-chemical and organoleptic analyses, being honey botanical classification based on a global interpretation of all results (Bogdanov and Gallmann 2008).

Phenolic compounds. The natural properties of honey are well known by consumers; they are mainly attributed to its antibacterial and antioxidant activities. Besides promoting human health and well-being, honey can also prevent deteriorative oxidation reactions in foods, such as lipid oxidation in meat and enzymatic browning of fruits and vegetables. Its antioxidant properties are derived from both enzymatic (catalase, glucose oxidase, peroxidase) and nonenzymatic substances (ascorbic acid, α-tocopherol, carotenoids, amino acids, proteins, Maillard reaction products, flavonoids, and phenolic acids). Among those, phenolics are considered the main responsible compounds for the antioxidant properties of honey. The type and amount of these compounds in honey can vary with seasonal, climatic, and processing factors, but mostly depend on the botanical origin of honey because they are transferred from plants to honey through the nectar collected by bees (Gheldof and others 2002; Silici and others 2010; Rosa and others 2011). For this reason, phenolic compounds have been considered useful markers in honey characterization and, consequently, their profile appears to be valuable to assess its botanical and/or geographical origins. In this context, the development of reliable analytical methods to obtain such information has been recently described as being of major importance (Sergiel and others 2014a).

The extraction of phenolic compounds is often performed by solid-phase extraction (SPE), with subsequent analysis performed by chromatographic methods (Kaškonienė and Venskutonis 2010). Due to the complexity of the honey matrix, sample preparation/extraction is considered a key step, since the identification of compounds can be affected by interferences that will compromise the sensitivity and selectivity of the analytical method (Michalkiewicz and others 2008). The use of SPE with different sorbents, including nonionic Amberlite XAD polymeric resins (Jasicka-Misiak and others 2012), hydrophobic C18 cartridges

(Pulcini and others 2006; Dimitrova and others 2007; Hussein and others 2011), and polymeric reversed-phase Strata X cartridges (Sergiel and others 2014b) has been suggested to separate phenolic acids and flavonoids from sugars and other polar substances. They have also been used to concentrate the compounds of interest prior to a more accurate identification (Sergiel and others 2014a). Compared to reversed-phase cartridges, nonionic resins, such as Amberlite XAD-2 and XAD-4, have been reported to effectively eliminate sugars, acids, pigments, and other interfering compounds (Petrus and others 2011). The influence of different factors, including the solid sorbents (Bond Elut octadecyl C18, Oasis HLB, Strata-X, and Amberlite XAD-2) and the composition of washing and elution solvents on the recovery of phenolic compounds from honey was evaluated by Michalkiewicz and others (2008). The selectivity of the procedure was assessed using spiked honeys and the performance of the method was tested in real honey samples from different botanical origins. Bond Elut C18 silica sorbent was found to be less appropriate for the isolation and pre-concentration of the tested compounds, while the best results were obtained with polymeric sorbent Oasis HLB, possibly due to $\pi-\pi$ interactions between aromatic structures. With the exception of rutin and quercetin, results were also better when compared with Amberlite XAD-2. As mentioned, the complexity of the matrix brings an added difficulty to the determination of a honey phenolic profile.

Besides the extraction step, the use of adequate separation and detection techniques enabling an unambiguous determination of as many components as possible is crucial (Keckes and others 2013). With this aim, different methods, namely ultra-high-performance liquid chromatography (UHPLC) coupled with hybrid mass spectrometry (Keckes and others 2013; Gašić and others 2014), HPLC-DAD (Jasicka-Misiak and others 2012; Jerkovic and others 2014; Sergiel and others 2014a), HPLC coupled with coulometric electrode array detection (CEAD) (Petrus and others 2011), and HPLC with electrospray ionization tandem mass spectrometry (ESI-MS/MS) (Sergiel and others 2014a) were suggested for the determination of phenolic compounds in honey. Recently, a different approach was proposed by Sergiel and others (2014b), who applied 3D-synchronous fluorescence spectroscopy (SFS) to identify phenolic compounds in honey. The authors concluded that 3D-SFS spectra obtained from the honey phenolic fraction could serve as a very good signature of a particular botanical origin and could be useful for authentication and/or identification in a very straightforward way.

Although most of the phenolic acids and flavonoids are simultaneously present in different monofloral honeys, several studies suggest the use of some of them as suitable markers for the floral origin of honey. Hamdy and others (2009) analyzed the flavonoids and phenolic acids of clover, cotton, and citrus honey samples and concluded that the flavanone hesperetin was present only in citrus honey, whereas quercetin, cinnamic acid, and *p*-hydroxy benzoic acid were detected in the 3 analyzed monofloral honeys. These results were in good agreement with previous studies that also suggested hesperetin as being a useful botanical marker for citrus honey (Ferreres and others 1993; Hamdy and others 2009). Other phenolic compounds also proposed as markers for the floral origin of honey include homogentisic acid for strawberry tree honey (Tuberoso and others 2010), methyl syringate for rapeseed honey (Kuś and others 2014), 8-methoxykaempferol for rosemary honey (Ferreres and others 1994), and ellagic acid for heather honey (Ferreres and others 1996a). Other compounds, such as abscisic acid in heather honey (Ferreres and others 1996b; Kuś and others 2014) and *p*-hydroxybenzoic acid in buckwheat honey (Kuś and others 2014), have also been suggested to serve as floral markers, though they have also been reported in honeys from other sources (Truchado and others 2008). Similarly, quercetin and kaempferol were proposed as markers for sunflower (Tomás-Barberán and others 2001) and rosemary (Gil and others 1995) honeys, respectively, yet both compounds have also been found in considerable amounts in other honeys (Petrus and others 2011).

Considering that some compounds previously suggested as floral markers for a particular honey are frequently found in other types of honey, the use of a phenolic profile instead of a single compound has been suggested as more adequate to identify the botanical origin of honey. Accordingly, Martos and others (2000) concluded that myricetin, tricetin, quercetin, luteolin, and kaempferol could be used for eucalyptus honey identification.

Bearing in mind that phenolic compounds are phytochemicals transferred from the plant to the nectar and then to the honey, they are mostly associated with botanical origin authentication, though their use has also been reported as suitable for geographical origin determination. Generally, such an approach requires the use of chemometrics as demonstrated by Karabagias and others (2014). The authors classified and differentiated Greek thyme honeys according to geographical origin based on selected phenolic compounds and quality parameter data combined with chemometrics.

Sugars. As mentioned, sugars (saccharides) are the main components of honey and are considered useful parameters to assess honey adulteration by the addition of syrups. Their use as floral or geographical origin markers of honey is not so frequent due to difficulties in specifying one or more carbohydrates present in honey that can serve this purpose (Kaškonienė and Venskutonis 2010). Nevertheless, some authors have suggested using the amount and ratio of particular carbohydrates, such as fructose and glucose, as well as oligosaccharides, as useful indicators to recognize monofloral honeys (Cotte and others 2004; Nozal and others 2005). Besides major mono- and disaccharides, it is known that tri- and tetrasaccharides can be present in small amounts in honeys. Therefore, different studies have been conducted to determine the carbohydrate profile of distinct honeys, either for botanical or geographical origin classification (Cotte and others 2004; Sanz and others 2004; Nozal and others 2005). With this aim, different methodologies based either on spectroscopic or chromatographic techniques have been suggested. They include diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) (Bertelli and others 2007), NMR (Lolli and others 2008), HPLC coupled to pulsed amperometric detection (PAD) (Nozal and others 2005), GC-MS based on the use of 2 different stationary phases (de la Fuente and others 2011), and a combination of HPLC-PAD and GC-FID (Cotte and others 2004). HPLC-PAD is considered advantageous because it does not require derivatization and allows high sensitivity (Nozal and others 2005). The analysis of low-molecular-weight carbohydrates in complex mixtures, when performed by conventional GC, frequently results on peak co-elution. To overcome the co-elution problem of anomeric structures, GCxGC-TOF-MS was recently applied for the separation and identification of saccharides previously converted to trimethylsilyl oxime derivatives (Brokl and others 2010). The authors evaluated different honeys, including 5 multifloral and 6 monofloral honeys marketed in New Zealand, obtaining similar qualitative profiles, but still with variations in the relative abundance of disaccharides.

Among spectroscopic methods, Consonni and others (2013) demonstrated the capability of NMR for saccharide analysis to discriminate, either the geographical origin or the floral source of

honey. According to the authors, NMR largely demonstrated its feasibility for the analysis of complex mixtures, such as honey, with the advantages of high reproducibility, and low sample amount and preparation time over chromatographic methods. The application of multivariate statistical analysis to the NMR saccharide data allowed the characterization and differentiation of honeys from 5 distinct botanical origins. High-mountain multifloral and rhododendron honeys partially overlapped, which could be related to their similar origin with respect to altitude (Consonni and others 2012). This is in good agreement with Kaškonienė and Venskutonis (2010), who reported that the interpretation of sugar composition data can become impracticable to determine the floral origin of honey, especially in the case of multifloral honeys since both botanical and geographical origins influence the composition of honey carbohydrates.

Volatile compounds. As previously mentioned, the botanical origin of honey depends on the pollen sources visited by the bees, making each honey unique on the basis of the nature, amount, and combination of various components and giving it a distinctive organoleptic character (Bertelli and others 2007). Considering that aroma is one of the distinguishable characteristics of honeys, the profile of volatile compounds can potentially be used as a fingerprint for their differentiation and authentication (Kaškonienė and Venskutonis 2010; Schievano and others 2013).

Volatile compounds in honey are grouped into chemical categories, including aldehydes, ketones, acids, alcohols, hydrocarbons, norisoprenoids, terpenes, benzene compounds, and their furan and pyran derivatives (Manyi-Loh and others 2011). Until now, more than 600 different compounds have been identified in volatile extracts or in the headspace isolates of honey (Kaškonienė and Venskutonis 2010). Using volatile analysis, typical compounds can be identified and related to a specific plant species, making it potentially useful as floral marker for certain honeys. Due to the presence of characteristic volatile organic components deriving from the original nectar sources, the monofloral honeys usually have high individual aroma profiles when compared with multifloral honeys (Kaškonienė and Venskutonis 2010). Notwithstanding, only a small number of compounds has been described as possible floral markers that allow recognizing honeys from different botanical origins. Methyl anthranylate (MA), a specific compound of citrus blossom nectar, has long been used as a marker for citrus honey, also being considered useful for the classification of this type of honey when citrus pollen is underrepresented (White and Bryant 1996; Juan-Borrás and others 2015). Since this compound is characteristic of citrus honeys, some laboratories for honey control require a minimum MA content of 2 mg/kg to classify a sample as being citrus unifloral honey, although that is not an official parameter. However, different authors have been showing that there is only a weak correlation between the content of MA and the percentage of citrus pollen, which can lead to false negatives if the level of 2 mg/kg is considered (Sesta and others 2008; Juan-Borrás and others 2015).

