CHAPTER 3

Chemical Composition, Characterization, and Differentiation of Honey Botanical and Geographical Origins

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Contents

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Abstract Botanical and biographical origins of honey are an important issue in food quality and safety. This chapter focuses on use of chemical components to determine botanical and geographical origins of honey. The botanical and geographical origins of the nectar are related with the chemical composition of honey. Honeys can originate from single and multiplant species. In general, the prices of honeys from single plant species are much higher than those of common polyfloral honeys because of consumer preferences. Single and multiple chemicals and components can well indicate the botanical and geographical origins of the honey. Marker chemicals and components include flavonoids, pollen, aroma compounds, oligosaccharides, trace elements, amino acids, and proteins. If multiple chemicals are used as markers, patterns of the chemicals are often used to detect the botanical and geographical origins of honey. Modern statistical software in combination with advanced analytical instrumentation provides high potential for the differentiation of the botanical and geographical origins of the honey.

I. INTRODUCTION

Honey is defined as ''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 honeycombs to ripen and mature'' according to the international standard about honey products such as the Codex Alimentarius (CA, 2010) and the European Community (EU) (EU Council, 2002). The CA standard defines the essential composition and quality in the following aspects. Honey sold shall not have added to it any food ingredient, including food additives, nor shall any other additions be made other than honey. Honey shall not have objectionable matter, flavor, aroma, or taint absorbed from foreign matter during its processing and storage. The honey shall not have begun to ferment or

effervesce. No pollen or constituent particular to honey may be removed except where this is unavoidable in the removal of foreign inorganic or organic matters. In addition, honey shall not be heated or processed to such an extent that its essential composition is changed and its quality impaired. Therefore, honey should be authentic (Bogdanov and Martin, 2002).

Honey is a natural and nutritious food that is produced by honeybees from the nectar and pollen of plants. Honey consists mostly of glucose, fructose, maltose, and sucrose; water; and other minor components including proteins, organic acids, amino acids, vitamins, flavonoids, and acetylcholine (Tewari and Irudayaraj, 2004). In general, pure honey contains extraneous matter, such as pollen, traces of wax, variable amounts of sugar-tolerant yeasts, and dextrose hydrate crystals. Honey containing less than 17.1% water will not ferment in a year. Most honey will crystallize in time unless action is taken to prevent it. The treatment processes of raw honey mainly include controlled heating to destroy yeasts and dissolve dextrose crystals, combined with fine straining or pressure filtration. Honey is usually warmed to 50–60 °C to lower its viscosity; this facilitates extraction, straining, or filtration. This temperature does not affect the honey very much during the relatively short processing period.

Mislabeling and adulteration of honey unfortunately have become a worldwide problem. Adulteration is commonly done by water dilution and addition with sugar and syrups (e.g., corn syrup, high-fructose corn syrup), while adulteration also includes bee feeding with sugars and syrup or artificial honey and deliberately mislabeling the floral or geographical origin. Food authentication is one of the most important issues in food safety and quality control. Regulatory authorities, food processors, retailers, and consumers are interested in knowing the origin and quality of honeys. Honey mislabeling and deliberate adulteration occur from time to time (Cordella *et al*., 2002; Lees, 2003; Sivakesava and Irudayaraj, 2001a,b; Tewari and Irudayaraj, 2004).

Many chemicals in honeys can be used to discriminate the geographical or botanical origin. For example, some researchers utilize fingerprinting of volatile and semivolatile chemicals present in honey to determine its variation with floral origins and honey processes (Anklam, 1998; Benedetti *et al*., 2004; Odeh *et al*., 2007). Saccharides represent the main components of honeys, and many papers have been published for using sugars as an indication of adulteration (Daniel-Kelly *et al*., 2004; Irudayaraj *et al*., 2003; Sivakesava and Irudayaraj, 2001a,b). Enzyme activities have been used to indicate honey botanical origins (Vorlová and Elechovská, 2002). Analyses of fermentation products such as glycerol and ethanol can also give some rough information about the processing of honey, which, however, could not be used to distinguish floral or geographical sources of honeys (Anklam, 1998; Huidobro *et al*., 1993, 1994).

The analysis of minerals and trace elements in honeys can be suitable for the detection of geographical origin, due to the fact that these values are connected very much by environmental pollution (Anklam, 1998; Rodriguez-Otero *et al*., 1994, 1995). Different floral and geographical origins of honeys may contain different organic acids and, therefore, the detection of the organic acid profiles may be helpful in obtaining information on honey floral and geographical sources (Wilkins *et al*., 1995). Carefully evaluating the patterns of phenolic acids, phenolic esters, and aromatic carbonyl compounds may give a good indication of the botanical origin of honeys (Anklam, 1998). Different amino acids in honey were used to detect the geographical source (Davies, 1975, 1976). It is noteworthy that the aforementioned methods have disadvantages to differentiate the geographical origins of honey. For example, the separation of trace volatile compounds from a complex mixture such as honey is very difficult. Moreover, volatile compounds in honey are not very useful for geographical origin differentiation, whereas they can be used to detect botanical origin. Saccharides in honey are suitable for adulteration detection but cannot be used to identify the floral or geographical origins of honeys. Enzyme activities and fermentation products can only give some information about honey processing and storage but are not suitable for the detection of the origin. Analyses of minerals and trace elements in honey can only offer slur discriminants of contaminated region. Profiles of organic acids and amino acids can only give some indirect information of honey origin. Proteins in honey can be used to detect the botanical and geographical origins of honey (Anklam, 1998; Rodriguez-Otero *et al*., 1994, 1995; Won *et al*., 2008).

Because global floral distributions vary among geographic regions, it is more credible to differentiate the botanical and geographical origins of honey based on proteins as markers than other honey compounds. Recently, some papers were published on geographical and botanical origin discrimination of honey based on trace proteins (Baroni *et al*., 2002; Ferreres *et al*., 1993; Lee *et al*., 1998; Marshall and Williams, 1987; Won *et al*., 2008). Marshall and Williams (1987) showed that at least 19 bands of proteins in honeys of different plant origins were detected with silver-staining sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recently, Maier *et al*. (2006) demonstrated the use of matrix-assisted laser desorption ionization tandem time of flight mass spectrometry (MALDI TOF MS) integrated with Biotyper software for fast and reliable classification and identification of bacteria and proteins in clinical diagnostics, environmental and taxonomical research, or food-processing quality control. This technique owes to its advantage of precise measurement, high speed, high sensitivity, and simple operation (Anke *et al*., 2008). Convenient protein fingerprinting treatment allows ones to perform this process for unambiguous identification of proteins in a very

short period of time (Chambery *et al*., 2009). Wang *et al*. (2009) developed a fast and reliable method of geographical origin discrimination of honey using MALDI TOF MS with Biotyper software. A protein profile spectrum can be acquired in a few seconds, and MS spectral data can be readily converted into protein fingerprinting barcodes via Biotyper software. This technique requires minimal sample preparation (Wang *et al*., 2009).

This chapter is primarily concerned with chemical components in honeys for geographical and botanical origin discrimination. Profiles and contents of organic pollutants in honeys have been found to correlate with the pollution status of the geographic origins of the honeys (Wang *et al*., 2010a,b). However, chemical contamination is not covered in this review. Different chemical compositions and analytical methods are compared for differentiation of the botanical and geographical origins of honey. Table 3.1 shows common marker chemicals and components and associated analytical techniques for discriminating geographical and botanical origins of honeys. Marker chemicals for a few unique honeys and associated analytical methods are also listed. References in this chapter were primarily cited from the databases of Elsevier, Springer, American Chemical Society publications, and Royal Society of Chemistry publications via keyword search. A review on a similar topic was recently published during revision of this chapter, which was also cited (Kaškonienė and Venskutonis, 2010). This chapter focuses on the research performed in the past four decades (1970–2010).

II. AUTHENTICITY ISSUES

Food authentication is one of the most important issues in food safety and quality control in every country. Regulatory authorities, food processors, retailers, and consumers are interested in knowing the origin and quality of foods. The authenticity of honey products and authenticity of honey descriptions as geographical, botanical origin, natural, organic, pure, and unheated honey, and so on, are two different aspects. There are excellent reviews on different aspects of honey authenticity (Anklam, 1998; Bogdanov and Martin, 2002; Martin *et al*., 1998; Molan, 1996; Singhal *et al*., 1997). Those reviews mainly focus on examination authenticity topics and the methods used to prove authenticity.

A. Industrial processing of honey

Common honey processing treatments are heat-assisted filtration and centrifugation. Some honey products might be mislabeled such as ''harvested in the cold,'' as honey is naturally harvested between 25 and 35 °C,

TABLE 3.1 Usual chemical compositions as markers and analytical techniques for discriminating geographical or botanical origins of honeys

Chemical markers	Analytical methods	References
Marker chemicals and components for most honeys		
Proteins	SDS-PAGE, MALDI TOF MS, 2-D Gel	Marshall and Williams (1987), Won *et al*. (2008), Wang *et al*. (2009)
Amino acids	HPLC, GC, GC–MS	Pawlowska and Armstrong (1994), Pirini and Conte (1992)
Aroma compounds	GC, GC–MS	Overton and Manura (1994), Bonaga *et al*. (1986)
Carbohydrates	HPLC, FTIR	Daniel-Kelly *et al*. (2004), Irudayaraj *et al*. (2003), Sivakesava *et al*. (2001)
Enzyme activity	Diastase analysis	Rendleman (2003)
Fermentation products	GC, enzymatic assay	Huidobro *et al*. (1993), Zucchi *et al*. (2006)
Flavonoids	LC, LC–MS, CE	Amiot *et al*. (1989), Ferreres *et al*. (1994a,e), Delgado *et al*. (1994)
Pollen	Pollen analysis	Von der Ohe *et al*. (2004), Sesta (2006)
Minerals and trace elements	AES and ICP-AES	Gonzalez-Miret *et al*. (2005), Nozal Nalda *et al*. (2005)
Organic aliphatic acids	LC, LC–MS, GC, GC–MS, enzymatic assay	Wilkins *et al*. (1995), Mato *et al*. (2006a,b), Mato *et al*. (1998a,b)
Phenolic compounds	TLC, GC, CE, LC	Gómez-Caravaca *et al*. (2006), Yao *et al*. (2003), Steeg and Montag (1988)
Stable isotopes	Isotope ratios analysis (H, C, N, S)	Schellenberg *et al*. (2010), Ghidini *et al*. (2006), Anklam (1998)
Marker chemicals for a few unique honeys		
Abscisic acid	LC, LC–MS	Ferreres *et al*. (1996a, 1996b)
Hesperetin and methyl anthranilate	GC, HPLC	Ferreres *et al*. (1994d)
3-Aminoacetophenone	GC	Bonaga and Giumanini, 1986

which is similar to beehive's temperature. Filter mesh sizes are generally not smaller than 0.2 mm to prevent pollen removal during the process. Such commercial honey products should be labeled as ''filtered honey'' according to the international honey regulation. If honeys are heated for pasteurization and liquefaction treatment, the processing would adversely affect honey quality such as the loss of volatile compounds and the reduction of enzyme activities. Pasteurization treatment (~10 min at 63 °C or for 1 min at 69 °C) is often performed to kill osmophilic yeasts. Pasteurization treatment involves rapid heating and cooling (Townsend, 1975). Figure 3.1 shows honey classification based on floral sources, packaging, and processing.

