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A review of the analytical methods to determine the geographical and botanical origin of honey

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This review is concerned with analytical methods to prove the authenticity of honey. A special emphasis is put on suitable methods for the detection of the geographical and botanical origin of honey. Whereas the determination of some single parameters, such as 5-hydroxymethylfurfural (HMF), moisture, enzyme activity, nitrogen, mono- and disaccharides, and residues from medicinal treatment or pesticides in honey does not lead to any information about the botanical and geographical origin, there are some suitable methods based on the analysis of specific components or on multi-component analysis. Mostly, such methods give indications of the botanical origin, investigating flavonoids patterns, distribution of pollen, aroma compounds and special marker compounds. There are some other profiles of components which could probably be used for the detection of the geographical origin (e.g. oligosaccharides, amino acids, trace elements). In particular, the combination of methods could be a promising approach to prove authenticity, especially when modern statistical data evaluation techniques will be applied. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

The Commission of the European Union (EU) has adopted a proposal to amend the Council Directive 74/409/EEC concerning honey. This Directive laying down common rules for the composition and manufacture of honey will be supplemented by not preventing information referring to the product's: 'floral or vegetable origin, being stated if the product comes essentially from the indicated source and possesses its organoleptic, physicochemical and microscopic characteristics; regional, territorial or topographical origin, if the product comes entirely from the indicated source; specific quality criteria.' The Commission of the EU is encouraging the development of harmonized analytical methods to permit the verification of compliance with the quality specifications for the different honeys.

This work is concerned with the investigation of the suitability of analytical methods which allow the determination of the botanical and geographical origin. The existing methods described in the literature are evaluated. The conclusions of this study may facilitate further analytical work in order to prevent fraud and to protect authentic honey samples.

Honey is produced by honey bees from nectar of plants, as well as from honey dew. Some of the components (carbohydrates, water, traces of organic acids, enzymes, amino acids, pigments, pollen and wax) are due to maturation of the honey, some are added by the bees and some of them are derived from the plants. Honey of the same floral source can vary due to seasonal climatic variations or to a different geographical origin. In addition to the definition of honey according to the Codex Alimentarius (1981), there are additional definitions in the regulations of various countries and in the EU (Molan, 1996). Various physical types (pressed, centrifuged, drained) and forms (comb, chunk, crystallized or granulated, creamed, heat processed) of honey are on the market. Raw honey contains extraneous matter, such as pollen, traces of wax, variable amounts of sugar-tolerant yeasts, and probably crystals of dextrose hydrate. Unless the moisture content is below 17%, no fermentation takes place. Most honey will crystallize in time unless action is taken to prevent it. The processing of honey, thus includes controlled heating to destroy yeasts and dissolve dextrose crystals, combined with fine straining or pressure filtration (White, 1978*a*). Honey is usually warmed to a temperature of 32–40°C in order to lower its viscosity, this facilitates extraction, straining or filtration. This temperature is similar to that in beehives and does not affect the honey very much during the relatively short processing period. However, some honey samples are heated to higher temperatures for liquefaction or pasteurization reasons.

Honey consists mostly of the monosaccharides glucose and fructose. The actual proportion of glucose to

fructose in any particular honey depends largely on the source of the nectar. The average ratio of fructose to glucose is 1.2:1 (White, 1978*a*; White, 1980). Saccharose (sucrose) is present in honey (approximately 1% of its dry weight). However, this level can be increased if the beekeeper has over-fed the bees with sugar during the spring. The mineral content varies from about 0.04% in pale honeys to 0.2% in some dark honey samples. This content is dependent on the type of soil in which the original nectar bearing plant was located. The protein content of honey is normally less than 0.5%. A small fraction of the proteins are enzymes, these include: invertase, diastase, glucose oxidase and catalase. There are many other minor constituents of honey, including very low concentrations of vitamins and plant acids. Amongst the compositional criteria prescribed in the existing EC honey directive are requirements relating to the concentrations of acidity, apparent reducing sugar (calculated as invert sugar) and apparent sucrose, 5-hydroxymethylfurfural (HMF), mineral content (ash), moisture and water-insoluble solids.

Adulteration techniques of honey are based on two different principles: 'dilution' of honey by water addition, and extension with sugar and syrups (e.g. corn syrup, high fructose corn syrup (HFCS)), other adulterations are due to bee feeding with sugars and syrup or artificial honey and mislabelling concerning the floral or geographical origin.

In the following, emphasis is put on methods for the detection of the last principle, in other words, for proof of compliance with labelling concerning the floral or geographical source.

ANALYSIS METHODS

Amino acids and proteins

The nitrogen content of honey is low and varies. The mean value is 0.04% (40 mg in 100 g honey with a high standard deviation (White, 1978*b*). About 33–55% can be lost by ultra-filtration (Paine *et al.*, 1934; Bergner and Diemair, 1975). The amino acid proline dominates in honey, representing 50–85% of the total (White, 1978*a*). Honey contains about 0.2% protein (White, 1978*b*) which is of bee and plant origin (Lee *et al.*, 1985; Stadelmeier and Bergner, 1986; Croft *et al.*, 1986). This includes bee α-amylase and other enzymes (Stadelmeier and Bergner, 1986).

