Physico-chemical methods for the characterisation of unifloral honeys: a review

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1. INTRODUCTION

The objective of this work is to review all known physico-chemical methods used for the determination of the botanical origin of honey. In earlier works special attention is ascribed to the classification power of different classification methods (Persano Oddo et al., 1995b, 2000; Anklam, 1998; Molan, 1998; Bogdanov and Martin, 2002). This review gives a critical evaluation of all physico-chemical methods, which have been used up to the present time for the classification of unifloral honeys. The review is divided into two parts. The first one provides a general survey of classical physico chemical methods. These methods are mostly validated and widely used in the routine control of honey. In the second part the utility of new methods for authentication of botanical origin is discussed. In most cases, these methods are not yet harmonised and validated for routine use. The principle of each new method is briefly discussed, without going into details, which can be found in the cited references.

2. ROUTINE PHYSICO-CHEMICAL ANALYSIS

Routine physico-chemical analysis yields quite reproducible results, even when data from many different laboratories are used to analyse unifloral honeys from different countries (Persano Oddo and Piro, 2004).

The analytical methods used for honey classification are mostly the same as the ones used for routine honey control. They have been validated and harmonised by the International Honey Commission (Bogdanov et al., 1997) and can be used within the scope of the Codex Alimentarius Honey Standard (Codex Alimentarius, 2001) and the European Union Honey Directive (European Commission, 2002). These methods are placed on-line on the website of the International Honey Commission (IHC)[1] and are constantly improved. Since the publication of the harmonised methods there are some changes (see discussion below), which have been included in the on-line version of the methods.

In this section, the discrimination power of the different routine quality criteria are discussed. Further information on the same quality criteria is given in other two papers of this issue (Persano Oddo and Piro, 2004; Piazza and Persano Oddo, 2004).

2.1. Colour

Colour is the physical property perceived most immediately by the consumer. The determination of colour is a useful classification criterion for unifloral honeys, varying from

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[1] http://www.apis.admin.ch/host/honey/ introduction. htm

Table I. Methods for the determination of the botanical origin of honey.

METHOD, PARAMETERS	STATUS, REMARKS
Classical methods	
Determination of routine physicochemical parameters electrical conductivity, sugars, fructose/glucose ratio, enzyme activity, proline, colour, optical rotation, pH, acidity	– Used together with pollen and sensory analysis; electrical conductivity and fructose/glucose ratio are most useful. – Chemometric approach is promising. – Routine parameters can also be determined by IR spectroscopy.
Other methods	
Determination of polyphenols by HPLC	Shows some good results, but present methods unsuitable for routine testing because of labour-intensiveness.
Determination of volatile compounds by dynamic head space or SPME, followed by GC-MS or electronical noses	Promising methods, which should be further improved and developed for quantitative analysis of volatiles. Electronic noses are also promising, but may not be commonly available in food analysis laboratories.
Amino acids	Has some discriminatory power, but depends also on geographical origin.
Immunoblot assays of honey proteins, originating from pollen.	Supplementary method to classical melissopalynology.
Trace elements	Express some discriminatory power, but may also depend on geographical origin and climate.
Aliphatic carboxilic acids	Limited discriminatory power as most acids are added to honey by the bees.
Infra red spectroscopy	Promising new and fast method, which should be further developed.
Pyrolysis mass spectroscopy	Promising new method, but demands expensive instrumentation.

water white, through amber tones, to almost black, with possible typical hues in some honey types, such as bright yellow, greenish or reddish. Indeed, the pricing of honey depends to a great extent on honey colour, light honeys like Acacia and Citrus generally achieving the highest prices. In Germany, Austria and Switzerland, dark honeydew honeys are especially appreciated.