Isophorone and its derivatives has been referred to as characteristic compounds in the volatile fraction of strawberry-tree (*Arbutus unedo*) honey (Bianchi and others 2005; de la Fuente and others 2007). Isophorone has also been reported in *A. unedo* flowers and in Corsican "autumn maquis" honey, which typically presents *A. unedo* in its composition (Yang and others 2014a). However, the presence of isophorone has also been described as indicative of a floral origin belonging to the heather family (Guyot and others 1999; Seisonen and others 2015). Moreover, isophorone and its derivatives have also been reported to occur in other types of honey, such as thyme (Alissandrakis and others 2007; Karabagias and others 2014), rare apple (*Malus domestica* Borkh.) (Kuś and others 2013), and *Saturana monteja* (Jerkovic and others 2015), thus indicating their utility for honey authentication. Identically, in other studies, the same volatile compounds were suggested as being characteristic for different honeys; as an example, lilac aldehydes were suggested as characteristic constituents of rhododendron (Senyuva and others 2009), citrus (de la Fuente and others 2005; Alissandrakis and others 2007; Papotti and others 2012), and rosemary (Castro-Vázquez and others 2009) honeys. Therefore, instead of a single compound, most studies refer to the use of several aroma compounds present at different levels to characterize honeys, as an attempt to distinguish their different botanical origins. A list of the main compounds suggested as possible markers of various honeys can be consulted in the review of Kaškonienė and Venskutonis (2010). Additional examples of volatile compounds that were recently suggested as markers for the botanical origin of honey are presented in Table 2.

Apart from the floral source, several factors can influence the volatile compounds of honey, such as plant maturity, edaphoclimatic conditions, and processing and storage of honey, among others. Overheating during processing or a long period of storage are reported to considerably affect volatile compounds in honey (Manyi-Loh and others 2011). Kaškonienė and others (2008) noticed remarkable changes in the content and composition of volatiles in honey samples analyzed after 3 months of storage, with the amount of the headspace volatiles decreasing by at least 70% in the majority of samples. The changes in heated or stored honey have been attributed mainly to compounds that are heat-labile and might be destroyed, and to volatile compounds produced by nonenzymatic browning (Maillard reaction) (Kaškonienė and others 2008; Manyi-Loh and others 2011). Recently, Agila and others (2013) studied the effect of both storage time and adulteration by the addition of HFCS on the volatile compounds of different honeys, concluding that the increase of HFCS content and storage period promoted a decrease in volatiles. To circumvent the variability in volatile composition frequently ascribed to these factors, Verzera and others (2014) proposed the use of enantiomeric ratios of some volatile constituents, which directly come from flowers, to authenticate the botanical origin of honey. It is known that chiral flavor and fragrance components of natural origin, generally, have a characteristic distribution of enantiomers that is attributable to stereo-selectively controlled biogenetic formation mechanisms. The authors demonstrated that the use of enantiomeric ratios of some volatiles can be a promising parameter for botanical authentication of honey, since the enantiomeric ratio of linalool and its oxides remained stable and was less influenced by production period, conditioning, packaging, and storage.

Another factor that can strongly affect the outcome of the analysis of volatile compounds in honey is the choice of the extraction method. Volatile compounds can be extracted by several methods depending on the matrix, the required selectivity, and effectiveness. In the particular case of honey, this process is difficult because it requires prior removal of sugars to facilitate the isolation of volatile compounds that are present at a very low concentration (Pontes and others 2007; Kaškonienė and others 2008; Manyi-Loh and others 2011). Moreover, the selection of the extraction method must also take into consideration that these compounds have a wide range of physicochemical properties, presenting different volatilities and polarities, which can differently affect their recovery (Plutowska and others 2011; Jerković and others 2011).

Table 2–Characteristic volatile compounds recently described for different types of honeys

Honey source	Country of origin [a]	Extraction method [b]	Compounds	Reference
Acacia	Slovakia (17)	SPME	Aldehydes from hexanal to decanal, 1-methylpyrrolidinone, 2,4-decadienal (trans isomer), 2-hexen-1-ol, (trans isomer), 3,4-dimethylhexan-3-ol, 3-ethylheptan-3-ol, heptan-3-one and dihydrolinalool	Špánik and others (2013)
Buckwheat	Italy (6), Russia (1), Poland (2), Nepal (1)		3-Methyl-2-buten-1-ol, 2-butanone, 2-hydroxy-3-pentanone, 4-methylpentanoic acid, 4-pentanoic acid, butanal, 2-methylbutanal, pentanal, dihydro-2-methyl-3(2H)-furanone, 5-methylfurfural and cis-linalool oxide.	Panseri and others (2013)
Chestnut	Greece (10)	USE	1-Phenylethanol and 2'-aminoacetophenone, *cis*-cinnamyl alcohol and *p*-hydrox-yacetophenone	Alissandrakis and others (2011)
	Corsican (50)	HS-SPME	2-Aminoacetophenone, benzaldehyde, acetophenone, nonanoic acid, octanoic acid, and 3-furaldehyde	Yang and others (2012a)
Cornflower	Poland	HS-SPME	3,4-Dihydro-3-oxoedulan	Kuś and others (2013)
Eucalyptus	Greece (10)	USE	2-Hydroxy-5-methyl-3-hexanone, 3-hydroxy-5-methyl-3-hexanone, exo-2-hydroxycineole, an unknown norisoprenoid, acetoin, nonanal, methyl nonanoate and dehydrovomifoliol	Alissandrakis and others (2011)
	Italy (2), South Africa (1)	SPME	Acetoin	Pažitná and others (2014)
Heather	Corsican (45)	HS-SPME	4-Propylanisol, *p*-anisaldehyde, benzaldehyde and 3-furaldehyde	Yang and others (2012b)
	Estonia (3)	SPME	Isophorone and 2-methylbutyric acid	Seisonen and others (2015)
Lavandin/ Lavander	Spain	SPE	γ-Nonalactone, farnesol, acetovanillone, 1-heptanol, decanal, 4-methoxyacetophenone and dehydrovomifoliol	Castro-Vázquez and others (2014)
Lime	Slovakia (6)	SPME	1-ethoxy-2-(2-ethoxyethoxy)ethane, 2,7-dimethyloxepine, 2-aminoacetophenone, 2-isobutylthiazole,2-isopropylidene-3-methylhexa-3,5-dienal, 3-hydroxy-β-damascone, 3-methylpyridazine, 4-(1-methylethyl)benzoic acid methyl ester, geranic acid, m-cymene, oxalic acid, hexyl propyl ester, p-mentha-1,8-dien-3-one, methyl ester of 2-hydroxypropanoic acid, α,4-dimethylbenzyl alcohol, α-hydroxyacetophenone, myrtanol, 2,6-dimethyl-3,5-heptadien-2-ol, citronellic acid, 1,2,5-trimethylpyrrole and 1,3,7,7-tetramethyl-9-oxo-2-oxabicyclo[4.4.0]dec-5-ene	Špánik and others (2013)
Orange	Greece (2), France (1)	SPME	Herboxid I, herboxide II, dill ether, 8-heptadecane, limonyl alcohol, nerolidol and 8-hydroxylinalool and lilac aldehydes.	Pažitná and others (2014)
	Sicilia (36)	SPME	Lilac aldehydes, linalool and its oxides, hotrienol, dill ether and the 2 isomers of p-menth-1-en-9-al and furfural	Verzera and others (2014)
Phacelia	Slovakia (2)	SPME	1-methyl-1,4-cyclohexadiene, 1-nitrohexane, 1-phenyl-1,2-propanedione, 2-(2-butoxyethoxy)ethanol, 2,3-octanedione, 2,4-hexadienal, 2,6-dimethyl-2,5-heptadien-4-one, 3,9-epoxy-p-mentha-3,8-diene, 4,4-dimethyl-2-cyclopenten-1-one, 5-(1-methylethylidene)-1,3-cyclopentadiene, acenaphthene, butanoic acid, 3-hexenyl ester (isomer i), methyl cinnamate, m-propyltoluene, 2,6-dimethylnaphthalene, valeric acid, 4-nitrophenyl ester, 4-decen-1-ol, 1,2-dimethoxybenzene and 1,3,8-p-menthatriene.	Špánik and others (2013)
Rape	Estonia (6)	SPME	Dimethyl trisulphide	Seisonen and others (2015)
	Slovakia (7)	SPME	2-Methyl-2-butenoic acid, 1-phenylpropan-2-ol, 2,4-dithiapentane, pentan-3-one, cinnamic alcohol, 2-phenethyl ester of formic acid, perillol and α-bisabolol	Špánik and others (2013)
Raspberry	Slovakia (2)	SPME	2-ethenyl-2-butenal, 3-methylhexane, 3-methylnonane, 3-pyridinemethanol, β-myrcene, cyclopentanemethanol, norbornane and undecanal	Špánik and others (2013)
Rosemary	Croatia (2)	SPME	Trimethyl-tetrahydronaphtalene, widdrene, 2-(2-ethoxyethoxy)-ethanol and p-propylanisole	Pažitná and others (2014)

(*Continued*)

Table 2–Continued.

