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Discrimination and classification of beet and cane inverts in honey by FT-Raman spectroscopy

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Abstract

As a natural product, honey has been prone to adulteration. Adulteration of honey by substituting with cheap invert sugars is a critical issue in the honey industry. Fourier Transform (FT) Raman Spectroscopy was used to detect adulterants such as cane and beet invert in honey. FT Ra man spectrum of adulterated samples were characterized and the region between 200 and 1600 cm^{-1} (representing carbohydrates and amino acid fractions) was used for quantitative and discriminant analysis. Partial least squares, and principal component regression analysis were used for quantitative analysis while linear discriminant analysis and canonical variate analysis (CVA) were used for discriminant analysis. FT-Raman spectroscopy was efficient in predicting beet and cane invert adulterants ($R^2 > 0.91$) in all three floral types of honey considered. Classification of adulterants in honey using CVA gave a minimum classification accuracy of about 96%. © 2002 Published by Elsevier Science Ltd.

Keywords: FT-Raman Spectroscopy; Adulteration; Honey; Sugars; Chemometrics

1. Introduction

Honey, a levorotatory carbohydrate rich product is produced by honeybees naturally form the nectar collected from flowers of different plants. Secretary products of bees convert nectar into honey. Normally honey contains 12.4–20.3% moisture and 60.7–77.8% sugars, of which about 0–2% may be sucrose, 25.2–35.3% glucose, and 33.3–43.0% fructose and less than 0.25% of ash. Honey has always been an easy target of adulterators for economic gains. Many adulterants including acid-invert syrups, corn syrups, sugar, starch, dextrin are reported (Singhal, Kulkarni, & Rege, 1997). The most known adulterants are cane and beet invert syrups, which can be tailored to mimic the natural sucrose–glucose–fructose profile of honey and are usually difficult to detect. Honey adulteration is difficult to detect due to high variability of composition among honey from different floral and geographical origins.

Detection of invert syrups in honey has been a problem for more than a century. The addition of a moderate amount of invert syrup does not cause fructose and glucose levels to fall outside the normal range of honey. Hydroxy methyl furfural (HMF), a product of acid inversion is used as an index to detect the presence of invert syrup in honey by many investigators (Singhal, et al, 1997). Recognizing the fact that HMF arises from heating or even storage of honey (Perez-Arquillue, Conchello, Arino, Juan, & Herrera, 1994) its validity as an adulterant indicator has been questioned. Other honey authenticity tests are being done by using different techniques such as spectroscopy, isotope ratio, chromatography, and trace metal analysis. Conventional sugar profiling of sucrose–glucose–fructose ratios by enzymatic and chromatographic methods such as high-performance liquid chromatography (HPLC) have been used to identify outliers from the prescribed standard, addition of invert syrup may also be detected indirectly by examining the oligosaccharides present. These oligosachharides are formed as impurities during sugar hydrolysis by a process called inversion. A simple and rapid method for spectrophotometric discrimination of monosaccharides from the oligosaccharide fraction in fruit juice, jam, syrup and honey was proposed (Caceres, Cardenas, Gallego, & Valcarcel, 2000). However, this approach was considered inadequate because

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of the amount of naturally occurring minor polysaccharide content, which are believed to rise from the transglucosylic activity of the natural enzymes produced by honeybees on plant sugars.

A microscopic procedure was described to detect adulteration of honey with cane sugar, acid-hydrolyzed cane sugar syrup, or with honey obtained from feeding sugars to bees (Kerkvlier, Shrestha, Tuladhar, & Manandhar, 1995). Profiles of different amino acids such as phenylalanine, aspartic acid and proline were also proved to be of great importance for authentication of honey (Davis, 1975). A recent approach is based on the differences in the ratio of ^{13}C to ^{12}C honey and invert syrups. Inverts such as corn syrup are slightly enriched in ^{13}C compared to honey. This difference is caused by the fractionation of carbon isotopes during photosynthesis (Singhal et al., 1997). Stable carbon isotope ratio analysis to detect the undeclared presence of cane and corn sugars exists (White, Winters, Martin, & Rossmann, 1998). However, it is time consuming and expensive.