B. Food additives and water removal

Pure honeys are comparatively costly. Some beekeepers may unlawfully use sweeteners to feed bees to increase honey sweetness. Another unlawful act is to directly add sugars into honey products. Some sweeteners that have been used include acid/ inverted sugar syrups, corn syrups, maple syrup, cane sugar, beet sugar, and molasses.

The water content in honey is generally less than 20%. Any excess of water can be removed by centrifugation or vacuum evaporation. If water is artificially added into honeys, it can cause honey fermentation. The water content in honey needs to be in a normal range such as 15.5–18.6% as recommended by the Eastern Apicultural Society of North America.

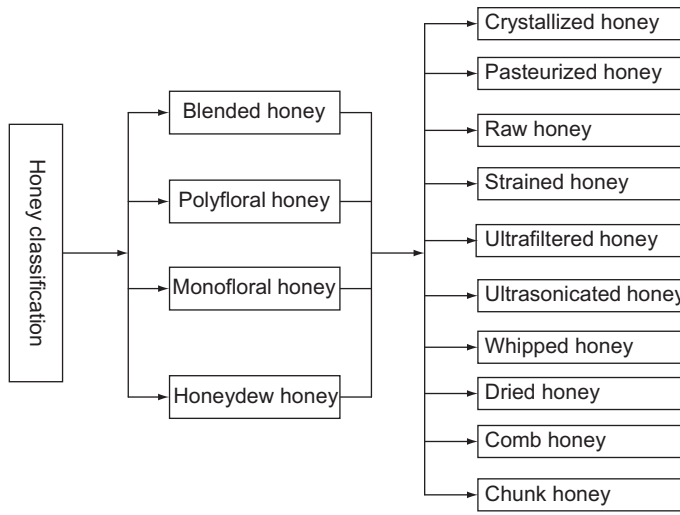

FIGURE 3.1 Honey classifications based on floral sources, and packaging and processing.

Highly diluted honey is unlikely a realistic adulteration practice. Honey water content can naturally be lower than 13.6% or higher than 23% based on the source of the honey, climate conditions, and other factors. Fermentation does not usually become a problem in honeys if water content is less than 18%.

C. Authenticity of botanical origin and mislabeling

Bees get nectars from different flowers, and thus, honey comes from different floral sources. When the botanical sources of honeys are considered to command higher prices, beekeepers may often describe different nectar sources to increase values. It is well known that light honeys such as orange blossom or acacia honey have higher prices than honey blends or other unifloral honeys. Currently, honey floral types are judged based on sensory analysis, pollen and chemical analysis, or a combination of several quality criteria. Honeys are often grouped as ''forest,'' ''honeydew,'' ''fir,'' etc. In some central European countries like Germany, Switzerland, and Austria, honeydew honeys have generally higher prices than blossom honeys. However, there are no internationally accepted quality criteria for the different types of honeydew honeys, but there are criteria in individual countries.

In recent years, new analytical techniques combined with multivariate data analysis have been used for the determination of the botanical origin of honey. They are, for example, physical and chemical characteristics determinations (Devillers *et al.*, 2004; Mateo and Bosch-Reig, 1998), mineral content analysis (Nalda *et al.*, 2005), carbohydrate composition (Terrab *et al.*, 2002), amino acid composition (Cotte *et al.*, 2004), mass spectrometry or metal oxide semiconductor-based gas sensing (Ampuero *et al.*, 2004; Benedetti *et al.*, 2004), differential scanning calorimetry (Cordella *et al.*, 2003a,b), pyrolysis mass spectrometry (Radovic *et al.*, 2001a,b), and Raman (Goodacre *et al.*, 2002) and near-infrared spectroscopy (Davies *et al.*, 2002). These methods allow one to clearly discriminate among several types of unifloral honeys, but not the polyfloral honeys that represent the majority of honeys. In reality, these methods may not be able to distinguish different polyfloral honeys on the market. This is why until now none of these methods are commonly used for the determination of the botanical origin of honey.

Ion chromatographic methods showed a potential to discriminate between unifloral and polyfloral honey samples by first classifying the honey samples into two groups (Cordella *et al.*, 2003a,b). However, only a few samples were analyzed in this study. The method remains to be verified in practical analyses. Currently, a reliable determination of the botanical and geographical origins can be achieved only by a global interpretation of sensory, pollen, and physicochemical analyses

carried out by experts (Bogdanov *et al.*, 2004; Persano Oddo and Bogdanov, 2004). However, the uncertainty is related to the interpretation of pollen analytical results because of a number of different factors, so it is an exigent task to develop new analytical methods (Molan, 1998).

D. Authenticity of geographical origin and mislabeling

In general, honeys produced in developing countries are cheaper than those in developed countries. There is a financial interest in mislabeling honeys. Pollens in honeys can reflect the identity of the flowers from which the honeybees collected the nectar. Pollen analysis has been used for the determination of the geographical origin of honey. The geographical origin is routinely checked by pollen analysis as it requires only inexpensive instrumentation. The pollen specialists have a precise knowledge of the pollen spectrum of the honeys of their country. Pollen analysis is very efficient for differentiating honey geographical origins. When the geographical differences are less pronounced, pollen spectra may not yield a confident authenticity proof. Pollen analysis itself often cannot satisfy the quality standards. Manual pollen analysis relies on personal experience, is subjective, and has not been tested by modern proficiency test trials, whereas computerized pollen analysis is very promising. Modern statistical methods make it possible to determine honeys from different topographical areas within definite, relatively small geographical regions.

Many analytical methods were studied for the determination of the geographical origin such as amino acid composition (Davies, 1975; Gilbert *et al.*, 1981), Raman spectroscopy (Goodacre *et al.*, 2002), mineral content (Latorre *et al.*, 1999, 2000), and sugar and mineral components combined with common chemical quality control data (Gomez Barez *et al.*, 2000; Gonzales Paramas *et al.*, 2000; Sanz *et al.*, 1995). Most studies dealt with a limited number of samples or samples from a small geographical area. The distinctions reported were, therefore, due to floral differences between the geographical regions and thus to the botanical origin of honey (Cometto *et al.*, 2003). Distinct floral differences allow a geographical discrimination.

Solid authentication of botanical origin normally requires a combination of several analytical methods, which is time consuming and costly. For example, highly specialized expertise is needed for the interpretation of the pollen spectra. New methods are needed for economical, rapid, and reproducible authentication of the botanical and geographical origins of honey (Bogdanov and Martin, 2002a; Bogdanov *et al.*, 2004). Wang *et al.* (2009) have recently reported use of MALDI TOF MS for honey geographical origin discrimination. It requires minimal sample preparation.

III. CHEMICAL COMPOSITION AND ANALYTICAL METHODS FOR DISCRIMINATION OF THE BOTANICAL AND GEOGRAPHICAL ORIGINS OF HONEYS

A. Proteins

The protein content in honey is about 0.2% (w/v). The proteins are from honeybees and plants. It has been suggested that protein and peptides in honeys can indicate geographical origin. One- and two-dimensional SDS-PAGE (1D, 2D) has been used to analyze proteins in honeys. At least 19 protein bands were detected in honeys of different botanical origins (Marshall and Williams, 1987). Those proteins were assumed to be predominantly of bee origin instead of floral origin. Proteins from Spanish (Galicia) honeys were separated on SDS-PAGE and 12 different bands have been observed, some of which were α- and β-glucosidase, amylase, and glucose oxidase (Rodriguez-Otero *et al.*, 1990). Baroni *et al.* (2002) reported that pollens from different plants can be distinguished by SDS-PAGE, and pollen proteins can be used as honey floral markers. Won *et al.* (2008) reported identification of the major proteins in honey produced by the bee *Apis mellifera* by SDS-PAGE. Lee *et al.* (1998) noted that the molecular weight of a major protein in honey from *Apis cerana* and *A. mellifera* was 56 and 59 kDa, respectively. Wang *et al.* (2009) reported a MALDI TOF MS method to determine the geographical origin of honey based on fingerprinting and barcoding of proteins in honey. The protein mass spectra of known Hawaii origin honeys were obtained. The spectral peak information was extracted to generate protein fingerprints. This information was transformed into a database library in a spectral barcode format. The barcode spectra in the library were used for differentiation of the geographical origin of honeys based on barcode pattern matching (Fig. 3.2). The differentiation ability of the database library of barcodes was validated by comparison between the database barcodes and those of known Hawaii origin honeys. Validation results showed that the protein fingerprints of honeys have better comparability with the honeys in the library known to be from the same region than with those from other regions. The protein fingerprints were used to differentiate the geographical origins of commercially purchased honey samples with labels indicating that they were produced in different countries and various regions in the USA. MALDI TOF MS can be a rapid, simple, and practical method for determining the geographical origin of honeys.

