JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Initial Study of Honey Adulteration by Sugar Solutions Using Midinfrared (MIR) Spectroscopy and Chemometrics

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Fourier transform infrared (FTIR) spectroscopy and attenuated total reflection (ATR) sampling have been used to detect adulteration of honey samples. The sample set comprised 320 spectra of authentic ($n = 99$) and adulterated ($n = 221$) honeys. Adulterants used were solutions containing both D-fructose and D-glucose prepared in the following respective weight ratios: 0.7:1.0, 1.2:1.0 (typical of honey composition), and 2.3:1.0. Each adulterant solution was added to individual honeys at levels of 7, 14, and 21% w/w. Spectral data were compressed and analyzed using k-nearest neighbors (kNN) and partial least squares (PLS) regression techniques. A number of data pretreatments were explored. Best classification models were achieved with PLS regression on first derivative spectra giving an overall correct classification rate of 93%, with 99% of samples adulterated at levels of 14% w/w or greater correctly identified. This method shows promise as a rapid screening technique for detection of this type of honey adulteration.

KEYWORDS: ATR; authenticity; FTIR; honey; chemometrics

INTRODUCTION

Many foods and food ingredients have the potential to be adulterated. Those that are expensive (e.g., vanilla, extra virgin olive oil) (*1*, *2*) and those whose composition or yield may vary as a result of fluctuations in weather during growth and harvest seasons (e.g., coffee, oranges) (*3*−*5*) may be particularly susceptible to this practice. Economic adulteration, i.e., the extension of a food or food ingredient by a cheaper and inferior product or component, is of considerable concern to food manufacturers, regulatory agencies, and consumers alike. Honey is highly prized by consumers as a natural sweet substance. It is defined as "the natural sweet substance produced by honeybees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in the honeycomb to ripen and mature" (*6*). While demand for honey is increasing, production is in decline for a variety of socioeconomic factors. Extension of honey by addition of other sweet substances such as sugars or industrial syrups at some stage during production or processing could be an attractive means of economic adulteration. Identifying this type of adulteration is important for financial reasons. Many different analytical techniques are employed in authenticity testing of honey. Among them are NMR spectroscopy (*7*), HPLC (*8*, *9*), GC (*10*), and carbon isotope ratio analysis (*11*, *12*). These techniques, while reported to be successful, are costly and require considerable analytical skill. With the exception of NMR spectroscopy, these techniques are also time-consuming and will destroy samples under test. A need therefore exists for a rapid, nondestructive, and less expensive method suitable at least for screening honey samples for authenticity confirmation.

Vibrational spectroscopic methods (near and MIR) have previously been applied to a range of authenticity problems (*1*, *3*, *13*−*17*). In combination with multivariate data analysis, they possess the speed, simplicity, and low cost per analysis required for screening techniques. MIR spectroscopy (2500−25 000 nm) may have particular benefits since it contains more spectral information than its NIR counterpart and the fundamental vibrational absorption bands in the MIR are better resolved than the broad overtone and combination absorption bands which arise in the NIR spectral region (750−2500 nm). The availability of ATR crystals simplifies sample handling in the MIR region.

Previous reports have described the use of these vibrational spectroscopic techniques to determine the chemical composition of honey samples (*18*−*21*) and also for detecting added sugar or syrups in honey (*22*−*25*). However, the experimental design used in the latter reports facilitated classification on the basis of alteration of the solids content of the honey or by adulteration with syrups possessing a very different chemical composition to that of honey. The work presented in this paper investigates the potential of MIR spectroscopy to identify honey samples adulterated with fructose:glucose solutions that have very similar sugar profiles to honey. Additionally, both the honey samples and the adulterant solutions were adjusted to a standard solids (°Brix) level.

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10.1021/jf034985q CCC: $27.50
Published on Web 12/16/2003

MATERIALS AND METHODS

Samples. Authentic honey samples were gathered from artisanal beekeepers throughout the island of Ireland. Samples were not independently tested for authenticity as each honey sample was collected directly from the apiarists. D-Fructose and D-glucose (Analar Grade) were obtained from Merck. Aqueous solutions were prepared using distilled water and containing fructose and glucose in the following (F:G) ratios by weight: 0.7:1.0, 1.2:1.0, and 2.3:1.0. Each adulterant sugar solution and all of the honey samples were diluted with distilled water to 70° Brix. Twenty-five authentic honey samples were adulterated at levels of 7, 14, and 21% w/w using each of the adulterant solutions. A further 74 honey samples were added to augment the authentic sample database, producing 99 authentic samples and 225 adulterated samples. It was necessary to remove four outlier samples from the adulterated spectral data set due to the presence of spikes in the spectra caused by electrical interference during data collection of these samples. Therefore, 221 adulterated sample spectra were used for analysis. Prior to spectral collection, samples were placed in an oven overnight at 40 °C to dissolve any crystalline material present and stirred manually to produce a homogeneous solution.