The most commonly used methods are based on optical comparison, using simple colour grading after Pfund (Fell, 1978) or Lovibond (Aubert and Gonnet, 1983). The values of these comparators give a measure of colour intensity, but only along the normal amber tone of honey. The Lovibond comparators are easier to handle than the Pfund graders, but honey is generally marketed according to the Pfund colour scale. That is why at present Lovibond graders with a Pfund scale are marketed. Other more objective methods have also been tested, as the determination of all colour parameters through the CIE L*a*b* *tristimu-* *lus* method (Aubert and Gonnet, 1983; Ortiz Valbuena and Silva Losada, 1990; Persano Oddo et al., 1995a), but none of these has been yet introduced in routine honey control. Recently, reflectance spectroscopy was also used for honey classification (Negueruela and Perez, 2000; Terrab et al., 2002a). However, before these methods can be applied to routine honey classification, they have to be validated by a comparison with the classical colour grading methods.

2.2. Optical activity

As a sugar solution, honey has the property of rotating the plane of polarised light. Some sugars (e.g. fructose) exhibit a negative optical rotation, while others (e.g. glucose) a positive one. The overall optical rotation depends on the concentration of the various sugars in honey. The determination of the specific rotation by means of a polarimeter is useful, mainly for the differentiation between honeydew honeys

(dextrorotatory, positive values) and blossom honeys (laevorotatory, negative values), but has also some classification power for different unifloral blossom honeys (Battaglini and Bosi, 1973; Piazza et al., 1991; Al-Khalifa and Al-Arify, 1999; Persano Oddo et al., 2000). The method for the determination of optical rotation by polarimetry is described in Bogdanov et al. (1997).

2.3. Electrical conductivity

The measurement of electrical conductivity (EC) was introduced a long time ago (Vorwohl, 1964). At present it is the most useful quality parameter for the classification of unifloral honeys, which can be determined by relatively inexpensive instrumentation. This has been confirmed by the data, published in this issue (Persano Oddo and Piro, 2004).

On the basis of an extensive survey of EC values on honeys originating from different parts of the world (Bogdanov et al., 1999), this parameter was included recently in the new international standards for honey (Codex Alimentarius, 2001; European Commission, 2002), replacing the determination of ash content. Indeed, EC correlates well with the mineral content of honey, (Accorti et al., 1987). In these standards maximal EC values for blossom honeys (except chestnut honey) are introduced for differentiation between honeydew and blossom honeys.

The method for the determination of electrical conductivity is described in Bogdanov et al. (1997). Honey EC values are expressed in milli Siemens/cm at 20 °C, while nowadays the international reference measurements should be carried out at 25 °C. This contradiction needs to be resolved.

2.4. Carbohydrates

Sugars are the main constituents of honey, comprising about 95% of honey dry weight. The relative amount of the two monosaccharides fructose and glucose is useful for the classification of unifloral honeys, as well as the fructose-glucose and glucose-water ratios (Talpay, 1985; Sabatini et al., 1989; Persano Oddo and Piro, 2004).

The minor sugars have a relatively low diagnostic value for the determination of botanical origin (Low et al., 1988; Sabatini et al., 1990; Goodall et al., 1995; Mateo and Bosch-Reig, 1997; Radovic et al., 2001c). The small differences between the minor sugar spectra of unifloral honeys are explained by the fact, that the oligosaccharides are mainly a product of honey invertase (Raude-Roberg, 1994). Only chemometrics of sugar spectra, determined by ion exchange chromatography has shown better results (see Sect. 2.11).

On the other hand, there are considerable differences between blossom and honeydew honeys, the latter containing a higher amount of oligosaccharides, mainly the trisaccharides melezitose and raffinose, both absent in blossom honeys. The differentiation between different types of honeydew honeys is difficult. An attempt to differentiate between honeydew honeys from various aphids was made by von der Ohe and von der Ohe (1996). Qualitative and quantitative differences in trehalose-, raffinose- and 2 oligosaccharides, called L1 and L2, were found. Metcalfa honey, a new honeydew honey type, produced mainly in Italy, can be distinguished from other honeydew honeys as it is rich in maltotriose and contains particularly high amounts of oligomers called dextrins (Barbattini et al., 1991; Fiori et al., 2000).

