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Authentication of the Botanical Origin of Honey

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Stjärnorna

När natten kommer
står jag på trappan och lyssnar,
stjärnorna svärma i trädgården
och jag står i mörkret.
Hör, en stjärna föll med en klang!
Gå icke ut i gräset med bara fötter;
min trädgård är full av skärvor.

Edith Södergran

Södergran, E. In *Dikter* ; Schildt: Borgå, 1916.

Contents

List of Abbreviations

ASCII	American standard code for information interchange
ATR	attenuated total reflection
CIE	Commission Internationale de l'Eclairage
cv	coefficient of variation
DTGS	deuterated triglycine sulfate
FT	Fourier transform
HMF	hydroxymethylfurfural
HPLC	high performance liquid chromatography
IR	infrared
LDA	linear discriminant analysis
MANOVA	multivariate analysis of variance
meq	milliequivalent
MIR	mid-infrared spectroscopy
mS	milliSiemens
n	number of samples
NIR	near-infrared spectroscopy
PC	principal component
PCA	principal component analysis
PCR	principal component regression
PLS	partial least squares regression
PRESS	predicted residual sum of squares
r	repeatability limit
R^2	coefficient of determination
s	standard deviation
SEP	standard error of prediction
SECV	standard error of crossvalidation
s.l.	sensu lato
sp.	species (plural spp.)
$\bar{x}$	mean
$\tilde{x}$	median
x_{min}	minimum
x_{max}	maximum

Zusammenfassung

Die botanische Herkunft des Nektars hat einen entscheidenden Einfluss auf die chemische Zusammensetzung des Honigs. Honige die überwieged von einer Pflanzenart stammen und die entsprechenden physikalischen, chemischen und pollenanalytischen Eigenschaften aufweisen, können als sogenannte Sortenhonige deklariert werden. Diese unterscheiden sich auch in ihren sensorischen Eigenschaften markant und erzielen auf Grund der unterschiedlichen Präferenzen der Konsumenten im Vergleich zu gewöhnlichen Mischblütenhonigen wesentlich höhere Preise.

Über 650 Akazien- (*Robinia pseudoacacia*), Alpenrosen- (*Rhododenron* spp.), Heide- (*Calluna vulgaris*), Kastanien- (*Castanea sativa*), Linden- (*Tilia* spp.), Löwenzahn (*Taraxacum* s.l.), Raps- (*Brassica* spp.), Metcalfa honigtau- (*Metcalfa pruinosa*), Eichen honigtau- (*Quercus* spp.) und Waldhonige (Abies spp., Picea spp.) sowie Mischblütenhonige wurden mit klassischen physikalischen, chemischen und pollenanalytischen Methoden untersucht und charakterisiert.

Um Alternativen für die zeitaufwendigen und mit Unsicherheiten behafteten klassischen Methoden zu finden, wurden neue analytische Ansätze gesucht. Es wurden Infrarot- und Front-Face Fluoreszenzspektroskopische Verfahren entwickelt und geprüft. Dabei erwiesen sich Infrarotspektren, die mit einer Messzelle in abgeschwächter Totalreflexion aufgenommen wurden und Fluoreszenz Anregungsspektren im Bereich zwischen 220 – 400 nm während die Emission bei 420 nm gemessen wurde, als besonders geeignet und zeigten die grössten Unterschiede zwischen den Sortenhonigen.

Bezüglich der Unterscheidung der verschiedenen Honigtypen erwiesen sich die Fluoreszenzspektroskopie und die Infrarotspektroskopie im mittleren Bereich in etwa ebenbürtig, während die Infrarotspektroskopie im nahen Bereich nur eine Unterscheidung von besonders charakteristischen Sortenhonigen und der Blüten- und Honigtauhonige zuliess. Die Auswertung der Spektren erfolgte mittels Hauptkomponentenanalyse und linearer Diskriminanzanalyse. Dabei zeigte sich, dass die verschiedenen Sortenhonige einfach voneinander zu unterscheiden sind, während es bedeutend schwieriger ist, die Mischblütenhonige von den Sortenhonigen zu unterscheiden. Mit mehreren aufeinanderfolgenden Klassifizierungsfunktionen konnte erstmals ein Verfahren beschrieben werden, das eine zuverlässige Unterscheidung zwischen einzelnen Sorten- und Mischblütenhonigen erlaubt. Die Fehlerraten (falsche Zuordnung einer Honigprobe unbekannter Herkunft) betrugen für die 11 untersuchten Honigtypen rund 3 % wobei für Alpenrosenhonig ein Wert von 10 % verzeichnet wurde.

Neben der Bestimmung der botanischen Herkunft erlaubt insbesondere die Infrarotspektroskopie im mittleren Bereich die Erstellung von quantitativen Kalibrationen zur zuverlässigen Bestimmung des Wasser-, Glukose-, Fruktose-, Saccharose- und Melezitosegehalts sowie der Fruktose/Glukose und Glukose/Wasser Verhältnisse sowie der elektrischen Leitfähigkeit, des pH-Werts und der freien Säure im Honig.

Zudem zeigten multivariate Auswertungen der Infrarot- und Fluoreszenzspektren im Hinblick auf eine Bestimmung der geografischen Herkunft der Honigproben sehr vielversprechende Resultate. Diese Fragestellung muss aber anhand eines geeigneteren Probensets weiter untersucht werden.

Die vorliegenden Untersuchungen zeigen, dass sich spektroskopische Verfahren für eine schnelle und zuverlässige Bestimmung von Sortenhonigen eignen und als Ersatz der klassischen Methoden in Betracht gezogen werden können.

Abstract

The botanical origin of the nectar has an outstanding influence on the chemical composition of honey. Honeys originating predominantly from a single plant species and exhibiting the corresponding physical, chemical and pollen analytical characteristics can be designated as unifloral honeys. They show considerable differences in their sensory properties as well and achieve remarkably higher prices than the common polyfloral honeys due to variable consumer preferences.

Over 650 acacia (*Robinia pseudoacacia*), alpine rose (*Rhododenron* spp.), heather (*Calluna vulgaris*), chestnut (*Castanea sativa*), lime (*Tilia* spp.), dandelion (*Taraxacum* s.l.), rape (*Brassica* spp.), Metcalfa honeydew (*Metcalfa pruinosa*), oak honeydew (*Quercus* spp.) and fir honeydew (*Abies* spp., *Picea* spp.) as well as polyfloral honeys were analysed and characterised with classical physical, chemical and pollen analytical methods.

In order to find alternatives to the time consuming and uncertain classical methods new analytical approaches were looked for. Infrared and front-face fluorescence spectroscopic methods were developed and evaluated. Mid-infrared spectra recorded using an attenuated total reflectance accessory and fluorescence excitation spectra registered between 220 – 400 nm with the emission measured at 420 nm showed the most characteristic differences between the unifloral honeys.

Fluorescence and mid-infrared spectroscopy proved to have an equal potential for the determination of the differnt honey types while near-infrared spectroscopy allowed only a classification of some characteristic unifloral honeys and blossom and honeydew honeys. Data evaluation with regard to a discrimination of the various honey types was performed by using principal component analysis and linear discriminant analysis. It was clearly demonstrated that the unifloral honeys can easily be distinguished from each other while it is much more difficult to differentiate between unifloral and polyfloral honeys. The approach using several subsequent classification functions allowed a reliable determination of both polyfloral and unifloral honeys. The error probabilities (misclassification of a sample of unknown botanical origin) for the eleven honey types studied were generally as low as 3 % with a maximum of 10 % found for alpine rose honey.

In addition to the determination of the botanical origin especially mid-infrared spectroscopy allowed a quantitative determination of water, glucose, fructose, sucrose and melezitose contents as well as fructose/glucose ratio, glucose/water ratio, electrical conductivity, pH-value and free acidity with a satisfying accuracy.

Chemometric evaluaton of the mid-infrared and fluorescence spectra in respect to a determination of the geographical origin of honey showed very promising results as well. However these findings have to be studied in more detail on a more appropriate set of samples.

The present study shows that spectroscopic techniques represent a valuable alternative to the classical methods for a rapid and reliable authentication of the botanical origin of honey.

Introduction

Currently the botanical origin of honey is determined by experts evaluating results from several analytical methods, in particular pollen analysis, electrical conductivity and sugar composition. Although the composition of unifloral honeys has been described in various studies, internationally accepted criteria and the measurands to be considered for their authentication have not been defined yet.

Pollen analysis has been considered to be the most important technique to classify different honey types. However changes in legislation have recently allowed the removal of pollen by filtration. The altered pollen content does no more allow reliable conclusions to be drawn on the botanical and geographical origin of honey therefore facilitating honey fraud. Moreover various factors influencing the presence of pollen in honey lead to uncertainties in the interpretation of pollen analytical results.

As several analytical techniques have to be used a reliable authentication is consequently very time consuming and costly. In addition very specialised expertise is required. This situation calls for alternative analytical methods for the determination of the floral origin of honey.

The aim of the present thesis is to characterise Swiss unifloral honeys by using classical physical, chemical and pollen analytical methods and to develop alternative techniques for a reliable and reproducible classification of unifloral and polyfloral honeys within a short time.

Among the numerous methods proposed, such as organic acid, amino acid, phenolic acid, volatile and mineral composition, spectroscopic techniques (i. e. near-infrared, mid-infrared and fluorescence spectroscopy) were selected and studied in more detail.

In order to share the gained knowledge as quickly as possible the chapters 4, 5, 6, 7 and 8 were published in peer reviewed journals. Chapter 3 has been accepted for publication and chapter 2 has been submitted for publication in Apidology. The thesis was therefore carried out as „paper-thesis" consisting of independent publications with the consequence that overlapping especially in the introduction and the material and method section were unavoidable. The chapters representing single publications are preceded by a comprehensive literature review and followed by a general concluding discussion and outlook.

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 metods 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 contamintion 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 conducti vity 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 (dextrorotatory, 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 satisfacory. 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 simultanously mentally considererd. 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 dected 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 (*Banksia 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 Portugese 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-

nilate 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 challening 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 differencies observed between geographical regions are rather due to variations in vegetation type i.e. the botanical origin.

Later on differencies 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 transflectance 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 3627-2971cm^{-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 14g/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 considerd 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-

ficult 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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CHAPTER 2

Authentication of the Botanical Origin of Honey Using Profiles of Classical Measurands and Discriminant Analysis*

ABSTRACT

The potential of physical and chemical measurands for the determination of the botanical origin of honey by using both, the classical profiling approach as well as chemometrics was evaluated for the authentication of ten unifloral (acacia, rhododendron, chestnut, dandelion, heather, lime, rape, fir honeydew, metcalfa honeydew,) and polyfloral honey types (in total n = 693 samples). The classical approach using a profile for the determination of the botanical origin of honey revealed that the physical and chemical measurands alone do not allow a reliable determination. Pollen analysis is therefore essential for discrimination between unifloral and polyfloral honeys. Chemometric evaluation of the physical and chemical data by linear discriminant analysis allowed however a reliable authentication with neither specialized expertise nor pollen or sensory analysis. The error rates calculated by Bayes' theorem ranged from 1.1% (rape and lime honeys) up to 9.9 % (acacia honey).

2.1 INTRODUCTION

2.1.1 DEFINITION OF UNIFLORAL AND POLYFLORAL HONEYS

The overwhelming majority of the honeys on the market contain significant nectar or honeydew contributions from several plant species and are therefore called polyfloral or multifloral honeys. Normally they are just labelled with the word "honey". The term unifloral honey is used to describe a honey in which the major part of nectar or honeydew is derived from a single plant species. Honey composition, flavour and colour varies considerably depending on the botanical source it originates from (*1*).

At present there is an increasing commercial interest to produce unifloral honeys. Indeed many consumers prefer unifloral to polyfloral honeys and appreciate the possibilty to choose between different honey types. The production of unifloral honeys offers the beekeepers also an opportunity to compete with low priced polyfloral honeys imported from abroad. Moreover the increasing interest in the therapeutic or technological uses of certain honey varieties may also contribute to the demand of a reliable determination of their botanical origin.

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According to the Codex Alimentarius Standard for Honey (*2*) and the EU Council Directive (*3*) relating to honey, the use of a botanical designation of honey is allowed if it originates predominately from the indicated floral source. Physical, chemical and pollen analytical characteristics of the most important European unifloral honeys have been described in various papers (*1, 4-7*).

2.1.2 TRADITIONAL CLASSIFICATION OF HONEYS BASED ON A PROFILE OF MEASURANDS

Traditionally the botanical origin of honey is determined by experts evaluating several physical, chemical, pollen analytical as well as sensory characteristics (*4, 8, 9*). The analytical results of a honey sample have relatively 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 together for this purpose.

This profiling approach used for decades, has recently been described in more detail by Persano Oddo and Piro (*1*). 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 generally 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. In other words the main problem in the authentication of unifloral honeys is to discriminate between unifloral and polyfloral honeys, rather than between different unifloral honeys.

2.1.3 DISCRIMINATION BETWEEN HONEY TYPES USING CHEMOMETRICS

Several attempts have already been made to predict the botanical origin of honey using their physical and chemical properties in combination with multivariate analysis. The first paper on classification of floral and honeydew honeys using discriminant functions considering pH-value, ash and monosaccharide content was published over forty years ago (*10*). Later electrical conductivity, monosaccharide content as well as glutamic acid concentration were found to be the most useful measurands for the discrimination between floral and honeydew honeys (*11, 12*). High fructose and glucose concentrations and low values in lactone and free acidity, electrical

conductivity, polyphenol content and net 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 net absorbance characterised honeydew honeys (*13*).

Numerous studies have treated the subject of the chemometric classification of unifloral honeys. Linear discriminant analysis on sugar composition data of rosemary (*Rosmarinus* sp.), orange (*Citrus* sp.), lavender (*Lavandula* sp.), sunflower (*Helianthus annuus*), eucalyptus (Eucalyptus sp.), heather (*Calluna vulgaris*) and honeydew honeys allowed only a discrimination between floral and honeydew honeys (*14*). When the same honey types were studied using water content, electrical conductivity, pH-value, colour (x, y, L chromatic coordinates) and sugar composition, jackknife classification rates higher than 90 % were found for all unifloral honeys. The most important characteristics were electrical conductivity followed by colour and fructose content. Classification functions were presented using water content, electrical conductivity, fructose, sucrose, and colour (*15*).

In a recents study 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) were reported. The average correct classification rate was as high as 89.6 % and all honey types except thistle 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 (*16*).

In a recent study on a large sample set fir (*Abies* spp.), cinder heather (*Erica carnea*), chestnut (*Castanea sativa*), lavender, acacia (*Robinia pseudoacacia*) rape (*Brassica* spp.) and sunflower honeys were analyzed. Principal component analysis (PCA) showed that samples of fir, chestnut, lavender and acacia honeys formed well separated groups in the plot of the first two PC's while samples of rape, cinder heather and sunflower honeys clustered together. Stepwise discriminant analysis was used to select the most important measurands among water, hydroxymethylfurfural (HMF), fructose, glucose, sucrose, erlose, raffinose and melezitose content 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 content (*17*).

The above mentioned approaches using physical and chemical measurands and chemometrics allow clear discrimination between the main honey types or even between several types of unifloral honeys, but none of them accounts for the polyfloral honeys that represent the most important majority (about 80 %) of the honeys produced. As already noted the main problem in the authentication of unifloral honeys is to discriminate between polyfloral and unifloral honeys, rather than between different unifloral honeys. This means that the above-mentioned methods are inadequate for analytical practice. This also explains why until now none of these methods is commonly applied to determine the botanical origin of honey and pollen analysis was referred to be the fundamental tool for authentication of the botanical origin of honey (*15*).

The only paper on chemometric evaluation of physical and chemical measurands considering unifloral and polyfloral honeys was pubished by Krause and Zalewski using PCA. Electrical conductivity, proline, free acidity and pH-value were found to be most important measurands for classifying honeys according to their botanical origin. The authors were able to distinguish between rape, acacia and honeydew honeys but failed to differentiate between polyfloral, lime (*Tilia* spp.) and heather honeys (*18*). Enzyme activities and HMF content are depending on honey processing and storage conditions and are therefore not useful for the determination of the botanical origin (*17, 18*).

As several analytical methods have to be simultaneously used for a reliable authentication of the botanical origin, it is consequently very time consuming and costly. In addition currently very specialised expertise is needed for the interpretation of the pollen analytical results and the physical and chemical measurands determined. Thus, there is a need for new analytical tools that allow a rapid and reproducible authentication of the botanical origin of honey (*19, 20*).

In this context the aims of the current work were to evaluate the potential of two different aproaches for authentication of unifloral and polyfloral honeys. These were on the one hand measurand profiles considering classical physical, chemical and pollen analytical characteristics and on the other hand chemometric evaluation of the physical and chemical measurands in order to verify the most important characteristics and to develop a mathematical procedure for the determination of the botanical origin of honey.

2.2. MATERIALS AND METHODS

2.2.1 SAMPLING

A total of 646 honey samples produced between 1998 and 2004 were collected and stored at 4°C until analysis. They originated predominantly from Switzerland (CH) but samples from Germany (D), Italy (I), France (F) and Denmark (DK) were also considered.

2.2.2 DETERMINATION OF PHYSICAL AND CHEMICAL MEASURANDS, POLLEN ANALYSIS

To classify these honey samples, the following measurands were determined according to the harmonized methods of the European Honey Commission (*21*): electrical conductivity, sugar composition, fructose/glucose ratio, pH-value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (*22, 23*).

2.2.3 BOTANICAL CLASSIFICATION BY REFERENCE METHODS

The honey samples were assigned to one of the following honey types according to their fructose/glucose ratio, melezitose content, electrical conductivity as well as pollen analytical results: acacia (*Robinia pseudoacacia*) (CH, n = 26; D, n = 7; F, n= 3), rhododendron (*Rhododendron* spp.) (CH, n = 24; I, n = 5), sweet chestnut (*Castanea sativa*) (CH, n = 52; I, n = 5; F, n = 3), rape (*Brassica napus* var. *oleifera*) (CH, n = 36), fir honeydew from (*Picea* spp. and *Abies* spp.) (CH, n = 110; D, n = 22), Metcalfa honeydew from *Metcalfa pruinosa* (I, n = 14), heather (*Calluna vulgaris*) (D, n = 19; DK, n = 3), lime (*Tilia* spp.) (CH, n = 22; D, n=12; I, n = 5), dandelion (*Taraxacum* s.l.) (CH, n = 22; D, n = 7; I, n = 2) and polyfloral honeys (CH, n = 294). The ranges of the physical

and chemical measurands mostly corresponded to the ranges presented by Persano and Piro (Persano Oddo and Piro, 2004). In case of uncertain classification based on physical, chemical and pollen analytical criteria the decision was made by sensory evaluation by experts.

2.2.4 CLASSIFICATION USING DIFFERENT PROFILES

Three types of profiles with three different sets of measurands were tested and compared for classification of unifloral and polyfloral honeys. The measurands considered in the Profiles I and II with the corresponding ranges defined for the different unifloral honeys are presented in **Table 1**. With profile I a classification of the honey types was attempted by using only physical and chemical characterstics. As this was known to be very difficult especially regarding the discrimination between unifloral and polyfloral honey types as many as possible measurands were included in the profile. Commonly used physical, chemical and pollen analytical measurands were incorporated in profile II. In profile III the ranges of the measurands presented by Persano Oddo and Piro (1) were used as far as they were available: i.e. fructose, glucose and sucrose content, fructose/glucose and glucose/water ratio, pH-value, free acidity and electrical conductivity. With respect to pollen analytical results only the minimum percentage of the specific pollen form of each unifloral honey type was considered.

The classification was achieved by comparing the values of the honey samples with each of the nine profiles of the unifloral honey types considered. They were assigned to the corresponding honey type if all values were within the ranges defined in the profile. Samples that did not fit into any of the profiles were regarded as polyfloral (principle of exclusion).

2.2.5 DATA PROCESSING AND CHEMOMETRICS

The following 17 measurands were originally included in data evaluation: fructose, glucose, total monosaccharides, sucrose, maltose, trehalose, isomaltose, erlose, melezitose, maltotriose, raffinose and water content, electrical conductivity, free acidity, pH-value, fructose/glucose and glucose/water ratio. Because of missing values the number of samples had to be reduced to a total of 646 considered in the chemomeric data evaluation. The values of each measurand were standardised (by subtracting the mean and subsequent division by the standard deviation). The equations for the standardisation of the variables are given in the Appendix A. This information is important for using the classification functions. The variables designated with a subscript uppercase “S” indicate standardised variables.

In order to select the most important variables for the classification of the unifloral honeys linear discriminant analysis (LDA) was applied. Backward elimination on the 17 initial variables was based on the partial F-values in the discriminant models. Forward selection on the same variables was used to confirm the results. The models were then optimised for maximum correct classification in jackknife classification. The validation was carried out with about one third of the samples, selected randomly, and not present in the group of samples used to build the model (SYSTAT® Version 11, Systat Software Inc., Richmond, USA).

Table 1. Data ranges of different unifloral honeys used in the profiles I and II.

		Considerd in		Acacia (n = 36)		Rhododen-drdon (n = 29)		Chestnut (n = 60)		Dandelion (n = 31)		Heather (n = 22)		Lime (n = 39)		Rape (n = 36)		Fir honeydew (n = 132)		Metcalfa honeydew (n = 14)	
	Unit	Profile I	Profile II	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
Electrical conductivity	$mScm^{-1}$	x	x	0.10	0.27	0.15	0.45	0.62	1.70	0.37	0.64	0.65	1.07	0.38	0.95	0.14	0.28	0.70	1.33	1.48	2.57
pH-Value		x		3.7	4.1	3.7	4.6	4.4	6.4	4.2	5.0	3.9	5.1	4.1	6.2	3.9	4.4	4.1	5.4	4.6	5.8
Free acidity	meq/kg	x		6	23	5	25	4	30	7	13	14	42	4	21	8	17	17	46	21	41
Fructose	g/100g	x	x	22.4	46.9	34.2	41.0	36.6	44.6	32.3	39.5	34.9	41.0	32.9	41.6	34.6	39.5	20.9	39.4	26.8	33.1
Glucose	g/100g	x	x	13.8	29.4	27.7	33.6	21.4	30.0	32.0	43.2	26.2	31.5	26.2	42.9	31.5	40.0	14.8	31.5	20.3	26.8
Sucrose	g/100g	x		0.0	8.8	0.0	2.6	0.0	3.6	0.0	0.3	0.0	0.6	0.0	4.5	0.0	2.0	0.0	2.7	0.0	0.1
Maltose	g/100g	x		0.0	3.6	0.0	8.6	0.0	5.6	0.0	5.7	0.0	1.9	0.0	5.7	0.0	2.2	0.0	4.9	4.7	7.7
Isomaltose	g/100g	x		0.0	1.2	0.2	2.5	0.0	2.4	0.0	1.7	0.0	1.3	0.1	2.2	0.0	1.5	0.0	3.4	0.9	4.4
Erlose	g/100g	x		0.4	2.8	0.0	3.7	0.0	4.3	0.0	0.8	0.0	0.2	0.0	0.9	0.0	1.4	0.0	4.5	0.1	1.2
Melezitose	g/100g	x		0.0	0.5	0.0	0.8	0.0	3.8	0.0	0.7	0.0	1.4	0.0	1.1	0.0	0.4	0.0	11.7	0.0	0.7
Maltotriose	g/100g	x		0.0	0.6	0.0	0.4	0.0	0.3	0.0	0.3	0.0	0.0	0.0	1.0	0.0	1.1	0.0	2.8	0.0	1.5
Raffinose	g/100g	x		0.0	0.6	0.0	0.5	0.0	0.6	0.0	0.3	0.0	0.0	0.0	0.3	0.0	0.2	0.0	2.8	0.0	0.4
Fructose/Glucose ratio		x	x	1.28	1.88	1.10	1.39	1.36	1.86	0.85	1.15	1.22	1.57	0.97	1.41	0.95	1.24	1.07	1.53	1.10	1.45
Robinia pollen	%		x	10.9	64.3	0.0	6.4	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0		
Rhododenron pollen	%		x	0.0	0.0	6.0	58.2	0.0	2.8	0.0	0.0	0.0	0.0	0.0	19.0	0.0	0.0	0.0	5.1		
Calluna pollen	%		x	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	8.0	81.0	0.0	0.0	0.0	0.0	0.0	0.0		
Castanea pollen	%		x	0.0	56.5	0.0	83.5	92.0	100.0	0.0	1.1	0.0	0.0	0.0	93.2	0.0	5.2	0.0	84.0		
Tilia pollen	%		x	0.0	0.6	0.0	34.0	0.0	3.6	0.0	0.0	0.0	0.0	2.0	80.0	0.0	0.0	0.0	11.5		
Taraxacum pollen	%		x	0.0	1.9	0.0	5.2	0.0	0.2	1.6	58.1	0.0	2.7	0.0	1.7	0.0	2.0	0.0	11.2		
Brassica pollen	%		x	0.0	9.2	0.0	8.3	0.0	0.0	0.0	41.5	0.0	0.0	0.0	35.1	68.1	97.8	0.0	73.9		

2.3. RESULTS

2.3.1 CLASSIFICATION BY PROFILE

In the profile considering only physical and chemical measurands it is obvious that most of unifloral honey samples are correctly assigned since almost the whole range of the values found was used (**Table 1**; **Table 2**, Profile I). Interestingly misclassifications between different unifloral honeys occurred rarely, clearly indicating that the physical and chemical properties of the unifloral honeys are distinctly different. Since nearly the whole data range found was considered, in principle all unifloral honey samples should therefore be correctly classified. Nevertheless, some unifloral honeys were assigned to polyfloral honeys due to reasons arising from truncation in the ranges used (limits set at 2.5 % and 97.5 % percentiles of the values observed). The correct classification rate for the polyfloral honeys was only 49 %. This means that approximately half of the polyfloral honey samples were misclassified to various unifloral honey types. Samples were especially assigned to rhododendron, lime and fir honeydew honeys. These unifloral honey types do express highly variable chemical compositions, thus showing a relatively broad data range for all measurands considered and therefore allow many samples to meet the requirements of the profile. Because of the high rate of misclassification of the polyfloral honey samples the profile using only physical and chemical measurands is inadequate for a reliable determination of the botanical origin of honey.

In Profile II that was based on the relative frequencies of the specific pollen and a reduced number of physical and chemical measurands, high correct classification rates were found again for the unifloral honeys (**Table 2**, Profile II). The classification rate of the polyfloral honeys was significantly improved up to 70 %, showing that pollen analysis plays a key role in the discrimination between unifloral and polyfloral honeys. Therefore pollen analytical, physical and chemical measurands should be included in the same profile used for the determination of the botanical origin.

The profile established with data ranges recently published (*1*) showed notably lower classification rates for the unifloral honey types studied than obtained with the profiles I and II (**Table 2**, Profile III). For example none of the heather honey samples were considered to be unifloral. Interestingly the highest classification rate was found for the polyfloral honeys.

2.3.2 CHEMOMETRIC EVALUATION

Multivariate explorative data analysis revealed that electrical conductivity, fructose, raffinose and glucose concentration, together with free acidity, contributed most to the classification of the different unifloral honeys using a single linear discriminant model. Total monosaccharide content was found to be redundant for classification when the individual glucose and fructose concentrations were considered. Most of the unifloral honeys revealed rates of correct classification of higher than 80%, by using the above-mentioned variables. The rates were similar in jackknife classification and validation thus demonstrating that the models used were robust (**Table 3**). Heather honey samples were partly classified as chestnut or polyfloral honeys and exhibited the second lowest classification rate (80 %). Fir honeydew honeys were mostly assigned to the correct group except a few samples that were misclassified as heather honeys. Among the unifloral honeys lime honeys showed with 71 % the lowest jackknifed classification rate.

Table 2. Classification rates of the different profiles studied

	Classification rates (%)												
	Profile I[1]											Profile II[2]	Profile III[3]
	Acacia	Rhodo-dendron	Chestnut	Dandelion	Heather	Lime	Rape	Fir honedew	Metcalfa honedew	Polyfloral	Correct class.	Correct class.	Correct class.
Acacia (n = 36)	100	0	0	0	0	0	0	0	0	0	100	92	83
Rhododendron (n = 29)	0	93	0	0	0	0	0	0	0	7	93	79	48
Chestnut (n = 60)	0	0	98	0	0	0	0	2	0	0	98	95	67
Dandelion (n = 31)	0	0	0	97	0	0	0	0	0	3	97	87	61
Heather (n = 22)	0	0	5	0	91	0	0	0	0	5	91	95	0
Lime (n = 39)	0	0	0	0	0	90	0	3	0	8	90	90	74
Rape (n = 36)	0	3	0	0	0	0	92	0	0	6	92	89	50
Fir honeydew (n = 132)	0	0	0	0	0	0	0	99	0	2	99	95	69
Metcalfa honeydew (n = 14)	0	0	0	0	0	0	0	0	93	7	93	93	64
Polyfloral (n = 294)	1	15	7	1	0	16	1	11	0	49	49	70	86

[1]Considering fructose, glucose, sucrose, maltose, isomaltose, erlose, melezitose, maltotriose, raffinose, fructose/glucose ratio, pH-value, free acidity and electrical conductivity.

[2]Considering fructose, glucose, fructose/glucose ratio, electrical conductivity. as well as Brassica-, Castanea-, Calluna-, Rhododenron-, Robinia-, Tilia- and Taraxacum pollen.

[3]Considering fructose, glucose, sucrose, fructose/glucose and glucose/water ratio, pH-value, free acidity and electrical conductivity and minimum percentage of the specific pollen.

Eighteen percent of the lime honey samples were classified as polyfloral and 11 % as dandelion honeys. Jackknife classification and validation revealed that polyfloral honeys were very often classified into the groups of the unifloral honeys while the latter were rarely misclassified into the polyfloral honeys (**Table 3**).

The high rate of misclassified polyfloral honeys made it impossible to use a single discriminant model for the authentication of the botanical origin of honey and lead to the idea to develop a two-step procedure. In the first step the sample was attributed to one of the ten honey types considered, using the classification functions of the overall discriminant model with as many groups as honey varieties. The sample was assigned to the honey type showing the highest value in the classification functions taken into account. In the second step this classification was verified by using one or several two-group models consisting of a group formed by samples of a given unifloral honey and a group called "non-unifloral" consisting of all the other samples. Each two-group model was separately built using LDA backward elimination and forward selection. They were optimised for maximum correct classification rate together with a minimum number of necessary variables. For the verification of the classification by the first model at least the two-group model of the corresponding honey type was used. In addition one to four two-group models (indicated by boldface numbers in **Table 3**) were used when a misclassification rate of higher than 3% was calculated in jackknifed classification or validation tables of the overall model. The probabilities for misclassification were calculated by applying Bayes' theorem on the conditional probabilities of disjoint events. The error probabilities cannot be directly taken from **Table 3**; they only quantify the conditional probabilities of correct classification given the corresponding honey type. By Bayes' theorem the posterior probabilities of finding the correct honey type given a distinct classification by the discriminant model was calculated, and the error rate being the complement to 1.

The classification rates for the unifloral honeys in the two-group models were generally > 90 %, except for lime honeys (**Table 4**). They showed with 79 % in jackknife classification respectively 56 % in validation the lowest rates. In general the high rates of correct classification for both, the unifloral and non-unifloral groups considered by the two-group models indicate that the botanical origin can be reliably determined by this procedure. Some overlapping is to be expected regarding dandelion, rape and lime honeys as about 15 % of the samples not belonging to these honey types are erroneously classified to these groups.

If a sample is assigned to the same honey type by the overall- and by the two-group model it is very likely that it belongs to this type of honey. If the classifications of the two models do not agree the sample has to be considered to be of polyfloral origin. When the sample is assigned to the same honey type by both, the overall model and the corresponding two-group model and is moreover considered to belong to the non-unifloral groups in all the other two-group models tested, the honey sample belongs almost certainly to the honey type indicated by the overall model. The respective error rates of this two-step procedure (for misclassification of a sample of unknown botanical origin), were found for the ten honey types studied except for acacia and fir honeydew honeys to be ≤ 5 % (**Table 5)**.

Table 3. Jackknife classification and validation tables of the honey samples classified by the overall discriminant model. Bold numbers indicate misclassification rates of > 3 % (two-group models to be used).

	Jackknife classification (%)										
	Acacia	Rhodo-dendron	Chestnut	Dandelion	Heather	Lime	Rape	Fir honedew	Metcalfa honeydew	Polyfloral	Total
Acacia (n= 28)	100	0	0	0	0	0	0	0	0	0	100
Rhododendron (n= 29)	0	93	0	0	0	0	**3**	0	0	**3**	93
Chestnut (n= 56)	2	0	91	0	0	**5**	0	0	0	2	91
Dandelion (n= 31)	0	0	0	84	0	**10**	**7**	0	0	0	84
Heather (n= 15)	0	0	**7**	0	80	0	0	0	0	**13**	80
Lime (n= 28)	0	0	0	**11**	0	71	0	0	0	**18**	71
Rape (n= 36)	0	**6**	0	0	0	0	94	0	0	0	94
Fir honeydew (n= 126)	0	0	0	0	**14**	1	0	85	0	0	85
Metcalfa honeydew (n= 13)	0	0	0	0	0	0	0	0	100	0	100
Polyfloral (n= 284)	3	**8**	**5**	**8**	**19**	**10**	**11**	**3**	0	34	34

	Classification rate in validation (%)										
	Acacia	Rhodo-dendron	Chestnut	Dandelion	Heather	Lime	Rape	Fir ho-neydew	Metcalfa honeydew	Polyfloral	Total
Acacia (n= 9)	100	0	0	0	0	0	0	0	0	0	100
Rhododendron (n= 10)	0	90	0	0	0	0	**10**	0	0	**0**	90
Chestnut (n= 18)	0	0	94	0	0	**6**	0	0	0	0	94
Dandelion (n= 11)	0	0	0	91	0	**9**	0	0	0	0	91
Heather (n= 4)	0	0	**0**	0	75	0	0	0	0	**25**	75
Lime (n= 9)	0	0	0	0	0	78	0	0	0	**22**	78
Rape (n= 13)	0	**0**	0	**8**	0	0	92	0	0	0	92
Fir honeydew (n= 42)	0	0	0	0	**14**	0	0	83	0	2	83
Metcalfa honeydew (n= 4)	0	0	0	0	0	0	0	0	100	0	100
Polyfloral (n= 96)	2	**17**	**1**	**1**	**19**	**9**	**10**	**4**	0	37	37

2.4. DISCUSSION

2.4.1 CLASSIFICATION USING MEASURAND PROFILES

Profiles based on only physical and chemical measurands were shown to be inadequate for a reliable classification of the botanical origin of honey because of the high rate of misclassification of polyfloral honeys (**Table 2,** Profile I). However in combination with pollen analytical results the number of physical and chemical measurands can be considerably reduced as the results using profile II showed (**Table 2**, Profile II). Pollen analytical data can be included in profiles by just defining a range for the relative frequency of the specific pollen of a given unifloral honey type (*1*). However a more reliable profile would probably consider ranges for all the specific pollen of the unifloral honey types. Such a procedure would considerably simplify pollen analysis since only the relative frequencies of the characteristic pollen forms of the unifloral honey types would have to be considered. For a classification of the most important honey types in Europe it would be sufficient to identify and calculate the total number of pollen, the sum of the nectarless species and about 15 pollen forms characteristic for the honey types considered. By the use of a profile that includes physical, chemical and pollen analytical measurands with well-defined ranges the ambiguity resulting from the correction of over- and underrepresented pollen could be avoided and fully comprehensible classifications could be obtained. For the correct classification of most honeydew honeys the physical and chemical measurands are generally sufficient. Although honeydew honeys do not contain specific pollen, the relative frequencies of the specific pollen of the floral honeys should be considered for a reproducible procedure.

The variability in the relative frequency of the pollen forms found in this study is considerable and may have to be adjusted when more samples of unifloral honeys have been studied. Among the samples considered in the present study some pollen forms were not detected at all in some unifloral honey types. Therefore the maximum percentage is for some pollen types equal to zero. In these cases it can supposedly be raised to the minimum value of the specific pollen of the corresponding unifloral honey type.

The high misclassification rates observed using profile III may be explained with the unfavorable definition of the range (*1*). The ranges presented were calculated using the standard deviation. This procedure implies a normal distribution of the data in order to make sense. However the values of several measurands show a highly asymmetric distribution. The authors must have also been aware of this problem, since minimum or maximum values were presented if the 95 % confidence interval exceeded the former. If the range used in profiles is calculated from the mean value using the standard deviation for asymmetrically distributed data, the range may be delicately clipped on one end of the distribution. The ranges published are based on a huge number of samples certainly having a considerable variability. Therefore the ranges of the individual measurands seem at first sight to be very liberal. But when the ranges are used in a profile considering as many as 9 different measurands it is very likely that a value of a sample lies outside the 95 % confidence interval. This is especially true in the case of asymmetrically distributed data. When the number of measurands included in the profile were reduced to 5 (i.e. electrical conductivity, fructose and glucose content, fructose/glucose ratio and specific pollen) the rate of correct classification rose considerably for most honey types except for lime and

polyfloral honeys (data not shown). The total monosaccharide content, glucose/water ratio and diastase activity are probably not very useful to determine the botanical origin of honey. It is clear that a data range used for classification should not include extreme values such as outliers. In an asymmetrically distributed dataset it would be better to define the range for example by the 2.5 and 97.5 percentiles. To classify honey samples according to their botanical origin using a profile it is not necessary to standardise the values as proposed by Persano Oddo and Piro (*1*).

2.4.2 CLASSIFICATION USING DISCRIMINANT FUNCTIONS

Our results indicating that the most importat measurands for a classification of unifloral and polyfloral honeys are electrical conductivity, fructose, raffinose and glucose concentration, together with free acidity are in agreement with those found in the literature (14, 17).

Table 4. Jackknife classification and validation table of the honey samples classified by the two-group discriminant models.

	Jackknife classification				Validation	
	Unifloral		Non-Unifloral		Unifloral	
	n	Correct (%)	n	Correct (%)	n	Correct (%)
Acacia	28	100	618	98	9	100
Rhododendron	29	90	616	92	10	90
Chestnut	56	95	590	97	18	100
Dandelion	31	90	615	85	11	100
Heather	15	93	631	94	4	88
Lime	28	79	582	84	9	56
Rape	36	92	610	85	13	100
Fir honeydew	126	92	517	97	42	88
Metcalfa honeydew	13	92	630	98	4	100
Polyfloral	282	79	329	65	96	82

The classification functions of the overall and two-group classification models are given in the Appendix B. The abbreviation “DG” designates the classification function values of the classification functions belonging to the general model. The honey samples were classified to the honey type whose corresponding classification function gave the highest classification function value. The quantity of the absolute values of classification function coefficients indicate which variables are particularly important for the discrimination between the honey types. Thus high fructose content, low electrical conductivity and glucose content are characteristic for acacia honeys and therefore the most important variables for the discrimination of acacia honeys as already shown by Piro et al. (*16*).

Interestingly electrical conductivity seems to be an important measurand for classification of metcalfa honeydew and chestnut honey but is not very relevant for the characterisation of fir honeys, while high raffinose content and high free acidity were found to be very characteristic for the latter. High glucose content was found to be important for the classification of rape and dandelion honeys

The classification functions of the two-group models are identified by the abbreviation "DT" in the classification function value. The difficulties in discrimination of lime honeys were indicated by the fact that eight variables were necessary in the model and none of the absolute values was particularly high. Maltose, isomaltose and erlose were found to be the most relevant measurands. The high rate of misclassification of lime honey samples to polyfloral and dandelion honeys may be explained by the variable chemical composition of this honey type as it often contains different amounts of honeydew and thus exhibits variable physical and chemical characteristics (**Table 3**). This makes it similar to polyfloral honey that may contain proportions of nectar and honeydew. In the two-group model, high glucose, low fructose concentration and a low fructose-glucose ratio were once again characteristic for rape honey. The most important variables for the classification of fir honeys were raffinose, melezitose and trehalose, which is in agreement with other studies (*14*, *17*). For the discrimination between acacia honey and all the other honey types the fructose/glucose ratio was found to be the most relevant variable. Chestnut honey was charaterised by a high fructose content, high electrical conductivity and pH-value. Glucose concentration was found to be the most important factor for classification between dandelion and other honey types. Metcalfa honeydew honey was characterised by high maltose and maltotriose contents as well as high electrical conductivity. The former findings confirm the results of previuos studies (*16*). For heather honey the water content and free acidity were found to be the most discriminating variables. For identifying rhododendron honey pH-value, free acidity and erlose content were found to be the most important measurands. The classification of polyfloral and lime honeys needs a high number of measurands and none of them seems to play a very decisive role. This is also reflected by the low classification rates. The sub-optimal classification of polyfloral honeys is of less importance, as we are interested in the authentication of unifloral honeys.

Table 5. Error probabilities for the classification of unifloral and polyfloral honeys calculated by Bayes' theorem (two-step approach)

	Error probability	
Honey type	Jackknife	Validation
Acacia	0.099	0.060
Rhododendron	0.038	0.047
Chestnut	0.031	0.013
Dandelion	0.023	0.017
Heather	0.015	0.019
Lime	0.011	0.001
Rape	0.012	0.011
Fir honeydew	0.044	0.055
Metcalfa honeydew	0.017	0.017
Polyfloral	0.017	0.020

Data evaluation showed that electrical conductivity is not a very reliable criterion to discriminate between floral and honeydew honeys although this measurand is defined as important in the Codex Alimentarius Standard for Honey and the European Honey Directive. However, several exceptions are listed in the above mentioned standards thus indicating the limited value of this measurand for the discrimination

of honey types. Thus multivariate data evaluation of traditional physical and chemical measurands may also be helpful to establish new criteria for a more reliable description of the honey types and for the determination of their botanical origin.

The chemometic analysis of physical and chemical data demonstrated that the botanical origin of honey can be determined without considering pollen analytical results. Unfortunately this approach does not save very much time and costs as only pollen analysis can be abandoned. Indeed 14 physical and chemical measurands have still to be determined. Nevertheless pollen analysis is the technique which requires the most professional expertise and skill, the most time and cannot be automated. In case of doubt the traditional approach using physical, chemical and pollen analytical results and the expertise required for their interpretation will so far remain the reference method.

2.4.3 CONCLUSIONS

The classical approach using a defined profile would allow a reliable and reproducible determination of the botanical origin provided that an international agreement can be made on the measurands as well as the corresponding data ranges to be taken into account. Using such a procedure pollen analysis cannot be discarded and will in principle express the same inconsistencies. However, the difficulties in the interpretation of pollen analytical results may be overcome by including pollen analytical characteristics together with physical and chemical measurands into a distinct profile and appropriate definition of the data ranges. For straightforward classification the profiles can be programmed in a spreadsheet software. However the ranges presented in this study should be reconsidered by an even larger set of unifloral honeys as especially the pollen ranges may need to be adjusted.

Chemometric evaluation of the physical and chemical measurands revealed that a determination of the botanical origin of honey can be achieved with a mathematical procedure without considering pollen analytical results. The classification functions published in the Appendix can be used for this purpose without special expertise and statistical software.

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GENERAL SUMMARY

Currently the botanical origin of honey is determined by experts by a global interpretation of several physical, chemical, pollen analytical and sensorial properties. Unifloral honeys express distinctly different physical and chemical characteristics and can thus be easily distinguished from each other. However, the challenge in the authentication of unifloral honeys is to distinguish the minority of unifloral honeys form the overwhelming majority of polyfloral honeys.

The aim of the current work was to evaluate the potential of different profiles and to develop a chemometric approach for a comprehensible determination of the botanical origin of honey using various physical and chemical measurands.

Traditionally the botanical origin of honey is determined by comparing the values of physical and chemical measurands of a honey sample with profiles of unifloral honeys consisting of defined ranges of the measurands considered (principle of exclusion). A profile consisting of as many as 13 different physical and chemical criteria showed high correct classification rates for the unifloral honeys but half of the polyfloral honey samples were misclassified to various unifloral honey types (**Table 2, Profile I**). This clearly indicates that a profile using only physical and chemical measurands is inadequate for a reliable determination of the botanical origin of honey. When the number of physical and chemical criteria was reduced to four and ranges for the relative frequencies of specific pollen werde included, the correct classification rate of the polyfloral honeys rose considerably (**Table 2**, Profile II). This demonstrates that pollen analysis plays a key role in the discrimination between unifloral and polyfloral honeys.

Multivariate explorative data analysis revealed that electrical conductivity, fructose, raffinose and glucose concentration, together with free acidity, contributed most to the classification of the different unifloral honeys. Correct classification rates of higher than 80 % were found for most of the unifloral honeys when the abovementioned variables were used. Again polyfloral honeys were very often misclassified to different unifloral honey types (**Table 3**). These difficulties were resolved by a two-step procedure. The sample was first classified to a honey type by the general model considering all the honey types. This attribution was thereafter verified by at least one two-group model consisting of a group formed by samples of a given unifloral honey and a group called "non-unifloral" consisting of all the other samples (**Table 4**). If the sample is assigned to the same honey type by the overall- and by the two-group model it is very likely that it belongs to this honey type. Chemometric evaluation of the physical and chemical measurands revealed that a determination of the botanical origin of honey may be achieved without pollen analysis by using the standardisation and classification functions shown in the **Appendix**.