Instrumentation. Solids content in honeys and adulterated solutions was measured by refractometry using an Abbé model 2WA benchtop refractometer. MIR spectra were collected at room temperature on a BIO-RAD Excalibur series FTS 3000 spectrometer (Analytica ltd., Dublin, Ireland); instrument control and spectral collection were performed using WIN-IR Pro (v 3.0) software supplied by the equipment manufacturer. Spectra were recorded on an in-compartment benchmark ATR trough top plate using a 45° ZnSe crystal with 11 internal reflections. Sixty-four scans were coadded at a nominal resolution of 4 cm^{-1}. Single beam spectra of the samples were collected and ratioed against a background of air. Spectra were truncated to the useful range of the ZnSe ATR crystal (800−4000 cm^{-1}) and then converted to a wavelength scale using the supplied Win-IR Pro software. Samples were applied to the ATR crystal to obtain a maximum absorption of approximately 1.0; this was achieved by variation of the crystal coverage by samples. This was carried out to avoid any nonlinearity in the spectral set, which can occur if the path length varies greatly from sample to sample. It also prevented saturation of the signal, which occurs around 1150 nm when the entire area of the crystal is covered by the test solution. The crystal was cleaned between samples with tepid water and dried with lens-cleaning tissue. The spectral baseline recorded by the spectrometer was examined visually to ensure that no residue from the previous sample was retained on the crystal. All spectra were recorded at room temperature between 20 and 25 °C. Duplicate spectra of each solution were collected using separate subsamples.

Data Processing. The means of duplicate spectra were used for statistical analysis. Spectra were exported from WIN-IR Pro as GRAMS files (ThermoGalactic, Salem, NH) and imported directly into The Unscrambler (v7.6; CAMO ASA, Norway) or Pirouette (v3.10; Infometrix Inc., WA). Models were developed using the spectral region between 6800 and 11 500 nm, which is dominated by information on the sugar composition (*26, 27*). Normalization of the spectra was carried out prior to analysis according to the equation below, where $X(i,k)$ is the absorbance value for sample i at wavelength k.

$$XN(i,k) = X(i,k)/Abs\{\text{mean } X(i,*)\}$$

This was performed to remove variance caused by small changes in the path length between samples. Calibrations were developed and evaluated on separate calibration and prediction sample sets. Samples were assigned to these sets based on their position in the spectral file. All odd-numbered samples were assigned to the calibration sample set and all even-numbered samples to the prediction sample set. PLS regression onto a dummy variable was used for discrimination of the authentic and adulterated samples. The dummy variable was assigned a value of −1 for an authentic honey sample and +1 for all other samples. For quantitative analysis, the y variable was assigned a value of 0 for an authentic sample, +7 for samples adulterated at a level of 7% w/w, +14 for 14%, and +21 for 21% adulteration level. Data pretreatments examined were first and second derivative spectral data using the Savitsky−Golay method and segment sizes of 13 points (157 nm) and 25 points (352 nm), respectively. Full cross-validation and variable weightings of 1/standard deviation were used in model development. In all cases, only optimal models are discussed in this paper. These models were then used to classify the prediction sample set. Cutoff points were arbitrarily chosen at 0 and 7 for the discrimination and quantitative predictions, respectively.

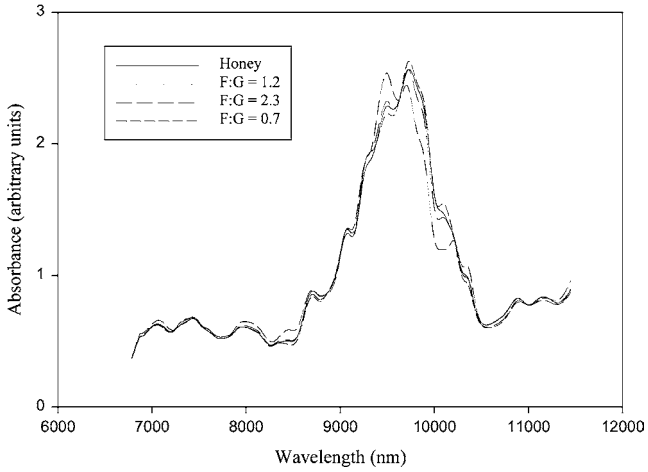

Figure 1. Average ATR spectra of unadulterated honeys and adulterant solutions (all adjusted to 70° Brix).