The carbon isotope ratio method also has some drawbacks. Nectars bearing flowering plants are almost exclusively C-3 whereas cane and corn are C-4 plants. Beet belongs to C-3 plants and hence beet invert will be difficult to detect using the stable carbon isotope ratio as the basis. In studies of authentication of maple syrup, a deuterium to hydrogen ratio was used to differentiate between compounds from two different plants with same photosynthetic pathway (Martin, Martin, Naulet, & McManus, 1996). Deuterium to hydrogen ratio for sugars vary among each individual C-3 plants. This can be a basis for discriminating between honey which is from a C-3 floral origin plant and beet invert, also a sugar from the C-3 plant.

Another quality issue in the honey industry is the authentication of honey based on its floral origin. Many researchers have used pollen analysis to distinguish honey types based on its floral origin (Singhal et al., 1997). Methods that use the flavanoids for floral identification of honey exists. The flavonoid pattern is believed to be more useful in determination of geographical origin than the botanical origin (Singhal et al., 1997). Gel filtration, ion exchange chromatography, and starch gel electrophoresis of proteins in honey can also differentiate floral honey and honey obtained from sugar-fed bees (White & Kushnir, 1967). However, the methods are not rapid and are expensive.

Recent attempts have been made to use Fourier transform infrared (FTIR) spectroscopy to detect adulteration in honey (Sivakesava & Irudayaraj, 2001). Raman spectroscopy is another most promising branch of vibrational spectroscopy and Fourier transform (FT) Raman spectroscopy is one of the fastest growing areas in analytical chemistry today. Since 1986, Raman spectroscopy using near infrared excitation has emerged in the Fourier domain, providing an exciting new pathway for material characterization. FT-Raman is based on the scattering of light from near infrared radiation due to the vibrational energy of the molecules in the sample. In the case of honey adulteration as discussed earlier, sugars of different origin differ in their stable carbon ratio as well as in deuterium to hydrogen ratio. Natural absorption of energy will be different for different isotopes, which can contribute to the distinct classification of adulterants. General advantages of Raman spectroscopy over FTIR are the non-interference of Raman measurement with the water present in the sample, ease of sampling and measurement, and minimal fluorescence interference (Ozaki, 1999). FT-Raman has been used for quantitative analysis of vitamin A (Hancewicz & Petty, 1995) and some compounds of pharmaceutical interest (Cutmore & Skett, 1993) and structure elucidation (Goral & Zichy 1990; Ozaki, Cho, Ikegaya, Muraishi, & Kawauchi, 1992). An FT-Raman approach for the authentication of edible oils (Aparicio & Baeten 1998; Marigheto, Kemsley, Defernez, & Wilson, 1998) and detection of virgin oil adulteration (Baeten, Meurens, Morales, & Apricio, 1996; LiChan, 1994) has been demonstrated.

FT-Raman spectroscopic methods are simple, rapid, cost effective, and non-destructive, and hence can be a method of choice for the detection of adulteration or for routine analysis. It is necessary to establish proper calibration and validation procedures with data acquisition protocols for FT-Raman methods. To extract information from the complex spectra containing overlapping absorption peaks, interference effects, and instrumental artifacts, multivariate analysis is often used.

Most commonly used multivariate statistical methods are partial least squares (PLS) and principal component regression (PCR). Data compression, calibration, and validation are the basis of these methods (Beebe, Pell, & Seasholtz, 1998). Other multivariate procedures commonly used for the classification of objects into groups or clusters based on a statistical measure are discriminant analysis: linear discriminant analysis (LDA) and canonical variate analysis (CVA). For a successful application of these methods certain factors such as proper choice of spectral range, stability of the spectra, and the number of variables employed in the calibration model should be given consideration. Detection of honey adulteration with an FT-Raman approach has not been attempted.

This study uses an FT-Raman approach to predict the degree of adulteration and the type of adulterant in honey. Differentiating similar sugars from different plant sources by traditional methods such as HPLC is not possible. The present research uses an FT-Raman approach to detect beet and cane invert sugar in honey. The main objectives of this study were to investigate the potential of FT-Raman spectroscopy (1) to characterize

the honey from three different floral sources, (2) to quantify the beet invert and cane invert adulterants and classify based on their levels for quality grading purposes, and (3) to develop the calibration and validation models for classification based on the type of adulterants.

