CHAPTER 1

Literature Review on the Determination of the Botanical Origin of Honey

1.1 INTRODUCTION

1.1.1 HONEY TYPES
The bees forage nectar and honeydew on the plants in the surroundings of their hive by maximising the energy efficiency (1). The different proportions of nectar or honeydew incorporated in honey vary depending on the vegetation type, flowering period of the plants or the honeydew production of plant sucking insects as well as the time when the honey is harvested by the beekeeper. Therefore the chemical composition and the sensory properties of honey vary considerably between different samples. This variability can be regarded as disadvantage if an absolutely uniform product is demanded. On the other hand the variability of other natural food such as wine or olive oil has been turned into an advantage by pointing out the specific differences and by appropriate marketing. As a matter of fact no one would like to abandon all the wine varieties we are offered today in favour of a uniform product.

Most of the honey produced worldwide is sold with just the designation honey. Generally this means that the honey contains nectar and honeydew contributions from several plant species and therefore is a blend of different kinds of honey. It is thus called polyfloral or multifloral honey. Honeys that originate predominantly from a single botanical source are called unifloral honeys.

The production of unifloral honeys generally implies bigger efforts by the beekeepers. Unifloral honeys are produced in places where the plant species producing the desired nectar or honeydew strongly prevail. Mostly this means that the bee colonies are moved to this location just before the flowering period starts. Under favourable climatic conditions the bees will collect large amounts of nectar or honeydew from the prevalent plant species in the surroundings and store them in the empty combs, thus producing a unifloral honey, which is separately harvested just after the flowering period. The possibilities to produce unifloral honey without moving the hives is very limited. Therefore migratory beekeepers that are specialised in the production of unifloral honeys move their colonies following the flowering period of the plants over thousands of kilometers during the season.

The number of unifloral honey types that can be produced depends on the geographical region and the climatic conditions. In the Mediterranean area the vegetative period of the flowering plants is considerably longer and their diversity larger compared to northern Europe. In the South the plants flourish more gradually, which facilitates the production of different kinds of unifloral honeys. In the North, in addition to the smaller diversity more plants flourish at the same time making it more difficult to produce pure unifloral honeys. However an advantage of the North is that the nectar flow is more intense during the short vegetation period resulting in larger crops.
In the Mediterranean countries about 50% of the honey is marketed with a botanical denomination. The use of a designation of the botanical origin is permitted by the current standards (2, 3) “if it comes mainly from the indicated source and possesses the organoleptic, physico-chemical and microscopic characteristics of the source”. The high rate (60%) of incorrect indications of the botanical origin made by the beekeepers show that one can not rely on conclusions drawn from field observations of foraging bees (4). Authentication by analytical methods is therefore absolutely necessary. As far as the surveillance of the botanical origin is concerned specific analytical criteria are only provided in terms of the electrical conductivity for the classification of the two main honey types, the blossom and honeydew honeys. All the other composition criteria given in the appendix of the standards are related to the detection of inappropriate honey processing techniques and adulteration (2, 3).

As legal criteria do not exist, an efficient control of the botanical designations is not assured. The national food control laboratories dealing with honey analytics have though established criteria of their own. Unfortunately they are to some extent varying between different countries and experts. This creates difficulties for the trade of unifloral honeys, as imported honey may be rejected because of non-compliance to national criteria. In order to protect consumers from being misled by wrong declaration of botanical origin and to preserve the reputation of the unifloral honey types, efforts should be made to harmonise the criteria used. An important step in this respect has been taken by the publishing of a monograph describing the physical, chemical as well as pollen analytical and sensory properties of the 15 most important European unifloral honeys (5).

1.1.2 THEORETICAL CONSIDERATIONS ABOUT THE DETERMINATION OF THE BOTANICAL ORIGIN OF HONEYS

Absolutely pure unifloral honeys do not exist, as bees never forage on a single plant species even if it dominates. It has nevertheless been tried to produce pure unifloral reference honeys in flight cage experiments especially when pollen analytical methods were developed (4, 6, 7). However it is questionable if these pure honeys are really useful as references especially considering the efforts needed to produce them under the artificial circumstances. The reference samples produced will just apply to samples produced under specific climatic conditions and from a certain plant cultivar. It may be difficult to relate “real world” samples from different parts of the world to these pure references. The approach to monitor the variability of the honey samples produced under natural circumstances and to define groups according to similar characteristics seems to be more promising from a practical point of view.

It is difficult to define the limit between polyfloral and unifloral honeys, because there are numerous nectar sources that can become mixed in variable ratios. Currently there is no single method that would allow to exactly measure the ratio of a given nectar in honey. By a global interpretation of results from several analytical techniques the most important source can be estimated. From the point of view of the consumer it is however more important that a certain honey type can be always recognized. In this respect probably the most promising approach is to gather as much as possible information on honey composition and to look for similar characteristics among these “real world” samples. The use of different analytical techniques will supply additional points of view on the various honey types. When the results of
several independent analytical methods are in agreement in respect of the characteristics of a unifloral honey type the more likely it will be that this group is correctly defined.

1.2 TRADITIONAL METHODS FOR THE DETERMINATION OF THE BOTANICAL ORIGIN OF HONEY

The classical approach to verify the botanical origin of honey is to use several complementary analytical methods. Traditionally the botanical origin of honey is determined by experts evaluating several physical, chemical, pollen analytical as well as sensory characteristics (8-10). The analytical results of honey samples have unconsciously been compared with profiles describing the data ranges of different unifloral honeys. When all the values of the measurands considered fit into the respective ranges described for a unifloral honey type, it is assigned to this corresponding honey type. On the contrary if the characteristics of the sample do not fit into the profiles of the unifloral honey types considered, the sample is classified as polyfloral honey. Thus the group of polyfloral honeys represents a miscellaneous pool of samples of various botanical origins with significant nectar or honeydew contributions from several plant species. However, the amount of honeydew should not prevail, otherwise it is regarded as honeydew honey. Unfortunately up to now neither the measurands to be considered nor their corresponding ranges for the individual unifloral honeys have been defined and internationally accepted. Usually only few physical and chemical measurands, in particular electrical conductivity, sugar composition and pollen analytical results are used for this purpose.

