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# Generalizability of PLS calibrations with FT-IR ATR spectrometry for the prediction of some physicochemical measurands of honey

Technical-scientific information

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# Contents

# Summary

This work had as main objective to evaluate the generalizability of Partial Least Squares (PLS) calibrations with Fourier Transform Infrared spectroscopy with an Attenuated Total Reflection accessory (FT-IR ATR) for the prediction of 16 physicochemical measurands of honey. More than 400 different honey samples from the Swiss National Honey Quality Program were analyzed using physicochemical reference methods adopted by the Swiss Bee Research Centre (Switzerland), as well as by FT-IR ATR. PLS regression was used to develop the calibration models (internal and external validation) for the measurands studied. Further 79 honey samples (different from the samples used for the calibration but the same as used in a previously published paper) were also used to evaluate the generalizability of the FT-IR ATR / PLS method. The predictions were of good accuracy for the determination of moisture (SECV = 0.197; RPD = 5.12; $R^2$= 0.9619), electrical conductivity (SECV = 0.0437; RPD = 6.32; $R^2$= 0.975), free acidity (SECV = 1.71; RPD = 4.75; $R^2$ = 0.9556), and melezitose (SECV = 0.315; RPD = 5.04; $R^2$ = 0.9607). For other sugars (fructose, glucose, turanose, trehalose and isomaltose), the sum of fructose plus glucose, fructose/glucose ratio, glucose/moisture ratio and pH, measurement uncertainties were acceptable. The method had poor performance only for the quantitative analysis of HMF and total nitrogen. Data presented here indicate that the procedure used previously by Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò (2006) can be reproduced with different equipments, different samples, different software, and different operators in different laboratory conditions, while keeping the selected spectral regions and algorithms the same. The 79 independent samples (same used by Ruoff et al., 2006) gave similar results in the present study.


**Keywords:** Honey; quality control; Fourier transform infrared spectroscopy; FT-IR ATR; chemometrics.

# 1. Introduction

Honey is the most important product from the apiary and is world-wide consumed and economically important (Almeida-Muradian, 2009).

Regarding honey regulation the European Community (Council Directive, 2001) and also the Brazilian regulation (Brasil, 2000) indicate many physicochemical measurands to assess honey quality, like water content (moisture), sugars, hydroxymethylfurfural (HMF) and free acidity. For each measurand a distinct analytical method has to be used. This takes much time and consequently limits the number of honey samples analyzed daily in a quality control laboratory. To improve this quality control it was necessary to develop rapid, simple and accurate methods for the routine analysis of honey (Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò, 2006; Ruoff, Luginbühl, Künzli, Bogdanov, Bosset, von OHE, K., von OHE, W., Amadò, 2006, Almeida-Muradian, 2009).

Infrared spectrometry has become a rapid technique that can be applied to various types of honey analysis (Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò, 2006) and especially mid-infrared spectroscopy in combination with multivariate data analysis has been used as a rapid, economic, and nondestructive procedure for honey analysis and the detection of honey adulteration (Kelly, Petisco and Downey, 2006, Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò, 2006). FT-IR ATR (MIR) was used to predict reducing sugars, moisture and acidity in Brazilian honeys (Pataca, Borges Neto, Marcucci and Poppi, 2007) . Also Etzold and Lichtenberg-Kraag (2008) used FT-IR to predict the botanical origin of honey from Germany.

The mid-infrared (MIR) region (400 $cm^{-1}$ to 4000 $cm^{-1}$) contains information arising from molecular vibrations, and MIR is particularly sensitive to the chemical and physical states of the sample. MIR spectroscopy can provide information regarding many analytes nondestructively (Irudayaraj and Tewari, 2003, Ruoff et al., 2006a).

Other authors also used FT-IR in honey analysis. Sivakesava and Irudayaraj (2001a) used a combination of FT-IR spectroscopy with multivariate procedures for determining the level of sugar addition to honey. Sivakesava and Irudayaraj (2001b) used FT-IR spectroscopy with an attenuated total reflection (ATR) accessory to determine invert sugar in 3 different varieties of honey. Sivakesava and Irudayaraj (2001c) used a combination of FT-IR spectroscopy and multivariate statistics as a screening tool for the determination of beet medium invert sugar adulteration in three different varieties of honey. Paradkar and Irudayaraj (2001) used FT Raman Spectroscopy to detect adulterants such as

cane and beet invert sugar in honey. Sivakesava and Irudayaraj (2002) used FT-IR as a screening tool for the determination of the type of sugar adulterant in honey. Irudayaraj,Xu and Tewari (2003) used FT-IR ATR combined with multivariate analysis to determine the level of invert cane sugar adulteration in honey. Kelly, Downey and Fouratier (2004) used FT- IR ATR to detect adulteration of honey samples. Tewari and Irudayara (2005) used FT-IR and z-Nose as screening tools for the identification and classification of honey from different sources. Spectral data were analyzed by principal component analysis, canonical variate analysis, and artificial neural network for classification of the different honey samples from a range of floral sources. Kelly, Petisco and Downey (2006) applied FT-IR Spectroscopy to discriminate between Irish artisanal honey and honey adulterated with various sugar syrups. Ruoff et al. (2006b) used FT-IR ATR for monofloral and polyfloral honey authentication.

Bertelli et al. (2007) published the identification and classification of 82 samples of Italian honeys from different flora source (robina, chestnut, citrus and polyfloral) using Diffuse Reflectance infrared FT Spectroscopy (DRIFTS) with multivariate statistical analysis. Ruoff et al. (2007) used FT-NIR to evaluate quantitatively 24 measurands in honey and to evaluate the potential of physical measurands for the determination of the botanical origin of honey by using the classical profiling approach and chemometrics for the authentication of unifloral (acacia, rhododendron, chestnut, dandelion, heather, lime, rape, fir honeydew, metcalfa honeydew) and polyfloral honey types.