2. Materials and methods

2.1. Samples

Pure honey samples of Clover, Orange and buckwheat (floral origin) were obtained from Sioux Honey Association, Sioux City, IA and adulterated with different quantities of medium invert beet and cane syrup. Liquid beet and cane invert sugar samples were obtained from Imperial Sugar Company (Sugar Land, TX). A set of 47 adulterated samples in the range between 2 and 25% (w/w) with increments of 0.5% were prepared for each adulterant. These ranges were chosen to demonstrate the adulteration detection limit in honey adulteration studies. Twelve of these were used for validation, the remaining were used for calibration. Samples were mixed well and kept at room temperature to equilibrate before FT-Raman measurements.

2.2. FT-Raman measurements

FT-Raman spectra were obtained using a Nicolet 870 spectrometer with the Nicolet Raman module 32B (Madison, WI, USA) and HeNe laser operating at 1064 nm with a maximum power of 2 W. The system was equipped with an InGaAs (Indium-Gallium Arsenide) detector, XT-KBr beam-splitter with 180° reflective optics, and a fully motorized sample position adjustment feature. A laser output power of 2 W was used, which was low enough to prevent possible laser induced sample damage and a high signal to noise ratio. Data were collected at 16 cm^{-1} resolution with 256 scans. Spectra were obtained in the Raman shift range between 200 and 4000 cm^{-1}. The system was operated using the OMNIC 5.1 software and the experiments were replicated three times.

2.3. Chemometrics

PLS and PCR algorithms were used for quantitative analysis. Discriminant analysis (LDA and CVA) was used to detect the presence of adulterants and to differentiate between the two different types of adulterants in honey.

2.3.1. Quantitative analysis

For quantitative analysis, the PLS (Haaland & Thomas, 1988) and PCR methods (Martens & Naes, 1988) from Grams 32 software package was used (Galactic Industries Corporation, Salem, NH). Original and 1st derivative transformed spectra were used for calibration models and the optimum number of calibration factors was selected based on predicted residual sum of squares (PRESS), which should be minimized, along with the R^2 values from regression. The predictability of the models was tested by computing the standard error of calibration (SEC) for the calibration data set and standard error of prediction (SEP) for validation data set. Cross validation was used to estimate the performance of the models developed (Beebe et al., 1998).

2.3.2. Discriminant analysis

The Win-DAS (Wiley, Chichester: United Kingdom) software package was used for discriminant analysis. Area normalization of spectroscopic data was performed to compensate for gross differences in the spectral response caused by the physical effects, such as instrumental artifacts. Based on the quantity of invert sugar adulterants added adulterated honey samples were classified into three groups of 2.0–8.0, 8.5–18.0, and 18.5–25.0% respectively. Such classification will be useful in assigning grades based on quality. LDA, and CVA were the two methods of discriminant analysis used for the purpose of multiple group classification. Multi-dimensional data, in which the number of variates was larger than the number of observations, cannot be used directly in the above methods. Hence, data compression methods such as PCA and PLS were employed to transform the data set comprising of a large number of inter-correlated variates (wave numbers) into a reduced new set of variates and then CVA was applied. This process was respectively denoted as PCA-CVA and PLS-CVA in the text. The purpose of using discriminant analysis was to classify based on the concentration of adulterants.

3. Results and discussion

3.1. Objective 1: characterization of honey using FT-Raman spectra

FT-Raman spectra of pure clover, buckwheat, and orange honey are shown in Fig. 1. Honey samples show a majority of the spectral peaks in the 300–1500 cm^{-1} region. Whereas two more peaks were observed at 2945 and 3384 cm^{-1} in the spectra. In the region between 300 and 1500 cm^{-1}, peaks were observed at 353, 423, 518, 630, 704, 775, 824, 866, 915, 981, 1072, 1126, 1267, 1374, and 1461 cm^{-1}. Matching peaks obtained for honey with those observed by Twardowski and Anzenbacher (1994) with the tentative bonds assignment corresponding to the fundamental group are given in Table 1.