This profiling approach used for decades, has recently been described in more detail by Persano Oddo and Piro (11). However, only physical and chemical measurands were considered and the presentation of the data ranges was not optimal. The classification with a profile works because unifloral honeys express at least in respect to some measurands specific properties that are generally not found in other honey types. The purest samples of unifloral honeys are therefore easily recognized. However, unifloral honeys are hardly ever pure and generally contain minor nectar or honeydew contributions from other botanical origins. The proportion of different sources continuously increases towards the polyfloral honeys. Where the limit between unifloral and polyfloral honeys is set, depends on definitions and is ultimately arbitrary. Consequently there will always be some overlapping between unifloral and polyfloral honeys.

1.2.1 POLLEN ANALYSIS

1.2.1.1 QUALITATIVE ANALYSIS

Honey contains pollen grains and other microscopic particles such as fungi spores and algae, originating from the plants from which the nectar or honeydew has been collected by the bees. Therefore the pollen composition of a honey sample reflects the vegetation type where the honey has been produced and is useful for the determination of the geographical as well as botanical origin of honey. During the microscopic examination, the honey sediment reveals valuable information on beekeeping practice (use of smoke, feeding of pollen substitutes and general hygiene) (12) as well as on honey extraction techniques, fermentation (13) and some kinds of adulteration (14, 15).
Pollen identification in honey is performed since the beginning of the last century, but the methodology has been improved and harmonised several times (12, 16, 17). The pollen grains are identified by light microscopy in a sediment prepared by centrifugation of diluted honey. In qualitative analysis 500 to 1000 pollen and honeydew elements are identified. The relative frequency of the different pollen forms is calculated thereafter. Recent interlaboratory studies show a satisfactory reproducibility of the method. The relative standard deviation for frequent pollen is generally small (3 %) while rare pollen forms show considerably higher coefficients of variation (up to 45 %). The precision of the method slightly increases when 1000 pollen grains are counted instead of 500 (17). Generally the plant species with the most frequent pollen found are considered to have predominantly contributed to the honey produced. To be considered unifloral a honey sample should contain at least 45 % of the corresponding pollen form, but unfortunately the pollen to nectar ratio varies considerably between different plant species (4, 7, 18). Some pollen forms are known to be over-represented while others are under-represented. Honeydew honeys do not contain any specific pollen but airborne pollen that become trapped in the sticky honeydew. Numerous factors may influence the pollen representation in honey, the most important are shortly discussed.

1.2.1.2 FACTORS INFLUENCING THE REPRESENTATION OF POLLEN IN HONEY

Influence of plant morphology, physiology and the bees
The amount of pollen present in the nectar depends first of all on the design of the flowers i.e. of the position of the anthers in respect to the nectaries. If the anthers are located higher than the nectaries, pollen are likely to fall into the nectar secreted and to contaminate it. The extent of this contamination depends among other factors on the amount of pollen produced, its size, whether nectar secretion coincides with anther maturation or not and on the foraging behaviour of the bee. Some plants produce very little pollen or may even be male sterile thus producing no pollen at all, e.g. some cultivars of orange (Citrus spp.). In the past decades pollen representation in honeys from new plant cultivars has considerably changed (19, 20).

During nectar foraging and honey processing, pollen and spores are very efficiently filtered from the honey sac of the bee by the proventriculus that serves as regulatory apparatus filtering and controlling the flow of food into the stomach. The removal of pollen depends on the duration of the nectar kept in the honey sac, the extent of honey processing, pollen size and structure of its exine. Large pollen and pollen with a spiny surface are more likely to be removed (6, 7, 21).

Contamination in the hive
Since pollen is the only protein source of the bees, they store it after foraging in their combs. During honey and pollen processing in the hive, pollen can be transferred into honey by the worker bees that fulfil different tasks. If the pollen originates from the same plant as the nectar, its proportion in honey is enriched. Similarly honey may also be contaminated with pollen from other plant species (22).
Contamination during uncapping and processing

Pollen can enter the honey by the actions of the beekeeper during uncapping and extraction of the honeycombs. Cells containing pollen are often cut especially during rigorous mechanical uncapping, releasing pollen from the cut cells into the honey. Some pollen may also be liberated during extraction. The most severe contamination occurs when honey is extracted by pressing, which is still used to extract heather honey (22). On the other hand pollen may be removed during honey processing by filtration (23).

1.2.1.3 INTERPRETATION OF POLLEN ANALYTICAL RESULTS

The factors affecting pollen representation resulting from plant morphology, physiology and the action of the bee can be taken into account in two ways. The more objective, but uncommon method, is to use corrective values, known as pollen coefficients, to compensate for pollen forms that are known to be under- or over-represented. These coefficients have been experimentally determined from honeys produced with caged bees foraging on single plant species or exceptionally pure unifloral honeys. Unfortunately up to now no agreement has been found which of the proposed coefficients should be used. The technique has not been commonly accepted because of disagreements in the methods used to generate the pollen coefficients and the numerous variables that influence the calibration of the coefficients (6, 7, 18, 24). It has recently been stipulated that research should be done to establish more reliable pollen coefficients (4).

When evaluating unifloral honeys, most of the melissopalynologists just consider descriptions on pollen forms that are over or under-represented in honey. In unifloral honeys from under-represented species, the minimum percentage of pollen is often as low as 10% or even lower, e.g. strawberry tree (Arbutus unedo), orange (Citrus spp.), dandelion (Taraxacum s.l.) and lime (Tilia spp.). On the other hand, honeys from over-represented plants, e.g. chestnut (Castanea sativa) and eucalyptus (Eucalyptus spp.) have to contain more than 90% pollen from the unifloral source before they can be considered as unifloral (10, 11, 17, 25, 26, 27).