The sugar composition can be determined by different chromatographic methods: HPLC with refractometric detection, ion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and gas chromatography (GC) with FID detection. These methods are described and validated by the IHC (Bogdanov et al., 1997). The results are comparable, as far as the main sugars fructose, glucose and sucrose are concerned. The quantitation of oligosacharides is less comparable when assessed by the different methods, due to the fact that they do not achieve the same level of separation and sensitivity.

2.5. pH and acidity

All honeys are acidic with a pH-value generally lying between 3.5 and 5.5, due to the presence of organic acids that contribute to honey flavour and stability against microbial spoilage. In honey the main acid is gluconic

acid, which is found together with the respective glucono-lactone in a variable equilibrium (White et al., 1958). Free acidity, total acidity and pH have some classification power for the discrimination between unifloral honeys, while lactones, due to their strong variability, do not provide useful information (Persano Oddo et al., 1986; Persano Oddo and Piro, 2004).

The methods for the determination of free acidity by titration to pH 8.3 or to an equivalence point have a poor reproducibility (Bogdanov et al., 1997), due to lactone hydrolysis during titration. The reproducibility of the measurement of total activity (free acidity + lactones) is slightly better (Conte et al., 2002).

2.6. Proline

Proline, the main amino acid of honey, added to honey by the bee, is a criterion of honey ripeness (von der Ohe et al., 1991). This parameter shows characteristic values in different unifloral honeys (Sabatini and Grillenzoni, 2002; Persano Oddo and Piro, 2004), roughly correlated with the enzyme activity (Sabatini and Grillenzoni, 2002). However, the variation of this parameter in different unifloral honeys is quite high and it is not possible to classify unifloral honey on the basis of proline content only (Sanchez et al., 2001; Sabatini and Grillenzoni, 2002; Persano Oddo and Piro, 2004).

The proline content is easily determined by photometry (Bogdanov et al., 1997).

2.7. Diastase and invertase

Even if enzymes are added to honey mostly by bees, the various honey types show considerable differences in enzyme activities (Persano Oddo et al., 1990; Persano Oddo et al., 1999; Persano Oddo and Piro, 2004), most likely due to the rate of the nectar flow and to the physiological stage of the bees' glands during the productive season. However, as the enzyme activity of honey decreases after storage and heating of honey, it can only be used for the classification of unifloral honeys if fresh honeys are examined.

The methods for the determination of diastase and invertase activity are described (Bogdanov et al., 1997). Later, another formula was found for the diastase determination with the Phadebas method in honeys with low enzyme content (Persano Oddo and Pulcini, 1999). For the expression of invertase results, international units (U/kg) were proposed instead of Hadorn numbers (von der Ohe et al., 1999). These changes were included in the online IHC methods.

2.8. Water content

The water content is a quality parameter, important above all for honey shelf life. It has a minor importance for the characterisation of unifloral honeys. However, depending on the production season and the climate, unifloral honeys show some typical differences in water content, which affect the physical properties of honey (viscosity, crystallisation) and also influence the value of the glucose/water ratio (Persano Oddo and Piro, 2004). However, water content can be artificially altered during honey processing.

Moisture is routinely determined by refractometry by an Abbé analogue refractometer (Bogdanov et al., 1997). Digital refractometers can also be used for the determination of water content, as the results achieved are not significantly different from those obtained with the analogue ones (Bogdanov, 1999). The values, determined by refractometry are somewhat lower than the true water content, which can be measured only by Karl Fischer titration (Zürcher and Hadorn, 1980; Bogdanov, 1999).

2.9. Hydroxymethylfurfural

Fresh honey does not contain hydroxymethylfurfural (HMF). Thus, HMF is not a criterion for the botanical classification of honey. However, before determining storage-dependent parameters like enzyme activity and colour, one should ensure that honeys are fresh and unheated. Before testing these parameters, it should be checked that the HMF content is below 15 mg/kg.

Three methods for the determination of HMF are described and validated by the IHC (Bogdanov et al., 1997). Only two of them are recommended for use: the HPLC and the White method. The Winkler method should not be used because one of the reagents (p-toluidine) is carcinogenic. Since the publication of the

IHC methods there is a change in the procedure of the HMF determination by HPLC: a Carrez treatment of the honey solution is necessary in order to prevent HMF break-down (Wunderlin et al., 1998; Känzig et al., 2001).