As the carbohydrate composition varies to a greater scale among the different types of honey compared to amino acids and organic acids, the analysis of the region representing carbohydrates may not be sufficient to characterize honey samples. Hence, selecting a region that shows a combination of absorption due to different compounds such as carbohydrates, proteins or amino acids, and organic acids for analysis is necessary. The spectral region between 200 and 1600 cm^{-1} was found to be of prime interest because it represents the vibrational modes of different bonds from carbohydrates, proteins, and organic acids. As shown in Fig. 1, the peaks observed at 353 cm^{-1} was minor, whereas the peak at 423 cm^{-1} was sharp and distinct. Both these peaks may be due to unknown bond vibrations from carbohydrates and proteins (amino acid) in honey. The major and sharp peaks at 518 and 630 cm^{-1} and minor peaks at 704 and 775 cm^{-1} were attributed to unknown bond vibrations of carbohydrates (Twardowski & Anzenbacher, 1994). The peak at 824 cm^{-1} was of

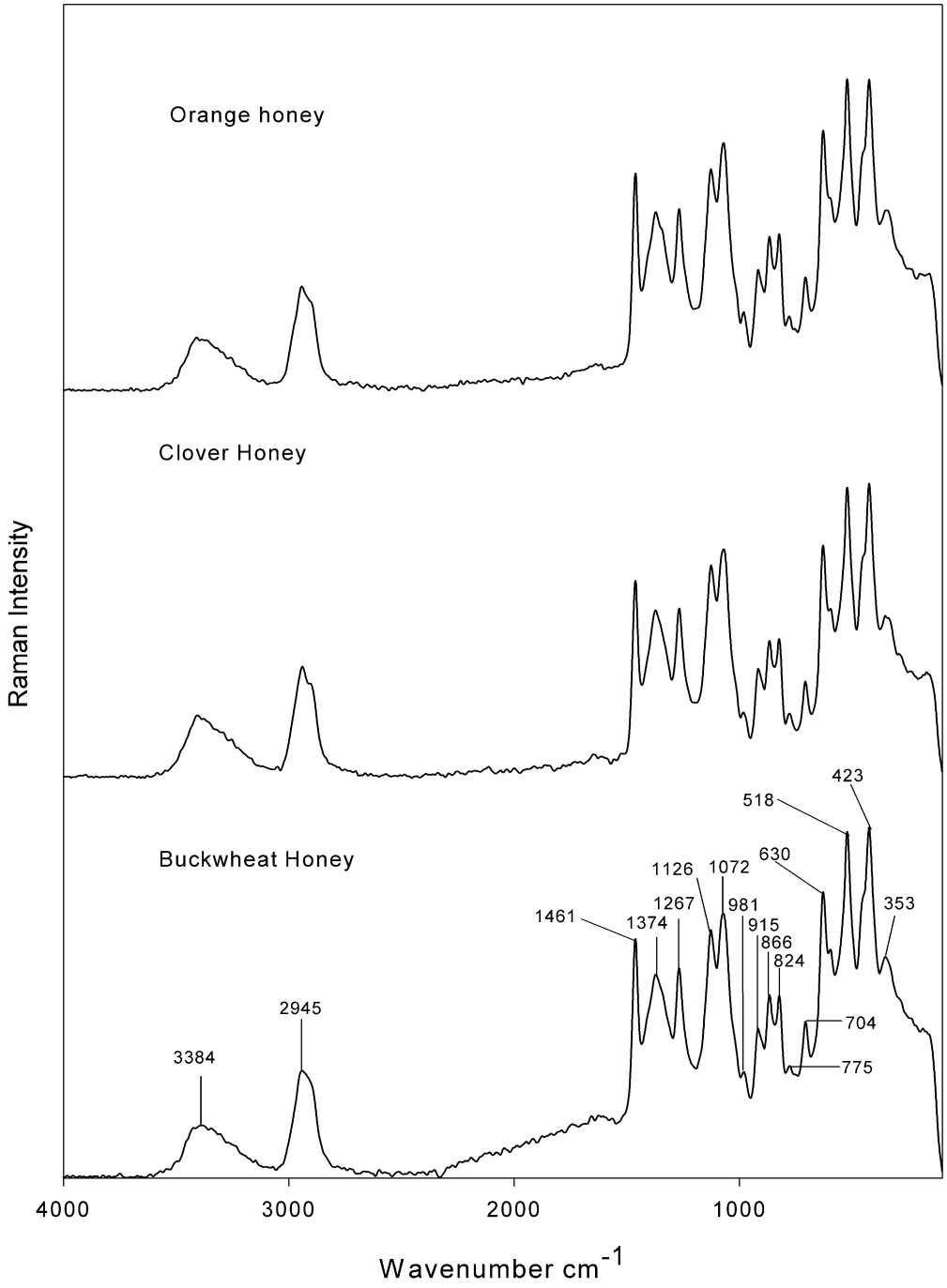

Fig. 1. FT-Raman spectra of pure honey from different floral sources.

moderate intensity and may be due to unknown vibrations. A moderate peak at 866 cm^{-1} was found to be due to the vibration of C (1)-H (i.e. bond at first carbon of sugars) of carbohydrates, whereas the minor peak at 915 cm^{-1} was attributed to the combination of the bending vibration of C (1)-H and COH and the peak at 981 cm^{-1} was due to an unknown vibration.

A strong peak at 1072 cm^{-1} could arise due to a major contribution by the bending vibration of C(1)-H and COH in carbohydrates and a minor contribution (due to low concentration in honey) due to the vibration of C–N bond in proteins and amino acids. Whereas a combined effect of vibration of an unknown bond of carbohydrates (major) and C–N bond of protein and amino acids (minor) may result in another strong peak at 1126 cm^{-1}. A strong and sharp peak at 1267 cm^{-1} was reported for the vibration of C (6)-OH and C (1)-OH of carbohydrate with minor contribution form amide III vibration of the peptide bond (Twardowski & Anzenbacher, 1994). Bending of C–H and O–H bonds resulted in a moderate and broad peak at 1374 cm^{-1}. The combination vibration of CH_2 group (bending) and minor contribution from