Different pollen representations related to plant morphology, physiology and the action of the foraging bee, can be taken into account when pollen analytical results are interpreted, but the influence of pollen contamination in the hive and during extraction and honey processing cannot be controlled. The bias resulting thereof is probably even larger than the one that applies to plant morphology and physiology. It is therefore questionable if efforts should be made to establish new pollen coefficients.

Another element of uncertainty for the interpretation of pollen analytical results is a consequence of the present European Union honey directive (2) and Codex Alimentarius (3) standards. Both indirectly allow the removal of pollen by filtration by a flexible paragraph saying that pollen may be removed by filtration if it is “unavoidable during removal of foreign inorganic or organic matter”. Although the use of a botanical designation is no more permitted when pollen have been removed, the allowance of honey filtration facilitates honey adulteration in respect to geographical and botanical origin as pollen analysis does no more allow reliable results to be obtained.
Despite of the above mentioned shortcomings, pollen analysis in combination with other techniques is still an indispensable method for the authentication of the botanical origin of honey (10, 17). It is so far the only instrumental technique that enables a discrimination between polyfloral and different types of unifloral honeys (28). It gives also an indication about the proportions of different nectar contributions in a honey sample.

As the trustworthiness of the pollen analytical results depends on the correctness of pollen identification, the expert’s ability and general knowledge of honey, it is important to look for complementary techniques that are less subjected to effects from honey processing (29).

1.2.2 SENSORY ANALYSIS

Sensory assessment is routinely used to identify defects in honey, i.e. fermentation, off-flavours and impurities. For the determination of the botanical origin the agreement of the sensory characteristics of a sample to a certain honey type is evaluated. Generally sensory analysis carried out by experts, provides a fairly precise evaluation of the botanical origin of honey.

The first attempts for descriptive sensorial analysis of unifloral honeys by an overall assessment of the sensations perceived in crude honey were made by Gonnet and Vache (30). Later on the descriptive techniques have been improved by standardising the terminology and by introducing reference compounds and flavours (31-33). The state of the art of honey sensory analytical methods has recently been reviewed and harmonised (34).

Although first attempts to introduce modern profiling techniques using a panel of trained experts, defined experimental protocols and statistical evaluation of the results have been made, most of the sensory evaluation of honey is still performed by single experts without any specific procedure. The modern sensory analytical methods should be further developed and harmonised in panels of different countries in order to obtain more objective and reproducible tools for honey characterisation. On the other hand the experts working in honey analytical laboratories have gathered an enormous amount of personal expertise in sensory evaluation of honey that should be incorporated into the more reproducible modern profiling techniques. A considerable handicap for the application of more advanced methods in laboratory practice are the limited financial and personal resources in the apicultural business.

The advantage of sensory analysis is that the same characteristics that are perceived by the consumer are evaluated. Despite of the shortcomings discussed, sensory analysis is an indispensable complementary technique for the determination of the botanical origin of honey together with pollen analysis as well as physical and chemical methods. Some qualitative defects like fermentation can also be detected by instrumental analysis but so far sensory analysis is the most adequate technique for the detection of minor off-flavours in unifloral honeys causing a non-conformity of the sample. This may be the case when as small proportion of a highly aromatic honey like chestnut honey becomes mixed into a mild honey like acacia honey. The sensory characteristics of the acacia honey will be considerably changed while the physical and chemical characteristics traditionally determined show no indication of non-conformity (34).
1.2.3 PHYSICAL AND CHEMICAL METHODS

Most of the physical and chemical methods used in honey analytics are principally intended for honey quality control and detection of honey adulteration, but some of them, particularly the determination of the electrical conductivity and the sugar composition allow as well conclusions on the botanical origin.

1.2.3.1 ELECTRICAL CONDUCTIVITY

Electrical conductivity depends predominantly on the mineral content of honey (35). This mesurand was recently included in the international standards replacing the determination of ash content (2, 3). Electrical conductivity can be determined with an inexpensive conductometer and was found to be the most important variable for the classification of unifloral honeys (28, 36-38). The range of electrical conductivity in honey lies between 0.06 and 2.17 mScm\(^{-1}\). Honeydew is directly sucked from the phloem by various insects and contains therefore considerably higher amounts of minerals compared to blossom honeys where the minerals are mostly resorbed before nectar secretion. Electrical conductivity is an important tool for the estimation of honeydew in honey. Generally honeydew honeys have an electrical conductivity higher than 0.8 mScm\(^{-1}\), blends between blossom and honeydew honeys have conductivity values between 0.51 and 0.79 mScm\(^{-1}\), and pure floral honeys exhibit conductivity values between 0.15 and 0.50 mScm\(^{-1}\). However various exceptions to these limits are known, i.e. chestnut (Castanea sativa), strawberry tree (Arbutus unedo), erica (Erica spp.), eucalyptus (Eucalyptus spp.), lime (Tilia spp.) and heather (Calluna vulgaris) honeys. Therefore a reliable determination of the botanical origin can not be based on electrical conductivity only.

1.2.3.2 CARBOHYDRATES

Sugars are the main constituents of honey, accounting for about 95 % of honey dry matter. Especially fructose and glucose concentration as well as the fructose/glucose ratio are useful for the classification of unifloral honeys (10, 11, 25). Considerable differences between the sugar composition of blossom and honeydew honeys exist, but much smaller ones within the blossom and honeydew honeys. Honeydew honeys contain a higher amount of di- and trisaccharides, especially melezitose and raffinose that are both absent in blossom honeys. Nectar and phloem sap contain only the sugars fructose, glucose and sucrose. The numerous di- and trisaccharides in honey are produced by microbial activity and enzymatic reactions in the intestinal tract of the aphids and during honey ripening (22, 39). The small differences in the sugar spectra of blossom honeys are explained by the fact, that the di- and trisaccharides are mainly produced through transglycosylation or enzymatic reversion by the alpha-glucosidase in honey (40). The determination of minor sugars has a low diagnostic value for the determination of botanical origin, generally only allowing a classification between honeydew and blossom honeys (41-43).