2.10. Determination of routine honey parameters by infrared spectroscopy

Infrared spectroscopy (IR) is based on the absorption of electromagnetic radiation at wavelengths in the range between 800 and 2500 nm. IR has several advantages: it is rapid, non destructive and allows a determination of multiple measurand in a single step. Recently it has been successfully applied for the quantitative determination of analytes in honey.

Quantitative infrared spectrometry is generally based on calibrations by partial least squares regression between the spectra and the results obtained by physico-chemical reference methods. Near Infrared spectroscopic (NIR) results correlate well with optical activity (Garcia Alvarez et al., 2002), as well as with moisture, fructose, glucose, sucrose and maltose content (Qiu et al., 1999; Pierard et al., 2000). Mid-infrared spectroscopy (MIR) was successfully applied for the determination of fructose, glucose, sucrose, maltose and erlose, electrical conductivity, pH value and free acidity (Lichtenberg-Kraag et al., 2001). Using the same method, invertase activity, moisture, proline and HMF content could not be accurately measured. Quantitative infrared spectrometry depends on calibration with results obtained by reference methods. Thus it is relatively time consuming to build the statistical model and is only profitable if a large number of samples can be analysed. On the other hand, once the calibration is established it can be transferred from one apparatus to another. The calibration is only valid within the concentration range of the analytes present in the samples used for the calibration and for the corresponding honey types. Thus, before applying infrared spectroscopic methods, the validity of the calibration should be checked by comparison with the reference methods, and adapted, if necessary. Before introducing the IR determinations as an international standard method, it should be harmonised and validated for use with all major commercial honey types.

2.11. Chemometric analysis of classic parameters

It is not possible to classify unifloral honeys by single routine parameters. However, by using statistical methods such as principal component analysis (PCA), linear discriminant analysis (LDA) and cluster analysis (CA) on different honey quality parameters (sugars, electrical conductivity, optical rotation, acidity, proline, nitrogen content) a good classification of unifloral honeys can be achieved (Krauze and Zalevski, 1991; Bogdanov, 1997; Vinci et al., 1997; Popek, 2002; Piro et al., 2002; Terrab et al., 2002b). Chemometry of sugars alone has also been examined, but it showed only a limited success in a Canadian (Goodall et al., 1995) and a Spanish study (Mateo and Bosch-Reig, 1997). Cordella et al. (2003) were more successful by using pattern recognition statistics on HPAEAC-PAD sugar profiles.

Different unifloral honeys can be correctly classified by chemometric tools, but their ability to differentiate between unifloral and multifloral honeys has not yet been shown. Also, the different statistical models have not yet been harmonised for routine use in different laboratories. Indeed, due to the immense variation of honey composition, and also to the interlaboratory analytical variation, chemometrics may not allow a routine classification of unifloral and multifloral honeys based on classic parameters in different laboratories. However, this approach might be useful as a classification tool for unifloral honeys in internal quality control by honey packers. The advantage of the method is that it allows a honey classification after normal routine quality control.

The chemometric methods are applied with the help of statistics software containing PCA, LDA and CA modules.

3. OTHER PARAMETERS, USED FOR THE CLASSIFICATION OF UNIFLORAL HONEYS

3.1. Phenolic acids and polyphenols

Phenolic acids and polyphenols are plant-derived secondary metabolites. These compounds have been used as chemotaxonomic

markers in plant systematics. They have been suggested as possible markers for the determination of botanical origin of honey. Considerable differences in composition and content of phenolic compounds between different unifloral honeys were found. Dark coloured honeys are reported to contain more phenolic acid derivatives but less flavonoids than light coloured ones (Amiot et al., 1989).