It has been suggested that certain ratios between concentrations of various amino acids could be used to determine the geographical source of a honey. In this respect, 98 honeys from six different geographical sources have been analysed (Davies, 1975, 1976). However, the botanical origin also varied. Regarding the ratio of aspartic acid/proline to amides/phenylalanine, it has been shown that there are variations in the ratios between samples of the same area. However, the variation between sources is much greater. Various kinds of honey from different botanical sources (acacia, citrus, chestnut, rhododendron, rosemary and lime) have been analysed by gas chromatography (GC) and the data evaluated statistically in order to gain knowledge about the possible use of amino acid patterns for classification (Pirini *et al.*, 1992). The results have shown that the presence of amino acids such as arginine, tryptophan, and cystine 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. Spanish honey samples from different botanical origins have been analysed by high performance liquid chromatography (HPLC; Perez Arquillue and Herrera Marteache, 1987). Sixteen protein amino acids were determined after acid hydrolysis of isolated protein fractions. Applying discriminant analysis of the results obtained, the authors could detect local and botanical differences with satisfactory results. Seventeen free amino acids have been determined by GC in 45 samples of honey obtained from the UK, Australia, Argentina and Canada (Gilbert *et al.*, 1981). The results were analysed statistically (using canonical variate analysis) in order to test the validity of the use of amino acid data for characterizing the geographical origin of honey. Good discrimination was shown between the samples from Australia, Argentina and Canada. Those from the UK were classified as a group, but fell between the samples from Argentina and Canada, and could be wholly discriminated only on further analysis when the Australian group was omitted. These results have shown that certain groups of honey samples from certain foreign countries could be distinguished from one another on the basis of free amino acids. HPLC methods have been used in order to determine the total amounts of proline, leucine and phenylalanine and their enantiomeric ratios in a variety of different honey samples (Pawlowska and Armstrong, 1994). Significant amounts of D-leucine and D-phenylalanine have been found in honeys of different botanical and geographical origins. The amino acid showing greatest variability within the samples analysed was leucine. The authors suggested that the enantiomeric ratios of amino acids could be used to test for storage effects, age, and the processing technique applied.

SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and high-resolution two-dimensional electrophoresis has been performed in order to investigate trace proteins of unconcentrated Australian honeys. The detected protein constituents were assumed to be predominantly of bee origin instead of floral origin. Only one sunflower sample showed proteins probably due to pollen origin (Marshall and Williams, 1987). Different protein fractions from Spanish (Galicia) honeys have been separated by electrophoresis in polyacrylamide gels and 12 different

fractions could be observed. Applying discriminant analysis, some classification could be carried out (Rodriguez-Otero *et al.*, 1990).

The content of nitrogen has been used to detect frauds in honey samples from Venezuela which had carbohydrates added to them. Samples containing less than 10 mg nitrogen per 100 g honey have been considered to be adulterated with sugars (Vit Olivier, 1987).

The analysis of the amino acid profiles seems to be more suitable for the detection of the botanical and geographical origin than that of the protein composition. However, the methods must be employed in conjunction with other techniques in order to obtain a reliable identification of the country of origin. The amino acid profiles could give an indication of the botanical source of honey samples. The amino acids arginine, tryptophan, and cystine have been shown to be characteristic for some floral honey types. Enantiomeric ratios of amino acids could be used for the detection of various different processing techniques and, therefore, geographical origin.

Aroma compounds

Flavour/fragrance qualities of food products and also of honey are very much dependent on the volatile and semivolatile organic compounds present in both the sample matrix and the headspace aroma. Volatiles contribute significantly to the honey flavour and to its variation with floral origin and the method of handling. The identification of volatile components is of importance to the understanding of flavour. An elucidation of the origin of aroma compounds should lead to a better understanding of factors causing flavour differences between honeys.

The isolation of volatile components from a complex mixture such as honey in order to obtain representative extracts is very difficult. In the specific field of honey, accurate quantification is essential in order to evaluate flavour changes linked to processing methods or long storage. Such knowledge would also be helpful in ascertaining a honey's floral origin. The simultaneous distillation–extraction (SDE) system developed by Likens and Nickerson (1964) and its modified version (Godefroot *et al.*, 1981) is one of the most applicable methods for the isolation of volatile compounds from a matrix. Since heat treatment can lead to artefacts, this extraction method has been again modified by the use of a vacuum which leads to the isolation of volatile compounds at room temperature (Maignial *et al.*, 1992). Comparing the results of an atmospheric SDE with those of SDE under vacuum from a non-specified honey sample, it could be seen that the atmospheric SDE led to a cooked honey flavour (the main components were furfural and HMF), whereas the latter gave a furfural-free extract with a fresh honey note. The corresponding GC exhibited a small peak of linalool oxide. A commercial Canadian honey was used for the optimization of the Likens–Nickerson method (Bouseta and Collin, 1995). Dichloromethane extraction under an inert atmosphere followed by simultaneous steam distillation–dichloromethane extraction appeared to be a useful method for honey flavour characterization. A complex mixture of hydrocarbons, alcohols, phenols, ethers, aldehydes, ketones, esters, furans and nitrogen compounds could be isolated and identified. The following 19 constituents could be identified by GC/mass spectrometry (MS) of the Canadian honey sample investigated: methylfuran, caproaldehyde, octane, furfural, furfuryl alcohol, l-hexanol, m-xylene, acetylfuran, HMF, benzaldehyde, α-pinene, phenol, β-pinene, benzyl alcohol, phenylacetaldehyde, phenethyl alcohol, camphor, coumarin, *trans*-caryophyllene. Volatile organic compounds were purged from nine commercially available honeys from various floral sources (wildflower, blueberry, orange, clover, tupelo, alfalfa, apple spread—geographical origin not indicated) and then trapped on an adsorbent resin (Overton and Manura, 1994). The adsorbent traps were subsequently analysed by thermal desorption GC/MS. This technique permitted the analysis of a wider range of both volatile and semivolatile organic compounds and was shown to be more sensitive by a factor of at least 100 as compared with the headspace technique. The honey samples analysed were found to contain numerous mono- and sesquiterpenoid compounds and flavours, such as benzaldehyde, furfural, isovaleraldehyde, and phenyllactaldehyde. The presence of the branched aldehydes methyl-butyraldehyde and 3-methyl-butyraldehyde in each of the honeys reflected the microbial quality and thermal treatment of honey. The author stated that this technique used together with pollen analysis may be used in floral source verification. The volatile components of a unifloral Italian chestnut honey have been isolated by steam distillation extraction and investigated by GC/MS (Bonaga *et al.*, 1986). Linear hydrocarbons, saturated and unsaturated, at an even and odd number of carbon atoms, from C1O to C37 were found in chestnut honey. *n*-Heptacosane, *n*-noncosane, *n*-tricosane, *n*-pentacosane, and *n*-hentriacontane were the largest gas chromatographic peaks in the *n*-alkane fraction of volatiles (about 40%), whereas *n*-tritriacontene and *n*-hentriacontene predominated in the unsaturated portion (about 60%). The positional and geometrical isomerism of the double bond in *n*-alkenes was investigated by the study of their epoxides. The same authors have shown that the extract of volatiles from Italian chestnut honey was a complex mixture of at least 50 compounds of which some of these, and particularly the main component of the mixture (3-aminoacetophenone) may be specific for the floral source (Bonaga and Giumanini, 1986). The flavour compound 1-(2,6,6-trimethyl- 1,3-cyclo-hexadienyl)-2-buten-1-one (β-damascenone) was quantified by a stable isotope dilution assay (SIDA) method in two honey samples (geographical origin not