However sugar composition may allow a classification between different honeydew honey types. An attempt to differentiate between honeydew honeys from various aphids was made by von der Ohe and von der Ohe (44). Qualitative and quantitative differences in trehalose, raffinose and oligosaccharide L2 content were found for the different aphid species. For aphids of the Coccidae family, a difference in oligosaccharide L1 content could be observed. The two unidentified oligosaccharides L1 and L2 could be identified by retention time, but the chemical nature of these compounds was not determined. It is supposed that L2 might be manninotriose.
(a sugar present in the phloem sap) as it is relatively stable to hydrolysis and present in all honeydew honeys. Metcalfa honeydew honey can be distinguished from other honeys by its high content of maltotriose and dextrins (45-47).

Recently polyalcohols such as (+) quercitol (1L-1,3,4/2,5-cyclohexanepentol) and perseitol (D-glyco-D-galacto-heptitol) have been reported to be characteristic for oak honeydew (Quercus spp.) (48) and avocado honeys (Persea americana) (49, 50) respectively and may thus present a promising approach for their authentication.

1.2.3.3 COLOUR
Honey colour varies from water clear, through amber tones, until almost black, some times with typical bright yellow, greenish or reddish hues. In most countries the pricing of honey depends to a great extent on colour: light honeys like acacia (Robinia pseudoacacia) and orange (Citrus spp.) generally realising the highest prices. On contrary in German-speaking countries dark honeydew honeys are especially appreciated.

The most commonly used methods for colour grading of honey are based on simple optical comparison, using the so called Pfund colour grader or the more sophisticated Lovibond instrument (51, 52). 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 scale. More objective spectroscopic techniques in transmission and reflectance mode have been used in a number of studies showing high correlation with results obtained with the classical methods (53-56). The determination of colour is a useful classification criterion for unifloral honeys. Unfortunately as honey colour darkens during storage it may therefore be only appropriate for the classification of fresh honeys. A strong interference of polyfloral honey with the unifloral honeys is also to be expected (57).

1.2.3.4 PH-VALUE 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 (58). Free acidity, total acidity and pH-value have some classification power for the discrimination between unifloral honeys, while lactones, showing very similar concentrations in various unifloral honeys may be less useful for a determination of the botanical origin (11, 27, 59).

1.2.3.5 OPTICAL ACTIVITY
Different sugars in honey have the property of rotating the plane of polarised light. Primarily fructose exhibits a negative optical rotation, while others (e.g. glucose), show a positive one. The overall optical rotation depends on the concentration of the various sugars present in honey. The determination of the specific rotation by means of a polarimeter is useful for the differentiation between honeydew (dextro-rotatory, positive values) and blossom honeys (laevorotatory, negative values), but may also be helpful for the classification of some unifloral honeys (11, 60, 61).
1.2.3.6 ENZYME ACTIVITY
Enzyme activities in honey are principally measured to evaluate possible heat defects. Even if alpha-amylase and alpha-glucosidase are derived mostly from the bees, the different honey types however show considerable differences in enzyme activities (11, 62, 63). The enzyme activities in honey depend on the intensity of the nectar flow and the amount of nectar processing by the honey bees. Therefore honey from very rich nectar sources e.g. acacia (*Robinia pseudoacacia*) often show low natural enzyme activities (64). Low enzyme activities may also indicate ultrafiltration of honey (23). However, as the enzyme activities in honey decrease during storage and heat treatment, indications to botanical origin can only be obtained from fresh honeys.

1.2.3.7 WATER CONTENT
The water content is the most important measurand related to honey quality, especially concerning the risk of spoilage due to fermentation. It has only a minor importance for the characterisation of unifloral honeys. However, according to 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 (10, 11, 65). Generally honeydew honeys have a lower water content than blossom honeys. Heather honeys are known for their higher water content. However, water content can be artificially altered during honey processing and is therefore not a reliable indicator for the botanical origin.

1.2.3.8 HYDROXYMETHYLFURFURAL
Fresh honey does not contain hydroxymethylfurfural (HMF). Thus, HMF is not a useful criterion for the botanical classification of honey. However, before determining storage dependent measurands such as enzyme activity or colour, one should ensure that honeys are fresh and do not express any heat defects by checking that the HMF content is below 15 mg/kg.

1.3 ALTERNATIVE METHODS FOR THE DETERMINATION OF THE BOTANICAL ORIGIN
The methods that are currently available for the identification of the botanical origin are not satisfactory. Especially the shortcomings in the interpretation of the pollen analytical results and the considerable time consumption resulting from the necessity to use several physical and chemical methods urge to find alternative analytical methods (29). Different approaches have been tested with variable success but none of the methods proposed has been accepted as a complementary technique not to mention as a substitute of the traditional methods. The most important approaches are discussed below.

1.3.1 CHEMOMETRIC EVALUATION OF TRADITIONAL PHYSICAL AND CHEMICAL MEASURANDS
The number of significant measurands to determine the botanical origin of honey easily exceeds the quantity that can be simultaneously mentally considered. This means that the decision is generally made using only a few measuands. Otherwise a special procedure has to be applied that helps to evaluate such data. This can be carried out by the traditional profiling approach where the values of the useful mea-
surands of a sample are compared with the corresponding ranges defined for the different honey types or even with more sophisticated mathematical models.

