

CHAPTER 9

General Discussion and Outlook

9.1 INTRODUCTION

Physical, chemical and pollen analytical characteristics of the most important European unifloral honeys have been described in various papers (1-4). Unfortunately neither the measurands to be considered nor their corresponding ranges for the individual unifloral honeys have been unequivocally defined and internationally accepted.

Traditionally the botanical origin of honey is determined by experts evaluating several physical, chemical, pollen analytical and sensorial criteria. The attribution of honey samples to different botanical origins is made by a profiling technique. Analytical data of an unknown honey sample are compared with the data ranges described for the different unifloral honeys. If all the values of the measurands considered fit into the ranges described for a given unifloral honey type, it is assumed to be identified. On the contrary if the characteristics of the sample do not fit into the profiles of the various unifloral honey types considered, the sample is classified as polyfloral honey. Thus the group of polyfloral honeys consists of a miscellaneous pool of honeys of various botanical origins with significant nectar or honeydew contributions from several plant species. Polyfloral honeys represent blends of several unifloral honeys.

The classification with a profile is possible because unifloral honeys generally express at least in respect to some measurands highly specific properties that are generally not found in other honey types. The purest samples of unifloral honeys are therefore the easiest to recognise. In practice unifloral honeys are hardly ever pure and generally contain minor nectar contributions from other botanical origins. Consequently there will always be some overlapping between unifloral and polyfloral honeys. Where the limit between these groups is set, depends on definition and is ultimately a matter of opinion. Therefore the main problem in the authentication of unifloral honeys is to discriminate between unifloral and polyfloral honeys, rather than between different unifloral honey types.

This was also shown by the chemometric evaluation of the data gathered with various analytical methods. Polyfloral honey samples were frequently misclassified as unifloral honeys when a single discriminant model considering all the different honey types was used. These difficulties could nevertheless be overcome by validating the classification of the first overall discriminant model by several two-group discriminant models just classifying between a given honey type and a group consisting of all the other honey types. The successive use of several discriminant models enabled a reliable classification of different unifloral and polyfloral honeys. However, absolutely correct classifications by chemometric models cannot be expected as these models are trained by uncertain reference sample sets.

9.2 DETERMINATION OF THE BOTANICAL ORIGIN USING CLASSICAL PHYSICAL AND CHEMICAL MEASURANDS

Traditionally relatively few physical and chemical measurands, in particular electrical conductivity and sugar composition, are used together with pollen analysis to classify honey samples according to the characteristics described for the various honey types. In the present study the prediction of the botanical origin of the honey samples was attempted by using different profiles and chemometrics. In multivariate data evaluation pollen and sensory analytical results were not considered.

9.2.1 CLASSIFICATION USING PROFILES

A profile for each honey type studied was set up by using the total range of the values available for the unifloral honeys previously classified by the traditional approach including pollen and sensory analysis. For this purpose fourteen different measurands were considered in the profiles. Classification was achieved by comparing the profile of each honey sample with each of the ten profiles established for the unifloral honey types considered.

Generally the unifloral honey samples were correctly classified to the honey type. Misclassifications between different unifloral honeys were rare. Numerous polyfloral honey samples were however misclassified to different groups of unifloral honeys. Most misclassifications happened to the honey types known to have a highly variable chemical composition like alpine rose, lime and fir. The determination of the botanical origin by profiles proved to be reliable only when pollen analytical criteria were included. This indicates that pollen analytical results play a key role in the discrimination between unifloral and polyfloral honeys.

When the same measurands and data ranges are used in a profile, the traditional approach allows to get reproducible results without any specialised expertise. In order to simplify the classification, the profiles can for example be programmed in a spreadsheet software. In the present study the number of samples considered for some honey types was not large enough to allow definitive ranges to be set. In future studies the number of samples should therefore be increased.

9.2.2 CLASSIFICATION USING CHEMOMETRICS

Multivariate explorative data analysis revealed that electrical conductivity, fructose, raffinose and glucose concentrations, together with free acidity, contributed most to the correct classification of the different unifloral honeys by a single model. This classification was verified by one or several two-group models. This validation step proved to be crucial for a reliable classification of the unifloral honeys and to reduce the misclassifications of polyfloral samples to unifloral honeys. When a sample was classified to the same honey type by both the overall and the respective two-group model the classification was in most cases correct, only few samples were assigned to the group of polyfloral honeys.

On contrary to the classification using a profile, the chemometric evaluation of the physical and chemical measurands demonstrated that a correct determination of the botanical origin of honey can be achieved without pollen analysis to a high degree of accuracy. The linear combinations and the standardisation functions given allow a correct classification of the botanical origin of unknown samples without statistical software.