the COO^- group may be exhibiting a strong and sharp peak at 1461 cm^{-1}. This COO^- group may be due to amino acids or organic acids in honey. Other moderate and broad peaks at 2945 and 3384 cm^{-1} are known to be due to stretching vibrations of CH and OH groups (Twardowski & Anzenbacher, 1994). Similarly, FT-Raman spectra for pure cane and beet invert syrups are shown in Fig. 2.

Table 1
Fuctional groups and vibrational modes obtained for the FT-Raman spectra of pure honey

Band position in Raman spectra, cm^{-1}	Assignment of bonds	Mode of vibration
353	Unknown carbohydrate and protein	–
423	Unknown carbohydrate and protein	–
518	Unknown carbohydrate	–
630	Unknown carbohydrate	–
704	Unknown carbohydrate	–
775	Unknown carbohydrate	–
824	Unknown	–
866	C(1)H	–
915	C(1)H and COH	Bending
981	Unknown	–
1072	C(1)H and COH	Bending
	C-N (protein or amino acids)	–
1126	Unknown carbohydrate	–
	C-N (protein or amino acids)	–
1267	C(6)OH and C(1)OH	–
	Amide III (peptide bond)	–
1374	CH and OH	Bending
1461	CH_2	Bending
	COO^-	–
2945	CH	Stretching
3384	OH	Stretching

3.2. Objective 2: quantification of adulterant and classification for quality grading using discriminant analysis

For predicting the quantity of adulterant in honey samples (i.e. quantitative analysis) calibration and validation models were developed. PLS was found to be superior to PCR analysis and hence used for quantitative studies. Table 2 lists the results of statistical analysis. For clover honey, the calibration and validation models for the prediction of adulterants such as beet and cane inverts had an $R^2 > 0.94$ and SEC < 2.52 and SEP < 2.20. Similar trends were observed for buckwheat and orange honey. For buckwheat honey, R^2 value for the model was more than 0.94 for beet as well as for cane invert adulteration prediction models (SEC was less than 2.32). An R^2 value of 0.93 and 0.91 was obtained for the cane and beet invert adulterated orange honey, respectively. In general, for the floral types analyzed, the "beet invert adulteration model" shows a slightly higher correlation compared with the "cane invert adulteration model". The number of factors for the "beet invert adulteration model" was found to be 4 whereas for the "cane invert adulteration model" it was 3. The predictive models had an acceptable accuracy with low errors and number of factors.