Chemometrics have been proposed for the classification of different honey types. Discriminant functions using pH-value, ash and monosaccharide contents were already presented in 1960 for the classification of blossom and honeydew honeys (66). Later electrical conductivity, monosaccharide content as well as glutamic acid concentration were found to be the most useful measurands for the discrimination of the main honey types (67, 68). High fructose and glucose concentrations as well as low values in lactone and free acidity, electrical conductivity, polyphenol content and absorbance (visible spectroscopy) were described to be characteristic for floral honeys. Low glucose and fructose and high melezitose concentrations as well as high values for free acidity together with high polyphenol content and absorbance characterised honeydew honeys (69).

Linear discriminant analysis applied on sugar composition data of various unifloral honeys allowed only a discrimination between blossom and honeydew honeys (42). When further measurands such as water content, electrical conductivity, pH-value, colour (x, y, L chromatic coordinates) and sugar composition were combined, jackknife classification rates higher than 90 % were found for all unifloral honeys. Electrical conductivity, colour and fructose content were shown to be the most important measurands. Classification functions were also presented using water content, electrical conductivity, fructose, sucrose, and colour (28). Piro et al. (37) presented classification functions for as many as 16 different unifloral honeys using diastase activity, electrical conductivity, specific rotation, total acidity, fructose, glucose and colour (Pfund scale and CIE L.a.b). The average correct classification rate reached 89.6 % and all honey types except thistle (Carduus spp.) honey were correctly classified at a rate higher than 80 %. Electrical conductivity, glucose and fructose concentration as well as colour were found to be the most important variables for the classification of unifloral honeys.

In a recent study stepwise backward linear discriminant analysis was used to select the most important measurands among water, hydroxymethylfurfural (HMF), fructose, glucose, sucrose, erlose, raffinose and melezitose contents as well as electrical conductivity, pH-value, free acidity, diastase activity and colour (Pfund scale). The botanical origin of the samples could be perfectly predicted using electrical conductivity, pH-value, free acidity, fructose, glucose and raffinose contents (38).

1.3.2 PHENOLIC ACIDS AND POLYPHENOLS
Phenolic acids and polyphenols are plant-derived secondary metabolites. These compounds have been used as chemotaxonomic markers in plant systematics. Some of them have also been proposed as possible markers for the determination of the botanical origin of honey. Considerable differences in both composition and content of phenolic compounds have been found in different unifloral honeys. Dark coloured honeys have been reported to contain more phenolic acid derivatives but less flavonoids than light coloured ones (70). Ellagic acid detected in Ericaceae nectar was found in heather (Calluna vulgaris) honey as well and was proposed as a marker indicating that phenolic compounds could be useful for the determination of the botanical origin of honey (71). These findings agree with results found in heather honeys from Erica and Calluna species (72-74).
Hesperetin (5,7,3’-trihydroxy-4’-methoxyflavanone) has been reported to be characteristic for orange (Citrus spp.) honeys (75). No consistent relationship could be found in the hesperetin and methyl anthranilate (a suggested volatile marker compound) content of orange honeys. Since hesperetin is more stable than methyl anthranilate, it was proposed as a complementary marker for orange honey (74, 76).

In a recent study the flavonoid profiles of nine European unifloral honeys were analysed. Hesperetin was confirmed as a marker of orange honey. No specific compounds could be detected in acacia (Robinia pseudoacacia) and lavender (Lavandula spp.) honeys. Abscisic acid, previously reported as a characteristic compound of heather honey (77) was also detected in rape (Brassica spp.), lime (Tilia spp.) and acacia honeys in similar concentrations. All honey samples contained variable amounts of propolis derived compounds that were not helpful for the determination of the botanical origin of honey i.e. the flavanones pinobanksin and pinocembrin, the flavones chrysin, galangin, techtochrysin, apigenin and genkwanin, several quercetin and kaempferol methyl ethers and the caffeic acid esters phenyl-ethyl-caffeate and dimethyl-allyl-caffeate (78).

The flavanoles myricetin, quercetin, tricetin and luteolin were detected in European and Australian eucalyptus (Eucalyptus spp.) honeys and proposed as characteristic markers as they were not found in other European unifloral honeys (79, 80). These findings were confirmed by a more recent study (81). However the same flavanols were detected as well in Australian tea tree (Melaleuca quinquenervia), heath (Bankzia ericifolia), brush box (Lophostemon conferta) (82) as well as in jelly bush and manuka (Leptospermum spp.) honeys (83). In addition to this, myricetin was formerly described to be a characteristic compound of Portuguese heather (Erica spp.) honey (71). The marker status of these compounds is thus very questionable if no characteristic concentration ranges can be set.

Although numerous phenolic compounds in honey are derived from the nectar sources and should therefore allow conclusions to be drawn on the botanical origin of honey, there seems to be some confusion about the compounds being relevant for the authentication. Possibly chemometric evaluation of the data could help to find the most significant components.

1.3.3 VOLATILES
Research on honey volatiles began in the early 1960’s. From the very beginning, the determination of volatiles was suggested to allow an objective characterisation and classification of unifloral honeys as it was assumed that the volatiles in honey originate from the plant species where the nectar had been collected. Indeed it has been shown that the precursors of the volatiles responsible for the specific flavour of unifloral honeys very often originate from the corresponding plants (84-86).

Various methods including solvent extraction (87) modified Likens-Nickerson steam distillation and solvent extraction (88, 89), dynamic headspace extraction (90, 91) solid phase micro extraction (68, 92-94) as well as gas sensors (95, 96) have been used to study the volatile composition of unifloral honeys. Until now about 600 compounds have been identified in various honey types and the list is certainly far from being exhaustive.