indicated) (Grosch *et al.*, 1990). The content was about 3 ppb ($\mu g\,kg^{-1}$) in acacia honey and about 8 ppb in lime honey. Aroma concentrates from an aqueous solution of haze honey have been analysed by two different techniques (Shimoda *et al.*, 1996). One hundred and thirty compounds were identified. The sensory importance of the volatile compounds was investigated by sniffing the fractions separated by preparative GC. As a result, benzenacetaldehyde, linalool, phenethyl alcohol, *p*-crosol, *p*-ansisaldehyde, methyl-*p*-anisaldehyde, trimethoxybenzene, 5-hydroxy-2-methyl-4H-pyran-4-one and lilac aldehydes seemed to contribute to the haze honey aroma. Chemical fingerprinting of Australian honey required information on the composition of natural honey volatiles (D'Arcy *et al.*, 1997). The naturally occurring volatiles from two varieties of eucalyptus honey were isolated and analysed by GC–MS. Fifty-five compounds could be identified. The nature of the volatiles and semivolatiles in these two Australian honeys suggested that Australian honeys are quite distinctive relative to other honeys that have been investigated by GC–MS.

The analysis results on aroma compounds of honey are dependent on the isolation techniques and the detection modes. In addition, the honey flavour depends on the methods of processing and storage and on the botanical source. A careful analysis of the volatiles in honey could be a useful tool for the characterization of the botanical source. Typical compounds could be identified for honeys from some floral sources to-date. However, aroma profile analysis should be combined with other methods for the determination of other constituents.

Carbohydrates (sugars)

Sugars (saccharides) represent the main components of honey. Besides the two main constituents, the monosaccharides glucose and fructose, there are the minor components consisting of about 25 oligosaccharides (disaccharides, trisaccharides, tetrasaccharides). Honey is an extremely variable and complex mixture of sugars and other components. Limited availability and the increased price of honey have provided major incentives for falsification with other carbohydrate materials. In addition to the traditional adulterants, such as invert syrup (IS) and conventional corn syrup (CCS), high fructose corn syrup (HFCS) is also used for adulteration. One test for the presence of added IS is the determination of HMF. This test 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 composition of honey is useful in judging its authenticity.

Saccharides can be determined by a number of different methods based on the use of their physical characteristics (White, 1980; Peris-Tortajada *et al.*, 1992) or by chemical (Kumar *et al.*, 1988; Gritzapis and Timotheou-Potamia, 1989) or enzymatic methods (Schwedt and Hauck, 1988; Le Marec and Lesgards, 1990). Chromatography, such as thin layer chromatography (Allegretti *et al.*, 1987; Sangiorgi, 1988; Patzsch *et al.*, 1988; Pukl and Prosek, 1990), GC (Deifel, 1985; Mateo *et al.*, 1987; Low and Sporns, 1988; Serra Bonvehi and Bosch Callis, 1989), ion chromatography with an amperometric pulsed detector (Peschet and Giacalone, 1991) and HPLC (Cirilli *et al.*, 1986; Bogdanov and Baumann, 1988; Campos, 1989; Bugner and Feinberg, 1992), is useful for the separation and detection of saccharides. Several authentic Ligurian (Italy) honey samples have been studied with respect to their sugar composition by GC (Zunin *et al.*, 1987). The aim of this study was to detect the addition of syrups to honey. The maltose/isomaltose ratio was shown to be unsuitable for the detection of adulteration with syrups. However, the determination of the sucrose and erlose content was shown to have a potential for this purpose. The addition of sucrose in concentrations of less than 5% (Lipp and Ziegler, 1989) or the distinction between authentic honey from honey produced by artificially fed bees has been detected by HPLC (Calcagno *et al.*, 1987). However, the amount of sucrose can decrease during the storage of honey due to the presence of the enzyme invertase (White, 1992).

Anion-exchange liquid chromatography has been shown to be a suitable tool for oligosaccharide profile analysis (Swallow and Low, 1994). Honey, chemically and enzymatically produced IS and HFCS contain a complex mixture of oligosaccharides which are formed during the production processes. The presence of fingerprint oligosaccharides can be used to detect the illegal use of HFCS and IS in honey. The oligosaccharide profiles of 91 authentic British honey samples were obtained by high performance anion-exchange liquid chromatography (HPAE) with pulsed amperometric detection (PAD) (Goodall *et al.*, 1995). Multivariate statistical techniques used to investigate these profiles have been shown to be useful tools for the investigation of their botanical source.

Most of the methods described above are suitable for the determination of various sugars in honey and also for the detection of the addition of sucrose, HFCS and IS. The analysis of the oligosaccharide profiles (based on GC or HPAE) in combination with multivariate statistical techniques could be a promising method for the detection of the botanical origin of honey.

Enzyme activity

Enzyme activity could be a measure of the exposure of honey to heat in processing and storage. However, this value is less exact than that of the HMF content, because enzyme activities vary a lot for various honey samples. This is due to the fact that different amounts of saliva containing enzymes can be added by the bees to honey under different conditions.

The activity of the enzyme diastase in honey is related to its heat treatment. However, its activity gives only an

indication about the processing (heat treatment) of honey samples, but is not suitable for the detection of the origin.