Honey source	Country of origin [a]	Extraction method [b]	Compounds	Reference
Sage	Croatia (2)	SPME	1,8-Cineole, thujone, camphore, borneol acetate, caryophyllene, alpha humulene, ledene, verbenone, ethyl geranate and veridiflorol	Pažitná and others (2014)
Sunflower	Slovakia (6)	SPME	α-bisabolene (cis isomer), γ-muurolene, copaene, δ-cadinene, levandulol, cis-p-menth-2-en-7-ol, p-methoxyanisole, α-amorphene, α-caryophyllene, α-gurjunene, α-selinene, trans-3-caren-2-ol, benzoic acid, 3-methylbutyl ester, 3,6-dimethylene-1,7-octadiene, decan-3-ol, β-cubebene, β-pinene, bornyl acetate, calamenene, calarene, β-caryophyllene	Špánik and others (2013)
Thyme	Greece (42)	HS-SPME	Formic acid ethyl ester, decanoic acid ethyl ester, formic and acetic acids, 1-hydroxy-2-propanone, 4,7,7-trimethylbicyclo[3.3.3]-octan-2-one, octane and terpinen-4-ol	Karabagias and others (2014)
Willow	Poland	HS-SPME and USE	Vomifoliol, methyl syringate and 4-hydroxy-3-(1-methylethyl)benzaldehyde	Jerkovic and others (2014)

[a] In brackets is presented the number of analysed samples;
[b] USE, ultrasound solvent extraction; HS, headspace; SPME, solid phase microextraction; SPE, solid phase extraction.

So far, different methodologies have been proposed for the extraction of honey volatile compounds, each of them having advantages and disadvantages. For instance, hydrodistillation (HD), liquid–liquid extraction (LLE), simultaneous steam distillation extraction (SDE) and Likens–Nickerson simultaneous distillation extraction (LNSDE), and micro-simultaneous steam distillation-solvent extraction (MSDE) require the use of heat that can lead to the formation of furan and pyran derivatives due to its effect on sugars or amino acids (nonenzymatic browning reaction/Maillard reaction) (Alissandrakis and others 2005; Cuevas-Glory and others 2007). The use of solvents, such as in ultrasound solvent extraction (USE) and the aforementioned techniques, can also affect the extraction of volatiles and GC analysis because solvents can solubilize nonvolatile compounds that contaminate the GC port. In addition, some volatiles can be lost during solvent removal and some analytes can be masked by the solvent inhibiting their detection (Cuevas-Glory and others 2007; Kaškonienė and others 2008; Manyi-Loh and others 2011). Despite this, USE does not require the use of heat and, thus, the generation of thermal artifacts is avoided. Moreover, USE allows the isolation of both low- and high-molecular-weight compounds, potentially providing interesting markers for the determination of honey origin (Alissandrakis and others 2005). Other methodologies, such as solid-phase dynamic extraction (SPDE), were proposed attempting to minimize the consumption of organic solvents and reduce the sample amount and sample preparation time. SPDE can entirely eliminate solvents, although it requires extensive modification of the GC injector or the addition of a desorption module (Cuevas-Glory and others 2007; Manyi-Loh and others 2011).

Nowadays, headspace solid-phase microextraction (HS-SPME) is considered the most popular technique for the analysis of volatile compounds from different food matrices, including honey (Silva and others 2013). Headspace extraction proved to be the most appropriate approach, taking advantage of differences in the Henry constants of volatile analytes and interfering compounds. Moreover, it protects the fiber from adverse effects caused by nonvolatile compounds that occur in the sample matrix (such as sugars in the case of honey) and allows modification of sample pH with no effect on the fiber (Cuevas-Glory and others 2007). HS-SPME has been widely implemented in semi-quantitative fingerprinting and profiling studies because it provides good sensitivity and selectivity for the determination of volatile nonpolar to mid-polar compounds, including flavors and off-flavors (Silva and others 2013). Fiber coating used in SPME is an important parameter having a great impact on the attained efficiency (Manyi-Loh and others 2011). Several studies testing different combinations of fiber coating have been performed in order to improve recoveries of honey volatiles. For instance, Čajka and others (2007) and, more recently, Plutowska and others (2011) have compared different SPME fiber combinations and both concluded that a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber combination provided the best sorption capacity and the broadest range of volatiles extracted from the headspace of a honey sample. Considering that the composition of volatile organic compounds can vary with the selected extraction method, the application of distinct techniques can be advised in order to obtain more relevant and comprehensive results. This was demonstrated by Jerkovic and others (2014) when evaluating several willow honeys from Poland as the composition for the same samples was completely different for the USE extractives compared to the ones obtained by HS-SPME. The headspace of samples was mainly characterized by the presence of linalool derivatives and bornane and pinane skeletons, while solvent extractives were dominated by vomifoliol and methyl syringate, with a less common compound (4-hydroxy-3-(1-methylethyl) benzaldehyde), thus showing the relevance of this type of approach (Jerkovic and others 2014). Apart from the used volatile extraction method, the aroma profile of honey is commonly determined by GC-FID or, more frequently, by GC-MS (Plutowska and others 2011; Jerkovic and others 2014; Yang and others 2014b). These methods combine high separation efficiency and sensitivity, providing qualitative and quantitative data. However, due to the complexity of the headspace volatile composition of honey, co-elution of compounds can occur when using 1D GC separation, which can compromise identification of volatiles. As a result, comprehensive GC × GC–TOF-MS has been recently proposed as an advanced separation and identification approach, allowing the rapid and comprehensive examination of a honey volatile profile. Compared to current GC-MS methods, several advantages can be pointed out, including the unbiased identification of sample components due to increased chromatographic

resolution and better spectral quality, higher sensitivity through enhanced signal-to-noise ratio and cryogenic focusing, and a higher analysis throughput (Čajka and others 2009; Chan and others 2012). Čajka and others (2007) implemented a HS-SPME followed by GC × GC–TOF-MS methodology for the analysis of volatile compounds in honey, which allowed the identification of a higher number of volatiles (164 compounds in a mixture made with 6 honey samples from different countries) when compared to studies using 1-dimension GC (35 to 110 compounds as previously reported in the literature). Moreover, the use of GC×GC-TOF-MS resulted in a faster separation of sample components (19 min) compared to chromatographic run times previously published for the analysis of honey volatiles by conventional GC methods (Čajka and others 2007). The developed methodology was further applied to the authentication of PDO honeys labeled as "Corsica" (Čajka and others 2009). A total of 374 honey samples, collected in 2006 and 2007, from Corsica ($n = 219$) and other non-Corsican French regions, namely Italy, Austria, Ireland, and Germany, was evaluated by GC×GC-TOF-MS. Considering the large volume of data, this technique allowed selecting 26 volatiles as marker compounds, performing smart chemometric analysis with a preliminary inspection of data structure by principal component analysis (PCA) followed by artificial neural networks with multilayer perceptrons (ANN-MLP). The proposed model can be considered appropriate for the geographical discrimination of PDO Corsican honeys due to the high prediction and classification abilities of 94.6% and 96.5%, respectively. The same authors applied supervised pattern recognition techniques to the same data, aiming at constructing classification/discrimination rules to predict the geographical origin of honeys on the basis of their profiles of volatile compounds (Stanimirova and others 2010). Among the applied techniques, which included LDA, soft independent modelling of class analogies (SIMCA), discriminant partial least squares (DPLS), and support vector machines (SVM), the best results in Corsican honey discriminations were obtained with the SVM classification model. Nevertheless, all techniques scored lower prediction and classification capacities when compared with ANN-MPL (Cajka and others 2007).

SIMCA was also applied to data obtained by selected ion flow tube mass spectrometry (SIFT-MS) aiming at classifying honeys from different geographical (Ohio and Indiana, USA) and different botanical origins (star thistle, blueberry, clover, wildflower, and cranberry), and determining the most important volatile compounds for their differentiation (Agila and others 2013). SIFT-MS has the advantage of requiring minimal sample preparation, providing real-time analysis of complex mixtures of volatile compounds using 3 positive precursor ions (H_3O^+, NO^+, and O_2^+) that quickly ionize volatile compounds in the sample headspace, without interacting with major components of air, such as N_2, O_2, H_2O, and CO_2 (Španěl and others 1999; Agila and others 2013). SIMCA analysis allowed differentiating honeys from Ohio and Indiana, but the same was not so evident regarding botanical origin because only Ohio wildflower honey was classified as being different from the other Ohio honeys. Nevertheless, caution should be taken when considering these results due to the small number of samples analyzed ($n = 10$).

Other studies have also attempted to use single or multiple volatile compounds for the identification of the geographical origin of honey. In this respect, 1-penten-3-ol was suggested as a specific volatile compound of English honeys (Radovic and others 2001). However, the restricted number of analyzed samples, namely 3 from England, 1 from Denmark, 2 from Portugal and 2 from Spain, precludes drawing any conclusions. Besides, for classification purposes a group of aroma constituents is more often used rather than a single compound. For example, furfuryl mercaptan, benzyl alcohol, δ-octalactone, γ-decalactone, eugenol, benzoic acid, isovaleric acid, phenylethyl alcohol, and 2-methoxyphenol were reported to be particularly important volatile compounds in the differentiation of Brazilian honeys (Moreira and others 2002). Although some studies attempted to use volatile compounds for the determination of the geographical origin of honey, most of them so far concluded that they do not provide, generally, enough information for such purpose, even when considering the ratio of enantiomeric compounds (Špánik and others 2014).

Organic acids. Organic acids are present in honey in small proportions (<0.5%), but they make an important contribution to some organoleptic, physicochemical and biological properties that are related to color and flavor, pH and acidity, and antimicrobial and antioxidant activities (Mato and others 2006b). Among the organic acids, gluconic acid, produced from glucose by the action of glucose-oxidase, is considered as being the most important and abundant in honey. Nonetheless, several other organic acids have been reported to be naturally present in honey, including acetic, butyric, oxalic, citric, formic, lactic, malic, citramalic, maleic, pyroglutamic, succinic, tartaric, and fumaric acids, among others (Wilkins and Lu 1995; Mato and others 2006b; Tezcan and others 2011; Haroun and others 2012). The increase of total acid content has been associated with fermentation phenomena and aging/storage (Mato and others 2006a). Additionally, organic acid profiles have also been suggested by some authors as being useful to assess the botanical and/or geographical origin of honey (Daniele and others 2012; Haroun and others 2012). Citric acid content has been referred to as a useful parameter to differentiate floral honey from honeydew honey (Suárez-Luque and others 2002). Wilkins and Lu (1995) identified 32 aliphatic dicarboxylic acids in monofloral honeys from New Zealand, and they suggested that *O*-methylmalic and 4-hydroxy-3-methyl-trans-2-pentenediocic acids could be used as floral markers for *Knightia excelsa* honeys.