In another study the flavonoid profile of *Citrus* honeys was studied and compared with the profiles of *Rosmarinus*, *Lavandula*, *Tilia*, *Helianthus*, *Prunus dulcis*, *Castanea*, *Trifolium repens*, *Robinia*, *Rhododendron*, *Prosopis*, *Eucalyptus*, *Calluna* and multifloral honeys (Ferreres et al., 1993). Hesperetin (5,7,3'-trihydroxy-4'methoxyflavanone) was detected in *Citrus* honeys only and was proposed as a marker substance. Hesperetin content of *Citrus* honeys was compared to the methyl antranilate concentration (Ferreres et al., 1994b) but no consistent relationship could be found. Since hesperetin is more stable than methyl antranilate it was proposed as a complementary marker for *Citrus* honey. Homogentisic acid was proposed as a marker of *Arbutus* honey (Cabras et al., 1999).

In a recent study (Tomas-Barberan et al., 2001) the flavonoid profile of 9 European unifloral honeys was analysed by HPLC. Hesperetin was confirmed as a marker of *Citrus* honey. No specific compounds could be detected in *Robinia* and *Lavandula* honeys. Abscisic acid, formerly reported as a characteristic compound of *Calluna* honey (Ferreres et al., 1994a) was also detected in *Brassica*, *Tilia* and *Robinia* honeys in similar concentrations. *Erica* honey was characterized by the presence of hydroxybenzoic, syringic, o-coumaric and ellagic acids. The gallic acid dimer ellagic acid was confirmed as a marker of *Calluna* honey. These findings agree with similar results found in heather honeys from *Erica* and *Calluna* species (Andrade et al., 1997a, b). All honey samples contained variable amounts of propolis-derived phenolic compounds that were not helpful for the determination of botanical origin. Thus, the determination of the flavonoid patterns is useful for the classification of some but not all unifloral honeys.

The methods used for flavonoid analysis are very time consuming as different purifica-tion steps are necessary before the determination, which is carried out most often by HPLC (Tomas-Barberan et al., 2001).

3.2. Analysis of volatile compounds

Research on honey volatiles started in the early 1960s. Recently, by studying volatiles isolated from the blossom and from the respective unifloral honey, it was found that most volatile compounds originate probably from the plant, but some of them are added by bees (Cepurnoi, 2000; Alissandrakis et al., 2003). Until the present time about 600 compounds have been characterised in different honeys, many of them being unifloral.

As unifloral honeys differ in respect of their sensory properties, it is probable that analysis of volatile compounds will allow classification of unifloral honeys. Because of their importance for the development of new methods for the classification of unifloral honeys, this section is dealt with in greater detail than the other parts of this review. The different methods for volatile extraction and analysis, used in practice, will be reviewed separately. Determination of volatiles is carried mostly by GC-MS. Attention should be given in volatiles studies to supply the correct name of the substance.

3.2.1. Extraction after Likens-Nickerson

The methodology that is capable of completely extracting honey volatiles is simultaneous solvent extraction, followed by a Likens and Nickerson steam distillation. This was adapted by Bouseta and Collin (1995) for the determination of honey volatiles of *Castanea*, *Tilia* and *Calluna* honeys (Bouseta and Collin, 1995; Guyot et al., 1999). Several marker compounds were identified. The volatile composition of nine additional unifloral honeys from various countries (*Abies*, *Lavandula*, *Citrus*, *Brassica*, *Robinia*, *Rosmarinus*, *Helianthus* and *Trifolium repens*) was studied as well, in order to confirm, that the identified markers are specific (Guyot et al., 1998, 1999). The method of Nickerson and Likens is very labour-intensive and is presently replaced by other, faster methods, e.g. purge and trap.

3.2.2. Headspace extraction

Dynamic headspace extraction coupled with a GC-MS system was introduced by Bouseta et al. (1992). The qualitative and quantitative composition of the volatile fraction of various unifloral honeys was found to be different. The results allowed a classification of *Lavandula*, *Abies*, *Eucalyptus*, *Taraxacum* and *Brassica* honeys. In a recent study several markers for the determination of botanical origin were proposed (Radovic et al., 2001a). However, in this study the number of samples per honey type was very limited.