Classification was also attempted by the kNN method using Pirouette software. This supervised classification method identifies an unknown sample on the basis of the identity of a predefined number of neighboring samples of known sample types modeled in the calibration development step. The technique is based on the assumption that the closer the samples lie in measurement space, the more likely they belong to the same category. One selects the k nearest samples from the calibration set to the unknown and applies a majority rule: the unknown is classified in the group to which the majority of k neighbors belong. The choice of k is determined by optimization. kNN is well-suited to data sets with small sample numbers and can function even with only one calibration set sample per category. It is sensitive to gross inequalities in the number of samples in each class. Calibration and prediction sample sets were the same as those used in PLS analyses. Samples were classified by three different approaches. First, into two groups, honey vs nonhoney, similar to the discrimination dummy variable in PLS. Second, into four groups classified on the basis of adulterant level; this approach is similar to the quantitative PLS analysis. Finally, into four classes grouped according to the adulterant solution used. In each case, all data pretreatments examined for PLS regression analysis were also used for kNN analysis. All of the above procedures for both PLS and kNN analysis were repeated with the calibration and prediction sample sets inverted, i.e., all even-numbered samples being assigned to a calibration sample set and all odd-numbered samples used as the prediction sample set.

RESULTS AND DISCUSSION

The average ATR spectrum of all of the authentic honey samples together with spectra of the three adulterant solutions are shown in **Figure 1**. Obvious differences are observed for adulterant solutions with either a high or a low F:G ratio. The main differences involve an increase in band absorption around 8450 and 9490 nm (F maximum ∼ 9490 nm) and a lowering of absorption intensity between 9800 and 10 200 nm (glucose maximum ∼ 9820 nm) for samples with a high F:G ratio. The opposite is observed for samples with a lower F:G ratio. However, as the spectral variations in honey samples can also be quite large (**Figure 2**), identification of authentic samples by visual analysis of test sample spectra is impossible. Chemometric techniques are therefore necessary to discriminate between authentic and adulterated samples.

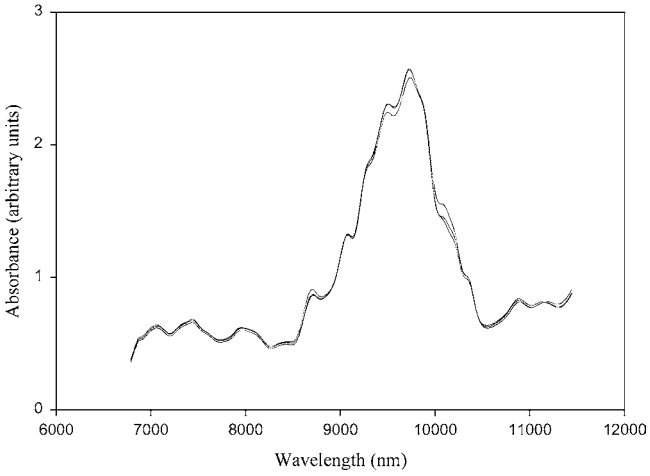

Figure 2. Individual ATR spectra of selected honey samples (adjusted to 70° Brix).

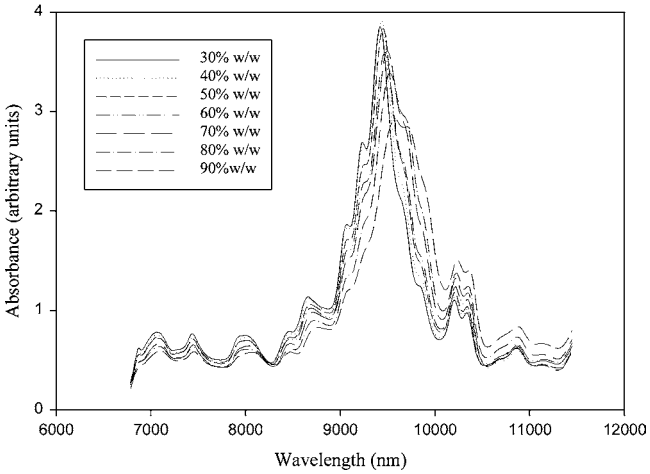

Figure 3. ATR spectra of aqueous solutions containing 30–90% w/w of fructose. A correction for water absorption was applied for comparison purposes.