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2.6 APPENDIX

A = Free acidity (meq/kg)
C = Electrical conductivity ($mScm^{-1}$)
E = Erlose (g/100 g)
F = Fructose (g/100 g)
FG = Fructose / Glucose-Ratio
G = Glucose (g/100 g)
GW = Glucose / Water-Ratio
I = Isomaltose (g/100 g)
MA = Maltose (g/100 g)
ME = Melezitose (g/100 g)
MT = Maltotriose (g/100 g)
P = pH-Value
R = Raffinose (g/100 g
S = Sucrose (g/100 g)
T = Trehalose (g/100 g)
W = Water (g/100 g)

2.6.1 STANDARDISATION FUNCTIONS USED:

$$A_s = \frac{A\text{-}18.68}{8.849} \qquad C_s = \frac{C\text{-}0.6911}{0.391} \qquad E_s = \frac{E\text{-}0.640}{0.848}$$

$$F_s = \frac{F\text{-}37.23}{3.458} \qquad FG_s = \frac{FG\text{-}1.268}{0.184} \qquad G_s = \frac{G\text{-}29.78}{3.884}$$

$$GW_s = \frac{GW\text{-}1.873}{0.277} \qquad I_s = \frac{I\text{-}0.831}{0.680} \qquad MA_s = \frac{MA\text{-}1.76}{1.29}$$

$$ME_s = \frac{ME\text{-}0.932}{1.40} \qquad MT_s = \frac{MT\text{-}0.0798}{0.286} \qquad P_s = \frac{P\text{-}4.48}{0.472}$$

$$R_s = \frac{R\text{-}0.234}{0.455} \qquad S_s = \frac{S\text{-}0.373}{0.592} \qquad T_s = \frac{T\text{-}0.850}{0.909}$$

$$W_s = \frac{W\text{-}16.05}{1.242}$$

2.6.2 CLASSIFICATION FUNCTIONS USED

DG_x = Classification function belonging to the general model
DT_x = Classification function belonging to the two group-model

$$DG_{Acacia} = -15.80 - 6.553\ C_S - 1.131\ A_S + 6.279\ F_S - 5.267\ G_S - 0.707\ R_S$$

$$DG_{Rhododendron} = -5.691 - 4.367\ C_S - 1.524\ A_S + 0.4233\ F_S - 1.908\ G_S - 1.04\ R_S$$

$$DG_{Chestnut} = -12.29 + 6.990\ C_S - 3.461\ A_S + 3.781\ F_S - 1.803\ G_S - 2.45\ R_S$$

$$DG_{Dandelion} = -6.676 + 0.5799\ C_S - 1.709\ A_S - 1.607\ F_S + 3.984\ G_S - 0.854\ R_S$$

$$DG_{Heather} = -4.095 + 0.6708\ C_S + 2.243\ A_S + 0.7035\ F_S - 0.2699\ G_S - 1.19\ R_S$$

$$DG_{Lime} = -3.140 + 0.2620\ C_S - 1.602\ A_S - 0.2838\ F_S - 0.2120\ G_S - 1.57\ R_S$$

$$DG_{Rape} = -6.758 - 3.399\ C_S - 0.7763\ A_S - 0.8761\ F_S + 2.513\ G_S - 0.465\ R_S$$

$$DG_{Fir} = -8.960 + 1.191\ C_S + 1.929\ A_S - 3.053\ F_S + 0.02895\ G_S + 4.02\ R_S$$

$$DG_{Metcalfa} = -24.65 + 10.97\ C_S - 0.3305\ A_S - 6.366\ F_S + 0.1213\ G_S - 2.86\ R_S$$

$$DG_{Polyfloral} = -2.724 - 1.169\ C_S + 0.4742\ A_S + 0.4872\ F_S + 0.2819\ G_S - 0.612\ R_S$$

$$DT_{Acacia} = -7.565 - 3.351\ C_S + 4.337\ FG_S$$
$$DT_{Non\text{-}Acacia} = -0.7060 + 0.1392\ C_S - 0.1910\ FG_S$$

$DT_{Rhododendron} = -4.336 - 3.792\ P_S - 3.612\ A_S - 2.879\ G_S + 1.99\ E_S - 2.032\ FG_S - 1.17\ S_S + 1.898\ C_S$
$DT_{Non\text{-}Rhododendron} = -0.7009 + 0.2182\ P_S + 0.1911\ A_S + 0.1065\ G_S - 0.0885\ E_S + 0.08556\ FG_S + 0.0316\ S_S - 0.1581\ C_S$

$DT_{Chestnut} = -7.521 + 2.260\ C_S + 3.273\ P_S + \cdot 3.999\ F_S$
$DT_{Non\text{-}Chestnut} = -0.7617 - 0.2497\ C_S - 0.3074\ P_S - 0.4043\ F_S$

$DT_{Dandelion} = -2.912 - 1.20\ P_S + 2.587\ G_S$
$DT_{Non\text{-}Dandelion} = -0.6987 - 0.0675\ P_S - 0.1292\ G_S$

$DT_{Heather} = -5.837 + 3.091\ W_S + 2.694\ A_S - 1.40\ ME_S$
$DT_{Non\text{-}Heather} = -0.7028 - 0.1547\ W_S - 0.07763\ A_S + 0.0266\ ME_S$

$DT_{Lime} = -2.074 - 0.6357\ A_S + 0.766\ S_S + 0.989\ MA_S + 0.964\ I_S - 1.35\ E_S - 0.931\ R_S - 0.4547\ C_S - 0.2105\ FG_S$
$DT_{Non\text{-}Lime} = -0.7095 + 0.09442\ A_S - 0.0134\ S_S - 0.0105\ MA_S + 0.0443\ I_S - 0.0949\ E_S - 0.196\ R_S - 0.06527\ C_S + 0.04987\ FG_S$

$DT_{Rape} = -2.862 - 1.280\ C_S - 5.501\ F_S + 7.363\ G_S - 0.515\ E_S + 7.2005\ FG_S$
$DT_{Non\text{-}Rape} = -0.7005 + 0.04757\ C_S + 0.3211\ F_S - 0.4674\ G_S + 0.0211\ E_S - 0.4397\ FG_S$

$DT_{Fir} = -6.811 + 0.8622\ A_S - 1.286\ F_S + 1.17\ T_S + 1.75\ ME_S - 0.595\ MT_S + 3.63\ R_S$
$DT_{Non\text{-}Fir} = -1.052 - 0.1992\ A_S + 0.3252\ F_S - 0.296\ T_S - 0.415\ ME_S + 0.150\ MT_S - 0.872\ R_S$

$DT_{Metcalfa} = -16.17 + 2.611\ C_S + 4.14\ MA_S + 3.38\ MT_S$
$DT_{Non\text{-}Metcalfa} = -0.7000 - 0.05311\ C_S - 0.0916\ MA_S - 0.0678\ MT_S$

$DT_{Polyfloral} = -0.9999 - 0.2374\ C_S + 0.5129\ A_S + 1.220\ F_S - 1.647\ G_S - 0.332\ MA_S - 0.276\ T_S + 0.222\ I_S - 0.514\ R_S - 1.527\ FG_S + 0.5209\ GW_S$
$DT_{Non\text{-}Polyfloral} = -0.8230 + 0.08931\ C_S - 0.2962\ A_S - 1.310\ F_S + 1.549\ G_S + 0.271\ MA_S + 0.243\ T_S - 0.0994\ I_S - 0.0681\ R_S + 1.626\ FG_S - 0.3674\ GW_S$

CHAPTER 3

Quantitative Determination of Physical and Chemical Measurands in Honey by Near-Infrared Spectrometry*

ABSTRACT

Fourier transform near-infrared spectroscopy (FT-NIR) was evaluated to quantitatively determine 24 different measurands in honey. The reference values of 421 honey samples of different botanical origins were determined by classical physical and chemical methods. Partial least squares regression was used to develop the calibration models for the measurands studied. These calibrations were then validated using independent samples and proved satisfying accuracies for the determination of water (standard error of prediction: 0.3 g/100 g), glucose (1.3 g/100 g), fructose (1.6 g/100 g), sucrose (0.4 g/100 g), total monosaccharide content (2.6 g/100 g) as well as fructose/glucose ratio (0.09) and glucose/water ratio (0.12). The prediction accuracy for hydroxymethylfurfural, proline, pH-value, electrical conductivity, free acidity and the minor sugars maltose, turanose, nigerose, erlose, trehalose, isomaltose, kojibiose, melezitose, raffinose, gentiobiose, melibiose, maltotriose was poor and unreliable. The results demonstrate that near-infrared spectrometry is a valuable, rapid and non-destructive tool for the quantitative analysis of some measurands related to the main components in honey.

3.1. INTRODUCTION

For the general quality control of honey according to the current standards of the Codex Alimentarius (*1*) and of the European Union (*2*), several physical and chemical measurands have to be determined, which mostly include water content, enzyme activities of invertase and a-amylase, hydroxymethylfurfural (HMF), electrical conductivity, and sugar composition. At present a specific analytical method has to be applied for each measurand of interest. Moreover, the methods commonly used to determine the chemical composition and the physical properties of honey are laborious and expensive, thus limiting the number of honey samples analysed. To further improve honey quality control it is necessary to develop rapid, simple and accurate methods for the routine quality assessment of honey.

Due to the increased computing performance in the last decades, infrared spectrometry has become a well-established technique for quantitative analysis of food. Infrared spectroscopy has been applied to different fields of honey analysis. The determination of botanical or geographical origin, quality control and detection of adulteration has been discussed in several papers dealing with infrared spectros-

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Ruoff, K.; Luginbühl, W.; Bogdanov, S.; Bosset, J. O.; Estermann, B.; Ziolko, T.; Kheradman-dan, S.; Amadò, R. Quantitative Determination of Physical and Chemical Measurands in Honey by Near-Infrared Spectroscopy. *Eur. Food Res. Technol.* **2007**, published online.

copy of honey as it presents a rapid and non-destructive approach (*3-6*).

Near-infrared (NIR) spectrometry has been successfully applied both in transmission and transflectance mode to the quantitative analysis of honey. Transmittance spectroscopy was found to yield sharper peaks and better resolution than reflectance spectroscopy and the calibration performance was found to be 30 – 70 % better. The shortest optical path length tested (1 mm) was found to produce the least saturated spectra in the region between 1300 and 2500 nm thus yielding the lowest standard errors of crossvalidation (SECV) for all components studied (*7*).

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 (*7-13*). 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 (*10, 14*). However, 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 (*7, 10*). In a calibration limited to avocado honey it was though possible to quantify low concentrations of perseitol (polyol of D-mannoheptulose) (*15*).

Some authors claim that the isotope ratio between ^{12}C and ^{13}C, used for the detection of cane sugar adulteration, can be determined by NIR. Unfortunately the calibration was restricted to two types of honey and was not validated with adulterated samples (*8, 11*).

The aim of the present work was to investigate NIR spectroscopy in transflection mode as a rapid analytical tool for the simultaneous quantitative determination of 24 different measurands, used in quality control of honey, based on a large calibration set with as much natural variability as can be expected in practice.

3.2 MATERIAL AND METHODS

3.2.1 HONEY SAMPLES

A total of 421 honey samples were used to establish the global calibration. 352 honey samples from Switzerland collected from seven different crops between 1997 and 2004, including unifloral, (i.e. *Castanea* sp. (n = 27), *Robinia* sp. (n = 19), *Tilia* spp. (n = 13), *Brassica* spp. (n = 25), *Taraxacum* sl. (n = 20), *Rhododendron* spp. (n = 14)), alpine polyfloral (n = 44) and polyfloral (n = 138) as well as honeydew honeys (n = 52) were analysed. Unifloral honeys from *Robinia* sp. (n = 4), *Tilia* spp. (n = 7), *Taraxacum* s.l. (n = 4) and polyfloral honeys (n = 15) of German provenience were included.

In addition polyfloral honey samples from Argentina (n = 3), Chile (n = 5), China (n = 1), Cuba (n = 2), France (n = 6), Greece (n = 1), Hungary (n = 1), Italy (n = 4), Mexico (n = 13), Slovakia (n = 1), Slovenia (n = 1) and Uruguay (n = 1) were included as well. These samples were used to evaluate the calibrations established with samples from Switzerland and Germany.

In order to be able to measure the water content in bakers honey, the calibration range of water content higher than 19 g/100 g was extended up to 24.6 g/100 g by adding water to 17 different polyfloral honey samples. All samples were stored at 4 °C before analysis. They were liquefied in a heating cabinet at 50 °C for 9 h and then allowed to cool to room temperature before analysis.

3.2.2 REFERENCE METHODS

The reference methods used for the quantitative determination of water, electrical conductivity, HMF, pH-value, proline, free acidity as well as various sugars (i.e. fructose, glucose, sucrose, turanose, nigerose, maltose, kojibiose, trehalose, isomaltose, erlose, melezitose and raffinose) were carried out according to the Harmonised Methods of the European Honey Commission (*16*). Pollen analysis was carried out according to von der Ohe et al. (*17*) and the botanical origin of the honey samples was determined according to Persano-Oddo and Piro (*18*). The range of the reference values of the honey samples analysed is indicated in **Table 1**.

Table 1. Reference data ranges of the honey samples

Measurand	Unit	n*	Mean	Minimum	Maximum
Water	g/100 g	382	16.3	13.4	24.6
Fructose	g/100 g	394	37.8	26.4	49.8
Glucose	g/100 g	392	30.1	18.5	40.0
Sucrose	g/100 g	387	0.5	0.0	6.7
Turanose	g/100 g	391	2.2	0.0	5.5
Nigerose	g/100 g	386	2.1	0.0	5.3
Maltose	g/100 g	392	1.6	0.0	4.9
Kojibiose	g/100 g	242	1.0	0.0	2.1
Trehalose	g/100 g	387	0.6	0.0	4.6
Isomaltose	g/100 g	377	0.7	0.0	3.4
Erlose	g/100 g	392	0.6	0.0	4.1
Melezitose	g/100 g	392	0.6	0.0	5.3
Raffinose	g/100 g	397	0.2	0.0	2.2
Gentiobiose	g/100 g	385	0.1	0.0	1.1
Melibiose	g/100 g	392	0.0	0.0	1.3
Maltotriose	g/100 g	392	0.1	0.0	1.9
Monosaccharides sum	g/100 g	393	67.9	44.9	78.2
Fructose/Glucose ratio		391	1.28	0.90	2.11
Glucose/Water ratio		374	1.90	1.09	2.60
Free acidity	meq/kg	376	17	5	44
HMF	mg/kg	388	10	0	112
Proline	mg/kg	370	476	158	1190
Electrical conductivity	$mScm^{-1}$	378	0.61	0.10	1.70
pH-value		376	4.4	3.5	6.1

*n: number of samples in cross-validation

3.2.3 NEAR-INFRARED SPECTROMETRY

NIR spectra were recorded using a Büchi NIRLab N-200 spectrometer operated with the NIRLabWare 3.0 software and equipped with a MSC 100 measuring cell with a rotating sample holder (Büchi Labortechnik AG, Flawil, Switzerland) to level out effects of sample inhomogeneity. The measurements were performed at room temperature without temperature control. About 10 g of liquefied honey was poured into a clean glass petri dish and covered with an aluminium plate so defining a 0.75 mm layer of honey between the bottom of the Petri dish and its surface and acting as reflection material. 64 scans with a resolution of 8 cm^{-1} were recorded in transflection mode for each spectrum in the wavenumber range between 4000-10000 cm^{-1}, **Figure 1** shows a typical FT-NIR spectrum of honey. Three replicates of each sample were averaged to obtain a mean spectrum.

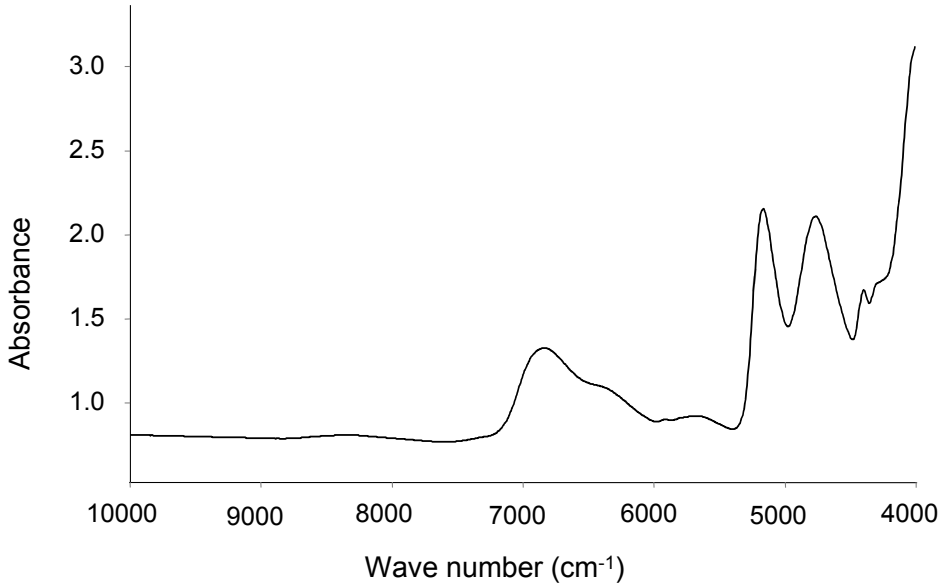

Figure 1. Typical FT-NIR spectrum of a honey sample.

3.2.4 DATA ANALYSIS

The primary interest was to study a 'global' calibration of all honey types considered and to evaluate its performance characteristics with respect to the application in practice where details on the samples are rarely known and are mostly not of interest in quantitative analysis of honey. For the chemometric evaluation, the GRAMS/32 AI Version 6.00 (Galactic Industries Corp., Salem NH, USA) software was used for quantitative analysis by partial least squares (PLS) regression: The calibration models were developed using the PLSplus/IQ add-on in the range between 4200-10000 cm^{-1} except for water, fructose, turanose, nigerose, kojibiose and isomaltose (see **Table 2**). Information on the interpretation of PLS loading vectors of various measurands can be found in the paper by Qiu et al. (*7*).

The optimised models were obtained by the "leave one out" cross validation technique based on the minimum predicted residual sum of squares (PRESS). The predictive quality of the models was evaluated by calculating the standard error of cross-validation (SECV) and the standard error of prediction (SEP) in the validation step with independent samples.

3.2.5 CALIBRATION AND VALIDATION

PLS cross-validations were performed to test different calibration models for the prediction of the various measurands. After elimination of spectral and concentration outliers (judged on the basis of Mahalanobis distance > 3) the models were set up with all averaged spectra. For validation (i.e. prediction of samples not included in the calibration) the spectra were split into two data sets. The criterion was to have a statistically sufficient number of validation samples while keeping as many as possible within the calibration set. The samples were arranged according to the numerical value of the measurand under consideration. About every tenth sample was selected for validation. This procedure produced random samples of 30-40 honey samples, which were representative for the distribution of the measurand's values and large enough for statistical validation of the respective PLS-model. The calibration was set up with the remaining spectra not included in the validation set. Validation SEP, coefficients of determination (R^2) between predicted and reference values and prediction bias were calculated (**Table 2**).

3.3 RESULTS AND DISCUSSION

3.3.1 REPEATABILITY LIMITS

The repeatability standard deviations (s_r) and limits (r_{IR}) of the NIR measurements were calculated based on eleven subsequent analyses of different aliquots of the same polyfloral honey sample (see **Table 2**; repeatability). For comparison the range of repeatability limits (r_{Ref}) from results of interlaboratory studies with the reference methods are listed as far as they are available (**Table 2**) (*16*).

3.3.2 PREDICTION OF THE MEASURANDS

The resulting standard errors from PLS cross-validation (SECV) and coefficients of determination (R^2) are given in **Table 2**. For the measurands studied, the coefficients of determination in calibration were between 0.009 (maltotriose) and 0.960 (water content) and in validation between 0.078 (HMF) and 0.970 (water content).

3.3.2.1 WATER CONTENT

The water content of honey is the most important measurand for the assessment of ripeness and shelf life, as a honey with a water content higher than 18 g/100 g may be spoiled by fermentation. The NIR method developed allows an accurate determination of this component. The repeatability limit r_{IR} of 0.108 g/100 g is equal to the lowest rRef of 0.110 g/100 g of the refractometric reference method (*16*). Moreover, the SEP and the R^2 in validation are with 0.3 g/100 g and 0.970, respectively, the best values of the calibrations performed (**Table 2**, **Figure 2**). The SEP is in the same range between 0.16 and 0.41 g/100 g as shown by a number of authors (*7-13*).

Table 2. Cross-validation and validation statistics; repeatability of the method.

			Calibration				Validation					[f]Repeatability		
Measurand	Unit	Spectral range	Samples in cross-validation	[a]Number of PLS-factors	[b]SECV	[c]R^2	Samples in calibration	Samples in validation	[d]SEP	R^2	[e]Prediction bias	[g]s_r	[h]r_{IR}	[i]r_{Ref}
Water	g/100 g	4200-7200	382	5	0.3	0.960	342	39	0.3	0.970	0.1	0.0	0.1	0.11 - 0.15
Fructose	g/100 g	4200-7200	394	6	1.6	0.759	354	40	1.6	0.810	0.2	0.1	0.4	0.8 - 1.0
Glucose	g/100 g	4200-10000	392	9	1.6	0.814	352	39	1.3	0.884	0.4	0.5	1.4	0.9 - 1.1
Sucrose	g/100 g	4200-10000	387	14	0.6	0.629	290	34	0.4	0.725	0.03	0.1	0.4	0.4
Turanose	g/100 g	4200-7200	391	7	0.7	0.134	350	39	0.6	0.153	0.1	0.0	0.1	0.3 - 0.4
Nigerose	g/100 g	4200-7200	386	13	1.1	0.227	321	38	1.1	0.149	0.3	0.1	0.4	
Maltose	g/100 g	4200-10000	392	10	0.9	0.197	313	37	0.9	0.233	0.2	0.1	0.3	0.5 - 0.6
Kojibiose	g/100 g	4200-7200	242	9	0.3	0.335	207	35	0.3	0.417	-0.03	0.1	0.3	
Trehalose	g/100 g	4200-10000	387	5	0.6	0.426	173	31	0.7	0.463	-0.02	0.0	0.1	
Isomaltose	g/100 g	4200-7200	377	11	0.5	0.313	283	39	0.5	0.420	0.1	0.1	0.1	
Erlose	g/100 g	4200-10000	392	12	0.5	0.462	257	34	0.5	0.664	0.05	0.1	0.2	
Melezitose	g/100 g	4200-10000	392	13	0.7	0.626	223	37	0.8	0.543	0.2	0.3	0.8	
Raffinose	g/100 g	4200-10000	394	11	0.3	0.554	106	31	0.4	0.465	0.04	0.0	0.1	
Gentiobiose	g/100 g	4200-10000	385	8	0.1	0.041	66	36						
Melibiose	g/100 g	4200-10000	392	6	0.1	0.029	79	30						
Maltotriose	g/100 g	4200-10000	392	1	0.2	0.009	58	32						
Monosaccharides sum	g/100 g	4200-10000	393	9	2.5	0.743	353	40	2.6	0.768	0.1	0.5	1.3	
Fructose/Glucose ratio		4200-10000	391	9	0.08	0.833	350	40	0.09	0.820	0.005	0.0	0.1	
Glucose/Water ratio		4200-10000	374	9	0.12	0.814	336	38	0.12	0.849	0.02	0.0	0.1	
Free acidity	meq/kg	4200-10000	376	17	5	0.636	338	38	4	0.737	-0.5	2	4	0.6 - 2.3
Hydroxymethylfurfural	mg/kg	4200-10000	388	15	12	0.435	323	37	13	0.078	0.9	2	5	0.9 - 2.2
Proline	mg/kg	4200-10000	370	18	125	0.588	331	38	125	0.650	7	40	111	6.6 - 24.4
Electrical conductivity	mS cm^{-1}	4200-10000	378	13	0.17	0.794	339	39	0.14	0.870	-0.003	0.098	0.274	0.002- 0.020
pH-value		4200-10000	376	14	0.3	0.622	338	38	0.3	0.657	-0.05	0.1	0.3	0.11 - 0.24

[a]PLS-factors: number of partial least squares factors used to build the model in crossvalidation and validation; [b]SECV: standard error of cross-validation, SECV $= \sqrt{\frac{\sum (\hat{y}_i - y_i)^2}{n}}$ where $\hat{y}_i$ predicted value of spectrum *i*, y_i reference value of spectrum *i* and *n* the number of spectra. [c]R^2: coefficient of determination; [d]SEP: standard error of prediction (equation see SECV; but with $\hat{y}_i$ representing a sample not used in calibration); [e]Prediction bias: mean difference between predicted and reference values; [f]Repeatability calculated from 10 predicted values obtained by applying the same calibrations as used for the estimation of the SECV; [g]s_r: repeatability standard deviation in FT-IR spectrometry; [h]r_{IR}: repeatability limit of FT-IR spectrometry; [i]r_{Ref}: repeatability limit of reference methods from (*16*)

3.3.2.2 SUGARS

As honey is a complex mixture of various sugars, it is particularly difficult to quantify all sugar types present at low concentrations by infrared spectroscopy. For the R^2 for the main sugars fructose respectively glucose, sufficiently high coefficients of determination of 0.810 and 0.884 and low standard errors both in cross-validation (SECV) and validation (SEP) of 1.6 and 1.3 g/100 g respectively were obtained, indicating that they can be determined by near-infrared spectroscopy with a satisfying accuracy (**Table 2, Figure 2**). The prediction accuracy of fructose and glucose concentrations found in this study is comparable to the findings of previous authors (*7, 9, 12, 13, 15*).

The sucrose content in honey is defined by maximum limits described in Codex Alimentarius (*1*) and European Union (*2*) standards. Moreover it is useful for the determination of the botanical origin (*18*). The prediction accuracy (SEP: 0.36 g/100 g; R^2: 0.725) is in the same range as found by Qiu et al. and Ha et al. (*7, 12*) allowing rough estimation of the sucrose content.

The fructose/glucose ratio and the glucose/water ratio are useful for the identification of the botanical origin of honey (*18, 19*). The prediction of the former with a SEP of 0.09 and a R^2 of 0.820 was accurate but slightly inferior to the findings of previous studies (SEP: 0.042 to 0.06) (*8, 11, 13*). However those calibrations were mainly established with acacia honey or based on very few samples. The glucose/water ratio could be predicted with an SEP of 0.12, which is higher than the one found by Pierard et al. (SEP: 0.047) (*13*) thus allowing only a rough estimation. These two measurands are used for the assessment of crystallisation tendency of honey. Honeys with a fructose/glucose ratio higher than 1.3 will crystallize slowly or remain liquid. Honeys with a glucose/water ratio of 1.7 or lower will not crystallise. Honeys with a ratio between 1.7 and 2.0 will crystallise slowly within one year and honeys with a glucose/ water ratio of 2.1 or greater will crystallise fast (*20 - 22*). However the crystallisation tendency of honey depends also on the amount of seed crystals, heat treatment and storage conditions (*22*).

The total monosaccharide content (sum of fructose and glucose) is useful for the discrimination of some unifloral honeys and between honeys of nectar and honeydew origin (*18, 23, 24*). The monosaccharide content could be determined with a satisfying accuracy (SEP: 2.6 g/100 g; R^2: 0.768). The squared standard error of prediction of the total monosaccharide content corresponds to the squared sum of the SEP of the individual sugars. Our finding corresponds to that found for acacia honey (SEP: 1.760; R^2: 0.772) by Cho et al. (*8*) and by mid-infrared spectrometry (SEP: 2.1; R^2: 0.816) (*3*).

Minor sugars may contribute to the authentication of some unifloral honeys (*25-29*) and to the determination of adulteration (*30-33*). The analysis of the disaccharides maltose, isomaltose, kojibiose, turanose, trehalose and nigerose present in small amounts as well as the trisaccharides erlose and melezitose show a SEP between 0.3-0.8 g/100 g and an R^2 between 0.149-0.664. Concerning gentiobiose, melibiose and maltotriose no calibration at all could be established. This means that near-infrared spectroscopy does not allow an accurate prediction of these minor sugars (**Figure 2**, melezitose). This is caused by the low concentration of these components, by the insufficient separation of these sugars by HPLC and the non-specific absorption bands in NIR. In a calibration with fewer samples a sufficiently accurate prediction of maltose (SEP: 0.28 g/100 g, R^2: 0.93) was obtained by Qiu et al. (*7*).

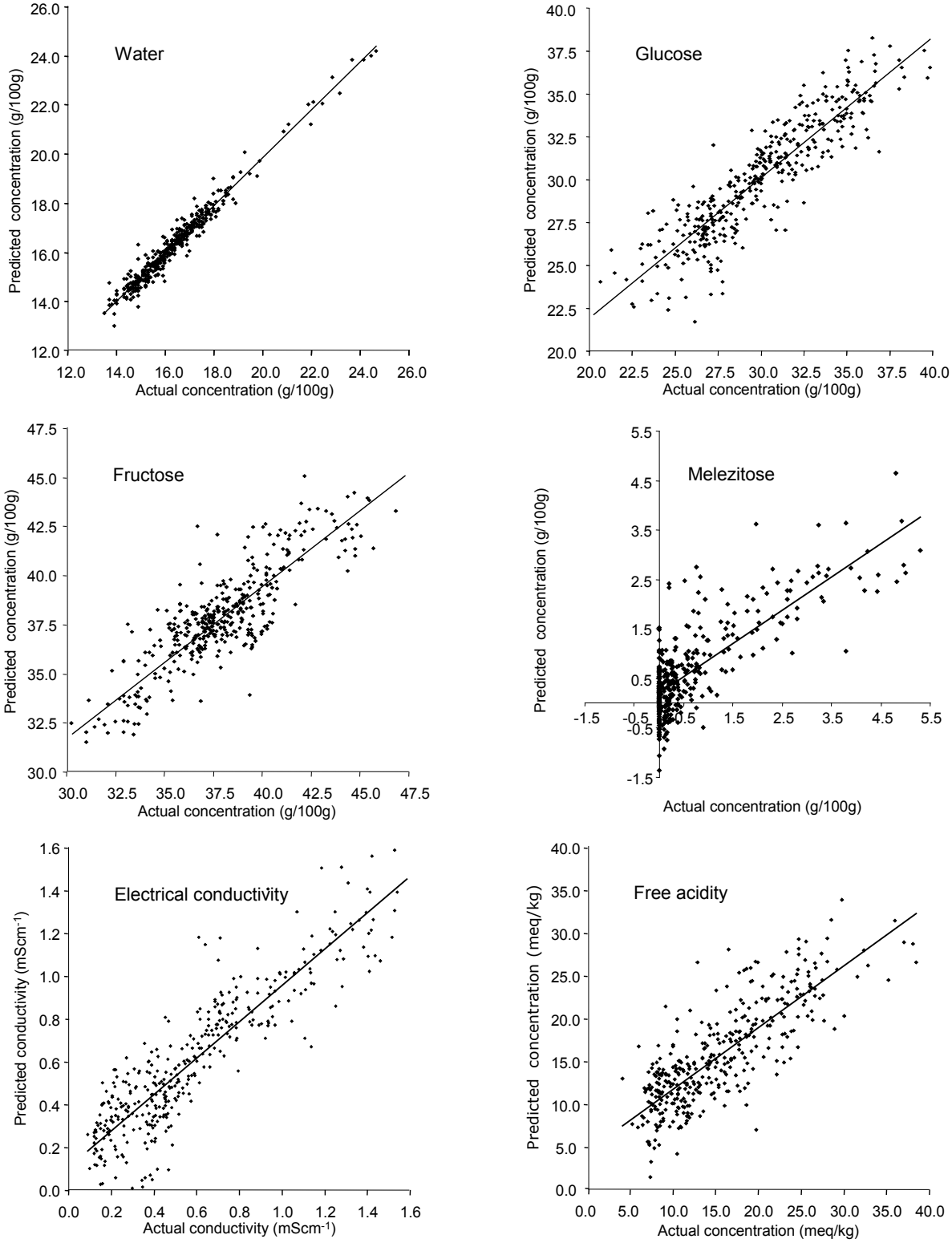

Figure 2. Calibration plots (predicted values from cross-validation)

In the present model the large number of samples considered and their diverse botanical origins are assumed to increase the spectral variability resulting in lower prediction accuracy. It may be improved when individual calibrations would be set up for different types of unifloral honeys. An example may be the good estimation of disaccharides, trisaccharides and perseitol in avocado honey where the calibration was restricted to this type of unifloral honey (*15*). In the analytical practice, however, this approach is not useful as the type of honey is rarely known or even completely unimportant.

The relatively long optical path of 1.5 mm resulting in very high absorbances, (low signal to noise ratio in the important spectral ranges) may explain the lower prediction accuracies found in the present study (*7*, *9*).

3.3.2.3 FREE ACIDITY

The organic acid content of honey is characterised by its free acidity. This measurand is useful for the evaluation of honey fermentation. A maximum of 50 meq/kg is defined by the current quality standards. Furthermore it is useful for the authentication of unifloral honeys and particularly allows differentiating nectar from honeydew honeys (*34*, *35*). The reference method using equivalence point titration is not very accurate because of lactone hydrolysis induced during titration. Free acidity in honey can be predicted by NIR with a moderate accuracy (SEP: 4 meq/kg; R^2: 0.737) (**Table 2**, **Figure 2**, free acidity). Our results confirm the findings of Qiu et al. (SEP: 4.39, R^2: 0.49)(*7*).

3.3.2.4 HYDROXYMETHYLFURFURAL (HMF)

Fresh honey contains only traces of HMF, which is an important criterion for the evaluation of storage time and heat damage. Most of the honey samples analysed were fresh as the median of the HMF content was 5 mg/kg. In order to extend the calibration range to some severely heat damaged samples with a HMF content of up to 112 mg/kg were also analysed. For the calibration range studied the predictive power was found to be very low and unreliable (SEP: 13 mg/kg; R^2: 0.078). NIR spectroscopy is therefore not adequate for the determination of the HMF content in honey (*10*). More promising findings (SEP: 1.72 and 3.32 mg/kg) of other authors are restricted to calibrations on the very light coloured acacia honey where the increase of HMF would probably positively correlate with a darkening of the colour during processing (*8, 11*).

3.3.2.5 PROLINE

The proline content in honey is related to the degree of nectar processing by the bees. It is therefore often used as an indicator of honey adulteration (*36*). The coefficient of determination is rather low (R^2: 0.650). The repeatability limit of the proline determination (r_{IR} = 111 mg/kg) is considerably higher than the lowest value of the photometric reference method (r_{Ref} = 6.6 mg/kg). The determination of proline by NIR is therefore not possible (SEP of 125 mg/kg).

3.3.2.6 ELECTRICAL CONDUCTIVITY AND PH-VALUE

Electrical conductivity and pH-value reflect the mineral content, and the hydronium ion activity of honey. The electrical conductivity is used to distinguish between floral and honeydew honeys according to the current standards (*1, 2*). Moreover it is also the most important physico-chemical criterion for the authentication of unifloral honeys (*37-39*).

The pH-value can be used for the discrimination between floral and honeydew honey (*35*), for the authentication of unifloral honeys (*19*) and for the differentiation of several honeydew honeys (*40*).

The non infrared active characteristics of honey such as electrical conductivity and pH-value are not accurate in validation, SEP's being 0.14 $mScm^{-1}$ and 0.3, and R^2 of 0.870 and 0.657, respectively (**Table 2, Figure 2** free acidity and electrical conductivity). These results partly confirm those obtained by Cozzolino and Corbella (electrical conductivity SEP: 0.010 $mScm^{-1}$, R^2 0.88; pH-value SEP: 0.21, R^2: 0.70) (*10*). The repeatability limits of determination by NIR (r_{IR} 0.274 $mScm^{-1}$ and 0.3) are distinctly different of the reference methods that are 0.002- 0.020 $mScm^{-1}$ respectively 0.11-0.24, indicating the basic difficulty of NIR spectrometry applied to the determination of properties not directly related to the gross composition of individual samples even if the correlation between IR absorption and reference values is not lower than for the abundant components. This difficulty arises from the physical principle of the NIR absorption as a very weak interaction between radiation and matter as well as the fact that conductivity and pH-value are properties induced by very small quantities of matter. The (weak) correlations observed between reference values and absorption in some spectral regions are examples of statistical 'nonsense correlations'. Near infrared spectroscopy therefore allows only a rough estimation of the electrical conductivity and pH-value in honey. These two measurands are highly correlated (r= 0.792; correlation matrix not shown). This is explained by the fact that the various organic acids in honey are at least partially dissociated and therefore act as electrolytes and proton donors.

3.3.3 VALIDATION OF A CALIBRATION ESTABLISHED ON THE BASIS OF SAMPLES FROM SWITZERLAND AND GERMANY WITH SAMPLES FROM OTHER COUNTRIES

A new calibration was set up using all samples except those collected outside Switzerland and Germany. The model was validated with the remaining 37 samples including polyfloral honeys from Argentina, Chile, China, Cuba, France, Greece, Hungary Italy, Mexico, Slovakia, Slovenia and Uruguay. For the measurands studied, all SEP values decreased considerably thus indicating that for maximum accuracy a calibration has to be set up with samples representing all honey types and geographical origins of interest (**Table 3**).

3.4 CONCLUSIONS

The calibration models developed proved satisfying accuracies for the determination of the content of water, glucose, fructose, sucrose, total monosaccharides as well as the fructose/glucose and glucose/water ratios. The prediction accuracies for minor compounds such as HMF and proline, free acidity and the sugars maltose, turanose, nigerose, erlose, trehalose, isomaltose, kojibiose, melezitose, raffinose, gentiobiose, melibiose and maltotriose as well as non infrared active measurands such as pH-value and electrical conductivity, were low and unreliable.

NIR showed for most measurands a better repeatability than mid-infrared spectroscopy (MIR) but only about half the accuracy (*3*) partially due to less specific absorption bands in the near-infrared region. These differences may also be due to the very high number of samples increasing the variability within the sample set of the NIR calibration (various geographical and botanical origins).

Table 3. Validation statistics of the prediction of measurands of honey samples collected outside Switzerland and Germany based on a calibration established using only Swiss and German samples.

	Validation with samples from outside Switzerland and Germany					
Measurand	Samples in calibration	Samples in Validation	Number of factors	SEP	R^2	Prediction bias
Water	350	37	6	1.1	0.277	0.25
Fructose	357	37	6	1.7	0.716	-0.23
Glucose	356	36	9	1.5	0.838	-0.04
Sucrose	352	37	14	1.1	0.071	1.74
Melezitose	200	37	13	0.8	0.316	0.30
Fructose/Glucose ratio	355	36	9	0.1	0.775	-0.01
Glucose/Water ratio	337	37	9	0.1	0.620	-0.01
Free acidity	339	37	16	7	0.376	13.86
Proline	333	37	17	192	0.349	223
Electrical conductivity	343	36	14	0.29	0.575	-0.04
pH-value	340	37	14	0.4	0.330	-0.62

For more accurate predictions separate calibration models could be set up for different types of unifloral honeys or at least for the main types honeydew and floral honeys. However, the botanical origin of honey is rarely known by the time when quantitative measurements are performed.

As several of the above mentioned measurands can be determined simultaneously with a satisfying accuracy, the technique is useful as a screening tool for the evaluation of the botanical origin of honey in combination with pollen analysis or may even allow a determination of some types of unifloral honeys by spectroscopic means alone (*4*). At least a reliable differentiation between floral and honeydew honeys can be assumed as an accurate prediction of polarimetric properties can be performed (*14*).

The determination of measurands such as sucrose and fructose/glucose ratio is valuable for assessing adulteration by sucrose and to predict honey crystallisation tendency. However near-infrared spectrometry does not allow a quantitative determination of HMF and enzyme activities, two criteria particularly important for honey trade, i.e. for the evaluation of storage and heat damage.

The main advantage of NIR combined with multivariate calibration algorithms such as PLS is to simultaneously gain quantitative information on several measurands used for quality control of honey within a short time and a single measurement. Once the calibrations are established NIR spectroscopy allows a rapid analysis of the water, glucose, fructose, sucrose, total monosaccharide contents, fructose/glucose ratio and glucose/water ratio in honey at low cost.

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CHAPTER 4

Quantitative Analysis of Physical and Chemical Measurands in Honey by Mid-Infrared Spectrometry*

ABSTRACT

Fourier transform infrared spectroscopy (FT-IR) was used to determine 20 different measurands in honey. The reference values for 144 honey samples of different botanical origin were determined by classical physical and chemical methods. Partial least squares regression was used to develop the calibration models for the measurands studied. They were validated using independent samples and proved satisfying accuracies for the determination of water (R^2 = 0.99), glucose (0.94), fructose (0.84), sucrose (0.91), melezitose (0.98) and monosaccharide content (0.82) as well as fructose/glucose ratio (0.98), glucose/water ratio (0.94), electrical conductivity (0.98), pH-value (0.87) and free acidity (0.96). The prediction accuracy for hydroxymethylfurfural, proline and the minor sugars maltose, turanose, erlose, trehalose, isomaltose and kojibiose was rather poor. The results demonstrate that mid-infrared spectrometry is a valuable, rapid and non-destructive tool for the quantitative analysis of the most important measurands in honey.

4.1 INTRODUCTION

Analytical methods applied to honey generally deal with five different topics: determination of botanical or geographical origin, quality control according to the current standards and detection of adulteration or residues. In all of these areas except residue analysis infrared spectroscopy has recently been applied as it presents a rapid, non-destructive and promising approach.

For the general quality control of honey according to the current standards of the Codex Alimentarius (*1*) and of the European Union (*2*), several physical and chemical measurands have to be determined, which mostly include water content, enzyme activities of invertase and a-amylase, hydroxymethylfurfural (HMF), electrical conductivity, and sugar composition. At present a specific analytical method has to be applied for each measurand of interest. Moreover, the methods commonly used to determine the chemical composition and the physical properties of honey are laborious and therefore expensive thus limiting the number of honey samples analysed daily. To further improve honey quality control it is necessary to develop rapid, simple and accurate methods for the routine quality assessment of honey.

Due to the increased performance of computers in the last decades infrared spectrometry (IR) has become a rapid and well established technique for quantitative food analysis. Infrared spectroscopy has been applied to different types of honey analysis.

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Near-infrared spectrometry (NIR) has been successfully applied both in transmission and transflectance mode to the quantitative analysis of fructose, glucose, sucrose, maltose and water content in honey samples from different crops (*3-6*). Furthermore, non-compositional characteristics such as electrical conductivity, colour and polarimetric properties have been also successfully calibrated (*6, 7*). However, near-infrared spectroscopic techniques have not been considered to be useful for the analysis of minor honey components such as HMF, free and lactone acidity and pH (*4, 6*).

Mid-Infrared spectroscopy (MIR) provides more specific and distinct absorption bands than NIR spectroscopy. Calibrations on a very large sample basis for different honey measurands have been developed by Lichtenberg-Kraag et al. (*8*). Reliable partial least squares (PLS) models were established for the quantitative analysis of fructose, glucose, sucrose, maltose, electrical conductivity, pH-value and free acidity. The dilution of the honey in the so-called Zero Liquid (FOSS, Hillerød, Denmark) which mainly consists of water, resulted in a strong noise in the water absorption bands (1717-1543 and 3627-2971cm^{-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. A further drawback of this method is that the honey sample has to be quantitatively weighed into the Zero Liquid.

Quantitative MIR spectrometry with a single reflection attenuated total reflection (ATR) accessory was recently applied to the analysis of fructose, glucose, sucrose and maltose in honey (*9*). In this study pure sugar solutions as well as a series of 60 honey samples from different botanical origin were analysed. Calibration with PLS and principal component regression (PCR) models for prediction of the 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 as reference method and by FT-IR were between 0.971 and 0.993. This indicates that FT-IR-ATR spectrometry seems to be adequate for rapid, non-destructive and accurate quantitative analysis of honey (*9*).

Recent publications (*10-14*) claim that honey adulteration with medium invert cane, beet and corn syrup 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 natural variation of the honey composition was not considered, as only three samples of different botanical origin were studied. In some of the experiments carried out by using artificially adulterated sugar solutions (*10, 11, 13*), the concentration of sucrose was so high that the adulteration could have been easily determined by analysing the sucrose content as it exceeded the limits defined by the European honey directive and the Codex Alimentarius (*1, 2*). In addition the experimental design facilitated the detection of such an adulteration because the water content was also changed when the honey samples were adulterated with the solutions of pure sugars (*13-14*). To prevent this problem Kelly et al (15) proposed to dilute all samples with water and to adjust the solid content to 70 °Brix. These authors also analysed 99 non-adulterated honey samples. 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 %.

The aim of the present work was to investigate FT-IR single reflection ATR spectroscopy as a rapid, simultaneous and non-destructive analytical tool for the determination of 20 different measurands used in quality control of honey.

4.2 MATERIAL AND METHODS

4.2.1 HONEY SAMPLES

144 honey samples obtained from seven different crops between 1997 and 2004 in Switzerland, including unifloral, (i.e. *Castanea* sp. (n = 8), *Robinia* sp.(n = 12), *Tilia* spp. (n = 7), *Brassica* spp. (n = 7), *Taraxacum* sl. (n = 6), *Rhododendron* spp. (n = 7) and *Abies* sp. (n = 8), polyfloral (n = 77) as well as honeydew honeys (n = 12) were analysed. In order to be able to measure the water content in bakers honey the calibration range of a water content above 19 g/100 g was extended to 24.6 g/100 g by adding water to 17 different honey samples. All samples were stored at 4 °C before analysis. They were liquefied in a water bath at 55 °C for 8 h and then allowed to cool to room temperature before analysis.

4.2.2 REFERENCE METHODS

The Harmonised Methods of the European Honey Commission (*16*) were used as reference methods for the quantitative analysis of water, electrical conductivity, HMF, pH-value, proline, free acidity as well as various sugars (i.e. fructose, glucose, sucrose, turanose, nigerose, maltose, kojibiose, trehalose, isomaltose, erlose, and melezitose).

Pollen analysis was carried out according to von der Ohe et al. (*17*). and the botanical origin of the honey samples was determined according to (*18*) The range of the reference values of the honey samples analysed is shown in **Table 1**.

4.2.3 FT-IR ATR SPECTROSCOPY

MIR spectra were recorded using a Bio-Rad FTS-7 (Bio-Rad, Cambridge MA, U.S.A.) equipped with a MKII Golden Gate TM single reflection ATR accessory (Specac Inc, Woodstock GA, U.S.A). The measuring cell consists of a diamond of 2.8 mm in dia-meter with a refractive index of 2.4 at 1000 cm^{-1}. The depth of penetration of the infrared radiation is 2.0 µm at 1000 cm^{-1} for a sample with a refractive index of 1.5 (which corresponds to the refractive index of honey). The spectrometer was equipped with a deuterated triglycine sulfate (DTGS) detector and operated with 4 cm^{-1} resolution. Single reflection ATR-accessories require only small amounts of sample and are much easier to clean than multiple reflection ATR-accessories but are consequently less sensitive because of the limited interaction of the infrared beam with the sample.

After applying a drop of the sample on the surface of the diamond, it was left to thermally equilibrate for 4 min. The number of scans per spectrum was selected on the basis of optimal signal to noise ratios and collection times required. 100 scans were then recorded for each spectrum in the wavelength range between 4000-550 cm^{-1}. Single-beam spectra of all samples were collected and ratioed against the background spectrum of the clean diamond surface (laboratory air) in order to present the spectra in absorbance. Two replicates of each sample were recorded at room temperature. After each measurement the diamond was thoroughly washed with demineralised water and dried with a soft tissue.

Table 1. Reference data ranges of the honey samples

Measurand	Unit	n	Mean	Minimum	Maximum
Water	g/100 g	144	16.6	13.4	24.6
Fructose	g/100 g	130	38.3	20.9	45.7
Glucose	g/100 g	130	29.4	21.5	38.2
Sucrose	g/100 g	127	0.8	0.0	9.7
Turanose	g/100 g	129	2.2	0.0	5.5
Nigerose	g/100 g	131	2.4	0.0	5.1
Maltose	g/100 g	131	1.8	0.0	4.6
Kojibiose	g/100 g	131	1.0	0.0	1.9
Trehalose	g/100 g	131	0.3	0.0	2.1
Isomaltose	g/100 g	128	0.7	0.0	3.7
Erlose	g/100 g	131	0.8	0.0	3.0
Melezitose	g/100 g	127	0.8	0.0	5.8
Monosaccharides sum	g/100 g	128	67.6	53.6	77.4
Fructose/Glucose ratio		129	1.32	0.97	1.86
Glucose/Water ratio		117	1.87	1.33	2.59
Free acidity	meq/kg	128	18	6	34
Hydroxymethylfurfural	mg/kg	128	8	0	40
Proline	mg/kg	126	499	187	1189
Electrical conductivity	$mScm^{-1}$	126	0.60	0.10	1.45
pH-value		127	4.5	3.8	6.0

*n: number of samples in cross-validation

The instrumental stability was monitored using a standard sample prepared by heating an acacia honey to 100 °C for 20 min. This standard was divided into a series of identical 2 ml vials and stored in the freezer until analysis. Spectra of this honey standard were recorded daily. The repeatability was determined by tenfold measurement of a honeydew sample (**Table 2**).

4.2.4 DATA ANALYSIS

For the chemometric evaluation, the GRAMS/AI (7.00) (Thermo Galactic, Salem NH, U.S.A.) software was used for quantitative analysis by PLS regression. The calibration models were developed using the PLSplus/IQ add-on (Thermo Galactic) to quantitatively predict the measurands on the basis of spectral information in the range between 3700-2400 cm^{-1} and 1800-700 cm^{-1}.

The optimised models were obtained by the "leave one out" cross-validation technique based on the minimum predicted residual sum of squares (PRESS). The predictive quality of the models was evaluated by calculating the standard error of cross-validation (SECV) and the standard error of prediction (SEP) in the validation step with independent samples.

4.2.5 CALIBRATION AND VALIDATION

PLS cross-validations were performed to test various calibration models for the prediction of the different measurands. These models were set up with all spectra and evaluated after outlier elimination. For validation (prediction for samples not included in the calibration) the spectra of all 144 samples were split into two data sets: for each measurand the spectra were sorted by quantity over the whole range of reference values and the two spectra of every 10'th sample from this list were used to validate the respective PLS-model. Consequently, the validation samples represented the whole concentration range of the measurands investigated. This procedure yielded about 25 – 28 samples for validation (not necessarily the same for each measurand). The calibration was set up with the remaining spectra not included in the validation set. Validation SEP, coefficients of determination and prediction bias were calculated (**Table 2**).

4.3 RESULTS AND DISCUSSION

4.3.1 REPEATABILITY LIMITS

The repeatability limits (s_r) of the FT-IR-ATR measurements were calculated based on 10 subsequent analyses of different aliquots of the same honey sample (see **Table 2**; repeatability). For comparison repeatability limits (r_{Ref}) from results of international interlaboratory studies with the reference methods are listed as far as they are available (**Table 2**) (*16*). The laboratory precision expressed as standard deviation of the results (not shown) from an acacia standard honey measured to monitor the instrumental stability was less than three times the repeatability standard deviation s_r. **Figure 1** shows a typical FT-IR-ATR spectrum of honey.

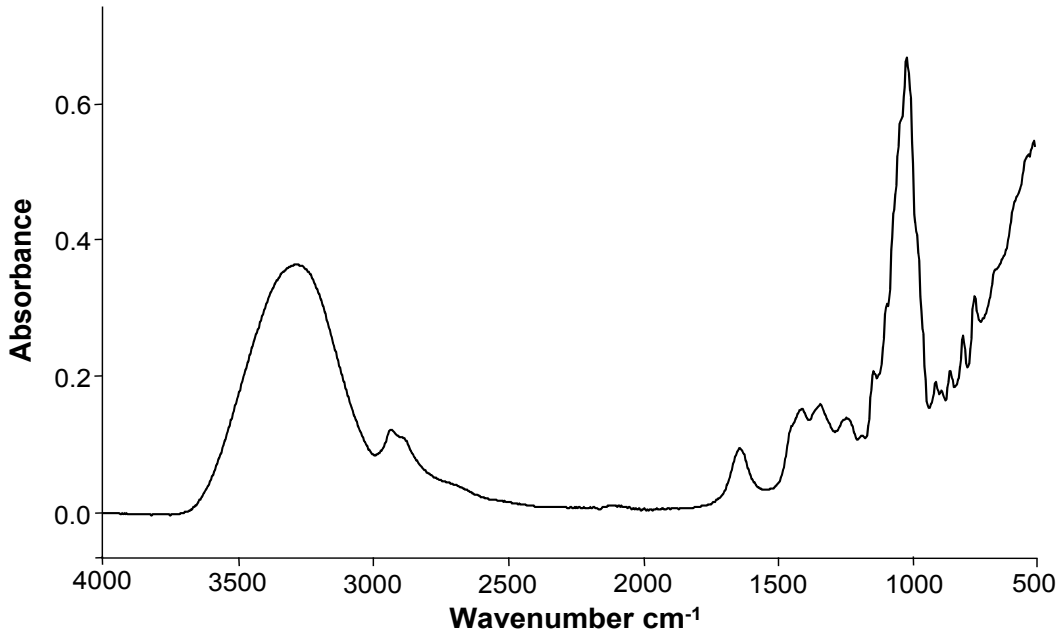

Figure 1. Typical FT-IR-ATR spectrum of a honey sample.