However, this approach does not save very much time and costs as only pollen analysis can be abandoned and the physical and chemical measurands have still to be determined by individual analytical methods. Nevertheless pollen analysis is the technique, which requires the most experience, time and in addition cannot be automated. The predictions based on the physical and chemical measurands were found to be not as accurate as the determinations using spectroscopic fingerprints developed in this work. However, the chemometric evaluation of the traditional physical and chemical measurands has the advantage that laboratories with a small sample throughput can determine the botanical origin using the existing laboratory equipment. They do not have to invest in expensive instruments and software. Also the tedious set up of the appropriate multivariate models may be avoided.

Moreover the data evaluation revealed that for example electrical conductivity is not a very reliable criterion to discriminate between floral and honeydew honeys although it is defined as such in the European honey directive (5). The latter indirectly recognises this inconvenience as several exceptions are indicated in the directive for different honey types. Consequently the multivariate data evaluation of traditional physical and chemical measurands may also be valuable to establish new criteria allowing a more reliable description of the honey types and the determination of the botanical origin.

9.3 DETERMINATION OF THE BOTANICAL ORIGIN USING FLUORESCENCE SPECTROSCOPY

Compared to other spectroscopic techniques based on absorption, front-face fluorescence spectroscopy offers a high sensitivity for fluorescent molecules and provides valuable information on their environment even in concentrated or opaque samples. Different emission and excitation wavelengths were tested during method development. The most adequate conditions for discrimination between honeys of different botanical origins were found using excitation spectra scanned from 220 to 400 nm with the emission measured at 420 nm and emission spectra obtained after excitation at 270 nm. The highest correct classification rates were obtained when these two types of spectra were combined to form a single fingerprint of a given honey sample. Fluorescence spectroscopy was the only spectroscopic technique that allowed to visually detect differences between the honey types by comparing the spectra. The shape of the fluorescence spectra and the intensity of fluorescence emission were found to be characteristic for the unifloral and polyfloral honeys studied. However the intensity varied considerably within a honey type. The normalisation used in the preliminary study was later found to be disadvantageous as the information regarding the intensity was lost.

Compared to infrared spectroscopy particularly front-face fluorescence spectroscopy is not a very popular and widespread technique. However, it proved to be the most promising technique for the classification of unifloral and polyfloral honeys. Our findings demonstrate that the classification based on classical criteria commonly used for the determination of the botanical origin of honey can be very well reproduced by front-face fluorescence spectroscopy and chemometrics.

Unlike infrared spectroscopy this technique can only be used for the determination of the botanical origin of honey and does not allow a simultaneous quantitative analysis of the main components. Therefore fluorescence spectroscopy will only be of interest for laboratories particularly interested in the determination of botanical

origin with a high sample throughput as the setup of the discriminant models is tedious and costly. This technique could gain more interest when it could be used for quantitative analysis of fluorescent compounds related to the therapeutic applications of certain unifloral honeys.

A small drawback of fluorescence spectrometry is that a special sample holder is needed and that quartz cuvettes have to be filled with highly viscous honeys and thoroughly cleaned after analysis. This inconvenience may be overcome by the development of more appropriate sample cells or by diluting the sample with water. The latter option would probably even allow an automated sampling (such as liquid flow analysis) otherwise difficult to achieve with spectroscopic techniques.

An important aspect that should be verified by future studies is the possibility to transfer spectra or whole discriminant models from an instrument to another as it has been already done for infrared spectroscopic applications (6-8). This will require a standardisation of the excitation radiation and the sensitivity of the detector. A material emitting a constant radiation would allow to calibrate instruments at different places.

Research application may be of interest for front-face fluorescence spectroscopy as the technique presents an independent point of view to the subject. Explorative data analysis on fluorescence spectra may allow to better define physical and chemical characteristics of certain unifloral honey types. The technique may as well be useful for the classification of unifloral honeys that are too similar to be discriminated by other physical and chemical techniques.

9.4 DETERMINATION OF THE BOTANICAL ORIGIN USING INFRARED SPECTROSCOPY

9.4.1 MID-INFRARED SPECTROSCOPY

This type of absorption spectroscopy reflects the overall chemical composition of the honey samples studied. Like in fluorescence spectroscopy the only sample preparation necessary is the liquefaction of the honey samples to be measured. The use of the attenuated total reflection single reflection sampling accessory proved to be very straightforward and fast. The technique does not imply the use of any quantitative measures or chemicals.

The largest variation between the honey types was found in the C-O and C-C stretching regions of the sugars between 950 and 1050 cm^{-1} . The classification obtained using mid-infrared attenuated total reflection spectroscopy and chemometrics was in agreement with the classifications using traditional measurands. Consequently it offers a very promising approach for the authentication of the botanical origin of honey. It is interesting to note again that the honey types with the most variable composition such as lime or alpine rose honeys showed the lowest classification rates in the discriminant models. Lime honey has a very distinct and dominant aroma that can be identified even at low concentrations. Therefore honey samples may often be regarded as lime honeys even if they are not very pure. Difficulties in the classification of alpine rose honeys are probably also related to its variable composition. As this honey type is produced in the mountains under difficult climatic conditions there is probably a considerable temptation to designate honeys as alpine rose honeys even if there is only a minor nectar contribution of alpine rose.