Predicting or classifying honey based on adulterant concentration will be a useful tool in overall quality assessment. Sometimes, when it is not essential to know the exact concentration of adulterants in the product, discriminating between different concentration ranges or groups might be important in overall quality assessment. This was demonstrated by classifying honey with adulterant concentration ranges between 2.0 and 8.0%, 8.5–18.0%, and 18.5–25.0% in the honey types considered. Each floral type of honey was studied separately with each of the adulterant (i.e. each honey with one adulterant at a time) and the model results are given in Table 3. The spectral range between 200 and 1600 cm^{-1} as used for discriminant analysis. CVA analysis of

Table 2
Quanitative analysis using PLS for predicting adulterant concentration

	Factors	R^2	SEC	R^2	SEP
Clover honey					
Beet invert	4	0.945	2.277	0.943	1.950
Cane invert	3	0.936	2.521	0.933	2.195
Buckwheat honey					
Beet invert	4	0.950	2.227	0.917	1.574
Cane invert	3	0.941	2.315	0.952	2.059
Orange honey					
Beet invert	4	0.935	2.348	0.928	2.151
Cane invert	3	0.914	2.765	0.909	2.014

PCA compressed data (CVA-PCA) was found to be superior to other methods. Hence, CVA-PCA was used for the discriminant analysis of honey into arbitrary grades based on the amount of adulterants. For clover honey, the classification accuracy was greater than 99 and 96% for calibration and validation models, respectively (for both beet and cane invert adulteration models). The classification accuracy for buckwheat honey was about 96 and 100% for the calibration and validation data sets, respectively. Whereas in the case of orange honey, the classification accuracy was greater than 96% for both calibration and validation models for the two adulterants studied. The number of factors for the "beet invert adulteration model" for all honey samples as in the range of 2–4 whereas those for the "cane invert adulteration model" were between 4 and 5; thus demonstrating the validity of the models to classify adulterated samples accurately into arbitrary groups.