Table 2. Cross-validation and validation statistics; repeatability of the method.

Measurand	Unit	Calibration				Validation				[e]Repeatability		
		Samples in calibration	Number of factors	[a]SECV	[b]R^2	Samples in Validation	[c]SEP	R^2	[d]Prediction bias	[f]s_r	[g]r_{IR}	[h]r_{Ref}
Water	g/100 g	144	3	0.46	0.955	28	0.24	0.989	0.07	0.08	0.22	0.11
Fructose	g/100 g	130	11	1.2	0.808	25	1.2	0.841	0.3	0.4	1.2	0.8
Glucose	g/100 g	130	13	1.0	0.925	26	0.9	0.943	0.2	0.3	1.0	0.8
Sucrose	g/100 g	127	16	0.3	0.964	25	0.3	0.907	-0.0	0.1	0.4	0.4
Turanose	g/100 g	129	13	0.4	0.704	25	0.2	0.774	0.0	0.2	0.4	0.3
Nigerose	g/100 g	131	7	0.7	0.633	23	0.5	0.880	-0.3	0.6	1.6	
Maltose	g/100 g	131	15	0.6	0.527	24	0.6	0.250	-0.0	0.3	1.0	0.5
Kojibiose	g/100 g	131	16	0.2	0.802	25	0.2	0.810	-0.1	0.2	0.5	
Trehalose	g/100 g	131	16	0.2	0.845	25	0.2	0.839	0.0	0.1	0.3	
Isomaltose	g/100 g	128	14	0.2	0.866	25	0.2	0.881	-0.01	0.09	0.25	
Erlose	g/100 g	131	15	0.3	0.841	27	0.3	0.886	-0.02	0.08	0.24	
Melezitose	g/100 g	127	15	0.2	0.971	26	0.2	0.975	0.0	0.1	0.4	
Monosaccharides sum	g/100 g	128	12	1.9	0.825	25	2.1	0.816	0.6	0.7	2.1	
Fructose/Glucose ratio		129	13	0.04	0.964	26	0.03	0.975	0.01	0.02	0.06	
Glucose/Water ratio		117	7	0.08	0.914	21	0.06	0.942	0.03	0.04	0.11	
Free acidity	meq/kg	128	14	2	0.958	25	2	0.958	0	1	3	0.7
Hydroxymethylfurfural	mg/kg	128	14	6	0.439	24	6	0.249	1	3	8	0.9
Proline	mg/kg	126	15	67	0.877	24	71	0.870	2	43	122	6.6
Electrical conductivity	$mScm^{-1}$	126	13	0.04	0.985	24	0.05	0.979	-0.01	0.03	0.07	0.002
pH-value		127	12	0.12	0.928	25	0.16	0.868	0.04	0.05	0.14	0.04

[a]SECV: standard error of cross-validation, SECV = $\sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}}$ where $\hat{y}_i$ predicted value of spectrum *i*, y_i reference value of spectrum *i* and *n* the number of spectra. [b]R^2: coefficient of determination; [c]SEP: standard error of prediction (equation see SECV; but with $\hat{y}_i$ representing a sample not used in calibration); [d]Prediction bias: mean difference between predicted and reference values; [e]repeatability calculated from 10 predicted values obtained by applying the same calibrations as used for the estimation of the SECV; [f]s_r: repeatability standard deviation in FT-IR spectrometry; [g]r_{IR}: repeatability limit of FT-IR spectrometry; [h]r_{Ref}: repeatability limit of reference methods from (*16*)

4.3.2 PREDICTION OF THE MEASURANDS

The resulting standard errors from PLS cross-validation and coefficients of determination (R^2) are given in **Table 2**. For the measurands studied, the coefficients of determination in calibration were between 0.439 (HMF) and 0.985 (electrical conductivity) and in validation between 0.250 (maltose) and 0.989 (water content). The variable coefficients of determination show that some measurands can be accurately predicted while a determination of others is not possible with a satisfying accuracy. The predictions of the individual measurands are discussed below.

4.3.2.1 WATER

The water content of honey is the most important measurand for the assessment of ripeness and shelf life, as a honey with a water content above 18 g /100 g may be spoiled by fermentation. The method developed allows an accurate determination of water. The r_{IR} is with 0.22 g/100 g in the same order of magnitude as the r_{Ref} of 0.11 g/100 g of the refractometric reference method (*16*). Moreover, the SEP and the R^2 in validation are with 0.24 g/100 g and 0.989, respectively, the best values of the calibrations performed. Thus, the water content in honey can be reliably determined by infrared spectroscopy.

4.3.2.2 SUGARS

As honey is a complex mixture of various sugars, it is particularly difficult to quantitatively measure the sugar types present at low concentrations by infrared spectroscopy. The results obtained for fructose, glucose, sucrose and melezitose, the typical trisaccharide of honeydew honey, show high coefficients of determination and low standard errors both in cross-validation (SECV) and validation (SEP) indicating that they can be accurately determined by mid-infrared ATR-spectroscopy (**Table 2, Figure 2**). The prediction accuracy of fructose, glucose and sucrose concentrations found in this study is comparable to the ones determined by NIR (*4, 5*) and MIR (*9*).

The prediction of the fructose/glucose ratio and the glucose/water ratio which are useful for the identification of the botanical origin of honey (*18, 19*) was very accurate with a SEP of 0.03 and 0.06 respectively as well as a R^2 of 0.975 and 0.942 respectively. These two measurands are also helpful for the assessment of crystallisation tendency of honey. Honeys with a fructose/glucose ratio larger than 1.3 will crystallize slowly or remain liquid. Honeys with a glucose/water ratio of 1.7 or lower will not crystallize at all, honeys with a ratio between 1.7 and 2.0 will crystallise slowly within one year and honeys with a glucose/ water ratio of 2.1 or greater will crystallise fast (*20, 21*). However the crystallisation tendency of honey depends also on the amount of seed crystals, heat treatment and storage conditions (*22*).

The total monosaccharide content (sum of fructose and glucose) is useful for the discrimination of some unifloral honeys and between honeys of nectar and honeydew origin (*18, 23, 24*). The monosaccharide content could be determined with a satisfying accuracy with a SEP of 2.1 g/100 g and a R^2 of 0.816. The standard error of precision of the total monosaccharide content corresponds to the sum of the SEP of the individual sugars.

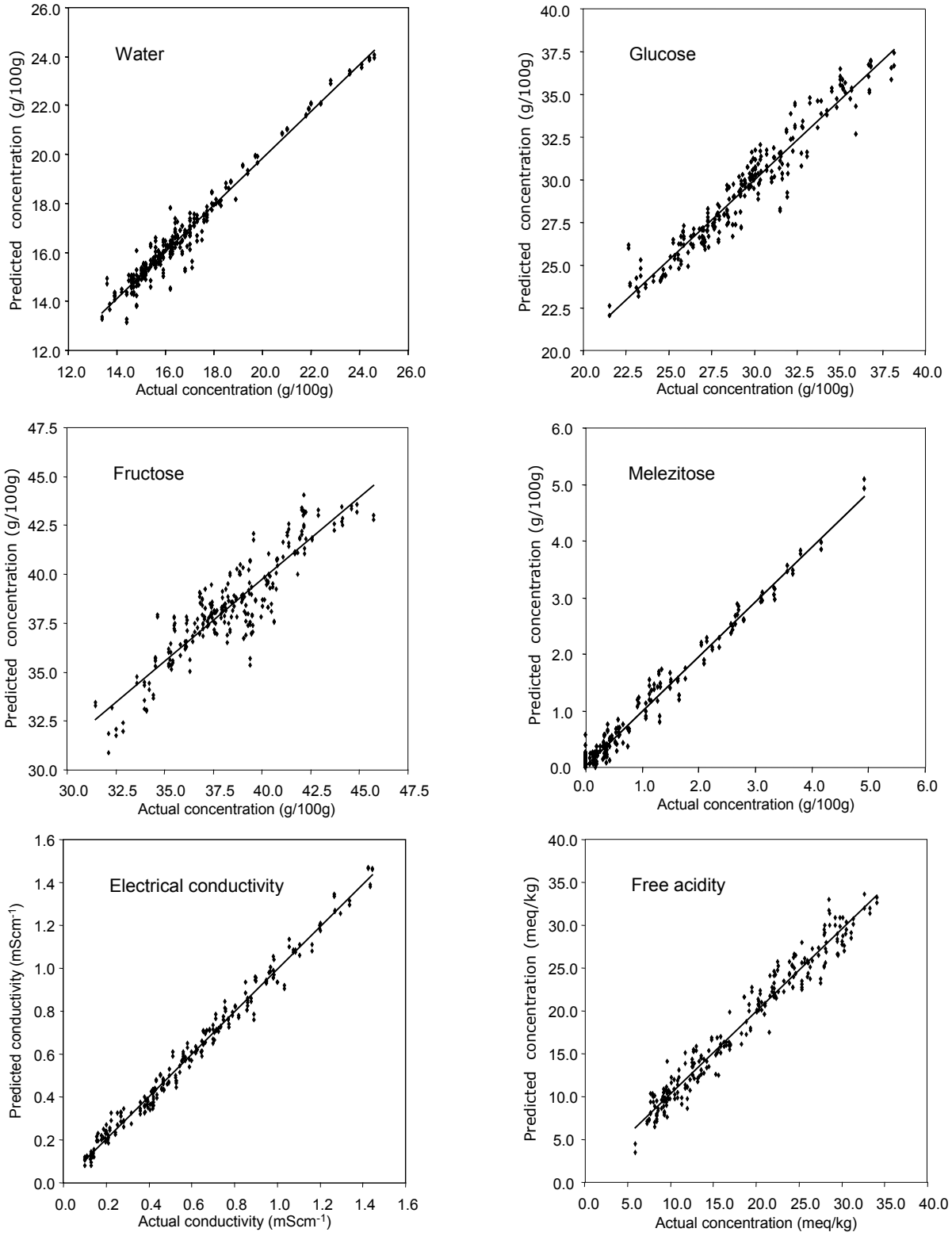

Figure 2. Calibration plots (predicted values from cross-validation)

Minor sugars may contribute to the authentication of some unifloral honeys (*25-29*) and to the determination of adulteration (*30- 33*). The analysis of turanose, nigerose, erlose show a SEP between of 0.2-0.5 g/100 g and a R^2 between 0.774-0.886. This means that a satisfactory measurement accuracy is hardly possible by FT-IR spectroscopy. However a gross estimation of these components in honey is possible. The prediction of maltose, kojibiose, trehalose, and isomaltose concentrations seems to be even less reliable. For maltose our results are inferior to those obtained by Tewari & Irudajaraj (*9*) and Qiu et al. (*4*) (NIR) but comparable to those of Lichtenberg-Kraag et al. (*8*) (r = 0.76).

The unsatisfactory measurement precision for the minor sugars is probably due to the insufficient separation capacity of the HPLC reference method used for the determination of the minor honey sugars in the complex sugar matrix.

4.3.2.3 FREE ACIDITY

The acid content in honey is characterised by the free acidity. The measurand is useful for the evaluation of honey fermentation. A maximum of 40 meq/kg is defined by the current standards. Furthermore it is helpful for the authentication of unifloral honeys and especially for the differentiation between nectar and honeydew honeys (*34*, *35*). The reference method of equivalence point titration is relatively poor because of lactone hydrolysis during titration. The free acidity in honey can be predicted by infrared spectrometry with a satisfying accuracy (SEP 2 meq/kg and R^2 0.958) and thus presents a valuable alternative to the reference method (**Table 2, Figure 2**).

4.3.2.4 HYDROXYMETHYLFURFURAL (HMF)

Fresh honey contains only traces of HMF which is an important criterion for the evaluation of storage time and heat damage. Most of the honey samples analysed were relatively fresh as the maximum HMF content was 39.51 mg/kg. At least for the calibration range studied the predictive model was with a SEP of 6 mg/kg and a R^2 of 0.249 rather poor. The infrared spectroscopic determination of the HMF content is not accurate enough in the range relevant for quality control of honey and may only allow a rough estimation.

4.3.2.5 PROLINE

The proline content in honey is related to the degree of nectar processing by the bees. It is therefore used as an indicator of honey adulteration (36). The coefficient of determination is high with 0.877. The repeatability limit of the proline determination (r_{IR} = 121.7 mg/kg) is poor compared to the photometric reference method (r_{Ref} = 24.4 mg/kg). This is not surprising because infrared spectrometry is generally not suitable for the determination of low concentrations. However the determination of proline by FT-IR with a SEP of 71.2 mg/kg is sufficient for a gross estimation of the proline content.

The proline content is highly correlated with free acidity (r = 0.794, correlation matrix not shown). This could be explained by the fact that some honeys have to be intensively processed by the bees resulting in a high proline concentration (e.g. honeydew honeys have a high free acidity).

4.3.2.6 ELECTRICAL CONDUCTIVITY AND PH-VALUE

Electrical conductivity and the pH-value reflect the mineral and acid contents of honey. The electrical conductivity is used to distinguish between floral and honeydew honeys according to the present standards. It is also the most important physicochemical measurand for the authentication of unifloral honeys (*37*, *38*, *39*). The pH-value can be used for the discrimination of floral and honeydew honey (*35*) as well and is also helpful for the authentication of unifloral honeys (*19*) and the differentiation of several honeydew honeys (*40*).

Interestingly, the non compositional and non infrared active characteristics of honey such as electrical conductivity and pH-value could also be predicted with high accuracies in validation, SEP's being 0.05 $mScm^{-1}$ and 0.16, and R^2 of 0.979 and 0.868, respectively (**Table 2, Figure 2**). The repeatabilities of the determination by infrared spectroscopy are with 0.073 $mScm^{-1}$ and 0.139 relatively close to the repeatabilities of the reference method that are 0.02 $mScm^{-1}$ respectively 0.06. Infrared spectroscopy presents therefore a rapid approach for the determination of electrical conductivity and pH-value with a satisfying accuracy. Electrical conductivity and the pH-value of honey are highly correlated (r= 0.852). This may be explained by the fact that the various organic acids in honey are at least partially dissociated and therefore act as electrolytes.

4.4 CONCLUSIONS

The advantage of mid-infrared spectroscopy compared to the current reference methods is to simultaneously obtain quantitative information on several measurands by a single measurement within short time. FT-IR-ATR spectrometry combined with multivariate calibration algorithms such as PLS is a very promising method for the quantitative analysis of the main measurands used for routine quality control of honey.

The calibration models developed proved satisfying accuracies for the determination of water, electrical conductivity, glucose, fructose, sucrose, melezitose, total monosaccharides, fructose/glucose ratio, glucose/water ratio, pH-value and free acidity. As several measurands can be determined at once with a satisfying accuracy, the technique is especially valuable for quality control of honey and could be simultaneously used as a screening tool for the evaluation of the botanical origin of honey. The determination of measurands like sucrose and fructose/glucose ratio is valuable for assessing adulteration by sucrose and to predict honey crystallisation tendency. However infrared spectrometry does not allow a quantitative determination of HMF and enzyme activities, two criteria particularly important for honey trade, i.e. for the evaluation of storage and heat damage. Infrared spectrometry is non-destructive, rapid, easy to use and requires only limited sample preparation which makes it a very efficient tool for honey quality control.

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CHAPTER 5

Authentication of the Botanical Origin of Honey by Near-Infrared Spectroscopy*

ABSTRACT

Fourier transform near-infrared spectroscopy (FT-NIR) was evaluated for the authentication of eight unifloral and polyfloral honey types (n = 364 samples) previously classified using traditional methods such as chemical, pollen and sensory analysis. Chemometric evaluation of the spectra was carried out applying principal component analysis (PCA) and linear discriminant analysis (LDA). The corresponding error rates were calculated by Bayes' theorem. NIR-spectroscopy enabled a reliable discrimination of acacia, chestnut and fir honeydew honey from the other unifloral and polyfloral honey types studied. The error rates ranged from lower than 0.1% to 6.3 % depending on the honey type. NIR proved also to be useful for the classification of blossom and honeydew honeys. The results demonstrate that near-infrared spectrometry is a valuable, rapid and non-destructive tool for the authentication of the above mentioned honeys, but not for all varieties studied.

5.1 INTRODUCTION

The vast majority of the honeys sold on the market contain significant nectar or honeydew contributions from several plant species and are therefore called polyfloral or multifloral honeys. Normally they are just designated with the word "honey". Probably no honey produced by free flying bees is purely unifloral. The term unifloral honey is used to describe honey in which the major part of nectar or honeydew is derived from a single plant species. Honey composition, flavour and colour varies considerably depending on the botanical source it originates from (*1*). According to the Codex Alimentarius Standard for Honey (*2*) and the European Union Council Directive (*3*) related to honey, the use of a botanical designation of honey is allowed if it originates predominantly from the indicated floral source and possesses the corresponding sensorial, physical, chemical and microscopic properties.

The physical, chemical and pollen analytical characteristics of the most important unifloral honeys have been described in various papers (*1, 4-6*). On contrary to unifloral honeys the polyfloral honeys do not express distinct physical or chemical characteristics apart from a huge variability, which makes their authentication particularly difficult.

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Ruoff, K.; Luginbühl, W.; Bogdanov, S.; Bosset, J. O.; Estermann, B.; Ziolko, T.; Amadò, R. Authentication of the Botanical Origin of Honey by Near-Infrared spectroscopy. *J. Agric. Food Chem.* **2006**, 54, 6867-6872.

The interest in the production of unifloral honeys is a higher consumer preference for some honey varieties leading to a commercial interest of the beekeepers. Recent applications in therapeutic or technological use of certain honey varieties also account for the requirement of reliable determination of the botanical origins (*7-10*).

Up to now a reliable authentiction of the botanical origin can only be achieved by experts by a global interpretation of sensory, pollen and physico-chemical analyses that include at least measurement of electrical conductivity and sugar composition (*4, 11, 12*). A specific analytical method has to be applied for each measurand of interest, thus resulting in laborious and expensive analyses. Especially the uncertainty related to the interpretation of pollen analytical results, originating from plant morphological differences, variable ratios of pollen and nectar from different plant species, the activity of the bees or even honey processing, filtration as well as new plant cultivars and sources such as honeydew without any relationship with pollen production, lead to search for new analytical methods (*13*).

In the last decades near-infrared spectrometry (NIR) has become a rapid and well established technique for quantitative and qualitative analysis of food. It has been successfully applied both in transmission and transflectance modes to the quantitative analysis of honey. 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 (*14-20*). Furthermore physical characteristics of honey such as electrical conductivity, colour and polarimetric properties have also been successfully calibrated (*20-21*).

The potential of near-infrared spectroscopy for the determination of the botanical origin of honey was recently evaluated using a reflectance probe (22). Principal component analysis (PCA) was used for data reduction. Linear discriminant analysis (LDA) was applied for the classification of the honey types studied. Over 80 % of acacia (*Robinia pseudoacacia*), chestnut (*Castanea sativa*) and rape (*Brassica* spp.) honeys were correctly assigned to the corresponding honey type on the basis of the spectra and mahalanobis distance in cross-validation, while only a third of the hea-ther (*Calluna vulgaris*) honeys considered, were correctly classified. 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. However, the number of samples per honey type was very restricted as only 13 different unifloral honeys from nine European countries were studied on a total of only 51 samples. No discrimination into groups according to geographical origin was found (*22*). These encouraging preliminary results should be validated with a larger set of samples.

Although near-infrared spectroscopy would allow to clearly discriminate between several types of unifloral honeys, this does not mean that the methodology will be useful in analytical practice because the great challenge in honey analytics is not to distinguish between several unifloral honey types but to discriminate the minority of approximately 20 % of unifloral honeys from the overwhelming majority of about 80 % of polyfloral honeys on the market. Unfortunately polyfloral honeys have so far not been considered in most of the recently developed analytical methods proposed for the authentication of the botanical origin of honey (*22-32*).

The aim of the present work was to investigate eight unifloral and polyfloral honey types by using FT-NIR spectroscopy in transflection mode in order to develop a rapid and reliable method for the authentication of unifloral and polyfloral honeys.

5.2 MATERIALS AND METHODS

5.2.1 SAMPLING AND BOTANICAL CLASSIFICATION BY REFERENCE METHODS

A total of 364 honey samples produced between 1998 and 2004 were collected and stored at 4 °C until analysis. They originated predominantly from Switzerland (CH), a few samples from Germany (D) were also included.

To classify these honey samples the following measurands were determined according to the harmonised methods of the European Honey Commission (*33*): electrical conductivity, sugar composition, fructose/glucose ratio, pH-value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (*34*, *35*).

Based on these analytical results, the honey samples were assigned to one of the following eight honey types according to the criteria of Persano and Piro (*1*): acacia (*Robinia pseudoacacia*) (CH, n = 19; D, n = 4), alpine rose (*Rhododendron* spp.) (CH, n = 14), chestnut (*Castanea sativa*) (CH, n = 27), rape (*Brassica* spp.) (CH, n = 25), fir honeydew (*Picea* spp. and *Abies* spp.) (CH, n = 52), lime (*Tilia* spp.) (CH, n = 13; D, n = 7), dandelion (*Taraxacum* s.l.) (CH, n = 20; D, n = 4) and polyfloral honeys (CH, n = 179). In the heterogenous group of the polyfloral honeys nectar or honeydew contributions from all of the above-mentioned sources were represented.

5.2.2 NEAR-INFRARED SPECTROSCOPY

The honey samples were liquefied in a heating cabinet at 50 °C for 9 h and then allowed to cool to room temperature before analysis, NIR spectra were recorded using a Büchi NIRLab N-200 spectrometer equipped with a MSC 100 measuring cell with a rotating sample holder (Büchi Labortechnik AG, Flawil, Switzerland) to level out effects of sample inhomogeneity. About 10 g of liquefied honey was poured into a clean glass petri dish and covered with the transflection plate so defining a 0.3 mm layer of honey between the bottom of the Petri dish and its surface and acting as reflector. 64 scans with a resolution of 4 cm^{-1} were recorded in transflection mode for each spectrum in the wavenumber range between 4000-10000 cm^{-1}, **Figure 1** shows a typical FT-NIR spectrum of honey. Three replicates of each sample were averaged to one average spectrum. The repeatability was determined by a ten-fold measurement of the absorbance of a polyfloral honey sample.

5.2.3 PROCESSING OF SPECTRA AND MULTIVARIATE ANALYSIS

To exclude random variability resulting from instrumental effects, the following spectral range was used for multivariate analysis: 4112 – 9947 cm^{-1}. After elimination of spectral outliers, principal component analysis (PCA) was applied to eliminate the spectral collinearity and to reduce the number of variables to 20 PC's (using GRAMS/32 AI with the PLSplus/IQ Add-on, Vs. 5.09, Thermo Galactic, Salem NH, U.S.A.).

In LDA, the 20 initial PC's were further reduced by backwards elimination on the basis of their partial F-values in the discriminant models (SYSTAT® Version 11, Systat Software Inc., Richmond, USA). The validation was accomplished with spectra of a third of the samples selected randomly and not present in the group of samples used to build the model.

5.3 RESULTS AND DISCUSSION

5.3.1 NIR-SPECTRA OF DIFFERENT HONEY TYPES AND REPEATABILITY LIMITS

The repeatability limit (r_{IR}) of the FT-NIR measurements was calculated based on 10 subsequent analyses of different aliquots of the same polyfloral honey sample determined at the maximum absorbance at 4761 cm^{-1}. The average of the maximum intensity of 2.236 au, a standard deviation of 0.069, a coefficient of variation of 3,1 % and a r_{IR} of 0.195 were found, indicating a satifying repeatability of the method.

The near-infrared spectra of the seven unifloral honeys studied are shown in **Figure 1**. Each spectrum displayed is a typical individual spectrum of the given honey type. Visible to the naked eye are mostly differences in absorbance intensity. Characteristic differences in shape were observed between 4200 and 7100 cm^{-1}. The largest variation among the spectra of the honey types considered were observed in C-O and C-C stretching regions of the saccharides between 4200 and 5200 cm^{-1} (**Figure 1**, enlargement A).

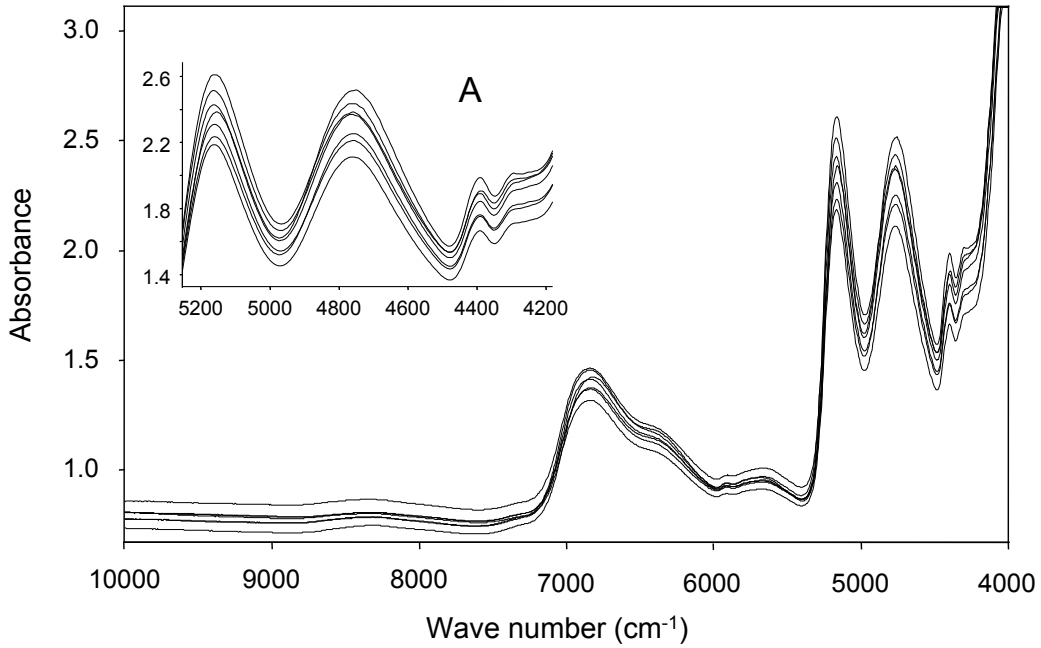

Figure 1. FT-NIR spectra of 7 different honey types (A: enlargement of the region between 4160 and 5260 cm^{-1})

5.3.2 LINEAR DISCRIMINANT ANALYSIS

When LDA was performed on the eight different honey types only chestnut and fir honeydew honeys were correctly classified with a rate of 90 % or higher in jackknife classification (**Table 1**). Some of the acacia honey samples were misclassified as alpine rose or polyfloral honeys, but were nevertheless correctly classified to 85 %. Generally a considerable number of samples were misclassified to groups of unifloral and polyfloral honeys showing rates of correct classification of only 39 – 63 % in jackknife classification. Dandelion honey showed with 39 % the lowest jackknife classification rate. The samples were predominantly misclassified to polyfloral and rape honeys.

Table 1. Jackknife classification and validation tables for the honey samples as classified by LDA (all honey types considered separately).

	Jackknife classification rate (%)								
	Acacia	Alpine rose	Fir honeydew	Chestnut	Dandelion	Lime	Rape	Polyfloral	Correct
Acacia (n = 20)	85	10	0	0	0	0	0	5	85
Alpine rose (n = 11)	9	46	0	0	0	27	9	9	45
Fir honeydew (n = 49)	0	0	90	0	2	2	0	6	90
Chestnut (n = 26)	0	0	0	96	0	4	0	0	96
Dandelion (n = 23)	0	9	0	0	39	9	17	26	39
Lime (n = 18)	0	11	0	0	0	44	0	44	44
Rape (n = 24)	0	4	0	0	29	0	63	4	63
Polyfloral (n = 172)	3	4	9	9	10	4	14	48	48
								Weighted average	60

	Classification rate in validation (%)								
	Acacia	Alpine rose	Fir honeydew	Chestnut	Dandelion	Lime	Rape	Polyfloral	Correct
Acacia (n = 7)	71	14	0	0	0	0	0	14	71
Alpine rose (n = 3)	0	100	0	0	0	0	0	0	100
Fir honeydew (n = 16)	0	0	88	0	0	0	0	13	88
Chestnut (n = 8)	0	13	0	75	0	13	0	0	75
Dandelion (n = 7)	0	29	0	0	29	29	14	0	29
Lime (n = 6)	0	17	0	0	0	83	0	0	83
Rape (n = 8)	0	0	0	0	50	0	50	0	50
Polyfloral (n = 57)	9	19	7	5	9	32	0	19	19
								Weighted average	45

Rape honey samples were vice versa often misclassified as dandelion honeys which resulted in a jackknife classification rate of only 63 %. Nectar contributions from dandelion and rape are prevalent in Swiss blossom honeys and may explain the misclassifications between polyfloral, rape and dandelion honeys. Lime honeys showed with 44 % a low rate of correct classification as well. Nearly half of the lime honey samples were assigned to the polyfloral honeys. This may be explained by the variable chemical composition of this honey type as it often contains different amounts of honeydew and thus nonuniform physical and chemical characteristics, similar to polyfloral honeys containing nectar and honeydew.

In validation the classification rates for all honey types diminished even more except for alpine rose and lime honeys. Probably this was due to the small number of samples in validation that happened to be very characteristic. Only 19 % of the polyfloral honeys were correctly classified; samples were misclassified to all groups except rape honey. Especially the high rate of misclassification of the polyfloral honeys into the groups of unifloral honeys makes it impossible to use the developed model for the determination of the eight unifloral and polyfloral honey types studied. The results show that NIR spectra contain too little information for a discrimination of most of the honey types considered.

If only unifloral honeys were considered for classification, all of the honey types studied showed correct classification rates in jackknife classification and validation of higher than 80 % except for dandelion (43 %) and rape honey (63 %) (detailed results are not shown). These findings indicate that analytical methods considering only the unifloral honeys (see introduction) are too optimistic.

The observation that acacia, chestnut and fir honeydew honeys could be nevertheless distinguished from the other unifloral and polyfloral honeys led to the idea to reduce the model to just four groups including acacia, chestnut and honeydew honeys and a so called pooled group combining samples of polyfloral, alpine rose, lime, rape and dandelion honeys. The LDA carried out showed that the above-mentioned unifloral honeys could be well distinguished from the samples of the pooled group (**Table 2**). The classification rates for the three unifloral honeys were considerably higher compared to the ones found for the model considering all honey types as separate groups (**Table 1**). The rates were similar in jackknifed classification and validation indicating that these models were robust. Again the unifloral honeys could be well distinguished from each other by this overall-model. Misclassifications only happened between the pooled group and the unifloral honeys.

The results in jackknife classification and validation (**Table 2**) revealed that honeys from the pooled group were often classified into the groups of acacia, chestnut and fir honeydew honeys. This observation lead to the development a two step procedure. In the first step the samples were classified to one of the four groups by an overall discriminant model. In the second step this classification was verified by using several models consisting of a group formed by samples of a given unifloral honey versus a group called “non-unifloral” consisting of all the other samples. For the verification of the classification by the first model at least the two-group model of the corresponding honey type was used. In addition one to four two-group models (indicted by bold numbers in **Table 2**) were used when a misclassification rate of higher than 3% was calculated in jackknife classification or validation tables of the overall model. The probabilities for misclassification were calculated by applying Bayes’ theorem on the conditional probabilities of disjoint events.

Table 2. Jackknife classification and validation tables for the honey samples as classified by LDA (the samples of dandelion, alpine rose, lime, rape and polyfloral honeys were combined in the pooled group).

	Jacknife classification rate (%)				
	Acacia	Fir honedew	Chestnut	Pooled group	Correct
Acacia (n = 20)	95	0	0	**5**	95
Fir honeydew (n = 49)	0	92	0	**8**	92
Chestnut (n = 26)	0	0	96	**4**	96
Pooled group (n = 248)	**3**	**7**	**7**	84	84
				Weighted average	87

	Classification rate in validation (%)				
	Acacia	Fir honeydew	Chestnut	Pooled group	Correct
Acacia (n = 7)	86	0	0	**14**	86
Fir honeydew (n = 16)	0	88	0	**13**	88
Chestnut (n = 8)	0	0	88	**13**	88
Pooled group (n = 81)	**7**	**5**	**9**	79	79
				Weighted average	81

The error probabilities cannot be directly taken from **Table 2**, they only quantify the conditional probabilities of correct classification given the corresponding honey type. By Bayes' theorem the posterior probabilities of finding the correct honey type given a distinct classification by the discriminant model was calculated, and the error rate is simply the complement to 1. The classification rates for the unifloral honeys in the two-group models were higher than 90 % (**Table 3**). The high rates of correct classification for both, the unifloral and non-unifloral groups considered by the two-group models indicate that the botanical origin of these three unifloral honey types can be reliably determined by this procedure. The classification rate for the samples of the pooled group was with 79 % respectively 65 % considerably lower. However, this is not very important, as we are principally interested in the authentication of unifloral honeys and the correct classification rate of 87 % respectively 84 % shows that unifloral honeys are rarely assigned to the pooled group.

If a sample is assigned to the same honey type by the overall and the two-group model it is very likely that it belongs to this type of honey. If the classifications of the two models do not agree the sample has to be considered to belong to the pooled group. When the sample is assigned to the same honey type by both, the overall model and the corresponding two-group model and is moreover considered to belong to the non-unifloral groups in all the other two-group models tested, the honey sample belongs almost certainly to the honey type indicated by the overall model. The respective error rates of this two-step procedure were calculated by Bayes' theorem.

Table 3. Jackknife and validation table for the honey samples classified by the two-group discriminant models

	Jackknife classification				Validation			
	Unifloral		Non-Unifloral		Unifloral		Non-Unifloral	
	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)
Acacia	20	95	323	96	7	86	81	93
Fir honeydew	49	92	294	94	16	94	81	91
Chestnut	26	100	317	93	8	100	81	85
Pooled group	248	79	95	87	81	65	31	84

Indeed, the approach in two steps allowed to further improve the reliability in discrimination of acacia, fir honeydew and chestnut honeys from the other honey types considered in the pooled group. The error probabilities calculated using Bayes' theorem (misclassification of a sample of unknown botanical origin) were found to be generally lower than 6 % (**Table 4**). Near-infrared spectroscopy can therefore be used for the determination of acacia, chestnut and honeydew honeys. The display of the first and the third linear discriminant scores shows that these three unifloral honeys form distinct groups that do not overlap at all. However, some overlap occurs between the unifloral honeys and samples of the pooled group (**Figure 2**). The interference of the samples of the pooled group, especially of the polyfloral honeys, with the unifloral honeys is characteristic and may be explained by their similar physical and chemical composition.

Table 4. Error probabilities for the classification of acacia, chestnut and fir honeydew honeys and samples belonging to the pooled group, calculated by Bayes' theorem.

	Error probability	
Honeytype	Jackknife	Validation
Acacia	0.022	0.045
Fir honeydew	0.031	0.044
Chestnut	0.030	0.058
Pooled group	$< 10^{-3}$	0.001

According to the current standards (*2, 3*) honeys can be classified into blossom and honeydew honeys according to the electrical conductivity (honeydew honeys having values >0.8mScm^{-1}). However some blossom honey types e.g. lime, chestnut and heather honeys are excluded from these classifications although expressing conductivity values >0.8mScm^{-1}. Therefore there is a need for alternative methods for the discrimination between blossom and honeydew honeys.

When the same samples were assigned to only two groups, i.e. into blossom and fir honeydew honeys, the samples were correctly classified at rates of over 90 % both in jacknifed classification and validation (**Table 5**). Near-infrared spectroscopy seems therefore to present a promising approach for the determination of the two main honey types.

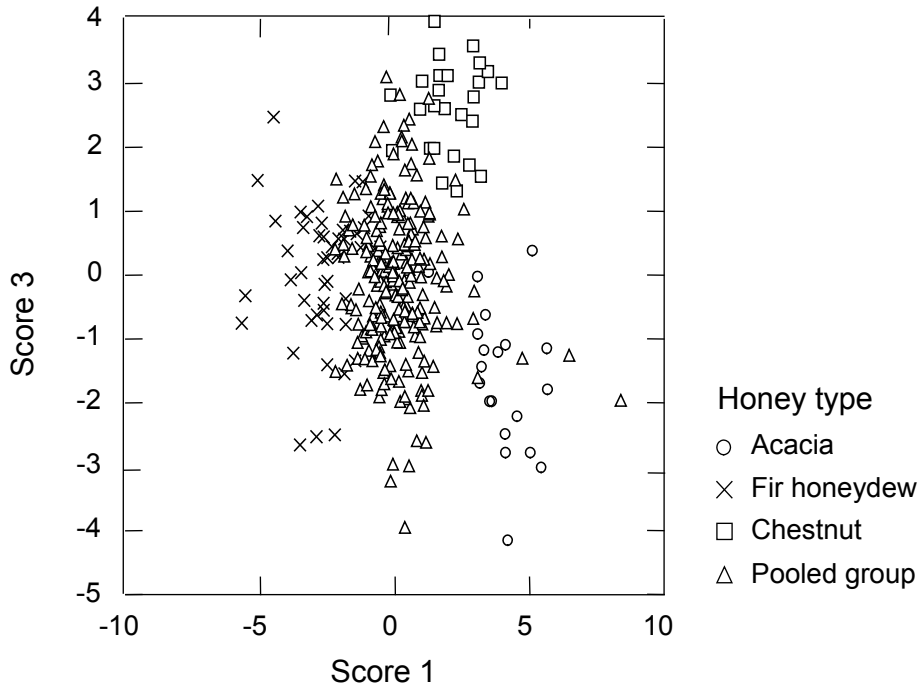

Figure 2. Scatterplot of the canonical discriminant scores

Table 5. Jackknife classification and validation tables for blossom and fir honeydew honeys as classified by LDA

	Jackknife classification rate (%)	
	Blossom	Fir honeydew
Blossom (n = 294)	94	6
Fir honeydew (n = 49)	8	92

	Classification rate in validation (%)	
	Blossom	Fir honeydew
Blossom (n = 96)	93	7
Fir honeydew (n = 16)	6	94

This study shows that near-infrared spectroscopy combined with chemometrics offers a promising approach for the authentication of certain unifloral honeys and that the problems related to the determination of the polyfloral honeys can be handled by the successive use of at least two mathematical models. The methodology permits to discriminate acacia, chestnut and fir honeydew honeys, expressing the most characteristic chemical compositions among the honey types studied. This means that near-infrared spectroscopy and the mathematical models developed agree with the characterisation based on the classical criteria for the above-mentioned honey types.

However the recorded NIR spectra generally show too small specific characteristics to allow a determination of the botanical origin of the eight unifloral and polyfloral honey types studied. The potential of the method could possibly be improved by measuring in transmission mode with a shorter path length where sharper bands and less saturated spectra in the region between 4000 and 7500 cm^{-1} nm were obtained (*16*).

Another way to gain more specific information would be to use an instrument scanning the spectrum from visible to the near infrared region as colour measurements have been shown to be useful for the authentication of some types of honey (*24, 36*). However, this approach may not help to solve problems related to the main obstacle in the determination of the botanical origin of honey, the discrimination between polyfloral and unifloral honeys, because the colour of polyfloral honeys is highly variable.

In addition to the possibility to determine the botanical origin of honey, the same spectra can be used to obtain quantitative information on several measurands important for the routine quality control. Using partial least squares regression models, calibrations proved satisfying accuracies for the determination of water, glucose, fructose, sucrose, the total monosaccharide contents as well as the fructose/glucose and glucose/water ratios (*37*).

A drawback of the current method is that before the botanical origin can be routinely determined , a considerable amount of work has to be carried out to build the chemometric models involved. The possibility to transfer the corresponding models or the spectra between different instruments and laboratories should be verified by future studies.

In conclusion the results demonstrate that near-infrared spectrometry is a valuable, rapid and non-destructive tool for the determination of the botanical origin of some honey types and for quantitative analysis of measurands related to the main components in honey.

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CHAPTER 6

Authentication of the Botanical Origin of Honey by Front-Face Fluorescence Spectroscopy. A Preliminary Study*

ABSTRACT

The potential of front-face fluorescence spectroscopy for the authentication of unifloral and polyfloral honey types (n = 57 samples) previously classified using traditional methods such as chemical, pollen, and sensory analysis was evaluated. Emission spectra were recorded between 280 and 480 nm (excit: 250 nm), 305 and 500 nm (excit: 290 nm) and 380 and 600 nm (excit: 373 nm) directly on honey samples. In addition excitation spectra (290 – 440 nm) were recorded with the emission measured at 450 nm. A total of four different spectral data sets were considered for data analysis. After normalisation of the spectra, chemometric evaluation of the spectral data was carried out using principal component analysis (PCA) and linear discriminant analysis (LDA). The rate of correct classification ranged from 36 % to 100 % by using single spectral data sets (250 nm, 290 nm, 373 nm, 450 nm) and from 73 % to 100 % by combining these four data sets. For alpine polyfloral honey and the unifloral varieties investigated (acacia, alpine rose, honeydew, chestnut and rape) correct classification ranged from 96 to 100 %. This preliminary study indicates that front-face fluorescence spectroscopy is a promising technique for the authentication of the botanical origin of honey. It is non-destructive, rapid, easy to use and inexpensive. The use of additional excitation wavelengths between 320 and 440 nm could increase the correct classification of the less characteristic fluorescent varieties.

6.1 INTRODUCTION

According to the Codex Alimentarius Standard for Honey (*1*) and the European Union Council Directive (*2*) relating to honey, the use of a botanical designation of honey is allowed if it originates predominately from the indicated floral source. At the current stage of knowledge, a reliable determination can be achieved by a global interpretation of sensory, pollen and physico-chemical analyses carried out by an expert (*3, 4)*. As several analytical methods are simultaneously necessary for a reliable authentication of unifloral honeys, such work is time consuming and costly. Thus, there is a need for new methods that allow a rapid and reproducible authentication of the botanical origin of honey at low cost (*5*). The use of front-face fluorescence spectroscopy seems to be a promising approach.

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Ruoff, K.; Karoui, R.; Dufour, E.; Luginbühl, W.; Bosset, J. O.; Bogdanov, S.; Amadò, R. Authentication of the Botanical Origin of Honey by Front-Face Fluorescence Spectroscopy A Preliminary Study. *J. Agric. Food Chem.* **2005**, *53*, 1343-1347.

Fluorescence spectroscopy provides information on the presence of fluorescent molecules and their environment in biological samples. Food products contain numerous intrinsic fluorophores, and are therefore suitable for fluorescence spectroscopic investigations. Honey contains small amounts of proteins, peptides and free amino acids which include tryptophan, tyrosine, and phenylalanine residues. Following excitation of a protein solution at 290 nm, characteristic fluorescence emission spectra of tryptophan residues can be recorded. When fluorescence of tryptophan, tyrosine and phenylalanine has to be considererd, the excitation wavelength is set at 250 nm and the fluorescence emission is recorded between 280 and 480 nm. However numerous fluorescent compounds such as nucleic acids and polyphenols found in food products may fluoresce following excitation in the 250-280 nm range. Food products contain also vitamins, some of which are fluorescent compounds. Riboflavin is due to its conjugated double bonds, a good fluorescent probe, exhibiting fluorescence emission spectra (400 – 640 nm) following excitation at 380 nm.

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.

With classical right-angle fluorescence spectroscopy, the measurements are carried out in dilute solutions where the absorbance is below 0.1. At a higher absorbance rate, a decrease of fluorescence intensity and a distortion of emission spectra are observed due to the inner filter effect. To overcome such problems, front-face fluorescence spectroscopy was developed (*6*) where only the surface of the material is illuminated and examined. The emitted photons are collected at an angle of 56° to the surface of the sample, in order to minimise artefacts generated by the photons of excitation reflected from the sample (*7*). This technique allows a quantitative investigation of fluorophores in powders as well as in concentrated or even opaque samples.

Food has 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 normalised fluorescence spectra in combination with multivariate statistics can be used for characterising and identifying different food. This has already been shown for processed milk (*8*), authenticating the geographical origin of cheese (*9*), as well as studying cheese ripening and structure (*10-12*).

Unifloral honeys are well known to contain numerous polyphenols (*13-16*) as well as other fluorophores such as amino acids (*17, 18*). Some of them have already been proposed as tracers for unifloral honeys, for instance ellagic acid for heather honey from Erica and Calluna species (*19*) or hesperetin for orange (*Citrus* spp.) honeys (*20, 21*). As polyphenols are strong fluorophores, fluorescence spectroscopy should be helpful for authenticating the botanical origin of honey.

Also, fluorescent amino acids have been proposed as markers for unifloral honeys. Phenylalanine and tyrosine were found to be characteristic for lavender honeys and allowed a differentiation from eucalyptus honeys (*22*). Tryptophan and glutamic acid were used for the differentiation between honeydew and blossom honeys (*23*). Therefore the aim of the current work is to study the fluorescence characteristics of seven different varieties and to develop a rapid, non-destructive, low-cost and reliable method for authentifying unifloral honeys.

6.2 MATERIALS AND METHODS

6.2.1 SAMPLING AND BOTANICAL CLASSIFICATION BY REFERENCE METHODS

A total of 57 honey samples produced in Switzerland between 1998 – 2001 were collected and stored at 4 °C until analysis. To classify these honey samples the following measurands were determined according to the harmonised methods of the European Honey Commission (*24*): electrical conductivity, sugar composition, fructose/glucose ratio, pH-value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (*25, 26*). Based on these analytical results and sensorial evaluation by four experts, the honey samples were assigned to one of the seven following honey types: acacia (*Robinia pseudoacacia*) (n = 7), alpine rose (*Rhododendron* spp.) (n = 5), chestnut (*Castanea sativa*) (n = 9), rape (*Brassica* spp.) (n = 10), honeydew (n = 8), alpine polyfloral (n = 7), and lowland polyfloral honeys (n = 11).

6.2.2 FLUORESCENCE SPECTROSCOPY

An aliquot part of 20 g of the honey samples was liquefied at 40 °C for 8 h, then allowed to cool to room temperature and pipetted into a 1 cm quartz cuvette. The latter was placed in the sample holder for the recording of the fluorescence spectra which was done by using a FluoroMax-2 (Spex-Jobin Yvon, F-91165 Longjumeau, France) spectrofluorometer equipped with a variable angle front-surface accessory, with the incident angle of the excitation radiation set to 56°. All spectra were corrected for instrumental distortions in excitation using a rhodamine cell in the reference channel.

Using the excitation wavelengths of 250 nm, 290 nm and 373 nm, the fluorescence emission spectra were recorded from 280 to 480 nm (increment 1 nm; slits at excitation: 3.5 and at emission: 2.0), 305 - 500 nm (increment 1 nm, slits at excitation: 2.5 and at emission: 2.0), and 380 - 600 nm (increment 2 nm; slits at excitation and emission 1.5), respectively. Fluorescence excitation spectra were recorded with excitation wavelength from 290 to 440 nm and measurement of light emission at 450 nm (increment:1 nm; slits at excitation: 2.0 and at emission: 1.5). Three spectra were recorded using different aliquots of each sample.

6.2.3 PROCESSING OF SPECTRA AND MULTIVARIATE ANALYSIS

First a normalisation of each spectrum was done in order to reduce the residual scattering effects according to Bertrand and Scotter (*27*) using the formulas:

$$c_i = F_i / \text{norm}$$

and

$$\text{norm} = \sqrt{\sum_{j=1}^{n} F_j^2}$$

where c_i is the normalised value at the emission wavelength i, F_i is the raw fluorescence intensity at the emission wavelength i, F_j is the fluorescence at wavelength j, and n is the number of data points for each spectrum.

Principal component analysis (PCA) was used to eliminate the spectral collinearity, random noise and to reduce the number of variables for subsequent analysis. It was performed on two different data sets. The subsequent linear discriminant analysis (LDA) was performed on the PC covering at least 99 % of the total spectral variability (SYSTAT® Version 10.2).