Unlike classical univariate calibration, the PLS calibration algorithm uses not only one spectral data point for the calibration, but the whole spectra or selected parts. The advantage of this type of calibration is the amount of spectral information used, so that even minor differences in the spectra of different samples can be identified (Conzen, 2006).

Generalizability theory is a statistical framework for conceptualizing, investigating, and designing reliable observations. It is used to determine the reliability (i.e., reproducibility) of measurements under specific conditions. It is particularly useful for assessing the reliability of performance assessments. It was originally introduced in Cronbach, L.J., Nageswari, R., and Gleser, G.C. (1963). Referring to this concept (and to the meaning of the word 'generalizability' in plain English), the idea of this research was to test if the procedure used by of Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò (2006) could be applicable in a different instrumental and software environment while keeping the spectral regions and the same algorithms, i.e. whether the method is generalizable with respect to the changed conditions. A different model of the FT-IR equipment, different software, different analyst, and more than 3 times samples was used in the present study, thus increasing the evidence of the findings.

Therefore, the basic objectives of this study was to develop multivariate calibration models (PLS) from FT-IR ATR spectra; to evaluate the performance of the method in predicting the honey quality control measurands through validations (internal and external); and to assess the possible generalizability of the calibration model developed in order to analyse different honey samples, as well as to compare the results obtained in the present study using the same samples from the previous work of Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò (2006).

# 2. Material and Methods

### 2.1 Honey Samples

416 randomly selected samples available from the Swiss Honey Quality Control Program were analysed using physicochemical methods (see 2.2 Reference Methods) and for the calibration of the FT-IR ATR method.

Further 79 honey samples (different from the samples used for the calibration but the same as used by Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò, 2006) were used as independent validation set in order to evaluate the generalizability of the FT-IR ATR / PLS method.

### 2.2 Reference Methods

The reference methods for the quantitative analysis of honey were the official methods of the Swiss Bee Research Centre laboratories and the majority follows the procedures described by the Harmonized Methods of the International Honey Commission (Bogdanov, Martin and Lüllmann, 1997).

#### 2.2.1 Water content

Refractometric method as recommended by AOAC (1990) was used. The equipment was an automatic refractometer Mettler Toledo RE40 with a printer Mettler Toledo LC-P45.

#### 2.2.2 Hydroxymethylfurfural (HMF)

Spectrophotometric method as recommended by AOAC (1990), using the wavelengths of 284 and 336 nm, was used.

#### 2.2.3 Sugars (HPLC)

HPLC method was used to measure the following sugars: fructose, glucose, sucrose, turanose, maltose, trehalose, isomaltose, gentiobiose, melibiose, erlose, melezitose, raffinose and maltotriose.

The HPLC system (Agilent®) is compound with mobile phase composed by a gradient of Phase A (water/ Acetonitrile, 80/20) and phase B (water/ Acetonitrile, 20/80)) according to Table1.

**Table 1:**
Gradient of mobile phase for sugar analysis

| Time (min.) | Phase A (%) | Phase B (%) |
|---|---|---|
| 0 | 0 | 100.0 |
| 18 | 8.3 | 91.70 |
| 28 | 8.3 | 91.70 |
| 28.1 | 0 | 100.0 |

Flow rate: 1.0 mL/min.
Time: 45 min.
Injection volume: 10 µL (Auto injector)
Injection temperature: 8°C
Column: Hypersil APS-2, 250* 4.6 mm, 3 µm (particle size)
Pre-column: Hypersil APS-2, 10* 4.6 mm, 3 µm (particle size)
Temperature of the oven: 40° C
Detector: Light Scattering Detection (ELSD) with Temp. 45°C, pressure 3.2 bar (SEDEX 85, AGILENT®)
Pump model: AGILENT. ® 1100 series

#### 2.2.4 pH and free acidity

Titrimetry as recommended by AOAC (1990) till pH 8.3.

#### 2.2.5 Total Nitrogen

Total Nitrogen was determined directly from a solution of 1 g honey to 10 mL by pyrochemiluminescence using a Skalar TN Analyser CA 10 from Contrec Technologies AG, Switzerland.

#### 2.2.6 Electrical Conductivity

Direct measurement in a water honey solution (Bogdanov, Martin and Lüllmann, 1997) was done using a conductivimeter (Radiometer CDM 92) with an electrode (Radiometer CDC641T). This specific International determination replaces the analysis of ash content (Boganov, Ruoff & Persano Oddo, 2004).

### 2.3 Fourier Transform Infrared Spectroscopy with Attenuated Total Reflection (FT-IR ATR) technique

Infrared spectra were collected by direct measurements using a Bruker Tensor 27 equipment using the same measuring conditions as the work done by Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò (2006).
The conditions of the analysis were:

**Equipment:**
Tensor 27 Sample Compartment RT-DLaTGS (Bruker Optik GmbH, Germany) equipped with a MKII Golden Gate single-reflection ATR accessory (Specac Inc., Woodstock, GA). 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 DigiTechTM 163 DLATGS detector which covers a spectral range from 12000 to 370 $cm^{-1}$ and operates at room temperature. The software used was Opus 6.5 (Bruker Optik GmbH, Germany).
Pre-treatment:

Honey samples were liquefied in an oven at 55 °C for 8 hours and then allowed to cool to room temperature before FT-IR analysis.

**Analysis:**
After pre-treatment, a drop of the sample was applied on the surface of the diamond, it was left to thermally equilibrate for 4 min. One hundred scans were recorded for each spectrum in the wavenumber range between 4000 and 550 $cm^{-1}$ with a spectral resolution of 4 $cm^{-1}$. Single beam spectra of all samples were recorded and ratioed against the background spectrum of the clean diamond surface (room temperature 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 Milli-Q water and dried with a soft tissue.