Dynamic headspace extraction of honey volatiles seems to be a promising approach for the determination of botanical origin of honey, also suitable for routine analysis. It can be carried out by an autosampler in situ, which makes it very convenient for routine studies. However, extraction conditions should be further optimised in order to extract more semi-volatiles.

3.2.3. Solid Phase Microextraction

Another new technique for the extraction of honey volatiles is Solid Phase Microextraction (SPME). It was first applied on several Italian unifloral honeys by Guidotti and Vitali (1998). The authors used a PDMS coated fibre. In the chromatograms of *Robinia*, *Castanea*, *Eucalyptus*, *Tilia* and *Thymus* honeys 20 to 30 peaks were detected. The differences between the chromatographic patterns and also peak heights of the same unifloral type were considerable. Many of the compounds were detected in all unifloral honeys but some of them were found in only one type of honey and were considered as markers.

The method described by Guidotti and Vitali was adapted by Piasenzotto et al. (2003). Many volatile compounds, reported by previous authors working with other methods, were detected in *Eucalyptus*, *Tilia*, *Citrus*, *Taraxacum*, *Castanea* and *Thymus* honeys. Most of the compounds were present in several types of unifloral honeys, but some were restricted to one unifloral source and could therefore be used as markers. High variability in concentration of the compounds was reported for different honey samples of the same unifloral source. This variation can be explained by the natural variation of the honeys. Since different samples of honey of the same botanical origin have similar chromatographic profiles, SPME was judged to be a good method for the determination of botanical origin by specific chromatographic fingerprints.

Another SPME method for the extraction of honey volatiles was developed by Verzera et al. (2001) by using a PDMS/DVB coated fibre. Unique chromatographic profiles were obtained for each type of the unifloral honeys studied (*Eucalyptus*, *Citrus*, *Hedysarum*, and *Castanea*).

In another work Carboxen/PDMS and PDMS/DVB coated fibers were used (Perez et al., 2002). The first fibre was shown to be able to extract more highly volatile compounds. *Citrus*, *Eucalyptus*, *Rosmarinus*, *Lavandula* and *Thymus* honeys were studied and a total of 35 components were detected. Eleven of them were found in all honeys investigated, while some others were proposed as characteristic for certain unifloral honeys. Canonical discriminant analysis was performed to find out the characteristic compounds of each unifloral honey studied. The honeys could be correctly classified by using the canonical functions. It is stated that this technique can be used for the authentication of unifloral honeys although further studies are necessary to confirm the utility of the technique. This is the only method that enables a quantitative determination of honey volatiles. Relatively few and highly volatile compounds were detected. However it should be mentioned that quantitation of different volatiles by external standards is very labour intensive.

In a recent study a triple phase DVB CAR/PDMS SPME fibre was tested for analysis of honey volatiles (Ruoff, 2003). Theoretically, this fibre should be capable of extracting more volatiles than the ones tested in previous studies. The major drawback of the method are the cracks in the fibre coating of most commercial fibre lots. The repeatability of the method is good for a broad range of compounds, as tested with a fibre with a smooth coating. With this technique 26 new compounds could be tentatively identified in *Brassica* honeys from Swiss winter rape and Finnish summer turnip rape (Ruoff, 2003). Benzenenitrile was detected in all winter rape and spring turnip rape honeys and might be a possible marker compound. Although being very similar, the volatile patterns

of the rape and turnip rape honeys were significantly different.

The above cited works shows that analysis of volatiles by SPME is a promising approach for the determination of the botanical origin of honey. However, there is still a long way to go before this method can be used for the determination of the botanical origin of honey in routine work. For this purpose the SPME-methods should be adapted for quantitative determinations of volatiles. In order to establish compositional criteria, a large number of unifloral honeys have to be collected and their volatile composition identified and quantified by GC-MS and/or GC-FID. The results should be compared to the results of the classical methods and the methods should be validated in ring trials.