In order to distinguish between different unifloral honeys, it has been proposed to search for unique and characteristic components in each unifloral honey type. Subsequently numerous marker compounds have been suggested e.g. methyl anthra-
nitate for orange (Citrus spp.) honeys (97-99), 3-amino acetophenone and 2-amino acetophenone for chestnut (Castanea sativa) honey (100, 101) benzoic acid, decanoic acid and dehydrovomifoliol for heather (Calluna vulgaris) honeys (102, 103). However only few compounds seem to be really specific for certain unifloral honeys and many of them can be found in variable concentrations in various honey types e.g. 3-amino acetophenone and dehydrovomifoliol have been later detected in tasmanian leatherwood (Eucryphia lucida) honey as well(84).

The use of individual marker compounds for the classification of unifloral honeys is probably only reasonable when they are quantitatively determined and specific concentration ranges are defined for the unifloral honeys. Otherwise there will be no possibility to distinguish polyfloral honeys with nectar contributions from a given plant from the unifloral honeys of the same source.

The use of a combination of several volatile components seems more promising since the results are less susceptible to variations of individual components. In this context chemometrics may be useful to determine the key components (93, 104) The whole chromatograms could also be used as characteristic fingerprints of the different honey types. However difficulties may arise with very sensitive techniques to handle chromatograms containing unknown volatiles resulting from a minor nectar source. Another drawback of the use of volatile composition is that the volatile composition may considerably change during honey processing and storage (105, 106).

Nevertheless the large amount of information obtained from a honey sample by analysing its volatile composition may be useful for very challenging classifications of the botanical origin, e.g. within the same plant family. It has been shown that honeys from different lavender (Lavandula spp.) species can be distinguished from each other and from other types of unifloral honey (107) and that different rape honeys can be classified according to their botanical origin (94).

Moreover the techniques are not very reproducible and very time consuming especially when the whole chromatographic separation is required. In this respect the use of gas sensors probably presents the most promising approach.

1.3.4 AMINO ACIDS AND PROTEINS

Proline, the main amino acid in honey, originates predominantly from the bee. Its concentration is used as an indicator of honey ripeness and for the detection of adulteration (108). Free amino acid profiles have primarily been proposed for the determination of the geographical origin of honey (109, 110). Cometto et al. (111) showed that the differences observed between geographical regions are rather due to variations in vegetation type i.e. the botanical origin.

Later on differences were also observed between various unifloral honeys (112). In a study on lavender (Lavandula spp.) and eucalyptus (Eucalyptus spp.) honeys high amounts of phenylalanine (906-1830 mg/kg) and tyrosine (229-382 mg/kg) were found to be characteristic for lavender honeys and allowed a differentiation from eucalyptus honeys (113).

Tryptophan and glutamic acid were used to distinguish honeydew from blossom honeys (67). Chemometric evaluation of free amino acid concentrations in combination with further measurands such as pH-value and sugar composition may also present a promising approach for the determination of unifloral honeys (111, 114).
Recently, a polymerase chain reaction based technique and an electrophoretic immunoblot assay for the study of pollen proteins in honey was described (115, 116). These very sensitive techniques allowed a reliable detection of pollen from different plant species and were proposed as alternative to traditional pollen analysis as pollen proteins were successfully used for the determination of botanical origin (116). Indeed such techniques are certainly valuable to detect transgene material in honey but since the analysed proteins originate from pollen these methods suffer from the same shortcomings as microscopic pollen analysis.

1.3.5 MINERAL COMPOSITION

Some authors claimed that mineral composition may be successfully used to classify different blossom honeys (117) while others did not succeed using mineral content alone. They had to use additional physical and chemical measurands i.e. free acidity and sugar composition (118). An investigation on a larger set of samples would probably show that the mineral composition is only useful for a distinction between blossom and honeydew honeys (119-121). This conclusion is also drawn by a recent study indicating a strong correlation between mineral content and honey color (122). Mineral content does not allow a more detailed classification between different unifloral honeys than the measurement of electrical conductivity does.

In a Canadian study on the mineral composition of honeys from different provinces, no discrimination was achieved between different floral origins. However, honeys from the coastal provinces with a more humid climate revealed a higher mineral content than those from central provinces with a continental climate (123). Mineral content was also successfully applied to authentify Galician honeys (124). Thus, mineral content seems to have some significance to determine the geographical origin of certain honeys.

1.3.6 ORGANIC ACIDS

In total 32 aliphatic dicarboxylic acids have been identified in some unifloral honeys from New Zealand by GC-MS. Methyl butanedioic acid and 4-hydroxy-3-methyl-trans-2-pentenedioic acid were proposed as floral markers for rewarewa (Knightea excelsa) honeys (125). Several mono-, di- and tricarboxylic acids such a formic, citric, pyruvic, malic, fumaric, pyro-glutamic, gluconic, galacturonic, citramalic and quinic acids have been identified by HPLC of sainfoin (Onobrychis viicifolia), rosemary (Rosmarinus officinalis), lavender (Lavandula spp.), thyme (Thymus spp.), oak honeydew (Quercus spp.) and heather (Erica sp.) honeys (126). Significant differences in the concentration of several acids between the honey types were also encountered in another study on acacia (Robinia pseudoacacia), eucalyptus (Eucalyptus spp.), rape (Brassica sp.), lime (Tilia spp.), lavender, rosemary, chestnut (Castanea sativa) and heather (Calluna vulgaris) honeys. However the number of samples was very limited (127). On the other hand, it has been reported, that many acids in honey are produced by the enzymes added by the bees during honey processing (128). Thus, it is questionable, if organic acids provide valuable information for the classification of unifloral honeys.
1.3.7 SPECTROSCOPIC TECHNIQUES

1.3.7.1 NEAR-INFRARED SPECTROSCOPY

In the last decades near-infrared spectrometry (NIR) has become a rapid and well established technique for quantitative and qualitative analysis of food (129). It has been applied both in transmission and transfectance modes to different fields of honey analysis, i.e. determination of botanical and geographical origin, quality control and detection of adulteration.