### 2.4 Chemometric modeling of the quantification

For the development of the chemometric PLS regression model, the IR spectra of honey samples with known composition were used to calculate a calibration function (mathematical model) which can be used for the analysis of future unknown samples, after evaluation of its capacity of prediction by internal and external validation.
This part was completely based on the model described by Ruoff, Iglesias, Luginbühl, Bosset, Bogdanov, Amadò (2006) applying different software (Opus 6.5, module Quant 2).

### 2.5 Calibration, validation and generalizability

To exclude noisy parts of the spectra only the ranges between 3700 and 2400 $cm^{-1}$ and from 1800 to 700 $cm^{-1}$ were used for PLS calibration. After elimination of obvious spectral outliers, PCA was applied to detect multivariate outliers on the basis of the Mahalanobis distance with a threshold value of 3.

PLS cross-validations were performed to assess different calibration models (i.e. different in the number of PLS factors) for the prediction of the various measurands. After elimination of spectral and concentration outliers the models were set up with two spectra per sample. The test set validation was carried out with spectra of half of the samples, selected randomly, and not present in the group of samples used to build the model. Validation standard error of prediction (SEP), coefficients of determination (R2) between predicted and reference values and the prediction bias were calculated.

In order to test the generalizability of the method as an indicator of the robustness, 79 independent samples (the same as used by Ruoff et al., 2006) were analysed in completely different conditions (different hardware, instruments, software, and different laboratory conditions). The FT-IR method was validated and the number of PLS factors optimized using the steps (calibration and the analysis of prediction) as recommended by Conzen (2006).

### 2.6 Statistical analysis

For the evaluation of the predictive quality and precision of the calibration model the Standard Error of Cross Validation (SECV), the Residual Prediction Deviation (RPD) and the coefficient of determination ($R^2$) were considered. The external validation (test set validation) was quantified by the Standard Error of Prediction (SEP), the prediction bias, and the coefficient of determination ($R^2$):

$$SECV = \sqrt{\frac{\sum_{i=1}^{N}(Y_{pred,i} - Y_{ref,i})^2}{N}}$$

$$RPD = \frac{SD_{ref}}{SEP_{bias}}$$

$$SEP = \sqrt{\frac{\sum_{i=1}^{n}(Y_{pred,i} - Y_{ref,i})^2}{n}}$$

$$bias = \frac{\sum_{i=1}^{n} Y_{pred,i} - Y_{ref,i}}{n}$$

where Y represents concentrations, SDref the standard deviation of the reference values, SEPbias the bias-corrected mean error of prediction, and N and n the numbers of validation and cross-validation samples, respectively.

The evaluation of the generalizability of the method was also based on paired t-tests for dependent samples comparing the results obtained by FT-IR ATR and the traditional physicochemical analysis. Differences were considered statistically significant at the level of 5% ($p < 0.05$) (Statistica 7.0).

# 3. Results

Physicochemical results from the reference methods are given in Table 2.

**Table 2:**
Data from honey samples using reference methods

| Measurand | Units | N of Cases | Minimum | Maximum | Median | Arithmetic Mean | Standard Deviation |
|---|---|---|---|---|---|---|---|
| Water | g/100g | 416 | 13.1 | 19.3 | 15.55 | 15.64 | 1.03 |
| Fructose | g/100g | 416 | 21.4 | 50.3 | 36.86 | 36.73 | 3.61 |
| Glucose | g/100g | 416 | 15.2 | 53.2 | 32.00 | 32.06 | 5.90 |
| Turanose | g/100g | 416 | 0.1 | 2.8 | 1.15 | 1.19 | 0.44 |
| Trehalose | g/100g | 416 | 0 | 2.5 | 0.40 | 0.49 | 0.43 |
| Isomaltose | g/100g | 415 | 0 | 2.7 | 0.44 | 0.49 | 0.38 |
| Erlose | g/100g | 395 | 0 | 7.4 | 0.263 | 0.451 | 0.642 |
| Melezitose | g/100g | 395 | 0 | 8.8 | 0.207 | 0.964 | 1.570 |
| Fructose+ Glucose | g/100g | 416 | 36.7 | 102.2 | 69.385 | 68.789 | 8.597 |
| Fructose/glucose ratio | | 416 | 0.8 | 2.0 | 1.163 | 1.172 | 0.179 |
| Glucose/water ratio | | 416 | 1.0 | 3.7 | 2.036 | 2.054 | 0.377 |
| Free acidity | meq/kg | 396 | 6.4 | 43.2 | 16.473 | 18.641 | 8.075 |
| HMF | mg/kg | 416 | 0 | 34.3 | 4.092 | 4.689 | 4.536 |
| Total nitrogen | mg/kg | 374 | 31.1 | 381.0 | 86.074 | 96.961 | 45.728 |
| Electical conductivity | $mScm^{-1}$ | 416 | 0.1 | 1.4 | 0.503 | 0.561 | 0.276 |
| pH- value | | 396 | 3.7 | 5.4 | 4.313 | 4.355 | 0.290 |

Internal validation (cross validation) results for FT-IR ATR measurements including SECV, RPD and of the corresponding $R^2$ are shown in Table 3.