Besides naturally occurring organic acids, the presence of some of them in honeys, in particular formic, oxalic and lactic acids, could also be attributed to their use against *Varroa* parasite, as an alternative to synthetic acaricides. Nevertheless, different studies have shown that these compounds are eliminated over time (Mato and others 2006a), without affecting the quality of honey, if they had been properly applied and in a timely manner (Bogdanov and others 1999, 2002).

The use of organic acids for honey authentication purposes has been suggested based on the approach of SCIRA (Daniele and others 2012). As already mentioned, when using SCIRA to assess the addition of syrups to honey, the carbon isotope ratio should also be determined in the extracted proteins. Considering that pollen is the main source of proteins in honey, its use as internal standard is not adequate if a honey has been filtered or when honey is not saturated with its own pollen, leading to misinterpretation/misclassification of authentic honeys. To overcome this problem, Daniele and others (2012) proposed to use the $^{13}C/^{12}C$ isotopic ratio of the acidic fraction of honey, replacing the use of proteins as internal standard, which could be especially useful when applied to ultra-filtrated honeys.

Proteins. Honey contains different minute amounts of proteins, including several enzymes originating from nectar and pollen, and from the secretions of cephalic glands of worker honeybees (Bilikova and Simuth 2010). To date, the studies on honey proteins

are still very limited, though enzyme content has been used in different countries as a quality indicator (Chua and others 2013). Still, some studies have been published, suggesting that honey proteins are mostly originated from the salivary and hypopharyngeal glands of bees and that honey proteome can be successfully used to differentiate between bee species (Chua and others 2013). Lee and others (1998) studied the major proteins of honeys produced by *Apis mellifera* and *A. cerana* and concluded that they have different molecular weights. Similarly, Won and others (2008) analyzed different honeys produced in Korea from *A. mellifera* (European honeys) and from the oriental bee *A. cerana* (Korean Native honey). Contrarily to *A. mellifera* honey, which can be obtained many times per year, Korean Native honey is very prone to be adulterated because it is harvested once a year and has a higher economic value. After honey centrifugation for pollen elimination, Won and others (2008) studied the major proteins in both types of honey by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) and MALDI-TOF MS, confirming that major proteins in honeys from distinct bee species have different molecular weights. A methodology based on the co-electrophoresis of artificial marker proteins with the same molecular weights of both the identified major proteins (56 kDa and 59 kDa) was proposed as a suitable approach for the discrimination of honeybee species in honey (Won and others 2008). Later, based on the immunological differences between the major proteins of both types of Korean honey, the same authors developed an enzyme-linked immunoassay (ELISA) for the discrimination of bee origin using polyclonal antibodies raised on white rats injected with the purified proteins extracted from honey (Won and others 2009). More recently, Ramón-Sierra and others (2015) proposed that the electrophoretic patterns obtained from the SDS-PAGE analysis of honey proteins could be used to determinate the entomological origin of honey, namely to differentiate honeys produced by *A. mellifera* and from the stingless-bees (*Melipona beecheii* and *Trigona* spp.).

According to some authors, honey proteins arising from pollen and/or as a result of the enzymatic reaction between bee saliva and plant pollen can also be considered useful markers for honey floral classification, when considering different types of honey produced by the same bee species (Baroni and others 2002). Baroni and others (2004) developed a sensitive ELISA for the evaluation of sunflower pollen in honey. After the isolation of two 33 to 36 kDa proteins, identified as being characteristic of sunflower pollen, they were used as coating antigens in a competitive ELISA. The results showed that the method was able to quantify sunflower pollen in honey down to 10%, with a linear response between 10% and 90%.

Šimúth and others (2004) reported that apalbumin 1, the major protein of royal jelly, is the dominant protein of honey. Subsequently, the same group developed an immunochemical method for the quantification of apalbumin 1 in honeys of different botanical and geographical origins, attaining an adequate sensitivity (Bilikova and Simuth 2010). A total of 25 monofloral honeys from 5 botanical origins (acacia, linden, rapeseed, dandelion, and chestnut honeys) and 2 honeybee lines and geographical origins (*A. mellifera ligustica* from Italy and *A. mellifera carnica* from Slovakia) were evaluated for their apalbumin 1 contents. The results showed differences mainly associated with the botanical origin of honey because no changes in apalbumin 1 contents were found for monofloral honeys produced in different regions or by different beelines. However, the authors concluded that the differences observed were not so significant to use them for the botanical origin determination of honey. The authors also concluded that low levels of apalbumin 1 in honey (below 50 μg/g) indicate adulteration with industrial glucose syrups or feeding the bees with sucrose syrup. Accordingly, the developed ELISA was proposed as a tool for preliminary testing for honey authenticity in what concerns adulteration by syrup addition (Bilikova and Simuth 2010). Di Girolamo and others (2012) analyzed different honeys (chestnut, acacia, sunflower, eucalyptus, and orange) for their proteome content to investigate whether plant proteins could be detected and used for the differentiation of botanical origin. Eight proteins were identified after SDS-PAGE separation and mass spectrometry analysis, showing that most of them were of animal origin and belonged to *A. mellifera* proteome, with only 1 protein being of plant origin (glyceraldehyde-3-phosphate dehydrogenase from *Mesembryanthemum crystallinum*). Five of the identified proteins belonged to the family of major royal jelly proteins and were also the most abundant in honeys. This finding suggests that honey proteome resembles the royal jelly proteome, although containing a considerably lower number of different proteins. The presence of major royal jelly proteins (MRJP) was also recently reported in acacia, tualang, and gelam honeys from Malaysia (Chua and others 2015). According to mass spectrometry results, MRJP-1 was the most abundant protein, particularly in acacia honey samples, with other proteins also being detected (including MRJP-2, MRJP-5, and MRJP-7), as well as a few uncharacterized proteins from *A. mellifera* (Chua and others 2015).

The use of protein barcoding for geographical origin determination was proposed by Wang and others (2009). The authors developed a fast and reliable method based on MALDI-TOF MS analysis that included 3 steps: rapid honey protein extraction, mass spectrometry analysis, and transformation of protein mass spectra into barcodes with the MALDI Biotyper 1.1 software. Protein fingerprints of 16 honeys collected in Hawaii were used to generate a database library of spectral barcodes that was further used to differentiate the geographical origins of 38 commercial honeys (including 15 labeled as being of Hawaii origin) based on pattern matching. Match scores obtained by comparing the masses and signal intensities of proteins from test samples with those in the database library were used to rank the confidence of the results obtained from commercial honey samples. High correlation values were obtained for Hawaii honeys and for 1 sample labeled as being from Canada and 2 from New Zealand. In general, the method allowed differentiating honeys according to their geographical origin since samples from Hawaii clustered together. The small number of samples ($n = 16$) used to construct the database library might explain those misclassifications, thus it is advisable to extend its range to increase the reliability of the method.

Amino acids. Amino acids present in honey are derived from the bees and from the plants visited by the bees, considering that the pollen is the most important source of proteins and the bees the source of free amino acids. The relevance of amino acids in honey regards their function as building blocks for proteins and as intermediates in the protein metabolism, besides their role as precursors for the production of some key flavor compounds (Janiszewska and others 2012).

The determination of an amino acid profile in honey is commonly performed by gas or liquid chromatographic methods (Janiszewska and others 2012). Spano and others (2009) used a RP-LC method with phenylisothiocyanate (PITC) precolumn derivatization to identify 14 free amino acids in strawberry-tree honey samples, with proline (65.63%) being the most abundant, followed by glutamic acid (6.49%), arginine (5.21%), alanine (5.17%), and phenylalanine (4.97%). Paramás and others (2006) optimized

a methodology using *o*-phthaldialdehyde (OPA) derivatization reagent in HPLC with fluorescence detection, which has made feasible the separation and quantification of 23 amino acids in bee pollen and Spanish monovarietal honey samples from ilex, oak, heather, and chestnut-tree. In all the evaluated honeys and pollen, proline was found to be the major amino acid. Similar results were reported by Hermosín and others (2003) for Spanish rosemary, eucalyptus, lavender, thyme, and orange blossom honeys, for which the main amino acids were found to be proline, phenylalanine, tyrosine, and lysine. In some samples, the content of phenylalanine was higher than that of proline. Lavender honey samples were differentiated from those of rosemary and thyme due to valine, α-alanine, and a high content of tyrosine, but rosemary, thyme, and orange blossom honey were very similar to each other. Rebane and Herodes (2008) evaluated several Estonian honeys from heather, dandelion, linden, rape, willow, phacelia-sweetclover, rosacean, and polyfloral origin, also identifying proline as the main amino acid, while sulfur-containing amino acids (Met and Cys) were not present. In opposition, Kivrak (2015) evaluated 58 monofloral honeys from 17 different botanical origins (cedar, eucalyptus, vitex, carob, clover, honeydew, sunflower, citrus, heather, thyme, flower, chestnut, sideritis, acacia, lavender, cotton and mad) from various regions across Turkey, using UPLC–ESI–MS/MS methodology, and they reported that phenylalanine was the major amino acid in almost all samples, followed by proline, tyrosine, and isoleucine. Acacia and heather honeys were also evaluated by Janiszewska and others (2012), together with raspebery, buckwheat, and goldenrod honeys collected from Poland. In this study, proline was found to be the major amino acid, with phenylalanine having much lower values in all the honeys evaluated, being the second major amino acid only in heather and goldenrod honeys. Although high concentrations of aspartic acid and asparagine were suggested as markers of raspberry and buckwheat honeys, the authors concluded that the variation in their composition was so large that amino acids cannot be used to clearly determine the botanical origins of Polish honeys (Janiszewska and others 2012).

As most of the amino acids are present at trace levels and the amount of proline could be used to represent the total amino acid content (Anklam 1998), only the proline content is nowadays included in the EU food laws for the quality parameters of honey (Chua and others 2013). Regarding authentication issues, even though some authors suggest that amino acids can be used for botanical origin identification, others have demonstrated that, in spite of the differences among honeys, their high variability disables its use as markers of plant origin (Hermosín and others 2003; Carratù and others 2011; Janiszewska and others 2012; Schievano and others 2015).