The potential of NIR for the determination of the botanical and geographical origin of honey was evaluated (130). Among the 13 different botanical origins studied only the acacia (Robinia pseudoacacia), chestnut (Castanea sativa), rape (Brassica sp.) and heather (Calluna vulgaris) honeys had sufficient samples for chemometric evaluation. After data reduction by principal component analysis (PCA), linear discriminant analysis (LDA) was used to build the discriminant models and applied for the classification of the honey types. In the plot of the principal component scores, the acacia honey samples grouped close together, while the samples of the other honey types did not present uniform clusters. In the discriminant score plots acacia and rape honeys formed two distinct groups while those of the other honey types overlapped. In average 67 % of the honey samples were correctly classified. All of the rape honey samples were correctly assigned while only 29 % of the heather honeys could be identified. The samples of the other botanical origins studied were mostly misclassified to the group of rape honeys. Half of the samples of various other unifloral origins were incorrectly assigned to the groups mentioned above and the other half of the samples were not assigned to a group. The number of samples per honey type was however very restricted as 13 different unifloral honeys from nine European countries were studied on a total of 51 samples. No classification according to the geographical origin of the samples could be observed (130).

A more recent study on 50 eucalyptus (Eucalyptus spp.) and polyfloral honeys showed that the LDA models developed correctly classified 75% of the polyfloral and 85% of the eucalyptus honeys (131). Despite of the limited number of samples, the preliminary results of the above mentioned studies are very encouraging and should be validated with a larger set of unifloral and polyfloral samples.

The quantitative analysis of honey components by NIR has been discussed in various studies. Accurate predictions were obtained for fructose, glucose, sucrose, maltose, water and ash contents as well as for the fructose/glucose and glucose/water ratios in honey samples from different crops (132-138). Furthermore non-compositional characteristics of honey such as electrical conductivity, colour and polarimetric properties (direct polarisation, polarisation after inversion, specific rotation in dry matter and polarisation due to non-monosaccharides) have also been successfully calibrated (138, 139). Near-infrared spectroscopic techniques have not been considered as adequate for the analysis of minor honey components such as HMF, free and lactone acidity as well as pH-value (135, 138). In a calibration limited to avocado honey it was though possible to quantify low concentrations of perseitol (49).

Some authors claimed that even the isotope ratio between $^{12}$C and $^{13}$C, used for the detection of cane sugar adulteration can be determined by NIR. Unfortunately this calibration was restricted to two types of honey and was not validated with adulterated samples (136, 137). Detection of adulteration by addition of beet and corn syrups was studied on Irish honeys (140). Falsifications could only be ascertained
above 20%. Therefore NIR does not seem to present a valuable alternative to the current isotope ratio mass spectroscopic and liquid chromatographic techniques. Particularly because the Irish honey samples do not allow to generalise for different honey types. The detection limit would supposedly be considerably higher when more authentic honey types would be considered.

1.3.7.2 MID-INFRARED SPECTROSCOPY

Mid-infrared spectroscopy (MIR) provides more specific and distinct absorption bands and thus more information on the sample than NIR. MIR has however rarely been applied to quantitative analysis of honey. Nevertheless a study based on a very large number of samples has shown that satisfying calibrations can be set up for the major honey components with accuracies generally exceeding those obtained by NIR (141). Reliable partial least squares (PLS) models were established for the quantitative analysis of fructose, glucose, sucrose, maltose, electrical conductivity, pH-value and free acidity. A drawback of the method, using a semi-automatic instrument designed for the analysis of liquids, is that the honey samples have to be quantitatively weighed into the so-called Zero Liquid (FOSS, Hillerød, Denmark) which mainly consists of water. This results in additional work and in a strong noise in the water absorption bands (1717-1543 and 3627-2971 cm\(^{-1}\)) thus preventing the determination of water content. Minor sugars present in concentrations lower than 2 g/100 g as well as proline, HMF content and invertase activity could not be determined (141).

This disadvantage could be overcome by using a single reflection attenuated total reflection (ATR) accessory which was recently applied to the analysis of fructose, glucose, sucrose and maltose in honey (142). In this study pure sugar solutions as well as 60 honey samples from different botanical origins were analysed. Calibration with PLS and principal component regression (PCR) models for the determination of sugar concentrations in honey were evaluated. The PLS model was shown to be more promising than the latter. Correlation coefficients calculated for the four sugars analysed by HPLC (reference method) and by MIR ranged from 0.971 to 0.993. This indicates that FT-IR-ATR spectrometry seems to be adequate for rapid, non-destructive and accurate quantitative analysis of honey (142).

Numerous studies carried out by the same authors (143-147) suggest that honey adulterated with various sugar syrups as well as pure glucose, fructose, and sucrose can be detected by infrared spectroscopy using a multiple reflection ATR-sampling accessory and chemometric models. However, the relevancy of these findings seems to be questionable as the natural variation of the honey composition was barely considered, since only three samples of different botanical origin were studied. In some of the studies the experimental design facilitated the detection of such an adulteration because the water content was also changed when the honey samples were adulterated with syrups and pure sugars (145, 146). To prevent this problem Kelly et al. (148) proposed to dilute all samples with water and to adjust the solid content to 70° Brix. In this study a considerable number of natural honeys was analysed as well. However adulterations below 14 g/100 g could not be reliably detected and the rate of false positives for adulterated samples in general was 7-10%.

Recently Tewari and Irudayaraj (149) claimed that ATR-MIR is very promising for the determination of the botanical origin of honey. However their display of the spectra of different botanical origins is surprising as they only differ in absorption and hardly in shape. On the display of the linear discriminant scores the samples group with an
exceptional perfection hardly ever reached by biological samples and could be the result of an overfitting. It would be expected that the so called “wild flower honeys” (polyfloral honeys) would be much more spread and overlap with the other groups at least in the display of the first discriminant scores. It seems therefore doubtful that the model presented will be valuable in practice (149).