**Table 3:**
Data from internal validation (cross-validation) used for the FT-IR ATR / PLS models to measure physicochemical measurands from honey samples

| Measurand | Units | Samples in calibration | Calibration range | number of factors | SECV | RPD | $R^2$ |
|---|---|---|---|---|---|---|---|
| Water | g/100g | 406 | 13.1 - 19.3 | 7 | 0.197 | 5.12 | 0.9619 |
| Fructose | g/100g | 368 | 21.4 – 50.3 | 10 | 1.290 | 2.09 | 0.7719 |
| Glucose | g/100g | 377 | 15.2 – 53.2 | 8 | 1.650 | 3.09 | 0.8952 |
| Turanose | g/100g | 403 | 0.1 - 2.8 | 13 | 0.225 | 1.94 | 0.7216 |
| Trehalose | g/100g | 327 | 0 - 2.5 | 8 | 0.221 | 1.73 | 0.6663 |
| Isomaltose | g/100g | 344 | 0 – 2.7 | 13 | 0.135 | 1.97 | 0.7411 |
| Erlose | g/100g | 131 | 0 – 7.4 | 15 | 0.219 | 2.35 | 0.8187 |
| Melezitose | g/100g | 277 | 0 – 8.8 | 13 | 0.315 | 5.04 | 0.9607 |
| Fructose+ Glucose | g/100g | 382 | 46 - 83 | 12 | 2.940 | 2.37 | 0.8223 |
| Fructose/glucose ratio | | 405 | 0.8 – 2.0 | 9 | 0.0532 | 3.05 | 0.8926 |
| Glucose/water ratio | | 407 | 1.0 – 3.7 | 7 | 0.0559 | 2.96 | 0.8858 |
| Free acidity | meq/kg | 388 | 6.4 – 43.2 | 14 | 1.71 | 4.75 | 0.9556 |
| HMF | mg/kg | 302 | 0 – 34.3 | 10 | 2.35 | 1.43 | 0.5109 |
| Total Nitrogen | mg/kg | 346 | 31.1 – 381.0 | 15 | 19.8 | 1.60 | 0.6116 |
| Electrical conductivity | $mScm^{-1}$ | 407 | 0.1 – 1.4 | 13 | 0.0437 | 6.30 | 0.9750 |
| pH-value | | 391 | 3.7 – 5.4 | 12 | 0.1180 | 2.41 | 0.8284 |

SECV = Standard Error of Cross Validation, RPD = Residual Prediction Deviation $R^2$ = coefficient of determination

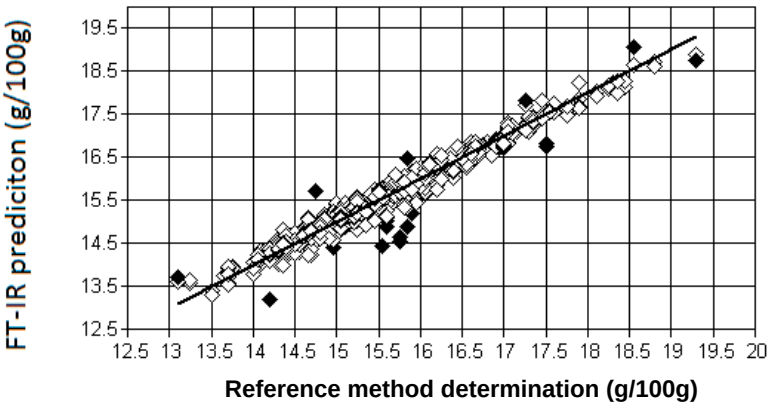
The external validation (test set validation) as well as the Standard Error of Prediction (SEP) and coefficients of determination ($R^2$) results are shown in Table 4.

**Table 4:**
Data from external validation (test set validation) used for the FT-IR ATR / PLS predictions of physicochemical measurands from honey samples

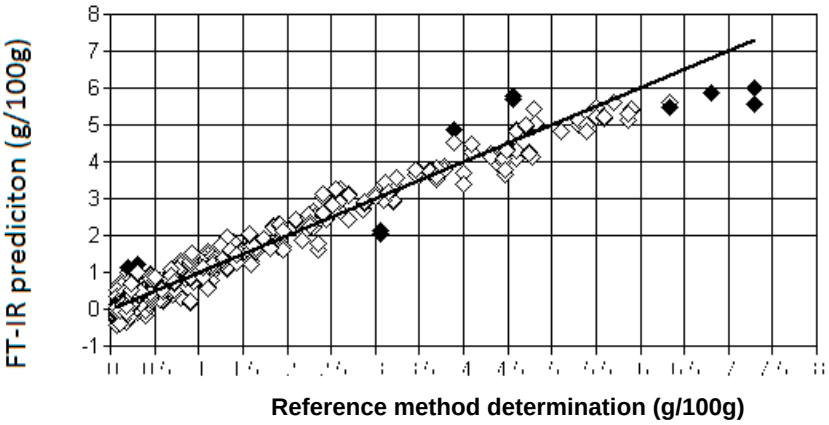
| Measurand | Units | N of Cases | Samples in calibration | SEP | $R^2$ | Prediction bias |
|---|---|---|---|---|---|---|
| Water | g/100g | 406 | 203 | 0.183 | 0.9683 | -0.00327 |
| Fructose | g/100g | 368 | 184 | 1.280 | 0.7730 | -0.113 |
| Glucose | g/100g | 377 | 188 | 1.770 | 0.8762 | 0.261 |
| Turanose | g/100g | 403 | 201 | 0.228 | 0.6996 | 0.00272 |
| Trehalose | g/100g | 327 | 165 | 0.219 | 0.6804 | 0.00447 |
| Isomaltose | g/100g | 344 | 172 | 0.151 | 0.6667 | -0.00709 |
| Erlose | g/100g | 164 | 164 | 0.238 | 0.7532 | 0.0272 |
| Melezitose | g/100g | 277 | 145 | 0.317 | 0.9599 | 0.0209 |
| Fructose+ Glucose | g/100g | 382 | 191 | 3.080 | 0.7965 | -0.0363 |
| Fructose/glucose | | 405 | 202 | 0.057 | 0.8633 | -0.00625 |
| Glucose/water ratio | | 407 | 203 | 0.061 | 0.8483 | -0.00747 |
| Free acidity | meq/kg | 388 | 194 | 1.540 | 0.9652 | 0.00213 |
| HMF | mg/kg | 302 | 151 | 2.440 | 0.4419 | 0.208 |
| Total nitrogen | mg/kg | 346 | 173 | 22.100 | 0.5188 | -0.424 |
| Electical conductivity | $mScm^{-1}$ | 407 | 203 | 0.0463 | 0.9719 | 0.00114 |
| pH- value | | 391 | 185 | 0.123 | 0.8101 | -0.0277 |