B. Amino acids

The nitrogen content in honey is about 0.04%. Amino acid content accounts for approximately 1% (w/w). Free amino acid profiles have been proposed for the determination of the botanical and geographical

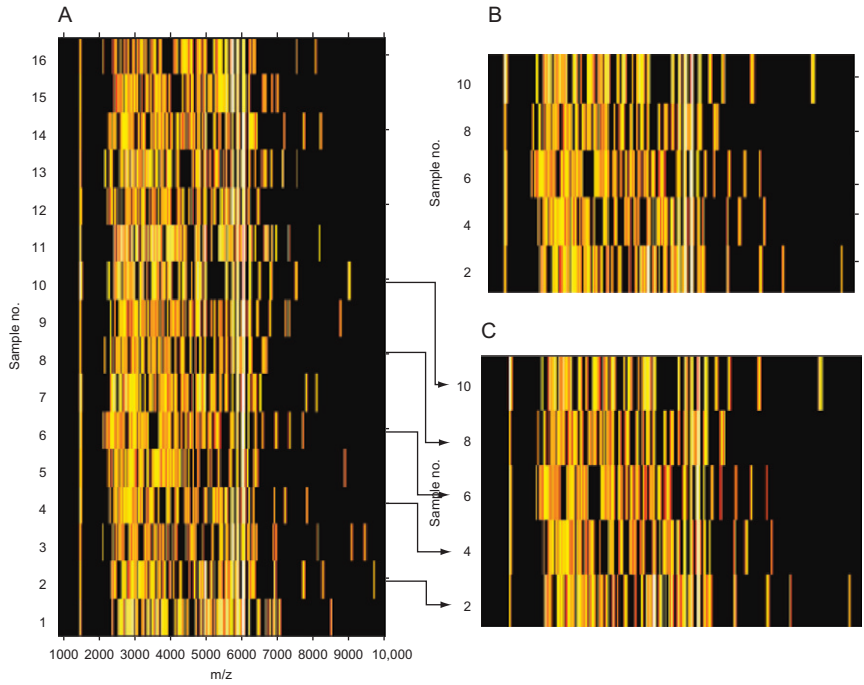

FIGURE 3.2 (A) MALDI TOF protein mass spectral barcodes of the 16 honeys of known Hawaii origin. (B) The protein barcodes of repeat analyses of the Hawaii origin honey samples 2, 4, 6, 8, and 10. (C) Enlarged display of the protein barcodes of the samples 2, 4, 6, 8, and 10 in the database library (cited from Wang *et al.*, 2009).

origins of honey (Anklam, 1998; Davies and Harris, 1982; Gilbert *et al.*, 1981). Pollen is the main source of honey amino acids. Davies (1975, 1976) found that the content ratios between some amino acids varied with honey geographical origins and floral origins, with the latter being even more distinct than the former. Proline is unique in honey and comes mainly from honeybees. The content of proline integrated with other components such as saccharide and glucose oxidase activities in honey has been proposed as an indicator of honey ripeness and adulteration (Von der Ohe *et al.*, 1991). In general, the proline content in honey must be more than 200 mg/kg, and at least 66% of the total free amino acids (usually 80–90%).

The application of linear discriminant analysis of the 16 amino acids found in Spanish honeys established both botanical and geographical differences (Pérez Arquillue and Herrera Marteache, 1987). Gas chromatography (GC) analyses of free amino acids showed obvious differences when honey samples from the UK, Argentina, Australia, and Canada were compared (Gilbert *et al.*, 1981). Pawlowska and Armstrong (1994)

have determined proline, leucine, and phenylalanine and their enantiomeric ratios in honey using high-performance liquid chromatography (HPLC). Leucine is the most variable amino acid found in the analyzed honey samples. Pirini and Conte (1992) used GC to analyze amino acids in honey samples from different botanical sources such as acacia, citrus, chestnut, rhododendron, rosemary, and lime. The presence of amino acids such as arginine, tryptophan, and cysteine is characteristic for some honey types. In some cases, the overall amino acid profile does enable differentiation between specific types. However, a single amino acid or a group of amino acids could not be selected as being suitable for the characterization of particular kinds of honey. Gilbert *et al*. (1981) used GC and determined 17 free amino acids in 45 honey samples from the UK, Australia, Argentina, and Canada. Canonical variate analysis (CVA) of the data showed good discrimination among the samples from Argentina, Australia, and Canada. Those from the United Kingdom were classified as a group; however, fell between the samples from Argentina and Canada; and could be discriminated only on further statistic analysis after removal of the honey samples from Australia. These results obviously showed that certain groups of honey samples originating in different geographical regions could be distinguished by free amino acid profiles. Pawlowska and Armstrong (1994) used HPLC to determine free amino acids such as proline, leucine, and phenylalanine, and their enantiomeric ratios in a variety of honey samples from different geographical regions. Significant concentrations of leucine and phenylalanine were detected in honeys from different botanical and geographical origins. Leucine content showed the greatest variability in these analyzed honey samples. It was suggested that the enantiomeric ratios of amino acids in the honey samples could be used to test for storage, age, and the processing technique. Hermosín *et al*. (2003) analyzed 48 honey samples from 6 different geographical regions of Spain and 10 botanical sources. Proline, phenylalanine, tyrosine, and lysine were the main amino acids found in 31 Spanish honeys of five different single botanical origins, followed by arginine, glutamic acid, histidine, and valine. Principal component analysis (PCA) and the Student–Newman–Keuls test were used to analyze the data to establish a range for the amino acid composition of Spanish honeys. Senyuva *et al*. (2009) used phenylalanine and tyrosine, as well as some other amino acids, to distinguish honeys from different regions and concluded that HPLC aided with PCA can verify both the botanical and geographic origins of honey. Pérez Arquillue and Herrera Marteache (1987) determined some Spanish honey samples from different botanical origins using HPLC. They detected 16 protein amino acids after acid hydrolysis of isolated protein fractions. Statistic discriminant analyses of the data could satisfactorily distinguish local and botanical differences.

Other similar research has also been made for attempting to establish a relationship between amino acid composition and characteristic aromas for eucalyptus (*Eucalyptus* spp.) and lavender (*Lavandula* spp.) honeys (Bouseta *et al.*, 1996). Eucalyptus honeys could be characterized based on seven volatile compounds, whereas lavender honeys had only five characteristic volatile compounds. The concentrations of phenylalanine and tyrosine in these eucalyptus and lavender honeys averaged 1238 and 440 ppm, respectively, which could characteristically differentiate lavender honeys from eucalyptus honeys. In addition, proline was the dominant amino acid in eucalyptus honeys but not in lavender honeys. High amounts of phenylalanine (906–1830 mg/kg) and tyrosine (229–382 mg/kg) were found to be characteristic for lavender honeys and allowed a differentiation from eucalyptus honeys (Bouseta *et al.*, 1996). In addition to the concentrations of phenylalanine and tyrosine being characteristic for differentiation of lavender and eucalyptus honeys (Bouseta *et al.*, 1996), tryptophan and glutamic acid were used to distinguish honeydew honeys from blossom honeys (Lglesias *et al.*, 2004). Chemometric evaluation of concentrations of free amino acids in combination with pH value and sugar composition is also a promising approach for the determination of unifloral honeys (Cometto *et al.*, 2003; Conte *et al.*, 1998).

The amino acid content of 92 honey samples from 17 botanical and 4 different geographical sources was analyzed with GC (Conte *et al.*, 1998). The majority of the samples showed proline as the dominant amino acid, and significant amounts of phenylalanine, aspartic acid plus asparagine, and glutamic acid plus glutamine. However, higher levels of serine, tyrosine, and lysine were found in thyme honeys. In rosemary honeys, tyrosine was the most abundant followed by proline and phenylalanine. A linear discriminate analysis was applied to differentiate thyme honeys from chestnut honeys. The authors suggested that a combination of amino acid analysis, determination of water activity, sugar content, pH value, sensory evaluation, and use of statistics could be the best method for distinguishing botanical and geographical origins of honeys. HPLC methods were used to determine up to 17 amino acids without derivatization (Bouseta *et al.*, 1996; Conte *et al.*, 1998) and 22 amino acids after derivatization with diethyl ethoxymethylenemalonate (Alaiz *et al.*, 1992; Chicón *et al.*, 2001).

C. Aroma compounds

Volatile and semivolatile compounds are present in honeys and are attributed to aroma qualities. Aroma compounds can indicate floral and geographical origins and processing treatments. Aroma compounds come from nectar or honeydew. Aroma components can be also formed during thermal processing and storage (Bonvehí and Coll, 2003; Soria *et al.*, 2003). More than 400 components have been detected in the volatile flavor fraction of honey

originated from different floral types (Bentivenga *et al.*, 2004). Either a single or multiple aroma compounds can be indicative of honey floral and geographical origins. 1-Penten-3-ol was a characteristic compound for English honeys (Radovic *et al.*, 2001a,b). However, a group of aroma constituents are more often used than a single aroma 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 Brazilian honeys (Moreira *et al.*, 2002). Phenylethyl alcohol can indicate lime honeys, and it is well known in the perfume industry as possessing floral, spicy, and herblike odor (Moreira *et al.*, 2002). However, Radovic *et al.* (2001a,b) found phenylethyl alcohol only in two of the four lime honey samples analyzed and concluded that the authenticity of such honeys may be confirmed by the presence of one of the following chemicals: 2-methylfuran, α-terpinene, α-pinene oxide, bicyclo[3.2.1]octane-2,3,4-trione, methyl isopropyl benzene, aromatic hydrocarbon, 3-cyclohexen-1-ol-5-methylene-6-isopropylene, and 4-methylacetophenone. The isolation and identification of volatile compounds are important for evaluating the variability of floral origin of honeys (Alissandrakis *et al.*, 2005, 2007; Anklam, 1998; Kaškonienė *et al.*, 2008).

The concentrations of volatile compounds in honey are very low. To analyze volatile components in honey, sugars must be removed. Various methods have been used to isolate volatile compounds, such as simultaneous steam distillation and extraction (SDE) (Bouseta and Collin, 1995), dynamic headspace extraction (Bianchi *et al.*, 2005; Radovic *et al.*, 2001a,b), ultrasound-assisted extraction (Alissandrakis *et al.*, 2005), hydrodistillation (Alissandrakis *et al.*, 2005), solvent extraction (Bonvehí and Coll, 2003), and solid phase microextraction (Bentivenga *et al.*, 2004). All these techniques have advantages and disadvantages. Isolation time requirement is an important issue, particularly when a large number of samples are to be analyzed. Use of solvents is associated with loss of volatiles during solvent removal. Heating may also result in loss of volatile components (Alissandrakis *et al.*, 2005, 2007). Various modifications of headspace can overcome some of the disadvantages. The profiles of headspace volatiles are more closely associated with sensory perceptions.