6.3 RESULTS AND DISCUSSION

6.3.1 FLUORESCENCE SPECTRA OF DIFFERENT HONEY TYPES

The recording of fluorescence spectra at various excitation and emission wavelengths was performed to study the differences between the seven honey types. **Figures 1-3** show their normalised fluorescence (emission) spectra. Every spectrum is more or less typical for a given honey type. As most of the spectra represent very similar shapes and can therefore visually hardly be distinguished only a few of the most different spectra are shown in the figures. The various spectra were recorded using different aliquots of the same sample.

For the spectra recorded following excitation at 250 nm (**Figure 1**), all honey types except chestnut honey exhibit broad and overlapping emission bands with at least two maxima located between 320-390 nm and 390-460 nm, respectively. The very characteristic fluorescence spectrum of chestnut honey shows a much narrower band with at least three shoulders and a maximum at approximately 380 nm. The two small peaks at 402 nm and 433 nm observed in all honey types (**Figures 1** and **2**) are artefacts probably due to instrumental interferences.

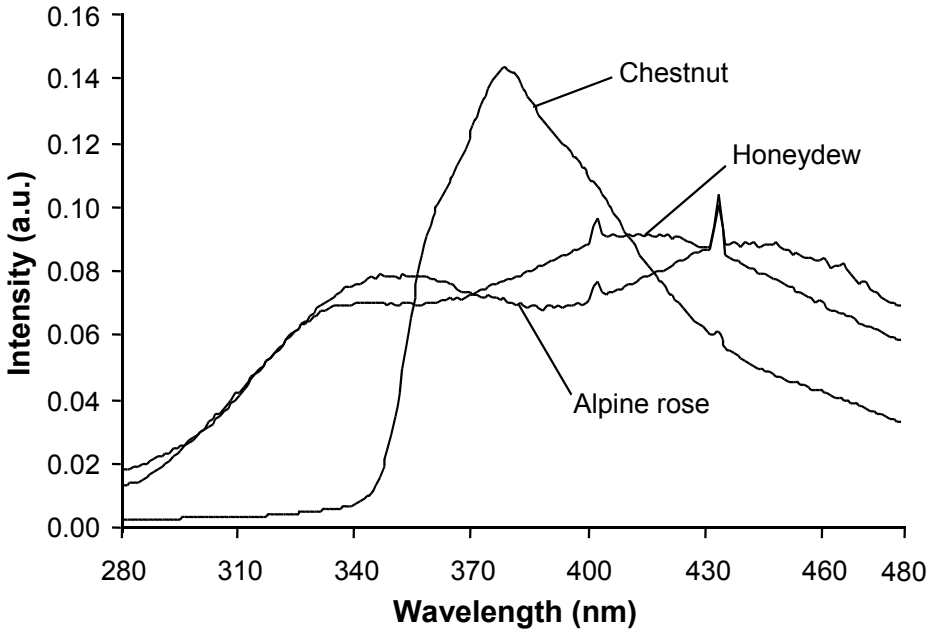

Figure 1. Normalized fluorescence spectra of different honey types (excitation at 250 nm)

The most significant differences between the spectra of the honey types under investigation were found at the excitation wavelength of 290 nm (**Figure 2**). The spectra of chestnut and honeydew honeys show maxima at about 375 nm and 410 nm, respectively, and express completely different shapes. But all spectra show at least two broad overlapping emission bands.

At the excitation wavelength of 373 nm (**Figure 3**) the spectra of chestnut honey again clearly differs from the other honey types investigated, but the shapes of the latter are much more similar, including a maximum at about 450 nm. The emission spectra shown in **Figures 1-3** are due to numerous fluorescent compounds occurring in the various honey types in different concentrations and in different environments leading to the various forms of these spectra.

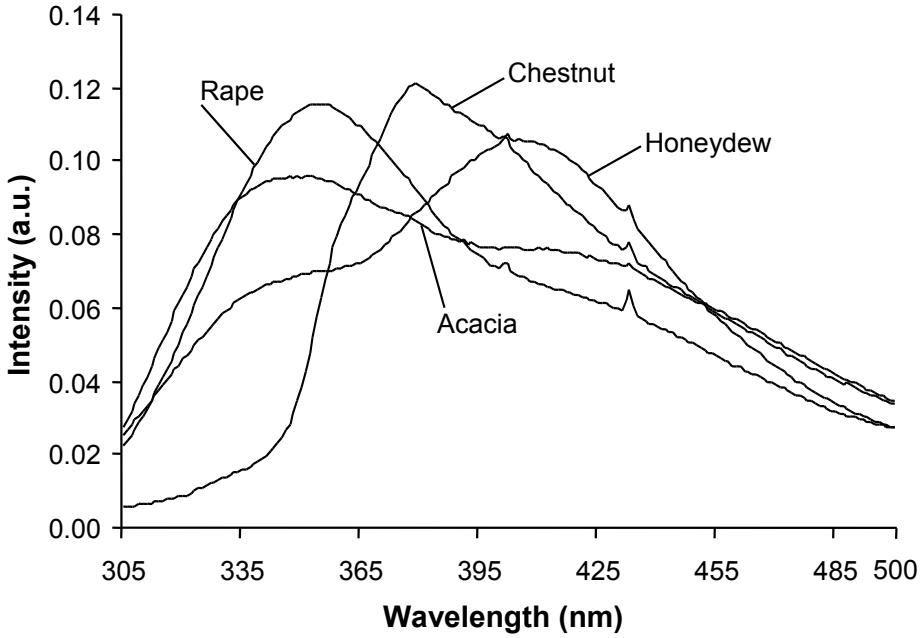

Figure 2. Normalized fluorescence spectra of different honey types (excitation at 290 nm)

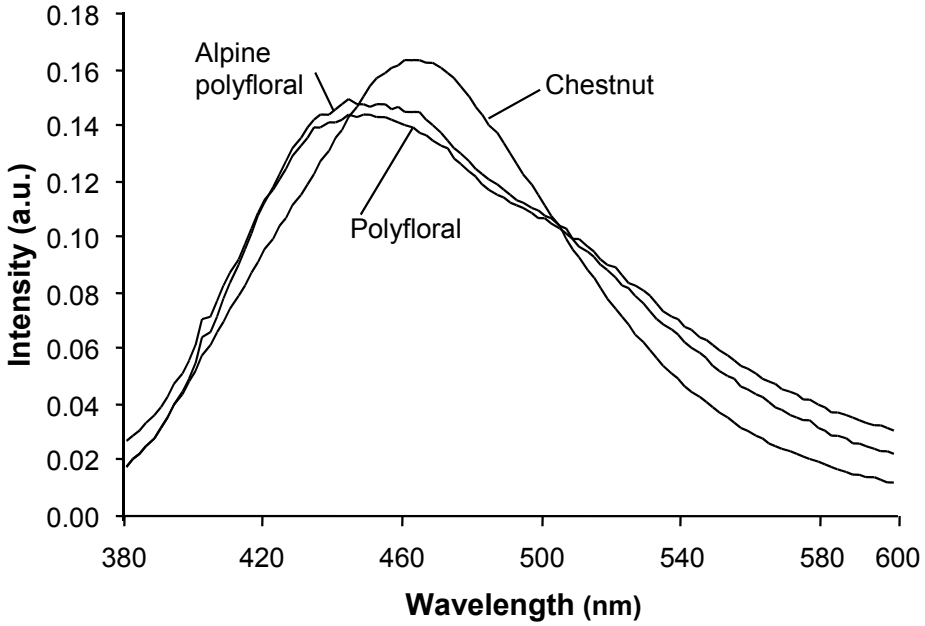

Figure 3. Normalized fluorescence spectra of different honey types (excitation at 373 nm)

When the excitation spectra were scanned from 290 to 440 nm with emission measured at 450 nm, several shoulders were observed between 330 and 370nm (**Figure 4**). For most honey types the maxima were located at about 370 nm, chest-

nut honey showing an additional maximum at about 390 nm. Chestnut, alpine rose, and acacia honeys were shown to be the most distinctly different.

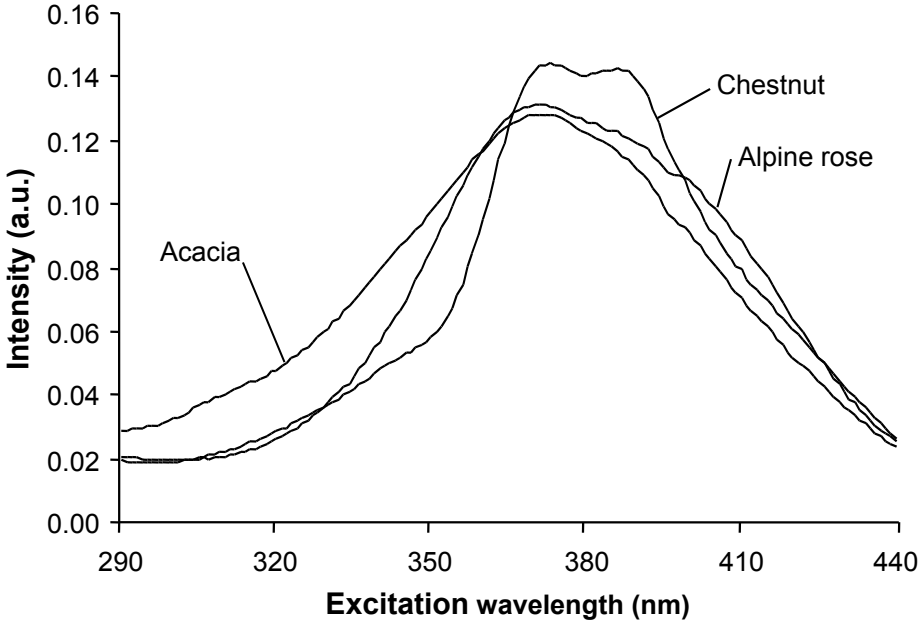

Figure 4. Normalized fluorescence spectra of different honey types (emission at 450 nm)

It has been shown that chestnut honey, compared to the other honey types analysed in this study, contains relatively high amounts of hydroxycinnamates such as caffeic, p-coumaric and ferulic acids as well as unidentified flavonoids (*13, 28*). Chestnut honey may also contain more phenylalanine than the other honey types analysed in this study (*18*). This may explain the differences in the fluorescence spectra.

6.3.2 LINEAR DISCRIMINANT ANALYSIS (LDA) ON THE FLUORESCENCE SPECTRA

LDA was performed on the principal component scores of each type of spectra as well as on the combination of the four different types of spectra. To build a classification model that potentially holds for single measurements of given samples all of the three spectra recorded per sample (instead of mean spectra) were statistically treated as independent objects in order to include instrumental measurement uncertainty and variation from replicate measurements (pure spectral random noise is eliminated by PC data reduction). Chemometric evaluation using single type of spectra resulted in rather poor classification rates except for chestnut honey (**Table 1**). The average rate of correct classifications was about 70 % ranging from 36 % for lowland polyfloral up to 100 % for chestnut and rape honeys. The most useful type of spectra for the discrimination between different honey types were the emission spectra recorded following excitation at 290 nm and the excitation spectra (290 – 440 nm). The spectra recorded at an excitation wavelength of 290 nm allow a correct classification of chestnut and rape honey. With a rate of 80 % these conditions were also the best ones for the authentication of alpine rose honey. Considering emission spectra recorded in the 380 – 600 nm range, honeydew honey could be recognised with a probability of 92 % from the other honey types. The excitation spectra (290 - 440 nm) were most useful for the authentication of acacia and alpine honeys (**Table 1**).

Table 1. Percentage of correct classification by using single data sets at different excitation and emission wavelengths and by combining of the data of the four different wavelengths (jackknifed classification by the "leave one out" method)[a].

	Correct classification (%)				
	by using a single data set				combining the four data sets
Botanical origin	250 nm	290 nm	373 nm	450 nm	
Acacia (n = 7)	57	71	71	81	100
Alpine rose (n =5)	60	80	53	73	100
Alpine polyfloral (n = 7)	76	57	52	86	100
Honeydew (n= 8)	75	75	92	83	96
Chestnut (n = 9)	100	100	100	100	100
Polyfloral (n = 10)	36	45	55	48	73
Rape (n = 10)	80	100	60	70	97
average	69	75	70	76	94

[a]The spectra were recorded after excitation at following wavelengths spectra 250 nm, 290 nm and 373 nm or by measuring the emission at 450 nm when scanning the excitation from 290 to 440 nm. (n = number of samples)

Combining the individually reduced data of the four types of spectra significantly improved the rate of correct classification of all honey types. The LDA was based on 19 principal components. The average classification rate rose to 94 % (**Table 2**). Acacia, alpine rose, alpine polyfloral and chestnut honey varieties were correctly assigned.

Table 2. Jackknifed classification of the combination of four data sets (n = no. of samples)

Honey type	Acacia	Alpine rose	Alpine polyfloral	Honey-dew	Chestnut	Lowland polyfloral	Rape	correct (%)
Acacia (n =7)	21							100
Alpine rose (n = 5)		15						100
Alpine polyfloral (n = 7)			21					100
Honeydew (n = 8)				23		1		96
Chestnut (n = 9)					27			100
Lowland polyfloral (n = 10)		3			3	24	3	73
Rape (n = 10)						1	29	97
total (n = 57)	21	18	21	23	30	26	32	94

Honeydew and rape honeys reached with 96 % and 97 %, respectively, high rates of correct assignment. The lowest correct classification rate (73 %) was found for the lowland polyfloral honeys. Three samples (nine spectra) were wrongly assigned (**Table 2**). One was assigned to alpine rose honey a second to the chestnut honey, and a third to rape honey.

The higher rate of misclassifications for the lowland polyfloral honeys can be well explained by the fact that the latter generally consist of small amounts of nectar or honeydew produced by various plants species. Therefore they do not have distinct physical or chemical properties that would correspond to their fluorescence characteristics. Rape is one of the most important nectar producing plants in Switzerland, and most lowland polyfloral honeys contain considerable amounts of this honey type. This explains why some lowland polyfloral honeys are misclassified as unifloral rape honeys and vice versa (see **Table 2** and **Figure 5**). One spectrum of a honeydew honey sample was classified as a lowland polyfloral honey.

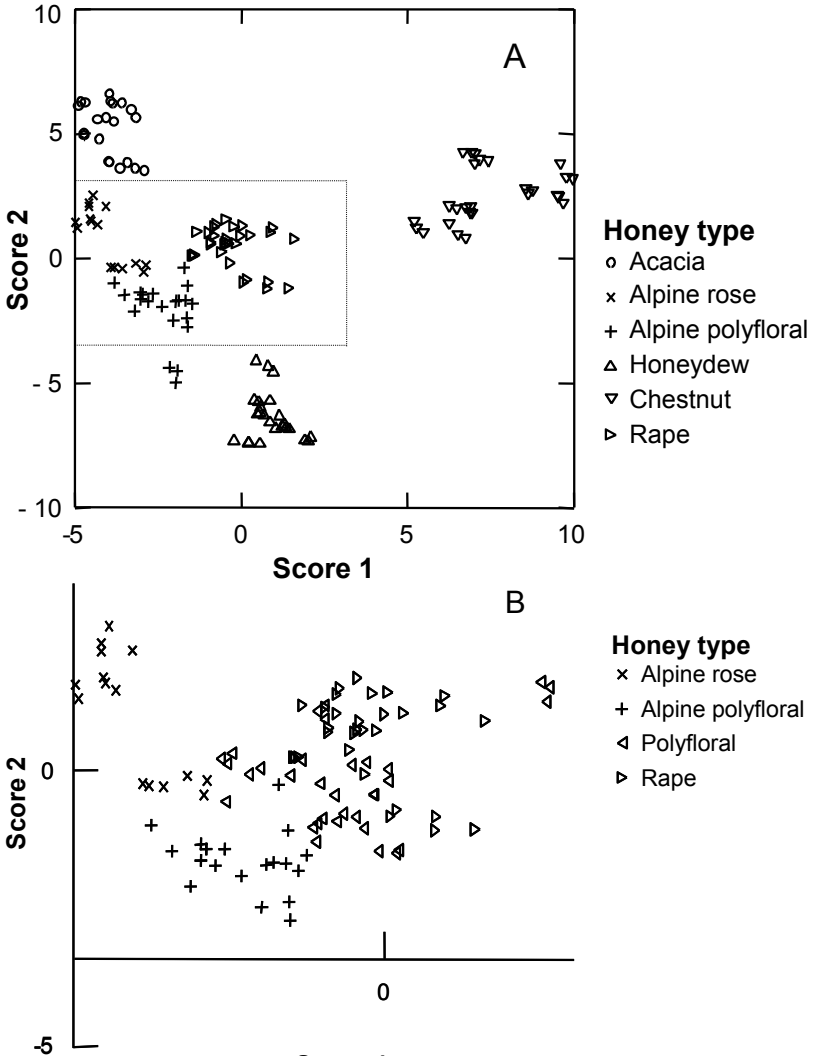

Figure 5.
(**A**) Scatterplot of canonical discriminant scores without lowland polyfloral honey, dashed line designates enlargement.
(**B**) Enlargement from scatterplot of canonical discriminant scores with lowland polyfloral honey samples included.

Furthermore the scatterplot of the scores of the first two discriminant functions from the LDA revealed some interesting characteristics of the honey samples analysed. As numerous plant species contribute to the characteristics of lowland polyfloral honeys, their corresponding cluster is the least homogeneous and is located between the clusters of alpine polyfloral and the rape honeys (**Figure 5B**).

One sample of lowland polyfloral honey was located among the rape honey samples. However, the pollen analysis revealed a relative frequency of 57 % of *Brassica* sp. pollen, in fact just below the 70 % threshold set in Switzerland for a unifloral rape honey based on traditional methods. Another so called lowland polyfloral honey was found between the clusters of rape and chestnut honey (**Figure 5B**). It was classified as lowland polyfloral honey as it predominantly consisted of apple and rape nectar but had also a minor contribution of chestnut. Alpine rose honey seemed to consist of two clusters that may be explained as follows: the samples which contain more alpine rose pollen, and are thus assumed to be purer honeys, are located in the neighbourhood of acacia honeys. As the other alpine rose honey samples contain less alpine rose pollen they are expected to have nectar contributions from other alpine plant species. Consequently they are located close to the group of alpine polyfloral honeys but were still, based on the classical criteria, considered as unifloral alpine rose honeys. Moreover one sample of alpine polyfloral honeys lies in the figure closer to the group of honeydew honeys. Its pollen analysis revealed that it contains a considerable amount of raspberry (*Rubus* spp.) honey.

Despite of the limited number of samples, another principal component matrix was generated for the honey types represented by 8-11 samples, i.e., honeydew, chestnut, lowland polyfloral and rape, to evaluate the potential of the discriminant function generated, by randomly assigning samples to a calibration and a validation set. About two thirds of the spectra were used as calibration spectra in order to build the model and about one third of the spectra as validation spectra for the evaluation of performance of the model. The classifications of the samples in the validation group are shown in **Table 3**. All samples but one (a sample of rape honey; three spectra) were correctly classified.

Table 3. Validation of the discriminant function (combining the four data sets, n = number of samples)

Honey type	Honeydew	Chestnut	Polyfloral	Rape	Correct (%)
Honeydew (n = 2)	6	0	0	0	100
Chestnut (n = 3)	0	9	0	0	100
Polyfloral (n = 3)	0	0	9	0	100
Rape (n = 3)	0	0	3	6	67
Total (n = 11)	6	9	12	6	91

This preliminary study shows that front-face fluorescence spectroscopy combined with chemometrics offers a promising approach for the authentication of the botanical origin of honey. The technique is non-destructive, rapid, easy to use and not expensive. It does neither need any particular sample preparation nor special qualification of the personnel. The current results show that there is a strong correlation between the classic methods for the authentication of different honey types and the fluorescence characteristics of the honey samples studied.

Unifloral honeys with very characteristic fluorescence spectra, such as chestnut honey, can be easily recognised using only one of the single spectra recorded. Honey types having less characteristic spectra, such as alpine polyfloral or lowland polyfloral honeys, need a combination of several spectra for a reliable authentication.

To a certain extent, the use of complementary excitation spectra (probably between 320 and 440 nm) could help to increase the correct classification rate of less typical honey varieties. However these preliminary findings should be confirmed with a larger set of samples and additional honey types.

ACKNOWLEDGEMENT

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CHAPTER 7

Authentication of the Botanical and Geographical Origin of Honey by Front-Face Fluorescence Spectroscopy*

ABSTRACT

Front-face fluorescence spectroscopy, directly applied on honey samples, was used for the authentication of eleven unifloral and polyfloral honey types (n = 371 samples) previously classified using traditional methods such as chemical, pollen, and sensory analysis. Excitation spectra (220 – 400 nm) were recorded with the emission measured at 420 nm. In addition emission spectra were recorded between 290 and 500 nm (excitation: 270 nm) as well as between 330 and 550 nm (excitation: 310 nm). A total of four different spectral data sets were considered for data analysis. Chemometric evaluation of the spectra included principal component analysis (PCA) and linear discriminant analysis (LDA), the error rates of the discriminant models were calculated by the Bayes' theorem. They ranged from < 0.1% (polyfloral and chestnut honeys) to 9.9 % (fir honeydew honey) by using single spectral data sets and from < 0.1% (metcalfa honeydew, polyfloral and chestnut honeys) to 7.5 % (lime honey) by combining two data sets. This study indicates that front-face fluorescence spectro-scopy is a promising technique for the authentication of the botanical origin of honey and may also be useful for the determination of the geographical origin within the same unifloral honey type.

7.1 INTRODUCTION

According to the Codex Alimentarius Standard (*1*) and the European Union Council Directive (*2*) relating to honey, the use of a botanical designation of honey is allowed if it originates predominately from the indicated floral source. Honey may also be designated by the name of a geographical region if it was produced within the area referred to (*1, 2*).

The vast majority of the honeys on the market contain significant nectar or honeydew contributions from several plant species and are therefore called polyfloral or multifloral honeys. Normally they are just designated with the word "honey". Probably no honey produced by free bees flying is purely unifloral. The term unifloral honey is used to describe honey in which the major part of nectar or honeydew is derived from a single plant species. Honey composition, flavour and colour varies considerably depending on the botanical source it originates from (*3*).

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The physical, chemical and pollen analytical characteristics of the most important European unifloral honeys have been described in various papers (*3-7*). On contrary to the unifloral honeys the polyfloral honeys do not exhibit distinct physical or chemical characteristics apart from a huge variability, which makes their authentication particularly difficult.

The interest in the production of unifloral honeys is caused by higher consumer preference for some honey types generating a commercial concern of the beekeepers. The recent interest in the therapeutic or technological use of certain honey types may also contribute to the demand of a reliable determination of the botanical origin.

7.1.1 BOTANICAL ORIGIN

A number of new analytical techniques combined with multivariate data analysis have been proposed for the determination of the botanical origin of honey. They are for example based on physical and chemical measurands determined during quality control of honey (*8, 9*) or the former combined with the determination of mineral content (*10*), as well as carbohydrate composition (*11*), amino acid composition (*12*), mass-spectrometry or metal oxide semiconductor based gas sensors (*13, 14*), differential scanning calorimetry (*15*), pyrolysis mass spectrometry (*16*), raman (*17*) and near-infrared spectroscopy (*18*).

Many of the methods mentioned above allow to clearly discriminate between several types of unifloral honeys, but none of these methods accounts for the polyfloral honeys that represent the majority of the honeys produced. This means that these methods may not be useful in analytical practice, as the great challenge in honey analytics is not to distinguish between several unifloral honey types but to discriminate the minority of unifloral honeys from the overwhelming majority of polyfloral honeys on the market. This also explains why until now none of these proposed methods are commonly used for the determination of the botanical origin of honey.

Only a single ion chromatographic method has been tested in the presence of polyfloral honeys and showed a potential to discriminate between several unifloral as well as polyfloral honey samples by first classifying the honey samples into two groups by colour measurements (*19*). However, only very few samples were analysed in this study and it remains to be verified if this methodology is useful in analytical practice.

Currently, a reliable determination of the botanical and geographical origin can only be achieved by a global interpretation of sensory, pollen and physico-chemical analyses carried out by experts (*4, 20, 21*). However, the uncertainty related to the interpretation of pollen analytical results, originating from a number of different factors demands the development of new analytical methods (*22*).

7.1.2 GEOGRAPHICAL ORIGIN

Pollen analysis is currently used to determine the geographical origin of honey as pollen in honey reflect the vegetation type where the nectar has been collected by the bees. In the past many analytical methods such as amino acid composition (*23, 24*), raman spectroscopy (*17*), mineral content (*25, 26*), sugar or mineral composition combined with common chemical quality control data (*27-29*) together with multivariate data evaluation have been proposed for the determination of the geographical origin.

Unfortunately in most of the above quoted studies the botanical origin of the honey samples was not determined, or the discrimination between the geographical origins was not verified on samples of the same botanical origin. Generally the sample sets analysed were small or limited to a small geographical area. The distinctions found are therefore rather due to differences of the vegetation type between the geographical regions and thus to the botanical origin of honey (30). A geographical discrimination will therefore be found when the differences are related to the vegetation type present in these areas.

As several analytical methods have to be used together for a reliable authentication of the botanical origin, such a work is time consuming and costly. Very specialised expertise is needed for the interpretation of the pollen spectrum used for the determination of the geographical origin of honey. Thus, there is a real need for new methods that allow a rapid and reproducible authentication of the botanical and geographical origin of honey at low cost (*21, 31*).

7.1.3 FLUORESCENCE SPECTROSCOPY

Compared to 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 inorganic and organic materials. In addition, front-face fluorescence spectroscopy allows an investigation of fluorophores in powders as well as in concentrated or opaque samples (*32, 33*).

Honey is known to contain fluorophores such as polyphenols (*34-37*) and amino acids (*38, 39*). Fluorescence spectroscopy should therefore be helpful for authenticating the botanical origin of honey. More detailed information on fluorescence spectroscopic applications to honey and other food can be found in our previous study that already showed that front-face fluorescence spectroscopy is a promising approach for the determination of the botanical origin of honey (*40*).

The aim of the current work was to study the fluorescence spectroscopic characteristics of eleven honey types and to develop a rapid, low-cost and reliable method for the authentication of unifloral and polyfloral honeys. As the physical and chemical characteristics of honey may be changed by adulteration the potential of fluorescence spectroscopy was also studied on this subject. As minor nectar contributions from plant species other than the unifloral source may contribute to regional characteristics of unifloral honeys the potential of fluorescence spectroscopy for the determination of the geographical origin of honey was studied as well.

7.2 MATERIALS AND METHODS

7.2.1 SAMPLING AND BOTANICAL CLASSIFICATION BY REFERENCE METHODS

A total of 371 honey samples produced between 1998 and 2004 were collected and stored at 4 °C until analysis. They originated predominantly from Switzerland (CH) but samples from Germany (D), Italy (I), Spain (E), France (F), Slovenia (SLO) and Denmark (DK) were also included.

To classify these honey samples corresponding to their botanical origin the following measurands were determined according to the harmonised methods of the European Honey Commission (*41*): electrical conductivity, sugar composition, fructose/glucose ratio, pH-value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (*42, 43*).

Based on these analytical results, the honey samples were assigned to one of the following eleven honey types, according to the criteria of Persano and Piro (3): acacia (*Robinia pseudoacacia*) (CH, n = 14; D, n = 4; F, n= 3); alpine rose (*Rhododendron* spp.) (CH, n = 14; I, n = 5); chestnut (*Castanea sativa*) (CH, n = 21; I, n = 5; F, n = 3); rape (*Brassica* spp.) (CH, n = 22); fir honeydew (*Abies* and *Picea* spp.) (CH, n = 56; D, n = 63; SLO, n = 2); oak honeydew (*Quercus* spp.) (E, n = 8); honeydew from *Metcalfa pruinosa* (I, n = 14); heather (*Calluna vulgaris*) (D, n = 21; DK, n = 2); lime (*Tilia* spp.) (CH, n =14; D, n = 9; I, n = 4); dandelion (*Taraxacum* s.l.) (CH, n = 10; D, n = 7; I, n = 2) and polyfloral honeys (CH, n = 68). In the heterogenous group of the polyfloral honeys nectar or honeydew contributions from all of the above-mentioned sources were represented.

7.2.2 ADULTERATED HONEYS

In order to evaluate the potential of fluorescence spectroscopy to detect beet sugar adulteration, an artificial honey was produced by feeding two colonies after the nectar flow, in autumn, with a sucrose solution of 62.5 g/100 g generally used as winter feed of bee colonies in Switzerland. The sucrose solution was converted into artificial honey by the bees and left to ripen in the combs until extraction. To evaluate the possibility to detect honey adulteration by fluorescence spectroscopy six chestnut and six acacia honey samples were adulterated with 50 % of the artificial honey produced.

7.2.3 FLUORESCENCE SPECTROSCOPY

An aliquot of 20 g of the honey samples was liquefied at 55 °C for 8 h, allowed to cool to room temperature and poured into a 1 cm quartz cuvette. The latter was placed into the sample holder of a Perkin Elmer LS 50 B Luminescence Spectrometer (Perkin Elmer, Beaconsfield, UK) equipped with a variable angle front-surface accessory, with the incident angle of the excitation radiation set to 56°. Spectra were recorded at a scan rate of 150 nm/min and saved as ASCII textfiles. Instrumental artifacts were corrected in excitation using a rhodamine cell in the reference channel.

7.2.4 METHOD DEVELOPMENT

In order to find additional wavelength ranges with specific emission or excitation for the honey types of interest in addition to those already used in the preliminary study (*40*), the following ranges were studied. An excitation scan between 220 and 440 nm and recording of the fluorescence intensity at 420 and 490 nm was carried out. Six further emission scans were recorded with wavelengths between 220 – 600 nm, with excitation wavelengths being 210, 270 nm, 310 nm, 350 nm, 390 nm and 440 nm, respectively. The following three instrumental settings yielded the most discriminating fluorescent spectra for the ten types of unifloral honeys studied: excitation scan between 220 and 440 nm with the fluorescence emission measured at 420 nm, (method A); using the excitation wavelenghts of 270 nm and 310 nm, fluoresecence emission spectra were recorded from 290 to 500 nm, (method B) and from 330 to 550 nm, (method C), respectively. The excitation slit-width was set to 10 nm and the scan speed to 150 nm/min for all of the three methods. Two spectra were recorded using different aliquots of each sample. The spectra of the honey types studied are shown in the **Figure 1**.

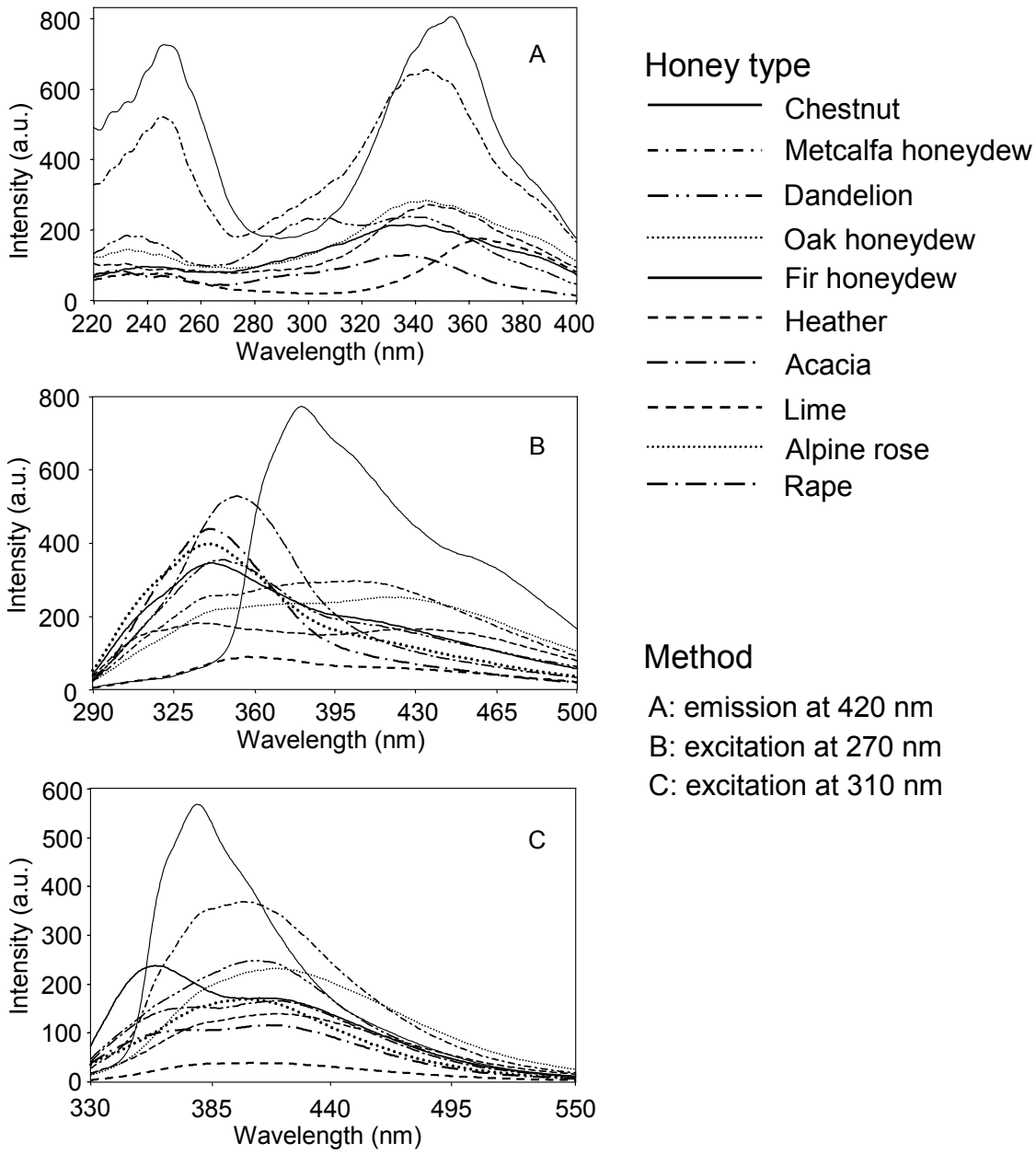

Figure 1. Fluorescence spectra of different honey types

A control honey sample for the evaluation of instrumental stability and determining the intermediate precision of the method was prepared by heating an acacia honey for 20 min up to 100 °C, then filtered to remove the pollen grains and partitioned into 2 ml glass vials, then stored at -20 °C until analysis. The intermediate precision was determined by recording spectra of the control honey sample on 18 days of analysis within 1.5 month. The small coefficients of variation indicate that instrumental conditions were reasonably stable over the duration of the measurements (**Table 1**).

7.2.5 PROCESSING OF SPECTRA AND MULTIVARIATE ANALYSIS

The spectra were converted into the GRAMS spc-format (GRAMS/32 AI Vs. 6.0, Thermo Galactic, Salem NH, U.S.A.) for more convenience in the visual examination and data reduction. It was found that a normalisation of the spectra was not necessary and that the consideration of the fluorescence intensities can even improve the possibilities in discriminating the different unifloral honeys (*40*).

To avoid random noise resulting from instrumental effects, only the following spectral ranges were used for multivariate analysis: method A: 224- 398 nm; method B: 290-500 nm and method C: 333- 547 nm. These ranges were also used for the combination of the spectra.

After elimination of spectral outliers, principal component analysis (PCA) was applied to eliminate the spectral collinearity and to reduce the number of variables to 20 PC's (using the PLSplus/IQ Add-on of GRAMS/32 AI Vs. 5.09). This was performed separately for each type of spectra and each combination of different types of spectra.

In linear discriminant analysis (LDA), the 20 initial PC's were further reduced by backward elimination of principal components on the basis of their partial F-values in the discriminant models (SYSTAT® Version 11, Systat Software Inc., Richmond, USA). The models were then optimised for maximum correct classification in jackknife classification. To account for the limited precision of single measurements, both spectra of each sample were used in the model of single types (A, B, and C) of spectra rather than the average. In the models using combined spectra, averaged spectra were used. The validation was carried out using spectra of one third of the samples selected randomly and not present in the group of samples used to build the model.

7.2.6 GEOGRAPHICAL ORIGIN

The applicability of fluorescence spectroscopy for the determination of the geographical origin of honey was evaluated for the honey types where samples originating from different countries were available. The differences resulting from the geographic origin were studied within the groups of unifloral honeys by using MANOVA (SYSTAT® Version 11, Systat Software Inc.) as well as LDA and are visualised by plots of the canonical discriminant scores (**Figure 3** and **4**).

7.3 RESULTS AND DISCUSSION

7.3.1 REPEATABILITY

The repeatability of the three different methods was determined by a six-fold measurement at the maximum intensity (I_{max}) of an acacia honey. With coefficients of variation (cv_r) between 1.1 and 2.6 % the methods showed a good repeatability which stayed in the same range over 43 days while the intermediate precision was determined (**Table 1**).

Table 1. Repeatability and intermediate precision of the three fluorescence spectroscopic methods

	Method		
Repeatability (n = 6)	A	B	C
Average I_{max} (au)*	179.8	185.8	100.9
reproducibility s_r (au)	1.9	2.1	2.6
coefficient of variation cv_r (%)	1.1	1.1	2.6
Repeatability limit (r) (au)	5.4	5.9	7.5
Relat. repeatability limit (%)	3	3.2	7.4
Intermediate precision (n = 18)			
Average I_{max} (au)	203	195	106
Laboratory reproducibility s_L (au)	7.2	2.7	2.4
Relat. laboratory reproducibility v_L (%)	3.6	1.4	2.2

*arbitrary units (au)

7.3.2 FLUORESCENCE SPECTRA OF DIFFERENT HONEY TYPES

The recorded fluorescence spectra at three different excitation and emission wavelengths for the ten unifloral honey types considered are displayed in **Figure 1** (for better legibility the spectra of alpine rose and rape honey are not shown in **Figure 1 A** as they are visually very similar to fir honeydew honey). Every spectrum is typical for a given honey type. The spectra obtained by the different methods were recorded using different aliquots of the same sample.

Excitation spectra were scanned from 220 to 400 nm with the emission measured at 420 nm (method A, **Figure 1 A**). For most honey types two maxima at about 240 nm and between 340 and 360 nm, respectively were observed, while lime honey exhibits its second maximum at about 365 nm. Most of the honey types investigated had their intensity within the same order of magnitude except for chestnut honey that shows a nearly two-fold intensity at the maximum. Metcalfa honeydew honey is also characterised by a more intense fluorescence. Dandelion honey shows an additional shoulder at about 300 nm.

For the spectra recorded using excitation at 270 nm (method B, **Figure 1 B**), all honey types except chestnut, rape and lime honeys exhibited broad and overlapping emission bands including at least two maxima located between 330-350 nm and 400-440 nm, respectively. The very characteristic fluorescence spectrum of chestnut honey showed a much narrower band with two shoulders and a maximum at approximately 380 nm. Rape and lime honeys showed both maxima at about 350 nm while the latter had a broader emission between 400 and 500 nm. Alpine rose honey showed a shoulder at about 310 nm and a maximum at about 340 nm. For heather, fir honeydew, dandelion, acacia, rape and alpine rose honeys the intensities at the maxima ranged between 150 and 520 arbitrary units while chestnut exhibited a considerably higher intensity of about 800 units. The lowest intensity was detected for lime honey. However, the intensities were found to vary considerably within the honey types.

Using an excitation wavelength at 310 nm (method C, **Figure 1 C**) the spectra of chestnut honey again clearly differed from the other honey types investigated, especially by the two-fold intensity compared to the others having a maximum at about 380 nm. Most of the honey types exhibited a maximum at about 400 nm and an intensity in the range from 100 to 200 arbitrary units. Lime honey showed again the lowest intensity. Rape and acacia honeys were characterized by a shoulder at 365 nm. The maximum of the spectra of fir honeydew honeys was located at about 355 and showed a shoulder at 420 nm. The band of the chestnut honey spectrum was narrower than by using method B and less intense, but nevertheless the most intense among the spectra recorded by method C. The spectra of metcalfa honeydew honeys expressed a broad band with an intensity of about 350 arbitrary units, being thus the second most intense spectra.

It has been reported that chestnut honey, compared to the other honey types analysed in this study, contains high amounts of hydroxycinnamates such as caffeic, p-coumaric and ferulic acids as well as unidentified flavonoids (*34, 44*). Chestnut honey may also contain more phenylalanine than the other honey types analysed in this study (*39*). The fluorescence of 2-aminoacetophenone, the main volatile component of chestnut honey, may also explain the characteristic spectra (*45, 46*).

Interestingly heather honey commonly known to contain high amounts of phenolic compounds (*47*) does not show spectra of high fluorescence intensity compared to the other honey types. This may be due to scattering, reflection and interference effects resulting from the numerous air bubbles present in heather honey.

7.3.3 LINEAR DISCRIMINANT ANALYSIS (LDA) APPLIED TO THE FLUORESCENCE SPECTRA: BOTANICAL ORIGIN

LDA was performed on the principal components of each type of spectra as well as on the combination of the two most significant types of spectra. In the evaluation of single spectra the highest average classification rate (weighted according the number of samples) of 85 % in validation was obtained for the method A (**Table 2**). The rates of correct classification were similar in both jackknife classification and validation, demonstrating that the models used were robust. Throughout the three methods studied the classification rate for the polyfloral honeys was with only 42 to 63 % very low. This can be explained by the lack of specific physical and chemical characteristics of this honey type. Thus the polyfloral honeys are classified into the groups of unifloral honeys with the smallest Mahalanobis distance (**Table 3**).

For the method A the lowest classification rate of 80 % was observed for metcalfa honeydew honey. Twenty percent of the samples were misclassified as chestnut honey. This can be explained by the important nectar contribution of chestnut often present in metcalfa honeydew honeys. In spite of the low number of samples (n = 5) used for validation, the 20 % of misclassification arises from a single misclassified sample. In the validation step all samples of acacia, alpine rose, chestnut, lime, dandelion and rape honeys were correctly classified. No validation was done for the oak honeydew honey due to the low number of samples available. In the jackknife classification some difficulties occurred to assign alpine rose and acacia honeys (**Table 3**). Some samples of heather honey were also misclassified to rape and polyfloral honeys.

Table 2. Percentage of correct classification by using single data sets at different excitation and emission wavelengths and by combining of the data of the methods (jackknife classification by the "leave one out" method and validation with independent samples)

[1]Method A: excitation scanned between 220 and 400 nm, emission measured at 420.
[2]Method B: excitation at 270 nm, emission measured between 290 and 500 nm.
[3]Method C: excitation at 310 nm, emission measured between 330 and 500 nm.

	Rates of correct classification by the different methods (%)							
	A[1]		B[2]		C[3]		Combination of spectra from methods A and B	
Honey type	Jackknife	Validation	Jackknife	Validation	Jackknife	Validation	Jackknife	Validation
Acacia	95	100	90	79	85	75	90	100
Alpine rose	87	100	50	80	63	50	93	100
Heather	98	88	100	100	91	100	100	100
Chestnut	97	100	96	100	96	100	96	100
Lime	96	100	98	100	98	100	95	100
Dandelion	100	100	97	100	100	100	100	100
Rape	88	100	100	100	93	43	95	100
Fir honeydew	92	86	91	84	84	76	96	97
Metcalfa honeydew	93	80	100	100	100	75	92	100
Oak honeydew	100		100		78		100	
Polyfloral	57	50	47	50	42	43	63	55
Average (weighted)	87	85	84	83	80	73	90	91

Interestingly a few samples of fir honeydew honey were classified as polyfloral or lime honeys. This could indicate that the value of 0.8 $mScm^{-1}$ in electrical conductivity is not always adequate to discriminate between polyfloral and honeydew honeys. Lime honeys very often contain some honeydew honey, which complicates their characterisation.

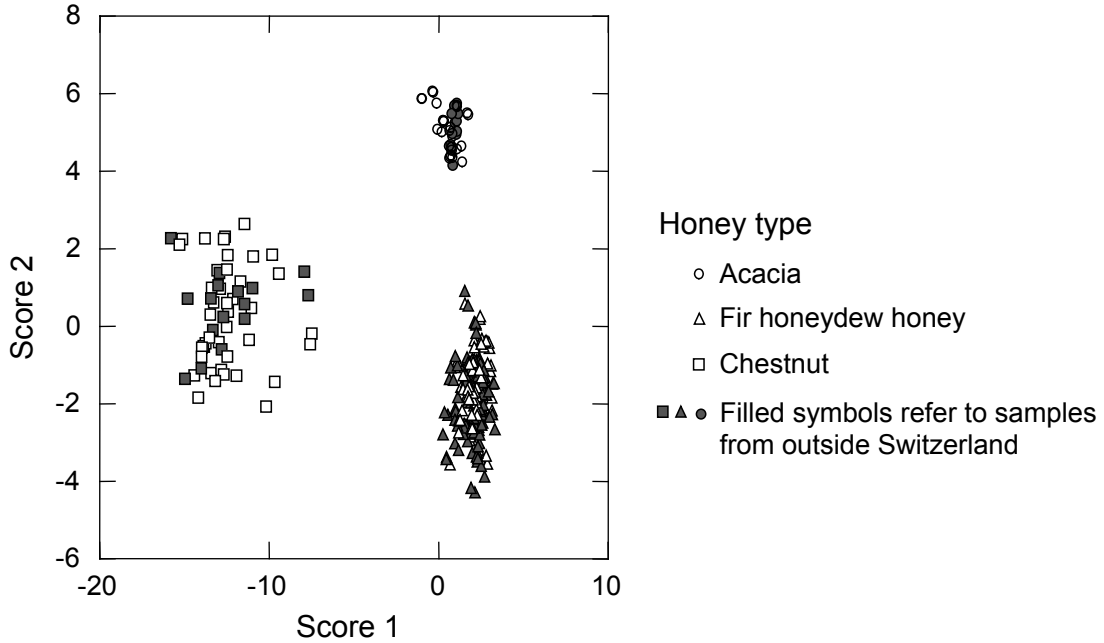

Figure 2. Scatterplot of canonical discriminant scores from method A (for better legibility only the spectra of three honey types are displayed)

Table 3. Jackknife classification and validation tables for the honey samples classified by LDA on the spectra of method A

	Jackknife classification rate for method A (%)										
	Acacia	Alpine rose	Heather	Chestnut	Lime	Dandelion	Rape	Fir honeydew	Metcalfa honeydew	Oak honeydew	Polyfloral
Acacia (n=21)	95	**5**	0	0	0	0	0	0	0	0	0
Alpine rose (n = 19)	**11**	87	0	0	0	0	0	0	0	0	**3**
Heather (n = 23)	0	0	98	0	0	0	**0**	0	0	0	**2**
Chestnut (n = 29)	0	0	0	97	0	0	0	0	2	2	0
Lime (n = 26)	0	0	0	0	96	0	0	0	0	0	**4**
Dandelion (n = 18)	0	0	0	0	0	100	0	0	0	0	0
Rape (n = 24)	0	0	0	0	0	0	88	0	0	0	**12**
Fir honeydew (n = 120)	0	0	0	0	1	0	0	92	0	0	**8**
Metcalfa honeydew (n = 14)	0	0	0	**7**	0	0	0	0	93	0	0
Oak honeydew (n = 8)	0	0	0	0	0	0	0	0	0	100	0
Polyfloral (n = 65)	0	**9**	2	**3**	**9**	**5**	**7**	**9**	0	0	57

	Classification rate in validation for method A (%)									
	Acacia	Alpine rose	Heather	Chestnut	Lime	Dandelion	Rape	Fir honeydew	Metcalfa honeydew	Polyfloral
Acacia (n=7)	100	**0**	0	0	0	0	0	0	0	0
Alpine rose (n = 6)	**0**	100	0	0	0	0	0	0	0	**0**
Heather (n = 8)	0	0	88	0	0	0	**6**	0	0	**6**
Chestnut (n = 10)	0	0	0	100	0	0	0	0	0	0
Lime (n = 9)	0	0	0	0	100	0	0	0	0	**0**
Dandelion (n = 6)	0	0	0	0	0	100	0	0	0	0
Rape (n = 7)	0	0	0	0	0	0	100	0	0	**0**
Fir honeydew (n = 40)	0	0	0	0	3	0	0	86	0	**11**
Metcalfa honeydew (n = 5)	0	0	0	**20**	0	0	0	0	80	0
Polyfloral (n = 22)	0	**7**	0	**9**	**0**	**0**	**21**	**14**	0	50

Even though samples originated from different geographical origins were correctly classified according to their botanical origin. Irrespective of their geographical origin the fluorescent characteristics of honey from various botanical origins seem to be uniform, as samples from outside Switzerland group among the samples from Switzerland (**Figure 2**, for better legibility the scores of only three different honey types are displayed).

The overall discriminating potential of method B is comparable to method A (**Table 2**). However for the discrimination between alpine rose and acacia honeys more difficulties were encountered using method B than method A. In spite of the fact that the two groups were mingled, some samples of alpine rose honey were even misclassified as polyfloral honeys (data not shown).

The potential of the method C for the classification of both unifloral and polyfloral honeys by using a single discriminant model was clearly inferior to that of the methods A and B. Beside the difficulties already mentioned for alpine rose and acacia honeys a considerable number of samples belonging to the groups of rape and honeydew honeys were not correctly classified in validation (**Table 2**).