The MIR spectrum of honey is dominated by sugar absorptions. The bands appearing between 6800 and 8700 nm are due to bending modes of C−C−H, C−O−H, and O−C−H groups (*26*). The more intense peaks in the region around 8700−11 000 nm arise mainly from C−O and C−C stretching modes (*26*), with a peak around 9400−9800 nm due to O−H vibrations (*27*). At longer wavelengths, bands due to C−H and O−H bending vibrations are also useful for discrimination and quantification purposes. Given the differences observed between the spectra of the major components of honey (fructose, glucose, and sucrose), it is not surprising that MIR spectroscopy can be used for accurate determination of the sugar composition of mixtures and syrups (*28*−*30*). However, it should also be noted that peak intensity and position for sugar solutions can vary dramatically with concentration. Spectra of aqueous solutions of fructose from 30 to 90% w/w were recorded, and the contribution from water was removed, thus leaving the absorbance due to the fructose molecules only. This was done by normalizing the spectra at the maximum of the water band at 6111 nm to a value of 1.0 and subtracting a similarly normalized spectrum of water. These modified spectra were then normalized over the range displayed in **Figure 3**. These spectra show a shift to longer wavelengths and a broadening of the absorption bands as the concentration of fructose is increased. Similar results were observed for glucose solutions and mixtures of fructose and glucose. To remove these complications arising from concentration effects, all samples were diluted to a constant °Brix value prior to analysis.

Table 1. Percentage Correct Classification of Prediction Sample Sets Using Discriminant PLS Regression

	data pretreatment		
sample groups	normalized only	normalized 1st derivative	normalized 2nd derivative
	odd-numbered data set[a]		
adulterated honey	96.4	95.5	93.6
unadulterated honey	84.0	84.0	86.0
loadings	5	4	4
	even-numbered data set[b]		
adulterated honey	96.4	95.5	92.7
unadulterated honey	91.8	95.9	91.8
loadings	5	6	6
	averaged values		
adulterated at 7%	90.4	89.0	84.9
adulterated at 14%	98.6	97.2	94.4
adulterated at 21%	100	100	100
adulterated honey	96.4	95.5	93.2
unadulterated honey	87.9	89.9	88.9
all samples	93.7	93.7	91.8

[a] Calibration models developed on even-numbered data set. [b] Calibration models developed on odd-numbered data set.

PLS. A summary of the results obtained using PLS regression to discriminate between authentic and adulterated honey samples is shown in **Table 1**. Results for prediction on the odd-numbered data set using the even-numbered data set for calibration are shown first followed by the results for prediction on the even-numbered data set using the odd-numbered data set for calibration. Both of these results were then combined and are labeled averaged values. Only small differences are observed for normalized, first, and second derivative data. An overall correct classification of 93.7, 93.7, and 91.8% was achieved with normalized, first derivative, and second derivative data, respectively. As further preprocessing of the data does not improve the results, normalized data is the preferred pretreatment in this case. Using this model, approximately 88% of the authentic honey samples and more than 96% of the adulterated honey samples are classified correctly. The vast majority of the misclassified samples belonged to the set, which were adulterated at the lowest level (i.e., 7%); more than 99% of samples adulterated at levels of over 7% are classified correctly.

The ability of discriminant PLS to differentiate between authentic honey samples and adulterated honey samples is compromised by the variability in the adulterated sample sets, i.e., three different levels of adulteration using three different adulterant solution compositions. To reduce this variability, PLS regression was carried out using the level of adulteration (0, 7, 14, and 21%) as the *y* variable.