The isolation of volatile components from honey that reflect its natural aroma, however, is very difficult. Flavor changes are usually associated with processing techniques and honey storage time. Accurate quantification of all volatile components is helpful for detecting honey floral origin. Classical SDE and its modified version (Godefroot *et al.*, 1981; Likens and Nickerson, 1964) are among the most popular methods currently used for the isolation of volatile compounds from honey samples. Atmospheric SDE was modified to SDE under vacuum for the isolation of volatile components at room temperature (Maignial *et al.*, 1992). Atmospheric

SDE can lead to detection of furfural, whereas SDE under vacuum gave a furfural-free extract with a fresh honey note. Bouseta and Collin (1995) reported that a complex mixture of hydrocarbons, alcohols, phenols, ethers, aldehydes, ketones, esters, furans, and nitrogen compounds could be isolated and identified from Canadian honeys using dichloromethane extraction under an inert atmosphere followed by simultaneous steam distillation–dichloromethane extraction. Excellent recoveries of about 70 tested compounds were obtained. Overton and Manura (1994) analyzed nine commercial honey samples from different floral sources (wildflower, blueberry, orange, clover, tupelo, alfalfa, apple). Volatile compounds were trapped on adsorbent resins and analyzed with GC/MS after thermal desorption. The results showed that this method was more sensitive than the headspace technique. By using this resin adsorption method, honey samples were found to contain many mono- and sesquiterpenoid compounds and flavors. The presence of the branched aldehydes, methyl-butyraldehyde, and 3-methyl-butyraldehyde in honey samples can reflect the microbial quality and thermal treatment of honey. A combination of this method and pollen analysis can be very useful for floral source identification. Bonaga *et al*. (1986) isolated the volatile components from unifloral Italian chestnut honey with SDE and analyzed with GC/MS. Linear hydrocarbons, saturated and unsaturated, from C_{10} to C_{37} were found in chestnut honey. *n*-Heptacosane, *n*-noncosane, *n*-tricosane, *n*-pentacosane, and *n*-hentriacontane were dominant saturated hydrocarbons (about 40%), whereas *n*-tritriacontene and *n*-hentriacontene were predominant unsaturated ones (about 60%). The volatile components from Italian chestnut honey were a complex mixture of more than 50 compounds of which 3-aminoacetophenone was a main component of the mixture and may be specific to the floral source. Grosch *et al*. (1990) quantified the flavor compound α-damascenone using a stable isotope dilution assay method in acacia and lime honey samples. The concentration of α-damascenone was about 3 ppb in acacia honey and about 8 ppb in lime honeys. Shimoda *et al*. (1996) detected 130 volatile compounds in haze honeys. The main aroma compounds included benzeneacetaldehyde, linalool, phenethyl alcohol, *p*-cresol, *p*-anisaldehyde, methyl-*p*-anisaldehyde, trimethoxybenzene, 5-hydroxy-2-methyl-4H-pyran-4-one, and lilac aldehydes. DÁrcy *et al*. (1997) identified 55 volatile compounds in two varieties of Australian eucalyptus honey, which had profiles that were quite distinctive in comparison with other honeys.

D. Carbohydrates

Honey is a supersaturated sugar solution; and sugar content accounts for more than 95% of the dry matter. Honey is an extremely varying and complex mixture of sugars and other minor components. Fructose is the

most dominant sugar followed by glucose in almost all types of honey. However, glucose is present at higher levels in honeys of rape (*Brassica napus*), dandelion (*Taraxacum officinale*), and blue curls (*Trichostema lanceolatum*) origin (Cavia *et al*., 2002). The ratio of fructose and glucose can be used as an indicator for the identification and classification of unifloral honeys (Low *et al*., 1988; Persano Oddo and Piro, 2004; Persano Oddo *et al*., 1995). Honeydew and blossom honeys have compositional differences. Honeydew honey usually contains higher content of oligosaccharides, mainly trisaccharides, melezitose, and raffinose; however, those oligosaccharides are not in blossom honeys (Bogdanov *et al*., 2004). Honey contains more than 20 oligosaccharides as minor components.

Honey has been produced in Lithuania since the ancient times. Baltrušaitytė *et al*. (2007a,b) recently reported antimicrobial and antioxidant properties of Lithuanian honeys. Kaškonienė *et al*. (2010) characterized the carbohydrate composition of Lithuanian honeys obtained from various sources and determined if there was any relationship between pollen content and the content of the carbohydrate in the honey.

The sucrose content of liquid sugar syrup can be 40 times higher than that in natural honey (Cotte *et al*., 2003). Maltose and maltotriose are usually present at high content in these syrups (29.8 g/100 g and 6.5 g/100 g, respectively), whereas the concentrations of maltose and maltotriose in honey are not so high. These sugars can be used as markers for the detection of adulteration. Maltose content in natural honey is generally less than 30 mg/g (Cotte *et al*., 2003; Joshi *et al*., 2000). Maltose in some honeys originating from certain plants can be up to 50 mg/g (Costa *et al*., 1999; Devillers *et al*., 2004). Ratios of fructose/glucose, maltose/isomaltose, sucrose/turanose, and maltose/turanose can be used an indicator to assess possible adulteration of honey with glucose or high-fructose syrups (Horváth and Molnár-Perl, 1997).

Higher profits are the major incentive for unlawful adulteration. The traditional adulterants are inverted sugar syrup, conventional corn syrup, and high-fructose corn syrup. Dehydration of fructose can produce hydroxymethylfurfural (HMF). HMF is usually indicative of adulteration with inverted sugar syrup. However, it is somewhat ambiguous because HMF can legally be present in honey that has been subjected to heat or abusive storage.

The knowledge of the carbohydrate components in honey is a useful index for distinguishing its authenticity. Saccharides can be determined according to their physical and chemical characteristics (Gritzapis and Timotheou-Potamia, 1989; Kumar *et al*., 1988; Peris-Tortajada *et al*., 1992; White, 1980) and enzymatic assays (Schwedt and Hauck, 1988). Chromatography methods include thin-layer chromatography (TLC) (Patzsch *et al*., 1988; Pukl and Prosek, 1990), GC (Low and Sporns, 1988; Mateo *et al*., 1987), ion chromatography-amperometric pulsed detection (Peschet and

Giacalone, 1991), and HPLC (Bogdanov, 1989; Bugner and Feinberg, 1992; Cherchi *et al.*, 1994). Zunin *et al.* (1987) analyzed sugar components in authentic Italian honey samples using GC. They found that the maltose and isomaltose ratio was not suitable for the detection of adulteration with syrups. However, measuring the sucrose content has a potential for judging adulteration purpose. Calcagno *et al.* (1987) used HPLC to distinguish authentic honeys from honeys produced by artificially fed bees and honeys with added sucrose. It is noteworthy that sucrose content will decrease during honey storage because of the presence of the enzyme invertase (White, 1992). Anion-exchange liquid chromatography is a suitable tool for oligosaccharide analysis (Swallow and Low, 1994). Inverted sugar syrup and high-fructose corn syrup are complex mixtures of oligosaccharides and usually are produced by chemical and enzymatic processes. The content of oligosaccharides can be used as an indicator of unlawful addition of the syrups in honeys. The oligosaccharide profiles of more than 90 British honey samples were obtained by using anion-exchange HPLC connected with pulsed amperometric detection (Goodall *et al.*, 1995). Spectroscopy methods such as FTIR have also been used to determine the sugar profiles and contents (Wang *et al.*, 2010c). Those methods are commonly used in a combination with statistical analyses to detect sugars and syrups for the investigation of honey adulteration.

E. Enzyme activity

Enzyme activities are a direct measurement of honey heating defects and honey age. Honey enzymes are from both nectars and honeybees. Enzyme activities vary largely with nectar and honey sources, even if α-amylase and α-glucosidase are mostly from honeybees (Low *et al.*, 1988; Persano Oddo and Piro, 2004; Persano Oddo *et al.*, 1990, 1999). Enzyme activities are related with the intensity of the nectar flow, concentration and composition of the nectar, and the honeybees. Therefore, honeys from rich nectar sources such as acacia often contain low natural enzyme activities (Wehling *et al.*, 2006). Enzyme activities decrease by overheating and long storage. Therefore, fresh honeys should be used to determine enzyme activities for distinguishing botanical origins. Pure honeys usually contain several classes of enzymes that are produced by honeybees' salivary secretion or are from the nectars or pollens. Amylases, invertases, glucosidases, catalases, phosphatases, and others are the most important enzymes in honey.

The activity of α-, β-, and γ-amylase is usually used as an important quality parameter of honey, according to the Directive 2001/110/CE (Voldřich *et al.*, 2009). Amylase in honey mainly originates from the salivary secretions of honeybees, which was concluded based on the presence of amylase in honey produced by sugar-fed honeybees (Stadelmeier and Bergner, 1986) and on similarities between honey and

honeybee amylases (Rinaudo *et al.*, 1973). The similarities included the need for chloride ions for catalytic activity, inhibition by acetylating agents, and similar sensitivity to pH by honeybee and honey amylases (Babacan and Rand, 2007).

Diastase (mixture of amylases) is used to evaluate honey freshness and heat treatment. Diastase is largely from honeybees with a contribution from pollen and nectar. It converses starch to maltose. The activity of diastase in honey is strongly affected by storage time and temperature conditions. Although natural levels vary with floral sources, a reduction of diastase activity from what is expected is a useful quality indicator. Legislation has set a minimum level for diastase activity; it should not be less than 8 diastase number (DN) units, where 1 DN unit hydrolyses 1 ml of 1% starch using 1 g of honey for 1 h at 37 °C. The reference equation from the International Honey Commission gives a definition of DN as: DN = (28.2 × absorbance change at 620 nm after 10 min) + 2.64. When natural honeys are added with syrups, such adulteration can reduce DN. Such adulteration can be masked by addition of foreign amylases such as bakery mold amylases (Voldřich *et al.*, 2009).

The diastase activity was traditionally determined according to the Schade method in the earlier years (Schade *et al.*, 1958). One unit of diastase activity (or more specifically, α-amylase), DN, is defined as that amount of enzyme that converts 0.01 g of starch to the prescribed endpoint in 1 h at 37 °C under the experimental conditions. In this assay, a standard solution of starch, which reacts with iodine to produce a color solution, is used as a substrate for honey enzymes under the standard conditions (Rendleman, 2003). A recently developed procedure uses an insoluble, dyed starch substrate (Persano Oddo and Pulcini, 1999). As this substrate is hydrolyzed by α-amylase, soluble dyed starch fragments are released into solution. After reaction termination and insoluble substrate removal by centrifugation, absorbance of the supernatant solution (at 620 nm) is measured. The absorbance is proportional to the diastase activity. This procedure has been widely adopted in the honey industry due to the convenience of a commercially available substrate and the simple assay format.

The amylase in honey has been used as a freshness indicator over the years (Oddo *et al.*, 1990). It has been received more attention due to wide use of honey in different food products (Ropa, 1999; Shimanuki, 1992). This diverse use of honey brings new challenges in food industry. Research has showed that honey amylase can reduce viscosity in starch-containing food products (Babacan and Rand, 2005). Barhate *et al.* (2003) studied amylase removal using ultrafiltration membranes to produce clarified honey and an enzyme enrichment using a combination of microfiltration and ultrafiltration membranes. Gatehouse *et al.* (2004) reduced the amylase activity in honeybee hypopharyngeal glands by RNA interference, indicating a possibility producing honey with low amylase activity.