To evaluate whether the rate of correct classification could be further increased by combining two of the most promising types of spectra, the ones of method A and B were averaged and concatenated for each sample. The rate of correct classification increased for alpine rose, fir honeydew and even for polyfloral honeys compared to the results obtained by using the individual methods A and B (**Table 2**).

The classification tables revealed that polyfloral honeys were very often classified into the groups of the unifloral honeys while the latter were rarely misclassified into the one of the polyfloral honeys. This observation lead to the development of a two step procedure. In the first step the sample was attributed to one of the eleven honey types considered using an overall discriminant model including all honey types. In the second step this classification was verified by using one or several two-group models consisting of a group formed by samples of a given unifloral honey versus a group called "non-unifloral" consisting of all the other samples. Each two-group model was separately built using LDA backward elimination and forward selection. For the verification of the classification by the first model at least the two-group model of the corresponding honey type was used. In addition one to six two-group models (indicated by bold numbers in **Table 3**) were used when a misclassification rate of higher than 3% was calculated in jackknife classification or validation tables of the overall model.

The classification rates for the unifloral honeys in the two-group models were generally > 90 % while the classification rate for the polyfloral honeys ranged between 48 and 75 % (**Table 4**). However, as far as the polyfloral honeys are concerned this is not very important, as we are principally interested in the authentication of unifloral honeys. The high rates of correct classification for both, the unifloral and non-unifloral groups considered by the two-group models indicate that the botanical origin can be reliably determined by this procedure. The respective error rates of this two-step procedure using the methods A and B as well as the combination of the two former types of spectra were calculated by applying Bayes' theorem on the conditional probabilities of disjoint events.

Table 4a. Jackknife and validation tabels for the honey samples classified by the two-group discriminant models of methods A and B.

Method A

	Jackknife classification				Validation	
	Unifloral		Non-Unifloral		Unifloral	
	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)
Acacia	21	100	343	96	7	100
Alpine rose	19	100	345	90	6	83
Heather	23	98	341	99	8	88
Chestnut	29	97	335	99	10	90
Lime	26	100	338	97	9	100
Dandelion	18	100	346	98	6	100
Rape	24	91	343	93	7	100
Fir honeydew	120	95	244	93	40	88
Metcalfa honeydew	14	100	350	100	5	100
Oak honeydew	8	94	356	99		
Polyfloral	65	74	300	65	22	48

Method B

	Jackknife classification				Validation	
	Unifloral		Non-Unifloral		Unifloral	
	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)
Acacia	21	100	341	96	7	100
Alpine rose	16	97	346	89	5	80
Heather	23	100	340	95	8	100
Chestnut	28	96	335	99	9	100
Lime	26	100	336	95	8	100
Dandelion	19	100	343	94	6	100
Rape	22	100	340	98	7	100
Fir honeydew	120	92	242	92	40	84
Metcalfa honeydew	12	100	350	98	4	88
Oak honeydew	8	100	354	95		
Polyfloral	68	65	294	72	22	75

Table 4b. Jackknife and validation tabels for the honey samples classified by the two-group discriminant models on the combination of the spectra A and B

Combination of spectra from methods A and B

	Jackknife classification				Validation	
	Unifloral		Non-Unifloral		Unifloral	
	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)
Acacia	20	100	327	97	7	100
Alpine rose	15	100	332	91	7	100
Heather	23	100	324	100	8	100
Chestnut	27	96	320	100	9	100
Lime	26	100	321	96	9	100
Dandelion	19	95	328	98	6	100
Rape	21	100	326	98	7	100
Fir honeydew	117	97	230	93	39	100
Metcalfa honeydew	12	100	335	98	4	100
Oak honeydew	8	100	339	99		
Polyfloral	59	69	288	71	15	75

Method A gave again the most promising results with an error probability (wrong classification of a sample of unknown botanical origin) < 5 % for all honey types except for fir honeydew where it was 10 % (Table 5). The error probabilities by using method B were higher for all honey types compared to those using method A except for the determination of metcalfa honeydew. By using the combination of the spectra of methods A and B, the error probability could be reduced to < 5 % (in validation) for the eleven honey types studied. It is interesting to notice that the error probabilities of the honey types that express the highest variability in physical and chemical characteristics such as lime and fir honeydew honeys are the highest in fluorescence spectroscopy as well. This can be interpreted that fluorescence spectroscopy reproduces well the characteristics of classical criteria.

Table 5. Error probabilities for the classification of unifloral and polyfloral honeys by the different methods

	Error probability					
	Method A		Method B		Combination of spectra from methods A and B	
Honey type	Jackknife	Validation	Jackknife	Validation	Jackknife	Validation
Acacia	0.029	0.006	0.109	0.030	0.022	0.005
Alpine rose	0.016	0.003	0.058	0.018	0.009	0.001
Heather	0.044	0.013	0.050	0.051	0.003	0.003
Chestnut	0.003	$< 10^{-3}$	0.034	0.053	$< 10^{-3}$	$< 10^{-3}$
Lime	0.067	0.037	0.096	0.054	0.075	0.039
Dandelion	0.037	0.008	0.075	0.072	0.021	0.019
Rape	0.003	0.002	0.046	0.070	0.033	0.042
Fir honeydew	0.088	0.099	0.107	0.090	0.047	0.045
Metcalfa honeydew	0.040	0.004	0.004	0.002	$< 10^{-3}$	$< 10^{-3}$
Oak honeydew	0.044		0.050		0.006	
Polyfloral	$< 10^{-3}$	$< 10^{-3}$	0.034	0.031	$< 10^{-3}$	$< 10^{-3}$

7.3.4 GEOGRAPHICAL ORIGIN

Differences in geographical origin were studied within the groups of samples of the same botanical origin when samples were available from at least two countries. Interestingly a statistically significant difference was found by MANOVA between the geographical origins of all honey types studied (**Table 6**). The lime honey samples originating from Switzerland, Germany and Italy formed groups in the plot of discriminant scores according to their geographical origin (**Figure 3**). The samples could also be correctly classified by LDA according to their geographical origin except for one Swiss sample that was classified to German provenience (data not shown). But the classification according to the geographical origin could only be observed within the groups of honeys of the same botanical origin. An LDA model of acacia, lime, dandelion and fir honeydew honeys of German and Swiss origin failed to classify the samples according to their geographical provenience (**Table 7**). This clearly indicates that the characteristics resulting from the botanical source are considerably stronger than the geographical aspects. The sample set of the lime honeys was small, a larger sample set would possibly lead to a less pronounced difference.

Table 6. Results from MANOVA for the geographical origin of the different unifloral honeys (method A).

Honey type, Country	Wilks' Lambda	p
Acacia (CH, D, F)	0.009	$< 10^{-3}$
Alpine rose (CH, I)	0.027	$< 10^{-3}$
Fir honeydew (CH, D)	0.696	$< 10^{-3}$
Chestnut (CH, F, I)	0.001	$< 10^{-3}$
Lime (CH, D, I)	0.004	$< 10^{-3}$
Dandelion (CH, D, I)	0.023	$< 10^{-3}$

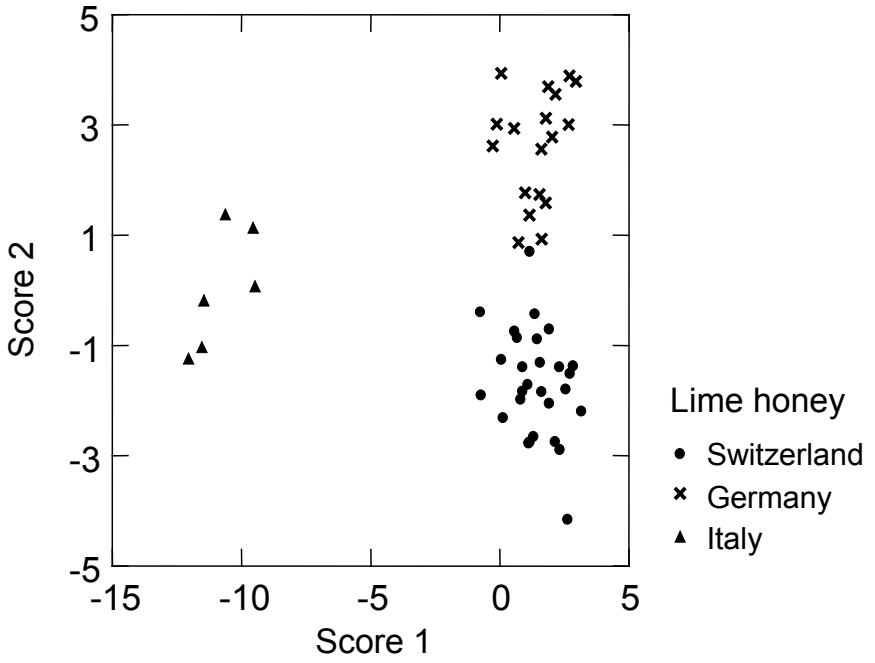

Figure 3. Scatterplot of canonical discriminant scores of lime honeys of different geographic origin (method A)

This may be illustrated on the example of the fir honeydew honeys from Germany and Switzerland where a classification according to geographical origin was not possible (**Figure 4**). However, the samples of fir honeydew honeys originated from an area of approximately 300 km in diameter belonging to Switzerland and Germany and therefore having a very similar vegetation.

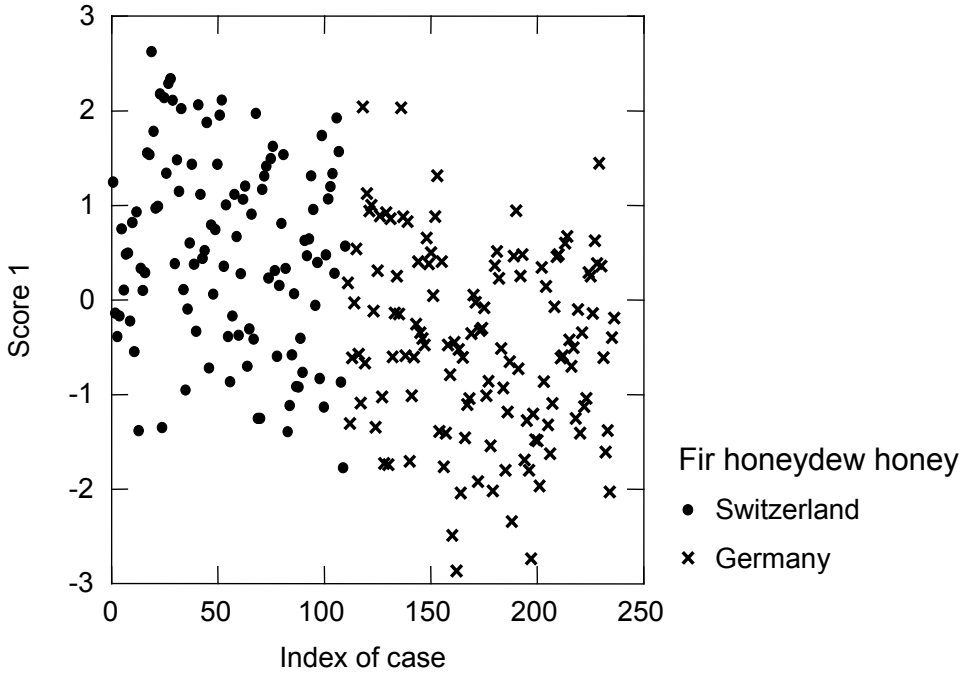

Figure 4. Scatterplot of the canonical discriminant score of honeydew honeys from spruce and fir of German and Swiss provenience (method A)

In future studies it should be verified if the geographical origin of honey could be determined by fluorescence spectroscopic techniques based on the minor contributions of accompanying flora that may be different in areas distant enough. The chemometric models should also be validated with samples of polyfloral provenience.

Table 7. Percentage of correct classification according to the geographical provenience by using the data set of method A

	Jackknifed classification matrix*		
	Switzerland	Germany	Correct (%)
Switzerland	117	69	63
Germany	59	109	65
Total	176	178	64

*Jackknife classification by the "leave one out" method on samples from acacia, lime, dandelion and honeydew honeys from spruce and fir.

7.3.5 ADULTERATION BY FEEDING OF BEES

The acacia and chestnut honey samples adulterated with as much as 50 % of artificial honey did not show any comprehensible changes in the spectra compared to the pure samples in any of the three methods studied. Generally the spectra of the adulterated samples remained in the range of the natural variation of the corresponding unifloral honeys. A detection of honey adulteration is therefore not possible except if the adulterant contains a characteristic fluorophore.

7.3.6 CONCLUSION

While absolutely pure unifloral honeys do not exist, the definition of unifloral honey is in fact based on the points of view and the descriptions of different analysts. However a consensus has been reached using the physical, chemical and pollen analytical characteristics of the unifloral honeys considered as internationally recognised criteria already published (*3-6*).

Of capital importance is certainly to ensure a uniform honey quality that can be recognised by consumers preferring a given type of honey. Currently the determination of the botanical origin of honey relies on the judgement of experienced experts who base their decision on the criteria of several analytical measurands. The challenge of new analytical methods that do not need such an expertise is to mathematically model and reproduce this decision making process. As the definition of a unifloral honey is ultimately a matter of opinion, absolutely correct classification by chemometric models can therefore not be expected as these models are trained by uncertain sample sets as reference.

As the characteristic physical and chemical differences between unifloral and polyfloral honeys are small and only very few compounds are specific to a given type of honey, the chemometric approach based on a fingerprint seems to be more promising than the search for individual marker compounds.

This study shows that front-face fluorescence spectroscopy combined with chemometrics offers a promising approach to the authentication of the botanical origin of honey and that the problems related to the determination of the polyfloral honeys can be overcome by the successive use of at least two mathematical models. The current results show that classifications 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. It depends on the cer-

tainty needed whether to base the classification on the single spectra of type A or to combine the spectra of methods A and B.

Of course the proposed fluorescence spectroscopic method needs a considerable amount of preliminary work to establish the chemometric models based on samples of known botanical origin. Once the classification models have been set the technique enables a rapid determination of the botanical origin without particular sample preparation and special qualification of laboratory personnel. It remains to be tested by future studies if these models can be transferred from one instrument to another, like in infrared spectroscopy when normalised fluorescence spectra are used (*40*) or the instruments are calibrated with reference materials.

In addition, the present work clearly shows that fluorescence characteristics of honey are much more depending on their botanical origin than on the geographical origin. Therefore the former should be determined before proposing a method for the determination of the geographical origin of honey. Such a method must be tested as well with samples of the same botanical origin.

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CHAPTER 8

Authentication of the Botanical and Geographical Origin of Honey by Mid-Infrared Spectroscopy*

ABSTRACT

The potential of Fourier-transform mid-infrared spectroscopy (FT-MIR) using an attenuated total reflectance (ATR) cell was evaluated for the authentication of eleven unifloral (acacia, alpine rose, chestnut, dandelion, heather, lime, rape, fir honeydew, metcalfa honeydew, oak honeydew) and polyfloral honey types (n = 411 samples) previously classified with traditional methods such as chemical, pollen, and sensory analysis. Chemometric evaluation of the spectra was carried out applying principal component analysis (PCA) and linear discriminant analysis (LDA), the error rates of the discriminant models being calculated by Bayes' theorem. The error rates ranged from less than 0.1% (polyfloral and heather honeys as well as honeydew honeys from metcalfa, oak and fir) to 8.3 % (alpine rose honey) both in jackknife classification and validation, depending on the honey type considered. This study indicates that ATR-MIR spectroscopy is a valuable tool for the authentication of the botanical origin and quality control, and may also be useful for the determination of the geographical origin of honey.

8.1 INTRODUCTION

According to the Codex Alimentarius Standard for Honey (*1*) and the European Union Council Directive (*2*) relating to honey, the use of a botanical designation of honey is allowed if it originates predominately from the indicated floral source. It may also be designated by the name of a geographical region if it was produced exclusively within the area referred to (*1, 2*).

The overwhelming majority of the honeys on the market contain significant nectar or honeydew contributions from several plant species and are therefore called polyfloral or multifloral honeys. Normally they are just designated with the word "honey". Probably no honey produced by free flying bees is purely unifloral. The term unifloral honey is used to describe honey in which the major part of nectar or honeydew is derived from a single plant species. Honey composition, flavour and colour varies considerably depending on the botanical source it originates from (*3*).

The physical, chemical and pollen analytical characteristics of the most important unifloral honeys have been described in various papers (*3-7*). Unlike the unifloral honeys the polyfloral honeys do not express distinct physical or chemical characteristics but a huge variability regarding all measurands, which makes their authentication particularly difficult.

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Ruoff, K.; Luginbühl, W.; Künzli, R.; Iglesias, M. T.; Bogdanov, S.; Bosset, J. O.; von der Ohe, K.; von der Ohe, W.; Amadò, R. Authentication of the Botanical and Geographical Origin of Honey by Mid-Infrared Spectroscopy. *J. Agric. Food Chem.* **2006**, 54, 6873-6880.

The interest for the production of unifloral honeys is related to higher consumer preference for some honey types leading to a commercial interest of the beekeepers. The increasing interest in the therapeutic or technological uses of certain honey varieties may also contribute to the demand of a reliable determination of their botanical origin.

8.1.1 BOTANICAL ORIGIN

Until now a reliable determination of the botanical origin can only be achieved by a global interpretation of sensory, pollen and physico-chemical analyses carried out by experts (*4, 8, 9*). However the uncertainty related to the interpretation of pollen analytical results, originating from a number of different factors demands the development of new analytical methods (*10*).

A number of new analytical methods combined with multivariate data analysis have been proposed to determine the botanical origin of honey. They are based on physical and chemical measurands for the quality control of honey (*11, 12*) sometimes in combination with the determination of mineral content (*13*), as well as carbohydrate composition (*14*), amino acid composition (*15*), mass spectrometry or metal oxide semiconductor based gas sensors (*16, 17*), differential scanning calorimetry (*18*), pyrolysis mass-spectrometry (*19*) and raman spectroscopy (*20*).

Recently the potential of near-infrared spectroscopy (NIR) to determine the botanical origin of honey was evaluated using a reflectance probe (*21*). Principal component analysis (PCA) and linear discriminant analysis (LDA) was applied for the classification of the honey types studied. Over 80 % of acacia, chestnut and rape honeys were correctly assigned to the corresponding honey type on basis of the spectroscopic data and Mahalanobis distance in cross-validation, but only a third of the heather honeys considered were correctly classified. However, the number of samples per honey type was very restricted as 13 different unifloral honeys from 9 European countries were studied on a total of 51 samples. No separation into groups according to their geographical origin was found.

Many of the methods mentioned above allow to clearly discriminate between several types of unifloral honeys (a minority of approximately 20 %), but none of them accounts for the polyfloral honeys that represent the most important majority (about 80 %) of the honeys produced. Thus, the main problem in the authentication of unifloral honeys is to discriminate between polyfloral and unifloral honeys. This means that the above mentioned methods are inadequate in analytical practice. This also explains why until now none of these methods is commonly applied to the determination of the botanical origin of honey.

Recently Tewari and Irudayaraj claimed that ATR MIR-spectroscopy 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 (*22*).

8.1.2 GEOGRAPHICAL ORIGIN

Pollen analysis is currently used to determine the geographical origin of honey as the pollen in honey reflect the vegetation type where the nectar has been collected by the bees. In the past many analytical tools such as raman spectroscopy (*20*), as well as determination of amino acid composition (*23, 24*), mineral content (*25, 26*), sugar or mineral composition sometimes combined with common chemical quality control data (*27-29*) together with multivariate data evaluation have been proposed for the same purpose.

Unfortunately in most of the above quoted papers the botanical origin of the honey samples has not been determined, or the discrimination between the various geographical origins has not been verified on samples of the same botanical origin. Moreover the sample sets considered were generally small or limited to a small geographical area. The distinctions found are therefore rather due to differencies of the local vegetation type (i.e. to the botanical origin of honey) than to the geographical regions (*30*).

Moreover, criteria related to the main components present in honey are more influenced by the botanical source than by the geographical region. This may explain why no geographical discrimination has been found by near-infrared spectroscopy (*21*). The same fact was also observed in a study using pyrolysis mass spectrometric data where the variability of the honey types within a country was found to be larger than between the geographical regions of interest (*19*). The presence or absence of certain volatiles analysed by dynamic headspace GC-MS have been proposed to be specific for some geographical origins as well (*31*). However the sample set used in this study was very limited and does therefore not allow to generalise. With relatively small sample sets a discrimination based on mineral or volatile composition between honeys originating from coastal and central provinces of Canada (*32*) and between Hungarian and Italian acacia honeys (*17*) have been shown. These methods have to be validated as analytical tools for the practice.

As several analytical methods have to be used together for a reliable authentication of the botanical origin, it is consequently very time consuming and costly. In addition very specialised expertise is needed for the interpretation of the pollen spectrum used for the determination of the geographical origin of honey. Thus, there is a need for new analytical tools that allow both a rapid and reproducible authentication of the botanical and geographical origin of honey (*9, 33*).

Due to the increased performance of computers in the last decades, infrared spectrometry (IR) has become a well established technique for quantitative food analysis. Concerning honey, it has predominantly been applied to the quantitative analysis of different measurands (*34-36*). In this context the aim of the current work was to study the infrared spectroscopic characteristics of eleven different honey types and to develop a rapid, low-cost and reliable method for the authentication of unifloral and polyfloral honeys. As minor nectar contributions from plant species other than the unifloral source may contribute to regional characteristics of unifloral honeys, the potential of MIR-ATR spectroscopy for the determination of the geographical origin of honey was studied as well.

8.2 MATERIALS AND METHODS

8.2.1 SAMPLING AND BOTANICAL CLASSIFICATION BY REFERENCE METHODS

A total of 411 honey samples produced between 1998 and 2004 were collected and stored at 4 °C until analysis. They originated predominantly from Switzerland (CH) but samples from Germany (D), Italy (I), Spain (E), France (F) and Denmark (DK) were also considered.

To classify these honey samples, the following measurands were determined according to the harmonised methods of the European Honey Commission (37): electrical conductivity, sugar composition, fructose/glucose ratio, pH-value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (38, 39).

Based on the results obtained with these classical methods, the honey samples were assigned to one of the following eleven honey types according to the criteria of Persano and Piro (3): acacia (*Robinia pseudoacacia*) (CH, n = 17; D, n = 6; F, n= 3), alpine rose (*Rhododendron* spp.) (CH, n = 18; I, n = 5), sweet chestnut (*Castanea sativa*) (CH, n = 23; I, n = 5; F, n = 3), rape (*Brassica* spp.) (CH, n = 23), fir honeydew (*Abies* spp. and *Picea* spp.) (CH, n = 74; D, n = 63), oak honeydew (*Quercus* spp.) (E, n= 8) honeydew from *Metcalfa pruinosa* (I, n = 14), heather (*Calluna vulgaris*) (D, n= 19; DK, n = 3), lime (*Tilia* spp.) (CH, n =13; D, n=9; I, n = 4), dandelion (*Taraxacum* s.l.) (CH, n = 19; D, n = 6; I, n = 1) and polyfloral honeys (CH, n = 75). In the heterogenous group of the polyfloral honeys nectar or honeydew contributions from all of the above-mentioned plant species were represented.

8.2.2 FT-IR-ATR SPECTROSCOPY

Fourier-transform MIR spectra were recorded using a Bio-Rad FTS-7 (Bio-Rad, Cambridge MA, U.S.A.) equipped with a MKII Golden Gate TM single reflection ATR accessory (Specac Inc, Woodstock GA, U.S.A). The measuring cell consisted of a diamond of 2.8 mm in diameter with a refractive index of 2.4 at 1000 cm^{-1}. The depth of penetration of the infrared radiation was 2.0 µm at 1000 cm^{-1} for a sample with a refractive index of 1.5 (approximately the refractive index of honey). The spectrometer was equipped with a deuterated triglycine sulfate (DTGS) detector and was operated at 4 cm^{-1} spectral resolution.

The honey samples were liquefied in a water bath at 55 °C for 8 h and then allowed to cool to room temperature before analysis. After applying a drop of the sample on the surface of the diamond, it was left to thermally equilibrate for 4 min. The number of scans per spectrum was selected on the basis of optimal signal to noise ratio and acquisition time required. 100 scans were recorded for each spectrum in the wave number range between 4000-550 cm^{-1}. Single-beam spectra of all samples were recorded and ratioed against the background spectrum of the clean diamond surface (laboratory air) in order to present the spectra in absorbance. Two spectra were recorded at room temperature using different aliquots of each sample. After each measurement the diamond was thoroughly washed with demineralised water and dried with a soft tissue. The repeatability was determined by ten-fold measurement of a honeydew sample.

8.2.3 PROCESSING OF SPECTRA AND MULTIVARIATE ANALYSIS

To exclude noisy parts of the spectra only the range between 3718 - 631cm^{-1} was used for multivariate analysis. After elimination of spectral outliers, principal component analysis (PCA) was applied to eliminate the spectral collinearity and to reduce the number of variables to 20 PC's (PCA with GRAMS/32 AI, PLSplus/IQ Add-on, Vs. 5.09, Galactic Industries Corporation, Salem NH, USA).

In linear discriminant analysis (LDA), the 20 initial PC's were further reduced by backwards elimination of principal components on the basis of their partial F-values in the discriminant models (SYSTAT® Version 11, Systat Software Inc., Richmond, USA). To include the variability of single measurements in the model, both spectra of each sample were used in PCA and LDA. The validation was carried out with spectra of one third of the samples, selected randomly, and not present in the group of samples used to build the model.

The results in jackknife classification ("leave one out" procedure) and validation (**Table 1**) revealed that polyfloral honeys were very often classified into the groups of the unifloral honeys while inversely the latter were rarely misclassified into the polyfloral honeys. This observation led to the idea to develop a two step procedure. In the first step the sample was attributed to one of the eleven honey types considered using an overall discriminant model with as many groups as honey varieties. In the second step this classification was verified by applying several models consisting of a group formed by samples of a given unifloral honey versus a group called "non-unifloral" consisting of all the other samples. Each two-group model was separately built using LDA backward elimination and forward selection. For the verification of the classification by the first model at least the two-group model of the corresponding honey type was used. In addition one to four two-group models were tested when a misclassification rate of higher than 3 % was calculated in jackknifed classification or validation tables of the overall model (indicated by bold numbers in **Table 1**). The probabilities for misclassification based on the spectra were calculated by applying Bayes' theorem on the conditional probabilities of disjoint events. The error probabilities cannot be directly taken from **Table 2**; they only quantify the conditional probabilities of correct classification given the corresponding honey type. By Bayes' theorem the posterior probabilities of finding the correct honey type given a distinct classification by the discriminant model was calculated, and the error rate is simply the complement to 1.

8.2.3 GEOGRAPHICAL ORIGIN

The applicability of FT-IR-ATR spectroscopy for the determination of the geographical origin of honey was evaluated for the honey types where samples from different countries were available. The differences resulting from the botanical origin were studied within the groups of unifloral honeys and between several honey types from Germany and Switzerland by using MANOVA and LDA (SYSTAT® Version 11, Systat Software Inc.).

Table 1. Jackknife classification and validation tables for the honey samples classified by LDA.

	Jackknife classification rate (%)											
	Acacia	Alpine rose	Heather	Chestnut	Dandelion	Lime	Rape	Fir honeydew	Metcalfa honeydew	Oak honeydew	Polyfloral	Correct
Acacia (n= 25)	100	0	0	0	0	0	0	0	0	0	0	100
Alpine rose (n = 22)	0	96	0	0	0	0	0	0	0	0	**5**	96
Heather (n = 21)	0	0	98	0	0	0	0	0	0	0	2	98
Chestnut (n = 31)	0	0	0	100	0	0	0	0	0	0	0	100
Dandelion (n = 23)	0	0	0	0	100	**0**	**0**	0	0	0	0	100
Lime (n = 25)	0	0	0	0	0	88	0	0	0	0	**12**	88
Rape (n = 22)	0	0	0	0	**7**	0	89	0	0	0	**5**	89
Fir honeydew (n = 130)	0	0	0	0	0	0	0	95	0	0	**5**	95
Metcalfa honeydew (n = 13)	0	0	0	0	0	0	0	**8**	92	0	0	92
Oak honeydew (n = 8)	0	0	0	0	0	0	0	0	0	100	0	100
Polyfloral (n = 75)	**2**	**6**	0	**3**	**11**	**9**	**5**	**5**	0	0	59	59

	Classification rate in validation (%)										
	Acacia	Alpine rose	Heather	Chestnut	Dandelion	Lime	Rape	Fir honeydew	Metcalfa honeydew	Polyfloral	Correct
Acacia (n= 8)	100	0	0	0	0	0	0	0	0	0	100
Alpine rose (n = 7)	0	100	0	0	0	0	0	0	0	**0**	100
Heather (n = 7)	0	0	100	0	0	0	0	0	0	0	100
Chestnut (n = 10)	0	0	0	100	0	0	0	0	0	0	100
Dandelion (n = 7)	0	0	0	0	71	**14**	**14**	0	0	0	71
Lime (n = 8)	0	0	0	0	0	100	0	0	0	**0**	100
Rape (n = 7)	0	0	0	0	0	0	100	0	0	**0**	100
Fir honeydew (n = 40)	0	0	0	0	0	0	0	98	0	**3**	98
Metcalfa honeydew (n = 4)	0	0	0	0	0	0	0	0	100	0	100
Polyfloral (n = 25)	**8**	**12**	0	**8**	**4**	**28**	**0**	**14**	0	26	26

8.3 RESULTS AND DISCUSSION

8.3.1 REPEATABILITY LIMITS

The repeatability limit (r_{IR}) of the FT-IR-ATR measurements were calculated at the maximum absorbance at 1024 cm^{-1} from 10 subsequently recorded spectra of different aliquots of the same honeydew honey sample. The average of the maximum intensity of 0.714, a standard deviation of 0.002, a coefficient of variation of 0.3 % and a r_{IR} of 0.006 were found, indicating an excellent repeatability of the method.

8.3.2 FT-IR-ATR SPECTRA OF DIFFERENT HONEY TYPES

The mid-infrared spectra of the ten unifloral honey types studied are shown in **Figure 1**. Each spectrum is typical for a given honey type. The most characteristic differences were observed between 800 and 1500 cm^{-1}. The largest variation in the spectra of the honey types were found in the C-O and C-C stretching regions of the saccharides between 950 and 1050 cm^{-1} (**Figure 1, enlargement A**). Indeed, differencies between the saccharide compositions of unifloral honeys have been reported (*3*, *11*, *40*). A more detailed discussion of the vibrational modes of the functional groups in honey can be found elsewhere (*22*).

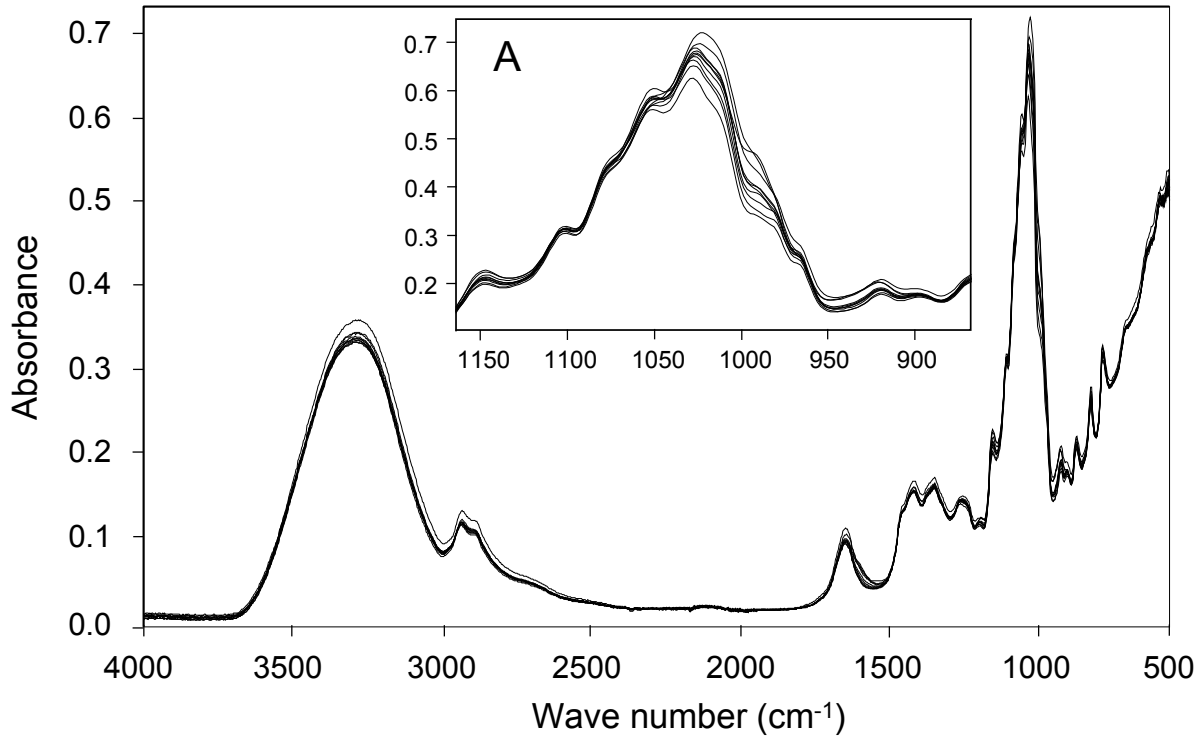

Figure 1. FT-MIR-ATR spectra of different honey types (A: enlargement of the region between 900 and 1150 cm-1)

8.3.3 BOTANICAL ORIGIN

Most of the unifloral honeys revealed very high rates of correct classification of more than 90% when classified using linear discriminant analysis (LDA) on PC's of the infrared spectra (**Table 1**). The rates were similar in jackknife classification and validation demonstrating that the models used were robust. Among the unifloral honeys the lime honeys showed the lowest jackknifed classification rate (88 %). Twelve per-

cent of the lime honey samples were classified as polyfloral honeys. This may be explained by the variable chemical composition of this honey type as it often contains different amounts of honeydew and thus exhibits variable physical and chemical characteristics. This makes it similar to polyfloral honey that may also contain nectar and honeydew contributions. Rape honey samples were partly classified as dandelion and polyfloral honeys and exhibited the second lowest classification rate (89 %). The misclassifications can be explained by the fact that dandelion and rape nectar contribute significantly to polyfloral honeys produced in Switzerland. In validation dandelion honey samples were misclassified to lime and rape honeys. However the relatively low number of samples does not allow a concluding evaluation. The different honeydew honeys were mostly assigned to the correct group except a few samples of metcalfa honeydew honey that were misclassified as fir honeydew honeys. However the number of oak honeydew samples was very small, therefore not allowing a validation.

Even though the samples originated from different geographical origins, they were nevertheless correctly classified according to their botanical origin. Irrespective of their geographical origin the infrared spectroscopic characteristics of honey from various botanical origins seem to be uniform, as samples collected from outside Switzerland grouped among those from Switzerland (**Figure 2**, for better legibility the discriminant scores of only five different honey types are displayed).

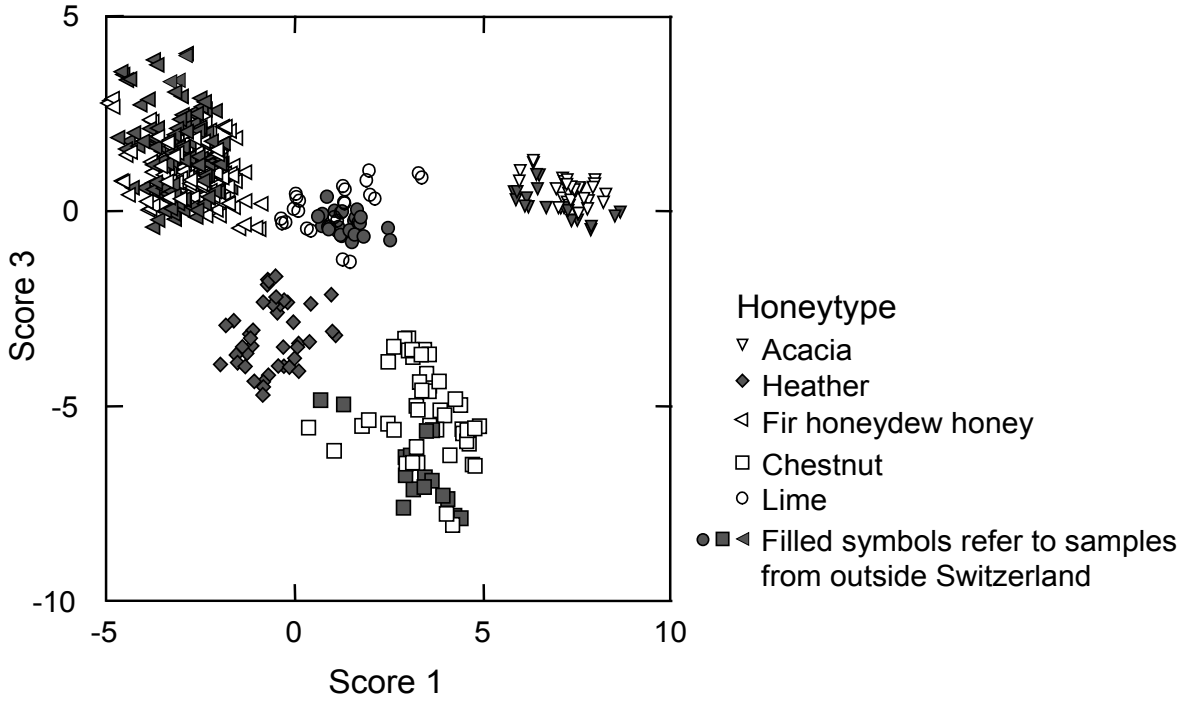

Figure 2. Scatterplot of canonical discriminant scores of different unifloral honeys from LDA (for better legibility only the scores of five honey types are displayed, all heather honeys originated form outside Switzerland).

It has been clearly shown that it is possible to discriminate between different types of unifloral honeys by infrared spectra and using a single mathematical model. However, this does not mean that the method will be useful in practice as polyfloral honeys are only correctly classified to 59 % and are very often misclassified into several types of unifloral honeys. Therefore the approach using two steps as described in

the methods section was tested. After the classification by the general model one to five two-group models (indicated by boldface numbers in **Table 1**) were used. The classification rates for the unifloral honeys in the two-group models were generally > 90 % while the classification rate for the polyfloral honeys ranged between 26 and 82 % (**Table 2**). However, as far as the polyfloral honeys are concerned this is not very important, as we are principally interested in figuring out the unifloral honeys. The high rates of correct classification for both, the unifloral and non-unifloral groups considered by the two-group models indicate that the botanical origin can be reliably determined by this procedure.

Table 2. Jackknife and validation table for the honey samples classified by the two-group discriminant models

	Jackknife classification				Validation	
	Unifloral		Non-Unifloral		Unifloral	
	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)
Acacia	25	100	370	98	8	100
Alpine rose	22	91	373	87	7	64
Heather	21	98	374	100	7	100
Chestnut	31	100	364	99	10	100
Lime	25	88	370	80	8	100
Dandelion	23	100	372	91	7	100
Rape	22	95	373	90	7	100
Fir honeydew	130	95	265	98	40	93
Metcalfa honeydew	13	92	382	100	4	100
Oak honeydew	8	100	387	100		
Polyfloral	75	69	320	82	25	26

If the sample is assigned to the same honey type by the overall and the corresponding two-group model it is very likely that it belongs to this type of honey. If the classifications of the two models do not agree the sample has to be considered to be of polyfloral origin. When the sample is assigned to the same honey type by both, the overall model and the corresponding two-group model, and is moreover considered to belong to the non-unifloral groups in all the other two-group models tested, the honey sample belongs almost certainly to the honey type indicated by the overall model. The respective error rates of this two-step procedure were calculated by the Bayes' theorem. The error probabilities (misclassification of a sample of unknown botanical origin) for the eleven honey types studied except for alpine rose honey were found to be ≤ 3 % (**Table 3**). The approach using two successive models allowed a reliable determination of both the polyfloral and unifloral honeys. The classification based on MIR-ATR-spectroscopic data and the mathematical models developed are in agreement with the classification using the traditional physical, chemical and pollen analytical criteria (*3*).

Table 3. Error probabilities for the classification of unifloral and polyfloral honeys calculated by Bayes' theorem

	Error probability	
Honey type	Jackknife	Validation
Acacia	0.027	0.031
Alpinerose	0.083	0.074
Heather	$< 10^{-3}$	$< 10^{-3}$
Chestnut	0.016	0.027
Lime	0.027	0.019
Dandelion	0.015	0.009
Rape	0.015	0.009
Fir honeydew	$< 10^{-3}$	$< 10^{-3}$
Metcalfa honeydew	$< 10^{-3}$	$< 10^{-3}$
Oak honeydew	$< 10^{-3}$	
Polyfloral	$< 10^{-3}$	$< 10^{-3}$

8.3.4 GEOGRAPHICAL ORIGIN

Differences in geographical origin were first studied by MANOVA within the groups of samples of the same botanical origin when such samples were available from at least two countries. A highly significant difference was thus found between the geographical origins of all the honey types considered (**Table 4**). When the geographical origins were modelled by LDA the spectra were correctly classified at high rates according to their geographical origin: alpine rose 95 %, heather 77 %, chestnut 98 %, lime 100 % and dandelion 76 %. The spectra of acacia honey samples originating from Switzerland, Germany and France were all correctly classified and formed groups according to their geographical origin (**Figure 3**). However the number of samples available from countries outside Switzerland was very limited. Therefore the effects observed should be verified with a larger set of samples.

Interestingly a difference between fir honeydew honeys of German and Swiss origin could be observed in a larger set of samples originating from several crops. The average jackknife classification rate was 92 %. In the plot of the first discriminant scores the Swiss samples generally had positive and the German samples negative values (**Figure 4**). The overlapping was small considering that all samples originated from an area of about only 300 km in diameter. In the average spectra of the German and Swiss honeydew honey samples differences were observed especially at the shoulder at 994 cm^{-1} of the distinct band with the maxium at 1024 cm^{-1} resulting from C-O and C-C stretching of the saccharides (**Figure 5**). The average spectra of the German honeydew honeys crossed the average spectra of the Swiss honeydew honeys at 1000 cm^{-1} and showed a more pronounced shoulder at 994 cm^{-1}. These subtle distinctions could be verified by multivariate analysis of the concentration of the various saccharides in honey, but probably lie within the measurement uncertainty of the reference method.

In order to verify whether the geographical origin can also be determined when samples of different botanical origins are considered, LDA was carried out on samples of acacia, lime, dandelion and the fir

Table 4. Results of MANOVA for the geographical origin of the different unifloral honeys.

Honey type	Wilks' Lambda	p
Acacia	0.002	$< 10^{-3}$
Alpinerose	0.073	$< 10^{-3}$
Heather	0.041	0.023
Fir honeydew	0.251	$< 10^{-3}$
Chestnut	0.016	$< 10^{-3}$
Lime	0.002	$< 10^{-3}$
Dandelion	0.330	0.014

honeydew honeys of both German and Swiss origins. The average rate of correct classification remained with 85 % quite high (**Table 5**). When average spectra of the unifloral honeys were compared, all except lime honey showed similar differences as observed between the honeydew honeys from Switzerland and Germany (**Figure 5**).

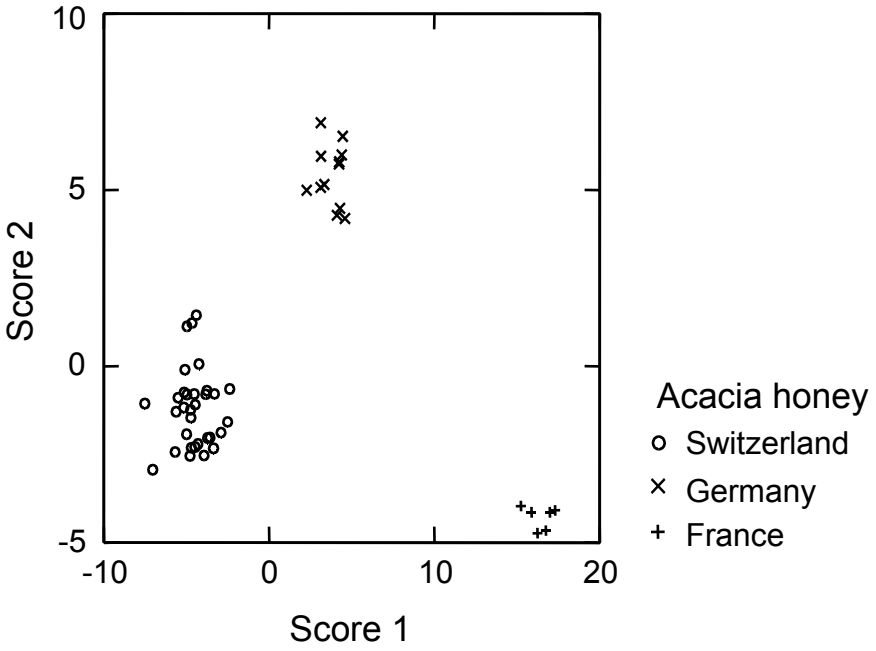

Figure 3. Scatterplot of canonical discriminant scores of acacia honeys of different geographical origin

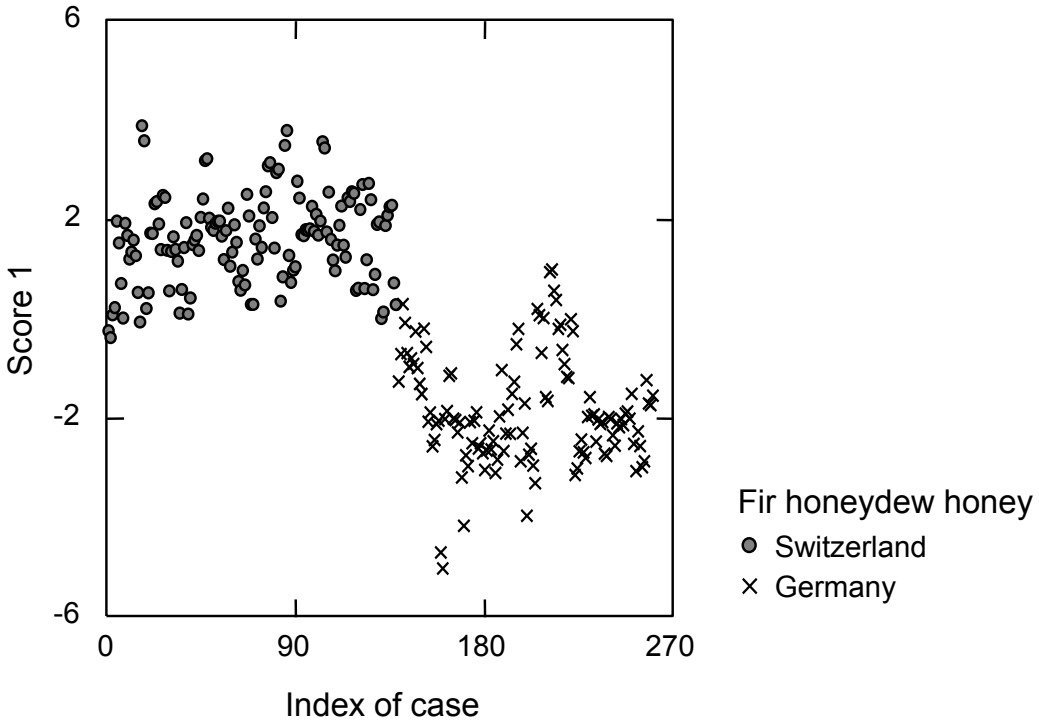

Figure 4. Scatterplot of the canonical discriminant score of fir honeydew honeys of German and Swiss provenience.

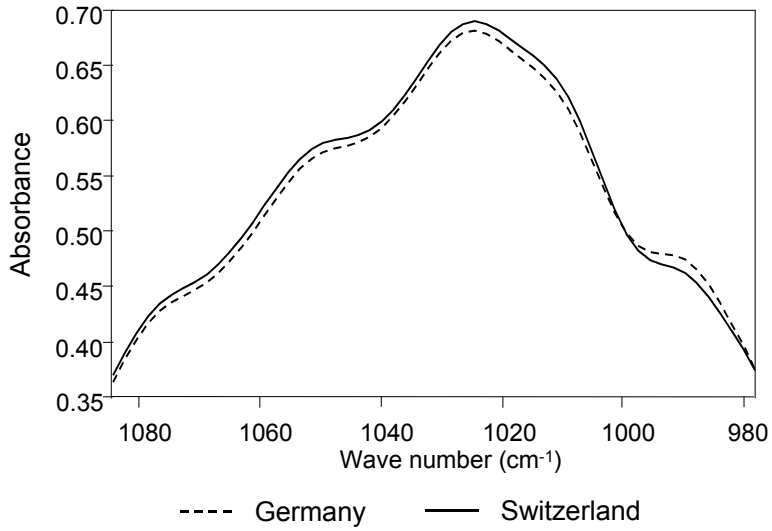

Figure 5. Enlargement of FT-MIR-ATR average spectra of fir honeydew honeys from Germany and Switzerland.