The results of this analysis are shown in **Table 2**. Overall, correct classification levels of 91.8, 92.8, and 92.5% were achieved with normalized, first derivative, and second derivative data, respectively. The preferred preprocessing in this case is for first derivative data. Almost 93% of both authentic honey samples and adulterated honey samples were predicted correctly. The largest portion of misclassifications arose from samples adulterated at the lowest level of adulteration. This classical application of PLS permits the prediction of adulterant content with a standard error of ∼4.75% (**Table 2**). The 95% confidence limit for this model is approximately 9.5% (∼2 × SEP). Therefore, this model will not be able to predict adulteration

Table 2. Percentage Correct Classification of Prediction Sample Sets Using Quantitative PLS Regression

	data pretreatment		
sample groups	normalized only	normalized 1st derivative	normalized 2nd derivative
	odd-numbered data set[a]		
adulterated honey	91.8	92.7	94.5
unadulterated honey	88.0	88.0	88.0
SEP (%)	4.8	4.8	4.7
loadings	6	4	6
	even-numbered data set[b]		
adulterated honey	91.8	92.7	90.9
unadulterated honey	95.9	98.0	95.9
SEP (%)	4.4	4.7	4.6
loadings	11	4	6
	averaged values		
adulterated at 7%	78.1	79.5	82.2
adulterated at 14%	97.2	98.6	95.8
adulterated at 21%	100	100	100
adulterated honey	91.8	92.7	92.7
unadulterated honey	91.9	92.9	92.0
all samples	91.8	92.8	92.5

[a] Calibration models developed on even-numbered data set. [b] Calibration models developed on odd-numbered data set.

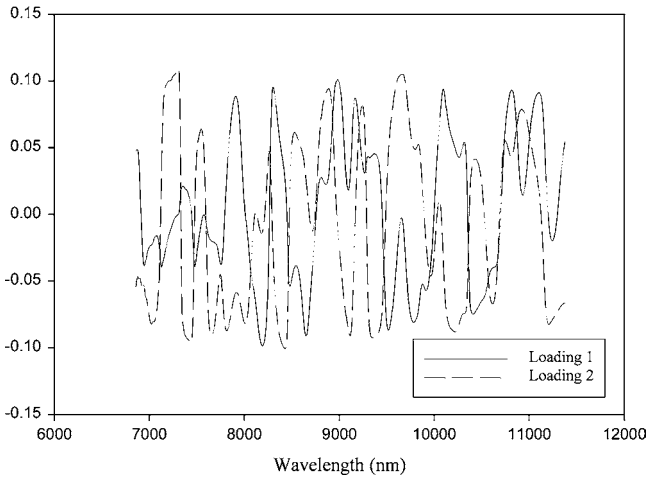

Figure 4. Loadings 1 and 2 of the quantitative PLS regression model using first derivative spectra of honey and adulterated solutions.

accurately at levels below this value and hence the misclassification of many samples adulterated at levels of 7%.

The results by both discriminant and quantitative PLS analysis techniques give similar results. The major difference observed is the compromise between the number of false positive and false negative results, which are related to the choice of cutoff point. Therefore, both techniques appear to discriminate based only on dilution of all of the minor components in honey that are absent in all of the adulterant solutions. This is further recognized through an examination of the loading plots.

Graphical illustrations of the first two loadings developed in the quantitative PLS model using first derivative data are shown in **Figure 4**. First derivative spectra of fructose and glucose (70° Brix) are shown in **Figure 5**. A loading can be calculated from these spectra to describe the difference in fructose and glucose concentration by subtracting the first derivative spectrum of glucose from that of fructose and dividing each variable in the resulting difference spectrum by the weighting used during development of the model (i.e., the standard deviation of each variable in the calibration sample set). This calculated loading describing the difference between fructose and glucose is shown

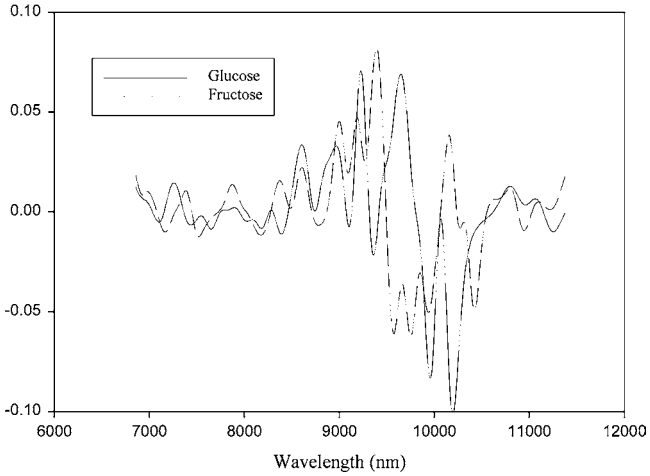

Figure 5. First derivative ATR spectra of glucose and fructose (70° Brix).