3.2.4. Analysis of volatile compounds by electronic nose

The principle of the electronic nose is to excite an array of sensors with the volatile fraction of the sample as a whole, without any separation into individual chemicals. This generates a "fingerprint" (response of the array of sensors) characteristic of each sample. The set of signals for all samples (set of fingerprints) is then statistically processed. Many applications have been reported for other foods, most of them using specific sensors. The electronic nose has been successfully used for the classification of Swiss unifloral honeys (Ampuero et al., 2004; Benedetti et al., 2004).

At present electronic noses are tested only in experimental research and there is a long way to go before they can be routinely used in food control laboratories.

3.2.5. Specific volatile markers

The first volatile marker for unifloral honeys was methyl anthranilate, found only in *Citrus* honey (Deshusses and Gabbai, 1962; White, 1966; Serra-Bonvehi, 1988). Methyl anthranilate can be quantitatively determined by distillation and subsequent photometry (White, 1966) by HPLC (Del Nozal et al., 2001) or by GC (Serra Bonvehi, 1988). Although many volatile compounds have been recently suggested as unifloral markers (see above) none of these has the status of an established marker compound, used for routine classification of unifloral honeys.

3.3. Amino acids and proteins

Honey contains different free amino acids, the main one being proline (see Sect. 2.6). The content of free amino acids of unifloral honeys was studied by many workers (Berger and Hahn, 1972; Petrov, 1974; Bosi and Battaglini, 1978; Kanematsu et al., 1982; Speer and Montag, 1986; Pirini et al., 1992; Pawlowska and Armstrong, 1994; Bouseta et al., 1996; Conte et al., 1998; Cotte et al., 2004; Iglesias et al., 2004). In most of these studies one or a few samples per honey type were analysed. In the honeys studied, the amino acid spectra were the same, but quantitative differences between the individual amino acids were encountered.

In other works, multivariate statistics was used to discriminate between the honey types. Floral and honeydew honeys (Iglesias et al., 2004) could be discriminated. In a study with 7 selected honey varieties only lavender honey could be differentiated, while there was no difference between the 6 other honey types (Cotte, 2004). A significant part of the free honey amino acids are added by the bees (Bergner and Hahn, 1972). This leads to a high variability of the amino acid content within honeys from the same botanical source (Davies, 1975; Gilbert et al., 1981). Honeys with different geographical, and thus also of different botanical origin, could be differentiated by canonical discriminant analysis of the amino acid data (Gilbert et al., 1981; Davies and Harris, 1982). All these results show that it is difficult to differentiate between individual unifloral honeys on the basis of the analysis of free amino acids. However, it seems that it is possible to differentiate between honeys of different geographical origin.

In two studies, amino acids were analysed after hydrolysis of the honey proteins (Bosi and Battaglini, 1978; Perez and Herrera, 1987), but in these studies, too, only one or a few samples per honey type were examined. Only quantitative differences between the amounts of the different amino acids were encountered. Multivariate statistics led to differentiation between the different honey types (Perez and Herrera, 1987). It should be borne in mind that protein amino acids are not specific

unifloral markers, as a major part of the honey proteins, the enzymes diastase, invertase and glucose oxidase are of bee origin (Bergner and Diemair, 1975).

Amino acids are generally determined by HPLC (e.g. Perez and Herrera, 1987) but GC can also be used (Pirini et al., 1992).

Recently, an immunoblot assay of honey proteins was successfully used for the determination of botanical origin (Baroni et al., 2002). As some of these proteins originate from pollen, these can be used as specific markers. This method, however, will have all the shortcomings of pollen analysis (see von der Ohe et al., 2004).

3.4. Trace elements

Different investigations showed that there are some differences in the trace elements of unifloral honeys, but the most pronounced ones were those between honeydew and blossom honeys. (Ivanov and Chervenakova, 1984; Feller-Demalsy et al., 1989; Sevimli et al., 1992). Some of the differences may also be due to the different geographical origin of the honeys. Indeed, trace elements are good indicators of the geographical origin of food, and thus probably also of honey.

The analysis of minerals is mostly carried out by radioactivation analysis (Sevlimi et al., 1992), while ICP-MS (inductively coupled-plasma mass-spectrometry) is the most recent analytical method (Caroli et al., 1999).