Nevertheless the results show that mid-infrared spectra contain valuable information on the botanical origin of honey and can be used for quantitative analysis of main components in honey. It presents thus a promising approach for a rapid analysis of honey.

1.3.7.3 RAMAN SPECTROSCOPY
Raman spectroscopy using laser light in the near-infrared region has been applied for the detection of beet and cane sugar syrups in honey (150). The authors suggest that invert sugar adulterations can be detected down to the plant source from which the sugar syrup has been produced and explain this by the change in the $^{12}$C/$^{13}$C ratio. It is however questionable if Raman spectroscopy is sensitive enough to detect such differences. In addition to this, the natural variation of honey composition has not sufficiently been considered as only three botanical origins were studied (150). Quantitative analysis of fructose and glucose showed poor repeatability compared to liquid chromatographic techniques (151). This may partly be due to the very low number of samples in calibration but probably quantitative analysis of honey with Raman spectroscopy will not produce more accurate predictions than NIR.

The prediction of the botanical origin using Raman spectroscopy and neuronal networks allowed 13 out of 14 honeys to be correctly classified in validation but the study allows not much conclusions to be drawn as seven different honey types were studied on a total of 43 samples (152). From a theoretical point of view the application of Raman spectroscopy has about the same potential as NIR.

1.3.7.4 FLUORESCENCE SPECTROSCOPY
Compared to the spectroscopic techniques based on absorption, fluorescence spectroscopy offers a 100- to 1000-fold higher sensitivity. It provides information on the presence of fluorescent molecules and their environment in organic materials. On contrary to the vibrational spectroscopic techniques discussed above fluorescence spectroscopy is an emission spectroscopic technique and can therefore provide a different approach to the determination of the botanical origin of honey.

To overcome decrease in fluorescence intensity at absorbances over 0.1 absorbance units and distortion of emission spectra due to quenching, front-face fluorescence spectroscopy was developed where only the surface of the material is illuminated and examined (153). This technique allows a quantitative investigation of fluorophores in powders as well as in concentrated or even opaque samples.

Food have complex matrices containing many different fluorophores. Their signals could overlap and make it impossible to measure the concentration of a single compound. Nevertheless, the shape of fluorescence spectra in combination with multivariate statistics can be used to characterise and identify different food as their fluorescence characteristics are strongly influenced by their environment.
Fluorescence spectroscopy has been used to study structural changes in triglycerides and proteins during cheese ripening (154, 155), determine different types of milk processing (156), classify different types of cheese (157), or to identify different bacteria species (158).

Unifloral honeys are well known to contain numerous polyphenols (70, 73, 78, 82) as well as other fluorophores such as amino acids (112, 159). Some of them have already been proposed as tracers for unifloral honeys, e.g. ellagic acid for heather honey from Erica and Calluna species (72) or hesperetin for citrus honeys (78, 160). Also, fluorescent amino acids have been proposed as markers for unifloral honeys. Phenylalanine and tyrosine have been found to be characteristic for lavender honeys and allowed a differentiation from eucalyptus honeys (161). Tryptophan and glutamic acid have been shown to be useful for the differentiation between honeydew and blossom honeys (67). As polyphenols and aromatic amino acids are strong fluorophores, fluorescence spectroscopy should be helpful for authenticating the botanical origin of honey.

1.4 CONCLUSIONS

The use of traditional methods for the authentication of the botanical origin of honey requires especially in regard of pollen analysis considerable knowledge on the different honey types and is therefore limited to experts working in this field. The uncertainty related to the interpretation of the pollen analytical results and the considerable amount of work involved as this technique has so far not been automated, demands to find complementary techniques for the authentication of the botanical origin of honey allowing a reproducible classification (29, 162).

The potential of various analytical techniques for the classification of pure unifloral honey has been shown. Unfortunately this is a trivial challenge as the pure unifloral honeys show considerably different physical and chemical characteristics. However the unifloral honeys account only for a minor proportion of the honeys produced, the majority of the honeys on the market contain important proportions of nectar or honeydew from different sources and are therefore considered as polyfloral honeys. Thus the major challenge in the authentication of the botanical origin is to distinguish the unifloral honeys from the polyfloral ones. Most of the numerous new analytical techniques proposed during the last decades for the authentication of unifloral honey, have not been tested in this respect. This may also explain why none of them is being routinely used in honey analytics.

As discussed above there are numerous techniques offering possibilities to obtain information related to the floral source of the honey. Since the composition of the different honey types is very similar, analytical techniques offering information on the overall composition such as spectroscopic techniques or techniques related to highly specific compositional properties offer the most promising approach when as few as possible techniques are intended to be used.

The ideal method would be fast and inexpensive, require little sample preparation, allow for automated sampling, and provide highly specific information related to the nectar sources the honey is derived from. From such points of view the approach using volatiles or spectroscopic techniques seem to present the most promising approach. Concerning volatiles a very large amount of information on a honey sample can be obtained but the methods are often very sensitive and the data may be dif-
difficult to handle because of shifts in retention times or the presence of unknown components in only part of the samples. The time required for the analysis of volatiles by gas chromatography depends especially on the completeness of the extraction and the separation needed. If their sensitivity is sufficient the spectroscopic techniques probably present the most promising approach. They do only require very little or no sample preparation, no harmful reagents, are fast, allow to get a fingerprint of the overall chemical composition of honey and show the ruggedness and the excellent repeatability of physical methods. In addition to the authentication of the botanical origin quantitative information on several honey components can be simultaneously obtained. Nevertheless a successful authentication of the botanical origin of honey probably depends less on the analytical method used than on the appropriate data evaluation procedure, chemometrics being indispensable in this respect. Once appropriate analytical techniques have been found it is important to harmonise the techniques and criteria to be used for a reproducible and reliable determination of the botanical origin of honey.

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