When LDA was performed on the same dataset using the botanical origin as grouping variable, all spectra were correctly assigned to the corresponding group of unifloral honey, thus indicating that the botanical origin is more significant than the geographical origin. In other words, differencies observed and interpreted as resulting from geographical origin may be indirect effects of the botanical origin. In uniforal 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.

While absolutely pure unifloral honeys do not exist, the definition of unifloral honey is in fact based on the points of view and descriptions of different analysts. Obviously a certain consensus has been found using the physical, chemical and pollen analytical criteria for unifloral honeys (*3-5*).

The characteristic physical and chemical differences between unifloral and polyfloral honeys are small and only a few compounds are specific to a given type of honey, the chemometric approach based on a spectroscopic "fingerprint" seems more promising than the use of certain marker compounds. The present study shows that MIR-ATR-spectroscopy combined with chemometrics offers a valuable approach to the authentication of the botanical origin of honey. The problems related to the determination of the polyfloral honeys can be overcome by the successive use of at least two discriminant models. While previous studies were only able to discriminate between different unifloral honeys this work demonstrates that unifloral honeys can be authenticated and distinguished from polyfloral honeys. The technique is non-destructive, rapid, easy to use and not expensive. It needs neither particular sample preparation nor special qualification of the laboratory personnel. Our results show that the authentication of the botanical origin of honey by MIR-ATR-spectroscopy and chemometrics is in agreement with the determination using classical criteria. In addition the same spectra can be used to obtain quantitative information on several measurands used for routine quality control of honey (41).

Table 5. Percentage of correct classification according to the geographical origin

	Jackknife classification matrix*		
	Switzerland	Germany	Correct (%)
Switzerland	197	32	86
Germany	27	136	83

*Jackknife classification by the "leave one out" method considering samples from acacia, lime, dandelion and fir honeydew honeys

The present work clearly shows that infrared spectroscopic characteristics of honey are much more depending on their botanical origin than on their geographical origin. 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 if 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.

A drawback of the current method is that before the botanical origin can be determined routinely, the proposed spectroscopic method needs a considerable amount of preliminary work, to be carried out by specialists, to build the chemometric models based on samples of known botanical origin. But these models could likely be transferred from an instrument to another as already demonstrated for quantitative analysis of various food constituents (*42-44*) and the substance identification by spectral databases. This remains to be verified in future studies.

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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 of 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 been 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 uniforal 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 an other. 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.

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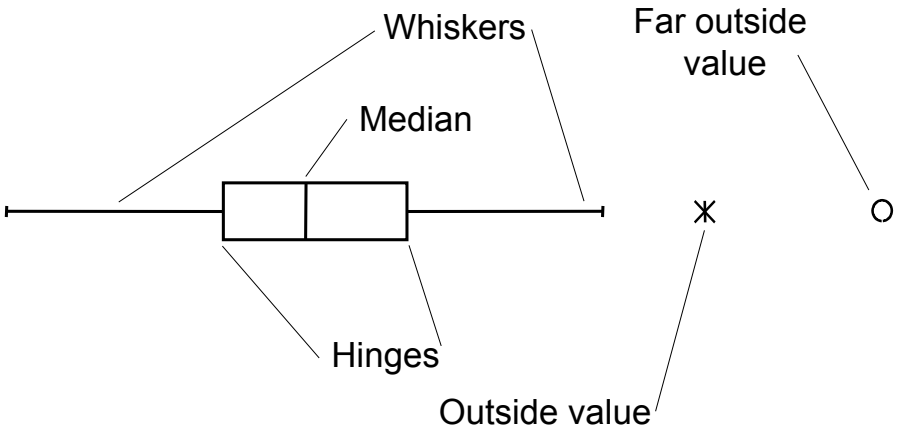
APPENDIX

Key to Figures and Tables in Appendix A & B

Box plots*



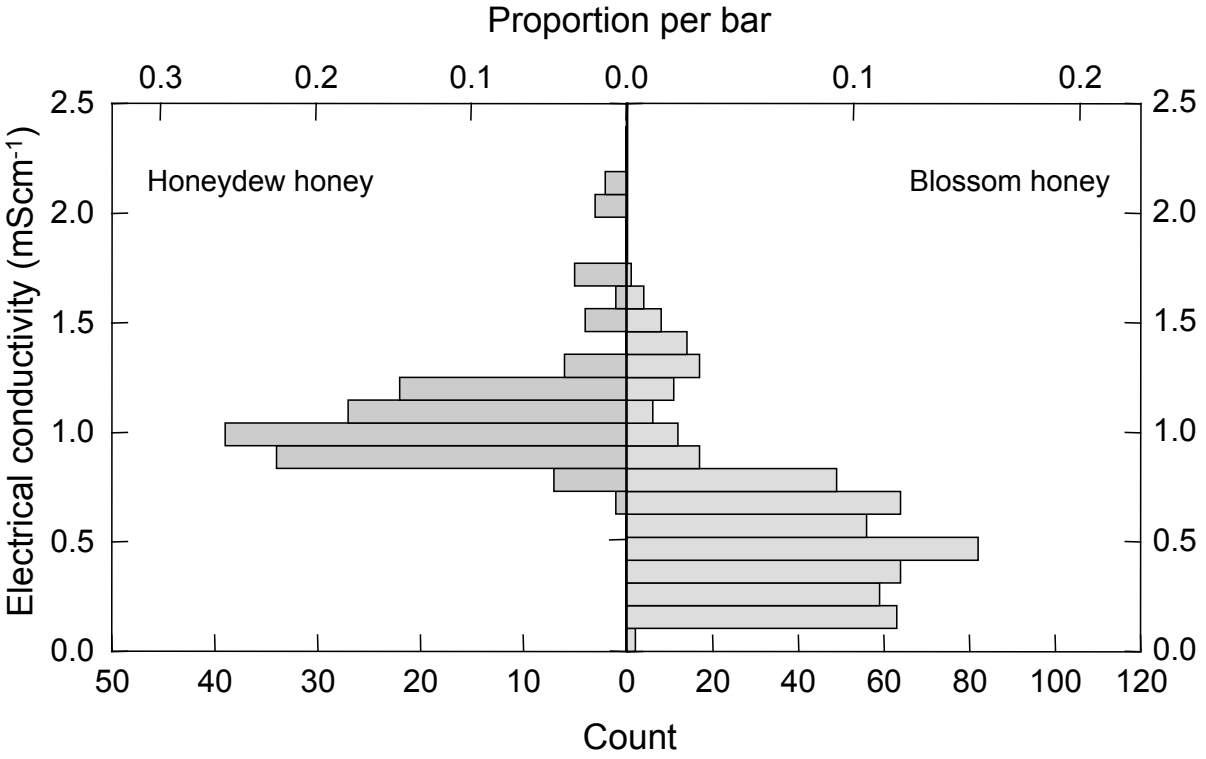
***The central vertical line marks the median of the sample. The lenght of each box shows the range within which the central 50 % of the values fall, with the box edges (hinges) at the first amd third quartiles. The whiskers display the ranges of values that fall within 1.5 interquartile ranges. Values between 1.5 and 3 interquartile ranges are plotted with asterisks. Values outside 3 interquartile ranges are plotted with circles.

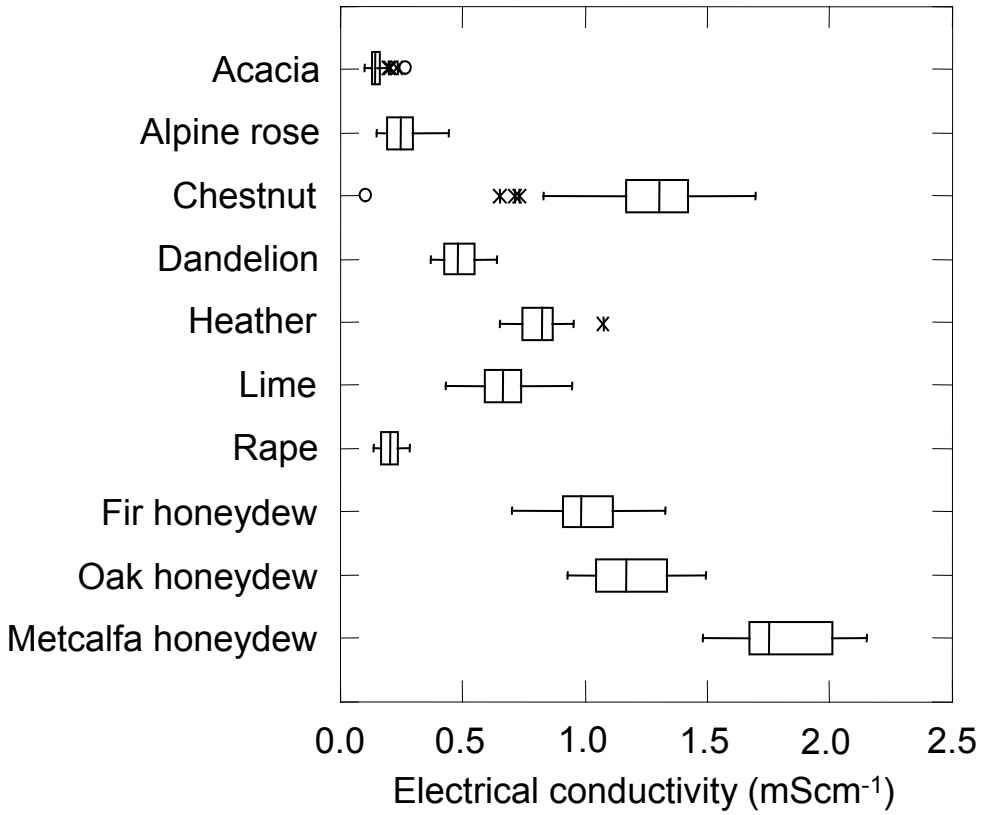
Symbols

n	number of samples
s	standard deviation
x_{min}	minimum
x_{max}	maximum
$\tilde{x}$	median
$\overline{x}$	mean

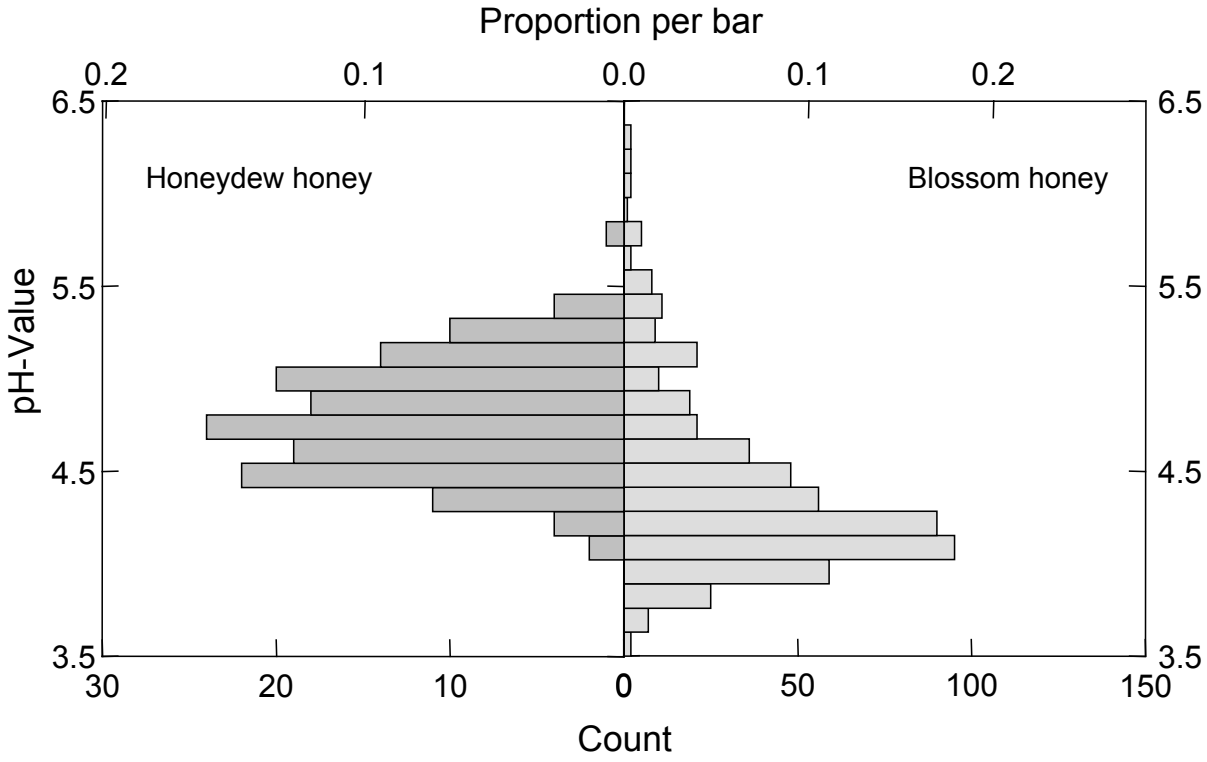
Appendix A

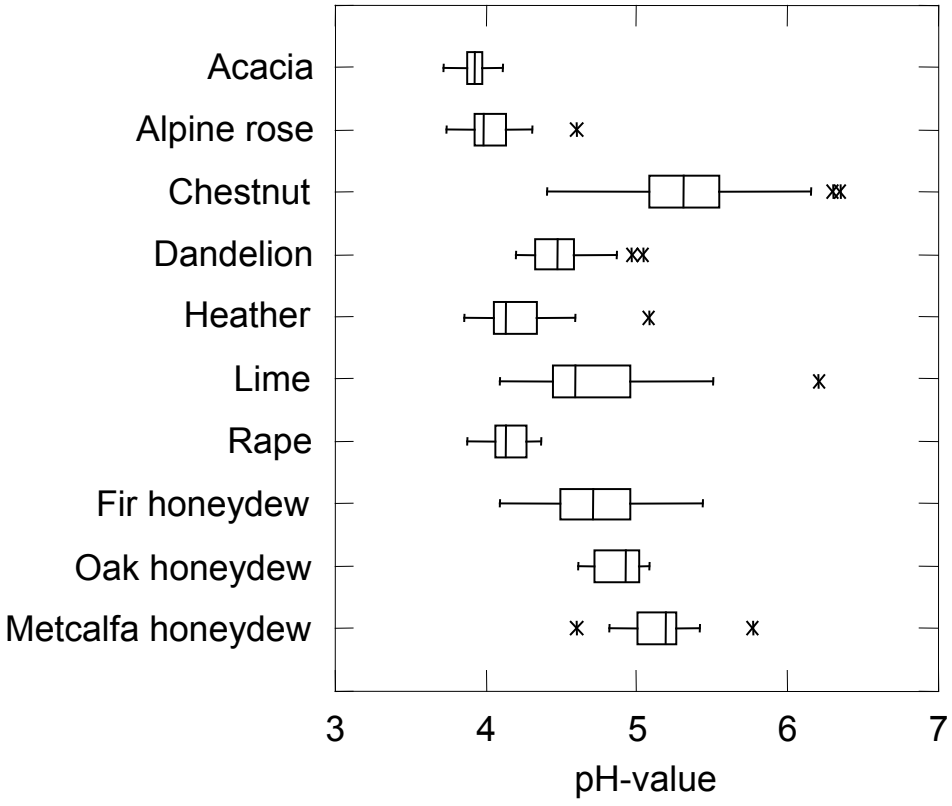
A.1 ELECTRICAL CONDUCTIVITY IN DIFFERENT HONEY TYPES



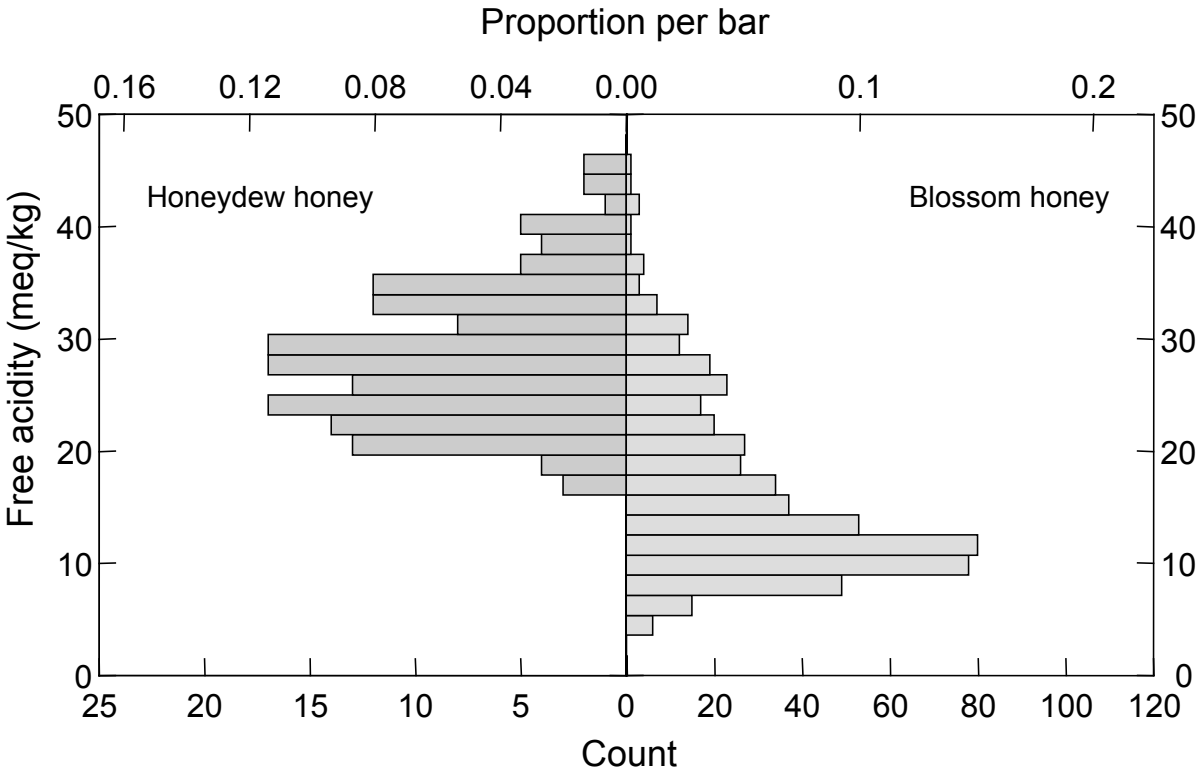


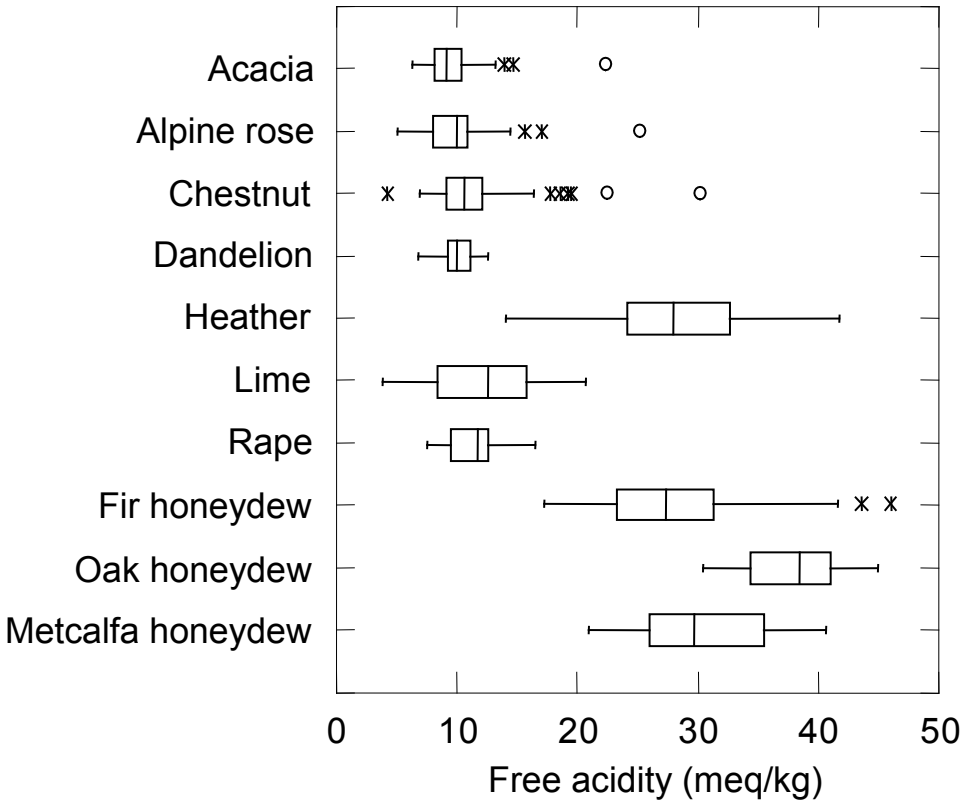
A.2 PH-VALUE IN DIFFERERNT HONEY TYPES





A.3 FREE ACIDITY IN DIFFERENT HONEY TYPES





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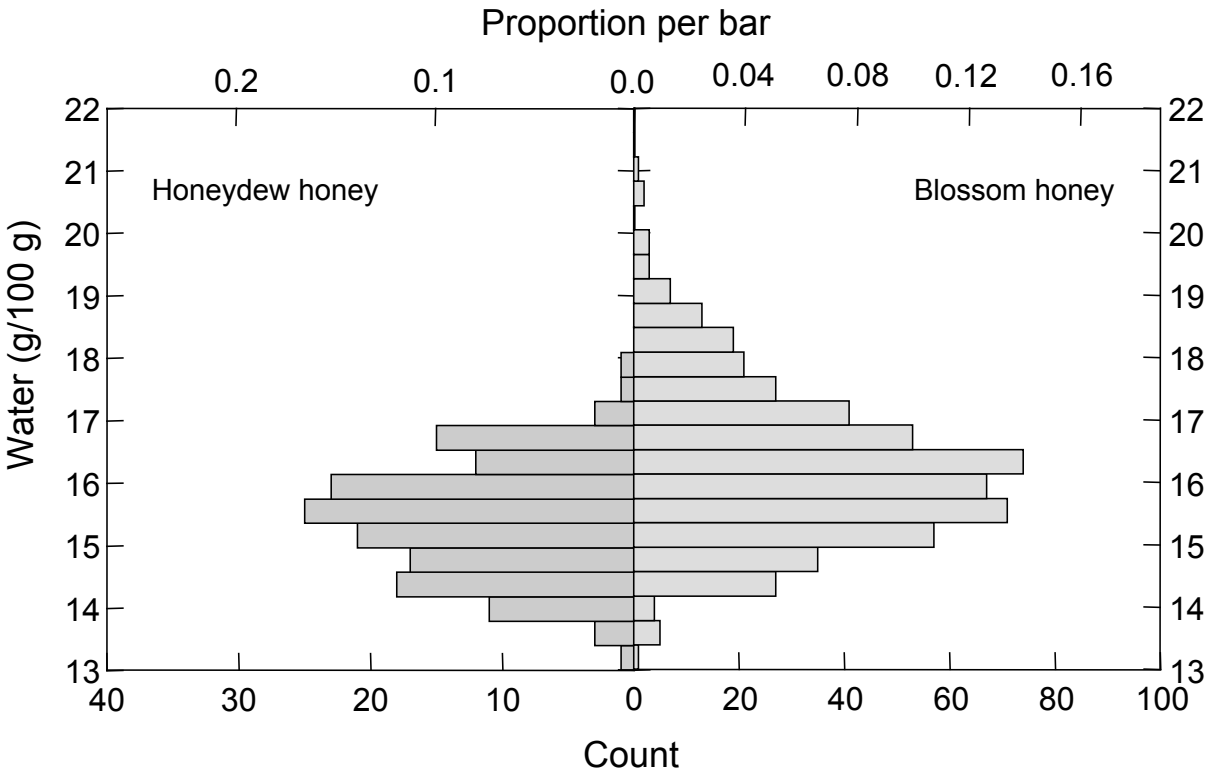

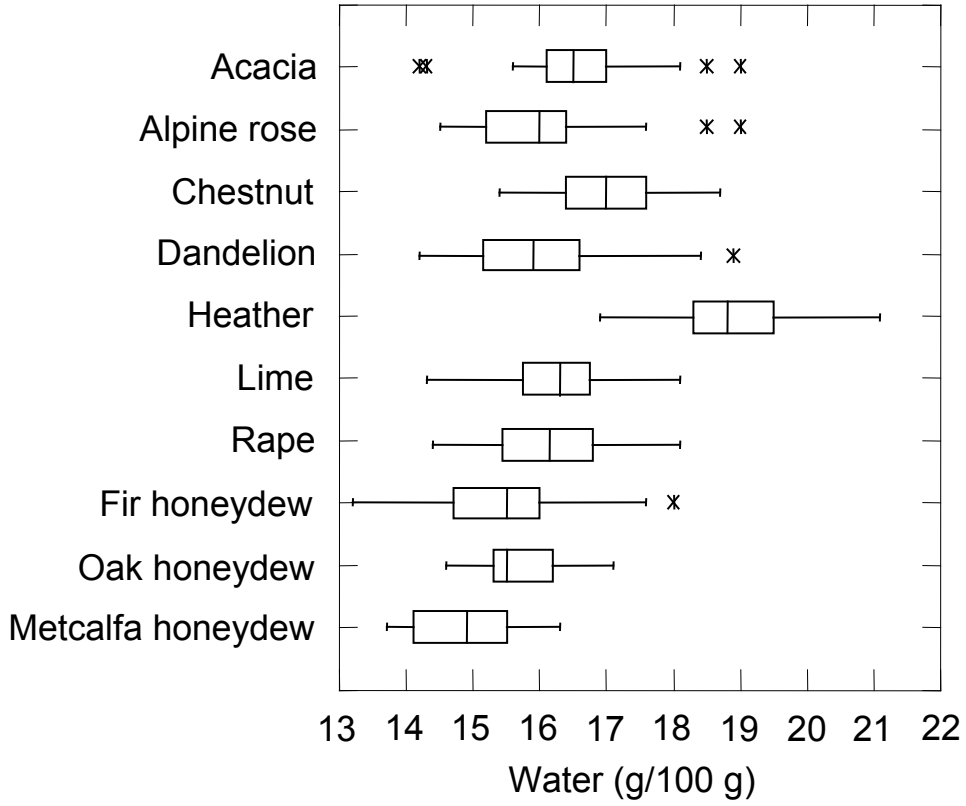

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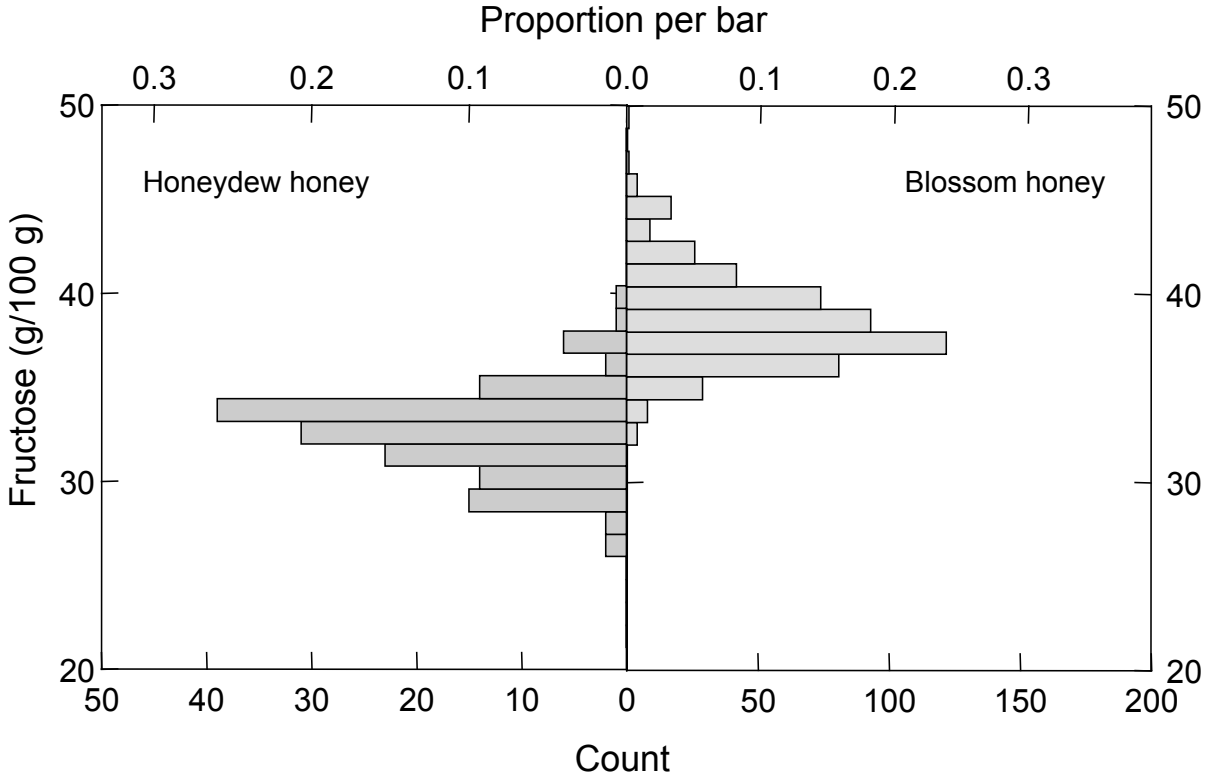
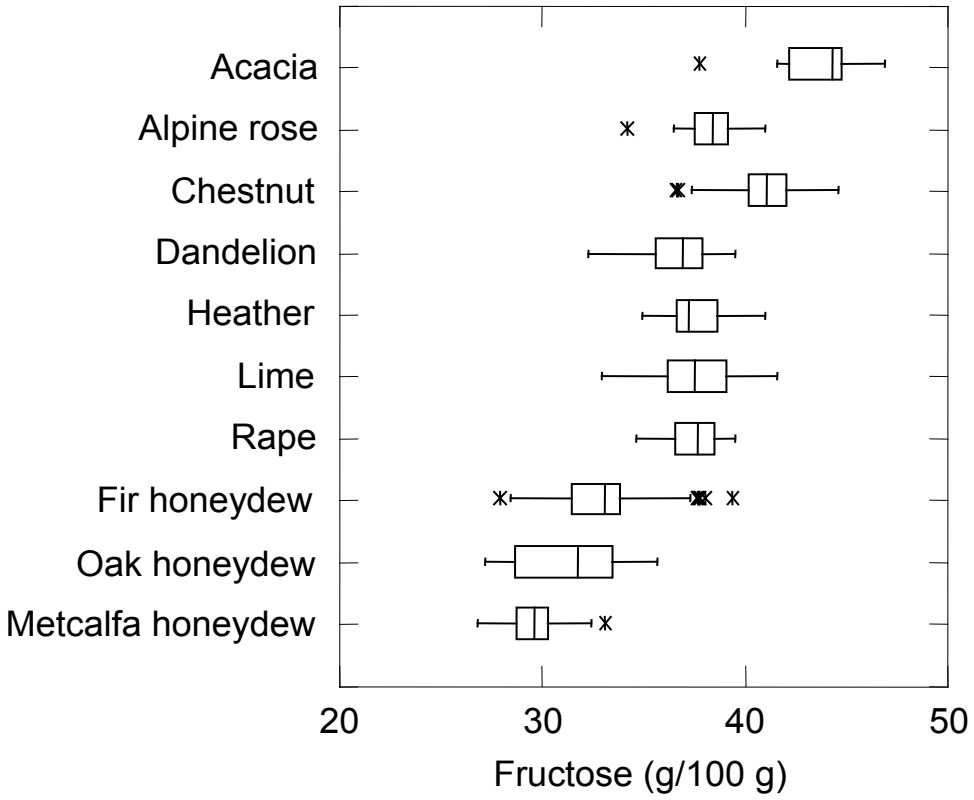
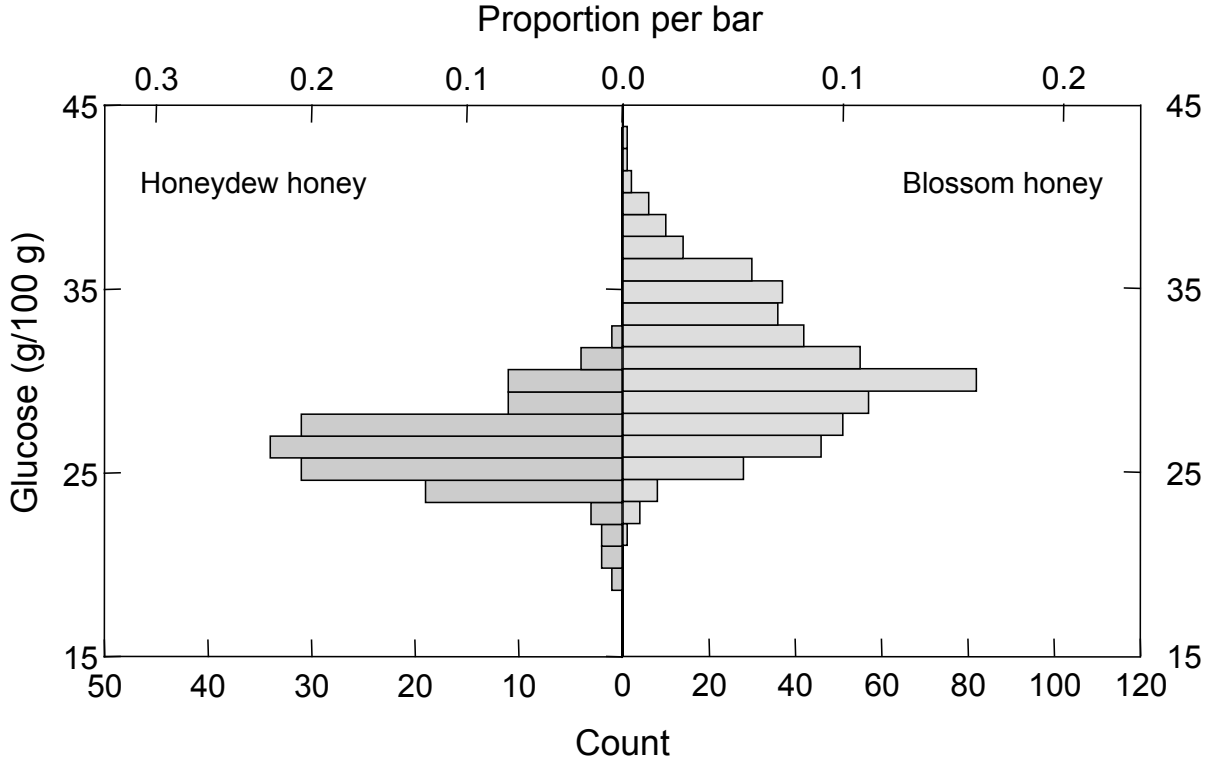
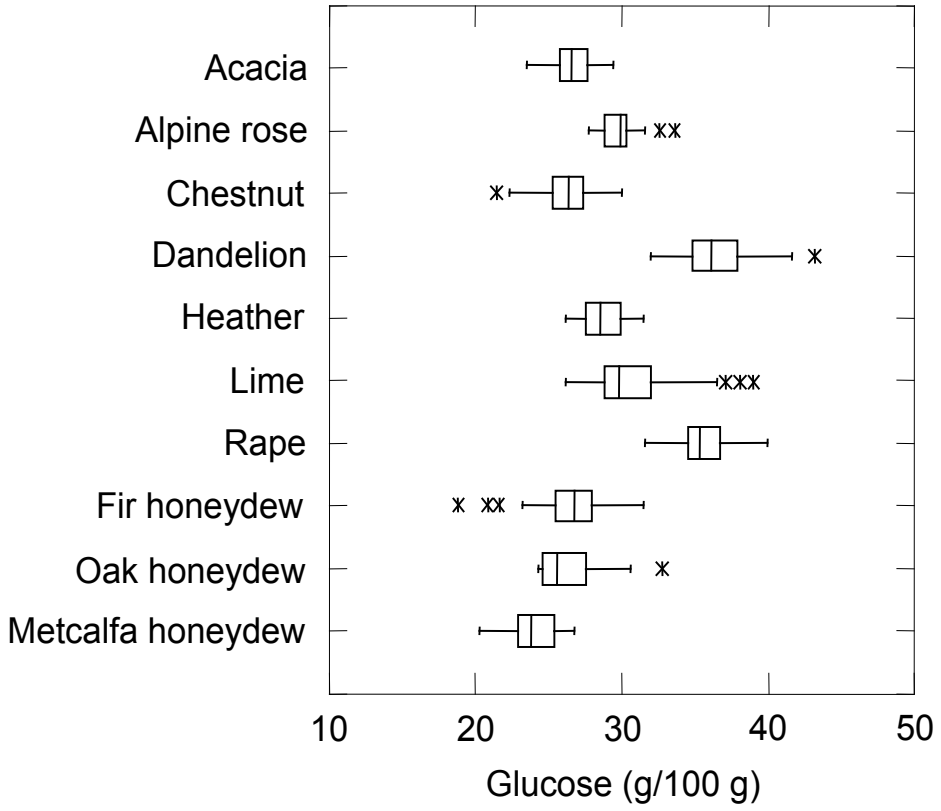


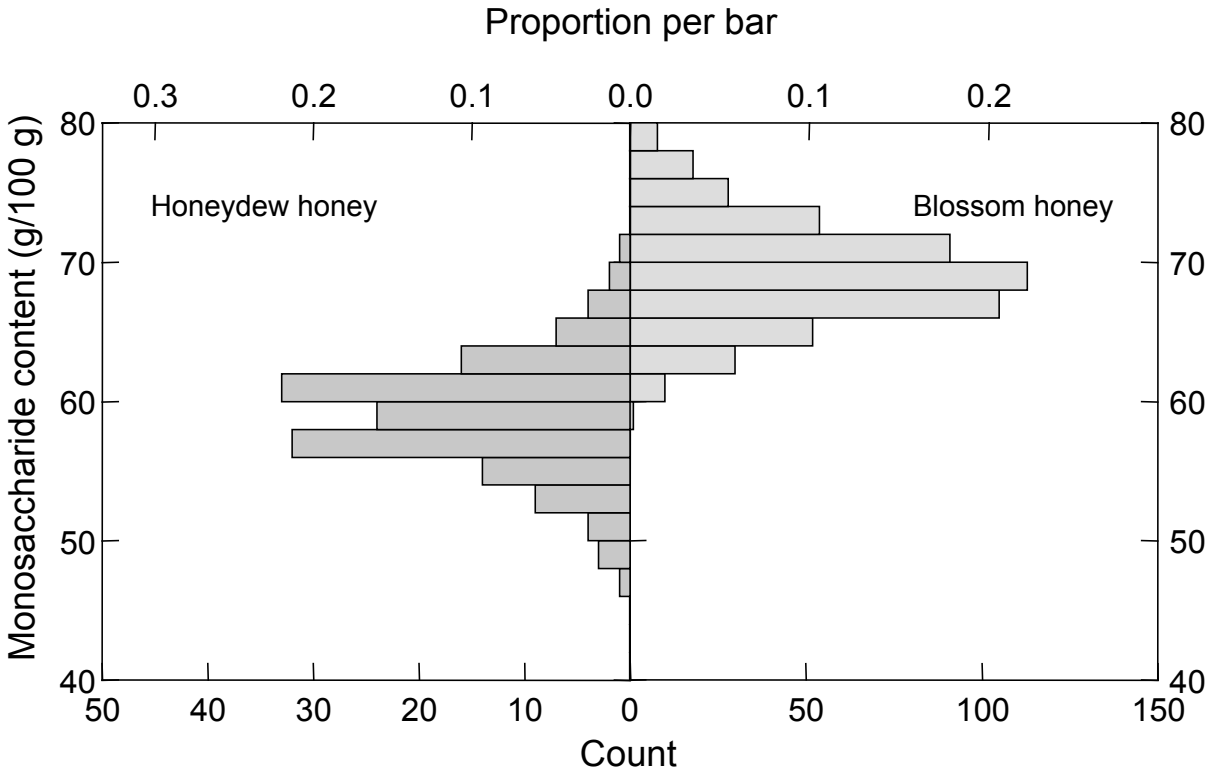


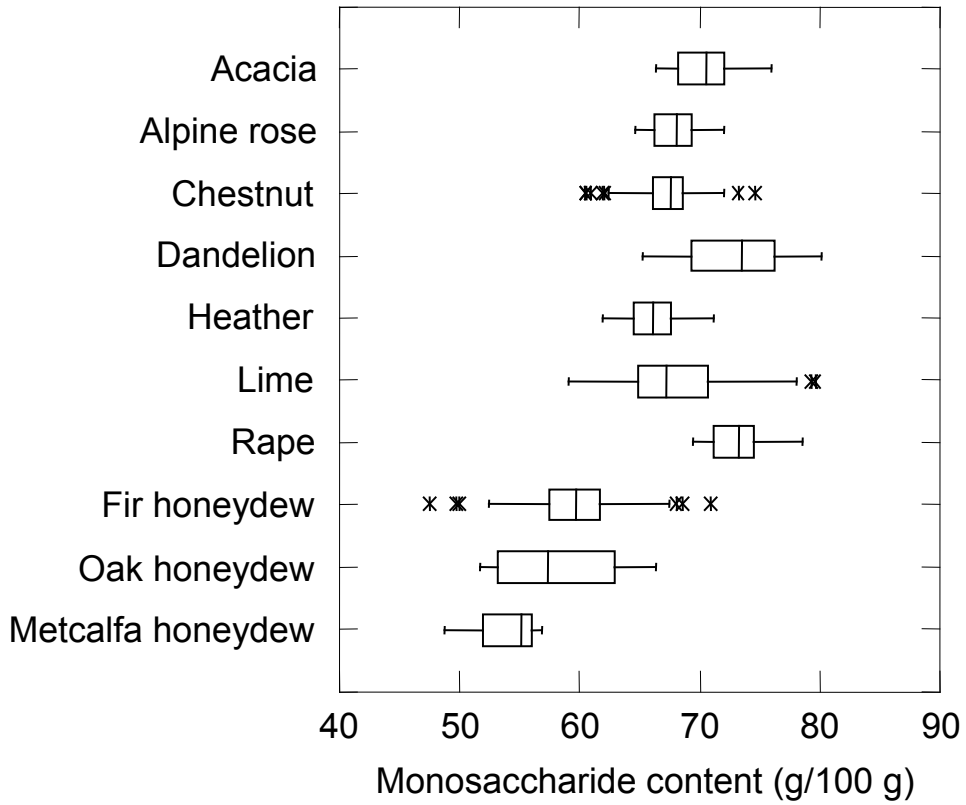
A.6 GLUCOSE CONTENT IN DIFFERENT HONEY TYPES





A.7 MONOSACCHARIDE CONTENT IN DIFFERENT HONEY TYPES





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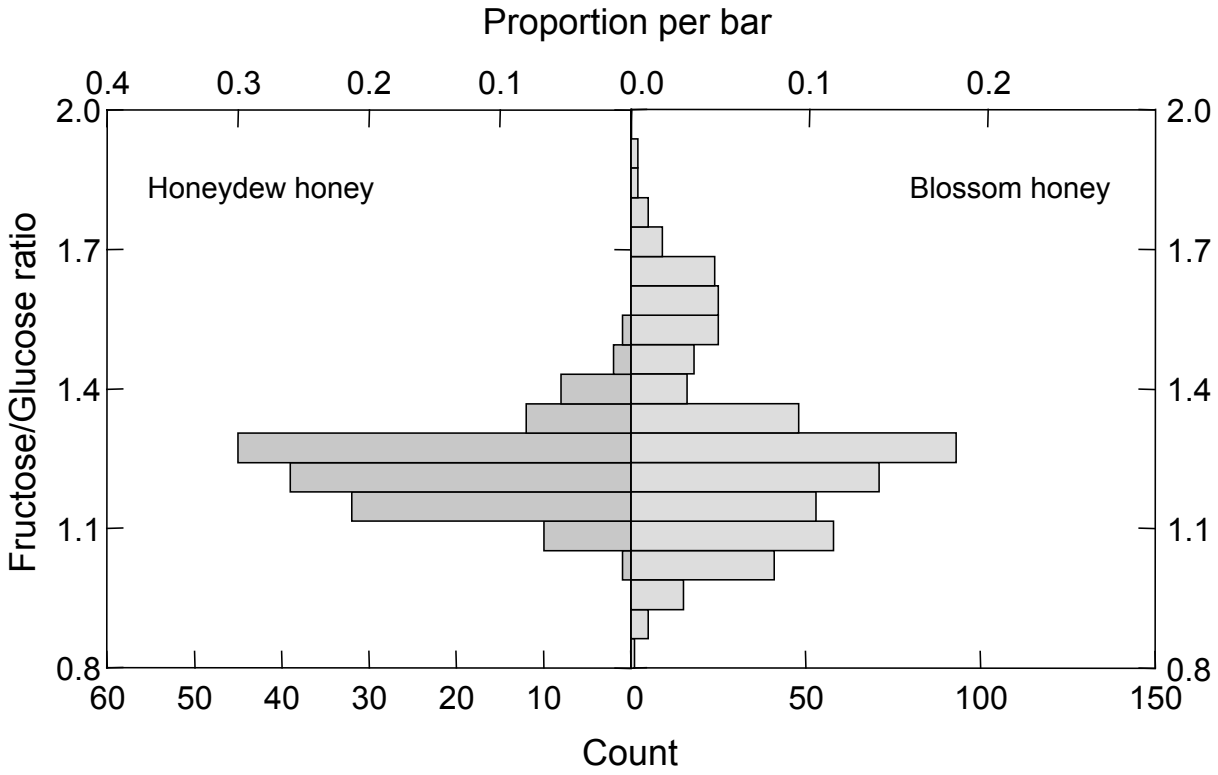

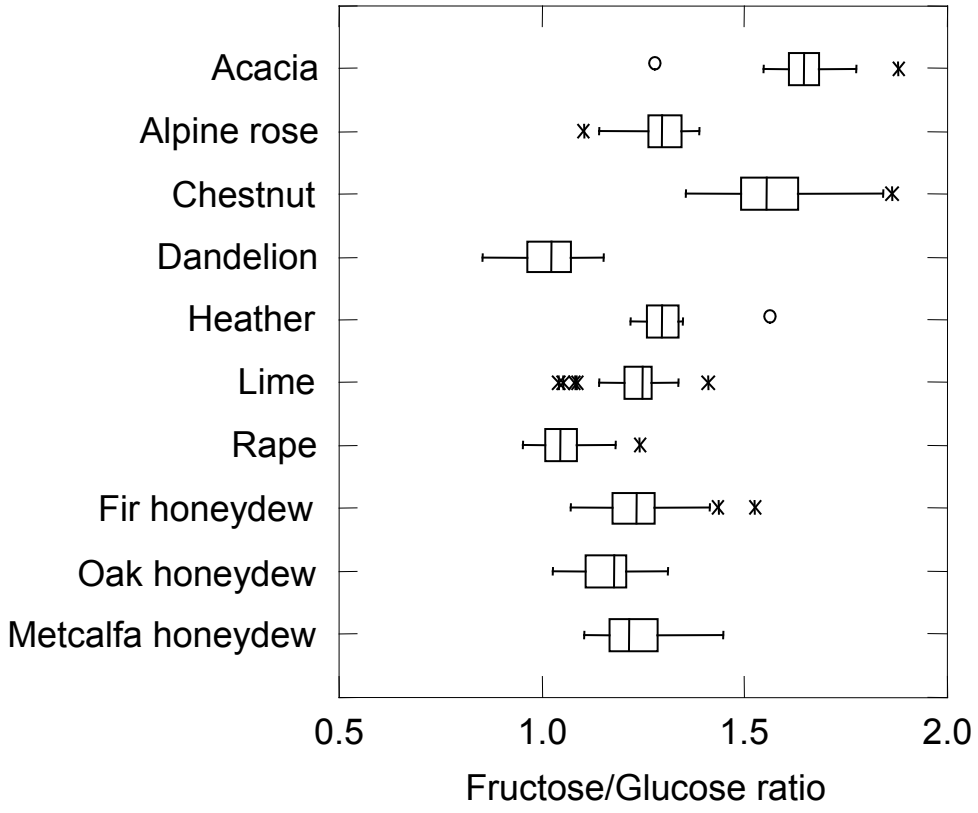

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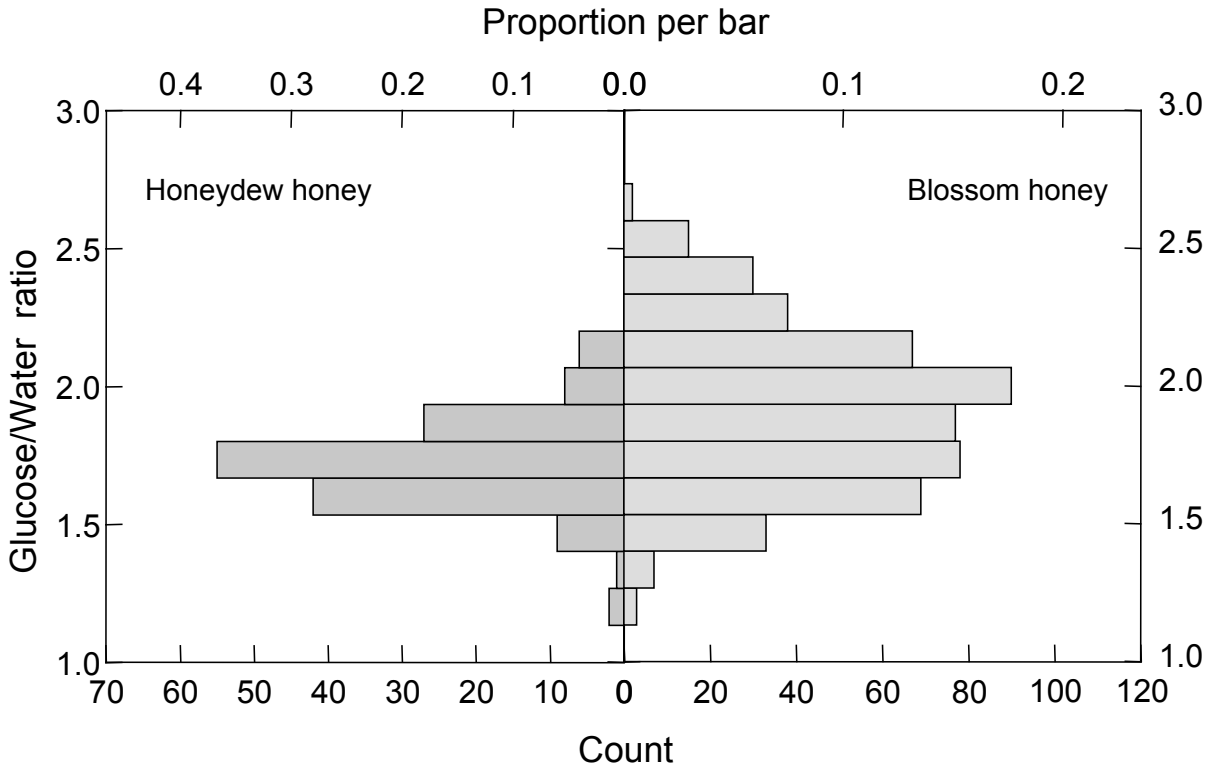