Fermentation products

The polyol glycerol occurs as a minor constituent in honey and is probably produced by micro-organisms present in the nectar and honeydew which are collected by the bees. Glycerol may, therefore, be considered a fermentation product. In the fermentation of a 20% glucose solution, aeration and low phosphate content favour the production of polyols such as glycerol, whereas anaerobic fermentation produces mainly ethanol. The glycerol contents of 33 honey samples from Galicia (Spain) have been determined using an enzymatic method ranging between 50 and 370 mg kg ^{1} (ppm) (Huidobro *et al.*, 1993). Primary alcohols of unpasteurized Galicain (Spain) honeys have been determined as apparent ethanol contents by means of an enzymatic method (Huidobro *et al.*, 1994). The contents were in the range of 14–50 ppm.

The content of the fermentation products is dependent on micro-organisms present in honeydew and nectar and gives information about the processing of honey (pasteurization). The determination of the fermentation products does not seem to be a suitable tool for the characterization of the floral or geographical source of honeys.

Flavonoids

Flavonoids constitute a large family of plant phenolic pigments. Many plant systems contain an extensive number of flavonoids and each plant tends to have a distinctive profile. The flavonoid content reaches about 0.5% in pollen, 10% in propolis and about 6000 μg kg ^{1} in honey. Only flavonoid aglycones seem to be present in propolis and honey, while bee 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 anti-microbially active flavanone pinocembrine was found to be present in 11 of 12 honey samples of different origins (Bogdanov, 1989). Four Swiss honey samples (two of floral, two of honeydew origin) were analysed by HPLC and the main flavonoid determined was pinocembrine. Its concentration varied between 2 and 3 ppm (200–300 μg 100 g ^{1} honey) (Bogdanov, 1989). However, flavonoid analysis of honey seems to be a promising technique in studies of the botanical and geographical origin of honey samples (Amiot *et al.*, 1989). The flavonoid composition in sunflower honey has been analysed by GC (Berahia *et al.*, 1993). Among various unidentified compounds, six flavone/flavols and four flavanone/flavols could be determined. The main peak again was pinocembrin. The characterization of the flavonoids present in sunflower honey and propolis was achieved in order to assess the relative effects of different components of honey and propolis (Siess *et al.*, 1996; Sabatier *et al.*, 1992). Honey and propolis contained the same major flavonoids: pinocembrin, chrysin, galangin and pinobanksin. The flavonoids present in 20 samples of Portuguese heather honey were analysed by HPLC (Ferreres *et al.*, 1994*a*). The total amount of flavonoids ranged between 60 and 500 μg 100 g ^{1} honey. However, in Spanish rosemary honey 500–2000 μg 100 g ^{1} honey were found. The main flavonoids are flavanones (pinocembrin, pinobanksin) and flavones (chrysin, galangin). All samples contained a similar flavonoid pattern composed of at least 22 compounds. For heather honey, the most characteristic substances found were myricetin, myricetin-3-methylether, myricetin-3′-methylether and tricetin. These four flavonoids have not been detected in other floral honeys so far. They could probably be used as markers for the botanical origin of heather honey. Another study by the same authors concerning the flavonoid fraction of nectar (collected from the honey-stomach of bees gathering nectar from heather flowers in Portugal), has shown that the following four main flavonoids were present: quercetin, kaempferol-3-rhamnoside, myricetin-3′-methylether and isorhamnetin-3-rhamnoside (Ferreres *et al.*, 1996*a*). Since the natural glycosides are hydrolyzed by bee enzymes to render the corresponding aglycones (metabolites detected in honey), acid hydrolysis was achieved. The aglycones quercetin, kaempferol, myricetin-3′-methylether and isorhamnetin were identified, as well as ellagic acid. It has been concluded that ellagic acid and myricetin-3′-methylether (which have not been identified in any of the monofloral honeys investigated so far) seem to be potential markers for the floral origin of heather honey.

Twenty-seven Spanish honey samples from the La Alcarria region have been analysed by HPLC (Ferreres *et al.*, 1992). The total flavonoid content ranged between 500 and 2000 μg 100 g ^{1} honey. The major flavonoids were pinocembrin, pinobanksin and chrysin. The honey samples were directly provided by the beekeepers and had not been industrially processed. However, the botanical origin was not specified. A total of 18 different flavonoids were detected in the honey samples analysed. Another study by the same authors has shown that correlation between botanical origin and flavonoid profiles is possible (Ferreres *et al.*, 1991). Therefore, 10 selected Spanish samples from the La Alcarria region were analysed (five rosemary, two lavender and three multifloral honeys). A common flavonoid pattern was observed in the different samples which led to the observation that pollen is not the main source of honey flavonoids. A close correlation between the flavonoids of honey and propolis has been found, suggesting that flavonoid analysis could be more useful in geographical origin determinations than in botanical origin studies. The same authors have developed a simple extraction technique for honey flavonoid HPLC analysis (Ferreres

et al., 1994*b*). By means of micellar electrokinetic capillary chromatography (MECC), correlations between flavonoid patterns and the botanical origin of various Spanish honey samples could be established (Ferreres *et al.*, 1994*c*). The analytical conditions have been applied to honeys from lavender, rosemary, citrus and heather. In citrus honey, an accumulation of hesperetin was found (Ferreres *et al.*, 1994*d*). The main compound in rosemary was found to be 8-methoxy-kaempferol and that in lavender luteolin. This MECC study has shown that the flavonoid pattern cannot be used for the determination of the geographical origin. Honey samples from Spain, Mexico and Canada were analysed using this technique and no significant differences were found. Capillary electrophoresis (CE) could represent an alternative technique in honey flavonoid analysis to HPLC (Delgado *et al.*, 1994).

Characteristic flavonoid patterns could be determined for honeys of special botanical origin (e.g. for heather honey, citrus honey, sunflower honey, etc.). In addition, the flavonoid patterns could also be a possible tool for determining the geographical origin. The analysis of flavonoids can be performed by HPLC or CE. Multivariate statistical data evaluation may improve the suitability of this method approach.