Minerals and trace elements. Honey contains a wide variety of minerals and trace elements, which depend on botanical and geographical origins. The mineral content of honey arises from the environment and soil by means of absorption by the flora from which bees collect nectar (Stankovska and others 2008). Many of the minerals present in honey, such as P, Fe, Al, Mg, Cu, Mn, Si, Cl, Ca, K, and Na are essential for human health (Stankovska and others 2008). Considering that honey is a highly consumed natural product, the determination of minerals and trace elements is of great interest for quality control and nutritional aspects. Besides, high levels of some elements, such as lead and cadmium, can be dangerous and cause toxicity (Moniruzzaman and others 2014). Honey has been recognized as a biological indicator of environmental pollution (Przybyłowski and others 2001) because the bees, during the collection of pollen, are in contact with the air, soil, and water of the surrounding area of their apiaries. As a result, the concentration of some trace elements in honey can reflect the level of contamination in the region (Ioannidou and others 2005; Moniruzzaman and others 2014).

Honey was described to contain between 0.04% and 0.2% (w/w) of trace elements (Anklam 1998). A large range of variation has been observed for the mean values of total mineral content among different honey types. However, most studies report K as being the main mineral element, with others, namely Ca, Na, and Mg being also present at high levels in some honeys (Nalda and others 2005; Pisani and others 2008; Vanhanen and others 2011; Uršulin-Trstenjak and others 2015). Several authors also suggested a general correlation between honey color and mineral composition, with lighter honeys, such as rosemary and lavender, having lower mineral contents compared to darker ones, such as heather and chestnut (Nalda and others 2005; Pisani and others 2008).

During the last decades, improvements in high-resolution methods and instrumentation allowed more comprehensive and detailed analyses of minerals and trace elements in honey (Kaškonienė and Venskutonis 2010). Different electroanalytical methods were used for this purpose, including potentiometric stripping voltammetry (Muñoz and Palmero 2006), differential pulse anodic stripping voltammetry (Sanna and others 2000), or ion chromatography and voltammetry (Buldini and others 2001). Nevertheless, atomic absorption spectrometry (AAS) proved to be the most frequently chosen method, being quite often used to assess minerals and trace elements in honey (Taddia and others 2004; Erbilir and others 2005; Hernández and others 2005; García and others 2005; Tuzen and others 2007; Silici and others 2008; Stankovska and others 2008; Alda-Garcilope and others 2012). More recently, other techniques, including inductively coupled plasma atomic emission spectrometry (ICP-AES) (Devillers and others 2002; Fernández-Torres and others 2005; Ioannidou and others 2005; Ozcan and Al Juhaimi 2012), inductively coupled plasma mass spectrometry (ICP-MS) (Chudzinska and Baralkiewicz 2010; Chudzinska and Baralkiewicz 2011; Chen and others 2014; Baroni and others 2015), and total reflection X-ray fluorescence (Golob and others 2005; Ribeiro and others 2015) are also being increasingly used to assess minerals and trace elements in honey. Although they proved to be effective and capable of identifying and differentiating trace elements in honey, some authors used a combination of 2 or more distinct techniques to analyze different minerals and trace elements separately. In this sense, Madejczyk and Baralkiewicz (2008) used ICP-MS and flame furnace AAS to determine trace elements and major elements, respectively. More recently, Moniruzzaman and others (2014) analyzed Ca, Mg, Fe, and Zn by flame AAS, and Na and K by flame atomic emission spectrometry (AES), and trace elements such as As, Pb, Cd, Cu, and Co were analyzed by graphite furnace AAS.

As mentioned, advances in instrumentation have provided a detailed mineral characterization of honey samples, which has then been used to assess the possibility of differentiating them according to their elemental composition. Some minerals, such as Na and K, are considered to be of floral origin because they are accumulated in the plant cells and depend on its enzyme content, while others are more associated with soil or environmental contamination. As a result, the composition of minerals and trace elements has been suggested as a useful parameter for the identification of both botanical and geographical origins of honey. Chen and others (2014) evaluated the potential of mineral elements

and chemometrics to classify Chinese honeys according to their botanical origin. For that aim, the content of 12 minerals in 163 honey samples from 4 botanical origins was evaluated and a back-propagation artificial neural network was explored to construct a classification model. After model training and cross-validation, an independent set of 42 honey samples was tested, showing a global prediction ability of 97.6%, with all samples being correctly classified, with the exception of 1 acacia honey that was classified as being rape. The use of quantitative and qualitative mineral profiles coupled to chemometric analysis, as a tool to distinguish the botanical origin of honey, was also suggested by Chudzinska and Baralkiewicz (2010) and Oroian and others (2015). Besides botanical origin, the mineral profiles and trace elements in honeys can also be used to verify geographical origin because of being largely affected by the regional environment and anthropogenic contamination (Wang and Li 2011). Di Bella and others (2015) studied 39 honey samples of different botanical origins (orange-blossom, lemon-blossom, chestnut, eucalyptus, acacia, sulla, and wildflower honeys) collected in distinct locations in Italy (Sicily and Calabria) to establish their geographical origins. An attempt for their differentiation was carried out by PCA of mineral composition data, which allowed differentiating between Sicilian and Calabrian honeys, despite their botanical origins. Conversely, samples were not separated based on botanical origin.

In addition to mineral composition, isotope ratio analysis can also bring relevant information concerning the geographical origin of food matrices, including (Schellenberg and others 2010). In particular, strontium isotopes in plants and in animals, which are fed with them, are related to the $^{87}Sr/^{86}Sr$ signature of the bioavailable strontium derived from water and soil (bedrock) (Baroni and others 2015). Accordingly, multielemental composition determined by ICP-MS together with isotopic signatures were used to develop a reliable fingerprint to assess the geographical origin of Argentinian honey (Baroni and others 2015). In addition to honey, samples of soil and water were also collected from 3 regions included in the study (Córdoba, Buenos Aires, and Entre Ríos). Besides considering data from the analysis of 31 elements, the ratios K/Rb and Ca/Sr were also included since the first can greatly differ among various types of rocks and soils, while the last can be used as a chemical tracer in geochemistry and bioavailability studies. Honey samples from the 3 regions were differentiated by classification trees and discriminant analysis using a combination of 8 key variables (Rb, K/Rb, B, U, $^{87}Sr/^{86}Sr$, Na, La, and Zn). Furthermore, this study clearly demonstrated that elemental and isotopic honey compositions are related to soil and water characteristics of the location of origin since the application of canonical correlation analysis and generalized procrustes analysis showed 91.5% consensus between soil, water, and honey samples, besides the clear differences between the 3 studied regions. Bontempo and others (2017) studied 265 honey samples of different botanical origin (polyfloral, citrus, rhododendron, eucalyptus, acacia, chestnut and honeydew) from Italy based on the determination of stable isotope ratios using IRMS and mineral element content using ICP with optical emission spectroscopy. The authors confirmed that the botanical origin was the most important factor affecting the isotopic and elemental characteristics of honey and that the initial results of combining isotopic and elemental profiles were shown to be encouraging with regard to geographical differentiation between botanical species. Therefore, the use of multielemental profile coupled with isotope ratio analysis seems to be a very promising approach for the discrimination of honeys from different geographical origins.

DNA markers. Considering the fact that plant DNA molecules are present in honey and that they possess high stability under environmental and production conditions, DNA-based methods can be considered as reliable and accurate tools to investigate the origin of pollen in honey (Cheng and others 2007; Galimberti and others 2013). Those methods allow reducing the time of analysis and increasing the level of species discrimination, thus representing suitable alternatives for botanical and geographical honey identification (Hawkins and others 2015).

Nonetheless, honey is a very complex matrix, mainly composed of different sugars and other substances, such as organic acids, polyphenols, pigments, enzymes, and such solid particles as waxes, which might interfere with DNA analysis. Furthermore, honey has only low amounts of target DNA since pollen is present at very low levels (Soares and others 2015). Therefore, the selection of an adequate DNA extraction protocol is a critical step and has to be optimized to ensure a sufficient amount of DNA, free of polymerase chain reaction (PCR)-inhibiting substances to accomplish a reliable and reproducible analysis (Guertler and others 2014; Lalhmangaihi and others 2014). For these reasons, some authors have suggested a sample preparation step to isolate pollen particles and eliminate undesirable compounds, such as sugars and flavonoids, prior to DNA extraction (Cheng and others 2007; Laube and others 2010; Soares and others 2015). Soares and others (2015) compared several sample pre-treatments in combination with different DNA extraction methods. The results showed different yields and purities for the tested combinations, but the treatment that included a mechanical pollen disruption step provided the best DNA yields.

Several studies have reported the DNA extraction of pollen from honey samples for subsequent botanical identification. The use of the DNeasy Blood and Tissue Kit (Qiagen GmbH) was reported with the aim of detecting DNA from different plant species in honey samples (Laube and others 2010; Valentini and others 2010). The classical CTAB-based method was also used for the extraction of DNA from honey by Jain and others (2013), obtaining extracts with adequate quality for honey traceability and molecular identification. Guertler and others (2014) reported a comparative study of a previously developed automated DNA extraction method (Guertler and others 2013), which was modified to cope with honey as a very complex matrix, using a manual CTAB buffer-based DNA extraction procedure, previously proposed by Waiblinger and others (2012). The study of Guertler and others (2014) combined a thorough CTAB buffer-based DNA extraction with the efficient DNA purification by the use of a Maxwell® 16 instrument. The results showed that automated DNA extraction was faster and resulted in higher DNA yield and sufficient purity, as well as equal or slightly lower Cq (quantification cycles of real-time PCR) values for all tested honey samples compared with the manual extraction method. In order to develop a simple and efficient method for the DNA extraction with a more reduced sample amount when compared with previous studies, Lalhmangaihi and others (2014) performed a conventional phenol-chloroform DNA isolation method. They DNA extracts were obtained with sufficient quality and amount for PCR amplification and sequencing of genetic markers routinely used for plant barcoding. To search for the best approach to isolate pollen DNA from honey, Soares and others (2015) compared 5 DNA extraction methods (NucleoSpin Plant kit (methods A and B), DNeasy Plant Mini Kit, CTAB-based and Wizard) combined with 3 different sample pretreatments. The Wizard method showed generally higher DNA yields, purity, and amplification capacity, mainly when combined