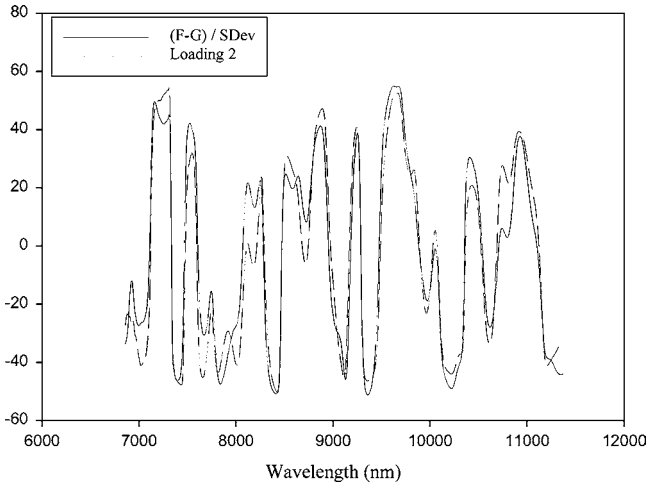

Figure 6. Comparison of the calculated loading describing differences between glucose and fructose and the second loading used in the quantitative PLS regression model.

in **Figure 6**. The second loading from the model is overlaid in the same figure. Loadings 1 and 2 account for 24 and 33% of the variation in the spectral data and 57 and 4% of the *y* data (adulterant concentration), respectively. It is evident from an examination of **Figure 6** that the variation in the spectral data being described by this second loading is due to the variation in the fructose and glucose concentrations. The largest portion of the spectral variation (33%) described by this loading accounted for a very small part of the classification (4%). Therefore, the regression model is unable to use this variation in the spectral data to the benefit of the prediction results. The first loading is likely to be related to absorptions of sucrose, maltose, and higher sugars that are present in honey but absent from the adulterant solutions.

Although this classical PLS analysis can take into account variations in the level of adulteration in the adulteration sample set, there is no information in the *y* variable relating to the adulterant solution composition. We can remove the complication in the adulterant sample set by using only samples adulterated with one of the three adulterant solutions and the authentic honey sample in the calibration. A classical PLS regression was carried out for each of the three adulterant solutions (F/G = 0.7, 1.2, 2.3) with the authentic honey samples, using normalized first derivative data and the level of adulteration as the *y* variable as before. A regression plot is shown for each of the three calibrations in **Figures 7**−**9**. The SEP for the

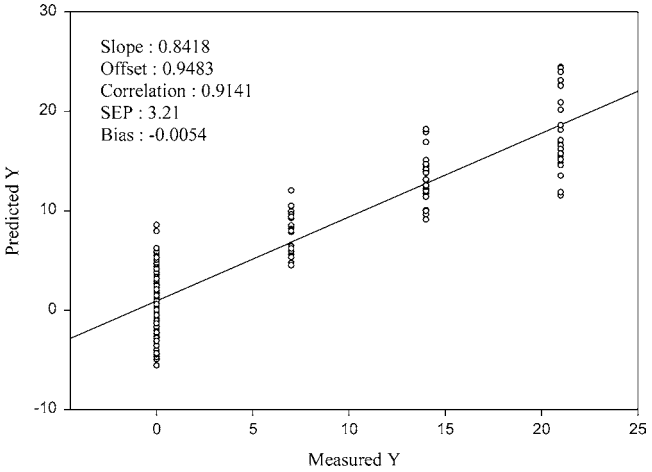

Figure 7. Plot of predicted vs measured values for the adulteration level with an adulterant solution of F:G ratio 0.7:1.0.

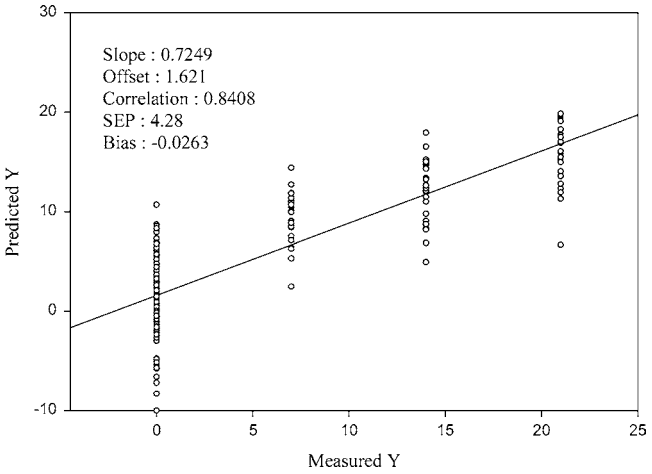

Figure 8. Plot of predicted vs measured values for the adulteration level with an adulterant solution of F:G ratio 1.2:1.0.