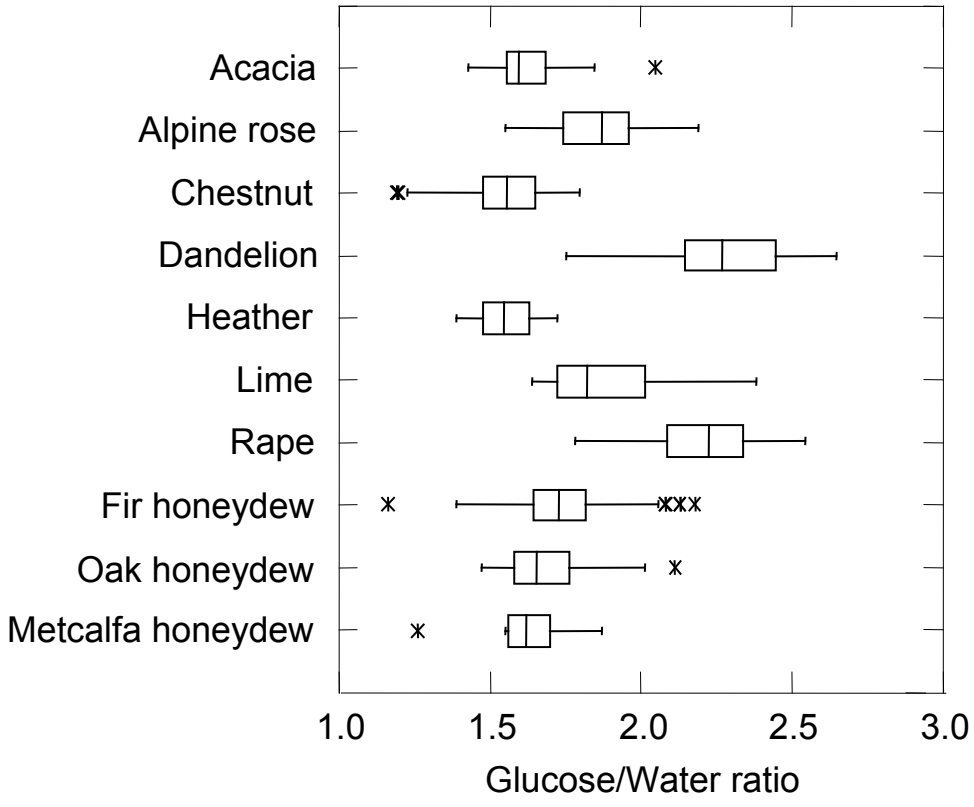

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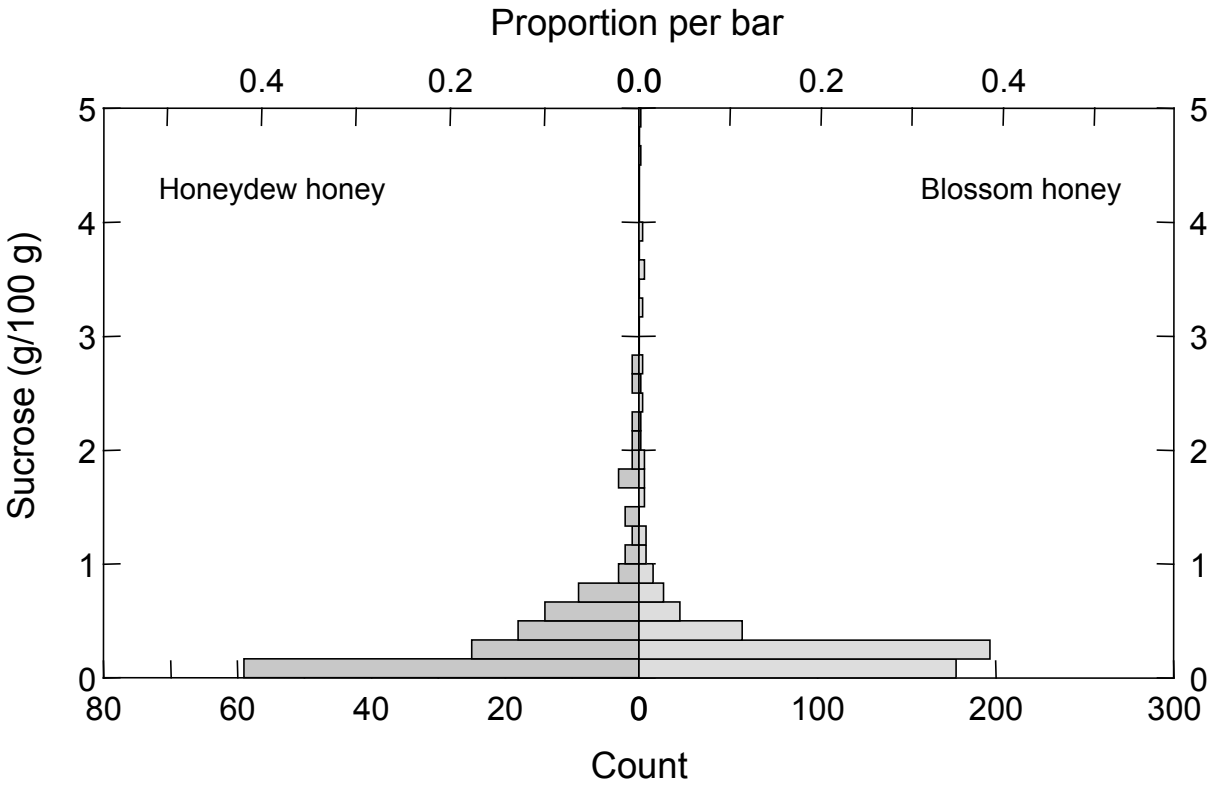

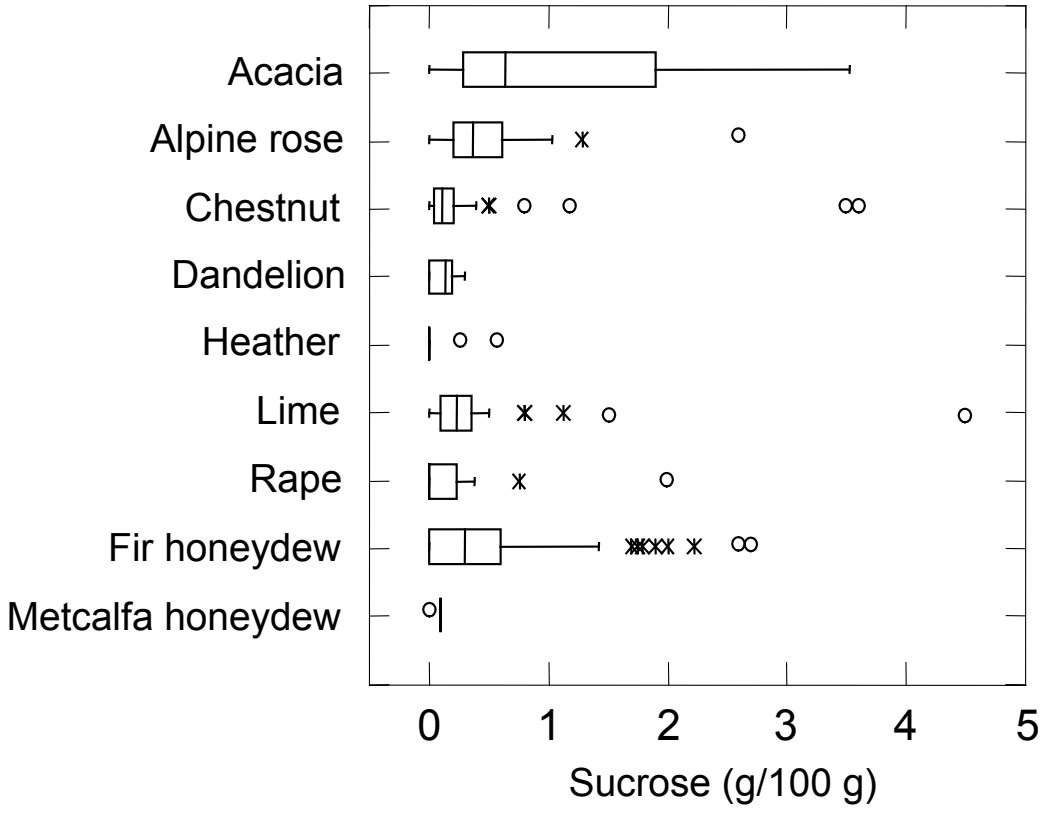

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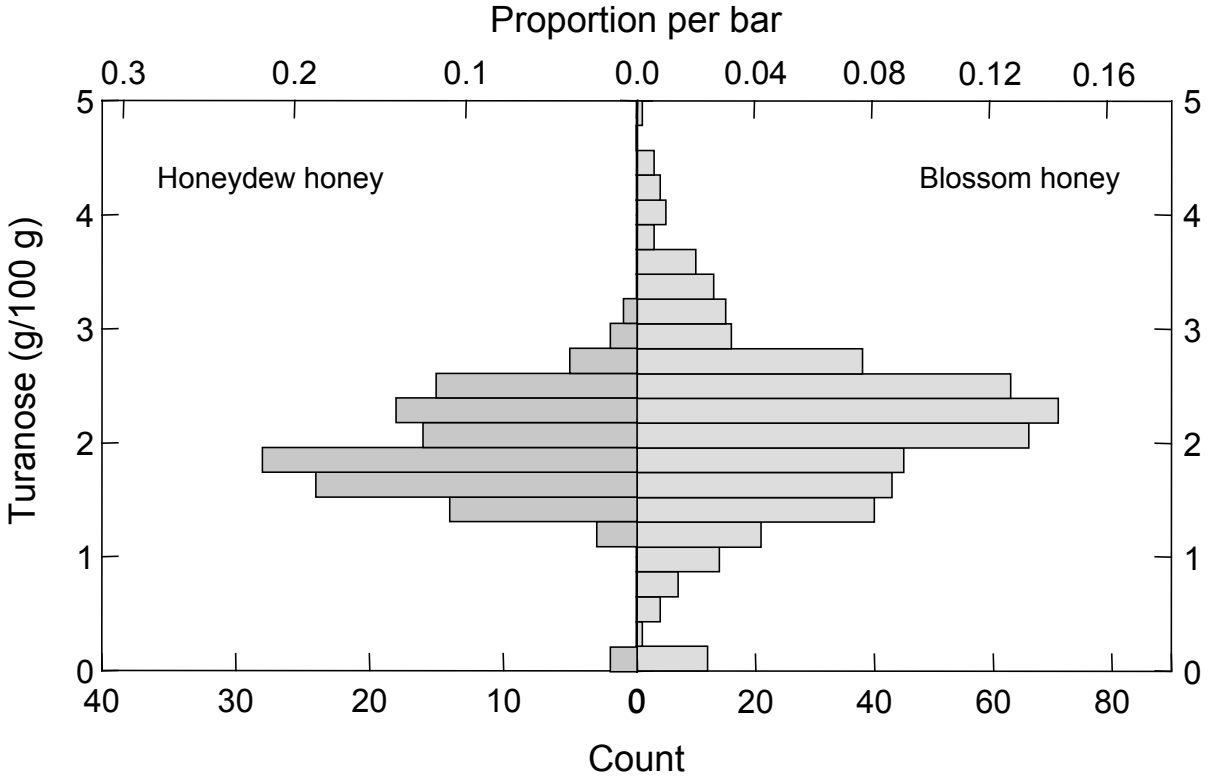
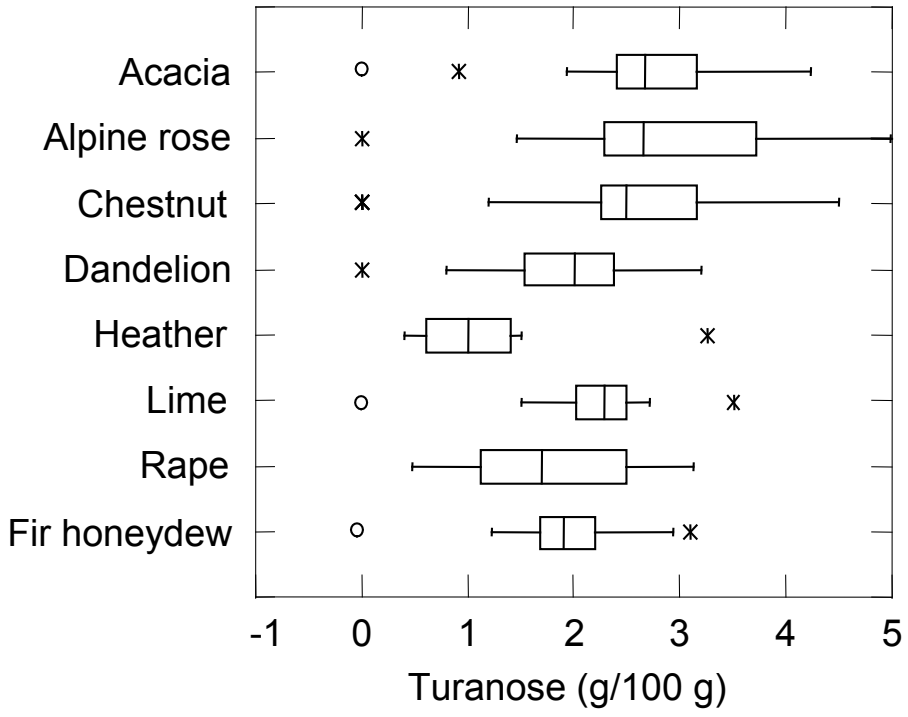
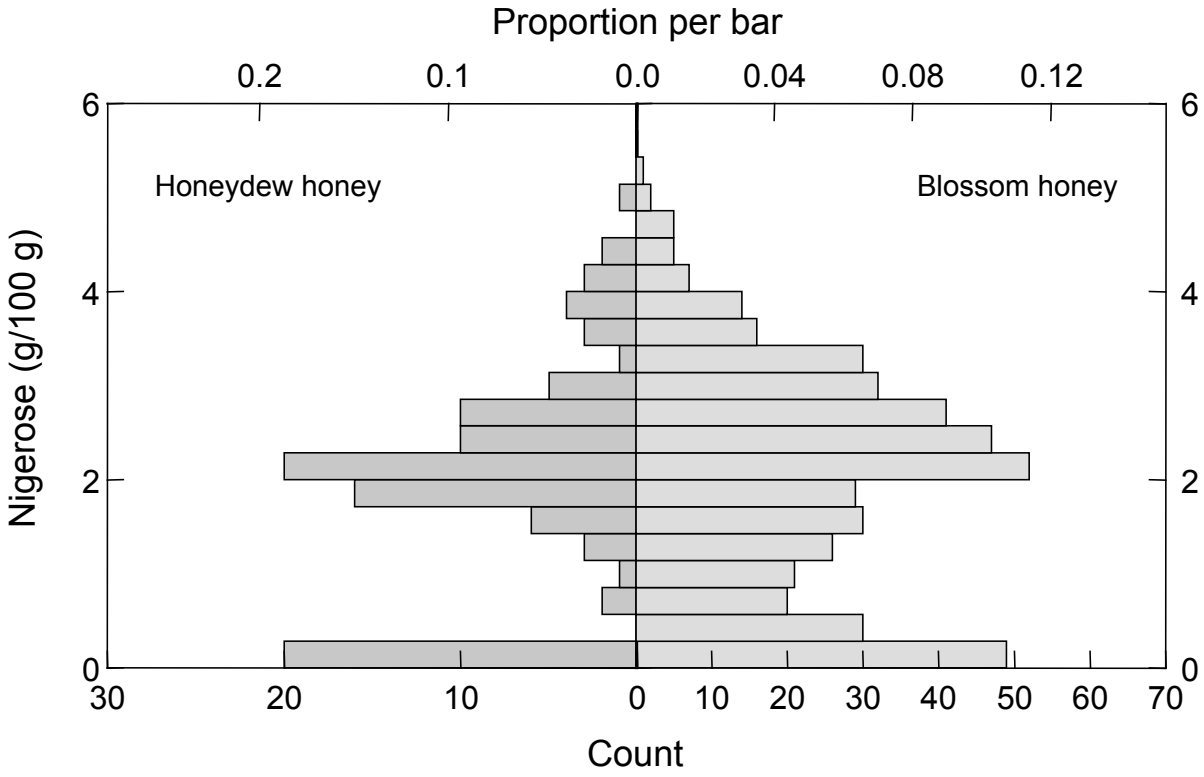
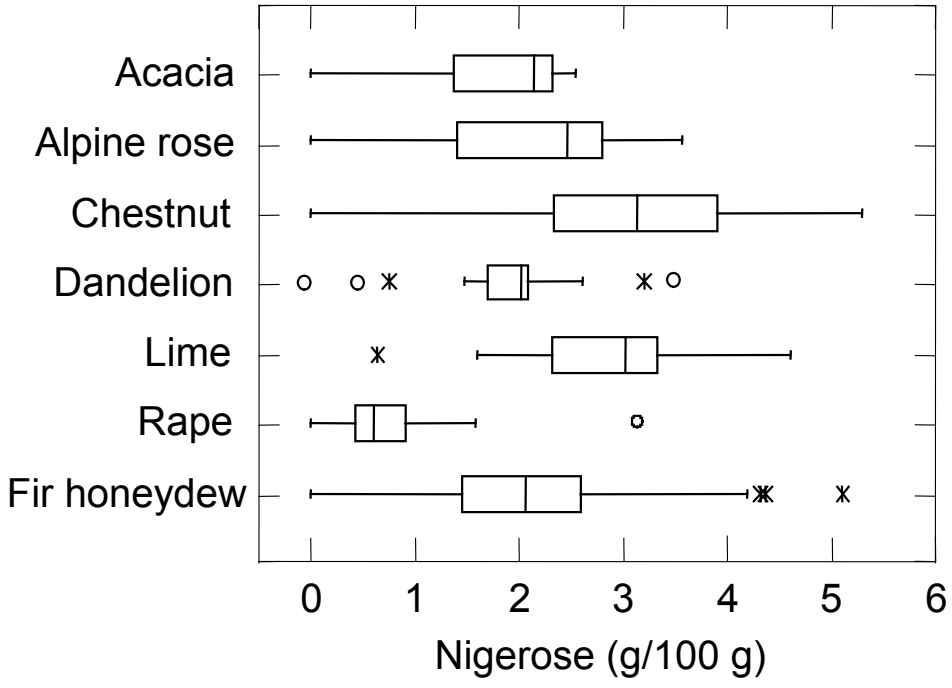




A.12 NIGEROSE CONTENT IN DIFFERENT HONEY TYPE





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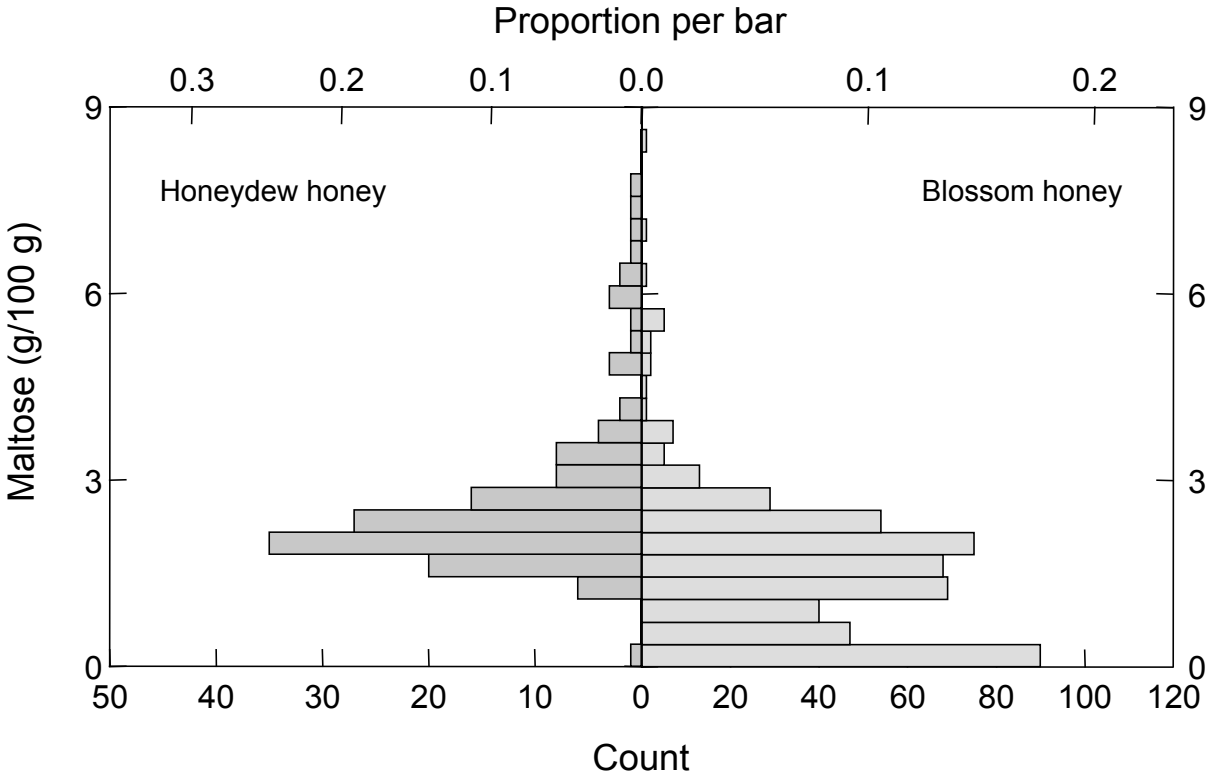

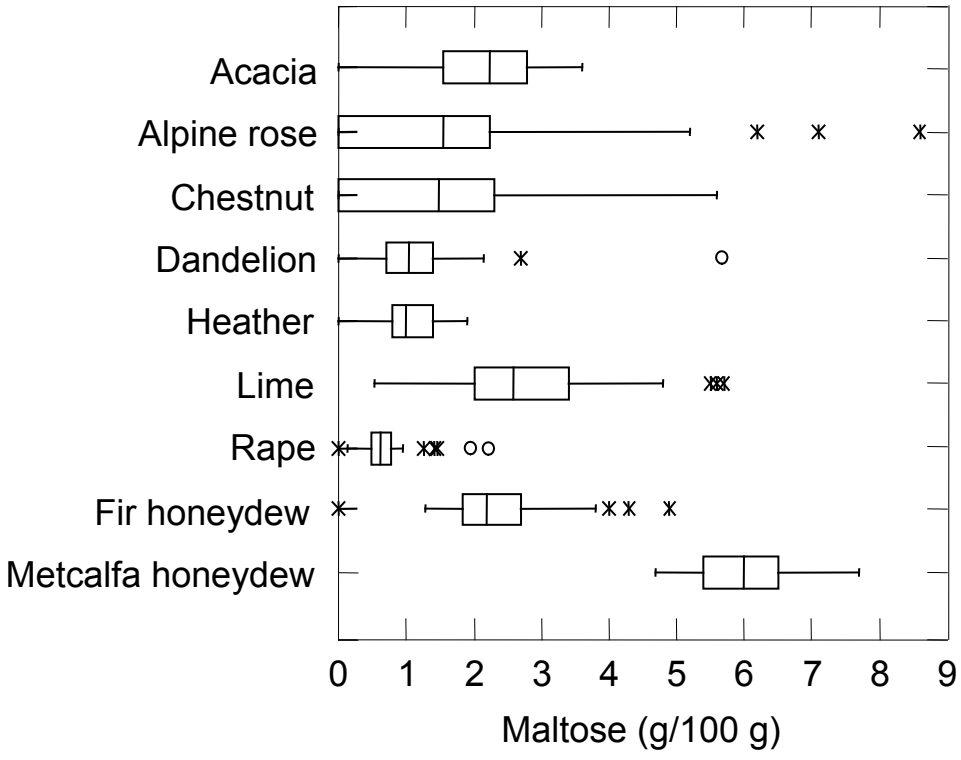

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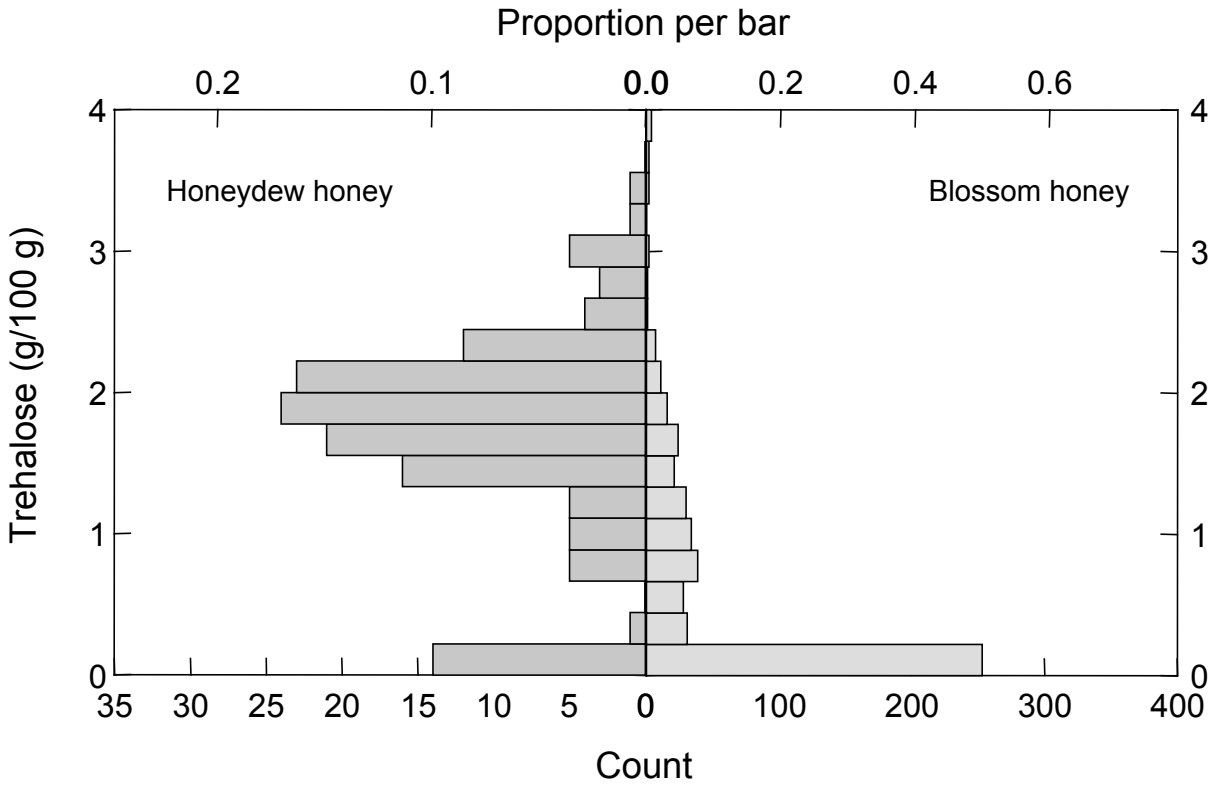
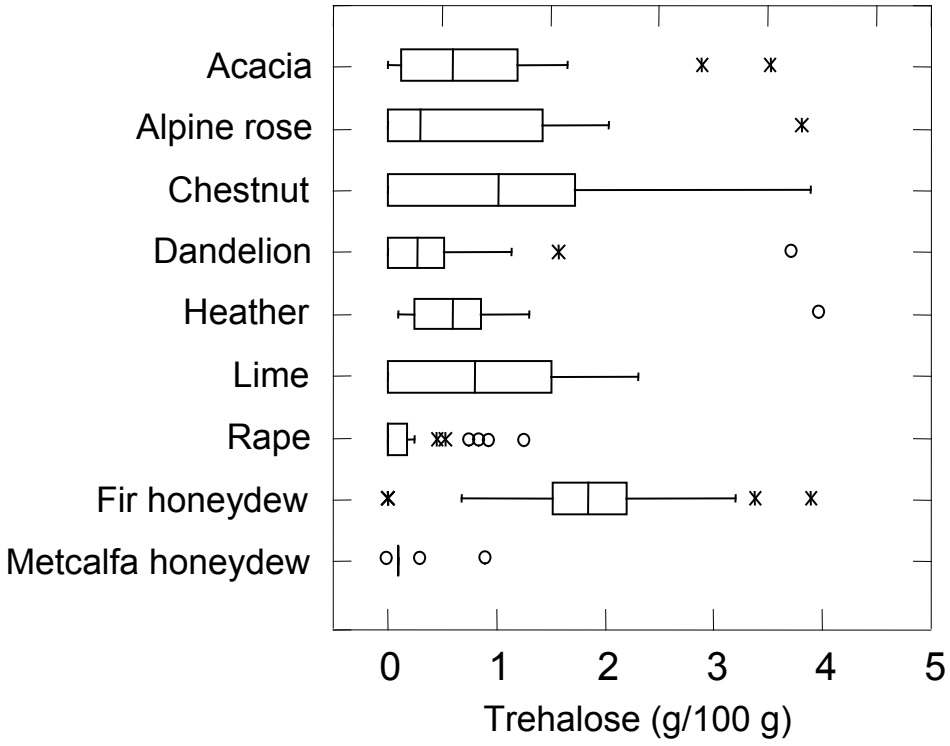
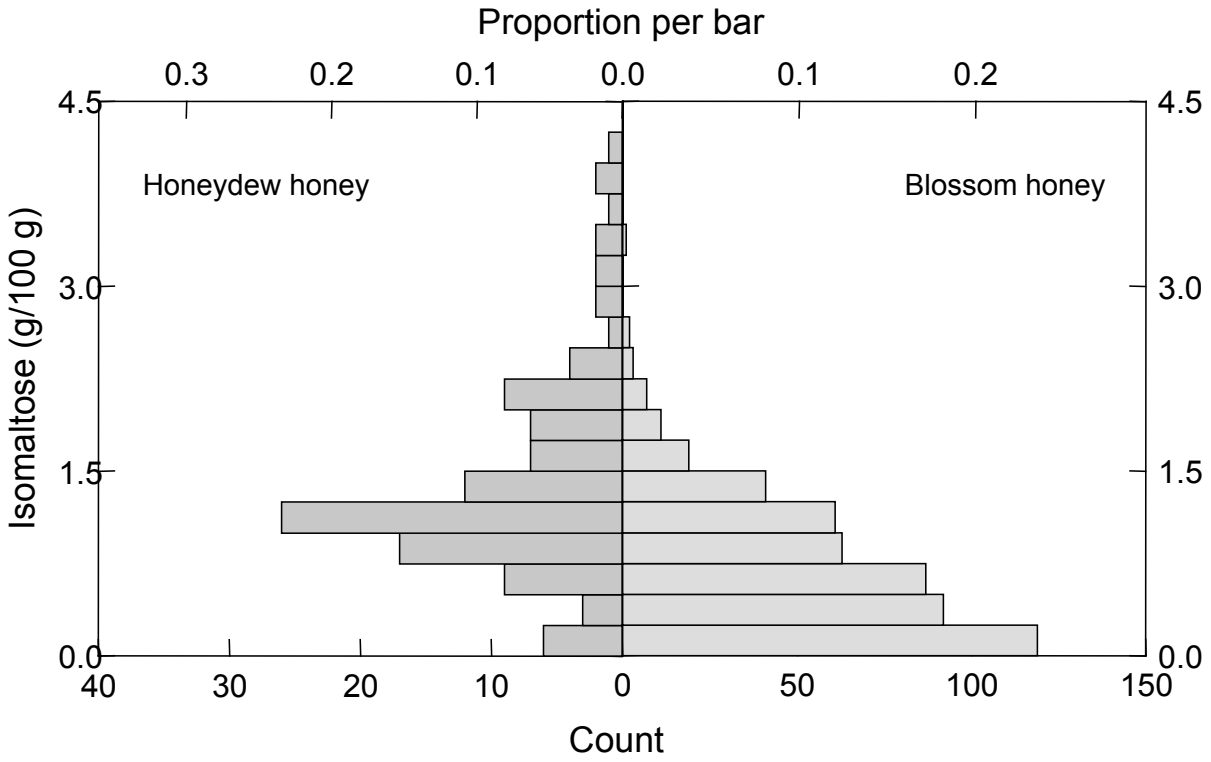
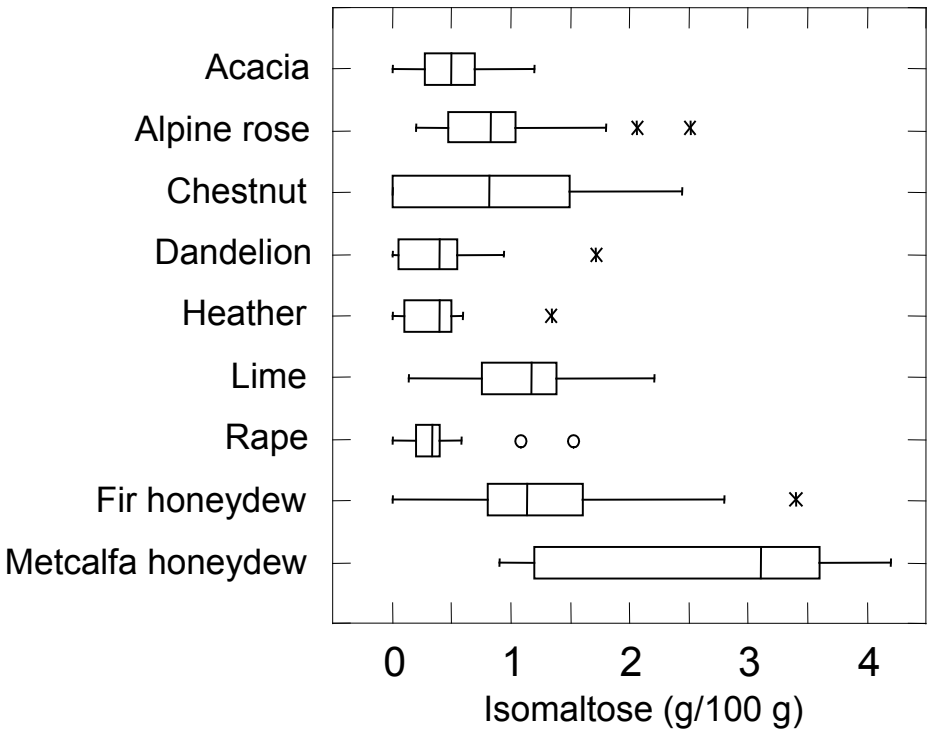




A.15 ISOMALTOSE CONTENT IN DIFFERENT HONEY TYPE





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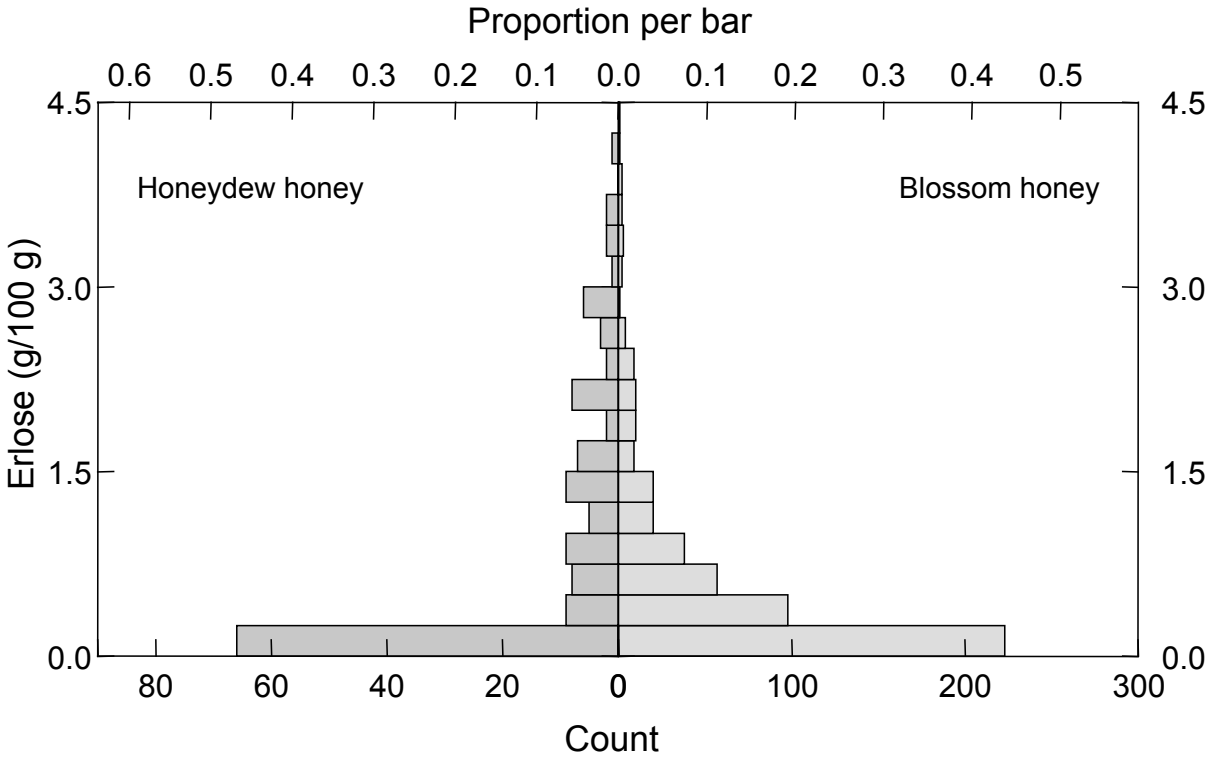
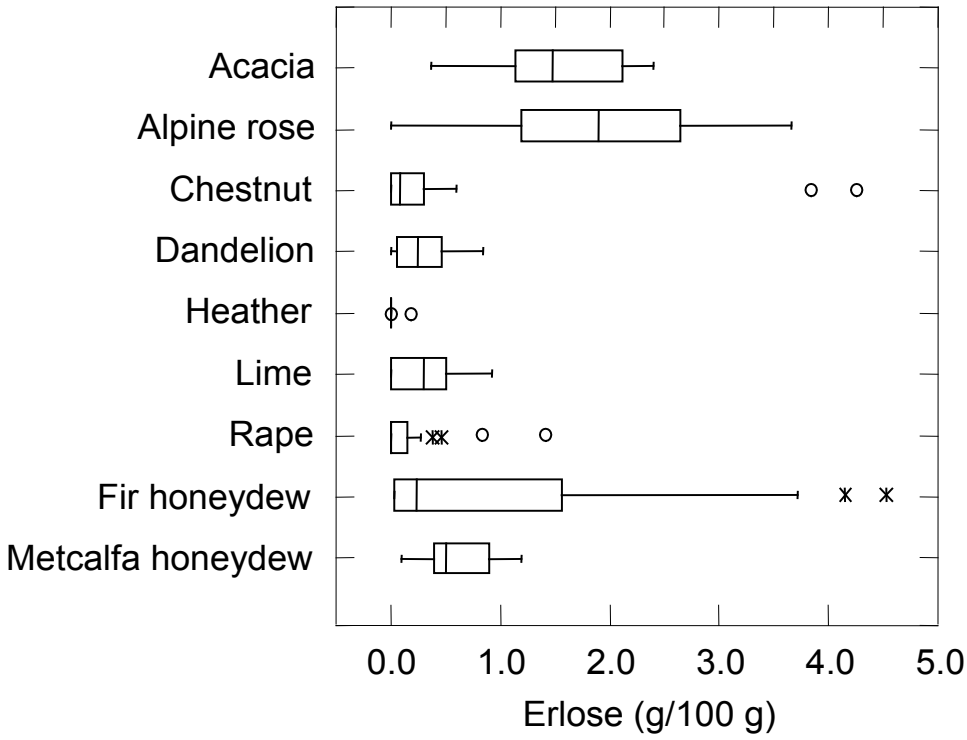
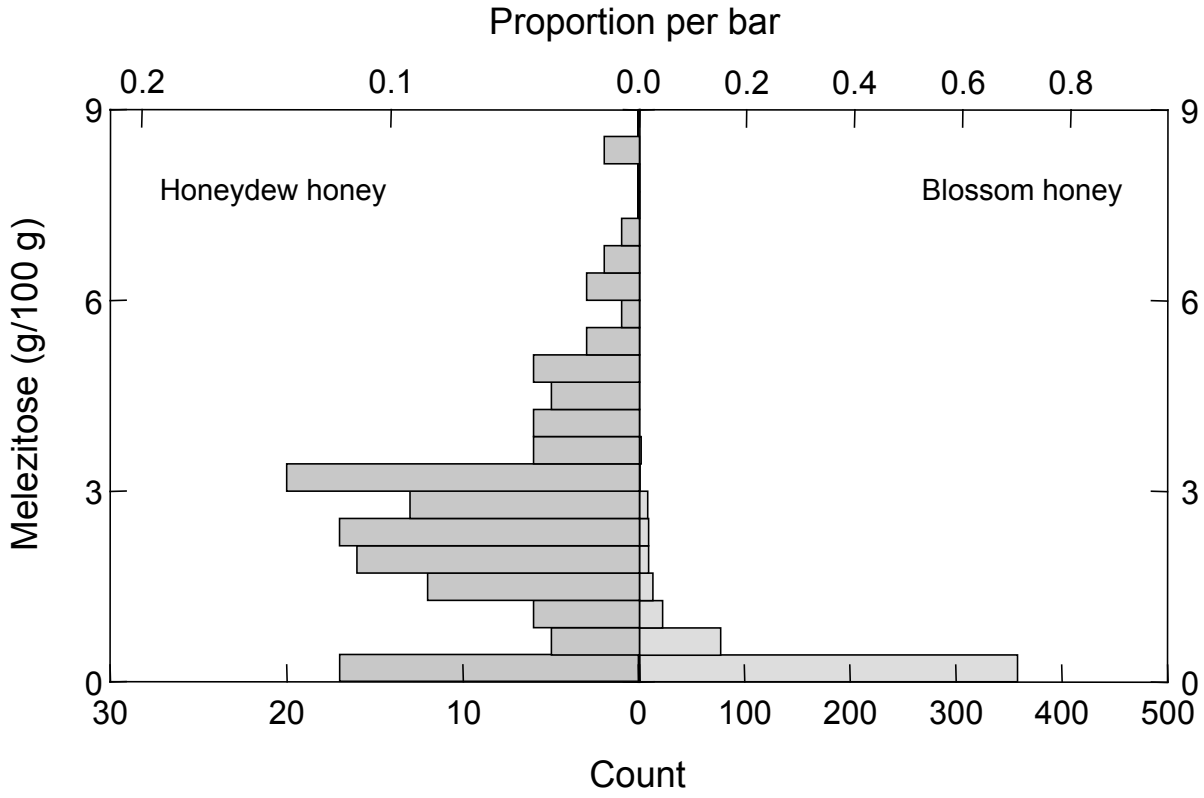
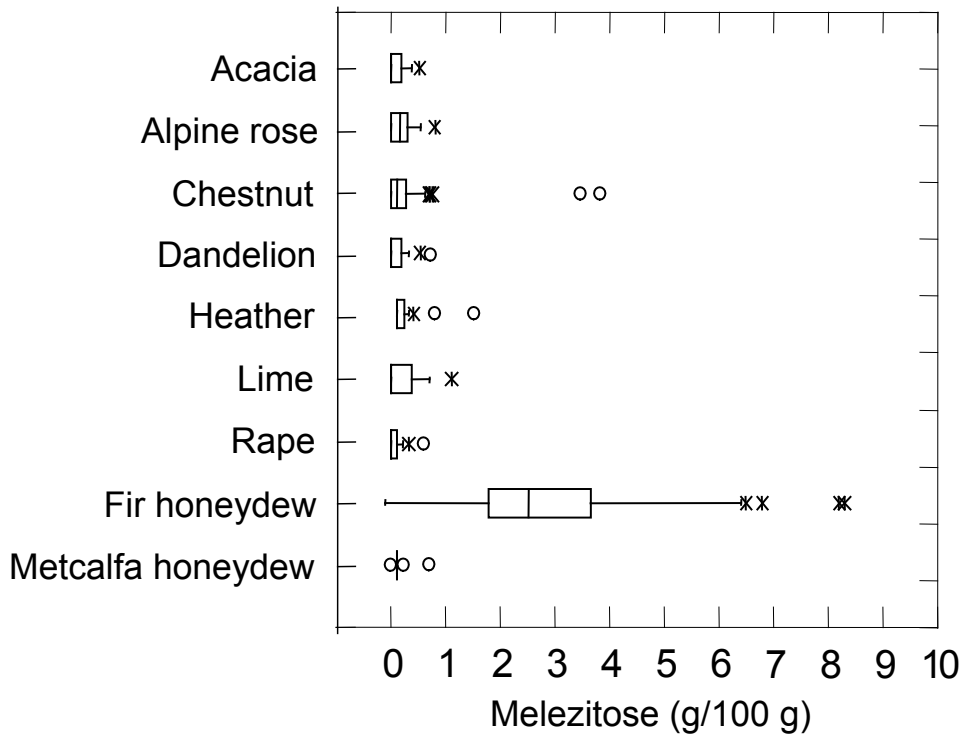