Generally this fact probably concerns many of the rare honey types or unifloral honeys exhibiting a strong and characteristic aroma. Infrared spectroscopy may be helpful for a better characterisation of these honey types.

In the present work the honey samples were classified using a reduced number of data, i.e. principal component scores. The classical approach using spectral libraries was also verified. It was found, with the algorithms tested, to be less efficient than the approach using linear discriminant analysis for the determination of the botanical origin. In future studies possibly more convenient algorithms could be found that would enable an equally good classification using spectral databases. This would possibly facilitate the data evaluation, as no special software applications have to be developed.

Infrared spectroscopic techniques give an additional and independent point of view on the topic of authentication of the botanical origin of honey. They may be advantageous to better characterise unifloral honeys that are not yet very well defined by the traditional physical and chemical criteria.

Multivariate data evaluation techniques such as cluster analysis that do not require a priori grouping but just classify the samples according to spectral similarities may be used to improve classification of honey samples.

Even though the samples originated from different geographical regions, they were nevertheless correctly classified according to their botanical origin. Irrespective of the geographical origin the spectroscopic characteristics of the honey types seem to be uniform and will consequently allow a classification of honey samples from different geographical origins according to their botanical origin. This finding should however be confirmed and extended by future studies in more detail.

9.4.2 NEAR-INFRARED SPECTROSCOPY

In contrast to mid-infrared spectroscopy near-infrared spectroscopy proved not to be useful for the authentication of the botanical origin of honeys. Near-infrared spectra contained too little characteristic information related to the botanical origin of honey, thus allowing only a discrimination between the most distinct unifloral honey types. The potential of near-infrared spectroscopy may be improved if additional information from honey colour would be included by using an instrument scanning the visible range as well.

9.5 QUANTITATIVE INFRARED SPECTROSCOPY

Mid-infrared attenuated total reflection spectroscopy combined with multivariate calibration algorithms such as partial least squares regression was successfully applied to set up calibrations allowing to accurately predict the concentrations of the main components in honey. Interestingly also non compositional and non-infrared active characteristics such as pH-value and electrical conductivity could be accurately measured. Satisfying accuracies were obtained for the prediction of water, electrical conductivity, glucose, fructose, sucrose, melezitose, total monosaccharides, fructose/glucose ratio, glucose/water ratio, pH-value and free acidity. Possibly the prediction accuracy could be further improved when the reference values of the "minor sugars" would be determined by ion- or gas chromatographic techniques.

Quantitative analysis using near-infrared allowed only the main components of honey to be determined with a satisfying accuracy.

Unfortunately infrared spectroscopic methods do not allow a quantitative determination of hydroxymethylfurfural and enzyme activities, two criteria particularly important for honey trade, i.e. for the evaluation of storage and heat damage. Reliable calibrations for the prediction of hydroxymethylfurfural content cannot be established because of its low concentration and lack of specific infrared absorption. Enzyme activities do not express any particular infrared absorption and could neither be indirectly measured.

A considerable advantage of infrared spectroscopy is that the same spectra may be used for both the determination of the botanical origin and to obtain quantitative information on several measurands used for routine quality control of honey. It allows to simultaneously predict concentrations of several measurands on the basis of single spectra within a few minutes. This is particularly important for routine quality control of honey at low cost (9).

9.6 GEOGRAPHICAL ORIGIN

A designation of the geographical origin may be used if the honey has been produced exclusively within the area referred to (5). Pollen analysis is currently used to determine the geographical origin of honey as the pollen in honey reflects the vegetation types where the nectar has been collected by the bees.

In the past many analytical methods have been proposed in combination with multivariate data evaluation for the determination of the geographical origin (10-16). Unfortunately in most of the methods presented the botanical origin of the honey samples was not determined, or the discrimination between the various geographical origins was not tested on samples of the same botanical origin. Consequently the distinctions found were rather due to differences of the vegetation type and thus to the botanical origin of honey, than due to the geographical regions considered.

In the present study differences in geographical origin were studied within some unifloral honeys as well as between different honey types. Using front-face fluorescence spectroscopy a classification according to the geographical origin was only observed within the groups of samples of the same botanical origin. The discriminant model failed e.g. to classify samples of German and Swiss origin according to their geographical provenience when samples of various botanical origins were considered. When the same samples were classified according to their botanical origin very high rates of correct classification were reached. This clearly indicates that the fluorescence characteristics resulting from the botanical source are considerably more prominent than characteristics related to the geographical region.