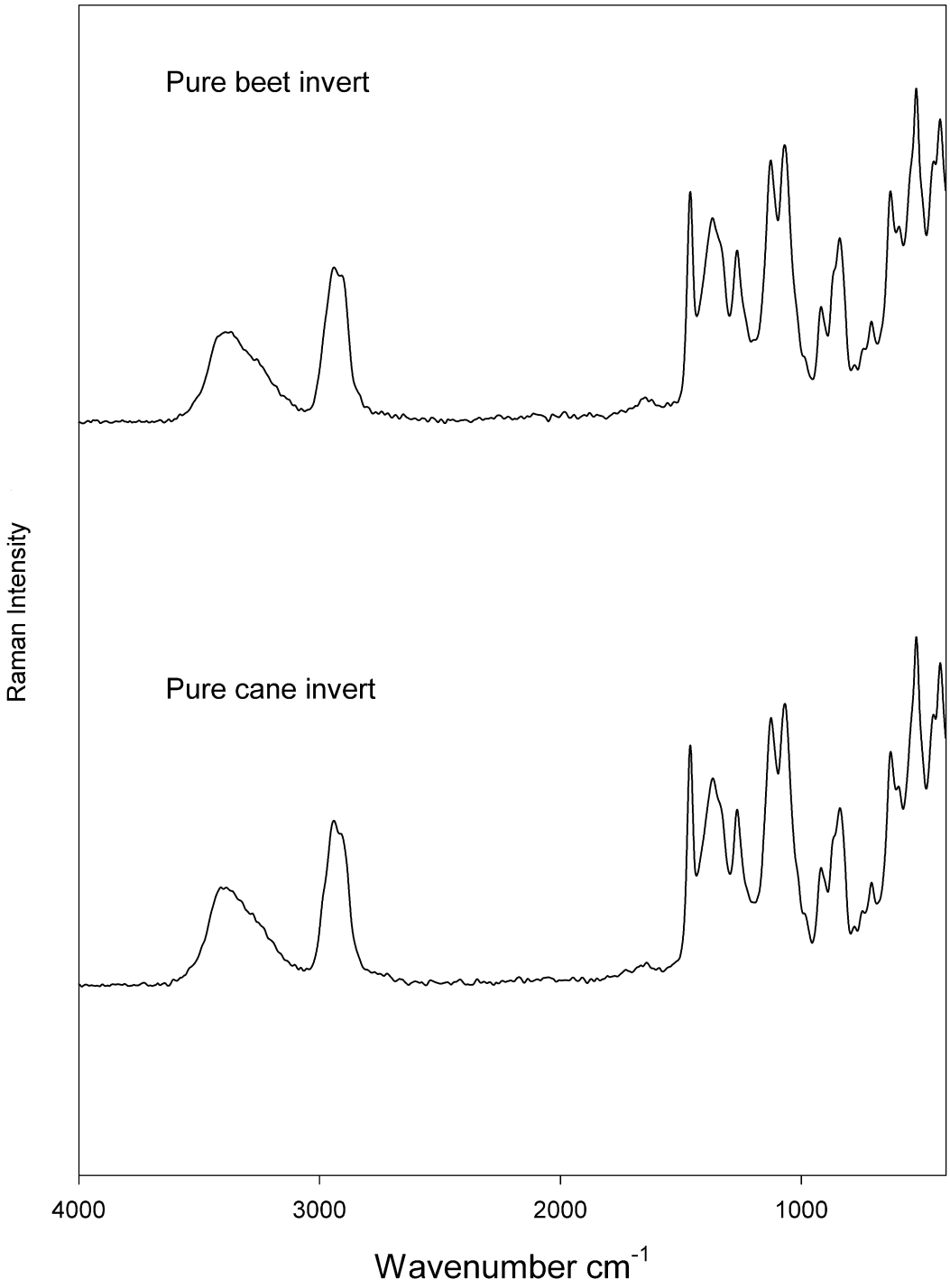

Fig. 2. FT-Raman spectra of pure cane and beet invert syrups.

Table 3
Discrimination analysis of honey using PCA compressed data in CVA: for classification based on quantity of adulteration

	Factors	% Correct discrimination of calibration samples	% Correct discrimination of validation samples
Clover honey			
Beet invert	2	98.57	100.00
Cane invert	4	98.57	95.83
Buckwheat honey	4	95.71	100.00
Beet invert	4	98.57	100.00
Cane invert			
Orange honey			
Beet invert	4	95.65	100.00
Cane invert	5	95.71	95.83

Such classification can be applied in quality grading of honey.

3.3. Objective 3: Classification of beet and cane invert in three-selected honey varieties

Two different analysis were conducted. In the first study, samples of adulterated honey from all floral sources were considered together for analysis based on the type of adulterant (i.e. beet invert or cane invert). In the second study, honey from each floral type was taken separately with its adulterants (beet and cane invert) and the base calibration and validation models were developed.

In the combined discriminant analysis study to classify beet and cane invert in honey, irrespective of floral