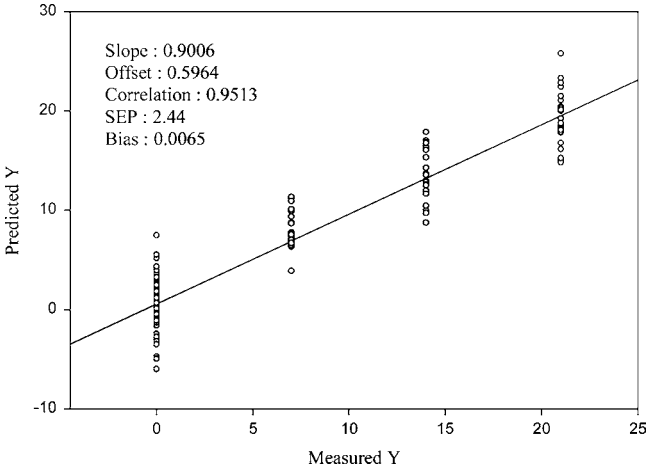

Figure 9. Plot of predicted vs measured values for the adulteration level with an adulterant solution of F:G ratio 2.3:1.0.

calibration using authentic honey samples and samples adulterated with the adulterant solution with a F:G ratio of 0.7:1.0 is 3.2%. A 95% confidence level (i.e., ~2 × SEP) is observed at a level of adulteration of 6.4%. For adulterant solutions with F:G ratio of 1.2:1.0, the limit for 95% confidence is 8.6% (2 × 4.3), and for the adulterant with a F:G ratio of 2.3:1.0, a value of 4.9% (2 × 2.44) is observed. These 95% confidence limits (6.4, 8.6, and 4.9%) compare favorably to the value of 9.5% (2 × 4.75, **Table 2**) obtained when samples from all three adulterant solutions were used in the calibration. The improvement in the calibrations is due to the ability of the model to use the variation in the fructose and glucose concentrations to separate the sets. As is expected, the smallest improvement is observed for the adulterant solution with a F:G ratio closest to that of honey (1.2). However, knowledge of the adulterant can lead to a much improved regression model.

Table 3. Percentage Correct Classification of Prediction Sample Sets Using kNN with Sets Grouped into Two Classes of Honey and Nonhoney

	data pretreatment		
sample groups	normalized only	normalized 1st derivative	normalized 2nd derivative
	odd-numbered data set[a]		
adulterated honey	85.5	92.7	92.7
unadulterated honey	92.0	88.0	86.0
k	9	9	7
	even-numbered data set[b]		
adulterated honey	90.9	92.7	90.9
unadulterated honey	91.8	93.9	85.7
k	8	3	3
	averaged values		
adulterated at 7%	74.0	83.6	80.8
adulterated at 14%	91.7	94.4	94.4
adulterated at 21%	98.7	100	100
adulterated honey	88.2	92.7	91.8
unadulterated honey	91.9	90.9	85.9
all samples	89.3	92.2	90.0

[a] Calibration models developed on even-numbered data set. [b] Calibration models developed on odd-numbered data set.