$R^2$ = coefficient of determination  SEP = standard error of prediction

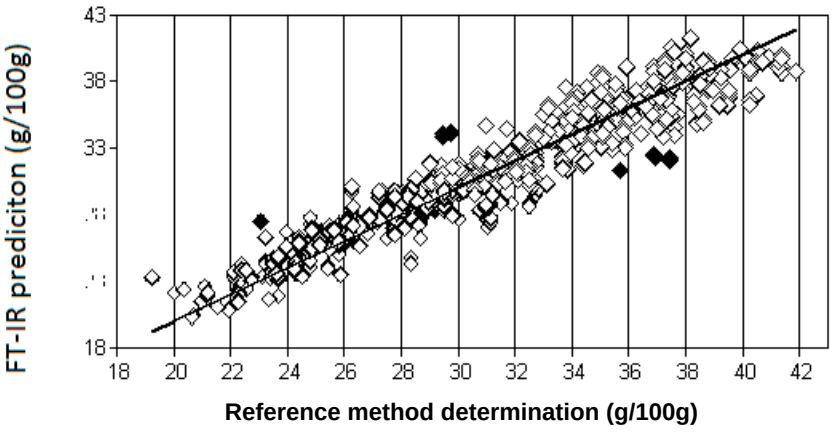
Figure 1 shows calibration plots (predicted values from cross validation) of honey measurands.