Honey amylase activity is susceptible to temperature and aging (Lampitt *et al*., 1929, 1930; Sancho *et al*., 1992; White *et al*., 1961, 1964). White *et al*. (1964) stored honey samples for periods up to 540 days at seven temperatures from −20 to 60 °C and determined loss of amylase activity at different time intervals. Half-life values were suggested over 4 years, 200 days, 31 days, and 1.05 days at storage temperatures of 20, 30, 40, and 60 °C, respectively. In an earlier study, White *et al*. (1961) had reported a half-life of 17 months for honey samples stored at temperatures ranging from about 23 to 28 °C. There was considerable variation in the rate of loss of amylase activity among honey samples. In addition, there were no obvious correlations between ash, total acidity, pH values, and moisture content and loss of enzyme activity. Babacan and Rand (2005) showed that honey amylase survived heat treatments at 85 °C for prolonged times (up to 11 min), which verified the heat resistance of amylase in honey.

Enzyme activity can indicate the exposure of honey to heating and long storage. This criterion is not more accurate than the HMF content value because enzyme activities vary with honey samples. The diastase activity is usually associated with heat treatment. However, its activity gives only an indication about the processing (heat treatment) of the honey but is not suitable for the detection of the origin.

F. Fermentation products

Raw honey having high moisture content or deliberate addition of water can cause fermentation and spoilage. Honey spoilage information can be obtained by the analysis of microscopic yeast count (Beckh and Lüllmann, 1999; Russmann, 1998). This count analysis does not produce conclusive results. A more reliable method is to determine the fermentation products such as glycerol and ethanol (Beckh and Lüllmann, 1999; Beckh *et al*., 2005; Zucchi *et al*., 2006). In fact, glycerol is often a minor component in honey and is probably produced by microorganisms presented in the nectar and honeydew. Glycerol can be considered a fermentation product. Under the condition of 20% glucose solution, aeration, and low phosphate content, fermentation products mainly are polyols such as glycerol, whereas in under anaerobic condition, fermentation products are mainly ethanol. Huidobro *et al*. (1993) analyzed the glycerol content in 33 honey samples originating from Galicia (Spain) using an enzymatic method and found that the glycerol content ranged between 50 and 370 mg/kg. They also analyzed some unpasteurized Galicain honey samples using the same enzymatic method and detected apparent ethanol content ranging from 14 to 50 mg/kg (Huidobro *et al*., 1994). The analysis of the fermentation products can only give some information about honey

processing. However, it does not seem to be a suitable method for the detection of the floral and geographical source of honeys.

G. Flavonoids

Flavonoids are a large family of plant phenolic pigments. Plants contain a large number of flavonoids. Each plant species has obviously distinctive profiles of flavonoids. Flavonoids can be used as floral origin markers of European eucalyptus honeys (Martos *et al*., 2000a,b). Honeys originating from *Eucalyptus camaldulensis* (red gum honey) contain tricetin as the main flavonoid marker, whereas the main flavonoid marker is luteolin in honeys originating from *Eucalyptus pilligaensis* (mallee honey). The flavonoid profiles in Australian and European eucalyptus honeys have large differences. The propolis-derived flavonoids pinobanksin (3,5,7-trihydroxyflavanone), pinocembrin (5,7-dihydroxyflavanone), and chrysin (5,7-dihydroxyflavone) in Australian eucalyptus honeys are seldom detected and in much smaller content in comparison with European eucalyptus honeys (Martos *et al*., 2000a,b).

Quinoline alkaloids can mark the floral origin of chestnut honey while they were not detected in any other unifloral honeys analyzed (Truchado *et al*., 2009). The terpenoid and flavonoid derivatives can be good complementary markers for the detection of the floral origin of chestnut honey. Truchado *et al*. (2008) used nectar flavonol rhamnosides as floral markers of acacia honey. These flavonoids were not detected in any of the honeys originating from different floral origins other than acacia. The presence of flavonoid glycosides in honey relating to its floral origin is particularly relevant as it considerably enlarges the number of potential markers for the detection of the floral origin of honeys.

Each plant tissue tends to have an obviously distinctive profile of flavonoids. The flavonoid content can reach about 0.5% in pollen, 10% in propolis, and about 6 mg/kg in honey. Flavonoid aglycones appear to be present only in propolis and honey, while pollen contains flavanols in herosidic forms. The flavonoids in honey and propolis have been identified as flavanones and flavanones/flavanols (Campos *et al*., 1990). The antimicrobially active flavanone pinocembrine was found to be a major flavonoid in honey (Bogdanov, 1989). Amiot *et al*. (1989) studied two blossom and two honeydew Swiss honey samples and found that pinocembrine was the main flavonoid. Pinocembrine concentration varied between 2 and 3 mg/kg (Bogdanov, 1989). Berahia *et al*. (1993) analyzed sunflower honey samples and detected six flavone/flavols, four flavanone/flavols, and pinocembrin, of which pinocembrin is the main flavonoid. The flavonoids in sunflower honey and propolis were characterized and assessed for their effects on hepatic drug-metabolizing enzymes and benzo[*a*]pyrene–DNA adduct formation (Sabatier *et al*., 1992; Siess *et al*., 1996).

Pinocembrin, chrysin, galangin, and pinobanksin are main flavonoids in honey and propolis.

Ferreres *et al.* (1994b) analyzed flavonoids in 20 Portuguese honey samples. The total content of flavonoids ranged from 0.6 to 5 mg/kg honey. However, the total content of flavonoids in Spanish rosemary honeys ranged from 5 to 20 mg/kg honey. Pinocembrin and pinobanksin are the main flavonoids, and chrysin and galangin are the main flavones. Almost all of honey samples included a similar flavonoid profile composed of more than 22 compounds. Myricetin, myricetin-3-methylether, myricetin-30-methylether, and tricetin are the main characteristic compounds in heather honeys. The four have not been detected in other floral honeys. These flavonoids can probably mark the botanical origin of heather honey. Ferreres *et al.* (1996a,b) reported that quercetin, kaempferol-3-rhamnoside, myricetin-30-methylether, and isorhamnetin-3-rhamnoside were the main flavonoids in nectar collected from the honey stomach of bees gathering nectar from heather flowers in Portugal. The natural glycosides in nectar are hydrolyzed by bee enzymes to produce the corresponding metabolites detected in honey. The aglycones quercetin, kaempferol, myricetin-30-methylether, and isorhamnetin and ellagic acids were detected in heather honey, while these compounds were not found in other monofloral honey samples. Ellagic acid and myricetin-30-methylether are potential markers for the floral origin of heather honey.

The total flavonoid content in 27 Spanish honey samples ranged from 5 and 20 mg/kg honey (Ferreres *et al.*, 1992). The main flavonoids included pinocembrin, pinobanksin, and chrysin. The honey samples (the botanical origin was not specified) were directly obtained from the beekeepers and had not been industrially treated. A total of 18 different flavonoids were detected in these honey samples. Ferreres *et al.* (1991) showed that flavonoid profiles and botanical origin have a good correlation in five rosemary, two lavender, and three multifloral Spanish honey samples from the La Alcarria region. The results suggest that pollen may not be the main source of honey flavonoids. The flavonoids in honey obviously correlated with those in propolis. A simple and fast extraction technique was developed to detect flavonoids in honey using HPLC (Ferreres *et al.*, 1994c). By using micellar electrokinetic capillary chromatography (MECC), correlations between flavonoid patterns and the botanical origin of various Spanish honey samples were established (Ferreres *et al.*, 1994d). The same analytical methods were also been applied to honey samples from lavender, rosemary, citrus, and heather floral origins.

A high content of hesperetin was detected in citrus honey samples (Ferreres *et al.*, 1993, 1994b,c,e). 8-Methoxy-kaempferol was the main compound in rosemary honey samples, whereas luteolin was the main compound in lavender honey samples (Ferreres *et al.*, 1994b, 1998). Quercetin was used as a marker for differentiating for sunflower honey

(Tomás-Barberán *et al.*, 2001). It appears that the flavonoid pattern cannot distinguish the geographical origin. Honey samples originating from Spain, Mexico, and Canada were analyzed by MECC, and no significant differences of the flavonoid pattern were found. In addition to HPLC, capillary electrophoresis (CE) is an alternative method for flavonoid analysis (Delgado *et al.*, 1994). Some characteristic flavonoid patterns could be used to determine special botanical origin of honeys such as heather, citrus, and sunflower honeys. Some characteristic flavonoid patterns may indicate the geographical origin of honeys.

H. Pollen analysis

Melissopalynology is the most commonly used method for the detection of honey botanical origin (Cotte *et al.*, 2004). However, this technique is time consuming and requires special personnel skill. Honey contains pollen grains and other microscopic particles like fungi spores and algae, originating from the plants from which the nectar or honeydew has been collected by the bees. Pollen in honey can reflect the botanical sources where the honey has been produced and can be also used as the detection method for the geographical origin of honey. Under microscopic observation, the honey sediment presents some valuable information on beekeeping practices such as use of smoke, feeding of pollen substitutes, and general hygiene (Louveaux *et al.*, 1978) and on honey extraction technique, fermentation (Russmann, 1998), and possible adulteration (Kerkvliet and Meijer, 2000; Kerkvliet *et al.*, 1995).

Pollen analysis can detect the geographical origin of royal jelly (Dimou *et al.*, 2007). Royal jelly is a product secreted by the hypopharyngeal and mandibular glands of nurse bees (*A. mellifera*). It can be produced by partial digestion of honey and pollen (Witherell and Graham, 1978). Royal jelly is mainly fed to queens and queen larvae, as well as to worker and drone larvae (Crailsheim, 1992; Free, 1957; Haydak, 1970; Witherell and Graham, 1978). The most important components of royal jelly include water, protein, lipids, and carbohydrates (Karaali *et al.*, 1988; Witherell and Graham, 1978). Royal jelly has a great nutrient value and offers important financial profits to beekeepers. Like honey, the detection of the geographical origin of royal jelly is important for commercial products. Although the physicochemical properties and composition of royal jelly have been studied (Boselli *et al.*, 2003; Chen and Chen, 1995; Nagai *et al.*, 2001; Sesta, 2006; Simüth, 2001), only a few melissopalynological studies of royal jelly have been reported (Barth, 2005). Royal jelly can be enriched by pollen grains that fall from bees, or from the pollen contained in the honeybee stomach (Renner *et al.*, 2003; Simpson, 1955). Thus, pollen analysis could be used to determine its geographical origin.