Honey samples were correctly classified according to their geographical origin using mid-infrared spectroscopy. Interestingly a difference between fir honeydew honeys from Switzerland and Germany was even observed when a larger set of samples was considered including samples of different crops. A difference was also observed in the average spectra of samples of various botanical origins from these two countries. The average rate of correct classification according to the geographical origin remained high when samples of different botanical origins were considered. However, when the linear discriminant analysis was performed on the same data set, using the botanical origin as grouping variable, all spectra were correctly assigned to the corresponding group of unifloral honey. This indicates again that the effects of the botanical origin are more relevant than those of the geographical origin. The differences observed and interpreted as resulting from geographical origin

may be indirect effects of the botanical origin. In unifloral honeys these differences could originate from small nectar contributions of the accompanying flora that may change with the geographical region where the honey is harvested.

The differences in geographical origin observed in this study should be verified in future investigations with larger sample sets better representing the honeys produced in different geographical regions and by including polyfloral honeys as well. It would certainly be helpful when the geographical origin could be determined within a unifloral honey type, but in principle a method for the determination of the geographical origin should be applicable and validated for all honey types.

9.7 DETECTION OF HONEY ADULTERATION

Fluorescence spectroscopy was not found to be useful for the detection of honey adulteration as the spectra of adulterated honeys did not show any comprehensible changes compared to those of the corresponding natural honeys. The spectra of the artificially adulterated samples remained in the range of the natural variation of the corresponding unifloral honeys. A detection of honey adulteration is therefore not possible unless the adulterant would contain a characteristic fluorophore. Fluorescence spectroscopy could be reconsidered if bee feed would be labelled with a characteristic fluorophore, like it has been done in some occasions by adding a dye to the sugar syrup. Consequently the detection of honey adulteration by feeding bees will remain an analytical challenge.

Infrared spectroscopy is neither useful for detection of honey adulteration with small amounts of sugar derived from sugar syrup. The sugar composition in honey is variable and the infrared absorption of the different sugars too similar. Using specific models for certain unifloral honeys might be a possibility to lower the detection limit. However it is not clear if the adulterated honeys would still be recognised as unifloral. Infrared spectroscopy may be useful in certain circumstances where for example high amounts of maltotriose indicate an addition of a starch hydrolysate. Adulteration with high amounts of sugar may be detected but can be more accurately identified by liquid chromatographic techniques.

9.8 OUTLOOK

There are several promising analytical methods for the determination of the botanical origin of honey. More crucial than the analytical technique used is certainly the appropriate data evaluation method. The measurands to be considered and the corresponding data ranges should be harmonised in order to achieve a reproducible classification of different honey types in various countries and to ensure a uniform honey quality that will be recognised by consumers preferring a given type of honey.

The various techniques developed and evaluated within this work for authentication of the botanical origin of honey showed consistent results indicating that they are adequate for recognising the different characteristics of the honey types studied. Especially front-face fluorescence and mid-infrared spectroscopy proved highly promising as fast analytical techniques for an authentication of the botanical origin. Moreover the classifications obtained by these new methods agree very well with the results obtained by traditional methods.

A considerable advantage of spectroscopic techniques is their ruggedness related to the physical measurements resulting in an excellent repeatability. Compared to chromatographic methods used for the determination of the volatile compounds of honey no problems such as shifts in retention time occur. Physical methods, such as spectroscopy, are generally also considerably faster than chemical analyses using gas or liquid chromatography. A further advantage of spectroscopic tools is that sample preparation is limited to the liquefaction of the honey sample and no harmful or expensive chemical reagents have to be used.

In conclusion the presented methods allow a reliable determination of the botanical origin of honey without the use of pollen analytical results requiring specialised expertise. Apart from the determination of the botanical origin the presented techniques may also be useful for the quality control of honey blends that are produced by various honey packers, as they express probably characteristic fingerprints as well.

The present work has to be considered as feasibility study. The application of the technique in routine analysis will need at least for some honey types a confirmation of the results with an even larger set of unifloral honeys. A drawback of the presented spectroscopic techniques is the considerable work involved in building the classification models before they can be used in analytical practice.

However in laboratories with a high sample throughput the investment will probably pay back soon as labour is the most cost intensive factor in honey analysis. In addition the botanical origin and the most important physical and chemical properties can be predicted within a few minutes instead of more than an hour. The spectroscopic techniques will gain even more value when the calibration models developed can be transferred from one instrument to another. At least regarding infrared spectroscopy calibration transfers should be possible, as has already been demonstrated in quantitative analysis of various food constituents. Finally, the necessity for recalibration and the analytical quality assurance measures to be taken should be verified by future studies.

9.8 LITERATURE CITED

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