3.5. Aliphatic organic acids

Thirty-two aliphatic dicarboxylic acids were identified in four unifloral New Zealand honeys by GC-MS. Methylbutanedioic acid and 4-hydroxy-3-methyl-trans-2-pentenedioic acid were proposed as floral markers for *Knightea excelsa* honeys (Wilkins et al., 1995). Analysis of formic, citric, pyruvic, malic, fumaric, pyro-glutamic, gluconic, galacturonic, citramalic and quinic acids of *Onobrychis*, *Rosmarinus*, *Lavandula*, *Thymus*, *Quercus* and *Erica* honeys were performed by HPLC (Del Nozal et al., 1998). Significant differences in the the concentration of the acids between the honey types were encountered. In another HPLC study analysis of oxalic, for-

mic, citric, ,pyruvic, malic, fumaric, pyro-glutamic and gluconic acids were studied in *Robinia*, *Eucalyptus*, *Brassica*, *Tilia*, *Lavandula*, *Rosmarinus*, *Castanea* and *Erica* honeys (Radovic and Anklam, 1999). There were some differences in the acid composition of the different honeys, but the number of samples was very limited. On the other hand, it was reported, that most honey acids originate from honey bees (Echigo and Takenaka, 1974). Thus, it is questionable, if acids are good markers for unifloral honeys.

Acids can be determined by HPLC (Del Nozal et al., 1998; Radovic and Anklam, 1999) or GC-MS (Wilkins et al., 1995).

3.6. Infrared spectroscopy

Infrared spectroscopy (IR) of different unifloral honeys and subsequent statistical analysis by PCA, and LDA or CA showed that a successful classification of the botanical origin of honey is possible. Recently, both NIR (Davies et al., 2002; Ruoff et al., 2003) and MIR, (Lichtenberg-Kraag, 2003) were successfully used for classification of unifloral and multifloral honeys.

NIR was also applied for the determination of a specific sugar of avocado honey, perseitol (Dvash et al., 2002). Although the average concentration of perseitol in honey samples was only 0.48 g/100 g, avocado honeys could be differentiated from other honeys, which do not contain this sugar.

It seems that IR can be used as a screening method for the classification of unifloral and multifloral honeys. Its advantage is that the determination of the botanical origin can be performed simultaneously with routine quality control (see Sect. 2.10).

3.7. Pyrolysis-mass spectroscopy

Pyrolysis-mass spectroscopy was successfully tested for discrimination of the botanical origin and also of the geographical origin of honey (Radovic et al., 2001b). The data was analysed statistically by PA and LDA. The instrumentation used for this method is very expensive and is thus mostly unavailable in food control laboratories.

4. CONCLUSIONS

Today, routine physicochemical methods are used in the classical approach for the authentication of botanical origin, together with pollen and melissopalynological analysis (see Introduction to this issue). These methods are mostly validated and widely used in the routine control of honey. However, this classical approach is laborious and depends on highly specialized personnel. In the last few years it has been often attempted to use a single analytical method for the determination of botanical origin. Some of the applied methods are very promising, e.g. the analysis of honey volatiles and IR spectroscopy. They should be further developed and tested for routine use with a sufficient number of authentic samples of unifloral and multifloral honeys.

The utility of the different methods is summarised in Table I.

Generally, two approaches for the determination of the botanical origin of honey are used. The first is to find and quantify specific parameters of unifloral honeys. In the second approach different quality parameters are first determined and then chemometric analysis is applied for honey classification. The first approach is easier and more straightforward, but due to the immense natural variation of honey composition, it may not be always possible to find specific markers for each unifloral honey and to define precise limits between unifloral and multifloral honeys. The second approach yields statistical models for the classification of a known group of unifloral honeys. The application of this approach in future routine work needs statistical models that are based on the analysis of a representative number of genuine unifloral honeys. In these models polyfloral honeys, too should also be included. Also, the chemometric techniques should be harmonised for routine use in control laboratories.

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