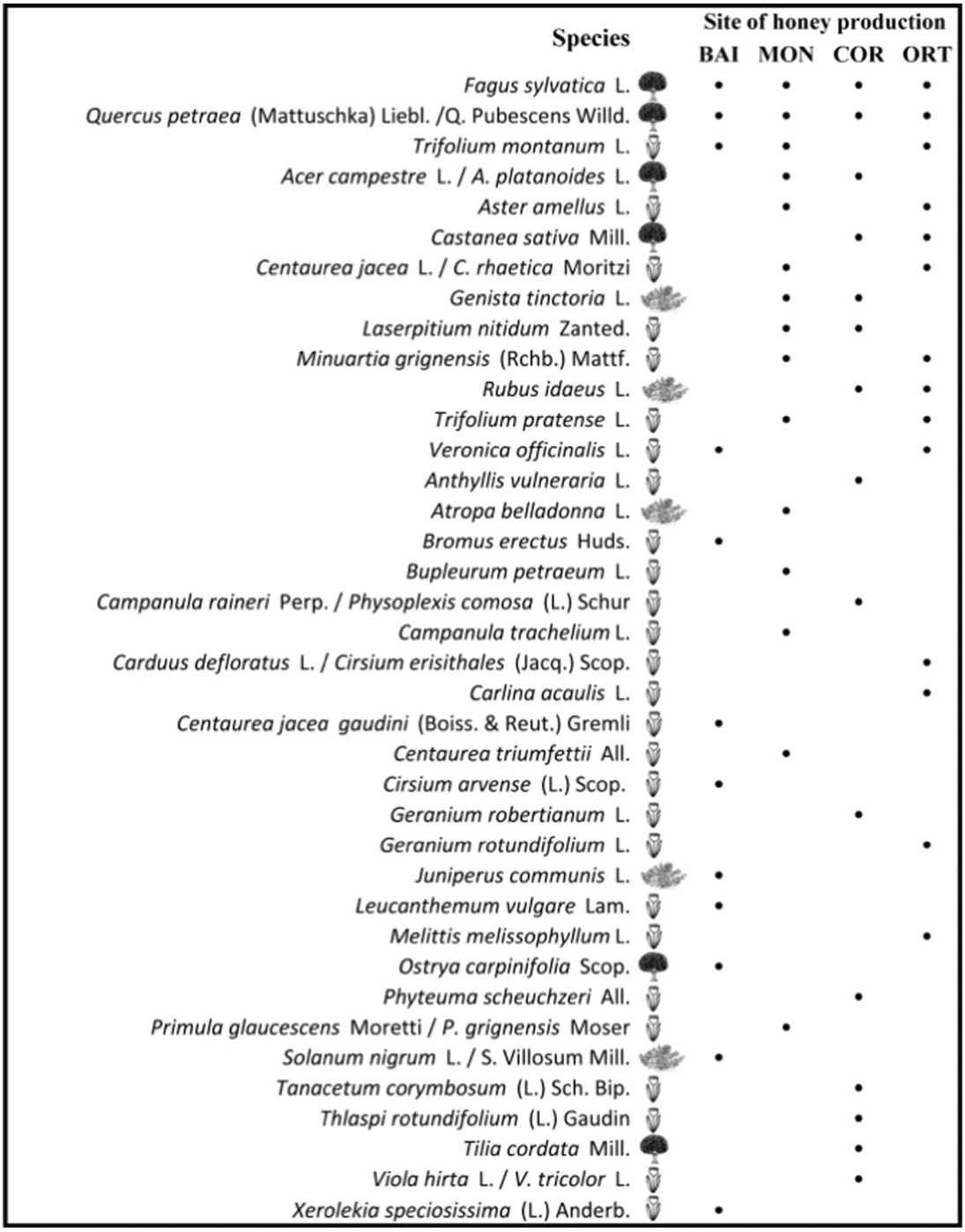

Figure 3–Dot blot distribution of plant species in the 4 tested honeys (multifloral honey from Northern Italy). The plant typology (tree, flower, and shrub) and the collection sites are indicated. BAI, Baita Amalia; MON, Alpe Moncodeno; COR, Cornisella; ORT, Ortanella. Reprinted from Bruni and others (2015) with permission from Elsevier Ltd.

with a mechanical disruption step of pollen to improve DNA yield. The CTAB and DNeasy methods were also successful, though with considerable lower yields, because both allowed clear amplification of heather DNA from the monofloral heather honey (Soares and others 2015).

Lately, a few DNA-based methods have been proposed for pollen identification. Laube and others (2010) successfully developed a combination of species-specific real-time PCR assays with TaqMan^{TM} probes for the detection of relevant species in Corsican honey (acacia, broom, citrus, clover, heather, eucalyptus, lavender, linden, oak, olive, rape, rockrose, rosemary, sunflower, and sweet chestnut). The authors were able to establish a plant species profile that was unique for Corsican honey when compared to honeys from Galicia, Germany, and England. Wilson and others (2010) investigated pollen foraging in endemic Hawaiian *Hylaeus* bees based on PCR followed by Sanger sequencing. Despite the

availability of pollen from multiple plant species, the authors revealed a high fidelity in pollen foraging.

Currently, DNA barcoding has been given increasing interest for taxonomic identification, having been recently suggested for the identification of plant and animal species in honey (Schnell and others 2010; Prosser and Hebert 2017). Among several barcode regions, the plastidial genes *mat*K and *rbc*L, as well as the internal transcriber spacers (ITS1 and ITS2) and the intergenic spacer *trnH-psbA* have been proposed for the differentiation of plants (CBOL Plant Working Group 2009; Hollingsworth and others 2011). Schnell and others (2010) used the approach of DNA barcoding based on PCR amplification followed by cloning and Sanger sequencing, and real-time PCR to characterize both plant and animal DNA present in honey. The authors targeted the *rbcL*, *adh1*, ITS1, and ITS2 sequences for plants and *COI* gene for animals. The results were promising, since they were able to identify some plant families combining the studied plant targets (for example, Brassicaceae, Fabaceae). Besides, they identified the honey bees as being *Apis mellifera ligustica* in all samples. Bruni and others (2015) targeted the *rbc*L and *trnH-psbA* plastid regions to identify the plant origins of 4 multifloral honeys from northern Italy. Based on the generation of an extensive reference database of local flora to determine the botanical origin of honey, 39 plant species were identified in the 4 honey samples, mostly from a mix of common plants belonging to *Castanea, Quercus, Fagus*, and other herbaceous taxa (Figure 3).

With the recent developments in high-throughput sequencing platforms, the so-called next-generation sequencing (NGS), combined with the use of universal markers has given rise to the approach of DNA metabarcoding (Hawkins and others 2015). This approach has been applied to identify and quantify airborne pollen based on a fragment of the *trn*L barcode (Kraaijeveld and others 2015). Keller and others (2015) and Richardson and others (2015) used ITS2 metabarcoding to characterize pollen collected from honey bee colonies, showing that it was superior for qualitative analysis than microscopy. Valentini and others (2010) exploited DNA metabarcoding targeting a *trn*L fragment to obtain the identity of plants in 2 commercial honeys (Pyrenean and wild flower), demonstrating the consistency of results with the type of sample. To investigate honeybee foraging, Hawkins and others (2015) assessed the potential of *trn*L metabarcoding to characterize the floral composition of honey. The results highlighted that DNA metabarcoding provided much better repeatability than melissopalinology, though without quantitative analysis, and with the pollen less likely to be detected if present at low levels. Prosser and Hebert (2017) exploited the DNA metabarcoding of 3 regions (ITS2, *rbc*La and *COI*) to identify the botanical and entomological origins of 7 honey types: light, medium dark, blended, pasteurized, creamed, and meliponine. They were able to identify both plant and insect sources in 5 samples, but 2 were misinterpreted. The authors highlighted as limitations of the methods such cases as filtered honey or the addition of pollen from the desired plant.

Nontarget approaches. In face of the increasing frauds concerning honey and products thereof, there is a growing need for simple, rapid, cheap, and reliable techniques that can be used for screening purposes and food authenticity surveillance. In the last few years, different research projects have been dealing with this issue and have already demonstrated the feasibility of using nontarget fingerprinting approaches as comprehensive tools for authenticity assessment (Riedl and others 2015). Nevertheless, the uptake and implementation of such techniques into routine analysis and official control is still very limited, which can be explained