Pollen from pollen traps is the most widely used method to record the flora sampled by bees in an area (Andrada and Telleria, 2005; Barth and Da Luz, 1998; Coffey and Breen, 1997; Nabors, 1997; Pearson and Braiden, 1990; Severson and Parry, 1981; Webby, 2004; Witherell and Graham, 1978). Witherell and Graham (1978) investigated the use of pollen analysis as a tool to detect the geographical origin of royal jelly. The pollen flora visited by bees was monitored by pollen traps for two consecutive years. The royal jelly samples from the same apiary were collected and examined. Honeybees usually used the freshly collected pollen for royal jelly production. Almost all main pollen types were found in the investigated area using pollen traps in the royal jelly samples.

The floral origin of honey can be determined by the analysis of the pollen in honey. The method mainly depends on the identification of pollen under microscopic examination. The different pollen types have been well described (Moore and Webb, 1978; Sawyer, 1988). However, there are some limitations (Molan, 1996) because plant species differ in amounts of pollen that can vary among seasons. The nectar yields also differ in male and female flowers, and pollen can be easily filtered out by the bee's honey sac (Maurizio, 1975). Another limitation is that honeybees can directly collect pollen without taking nectar and can collect almost all the pollen from all plants. Pollen may not be present in honey because some commercial honeys are filtered. Finally, pollen analysis cannot be used to prove authenticity of honey because pollen can be added fraudulently. Like citrus honey, pollen analysis is not suitable to distinguish it from other floral origins because the amounts of pollen are generally small and vary largely (Serra Bonvehi *et al*., 1987).

Honey usually originates from multiple botanical sources rather than a single source. So-called unifloral honey can be only used to describe honey originating from one plant species. The honey was considered as ''unifloral'' when more than 45% of the pollen was collected from one plant species (Maurizio, 1975). However, the distinguishing level is a limitation when a floral source leads to nectar with a higher or lower content of pollen grains than the average. Chestnut honey can be called unifloral honey only when more than 90% of the pollen directly originates from *Castanea*, whereas unifloral citrus honey needs only 10% of pollen to be from citrus. Pollen analysis can be used as the identification of the floral and geographical origin of honey. The geographical origin is the case when a particular floral species is only growing in specific areas.

Pollen analysis in combination with other techniques is still an effective tool for the authentication of the botanical origin of honey (Persano Oddo *et al*., 1995; Von der Ohe *et al*., 2004). It can distinguish polyfloral and different types of unifloral honeys (Mateo and Bosch-Reig, 1998). It can also indicate the percentages of different nectar contributions in honey products. A polymerase chain reaction technique and an

electrophoretic immunoblot assay were used to detect specific genes and pollen proteins, respectively, for the assessment of floral origin in honey samples (Baroni *et al*., 2002; Siede *et al*., 2004). These very sensitive techniques allowed a reliable detection of pollen, as pollen proteins were successfully used for the determination of botanical origin (Baroni *et al*., 2002). Such techniques are certainly valuable to detect transgene material in honey. As the analyzed proteins originate from pollen, these methods suffer from the same shortcomings as microscopic pollen analysis.

I. Minerals and trace elements

The content of minerals and trace elements in honey products can be used as an indicator of environmental pollution and an indirect indicator of the geographical origin of honey. Rodriguez-Otero *et al*. (1994) determined sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), copper (Cu), iron (Fe), manganese (Mn), phosphorus (P), chlorine (Cl), silicon (Si), sulfur (S), and ash content of 91 original honey samples from Spain Galicia region, but all samples without labeling floral sources. Potassium was the most abundant of the elements with an average content of 1500 mg/kg. Rodriguez-Otero *et al*. (1995) also determined Si, P, S, Cl, and ash content of 24 commercial honey products originating from Spain with a mean content 3 mg/kg for Si, 80 mg/kg for P, 45 mg/kg for S, and 260 mg/kg for Cl. The P and Cl values were higher than those of honeys from other regions. Perez-Cerrada *et al*. (1989) applied an ion chromatographic (IC) technique to determine inorganic anions such as chloride, hydrogen phosphate, and sulfate in Spanish honey. Pietra *et al*. (1993) determined trace elements in heather honey samples originating from Italy using preseparation neutron activation analysis. The same method was used to detect trace elements in Turkish honey samples originating from various floral origins such as mixed flower, sunflower, thyme, and citrus flower (Sevlimli *et al*., 1992). Other trace elements such as lead (Pb), cadmium (Cd), and Mn in honey samples produced in different seasons were determined with graphite furnace atomic absorption spectrometry (ETAAS), indicting slightly higher levels in the summer (Stein and Umland, 1986). The elements selenium (Se), Fe, and Ca in honeys were also analyzed with ETAAS (Dabeka and McKenzie, 1991; Siong *et al*., 1989a,b; Szymozyk *et al*., 1986). The trace element content in honey collected from a large area in Hungary is available (Fodor and Molnar, 1993). In general, the trace element content in honeys from industrial areas is higher than that of nonindustrial areas. Minerals and trace elements in honeys can suggest the geographical origin because of the fact that their content is largely affected by the regional environment.

The content of mineral substances in honey samples ranges from 0.2 to 10.3 g/kg (White, 1975). Potassium is the main mineral element with an

average of one-third of the total. Some research has suggested that the trace element content in honey is mainly based on the botanical origin of honey, for instance, light blossom honeys having a lower content than dark honeys such as honeydew, chestnut, and heather (Feller-Demalsy *et al*., 1989; Gonzalez-Miret *et al*., 2005; Sevlimli *et al*., 1992). It is possible to differentiate between ling (*Calluna vulgaris*), heather (*Erica* sp.), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), lavender (*Lavandula* sp.), and oak (*Quercus* sp.) honeydew honeys by determining Mg, Ca, aluminum (Al), Fe, Mn, zinc (Zn), boron (B), Cu, cobalt (Co), chromium (Cr), nickel (Ni), Cd, and P (Nozal Nalda *et al*., 2005). Electrical conductivity correlates significantly to honey mineral content and is frequently used for the characterization of the botanical origin of honey (Bogdanov *et al*., 2004). The mineral content in honey is also related to the geographical origin, and it is possible to be used to differentiate between honeys of different geographical origins (Hernandez *et al*., 2005; Lasceve and Gonnet, 1974; Latorre *et al*., 1999). Heavy metals such as Cd, Pb, Cr, and Ni in honey can suggest the pollution status in the region (Porrini *et al*., 2003). It has been reported that Pb contamination of honey in polluted and nonpolluted areas was not significantly different because of the high variability of the data, whereas the highest values were often found in polluted areas (Bogdanov, 2006; Bogdanov *et al*., 2007). Fernandez-Torres *et al*. (2005) reported that the concentrations of Zn, Mn, Mg, and Na in eucalyptus, heather, orange blossom, and rosemary honeys from Spain were strongly dependent on their botanical origin. Chudzinska and Baralkiewicz (2010) used inductively coupled plasma-mass spectrometry (ICP-MS) to determine trace elements (microelements) in honey from Poland; honeydew, buckwheat, and rape honey samples were consequently classified by this technique into two main groups, honeydew honey and nectar honey. The data variability of heavy metal content is probably due to the different botanical origins of the honey samples analyzed. All environmental, geographical, and botanical factors thus have influences on the trace elements of honey. It is important to consider all factors for studying honey trace element components.

J. Organic aliphatic acids

The amount of organic acids in honey is less than 0.5%. Organic acids can be used as an important indicator of organoleptic properties like color and flavor and physical and chemical properties such as pH, acidity, and electrical conductivity (Crane, 1990). The organic acids in honey also have antibacterial and antioxidant activities (Gheldof *et al*., 2002; Weston *et al*., 1998). Organic acids in honey can be used as fermentation indicators, or for the treatment of Varroa mite infestation (Calderone, 2000; Mutinelli *et al*., 1997) and discriminating between honeys based on their

botanical and geographical origins (Cherchi *et al.*, 1994; Del Nozal *et al.*, 1998; Ferreres *et al.*, 1996a,b; Wilkins *et al.*, 1995). The significance of nonaromatic organic acids in honey was recently reviewed (Mato *et al.*, 2003); enzymatic, chromatographic, and electrophoretic methods were summarized for the analysis of the most important organic acids in honey.

Thirty-two aliphatic acids in honeys from New Zealand were extracted with diethyl ether, methylated with diazomethane, and identified with GC–MS. 2-Methylbutanediocic acid and 4-hydroxy-3-methyl-*trans*-2-pentenediocic acid were suggested as floral marker compounds to distinguish New Zealand rewarewa (*Knightea excelsa*) honeys (Mato *et al.*, 2006a,b; Wilkins *et al.*, 1995). HPLC solid phase extraction (SPE) was used to characterize organic aliphatic acids in honey samples. The average recoveries ranged from 89% to 104% with the detection limits from 0.002 to 3 mg/kg (Cherchi *et al.*, 1994). Polyfloral and unifloral Italian honey samples contained gluconic acid (2–12 g/kg), pyruvic acid (9–78 mg/kg), malic acid (69–145 mg/kg), citric acid (64–160 mg/kg), succinic acid (12–48 mg/kg), and fumaric acid (0.5–2.6 mg/kg) (Bogdanov *et al.*, 2004).

Enzymatic methods have been mainly used for the quantification of citric, malic, formic, D- and L-lactic, oxalic, and total D-gluconic acids in honeys. It can be also used for the determination of other acids such as acetic, L-ascorbic, and succinic acids (Mato *et al.*, 1998a,b). Tourn *et al.* (1980) compared an enzymatic assay with other analytical methods and showed remarkable advantages of the enzymatic assay including its specificity, sensitivity, rapidity, and simplicity. Stoya *et al.* (1986) analyzed the formic acid content of honey using an enzymatic method to distinguish which honey samples came from hives untreated and from hives treated with formic acid against Varroa mite infestation (residual formic acid, after treatment). Stoya *et al.* (1987) also determined D-(–) and L-(+)-lactic acid content in honeys untreated against varroa mites (natural content of these acids) and in honeys from hives treated with D-(–) and L-(+)-lactic acids against varroa mite infestation (residual acids, after treatment). Hansen and Guldborg (1988) analyzed the formic acid content of Danish honeys. Talpay (1988) determined the citric acid content in 153 floral honeys, 97 honeydew honeys, and 18 samples from bees that had been artificially fed with sugar. Talpay (1989) analyzed the formic acid content of 306 honeys from different botanic and geographic origins. Sabatini *et al.* (1994) applied an enzymatic method to determine formic acid, D-lactic, and L-lactic acids content in 42 unifloral honeys from 7 different botanical sources. Mato *et al.* (1997, 1998) developed an enzymatic micromethod to quantify total D-gluconic acid, citric acid, and L-malic acid in honey. Oxalic acid content was also determined with an enzymatic assay (Bogdanov *et al.*, 2002; Mutinelli *et al.*, 1997).