Melissopalnyology (pollen analysis)

Traditionally, the determination of the floral origin of honey has been achieved by the analysis of the pollen present in honey. The method is based on the identification of pollen by microscopic examination. The different pollen types are described in the literature (e.g. D'Albore and Oddoo, 1978; Moore and Webb, 1978; Sawyer, 1988).

However, there are some problems related to this method (Molan, 1996): different plant species produce different proportions of pollen, the amount of pollen can vary from season to season, the nectar yield can be different in male and female flowers, pollen can be filtered out in the bee's honey sac (Maurizio, 1975), bees can take pollen without collecting nectar, most of the pollen can be collected from plants that cannot be the sources of honey, filtering of honey for packaging for sale, and the straining of honey. Another limitation of this method for proving authenticity is that pollen can be added fraudulently. In the case of citrus honey, pollen analysis is not as useful as in honeys of some other floral origins, due to the fact that the amount of pollen is generally small and very variable (Serra Bonvehi *et al.*, 1987).

Honey can never be derived from a single botanical source. The term 'unifloral' honey is used to describe honey produced mostly from one plant species. Generally, the pollen content for a honey to be called 'unifloral' should be at least 45% of the total pollen content (Maurizio, 1975). This percentage is not valid when a floral source leads to nectar with a higher or lower content of pollen grains than the average. For example, unifloral chestnut honey requires at least 90% of the pollen to be from Castanea, but unifloral citrus honey needs only 10% of pollen to be from citrus.

Pollen analysis can be used for the identification of the floral and in some cases also for the geographical origin of honey. The latter is the case when a particular floral species is only growing in specific areas.

Minerals and trace elements

The mineral and trace element content in honey samples could give an indication of environmental pollution and herewith also an indication of the geographical origin of honey. The minerals sodium, potassium, calcium, magnesium, copper, iron, manganese, phosphorus (phosphate), chlorine (chloride), silicon (silica), sulphur (sulphate) and ash contents of 91 samples of raw honey from Galicia (Spain; floral source not indicated) were determined (Rodriguez-Otero *et al.*, 1994). Potassium was shown to be the most abundant of the elements determined, with an average content of 1500 mg kg ^{1} (ppm). In general, the honeys investigated have shown higher mineral contents in comparison with honeys reported in the literature. The same authors have analysed 24 commercial Spanish honey samples regarding their silicon, phosphorus, sulphur, chlorine and ash contents (Rodriguez-Otero *et al.*, 1995). The mean contents were 3 ppm for silicon, 80 for phosphorus, 45 for sulphur and 260 for chlorine. The values for phosphorus and chlorine were high with respect to honeys from other regions. An ion chromatographic (IC) method was applied for the analysis of inorganic anions (chloride, hydrogen phosphate and sulphate) in Spanish honey (not specified) (Perez-Cerrada *et al.*, 1989).

Trace elements in Italian heather honey samples have been analysed by preseparation neutron activation analysis (PNAA) (Pietra *et al.*, 1993). Instrumental neutron activation analysis has also been applied to the determination of various trace elements in Turkish honey samples from various floral origins (mixed flower, sunflower, thyme and citrus flower) (Sevlimli *et al.*, 1992). The trace elements lead, cadmium and manganese in honey samples (floral source not indicated) from various seasonal origins (spring, summer, autumn) have been analysed directly with minimal sample preparation (dilution with water) by graphite furnace atomic absorption spectrometry (ETAAS) (Stein and Umland, 1986). The lead contents were found to be slightly higher in the summer honey samples. The elements selenium, iron and calcium have also been determined by ETAAS in honey samples (origin not specified) (Dabeka and McKenzie, 1991; Szymozyk *et al.*, 1986; Siong *et al.*, 1989*a*,*b*). Trace element concentrations in honey collected from a wide area in Hungary were shown to be useful as an environmental indicator (Fodor and Molnar, 1993). The efficiency and reproducibility of sample preparation (dilution, ashing and

digestion) for simultaneous multi-element analysis have been compared for 13 elements in honey. In almost all cases, the trace element concentrations in honey samples from industrial areas were higher. This was shown to hold especially true for the elements cadmium, copper, lead and zinc.

The determination of minerals and trace elements in honey could be suitable for the detection of geographical origin, due to the fact that these values are affected very much by environmental pollution. The investigation of trace element profiles in combination with modern statistical data evaluation techniques could be a promising approach.

Organic acids (aliphatic)

Thirty-two aliphatic dicarboxylic acids were identified as methylesters in extracts of four unifloral New Zealand honeys by GC–MS. The constituents 2-methylbutanediocic acid (*O*-methylmalicacid) and 4-hydroxy-3-methyl-*trans*-2-pentenediocic acid were proposed as floral marker substances for New Zealand rewarewa (*Knightea excelsa*) honeys (Wilkins *et al.*, 1995). A method based on HPLC was described in order to characterize organic aliphatic acids in honey samples after purification by solid phase extraction (SPE) (Cherchi *et al.*, 1994). The average recoveries of the acids ranged from 89 to 104% and the detection limits from 0.002 to 3 ppm. Italian honey samples analysed were multifloral and unifloral, such as strawberry-tree, asphodel, and red gum and their botanical origin was checked by pollen analysis. The mean concentrations of organic acids in these samples were: gluconic acid (2–12 g kg $^{-1}$), pyruvic acid (9–78 mg kg $^{-1}$), malic acid (69–145 mg kg $^{-1}$), citric acid (64–160 mg kg $^{-1}$), succinic acid (12–48 mg kg $^{-1}$), fumaric acid (0.5–2.6 mg kg $^{-1}$).

Relatively little work has been published on the composition of organic acids in various types of honey. However, the investigation of the organic acid profile and pattern evaluation by statistical methods could be helpful in providing additional information on honey samples from various sources.

Phenolic compounds (other than flavonoids)

During the second metabolism of plants, various hydroxybenzoic, and hydroxycinnamic acids are formed (Gross, 1981). It has been shown that concentrations of such substances differ in various plants (Herrmann, 1979, 1989).