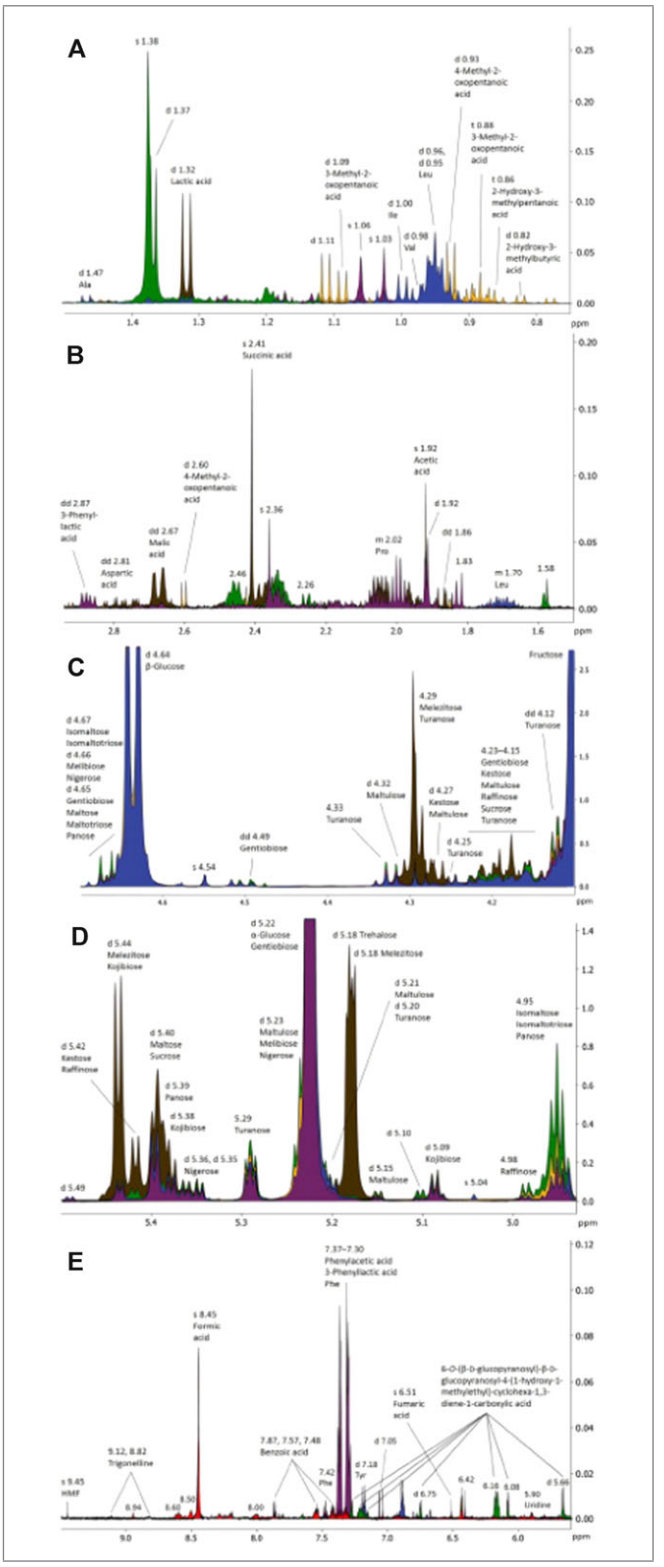

Figure 4–Highlighted NMR fingerprints for buckwheat (blue), dandelion (yellow), heather (purple), honeydew (brown), linden (green), and lingonberry (red) honeys. Spectral regions (A) 1.50 to 0.75 ppm, (B) 2.94 to 1.50 ppm, (C) 4.70 to 4.10 ppm, (D) 5.50 to 4.93 ppm, and (E) 9.50 to 5.60 ppm. Abbreviations: Ala, alanine; HMF, 5-hydroxymethylfurfural; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Pro, proline; Tyr, tyrosine; Val, valine. Peak multiplicities: s, singlet; d, doublet; dd doublet of doublets; m, multiplet; t, triplet. Reprinted from Kortesniemi and others (2016) with permission from Elsevier Ltd.

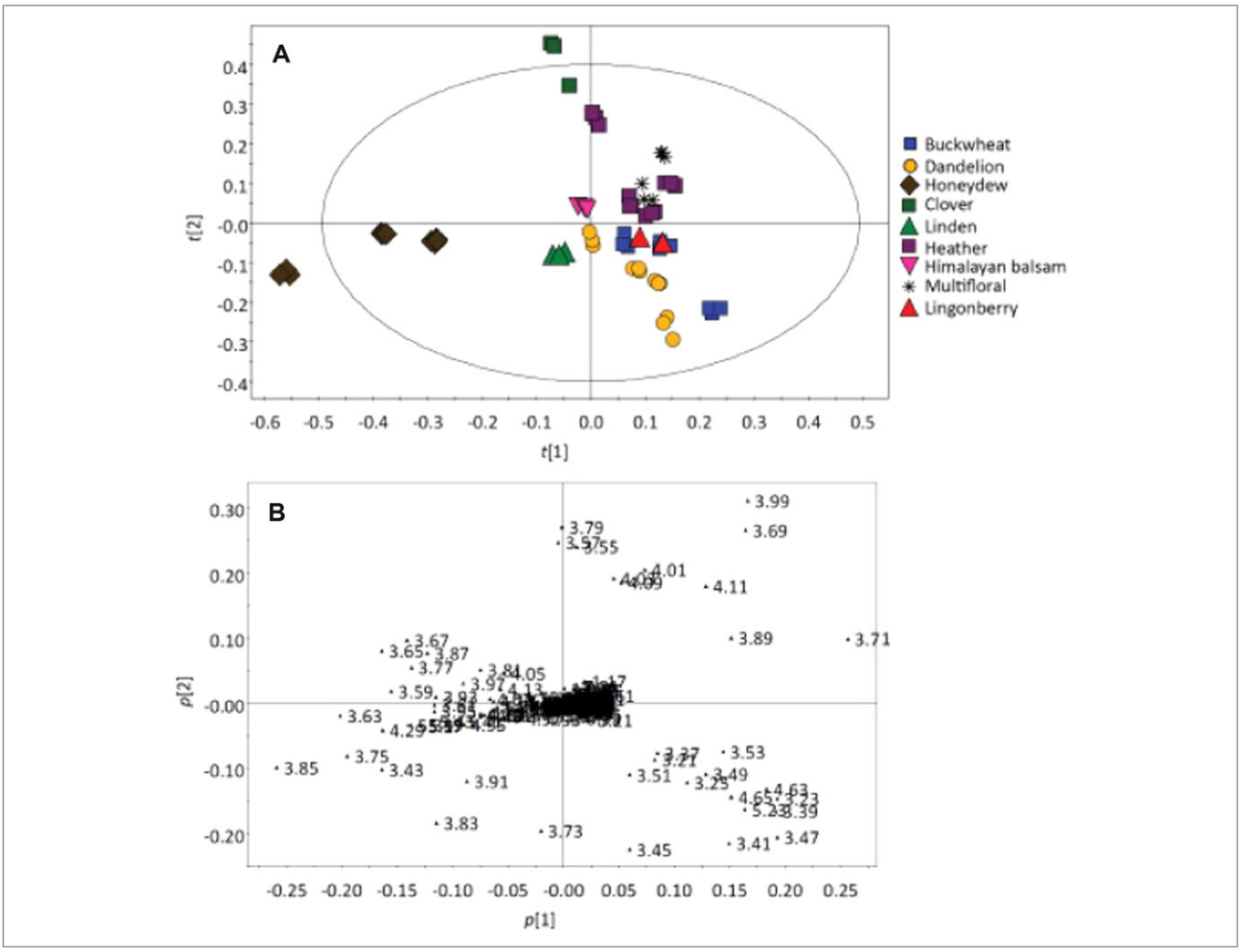

Figure 5–PCA model (t[2] vs. t[1]). The $R^2X_{(cum)}$ and $Q^2_{(cum)}$ for the model were 0.99 and 0.96, respectively. (A) Scores plot (R^2X[1] = 0.50 and R^2X[2] = 0.33). The legend indicates the honey type. (B) Loadings plot. Reprinted from Kortesniemi and others (2016) with permission from Elsevier Ltd.

in part by the fact that several studies have been conducted as proof-of-principle studies and by the on-going need for validation, harmonization, and standardization of nontarget approaches.

Among food fingerprinting techniques, nontarget spectrometric and spectroscopic chemical approaches, followed by the application of multivariate statistical analysis, have been successfully applied for the detection of adulterations in different food matrices (Riedl and others 2015). Spectroscopy, in particular, presents several advantages over target approaches, namely its overall simplicity (with relatively easy sample preparation and suited to be used by untrained personnel), rapidity, nondestructive character and, generally, low to moderate cost of instrumentation. The use of spectroscopic techniques, including NMR, NIR, and MIR, has been reported in several studies for honey characterization and botanical origin identification, taking in consideration the above advantages over traditional methods, such as melissopalinology (Ruoff and others 2005, 2007; Cozzolino and others 2011; Ohmenhaeuser and others 2013). Aiming at differentiating botanical origins of Turkish honeys, Gok and others (2015) used ATR-FTIR spectroscopy to compare honey samples based on their spectral differences in the 4000 to 650 cm^{-1} region. In addition to 120 honeys from different botanical origins, 24 "fake" samples, including maple syrups, grape molasses, and adulterated honey, were also included in the study. The vector-normalized first derivative of each spectrum in the range of 1800 to 750 cm^{-1} was used as input data, and PCA and hierarchical cluster analysis were applied for sample classification. The results showed variations in the spectral parameters associated with the diverse botanical origins and that could be possibly explained by the differences in water, carbohydrate, and protein contents. However, for some honeys, the number of evaluated samples was low and the proposed model was not submitted to any kind of validation.

The use of NMR spectroscopy for the botanical origin differentiating of honey has also been suggested in different studies. Schievano and others (2010) used the ^{1}H NMR spectra of organic extracts as a "fingerprint" to differentiate the botanical origins of 118 distinct honeys (chestnut, acacia, linden, and multifloral). Based on PCA and supervised techniques, namely projection to latent structures by PLS-discriminant analysis, a classification model according to floral origin was obtained with high predictability power. More recently, a higher number of samples (n = 328) was evaluated by a combined NMR spectroscopy (^{1}H and ^{13}C) and chemometric data analysis aiming at differentiating the botanical origins of honey samples (Ohmenhaeuser and others 2013). In this study, high-resolution ^{1}H NMR was also used for the identification and the quantification of 13 selected components in

Table 3–Summary of pros and cons of the analytical methods applied to honey authentication

Parameter/approach	Pros	Cons
Melissopalynology	• Allow a good species identification and differentiation	• Time consuming • Require experts with adequate skills and experience • Unable to apply in filtered honeys or adulterated by pollen addition • Difficult to apply in honeys with "under represented" pollen
Phenolic compounds	• Present in all plants and dependent on botanical origin • Easily transferred to honey trough bees during honey production	• Dependent on the extraction step and detection techniques used • The same compound could be present in more than 1 type of honey
Sugars	• Main components of honey	• Difficult to identify and specify in honey samples
Volatile compounds	• Distinguishable characteristic of honeys • Depending on the source plants of honey origin	• Requires a previous removal of sugars • Questionable results in overheated or long storage honeys • The same compound could be present in more than 1 honey type • Individual volatile compounds can be present in a wide range of concentration
Organic acids	• Depending on the botanical and geographical origin	• The presence of formic, oxalic and lactic acids could be attributed to its use as an alternative to synthetic acaricides against *Varroa* parasite • Pollen is the main source, thus, unable to apply in filtered honeys or adulterated by pollen addition
Proteins	• Differentiation of entomological, botanical and geographical origins • Indirect detection of sugar addition	• No significant differences in the major protein (apalbumin 1) • Requires a large number of samples
Amino acids	• Pollen is the main source of honey amino acids	• Requires the use of the overall amino acid profile to differentiate between specific types.
Minerals and trace elements	• Dependent of the composition of soil and the environment • Determination of botanical and geographical origins • Quality control and nutritional determination because high levels can be dangerous and cause toxicity	• Some metals may be present in honey due to the environmental contamination
DNA markers	• Stability under environmental conditions and production techniques • More reliable and accurate for botanical and geographical origin	• Complexity of honey matrix might influence the DNA extraction • Unable to apply in filtered honeys or adulterated by pollen addition • Difficult to apply in honeys with "under represented" pollen
Nontarget	• Short time of analysis required • Noninvasive technique • Minimal or null sample preparation • Simple procedure	• Require large number of samples • Multivariate data analysis is required. • High-cost equipment is generally required.