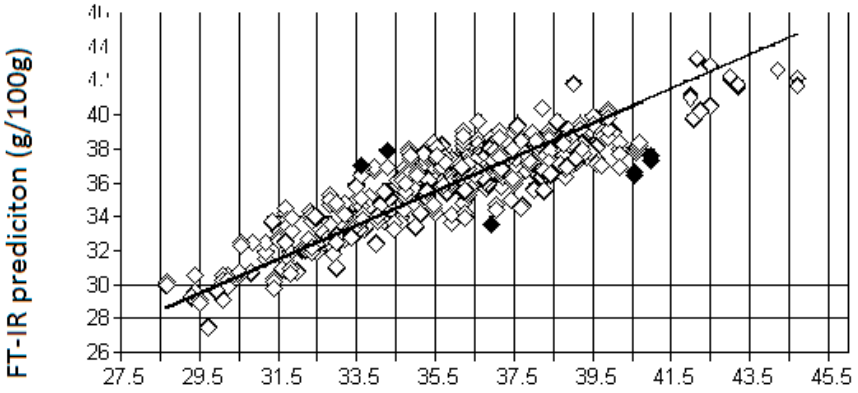

**(a) Water**



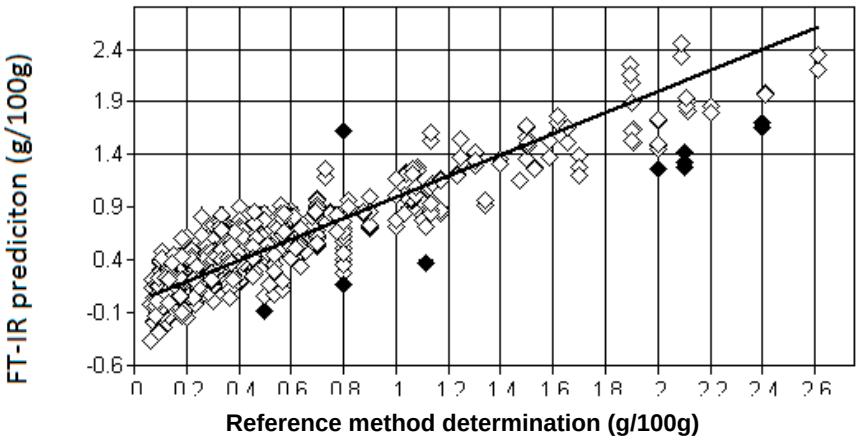

**(b) melezitose**



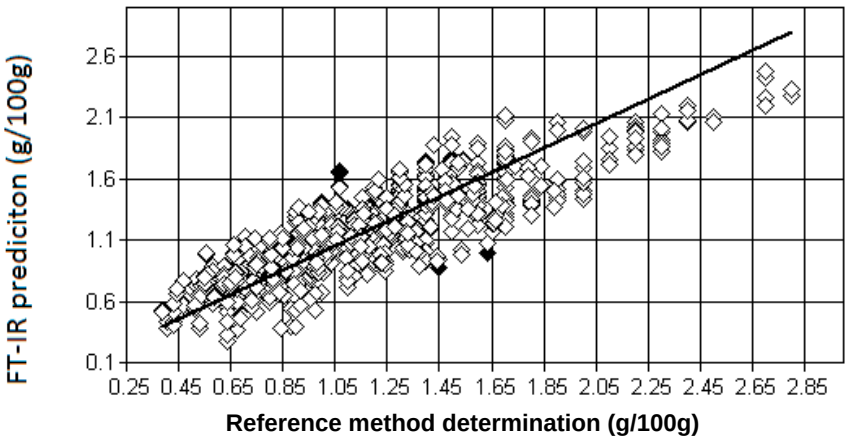

**(c) glucose**



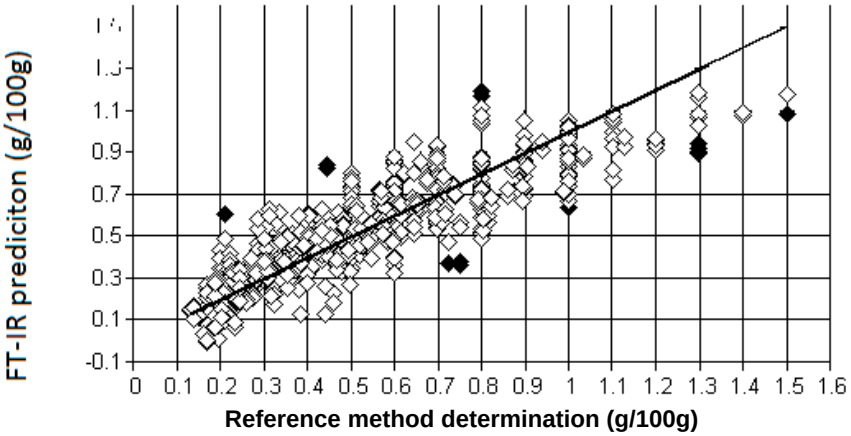

**(d) fructose**



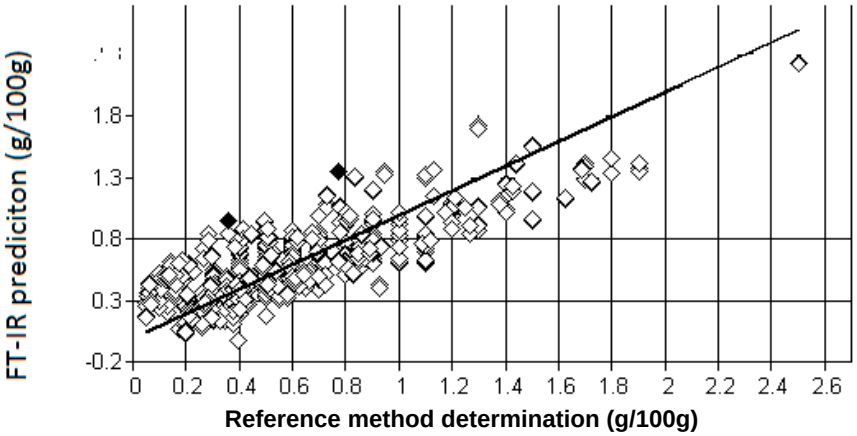

**(e) erlose**



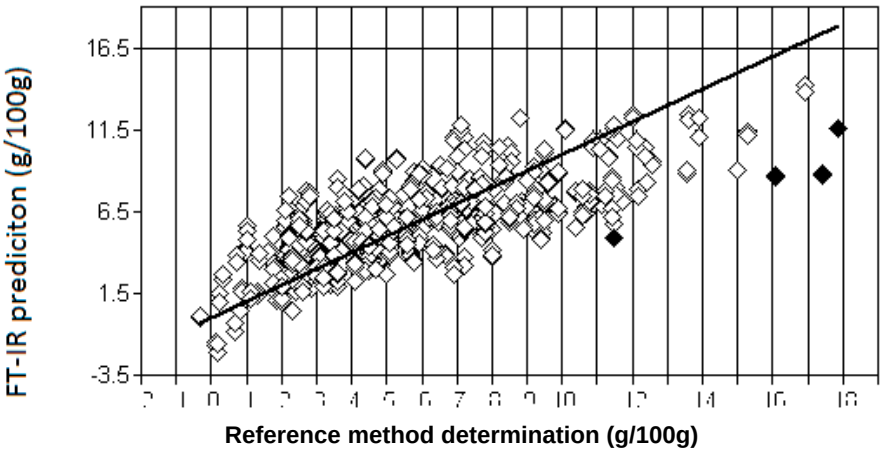

**(f) turanose**



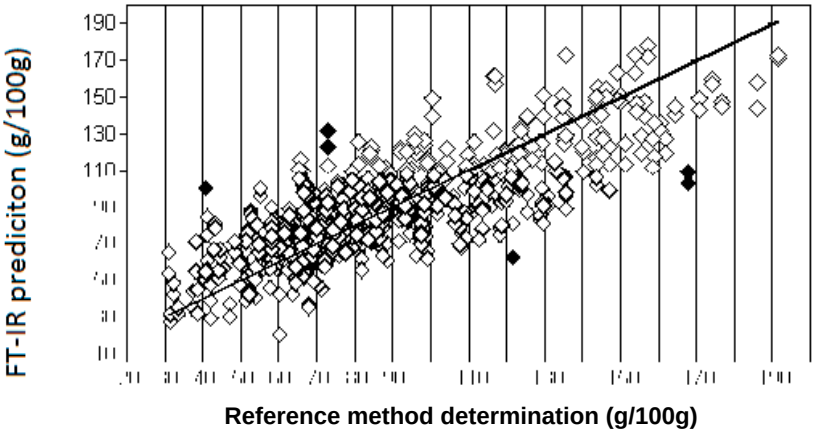

**(g) isomaltose**



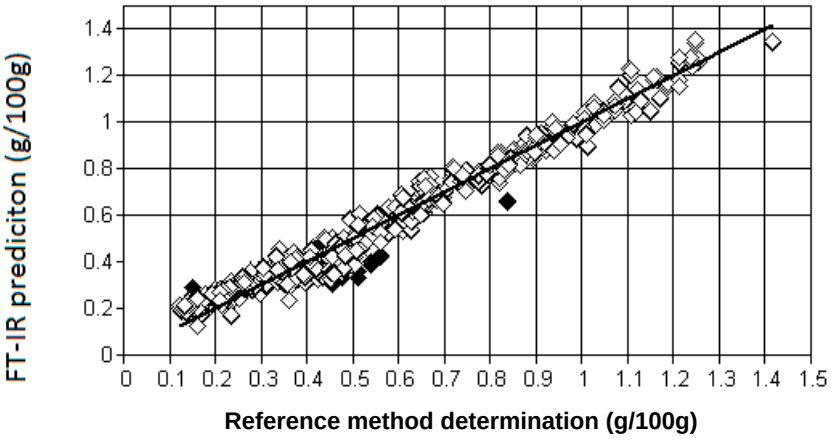

**(h) trehalose**



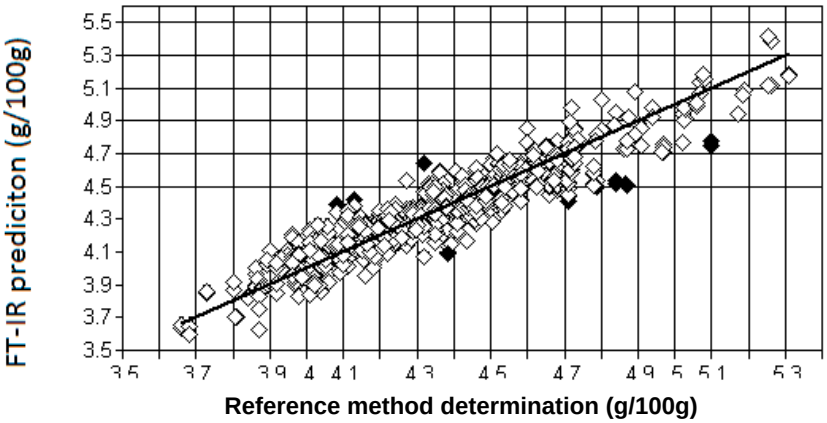

**(i) HMF**




**(k) total nitrogen**




**(l) electrical conductivity**




**(m) pH**

Figure 1. Calibration plots (predicted values from cross-validation) for water (a), melezitose (b), glucose (c), fructose(d), erlose (e), turanose (f), isomaltose (g), trehalose (h), HMF (i), total nitrogen (k), electrical conductivity(l) and pH (m).