Aromatic carbonic acids (phenolic acids) arise from the phenyl-propanoid metabolism in plants. Various honeys from different floral sources have been analysed regarding their phenolic acid contents by GC after methylation (Steeg and Montag, 1988*a*). Rape honeys have been characterized by the occurrence of phenylpropanoic acid and buckwheat honeys had a higher content of 4-hydroxybenzoic acid and no phenylacetic acid. Heather honeys could be identified by the presence of a high concentration of benzoic acid, phenylacetic acid, mandelic acid and β-phenyllactic acid. The differentiation of honeydew honeys and lower honeys was shown to be possible because of the difference in the concentration of protocatechuic acid.

Phenolic acids in honey samples have also been analysed by HPLC with coulometric detection. The concentration in honeys was between 0.01 and 10 ppm (10–1000 μg 100 g $^{-1}$ honey) (Sontag *et al.*, 1989). A buckwheat honey extract has been analysed and the following compounds could be identified: 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. Applying similar method conditions, honeys of various floral types were compared and characterized by the same authors (Jörg and Sontag, 1992). The distribution pattern of phenolic acids allows to differentiate between honey dew, chestnut and forest blossom honey.

Phenolic esters have been analysed by HPLC in chestnut, clover, dandelion, fir, linden, orange, rape, robinia (false acacia) and sunflower honey (Jörg and Sontag, 1993). The characteristic compounds were: methyl-4-hydroxybenzoate, methyl-vanillate and methyl-syringate. These compounds could be detected in most of the honey samples analysed. In robinia honey, only methyl-syringate was found. Rape honey also had a very high concentration of methyl-syringate. The content of methyl-4-hydroxybenzoate was higher in rape and orange honey. Some small differences were observed between the varieties chestnut, clover, dandelion, linden and sunflower. The results allowed the clear differentiation between rape and robinia honey.

Aromatic carbonyl compounds leading to strong flavour are produced in secondary enzymatic reactions from the respective phenolic acids mentioned above and are present in honey in minor concentrations (Steeg and Montag, 1988*b*). They have been isolated from solvent extracts of honey and analysed by GC (Häusler and Montag, 1989). Such compounds detected in honey were: benzaldehyde, phenylacetaldehyde, acetophenone, *trans*-cinnamic aldehyde, 2-anisaldehyde, 4-anisaldehyde, vanillin and 3,4-dimethoxy-5-hydroxybenzaldehyde. There was no indication of the botanical origin given in this investigation. The same authors have analysed various carbonyl compounds in honey from different floral sources, such as chestnut, acacia, buckwheat, eucalyptus, orange and sunflower (Häusler and Montag, 1990). The phenylpropane metabolites salicylaldehyde, *p*-toloylaldehyde, vanillin, 2,5-dimethoxybenzaldehyde and 3,4-dimethoxybenzaldehyde could be detected as naturally occurring minor components (5–180 ppb).

A careful evaluation of the patterns concerning phenolic acids, phenolic esters and aromatic carbonyl

compounds could probably give an indication of the botanical origin of honeys.

Stable isotopes

As already mentioned, honey is often adulterated with the relatively cheap HFCS. Carbon stable isotopic ratio analysis (SIRA) can be used to detect honey that has been adulterated with other sugars. The natural abundances of the stable isotopes of the main bioelements in biogenic material are submitted to small variations caused by isotopic effects of physical processing and chemical reactions in the natural cycles of these elements. The resulting typical relative abundances (δ-values) of food can permit assignment to their origin and treatment and could represent in some cases, proof of adulteration (e.g. honey with HFCS) (Schmidt, 1986; Croft, 1987). SIRA has been used for the detection of adulteration (with corn syrup or cane sugar) of various foods (honey, maple syrup, apple juice, etc.). Applying this method, small quantities of the ^{13}C content of the carbon of different plant types produced by different photosynthetic pathways are measured. Most fruits and grains are Calvin cycle (C3) pathway plants yielding ^{13}C values near $-25‰$; cane and corn are Hatch-Slack (C4) pathway plants with ^{13}C values near $-10‰$. The coupling of an elemental analyser with an isotopic ratio mass spectrometer (IRMS) allows on-line isotopic measurements (Pichlmayer and Blochberger, 1988). The $\delta^{13}C$ values of various honey samples analysed were about -23.2 to $-24.6‰$.

As the $\delta^{13}C$ values alone for honey cannot always definitively be used to prove adulteration by addition of C4 plant sugars, they have been determined in correlation 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 indicate the addition of C4 plant sugars (Roßmann *et al.*, 1992; White, 1992). The limit for the detection of adulteration is 7%. The addition of C3 plant sugars (beet sugar) cannot be proved by this method. However, for certain types of honeys, authenticity can be confirmed by the δD-values. (Roßmann *et al.*, 1992).

Using the difference in stable carbon isotope ratio (SIRA) between a honey and its protein fraction, an evaluation of honey adulteration with amounts of 7–20% and larger of corn or cane sugar can be carried out. Fifty authentic honey samples were used to establish the purity criteria and 38 other samples with $\delta^{13}C$-values in 'questionable' or 'adulterated' range were tested. A difference of 1.0‰ or more between honey and protein fractions was proposed to indicate adulteration (White and Winters, 1989).

Carbon-13 nuclear magnetic resonance (^{13}C-NMR) has been applied for the qualitative and quantitative analysis of structurally similar disaccharides in honey (Low *et al.*, 1988). The disaccharides were: glucose–glucose and glucose–fructose. ^{13}C-NMR has also been applied for the analysis of a complex mixture of minor disaccharides in honey. Disaccharide ratios (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 with respect to the isotopic ratio parameters $\delta^{13}C$, measured by MS and deuterium/hydrogen (D/H) of the methyl group of the ethanols produced by alcoholic fermentation, measured by deuterium NMR (Lindner *et al.*, 1996). Ethanol samples obtained from fermentation of citrus honeys have D/H values similar to ethanols from fermented citrus juice and that exceed the values obtained from other honeys by 5 ppm. This difference in D/H can be used to confirm the authenticity of citrus honey. The $\delta^{13}C$ values of all honeys tested were similar and typical to C3 plants.