honeys. Spiteri and others (2015) presented a large and systematic NMR profiling study, including a worldwide range of honeys from different botanical and geographical origins, to establish a general target and nontarget methodology for honey authentication. The authors used H^1-NMR profiling coupled to suitable quantification procedures and statistical models, with analytical criteria being defined to verify the authenticity of mono- and multi-floral honeys based on a reference data set of more than 800 honeys. The results enabled the identification of typical plant nectar markers in monofloral honeys, with spectral patterns and natural variability being established for multifloral honeys. Based on a statistical comparison with a commercial data set of about 200 honeys, they were able to identify marker signals for sugar syrups and to detect sugar addition down to 10% of spiking levels. Still in the same report, quantifications of glucose, fructose, sucrose, and HMF were performed, and markers showing the onset of fermentation were presented (Spiteri and others 2015). ^{1}H NMR metabolomics was applied to Finnish honeys to determine their botanical origin and the key markers of authentication (buckwheat, clover, dandelion, heather, Himalayan balsam, honeydew, linden, lingonberry, multifloral) (Kortesniemi and others 2016). The fingerprints of some samples are presented in Figure 4, from which the authors highlighted 2-hydroxy-3-methylbutyric, 2-hydroxy-3-methylpentanoic, 3-methyl-2-oxopentanoic, and 4-methyl-2-oxopentanoic acids as specific markers for dandelion (*Taraxacum* spp.) honeys. Full discrimination of buckwheat, dandelion, heather, and honeydew honeys was accomplished with PCA (Figure 5) and orthogonal partial least squares discriminant

analysis (OPLS-DA). In the PCA, melezitose, glucose, and fructose explained 83% of the variation, while the origin-specific nonsaccharide metabolites were the discriminatory compounds in OPLS-DA loadings (Kortesniemi and others 2016).

The use of spectroscopic techniques has also been attempted, with promising results for the geographical origin differentiating of honey. Woodcock and others (2007) evaluated the potential of NIR spectroscopy to confirm the geographical claim of several samples of honey, including 167 unfiltered (88 Irish, 54 Mexican, and 25 Spanish) and 125 filtered (25 Irish, 25 Argentinean, 50 Czech, and 25 Hungarian) honey samples. After a preliminary examination of data using PCA, classification models were obtained by PLS regression and SIMCA. Both developed models showed encouraging results as they produced correct classification rates ranging from 91.7% to 100%, even though better results were achieved with PLS regression (100% correct classification for all honeys, with the exception of 96% for Argentinean filtered samples). Later on, the same authors applied NIR spectroscopy to confirm the geographical origin of honey labeled as authentic Corsican (Woodcock and others 2009). The employed strategy was based on the collection of NIR spectra from authentic Corsican ($n = 219$) and non-Corsican samples from 5 EU countries ($n = 154$), which were mathematically processed to develop a univariate specification based on fingerprint spectroscopy from a multivariate data set (Woodcock and others 2009). The best performing discriminant PLS regression models, developed using cross-validation (a variable selection algorithm and a second derivative data pretreatment), were tested by challenging the model with spectra of separate samples of authentic Corsican and non-Corsican honeys. Using separate calibration and validation samples and a variable selection procedure, the highest correct classification values were 90.4% and 86.3% for Corsican and non-Corsican honey samples, respectively. The results of both studies suggest that NIR spectroscopy coupled to chemometrics could be a suitable approach to verify the geographical provenance of honey, with a high degree of certainty. However, problems might arise if honeys from countries not included in the models are analyzed. In this sense, the inclusion of as many samples as possible from worldwide origins and several production seasons is advisable in order to construct a more robust and accurate model that could be useful as a screening technique.

Recently, the use of electronic nose and tongue has been suggested as promising techniques for honey characterization and authentication (Benedetti and others 2004; Dias and others 2008; Escriche and others 2011, 2012; Dymerski and others 2014). Electronic nose is an analytical tool consisting of an array of weakly specific or broad-spectrum chemical sensors that are intended to mimic the human olfactory system, being able to convert sensor signals to data that can be analyzed with appropriate statistical software. This device provides a rapid monitoring of the volatile components of food, providing real-time information about various characteristics of the matrix under study (Schaller and others 1999; Benedetti and others 2004; Dymerski and others 2014). The usefulness of this type of equipment coupled to chemometrics, such as PCA, ANN, and LDA, as a simple and fast approach for the correct classification of different types of honey has been demonstrated by Benedetti and others (2004) and Dymerski and others (2014). Electronic tongue is a novel device for the analysis of liquid samples, composed of an array of sensors combined with pattern recognition tools, presenting several advantages, such as short time of analysis, simplicity, low cost, noninvasive character, and minimal or no sample preparation. So far, the use of electronic tongue coupled to different multivariate data analysis, such as PCA, PLS, and ANN, for honey characterization and authentication has been successfully described in different studies (Dias and others 2008; Wei and others 2009; Major and others 2011; Escriche and others 2012).

Concluding remarks

Consumer demands for authentic and natural products with beneficial health properties have positioned honey as an important food commodity subjected to numerous studies. The importance of honey starts in its production, for which the bees are responsible, with the whole pollination process vital to global biodiversity. The nectar-providing plant species, the bee species, the geographical area, and the harvesting conditions influence the honey properties and honey classifications. Honeys classified as monofloral, PDO, and PGI honeys are generally perceived as premium and valued products because of their refined and unique flavor and taste. In opposition, the decline of bee populations, the progressive increase of imported honey with lower prices and lower-quality has prompted honey adulteration through incorrect labeling of origin and fraudulent admixing with lower quality honeys or with sugar syrups. Accordingly, honey authentication addresses several issues that are mainly related to its production and origin, which have been the subjects of a huge number of studies, focusing mainly on several chemical markers and quality control parameters. The legislation regarding honey suggests composition criteria (sugar content, moisture content, water-insoluble content, electrical conductivity, free acid, diastase activity, and HMF content) to determine the quality of honey. However, the authenticity of honey has been a difficult and challenging task to perform due to the high complexity of the matrix and due to the different types of adulterations possible.

Regarding adulterations with sugar addition to increase honey production or overfeed the bees, SCIRA has been used as an official analytical method in many countries based on the fact that monocotyledonous plants (C4), such as sugar cane or corn, and dicotyledonous (C3), the main plants used as nectar sources, have distinct carbon isotopic ratios from different photosynthetic cycles. However, the addition of C3 plant sugars, such as sugar beet or rice syrups is not feasible to detect based on such analysis. As alternatives, several chromatographic approaches have been proposed to determine oligosaccharides with different DP and to establish trace polysaccharide fingerprints. Still as alternatives, the recent advances on spectroscopic techniques have proposed powerful tools to identify adulterations by sugar addition in honey when combined with chemometrics. Despite the advantages of such approaches, a main drawback is the need for large sets of samples.

Although the application of high/uncontrolled heat treatment to honey is not allowed, some producers do it intentionally for pasteurization purposes and/or liquefaction, thereby affecting negatively the quality of honey. Therefore, freshness and overheating are important quality control and authenticity parameters of honey, whose assessment is performed by the standard methods that rely on the determinations of diastase activity and HMF content.

Nowadays, the main concerns related to honey authenticity are focused on the mislabeling of both botanical and geographical origins. Currently, organoleptic properties, physicochemical analysis, and pollen spectrum are the main criteria for honey classification. However, so far, numerous new analytical methodologies based on target and nontarget approaches have been developed and applied to assess the origin of honey. Although several chemical and

molecular markers have been successfully targeted, all of them have their own advantages and limitations for honey authentication (summarized in Table 3). The chemical markers have been widely analyzed by chromatographic techniques. More recently, spectroscopic techniques, namely NMR, Raman spectroscopy and IR in combination with chemometrics have been proposed to target those markers. Moreover, their usefulness has been effectively demonstrated as fingerprint nontarget approaches for the botanical and geographical origin identification of honey. Having in mind the inherent advantages of spectroscopic techniques, whether as target or nontarget strategies, more efforts should be devoted to validate and include them as official methods for honey authentication. Concerning the use of molecular markers based on DNA analysis, recent reports show promising results, which highlight their high potentiality for botanical and even entomological origin identification of honey. The recent advances on high-throughput sequencing platforms (NGS) combined with the DNA barcoding approach offer highly reliable tools to be applied to botanical and entomological authentication of honey.

In summary, several recent advances have emerged, mainly to differentiate the botanical and geographical origins of honey and to detect the addition of syrups. The spectroscopic methods can offer reproducible, low-cost, expedite and simple analysis based on target or nontarget approaches to determine both botanical and geographical origins of honey. However, the initial investment on the equipment can be very high, depending on the instrument, a great number of samples are required for method development and validation, and chemometric approaches are also needed. The DNA-based methods can offer affordable, high specific and sensitive tools, for the botanical origin identification, but they cannot be applied to filtered honeys. Additionally, if based on NGS, it requires the acquisition of high cost equipment and expertise personnel on bioinformatics. Multi-elemental and trace analysis appears to be the most promising approach to discriminate the geographical origin of honey, particularly using ICP-MS, with advantages and drawbacks as for spectroscopic methods. Stable isotope ratio analysis using IRMS is surely the most appropriated approach to detect sugar adulteration in honey. In this last case, as for the botanical and geographical determination, the recommended approach is the combination of at least 2 techniques.

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Author Contributions

Sónia Soares, Joana S. Amaral, and Isabel Mafrawrote the manuscript with critical input and corrections by Maria Beatriz P.P. Oliveira. Isabel Mafra did the final editing. All authors contributed to locating and to interpreting the literature sources.

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