Figure 2 shows the relation between moisture obtained by Ruoff et al. ($Water_{RefRuoff}$) using the reference method versus moisture obtained in the present work using the same reference method ($Water_{Ref08}$).

**Figure 2.**
Scatterplot correlating water content values from Ruoff et al. (2006a) and the values obtained in the present study using reference method.

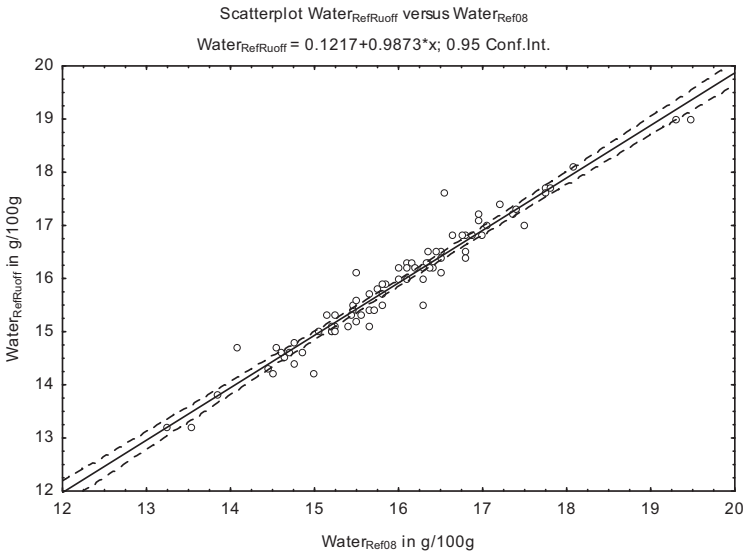
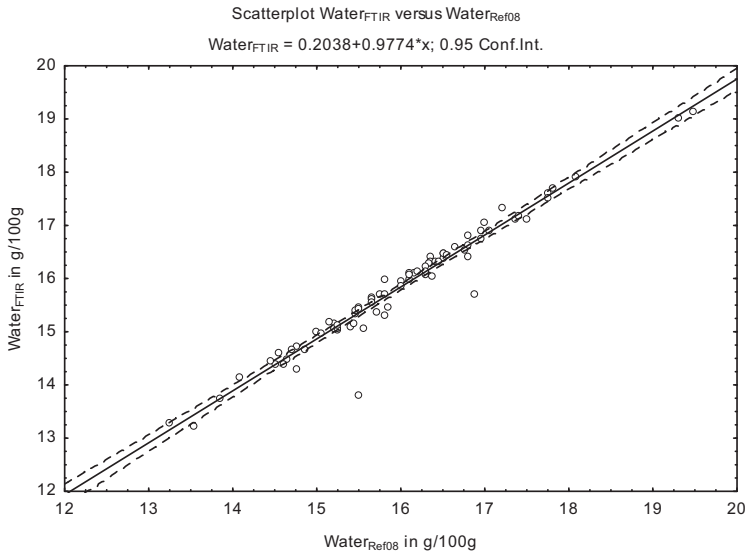



Figure 3 shows the relationship between water content predicted by FT-IR (WaterFTIR) and the values obtained from Ruoff (WaterRef08).

**Figure 3.**
Scatterplot correlating water content values from FT-IR / PLS prediction and the values of Ruoff et al.(2006a).



The comparison of the independent 79 samples (same as used by Ruoff et al. (2006a) and symbolized as $X_{RefRuoff}$) with the predicted values (11 measurands) as obtained by FT-IR ($X_{FT-IR}$) can be seen at Table 5.