Carbon SIRA regarding the $\delta^{13}C$ values allows 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 origin. However, the ratio deuterium/hydrogen (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 in the samples, such as $^{18}O/^{16}O$.

Special compounds (possible marker compounds)

Abscisic acid in heather honey

HPLC analysis of Portuguese heather honey fractions have shown that two organic acids (non-flavonoid compounds) were the main constituents (Ferreres *et al.*, 1996*b*). These compounds were isolated and identified as *cis*,*trans*-abscisic acid and *trans*,*trans*-abscisic acid. Their content ranged between 0.3 and 17 ppm in honey. These compounds have not been detected in any of the different monofloral honey samples analysed so far, and, therefore, could be useful markers of heather honey. More heather honey samples from other geographical and botanical origins should be analysed in order to prove that abscisic acid could be a useful marker of heather honey.

Hesperetin in citrus honey

The flavanone hesperetin has been suggested as a possible marker for the floral origin of citrus honey, since this compound has so far not been detected in honey of any other origin (Ferreres *et al.*, 1993). It is a constitutive phenolic compound of citrus nectar, where it is present as a glycoside (hesperidine).

Methyl anthranilate in citrus honey

Methyl anthranilate is a characteristic volatile component of citrus nectar and honey and has been used as a

marker for citrus honey. The analysis has been carried out by GC or spectrophotometrically (White, 1966; Graddon *et al*., 1979; Bicchi *et al*., 1983; Serra Bonvehi, 1988). A HPLC method has been described which allows the simultaneous determination of HMF and methyl anthranilate in honey samples (Vinas *et al*., 1992). However, as methyl anthranilate is a volatile compound, it suffers significant changes in concentration under varied environmental conditions and under different honey storage conditions (White *et al*., 1964; Serra Bonvehi, 1988). The contents of hesperetin and methyl anthranilate were obtained by GC and HPLC, respectively, in 18 honey samples produced in Mediterranean Spain (Ferreres *et al*., 1994*d*). There was no correlation found between the contents of both compounds. The concentration of methyl anthranilate ranged between 1.4 and 3.6 ppm, while hesperetin ranged between 0.3 and 0.9 ppm. These results support the hypothesis that hesperetin could be used as an additional marker in the determination of citrus honey origin.

3-Aminoacetophenone in chestnut honey

This volatile compound has been found to be the main constituent of the complex mixture of volatiles in (Italian) chestnut honey and could be specific to the floral source (Bonaga and Giumanini, 1986).

The analysis of special marker compounds facilitates the detection of the origin. However, such special compounds are not available or known for all botanical varieties or if they exist, often only in very small quantities. In addition, the quantification of such components could raise problems due to the fact that honey is a natural product and the concentrations can vary. Some of these marker compounds are easily available and could, therefore, be fraudulently added to honey.

MULTI-COMPONENT ANALYSIS

Compliance with legal aspects

A collaborative trial concerning the analysis of various parameters as prescribed in the existing EU Honey Directive has been carried out in order to evaluate the suitability of the analytical methods (Lord *et al*., 1988). The results indicated that the proposed methods of analysis for the determination of mineral content, moisture, acidity, apparent reducing sugar content and water-insoluble solids content were satisfactory, while those for HMF and apparent content required further investigation. The values determined were not used in terms of floral or geographical authenticity proof. The methods for the determination of carbohydrates, HMF and proline were established and evaluated (White, 1979). The adulteration of honey samples with HFCS was detected by applying different methods (Abdel-Aal *et al*., 1993). Pure honey was adulterated with HFCS at levels of 10–50%. The sugar composition as a fingerprint was determined by HPLC. The following compositional properties were also determined for pure and adulterated honey samples: moisture, total soluble solids, nitrogen, apparent viscosity, HMF, ash, sodium, calcium, potassium, proline, refractive index and diastase activity. Statistical analysis revealed that the following compositional properties were highly significantly negatively correlated with sugar composition, dry matter, apparent viscosity, sodium, potassium, proline and nitrogen. In contrast, ash, calcium, HMF and moisture were highly significantly positively correlated with sugar composition of pure and adulterated honey. The water activity (a_w) of various Italian honey samples (prune, sunflower, acacia and orange) was determined, together with other parameters, such as sugar and water contents, and the physical characteristics, such as fluid or crystallized status (Piana *et al*., 1991). The various honey types could be characterized by their different a_w/water content values. Italian honeys from the market were tested for their physical, chemical and organoleptic characteristics (Bolchi Serini, 1981). Parameters such as refraction index, colour, viscosity, impurity, reductive sugars, acids, ash, enzymes and HMF were determined, but only with respect to the legal requirements and not for use as a control of origin. The carbohydrates glucose, fructose and sucrose and two organic acids (malic and citric acid) in two Italian flower honeys were analyzed by enzymatic methods (Tourn *et al*., 1980). Eleven parameters for honey quality were determined in 29 honey samples from Spain (Lopez *et al*., 1996). Multivariate chemometric techniques, such as principal component analysis, cluster analysis and linear discriminant analysis, were used to classify honey samples on the basis of their compositional data. Using the values of total acidity and diastase activity, a mostly correct classification of natural authentic honeys and processed honeys could be achieved. However, no indications on the floral or geographical origin could be made. The contents of sugars and minerals in Spanish honey were determined by polarimetric, reductometric, HPLC and enzymatic methods (Frias and Hardisson, 1992). The physicochemical characteristics of 25 samples of commercial Spanish eucalyptus honey were analysed and 35 parameters were measured, including sugar and mineral contents, total nitrogen, proline, water content, pH, acidity, HMF, diastase activity, colour, ash, insoluble solids and electrical conductivity (Gomes *et al*., 1993). The samples contained a mean of 18 pollen types. Samples with more than 70% of eucalyptus pollen were considered to be unifloral. In 92% of the samples analysed, eucalyptus was the most abundant pollen, at over 45% of total pollen found. The quality of 27 rosemary honey samples from Spain was evaluated (Perez Arquillue *et al*., 1994). Most samples showed a proper maturity considering the low moisture content. The low electrical conductivity and ash content were typical of pale honeys. Other parameters analysed were

