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.

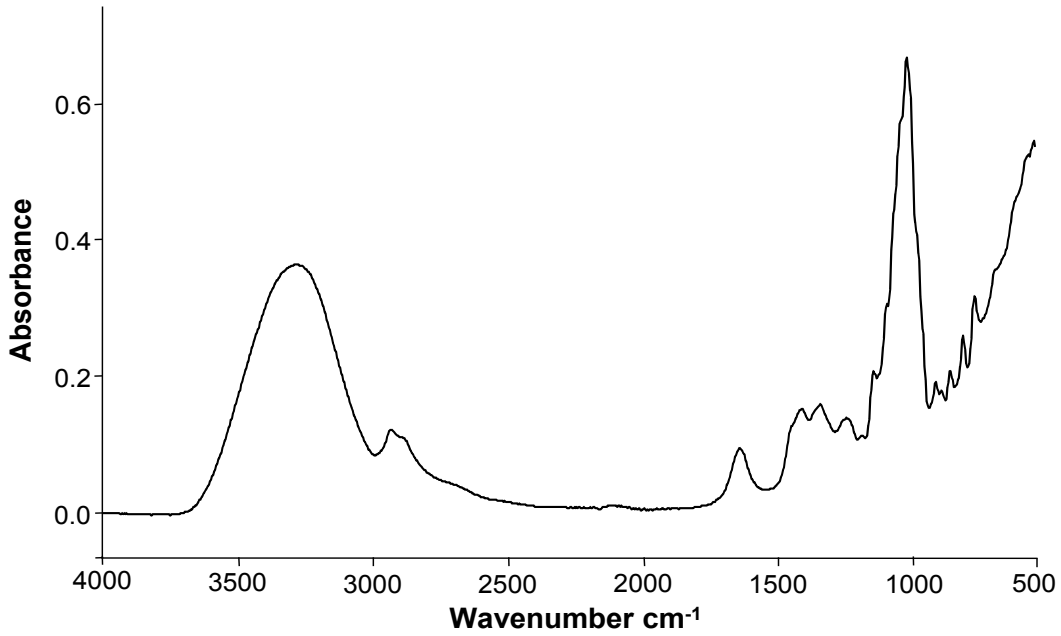

Figure 1. Typical FT-IR-ATR spectrum of a honey sample.

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.

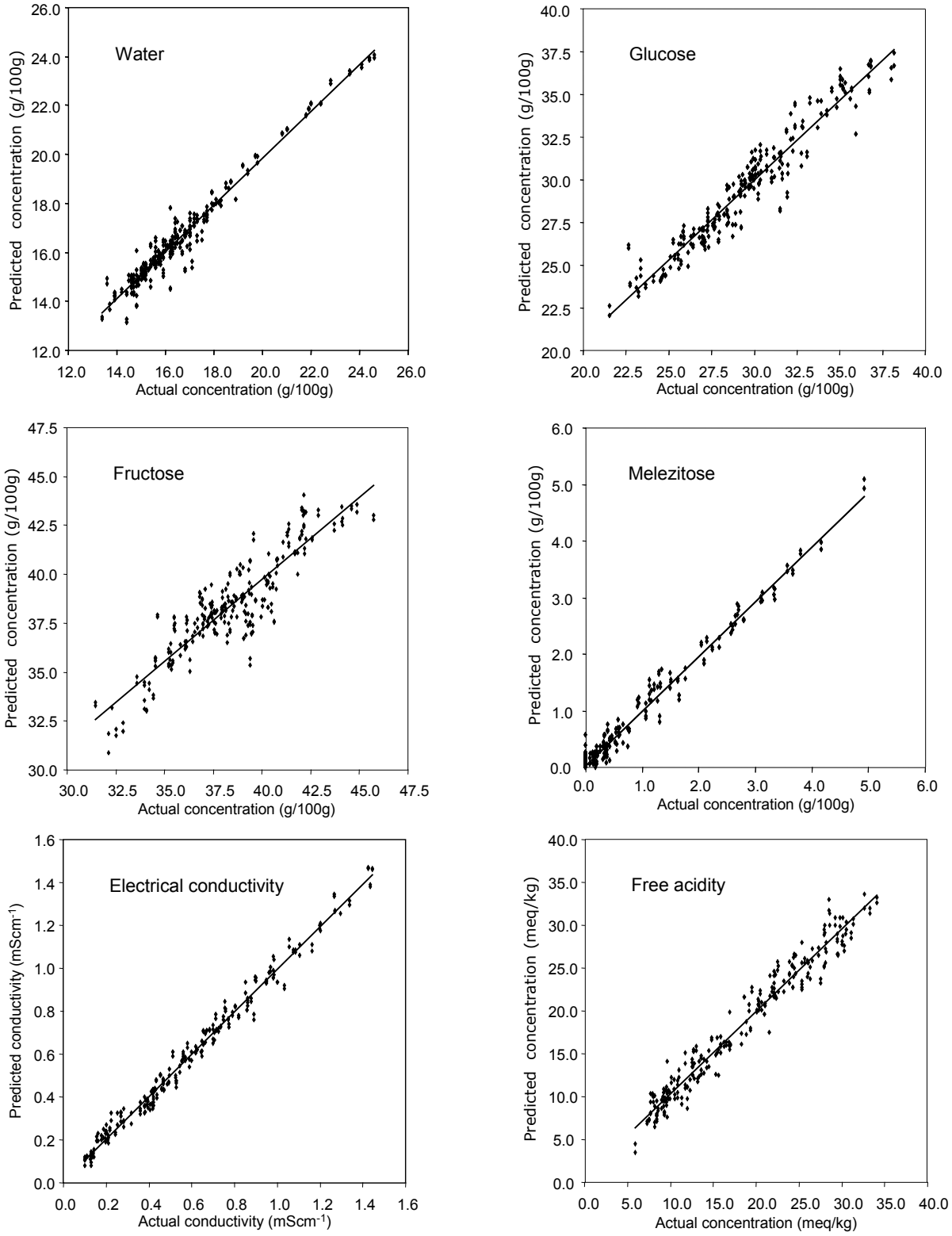

Figure 2. Calibration plots (predicted values from cross-validation)

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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