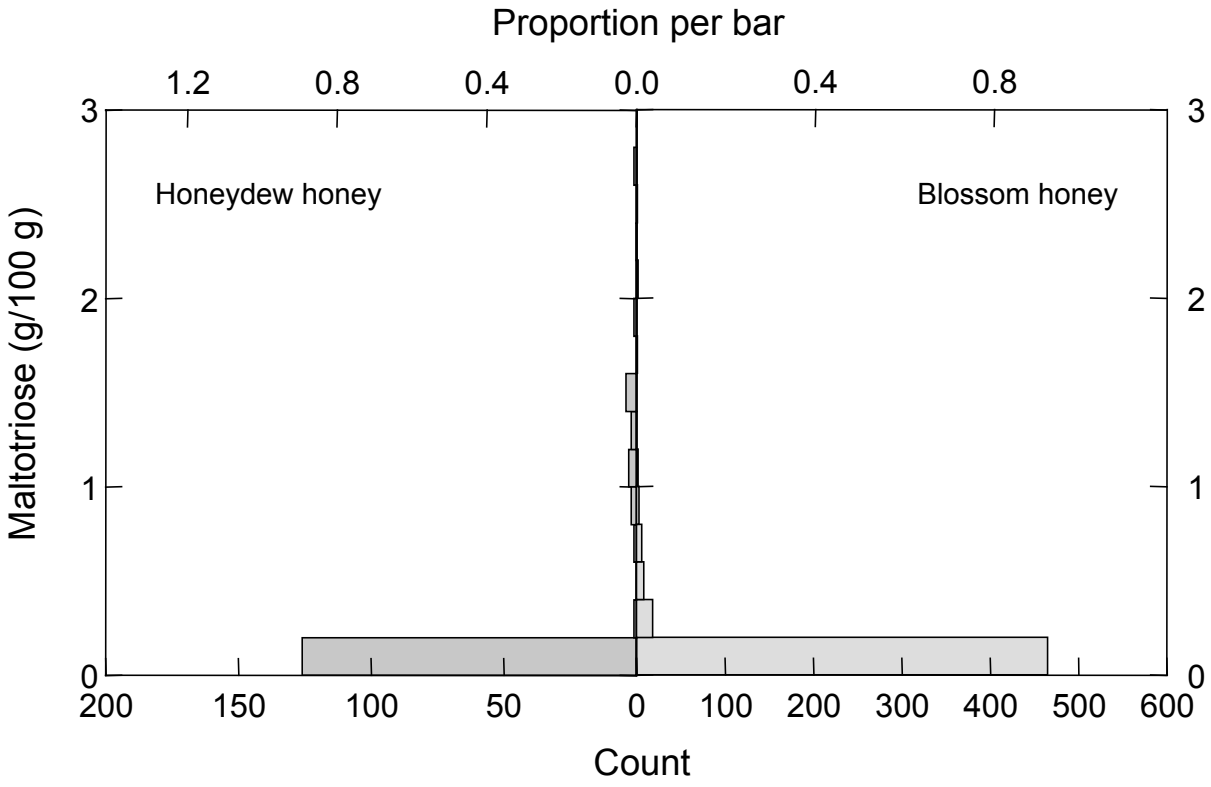


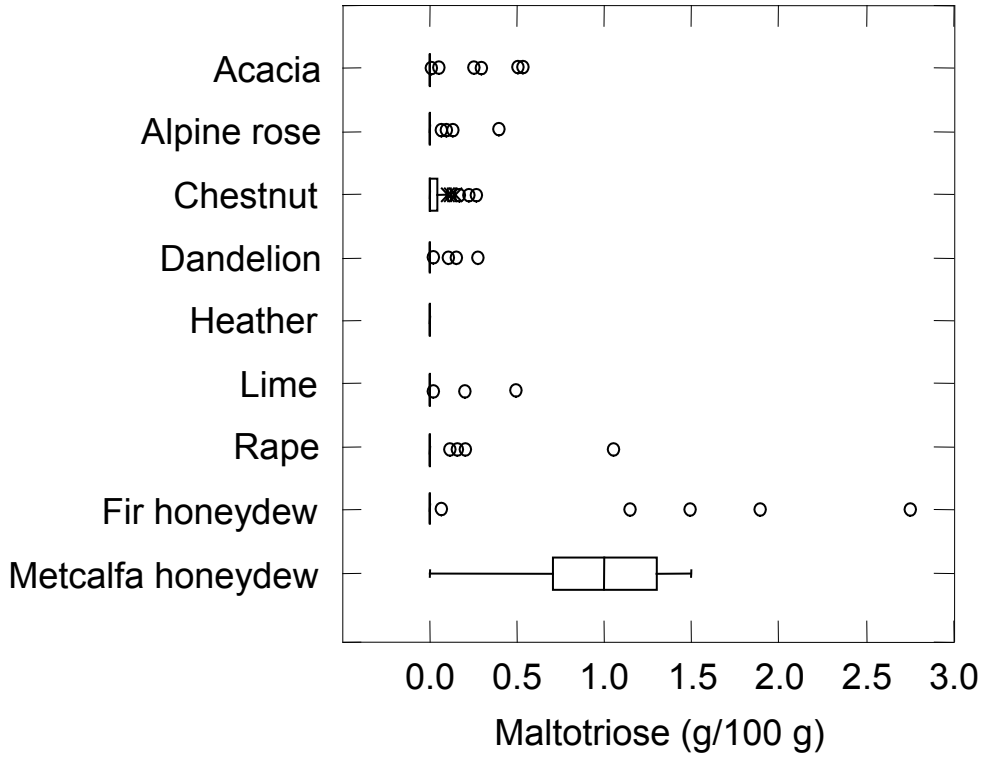
A.17 MELEZITOSE CONTENT IN DIFFERENT HONEY TYPES



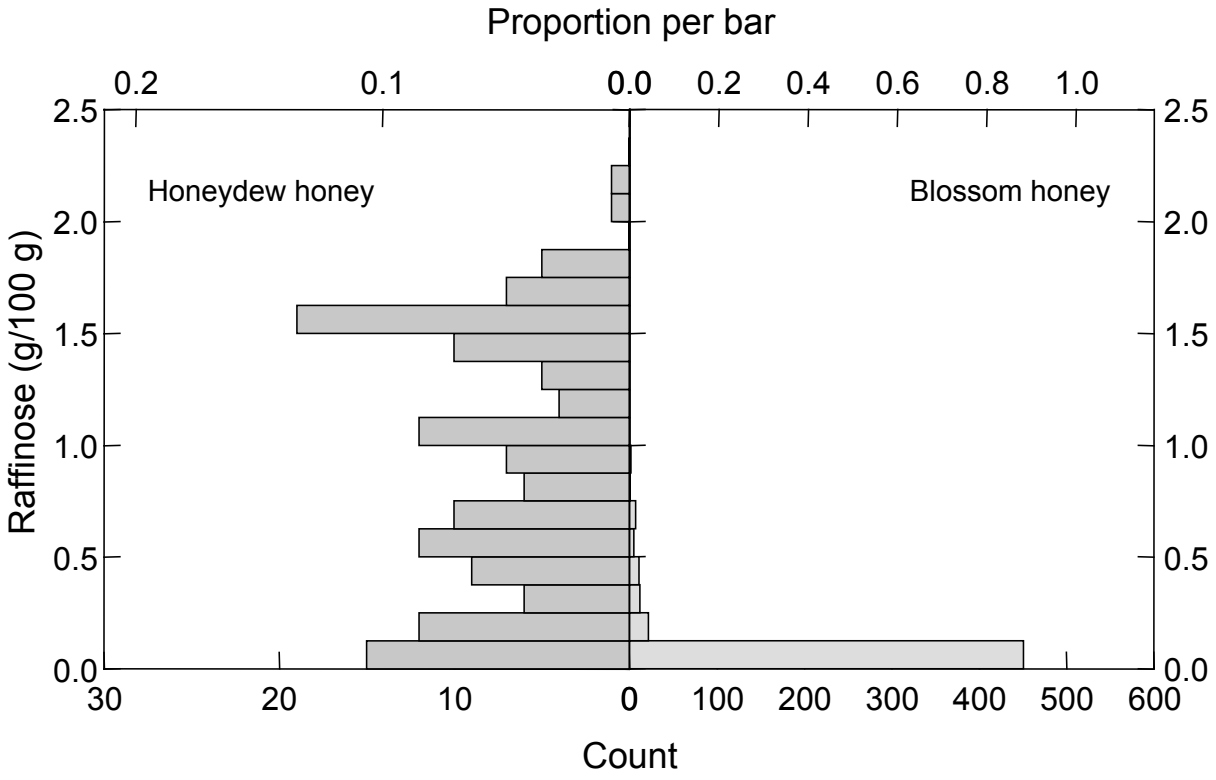


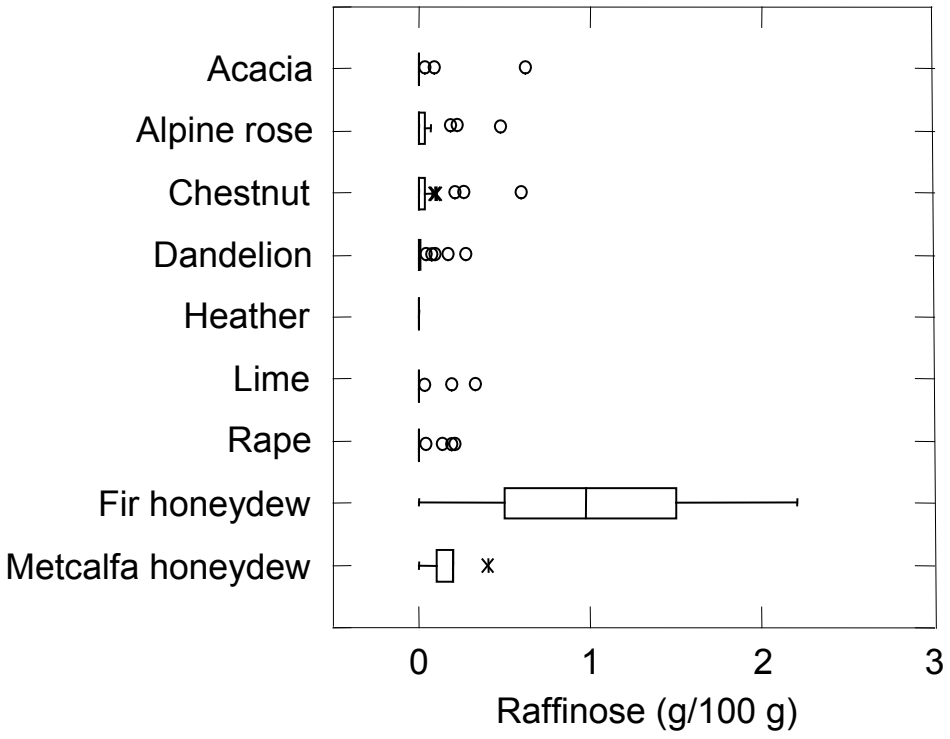
A.18 MALTOTRIOSE CONTENT IN DIFFERENT HONEY TYPES



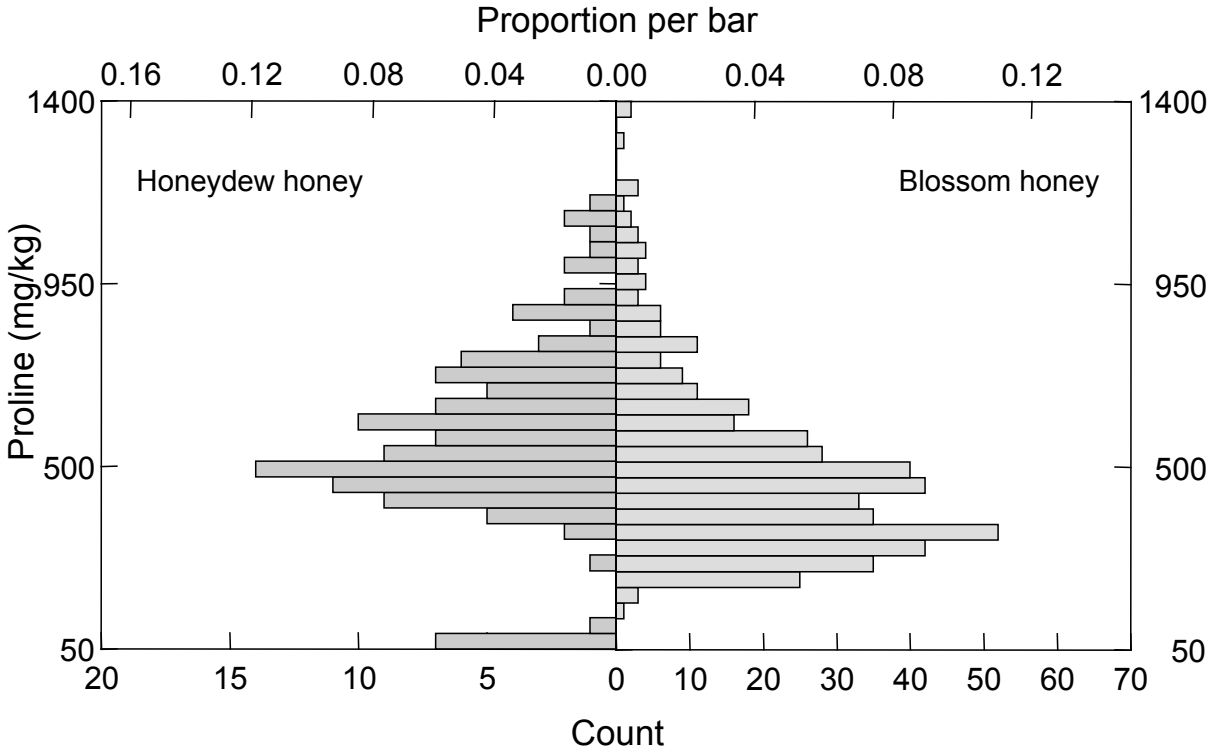


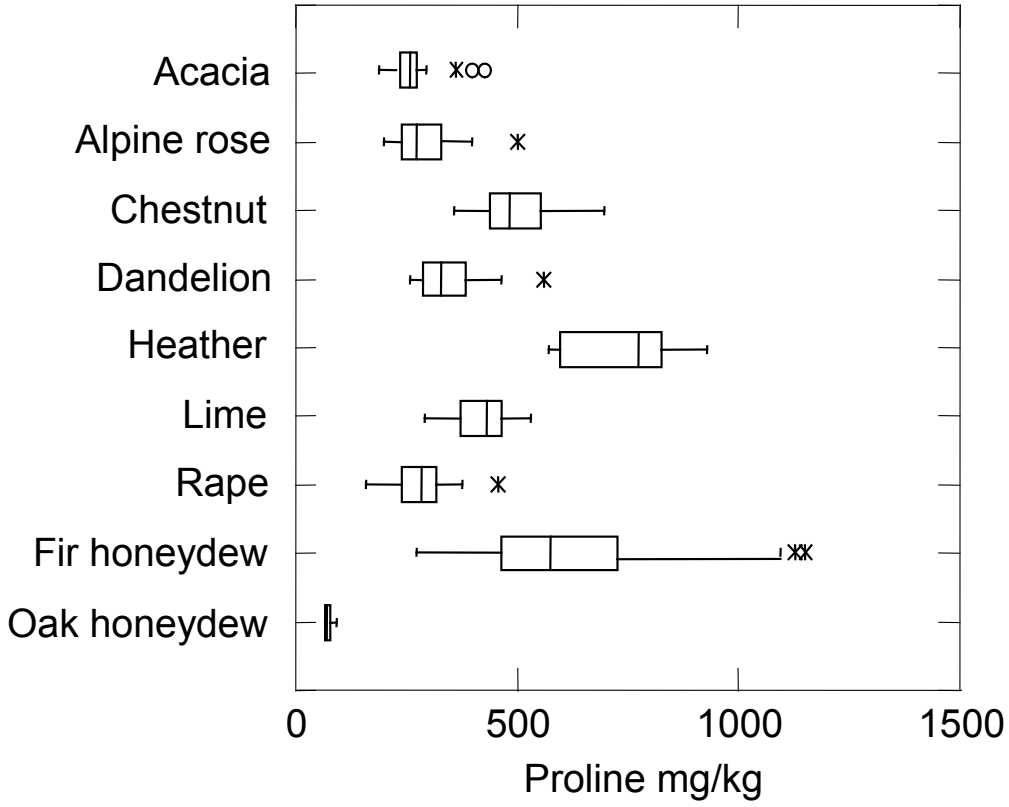
A.19 RAFFINOSE CONTENT IN DIFFERENT HONEY TYPES





A.20 PROLINE CONTENT IN DIFFERENT HONEY TYPES





Appendix B

B.1 PHYSICAL AND CHEMICAL COMPOSITION OF HONEY

The following honey types are considered: acacia, alpine rose, chestnut, dandelion, heather, lime, rape, oak honeydew, fir honeydew, metcalfa honeydew, polyfloral.

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	680	0.652	0.10	2.15	0.14	0.40	0.95	1.55	0.698	0.394
pH-value		680	4.39	3.5	6.4	3.8	4.1	4.8	5.6	4.49	0.47
Free acidity	meq/kg	680	17.3	4	46	7	11	26	39	18.9	9.1
Water	g/100 g	682	15.90	13.2	21.1	13.9	15.2	16.8	18.8	16.05	1.24
Fructose	g/100 g	661	37.40	26.8	49.8	29.5	35.3	39.3	44.4	37.15	3.52
Glucose	g/100 g	661	29.40	18.8	43.2	23.6	26.8	32.2	38.1	29.74	3.89
Monosaccharides	g/100 g	661	67.81	47.5	80.1	53.9	63.5	70.9	76.7	66.89	5.75
Fructose/Glucose ratio		661	1.245	0.85	1.90	0.98	1.14	1.34	1.69	1.266	0.184
Glucose/Water ratio		659	1.833	1.16	2.65	1.43	1.66	2.06	2.48	1.871	0.277
Sucrose	g/100 g	652	0.21	0.0	4.8	0.0	0.1	0.4	2.3	0.37	0.59
Turanose	g/100 g	623	2.10	0.0	5.0	0.5	1.7	2.5	3.7	2.09	0.74
Nigerose	g/100 g	564	2.12	0.0	5.3	0.0	1.1	2.8	4.3	1.99	1.20
Maltose	g/100 g	652	1.76	0.0	8.6	0.0	0.9	2.3	5.5	1.76	1.29
Trehalose	g/100 g	647	0.63	0.0	4.0	0.0	0.0	1.6	2.9	0.85	0.91
Isomaltose	g/100 g	617	0.73	0.0	4.2	0.0	0.3	1.2	2.4	0.83	0.68
Erlose	g/100 g	651	0.32	0.0	4.5	0.0	0.0	0.9	3.1	0.64	0.85
Melezitose	g/100 g	652	0.26	0.0	8.4	0.0	0.0	1.3	4.9	0.93	1.40
Maltotriose	g/100 g	647	0.00	0.0	2.8	0.0	0.0	0.0	1.0	0.08	0.29
Raffinose	g/100 g	652	0.00	0.0	2.2	0.0	0.0	0.2	1.6	0.23	0.46
Proline	mg/kg	589	463.7	65	1385	213	347	609	1062	504	219
HMF	mg/kg	593	3.2	0	83	0	1	7	28	5.8	8.6

B.2 PHYSICAL AND CHEMICAL COMPOSITION OF BLOSSOM HONEY

The following honey types are considered: acacia, alpine rose, chestnut, dandelion, heather, lime, rape, polyfloral.

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	465	0.467	0.10	1.70	0.14	0.29	0.73	1.49	0.573	0.370
pH-value		467	4.26	3.5	6.4	3.8	4.1	4.6	5.7	4.41	0.50
Free acidity	meq/kg	467	12.7	4	42	7	10	18	33	14.9	7.0
Water	g/100 g	467	16.20	13.4	21.1	14.3	15.4	17.1	19.0	16.33	1.25
Fructose	g/100 g	447	38.20	32.3	49.8	34.9	37.0	40.2	44.7	38.69	2.53
Glucose	g/100 g	447	30.40	21.4	43.2	24.4	27.7	33.7	38.6	30.84	3.90
Monosaccharides	g/100 g	447	69.15	59.1	80.1	62.5	67.1	71.8	77.4	69.52	3.72
Fructose/Glucose ratio		447	1.247	0.85	1.90	0.96	1.11	1.40	1.73	1.279	0.214
Glucose/Water ratio		445	1.920	1.19	2.65	1.41	1.68	2.13	2.51	1.914	0.303
Sucrose	g/100 g	447	0.20	0.0	4.5	0.0	0.1	0.3	2.5	0.37	0.61
Turanose	g/100 g	431	2.19	0.0	5.0	0.1	1.6	2.6	4.0	2.14	0.82
Nigerose	g/100 g	393	2.13	0.0	5.3	0.0	1.0	2.8	4.3	2.00	1.21
Maltose	g/100 g	447	1.43	0.0	8.6	0.0	0.6	2.1	4.8	1.50	1.21
Trehalose	g/100 g	442	0.12	0.0	4.0	0.0	0.0	1.0	2.5	0.59	0.80
Isomaltose	g/100 g	442	0.58	0.0	2.6	0.0	0.2	1.0	2.0	0.67	0.55
Erlose	g/100 g	447	0.30	0.0	4.3	0.0	0.0	0.7	3.0	0.56	0.78
Melezitose	g/100 g	447	0.12	0.0	3.8	0.0	0.0	0.4	2.4	0.33	0.57
Maltotriose	g/100 g	442	0.00	0.0	2.0	0.0	0.0	0.0	0.5	0.05	0.17
Raffinose	g/100 g	447	0.00	0.0	0.7	0.0	0.0	0.0	0.4	0.04	0.10
Proline	mg/kg	407	415.0	158	1378	218	317	541	902	449	180

B.3 PHYSICAL AND CHEMICAL COMPOSITION OF HONEYDEW HONEY

The following honey types are considered: oak honeydew, fir honeydew, metcalfa honeydew.

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	151	1.026	0.70	2.15	0.80	0.92	1.15	2.01	1.090	0.267
pH-value		149	4.77	4.1	5.8	4.2	4.5	5.0	5.4	4.78	0.31
Free acidity	meq/kg	149	28.2	17	46	19	24	33	43	28.5	6.3
Water	g/100 g	151	15.50	13.2	18.0	13.7	14.6	16.0	17.1	15.38	0.94
Fructose	g/100 g	150	32.88	26.8	39.4	28.3	31.0	33.7	37.7	32.50	2.30
Glucose	g/100 g	150	26.70	18.8	32.8	21.7	25.3	27.7	30.7	26.47	2.18
Monosaccharides	g/100 g	150	59.04	47.5	70.9	49.8	56.4	61.5	67.3	58.97	4.05
Fructose/Glucose ratio		150	1.231	1.03	1.53	1.09	1.17	1.28	1.42	1.232	0.084
Glucose/Water ratio		150	1.718	1.16	2.18	1.44	1.63	1.81	2.11	1.726	0.163
Sucrose	g/100 g	141	0.20	0.0	2.7	0.0	0.0	0.6	2.0	0.40	0.52
Turanose	g/100 g	128	1.90	0.0	3.1	1.3	1.7	2.2	2.8	1.94	0.46
Nigerose	g/100 g	107	2.06	0.0	5.1	0.0	1.4	2.6	4.3	1.96	1.21
Maltose	g/100 g	141	2.30	0.0	7.7	1.3	1.9	2.9	6.5	2.67	1.31
Trehalose	g/100 g	141	1.80	0.0	3.9	0.0	1.5	2.1	3.1	1.70	0.76
Isomaltose	g/100 g	111	1.20	0.0	4.2	0.1	0.9	1.8	3.7	1.41	0.85
Erlose	g/100 g	141	0.40	0.0	4.5	0.0	0.0	1.5	3.5	0.87	1.08
Melezitose	g/100 g	141	2.50	0.0	8.4	0.0	1.5	3.4	6.6	2.67	1.74
Maltotriose	g/100 g	141	0.00	0.0	2.8	0.0	0.0	0.0	1.5	0.13	0.43
Raffinose	g/100 g	141	0.90	0.0	2.2	0.0	0.4	1.5	1.8	0.89	0.58
Proline	mg/kg	118	552.0	65	1153	66	451	723	1082	574	226

B.4 PHYSICAL AND CHEMICAL COMPOSITION OF ACACIA (*Robinia pseudoacacia*) HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	31	0.140	0.10	0.27	0.10	0.13	0.16	0.26	0.150	0.036
pH-value		31	3.92	3.7	4.1	3.7	3.9	4.0	4.1	3.91	0.01
Free acidity	meq/kg	31	9.1	6	23	6	8	11	20	9.8	3.1
Water	g/100 g	31	16.50	14.2	19.0	14.2	16.1	17.0	18.9	16.58	1.03
Fructose	g/100 g	30	44.30	37.8	46.9	38.7	42.1	44.8	46.6	43.69	1.75
Glucose	g/100 g	30	26.52	23.5	29.4	23.8	25.7	27.6	29.3	26.59	1.42
Monosaccharides	g/100 g	30	70.51	66.3	75.9	66.3	68.2	72.0	75.6	70.28	2.43
Fructose/Glucose ratio		30	1.646	1.28	1.88	1.35	1.61	1.68	1.86	1.647	0.099
Glucose/Water ratio		28	1.597	1.43	2.05	1.43	1.55	1.69	2.01	1.624	0.130
Sucrose	g/100 g	30	0.63	0.0	3.5	0.0	0.3	1.9	3.5	1.06	1.01
Turanose	g/100 g	30	2.66	0.0	4.2	0.2	2.4	3.2	4.2	2.65	0.82
Nigerose	g/100 g	25	2.14	0.0	2.5	0.0	1.4	2.3	2.5	1.73	0.88
Maltose	g/100 g	30	2.23	0.0	3.6	0.0	1.5	2.8	3.6	1.97	1.01
Trehalose	g/100 g	30	0.60	0.0	3.5	0.0	0.1	1.2	3.4	0.80	0.84
Isomaltose	g/100 g	30	0.49	0.0	1.2	0.0	0.3	0.7	1.2	0.48	0.33
Erlose	g/100 g	30	1.47	0.4	2.4	0.4	1.1	2.1	2.4	1.53	0.59
Melezitose	g/100 g	30	0.00	0.0	0.5	0.0	0.0	0.2	0.5	0.08	0.13
Maltotriose	g/100 g	30	0.00	0.0	0.6	0.0	0.0	0.0	0.5	0.06	0.15
Raffinose	g/100 g	30	0.00	0.0	0.6	0.0	0.0	0.0	0.5	0.03	0.12
Proline	mg/kg	23	256	187	424	189	233	274	422	265	58
Asteraceae T	%	4	2	0	2	0	1	2	2	1	1
Brassica	%	4	5	2	9	2	3	7	9	5	3
Rhododenron	%	0	0	0	0					0	
Calluna	%	0	0	0	0					0	
Robinia	%	29	30	11	64	11	21	38	63	31	14
Castanea	%	19	14	9	57	9	11	29	57	22	15
Tilia	%	1	1	1	1					1	

B.5 PHYSICAL AND CHEMICAL COMPOSITION OF ALPINE ROSE (*Rhododenron* spp.) HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	29	0.243	0.15	0.45	0.15	0.19	0.31	0.44	0.264	0.087
pH-value		29	3.98	3.7	4.6	3.8	3.9	4.1	4.5	4.03	0.19
Free acidity	meq/kg	29	10.0	5	25	5	8	11	24	10.3	4.0
Water	g/100 g	29	16.00	14.5	19.0	14.6	15.2	16.4	18.9	16.07	1.07
Fructose	g/100 g	29	38.40	34.2	41.0	34.7	37.5	39.2	40.8	38.31	1.38
Glucose	g/100 g	29	29.85	27.7	33.6	27.7	28.8	30.4	33.4	29.80	1.36
Monosaccharides	g/100 g	29	68.01	64.7	72.0	64.8	66.2	69.5	71.8	68.11	2.07
Fructose/Glucose ratio		29	1.298	1.10	1.39	1.11	1.26	1.34	1.38	1.288	0.067
Glucose/Water ratio		29	1.872	1.55	2.19	1.57	1.74	1.96	2.18	1.863	0.154
Sucrose	g/100 g	29	0.37	0.0	2.6	0.0	0.2	0.6	2.3	0.49	0.51
Turanose	g/100 g	24	2.65	0.0	5.0	0.1	2.3	3.7	4.9	2.89	1.01
Nigerose	g/100 g	24	2.45	0.0	3.6	0.0	1.4	2.8	3.5	2.04	1.19
Maltose	g/100 g	29	1.55	0.0	8.6	0.0	0.0	2.3	8.3	1.89	2.36
Trehalose	g/100 g	29	0.31	0.0	3.8	0.0	0.0	1.5	3.4	0.79	0.95
Isomaltose	g/100 g	29	0.83	0.2	2.5	0.2	0.5	1.0	2.4	0.87	0.54
Erlose	g/100 g	29	1.90	0.0	3.7	0.0	1.1	2.7	3.6	1.81	1.06
Melezitose	g/100 g	29	0.15	0.0	0.8	0.0	0.0	0.3	0.7	0.19	0.21
Maltotriose	g/100 g	29	0.00	0.0	0.4	0.0	0.0	0.0	0.4	0.03	0.08
Raffinose	g/100 g	29	0.00	0.0	0.5	0.0	0.0	0.0	0.4	0.04	0.10
Proline	mg/kg	24	272	197	502	199	241	327	492	289	68
Asteraceae T	%	8	1	0	5	0	1	1	5	2	2
Brassica	%	5	3	0	8	0	1	7	8	4	3
Rhododenron	%	24	25	6	58	6	18	38	57	29	14
Calluna	%	2	2	1	2	1	1	2	2	2	1
Robinia	%	1	6	6	6					6	
Castanea	%	8	56	1	84	1	14	78	84	48	34
Tilia	%	1	1	1	1					1	

B.6 PHYSICAL AND CHEMICAL COMPOSITION OF CHESTNUT (*Castanea sativa*) HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	59	1.302	0.12	1.70	0.64	1.16	1.42	1.64	1.250	0.277
pH-value		59	5.31	4.4	6.4	4.7	5.1	5.5	6.3	5.34	0.40
Free acidity	meq/kg	59	10.3	4	30	7	9	12	23	11.2	4.2
Water	g/100 g	59	17.00	15.4	18.7	15.7	16.4	17.6	18.7	16.99	0.78
Fructose	g/100 g	56	41.09	36.6	44.6	36.7	40.2	42.1	44.2	41.02	1.63
Glucose	g/100 g	56	26.31	21.4	30.0	22.2	25.3	27.3	29.9	26.21	1.88
Monosaccharides	g/100 g	56	67.58	60.5	74.6	60.5	66.1	68.6	73.3	67.23	2.93
Fructose/Glucose ratio		56	1.556	1.36	1.86	1.40	1.49	1.63	1.85	1.572	0.109
Glucose/Water ratio		56	1.556	1.19	1.80	1.20	1.48	1.65	1.76	1.547	0.144
Sucrose	g/100 g	56	0.11	0.0	3.6	0.0	0.0	0.2	3.5	0.29	0.67
Turanose	g/100 g	51	2.50	0.0	4.5	0.0	2.2	3.2	4.1	2.52	0.91
Nigerose	g/100 g	51	3.13	0.0	5.3	0.0	2.3	3.9	5.1	2.99	1.34
Maltose	g/100 g	56	1.49	0.0	5.6	0.0	0.0	2.3	5.2	1.48	1.47
Trehalose	g/100 g	56	1.02	0.0	3.9	0.0	0.0	1.7	2.6	0.93	0.95
Isomaltose	g/100 g	56	0.81	0.0	2.4	0.0	0.0	1.5	2.4	0.85	0.80
Erlose	g/100 g	56	0.09	0.0	4.3	0.0	0.0	0.3	3.9	0.27	0.75
Melezitose	g/100 g	56	0.10	0.0	3.8	0.0	0.0	0.3	3.5	0.28	0.68
Maltotriose	g/100 g	56	0.00	0.0	0.3	0.0	0.0	0.0	0.3	0.04	0.07
Raffinose	g/100 g	56	0.00	0.0	0.6	0.0	0.0	0.0	0.3	0.04	0.01
Proline	mg/kg	51	484	359	697	371	439	554	692	508	89
Asteraceae T	%	1	0	0	0					0	
Brassica	%	0	0	0	0					0	
Rhododenron	%	7	1	0	3	0	1	2	3	1	1
Calluna	%	0	0	0	0					0	
Robinia	%	1	0	0	0					0	
Castanea	%	54	98	92	100	93	96	99	100	98	2
Tilia	%	21	1	0	4	0	1	1	4	1	1

B.7 PHYSICAL AND CHEMICAL COMPOSITION OF **DANDELION** (Taraxacum s.l.) **HONEY**

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	31	0.482	0.37	0.64	0.37	0.42	0.55	0.63	0.493	0.072
pH-value		31	4.47	4.2	5.0	4.2	4.3	4.6	5.0	4.50	0.21
Free acidity	meq/kg	31	10.0	7	13	7	9	11	13	10.1	1.6
Water	g/100 g	31	15.90	14.2	18.9	14.3	15.1	16.6	18.8	16.03	1.16
Fructose	g/100 g	31	36.95	32.3	39.5	32.5	35.5	38.0	39.5	36.74	2.01
Glucose	g/100 g	31	36.13	32.0	43.2	32.0	34.7	38.0	42.8	36.37	2.79
Monosaccharides	g/100 g	31	73.51	65.2	80.1	65.8	69.3	76.3	79.8	73.11	3.97
Fructose/Glucose ratio		31	1.025	0.85	1.15	0.87	0.96	1.07	1.15	1.014	0.075
Glucose/Water ratio		31	2.267	1.75	2.65	1.78	2.15	2.45	2.64	2.279	0.218
Sucrose	g/100 g	31	0.13	0.0	0.3	0.0	0.0	0.2	0.3	0.13	0.01
Turanose	g/100 g	29	2.00	0.0	3.2	0.2	1.5	2.4	3.2	1.92	0.73
Nigerose	g/100 g	22	2.08	0.0	3.5	0.0	1.8	2.1	3.5	1.95	0.79
Maltose	g/100 g	31	1.05	0.0	5.7	0.0	0.7	1.4	4.9	1.21	1.04
Trehalose	g/100 g	31	0.28	0.0	3.7	0.0	0.0	0.5	3.1	0.43	0.72
Isomaltose	g/100 g	31	0.40	0.0	1.7	0.0	0.0	0.6	1.5	0.39	0.38
Erlose	g/100 g	31	0.25	0.0	0.8	0.0	0.1	0.5	0.8	0.29	0.26
Melezitose	g/100 g	31	0.00	0.0	0.7	0.0	0.0	0.2	0.7	0.11	0.17
Maltotriose	g/100 g	31	0.00	0.0	0.3	0.0	0.0	0.0	0.3	0.03	0.07
Raffinose	g/100 g	31	0.00	0.0	0.3	0.0	0.0	0.0	0.3	0.03	0.06
Proline	mg/kg	25	327	257	560	258	285	390	548	347	75
Asteraceae T	%	29	15	2	58	2	10	22	55	18	12
Brassica	%	11	8	0	42	0	1	32	42	15	17
Rhododenron	%	0	0	0	0					0	
Calluna	%	0	0	0	0					0	
Robinia	%	0	0	0	0					0	
Castanea	%	1	1	1	1					1	
Tilia	%	0	0	0	0					0	

B.8 PHYSICAL AND CHEMICAL COMPOSITION OF HEATHER (*Calluna vulgaris*) HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	24	0.825	0.65	1.07	0.65	0.75	0.87	1.06	0.821	0.098
pH-value		24	4.13	3.9	5.1	3.9	4.1	4.3	5.0	4.21	0.27
Free acidity	meq/kg	24	28.0	14	42	15	24	33	41	28.0	6.3
Water	g/100 g	24	18.80	16.9	21.1	17.0	18.3	19.5	21.1	18.93	0.99
Fructose	g/100 g	15	37.20	34.9	41.0	34.9	36.6	38.8	41.0	37.61	1.65
Glucose	g/100 g	15	28.50	26.2	31.5	26.2	27.5	30.1	31.5	28.79	1.55
Monosaccharides	g/100 g	15	66.10	61.9	71.2	61.9	64.5	67.7	71.2	66.41	2.54
Fructose/Glucose ratio		15	1.296	1.22	1.57	1.22	1.26	1.34	1.57	1.309	0.083
Glucose/Water ratio		15	1.546	1.39	1.72	1.39	1.47	1.64	1.72	1.549	0.103
Sucrose	g/100 g	15	0.00	0.0	0.6	0.0	0.0	0.0	0.6	0.06	0.17
Turanose	g/100 g	15	1.00	0.4	3.3	0.4	0.6	1.4	3.3	1.14	0.72
Maltose	g/100 g	15	1.00	0.0	1.9	0.0	0.8	1.4	1.9	1.09	0.48
Trehalose	g/100 g	15	0.60	0.1	4.0	0.1	0.2	0.9	4.0	0.79	0.95
Isomaltose	g/100 g	15	0.40	0.0	1.3	0.0	0.1	0.5	1.3	0.35	0.34
Erlose	g/100 g	15	0.00	0.0	0.2	0.0	0.0	0.0	0.2	0.02	0.05
Melezitose	g/100 g	15	0.00	0.0	1.4	0.0	0.0	0.2	1.4	0.18	0.39
Maltotriose	g/100 g	15	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
Raffinose	g/100 g	15	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
Proline	mg/kg	10	774	571	928	571	598	825	928	745	124
Asteraceae T	%	1	3	3	3					3	
Brassica	%	0	0	0	0					0	
Rhododenron	%	0	0	0	0					0	
Calluna	%	10	45	8	81	8	22	59	81	44	26
Robinia	%	0	0	0	0					0	
Castanea	%	0	0	0	0					0	
Tilia	%	0	0	0	0					0	

B.9 PHYSICAL AND CHEMICAL COMPOSITION OF LIME (*Tilia* spp.) HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	34	0.665	0.43	0.95	0.43	0.59	0.74	0.91	0.663	0.115
pH-value		36	4.60	4.1	6.2	4.1	4.4	5.0	5.9	4.72	0.43
Free acidity	meq/kg	36	12.6	4	21	4	8	15.7	20.5	12	5
Water	g/100 g	36	16.30	14.3	18.1	14.4	15.8	16.8	17.9	16.33	0.88
Fructose	g/100 g	30	37.52	32.9	41.6	33.4	36.2	39.1	41.5	37.66	2.07
Glucose	g/100 g	30	29.80	26.2	39.0	26.4	28.8	32.0	38.8	30.83	3.30
Monosaccharides	g/100 g	30	67.21	59.1	79.6	59.9	64.9	70.6	79.5	68.49	5.07
Fructose/Glucose ratio		30	1.250	1.04	1.41	1.04	1.20	1.27	1.39	1.229	0.082
Glucose/Water ratio		30	1.822	1.64	2.38	1.65	1.72	2.01	2.38	1.909	0.217
Sucrose	g/100 g	30	0.23	0.0	4.5	0.0	0.1	0.4	3.8	0.45	0.84
Turanose	g/100 g	26	2.29	0.0	3.5	0.2	2.0	2.5	3.4	2.22	0.60
Nigerose	g/100 g	15	3.02	0.6	4.6	0.6	2.3	3.3	4.6	2.85	1.01
Maltose	g/100 g	30	2.58	0.5	5.7	0.6	2.0	3.4	5.7	2.80	1.31
Trehalose	g/100 g	30	0.80	0.0	2.3	0.0	0.0	1.5	2.3	0.80	0.80
Isomaltose	g/100 g	30	1.17	0.1	2.2	0.2	0.8	1.4	2.2	1.14	0.54
Erlose	g/100 g	30	0.30	0.0	0.9	0.0	0.0	0.5	0.9	0.29	0.27
Melezitose	g/100 g	30	0.00	0.0	1.1	0.0	0.0	0.4	1.0	0.21	0.29
Maltotriose	g/100 g	30	0.00	0.0	0.5	0.0	0.0	0.0	0.4	0.03	0.10
Raffinose	g/100 g	30	0.00	0.0	0.3	0.0	0.0	0.0	0.3	0.02	0.07
Proline	mg/kg	17	432	292	530	292	369	468	530	414	71
Asteraceae T	%	8	1	0	2	0	1	1	2	1	1
Brassica	%	2	21	6	35	6	6	35	35	21	20
Rhododenron	%	4	1	1	3	1	1	2	3	1	1
Calluna	%	0	0	0	0					0.	
Robinia	%	1	1	1	1					1	
Castanea	%	14	2	0	93	0	1	17	93	17	27
Tilia	%	32	14	2	80	3	8	25	68	18	15

B.10 PHYSICAL AND CHEMICAL COMPOSITION OF RAPE (*Brassica* spp.) HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	36	0.200	0.14	0.28	0.14	0.16	0.23	0.27	0.200	0.040
pH-value		36	4.13	3.9	4.4	3.9	4.1	4.3	4.4	4.14	0.13
Free acidity	meq/kg	36	11.6	8	17	8	10	13	16	11.2	2.1
Water	g/100 g	36	16.15	14.4	18.1	14.6	15.5	16.8	17.9	16.22	0.92
Fructose	g/100 g	36	37.65	34.6	39.5	34.8	36.5	38.5	39.4	37.47	1.30
Glucose	g/100 g	36	35.31	31.5	40.0	32.1	34.5	36.7	39.8	35.70	1.84
Monosaccharides	g/100 g	36	73.29	69.5	78.5	69.6	71.2	74.5	78.4	73.16	2.38
Fructose/Glucose ratio		36	1.047	0.95	1.24	0.95	1.00	1.09	1.22	1.052	0.063
Glucose/Water ratio		36	2.224	1.78	2.54	1.83	2.08	2.34	2.53	2.209	0.178
Sucrose	g/100 g	36	0.00	0.0	2.0	0.0	0.0	0.2	1.5	0.16	0.36
Turanose	g/100 g	36	1.70	0.5	3.1	0.6	1.1	2.5	3.1	1.79	0.78
Nigerose	g/100 g	35	0.60	0.0	3.1	0.0	0.4	0.9	2.6	0.75	0.57
Maltose	g/100 g	36	0.62	0.0	2.2	0.1	0.5	0.8	2.1	0.73	0.45
Trehalose	g/100 g	33	0.00	0.0	1.3	0.0	0.0	0.2	1.2	0.17	0.33
Isomaltose	g/100 g	33	0.34	0.0	1.5	0.0	0.2	0.4	1.4	0.35	0.30
Erlose	g/100 g	36	0.00	0.0	1.4	0.0	0.0	0.1	1.2	0.13	0.28
Melezitose	g/100 g	36	0.00	0.0	0.4	0.0	0.0	0.1	0.4	0.06	0.11
Maltotriose	g/100 g	33	0.00	0.0	1.1	0.0	0.0	0.0	0.8	0.06	0.19
Raffinose	g/100 g	36	0.00	0.0	0.2	0.0	0.0	0.0	0.2	0.02	0.06
Proline	mg/kg	36	285.1	158	456	175	238	317	424	284	58
Asteraceae T	%	9	1	0	2	0	0	2	2	1	1
Brassica	%	36	86	68	98	68	82	92	98	85	9
Rhododenron	%	0	0	0	0					0	
Calluna	%	0	0	0	0					0	
Robinia	%	0	0	0	0					0	
Castanea	%	3	0	0	5	0	0	4	5	2	3
Tilia	%	0	0	0	0					0	

B.11 PHYSICAL AND CHEMICAL COMPOSITION OF FIR (*Picea* spp.and *Abies* spp.) HONEYDEW HONEY

						Percentiles (%)					
	Unit	n	$\bar{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	129	0.983	0.70	1.33	0.79	0.91	1.11	1.30	1.011	0.132
pH-value		127	4.71	4.1	5.4	4.2	4.5	5.0	5.3	4.73	0.29
Free acidity	meq/kg	127	27.4	17	46	18	23	31	40	27.63	5.82
Water	g/100 g	129	15.50	13.2	18.0	13.8	14.7	16.0	17.1	15.41	0.94
Fructose	g/100 g	128	33.06	27.9	39.4	28.7	31.5	33.8	37.7	32.86	2.01
Glucose	g/100 g	128	26.80	18.8	31.5	22.7	25.5	27.9	30.6	26.68	2.02
Monosaccharides	g/100 g	128	59.72	47.5	70.9	51.7	57.6	61.7	67.6	59.54	3.70
Fructose/Glucose ratio		128	1.235	1.07	1.53	1.09	1.18	1.28	1.41	1.235	0.079
Glucose/Water ratio		128	1.729	1.16	2.18	1.45	1.64	1.82	2.01	1.737	0.157
Sucrose	g/100 g	128	0.29	0.0	2.7	0.0	0.0	0.6	2.1	0.43	0.54
Turanose	g/100 g	128	1.90	0.0	3.1	1.3	1.7	2.2	2.8	1.94	0.46
Nigerose	g/100 g	107	2.06	0.0	5.1	0.0	1.4	2.6	4.3	1.96	1.21
Maltose	g/100 g	128	2.19	0.0	4.9	1.3	1.8	2.7	3.9	2.32	0.69
Trehalose	g/100 g	128	1.84	0.0	3.9	0.5	1.5	2.2	3.1	1.86	0.61
Isomaltose	g/100 g	98	1.14	0.0	3.4	0.0	0.8	1.6	2.7	1.25	0.64
Erlose	g/100 g	128	0.20	0.0	4.5	0.0	0.0	1.5	3.6	0.90	1.13
Melezitose	g/100 g	128	2.60	0.0	8.4	0.2	1.9	3.8	6.7	2.92	1.62
Maltotriose	g/100 g	128	0.00	0.0	2.8	0.0	0.0	0.0	1.3	0.06	0.34
Raffinose	g/100 g	128	0.97	0.0	2.2	0.0	0.5	1.5	1.8	0.97	0.56
Proline	mg/kg	110	576.6	272	1153	353	464	727	1089	610.3	187.5
Asteraceae T	%	66	1	0	11	0	0	2	4	1	2
Brassica	%	68	6	0	74	0	2	15	60	13	17
Rhododenron	%	2	4	2	5	2	2	5	5	4	2
Calluna	%	0	0	0	0					0	
Robinia	%	0	0	0	0					0	
Castanea	%	42	3	0	84	0	1	8	64	8	15
Tilia	%	15	2	1	12	1	1	5	12	3	3

B.12 PHYSICAL AND CHEMICAL COMPOSITION OF OAK (*Quercus* spp.) HONEYDEW HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	9	1.166	0.93	1.49	0.93	1.03	1.37	1.49	1.193	0.203
pH-value		9	4.93	4.6	5.1	4.6	4.7	5.0	5.1	4.86	0.18
Free acidity	meq/kg	9	38.5	30	45	30	34	42	45	37.9	4.7
Water	g/100 g	9	15.50	14.6	17.1	14.6	15.3	16.3	17.1	15.73	0.79
Fructose	g/100 g	9	31.75	27.2	35.7	27.2	28.6	33.5	35.7	31.22	2.99
Glucose	g/100 g	9	25.57	24.3	32.8	24.3	24.5	28.3	32.8	26.90	2.99
Monosaccharides	g/100 g	9	57.32	51.7	66.4	51.7	53.1	63.2	66.4	58.12	5.53
Fructose/Glucose ratio		9	1.178	1.03	1.31	1.03	1.10	1.22	1.31	1.165	0.086
Glucose/Water ratio		9	1.655	1.47	2.11	1.47	1.56	1.83	2.11	1.715	0.222
Proline	mg/kg	8	69	65	93	65	66	78	93	73	10

B.13 PHYSICAL AND CHEMICAL COMPOSITION OF METCALFA (*Metcalfa pruinosa*) HONEYDEW HONEY

						Percentiles (%)					
	Unit	n	$\tilde{x}$	x_{min}	x_{max}	2.5	25.0	75.0	97.5	$\bar{x}$	s
Electrical conductivity	$mScm^{-1}$	13	1.750	1.48	2.15	1.48	1.66	2.01	2.15	1.802	0.224
pH-value		13	5.19	4.6	5.8	4.6	5.0	5.3	5.8	5.16	0.29
Free acidity	meq/kg	13	29.7	21	41	21	26	36	41	30.6	6.6
Water	g/100 g	13	14.90	13.7	16.3	13.7	14.1	15.6	16.3	14.85	0.90
Fructose	g/100 g	13	29.60	26.8	33.1	26.8	28.6	30.5	33.1	29.78	1.69
Glucose	g/100 g	13	23.80	20.3	26.8	20.3	22.9	25.5	26.8	24.15	1.88
Monosaccharides	g/100 g	13	55.20	48.7	56.9	48.7	51.8	56.1	56.9	53.93	2.67
Fructose/Glucose ratio		13	1.217	1.10	1.45	1.10	1.16	1.31	1.45	1.240	0.115
Glucose/Water ratio		13	1.620	1.26	1.87	1.26	1.56	1.71	1.87	1.631	0.153
Sucrose	g/100 g	13	0.10	0.0	0.1	0.0	0.1	0.1	0.1	0.08	0.04
Maltose	g/100 g	13	6.00	4.7	7.7	4.7	5.3	6.7	7.7	6.11	0.94
Trehalose	g/100 g	13	0.10	0.0	0.9	0.0	0.1	0.1	0.9	0.17	0.23
Isomaltose	g/100 g	13	3.10	0.9	4.2	0.9	1.2	3.7	4.2	2.66	1.20
Erlose	g/100 g	13	0.50	0.1	1.2	0.1	0.4	0.9	1.2	0.60	0.33
Melezitose	g/100 g	13	0.10	0.0	0.7	0.0	0.1	0.1	0.7	0.13	0.18
Maltotriose	g/100 g	13	1.00	0.0	1.5	0.0	0.6	1.3	1.5	0.89	0.51
Raffinose	g/100 g	13	0.20	0.0	0.4	0.0	0.1	0.2	0.4	0.16	0.10

Curriculum Vitae

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1990 – 1995	Grammar school, in Zurich, Matura Typus C
1996 – 2003	Dept. of Applied Biology, University of Helsinki, Finland major subject: apiculture, minor subject: food chemistry degree: Master of Science in Agriculture und Forestry
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