kNN. The application of kNN to this data set was used to try and overcome the problems encountered with PLS and to use more of the information in the spectra related to the concentration of fructose and glucose in the classification procedure. As kNN attempts to categorize samples based on proximity, it should be sensitive to this variation. Three prediction approaches were attempted. First, to classify according to honey vs nonhoney samples; second, to classify according to the level of adulteration (which can therefore assign a quantity to the adulteration); and finally, to classify according to the adulterant solution used. This final classification could therefore be used to identify an adulterant. The results of each prediction are shown in **Tables 3**−**5**. The best results were obtained using two classes (honey vs nonhoney) with first derivative spectra. This is not surprising as in the other two methods votes for the adulterated samples were divided into three thus increasing the competitiveness of the authentic sample set for both models. In the case of the best model (two classes, first derivative data), an overall correct classification rate of 92% was achieved, with 90.9% of honey samples and 92.7% of the adulterated samples correctly classified. The kNN classification technique does show discrimination based on the F:G ratio. This is demonstrated by the fact that 100% of samples adulterated with the 2.3 F:G adulterant solution were detected as nonhoneys. This did not occur with PLS regression. The results were not as accurate for the 0.7 F:G solution as compared to the 2.3 F:G solution due to the larger variation of glucose concentration in honey as compared to that of fructose and the similarity between the maltose and the glucose spectra. These factors reduce the accuracy of identification of this adulterant, and in all of the predictions, some samples adulterated were misclassified; however, this occurred only at the lowest level (7%). As a general screening technique using models with many different

Table 4. Percentage Correct Classification of Prediction Sample Sets Using kNN with Sets Grouped into Four Classes Related to Level of Adulteration

	data pretreatment		
sample groups	normalized only	normalized 1st derivative	normalized 2nd derivative
	odd-numbered data set[a]		
adulterated honey	80.9	87.3	85.5
unadulterated honey	100	94.0	92.0
k	8	4	4
	even-numbered data set[b]		
adulterated honey	87.3	90.9	89.1
unadulterated honey	93.9	93.9	89.8
k	1	3	4
	averaged values		
adulterated at 7%	65.8	74.0	72.6
adulterated at 14%	86.1	93.1	90.3
adulterated at 21%	97.3	100	98.7
adulterated honey	84.1	89.1	87.3
unadulterated honey	97.0	93.9	90.9
all samples	88.1	90.6	88.4

[a] Calibration models developed on even-numbered data set. [b] Calibration models developed on odd-numbered data set.

Table 5. Percentage Correct Classification of Prediction Sample Sets Using kNN with Sets Grouped into Four Classes Related to Adulterant Solution Added

	data pretreatment		
sample groups	normalized only	normalized 1st derivative	normalized 2nd derivative
	odd-numbered data set[a]		
adulterated honey	86.4	87.3	89.1
unadulterated honey	94.0	94.0	92.0
k	3	10	6
	even-numbered data set[b]		
adulterated honey	88.2	90.0	90.0
unadulterated honey	93.9	93.9	85.7
k	4	3	3
	averaged values		
adulterated at 7%	74.0	74.0	74.0
adulterated at 14%	87.5	91.7	94.4
adulterated at 21%	100	100	100
adulterated honey	87.3	88.6	89.5
unadulterated honey	93.9	93.9	88.9
all samples	89.3	90.3	89.3

[a] Calibration models developed on even-numbered data set. [b] Calibration models developed on odd-numbered data set.

adulterants, this method may have more value than the regression methods as no knowledge of the adulterant is necessary and it can also be used to identify and quantify the adulterant. However, when searching for one specific adulterant, PLS regression produces better results.

Conclusion. In this work, we have shown that it is possible to distinguish infrared spectra of honey from those of honey samples adulterated with sugar solutions. Two complementary techniques have been described. The application of PLS regression has shown more sensitivity to the minor components in honey, and kNN has shown the ability to distinguish samples adulterated at low levels with solutions with different sugar composition from that of honey, such as medium invert syrups and dextrose syrups. MIR spectroscopy has the potential to be used as a rapid screening technique for the identification of adulterated honey samples and is likely to be sensitive to a broad range of sugar syrups.

Future work will be aimed at identifying the limits of this technique. Fully inverted syrups and high fructose corn syrups will be examined, and the combination of both MIR and NIR spectra will be used to identify samples.

ABBREVIATIONS USED

ATR, attenuated total reflectance; F, fructose; FTIR, Fourier transform infrared; G, glucose; GC, gas chromatography; HPLC, high-performance liquid chromatography; kNN, k nearest neighbors; MIR, midinfrared; NIR, near-infrared; NMR, nuclear magnetic resonance; PLS, partial least squares; SEP, standard error of prediction.

ACKNOWLEDGMENT

We thank the members of the Irish Federation of Beekeepers' Associations and those of the Ulster Beekeepers' Association for supplying samples of honey.

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Received for review September 1, 2003. Revised manuscript received November 11, 2003. Accepted November 11, 2003. We acknowledge the Irish Department of Agriculture and Food (FIRM program) for financial support.

JF034985Q