the sucrose, glucose, fructose, trisaccharides and HMF content. Unifloral Spanish honey samples (willow, sainfoin, chickweed, crucifer, fruiter, thyme, blueweed, lavender and vetch) were analysed for their moisture content, optical rotation, electrical conductivity, ash and HMF contents, diastase activity, pH, acidity and carbohydrate composition Perez Arquillue *et al*., 1995). The samples were considered to be unifloral when more than 45% of the pollen of the respective flowers were found. No classification on, and no evaluation of, these parameters was carried out with a view to determine the floral origin of the samples analyzed. These samples were found to just meet the major national and international honey specifications. Cluster analysis was applied to physical and chemical parameters obtained from Spanish honeys from the Basque country (Sancho *et al*., 1992). The parameters were combined into five clusters: free acidity, total acidity, fructose content, glucose content and diastase activity. Proline content and the total reducing sugars content could also be formed into clusters. The samples themselves have also been combined into two clusters: one cluster had only one sample which had the higher diastase activity. The adulteration of Spanish honey samples with corn syrup was proven by analysis of various parameters, such as HMF content, isomaltose/maltose ratio, moisture content, pH, ashes, sulphur dioxide, viscosity and density (Serra Bonvehi and Pajuelo, 1986). Based on a comparative study of humidity analysis and the ash and the sugars in a sample of Portuguese honey, a recommendation has been made for the introduction of analysis methods in the Portuguese legislation concerning honey (Pena Ferreira *et al*., 1989). The physico-chemical and organoleptic assessment of 55 honey samples produced in a special Italian region were investigated (Esti *et al*., 1997). The data obtained have shown that the analytical parameters of all samples were in conformity with the limits set by the Italian regulations. The high values of the coefficient of variation for HMF, diastase activity and total acidity indicated that there were qualitative differences in the samples, due to the non-uniform distribution of the correct beekeeping techniques in the region.

Origin control

Geographical

Honey samples from two production areas in the northwestern part of Spain (Galicia) were analysed according to 11 parameters of legal quality control (Pena Crecente and Herrero Latorre, 1993). The samples were from mixed floral origins from the 1990 harvest. Classification of these honeys according to their geographical origin was achieved by pattern recognition techniques applied to the chemical data. Humidity and free acidity were found to be the most important parameters for classification. Authentic honey samples from the province of Alberta, Canada, were analysed regarding their moisture, enzymes (α- and β-glucosidase, diastase), HMF, proline, free and lactone acidity, ash and carbohydrate composition in order to establish original values (Sporns *et al*., 1992). The samples were found to meet all major national and international honey specifications. By comparing these results to those obtained from other world floral honeys, these samples were found to be lower in moisture, α-glucosidase, proline, acidity and ash. The proline levels were often lower than 200 ppm which has been used as a minimum level for honey authenticity by some Canadian honey importers. Thirty-seven honeys of Swiss and other geographical origin were analysed concerning the content of trace insecticides, water, protein, sugars and HMF, water activity (a_w), and the activity of diastase, saccharase and heat-stable inhibines and light absorption, colour intensity and electric conductance (Bogdanov, 1989). No detectable amounts of the insecticides fumidile and sulphathiazol could be detected. There was no correlation between inhibines and the botanical (honeydew or floral) and the geographical (Swiss or foreign) honey origin. Swiss honey samples had significantly less water, lower water activity, higher activities of saccharase and diastase and a lower content of HMF than the foreign honeys (Bogdanov *et al*., 1987).

Botanical

Eighteen chemical and physical parameters of nectar and honeydew honeys have been determined and the results analyzed statistically by the method of principal component analysis (Krauze and Zalewski, 1991). The honeys could be divided into the following groups: acacia, rape, lime and heather, and honeydew. The most important first principal component was strongly associated with the value of electrical conductivity, the content of ash, free acids and proline, as well as with the pH and the diastase number. The principal component loadings and linear correlation suggested that these parameters contributed much more to the classification of honeys than apparent reducing sugars, apparent sucrose, mono-, di- and trisaccharides, glucose and fructose. The palynological (pollen) and physicochemical properties of 15 citrus honey samples commercially produced in Spain have been reported (Serra Bonvehi and Ventura Coll, 1995). Sugar profiles and the flavour component methyl anthranilate were shown to change with the transition period before honey commercialization. Therefore, the recommendation was made to classify fresh Spanish citrus honey with the group of abundant nectariferous flower honeys in order to admit its higher sucrose content which was shown to largely exceed the legal limits established for honey by the EU.

As demonstrated by the examples given above, an approach using various methods and data evaluation with modern statistical techniques could be promising in order to detect the botanical and geographical origin of honeys. The parameters taken into account have to be

chosen very carefully and should be limited to a minimal number in order to keep the laboratory costs low, but to obtain optimal results.

CONCLUSION

Whereas the determination of some single parameters (HMF, residues, enzyme activity, SIRA, moisture, nitrogen, mono- and disaccharides) in honey does not lead to any information about the botanical and/or geographical origin, the analysis of profiles of some components could be suitable for this purpose: botanical origin: aliphatic organic acids, amino acids, aroma compounds, aromatic carbonyl compounds, flavonoids,oligosaccharides, phenolic acids and esters, proteins, and specific stable isotopic ratios (e.g. D/H); geographical origin: amino acids, aroma compounds, flavonoids, minerals and trace elements, oligosaccharides, protein, and specific stable isotopic ratios (e.g. $^{18}O/^{16}O$).

The combination of methods (multi-component analysis) especially with the support of modern statistical data evaluation techniques seems to be a promising approach for the authenticity proof of honeys.

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