Chromatographic methods that are used to determine organic acids in honey include paper and on-column ion exchange chromatography, GC, and HPLC. Stinson *et al*. (1960) first developed a chromatographic separation and simultaneous quantification of several organic acids in honey samples. Speer and Montag (1985) quantified phenylacetic and benzoic acids in 32 honeys from different botanical origins using GC–flame ionization detector (GC-FID). Speer and Montag (1987, 1988) determined the concentrations of 24 aromatic organic acids in honeys from different floral sources. Echigo and Takenaka (1974) analyzed oxalic, malonic, succinic, fumaric, malic, α-ketoglutaric, tartaric, *cis*-aconitic, citric, and gluconic acids in approximately 45 min using GC. Wilkins *et al*. (1995) analyzed 32 honeys and identified dicarboxylic aliphatic acids using GC–MS. Verzera *et al*. (2001) developed a solid phase microextraction-GC–MS method and confirmed 113 volatile compounds in honeys from different botanical sources originating from Sicily (Italy). Pilz-Güther and Speer (2004) analyzed D,L-lactic, citric, succinic acid, and L-malic acids in honey using GC–FID after SAX-cartridge cleanup and concentration.

Perez-Cerrada *et al*. (1989) developed an ionic chromatography–conductivity detection (IC–CD) method to determine inorganic anions and malic, tartaric, and oxalic acids. Jörg and Sontag (1992) developed a HPLC method for qualitative and quantitative analysis of phenolic acids (2-hydroxybenzoic, 3-hydroxybenzoic, 4-hydroxybenzoic, 3,4-hydroxybenzoic, 4-hydroxycinnamic, and 4-hydroxy-3-methoxycinnamic acid) in honey samples from different botanical origins. Cherchi *et al*. (1994) identified several organic acids in honey samples and quantified gluconic, pyruvic, malic, citric, succinic, and fumaric acids in 48 floral honeys using HPLC-UV. Defilippi *et al*. (1995) determined formic acid in honey samples by IC–CD. Ferreres *et al*. (1996a,b) isolated, identified, and quantified *cis*/*trans*-abscisic acid and *trans*/*trans*-abscisic acid in Portuguese bell heather honeys using reversed-phase HPLC with diode array detection (DAD). Del Nozal *et al*. (1998) determined citric, pyruvic, galacturonic, gluconic, malic, citramalic, quinic, succinic, fumaric, and formic acids in 57 honeys from different botanical sources. Alamanni *et al*. (2000) analyzed oxalic, lactic, and formic acid contents in 49 unifloral Sardinian (Italy) samples. Del Nozal *et al*. (2000) determined oxalic acid and other inorganic anions such as sulfate and nitrate in 99 floral and honeydew honeys using IC–CD. Casella and Gatta (2001) determined organic acids such as gallic, ascorbic, gluconic, lactobionic, galacturonic, and glucuronic acids by an anionic exchange chromatography. Suárez-Luque *et al*. (2002a, b) used HPLC-UV and determined malic, maleic, citric, succinic, and fumaric acids in 15 min. Del Nozal *et al*. (2003a,b) separated oxalic, D-glucuronic, citric, galacturonic, propionic, pyruvic, malic, citramalic, quinic, D-gluconic, lactic, formic, glutaric, fumaric, succinic, and butyric acids in 39 honeys and 58 honeys.

CE is widely used for separation and quantification of organic acids (Stover, 1997). Many CE studies were performed to quantify organic acids in some food matrices (Frazier, 2001; Galli *et al*., 2003; Klampfl *et al*., 2000; Lindeberg, 1996). Many small organic acids can be well separated with CE (Boden *et al*., 2000; Mato *et al*., 2006a,b; Navarrete *et al*., 2005). Those acids include acetic, citric, fumaric, lactic, maleic, malic, oxalic, pyruvic, succinic, and gluconic acids which can be separated by CE in a short time.

In general, CE is simple, rapid, and low cost because it needs neither laborious treatment of the samples nor long times of analysis. However, its high detection limit is a major limitation of CE. CE is often poorly reproducible. Enzymatic assay is more suitable for quantifying one organic acid in honey samples because it is specific, precise, and accurate. GC is more suitable for analyzing volatile or semivolatile chemicals. HPLC is versatile and reproducible. However, common HPLC detectors such as UV-VIS are not very sensitive for organic aliphatic acids.

K. Phenolic compounds

Phenolic compounds including polyphenols are one of the most important groups of compounds in plants, comprising more than 8000 known structures (Gómez-Caravaca *et al*., 2006). Many methods such as colorimetric assays, TLC, GC, HPLC, and CE have been developed to analyze polyphenols in honey and propolis. Chemical identification might be more accurate and more convenient for the characterization of honey floral sources (Alvarez-Suarez *et al*., 2009; Tan *et al*., 1989; Yao *et al*., 2003). The analysis of phenolic compounds and flavonoids in honeys has been studied to distinguish honey floral and geographical origins (Amiot *et al*., 1989; Davies, 1976; Ferreres *et al*., 1992; Sabatier *et al*., 1992; Tomás-Barberán *et al*., 1993a,b).

Some phenolic acids such as ellagic acid can be used as floral markers of heather honey (Cherchi *et al*., 1994; Ferreres *et al*., 1996a,b), and the hydroxycinnamates (caffeic, *p*-coumaric, and ferulic acids) as floral markers of chestnut honey (Cherchi *et al*., 1994). Pinocembrin, pinobanksin, and chrysin are the characteristic flavonoids of propolis, and these flavonoid compounds have been found in most European honey samples (Tomás-Barberán *et al*., 2001). However, for lavender and acacia honeys, no specific phenolic compounds could be used as suitable floral markers (Tomás-Barberán *et al*., 2001). Other potential phytochemical markers like abscisic acid may become floral markers in heather honey (Cherchi *et al*., 1994). Abscisic acid was also detected in rapeseed, lime, and acacia honey samples (Tomás-Barberán *et al*., 2001). Snow and Manley-Harris (2004) studied antimicrobial activity of phenolics.

Various hydroxybenzoic and hydroxycinnamic acids are present in plants (Gross, 1981; Herrmann, 1979, 1989). Steeg and Montag (1988)

determined the phenolic acid content in honey samples originating from different floral sources using GC. Rape honeys contained a higher content of phenylpropanoic acid, while buckwheat honeys had a higher content of 4-hydroxybenzoic acid and no phenylacetic acid was found. Heather honeys contained a higher content of benzoic acid, phenylacetic acid, mandelic acid, and phenyllactic acid. Protocatechuic acid can be used as a marker to distinguish honeydew honeys and other honeys because of the difference in the concentration of protocatechuic acid. The concentrations of phenolic acids in honeys ranged from 0.01 to 10 mg/kg (Sontag *et al*., 1989). 3,4-Dihydroxybenzoic acid, 4-hydroxyphenyllactic acid, 2,5-dihydroxybenzoic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 3,4-dihydroxycinnamic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 2-hydroxybenzoic acid, 4-hydroxycinnamic acid, and 4-hydroxy-3-methoxycinnamic acid were detected in buckwheat honeys (Jörg and Sontag, 1992). Jörg and Sontag (1992) characterized the profiles of these compounds in different honeys and used the patterns to distinguish honeydew, chestnut, and forest blossom honeys. Jörg and Sontag (1993) analyzed phenolic esters in chestnut, clover, dandelion, linden, orange, rape, and sunflower honeys and found that the characteristic compounds included methyl-4-hydroxybenzoate, methyl-vanillate, and methyl-syringate. Only methyl-syringate was found in robinia honey. The content of methyl-4-hydroxybenzoate was relatively higher in rape and orange honeys. Using GC, Häusler and Montag (1989, 1990) detected the aromatic carbonyl compounds such as benzaldehyde, phenylacetaldehyde, acetophenone, *trans*-cinnamic aldehyde, 2-anisaldehyde, 4-anisaldehyde, vanillin, and 3,4-dimethoxy-5-hydroxybenzaldehyde in honeys from different floral sources like chestnut, acacia, buckwheat, eucalyptus, orange, and sunflower. The phenylpropane metabolites salicylaldehyde, *p*-tolyl-aldehyde, vanillin, 2,5-dimethoxybenzaldehyde, and 3,4-dimethoxybenzaldehyde were found as natural minor components ranging from 5 to 180 mg/kg.

Spectrophotometric, chromatographic, and electrophoretic methods are commonly used for the determination of phenols in honey. The colorimetric assay based on the reaction of Folin–Ciocalteu reagent is widely used for the determination of total phenols in honey (Aljadi and Kamaruddin, 2004; Al-Mamary *et al*., 2002; Stoya *et al*., 1987). The main disadvantage of this assay is its low specificity, as the color reaction occurs with any oxidizable phenolic hydroxy group. A typical Folin–Ciocalteu method can be described as follows. Each honey sample is diluted with distilled water and filtered. This solution is then mixed with Folin–Ciocalteu reagent for 5 min followed by addition of sodium carbonate. After incubation at room temperature, the absorbance is measured at 760 nm against a methanol blank. Gallic acid is used as a standard to produce the calibration curve (Singleton *et al*., 1999).

A modified Folin–Ciocalteu method is also available (Vinson *et al.*, 2001). GC is not an ideal method for phenolics analyses because most phenolics are not volatile. Few studies of GC and GC–MS for the analysis of phenolics in honeys were published (Aljadi and Yusoff, 2003; Berahia, 1993; Ferreres *et al.*, 1993). HPLC is the most useful method for analyzing polyphenolic compounds. Reversed-phase HPLC with UV–VIS or a diode array detector is most widely used, although electrochemical detection systems (Inoue *et al.*, 2005; Long *et al.*, 2003; Sabatier *et al.*, 1992) and mass detectors (Cabras *et al.*, 1999; Inoue *et al.*, 2005) have been used. Polyphenols are usually identified by comparing retention times of HPLC chromatograms and UV spectra. NMR spectrometry is often used as a complementary technique for structural assignment (Cabras *et al.*, 1999; Ferreres *et al.*, 1996a,b; Martos *et al.*, 1997, 2000a,b). The speed, resolution, simplicity, and low operating costs of CE make it become an attractive option for determining phenolics in honey (Andrade *et al.*, 1997; Delgado *et al.*, 1994; Ferreres *et al.*, 1994f). A key to applying the CE method to determine phenolics is CE optimization conditions and a decrease of the limit of detection.