**Table 5:**
Comparison between data from 79 independent samples using reference methods and FT-IR ATR / PLS prediction (paired t-test )

| Measurand | Mean | Standard Deviation | p |
|---|---|---|---|
| $Water_{RefRuoff}$ | 15.901 | 1.160 | |
| $Water_{FTIR}$ | 15.825 | 1.144 | 0.0345 |
| $Glucose_{RefRuoff}$ | 29.624 | 4.981 | |
| $Glucose_{FTIR}$ | 29.304 | 7.169 | 0.3494 |
| $Fructose_{RefRuoff}$ | 37.602 | 4.541 | |
| $Fructose_{FTIR}$ | 36.594 | 4.125 | 0.0000 |
| $Melezitose_{RefRuoff}$ | 0.952 | 1.496 | |
| $Melezitose_{FTIR}$ | 0.637 | 1.493 | 0.0000 |
| $Turanose_{RefRuoff}$ | 2.275 | 1.066 | |
| $Turanose_{FTIR}$ | 1.024 | 0.393 | 0.0000 |
| $El.Conductivity_{RefRuoff}$ | 0.656 | 0.439 | |
| $El.Conductivity_{FTIR}$ | 0.668 | 0.451 | 0.1483 |
| $pH_{RefRuoff}$ | 4.473 | 0.502 | |
| $pH_{FTIR}$ | 4.653 | 0.837 | 0.0201 |
| $Free\ Acidity_{RefRuoff}$ | 15.577 | 9.143 | |
| $Free\ Acidity_{FTIR}$ | 14.446 | 8.501 | 0.0004 |
| $Erlose_{RefRuoff}$ | 0.317 | 0.743 | |
| $Erlose_{FTIR}$ | 0.393 | 0.801 | 0.4249 |
| $Fruct/Gluc_{RefRuoff}$ | 1.274 | 0.242 | |
| $Fruct/Gluc_{FTIR}$ | 1.314 | 0.287 | 0.0294 |
| $Gluc/Water_{RefRuoff}$ | 1.898 | 0.334 | |
| $Gluc/Water_{FTIR}$ | 1.847 | 0.377 | 0.0320 |

$_{Ref\ Ruoff}$ = results from Ruoff et al. (2006a)
$_{FTIR}$ = Fourier Transform Infrared / PLS predicted values

# 4. Discussion

### 4.1 Prediction of the measurands

The results from Table 2 and 3 showed that some measurands can be accurately predicted while others have large uncertainties. This can also be observed looking at Figure 1. The best prediction was obtained for electrical conductivity with SEP = 0.0463 $mScm^{-1}$ and $R^2$ = 0.9719.

The FT-IR ATR method used in this study allowed an accurate determination of water content (moisture) as indicated by the SEP of 0.183 g/100 g and $R^2$ of 0.9686 for test set validation.

The only sugar that could be predicted with a very good accuracy in the present work was melezitose with SEP = 0.317 g/100 g and $R^2$ = 0.9599 which can be considered similar to those obtained by Ruoff et al. (2006a). The other sugars had lower coefficients of determination, the highest ($R^2$ = 0.8953) for glucose and the lowest ($R^2$ = 0.7411) for isomaltose.

Free acidity was predicted by FTIR with good accuracy (SEP = 1.540 meq/kg and $R^2$ = 0.9652).

The prediction of HMF with the proposed model was poor (SEP = 2.440 mg/kg and $R^2$= 0.4419) indicating, and confirming the conclusion obtained by Ruoff et al. (2006a), that FT-IR is not suitable for measuring very low concentrations of this measurand. The same problem (very low content) and unsatisfactory prediction was obtained for total nitrogen FT-IR analysis (SEP = 20.100 mg/kg and $R^2$= 0.5188).

### 4.2 Evaluation of the generalizability

The evaluation of the generalizability of the present calibration was also performed comparing the data obtained from 79 independent samples (same samples as used by Ruoff et al., 2006a).

As the samples were stored some time their moisture content was measured using refractometry and FT-IR ATR. The present results suggest that the honey samples stored at 4°C have not changed in water content within 3 to 6 years (Figure 2). Figure 3 shows a close correlation between FT-IR water content prediction and the results obtained by Ruoff et al (2006a).

Although some significant differences can be observed between the reference method and FT-IR ATR (Table 4) results, these do not generally indicate a lack of generalizability, because the most important model parameters such as SEP and standard deviation of the concentrations obtained by the reference methods versus FT-IR predicted values had shown satisfactory results (i.e. the statistical significance is not the same as the practical relevance).

Due to the fact that HMF increases throughout time, it was necessary to make a new quantification for the reference values in order to compare with the prediction by FT-IR. No difference was observed between the determination using the reference method (6.554 ± 5.2) and FT-IR method (6.114 ± 3.72) ($p < 0.05$).

# 5. Conclusions

Mid-infrared spectroscopy is a technique that can be used for the quantitative physicochemical quality control of honey with the advantage of being rapid and non destructive. It only needs a very small quantity of sample, predicts many measurands at the same time and its predictions are comparable with the results of traditional reference methods.

FT-IR ATR spectrometry combined with PLS regression provides good or at least satisfying screening results for many measurands used in routine quality control of honey.

The PLS predictions were of very good accuracy for the determination of water, electrical conductivity, free acidity and melezitose; measurement uncertainty was larger for the other sugars (fructose, glucose, turanose, trehalose, isomaltose), the sum of fructose plus glucose, fructose/glucose ratio, glucose/water ratio, pH-value and poor performance was found for the quantitative analysis of HMF and total nitrogen.

FT-IR ATR / PLS can be considered a valuable screening method for physicochemical honey quality control.

Results of the present generalizability study indicate that the procedure used previously by other authors (Ruoff et al, 2006a) can be reproduced with different equipment, different samples, as well as different software, in different laboratory conditions, indicating the robustness of the FT-IR ATR / PLS method for honey analysis.

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