L. Stable isotopes

Stable isotopes are referred to as element isotopes that are stable and do not decay over time. Most elements have at least one stable isotope. For example, hydrogen exists as three isotopes ^{1}H, ^{2}H, and ^{3}H (^{1}H and ^{2}H are stable isotopes), and carbon exists as three isotopes, ^{12}C, ^{13}C, and ^{14}C (^{12}C and ^{13}C are stable isotopes). In most cases, the more abundant stable isotope species typically contain the fewest number of neutrons for that element. Stable isotopes can be distinguished from the radioactive isotopes of an element. Radioactive isotopes have finite lives and undergo a decay to become a new element. The decay time may vary widely ranging from less than a second to thousands of years. For example, carbon has six radioactive isotopes (^{9}C, ^{10}C, ^{11}C, ^{14}C, ^{15}C, and ^{16}C) and the half-life of ^{14}C radioactive isotopes is 5730 years and it can be used in dating biological materials (Ghidini *et al.*, 2006). Schellenberg *et al.* (2010) have determined multielement stable isotope ratios (H, C, N, S) of honey from different European regions (Table 3.2).

Stable isotopes are among the most reliable techniques for the detection of the geographical origin of honey (Anklam, 1998). Some researchers applied this technique to detect adulterations in milk. In particular, the $^{13}C/^{12}C$ profiles can be used to detect honey adulterations such as adding exogenous sugars. It was suggested that the range of values $\delta^{13}C$ found for bee-produced honey was $-21.96‰$ to $-30.47‰$ for C3 plants, $-11.82‰$ to $-19.00‰$ for C4 plants, and $-11.33‰$ to $-11.78‰$ for cane sugar (Padovan *et al.*, 2003).

TABLE 3.2 Summary of $\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$, and $\delta^{34}S$ values of honey proteins from different geographical regions (modified from Schellenberg *et al.*, 2010)

Region of origin	$\delta^{13}C$ [‰] versus V-PDB[a]	$\delta^{2}H$ [‰] versus V-SMOW[b]	$\delta^{15}N$ [‰] versus air	$\delta^{34}S$ [‰] versus V-CDT[c]
Algarve (Portugal)	–25.4	–73	2.0	5.9
Allgä (Germany)	–26.1	–121	1.8	2.0
Barcelona (Spain)	–25.2	–105	0.0	4.7
Carpentras (France)	–24.6	–106	1.9	4.1
Chalkidiki (Greece)	–25.1	–111	1.3	5.6
Cornwall (UK)	–25.8	–86	5.4	9.5
Franconia (Germany)	–26.2	–118	2.6	3.6
Gäboden (Germany)	–26.3	–118	3.3	4.8
Iceland	–26.8	–107	0.5	8.0
Ireland	–25.7	–99	4.2	9.3
Jylland (Denmark)	–26.3	–112	4.5	4.2
Lakonia (Greece)	–25.8	–96	2.0	9.2
Limousin (France)	–25.4	–106	2.0	5.2
Marchfeld (Austria)	–25.9	–108	4.3	4.0
Mühlviertel (Austria)	–26.7	–114	3.2	4.5
Orkneys (UK)	–26.5	–88	0.9	11.1
Poland	–26.2	–101	3.9	3.5
Sicily (Italy)	–24.2	–99	3.9	2.0
Trentino (Italy)	–24.8	–110	0.8	5.2
Tuscany (Italy)	–24.4	–99	1.7	3.7

[a] V-PDB, Vienna Pee Dee Belemnite.
[b] V-SMOW, Vienna Standard Mean Ocean Water.
[c] V-CDT, Vienna Canyon Diablo Troilite.

Honey may be adulterated with the relatively cheap high-fructose corn syrup. Analysis of the carbon stable isotopic ratio can be used to detect whether honey is adulterated with sugars or syrups. The natural abundances of the stable isotopes of the main bioelements in biogenic material can produce small variations, which can be caused by isotopic effects of physical processing and chemical reactions in the natural cycles of these elements. Information of origin, treatment, and adulteration of honey can be identified by determining typical relative abundances (δ-values) (Croft, 1987; Schmidt, 1986). Stable isotopic ratio analysis (SIRA) has been used as the detection of adulteration with corn syrup or cane sugar to various foods. Small quantities of the ^{13}C content of different plant types (C3 vs. C4) are determined. Most fruits and grains are Calvin cycle pathway (C3) plants yielding ^{13}C values near $-25‰$; cane

and corn are Hatch–Slack pathway (C4) plants with ^{13}C values near $-10‰$. The coupling of an elemental analyzer with an isotopic ratio mass spectrometer allows online isotopic measurements (Anklam, 1998). The ^{13}C values of various honey samples were about $-23.2‰$ to $-24.61‰$. As the ^{13}C values alone for honey cannot always definitively prove adulteration by addition of C4 plant sugars, they have been determined in conjunction with those of the protein from honey. The protein value could be used as an internal standard. For authentic honey samples, a mean difference of $+0.1‰$ (range: $+1.1‰$ to $-0.9‰$) has been measured. More negative differences suggest the addition of C4 plant sugars (White, 1992). The limit for the detection of adulteration is about 7% of the adulterated sugars. The addition of C3 plant sugars (beet sugar) cannot be proved based on this method. However, authenticity can be confirmed by the δ-values for certain types of honeys (Anklam, 1998). Using the difference in stable carbon isotope ratio between a honey and its protein fraction, confirmation of honey adulteration with amounts of 7–20% or larger of corn or cane sugar can be carried out. Fifty authentic honey samples were used to develop the purity calibration and validation sets, and 38 other honey samples with ^{13}C values in ''questionable'' or ''adulterated'' range were used as testing samples. Adulteration can be confirmed based on a difference of more than 1.0% between honey and protein fractions (White and Winters, 1989). ^{13}C NMR has been also applied for the qualitative and quantitative analysis of structurally similar disaccharides in honey such as glucose–glucose and glucose–fructose (Low and Sporns, 1988). ^{13}C NMR has also been applied for the analysis of a complex mixture of minor disaccharides in honey. Disaccharide ratios like maltose, sucrose, kojibiose, palatinose, turanose, gentiobiose, neotrehalose, nigerose, and isomaltose in alfalfa honey and in sweet clover honey obtained by ^{13}C NMR were compared with those obtained by GC analysis. Honey samples from Israel have been characterized according to the isotopic ratio parameters $\delta^{13}C$, measured by MS and deuterium/hydrogen (D/H) of the methyl group of the ethanol produced by alcoholic fermentation, measured by deuterium NMR (Lindner *et al*., 1996). Beretta *et al*. (2008) studied ^{1}H NMR techniques to assign reliable markers for European honeys to distinguish honeydew honeys from blossom honeys. Truchado *et al*. (2009) used ^{1}H NMR and ^{13}C NMR to analyze chestnut nectar which is collected by the bees to make honey and identified kynurenic acid and 4-quinolone-2-carboxylic acid as useful chemical markers for honey floral origins. Donarski *et al*. (2010) used ^{1}H NMR to analyze kynurenic acid as a biomarker of sweet chestnut honey. Ethanol samples obtained from the fermentation of citrus honeys have D/H values almost similar to ethanol from fermented citrus juice and that exceed the values obtained from other honeys by 5 mg/kg. This difference in D/H can be used to confirm the authenticity of citrus honey. The

^{13}C values of all honeys tested were similar and typical to C3 plants. Carbon SIRA regarding the ^{13}C values can be used for the detection of the addition of sugars (cane sugar, corn syrup) to honey. This method is not suitable for the determination of the botanical or geographical origins of some honey samples. However, the ratio D/H could represent a useful method for the determination of citrus honeys. This method could probably be extended to various other floral honey samples and to other stable isotopes such as $^{18}O/^{16}O$ in the samples.

Isotope ratio mass spectrometry is a promising tool for origin assignation of food samples. It sometimes can distinguish the geographical origin of samples by itself. More frequently, it is effective to use it with other determinations and then combined via multivariate statistics. One of the greatest limitations to the application of the technique in origin assignation is the lack of large databases of isotopic abundances in food items.

IV. SPECIAL MARKER COMPOUNDS

A. Abscisic acid in heather honey

cis/*trans*-Abscisic acids are characteristic in Portuguese heather honey, and their content ranged between 0.3 and 17 mg/kg in honey (Ferreres *et al*., 1996a,b). These compounds have not been detected in any of the different monofloral honey samples analyzed so far and, therefore, can be useful markers for heather honey.

B. Hesperetin and methyl anthranilate in citrus honey

The flavanone hesperetin has been detected in citrus honeys but not in honey samples of any other origins (Ferreres *et al*., 1993). It is a constitutive phenolic compound of citrus nectar, where it is present as a glycoside (hesperidin).

Methyl anthranilate is a special volatile compound of citrus nectar and honey and has been used as a marker for citrus honey (Bicchi *et al*., 1983; Ferreres *et al*., 1994e; Graddon *et al*., 1979; Nozal Nalda *et al*., 2005; Serra Bonvehi, 1988; Serra Bonvehi and Ventura Coll, 1995; Vinas *et al*., 1992; White, 1966). Methyl anthranilate is volatile; significant changes in concentration can occur under various environmental conditions and under different honey storage conditions (Serra Bonvehi, 1988; Serra Bonvehi and Ventura Coll, 1995; White *et al*., 1964). Ferreres *et al*. (1994c) measured the content of hesperetin and methyl anthranilate in 18 honey samples from Spain. No correlation was obtained between the content of the two compounds. The concentration of methyl anthranilate ranged from 1.4 to 3.6 mg/kg, while hesperetin ranged from 0.3 to 0.9 mg/kg. Hesperetin is

proposed to serve as an additional marker in the determination of citrus honey origin.

C. 3-Aminoacetophenone in chestnut honey

3-Aminoacetophenone was detected to be specific to Italian chestnut honeys (Bonaga and Giumanini, 1986).

V. CONCLUSIONS

Honey authentication can ensure honey quality and safety and can facilitate the advances of apicultural industry. The identification and characterization of honey botanical and geographical origins are complicated. It often requires the use of several criteria and chemical markers and a combination of several analytical methods as well as appropriate statistical analyses. There are many diagnostic compounds and analytical techniques that can be used to obtain information relevant to the botanical and geographical sources of honey. Because the components of the different honey types are generally similar, spectroscopic techniques such as IR can offer overall compositions and profiles. Techniques offering highly specific compositional properties are desirable for identifying botanical and geographical sources. In addition, an ideal method would be fast and economical, require little sample preparation, automation, and provide highly specific information relevant to botanical and geographical sources of honey. MALDI TOF MS analysis represents a new promising method for botanical and geographical source identification. Other new analytical methods include various GC tandem MS and HPLC tandem MS.

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