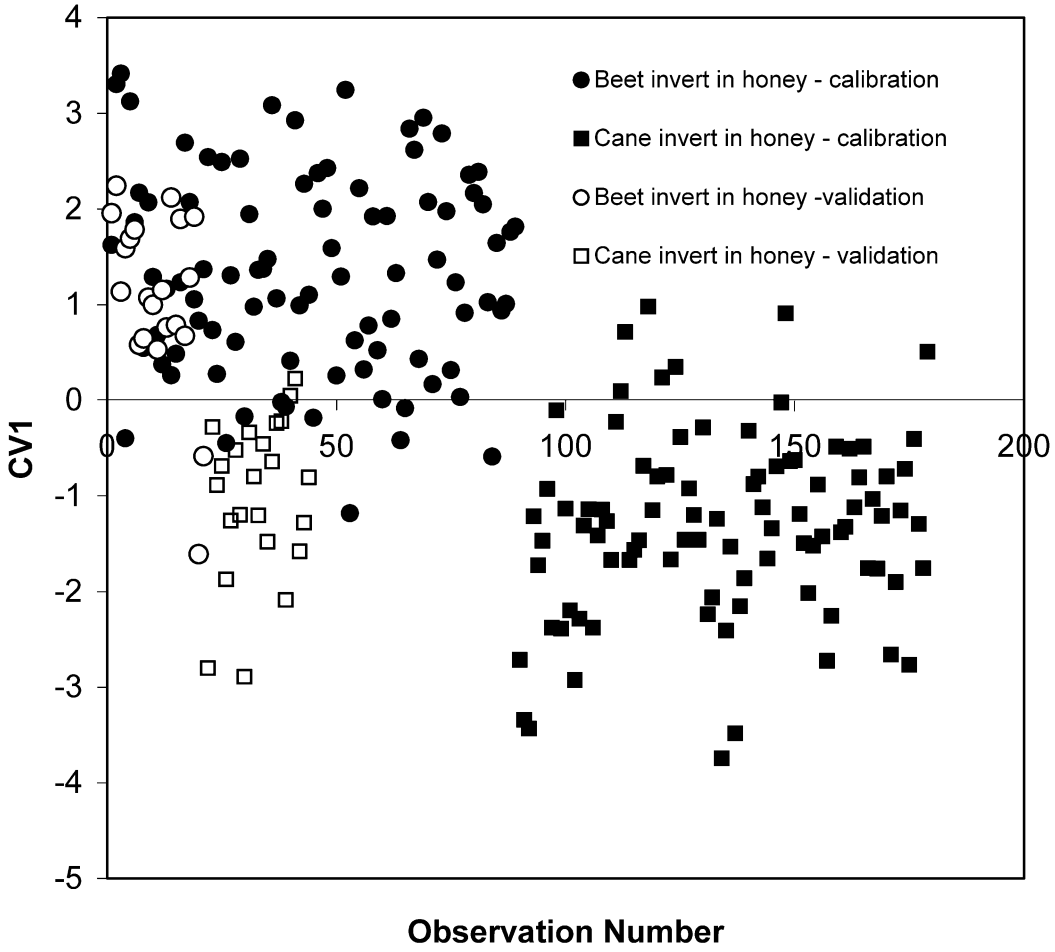

Fig. 3. Combined model to discriminate honey based on the type of adulterant.

Table 4
Discrimination of honey with different adulterants for classification based on the type of adulterant in each floral type (PCA compressed data was used in CVA)

	Factors	% Correct discrimination of calibration samples	% Correct discrimination of validation samples
Clover honey	6	96.43	95.83
Buckwheat honey	5	95.71	97.92
Orange honey	5	96.40	97.87

types, PLS compressed data with CVA (PLS-CVA) using the spectra in the range of 200–1600 cm^{-1} gave better prediction and hence used in analysis. Here, beet invert adulterated honey samples of clover, buckwheat, and orange were used as one group, and the cane invert adulterated honey samples from the same floral origins were used as the second group. Fig. 3 presents, a plot of CV1 with respect to observation numbers for the combined discrimination analysis with samples of different floral groups of honey separating together based on the type of adulterant in honey (i.e. beet invert adulterated honey as classified as one group and cane invert adulterated honey samples as the other). Discriminant analysis by PLS-CVA shows a classification accuracy of about 90% for calibration data set and 91% for the validation data set with a factor of 10. In this study, a discrimination model for the discrimination of honey adulterants was developed irrespective of its floral sources. Such a model can be used to predict adulteration in honey when the floral source is not known. The accuracy can be improved if separate adulterant classification models could be developed for honey from each honey variety.

The second study, which examines the development of discriminant models for honey from each floral source in the spectral range 200–1600 cm^{-1} was accomplished using the PCA-CVA method (Table 4). The percentage of correct classification of honey samples from all the floral sources was about 96% for both calibration and validation data sets. The number of factors in the discriminant model were between 5 and 6. Development of models for each of the honey varieties from different floral sources will help to quantify beet or cane invert adulteration in honey of known and mono-floral source. Here we can directly use the respective model developed for specific floral source.

The differences observed by the FT-Raman spectroscopy between honey and invert syrup adulterants may be due to the stable carbon (^{13}C–^{12}C) and/or hydrogen (deuterium to hydrogen) ratios, oligosaccharides, amino and organic acids acid as explained earlier. The most important contributor might be the differences in the stable isotope ratios. Since Raman spectroscopic measurements are based on the vibrational energy of the molecules that constitute the sample, natural absorption of energy is expected to be different for different isotopes and would contribute to the classification of adulterants (Twardowski & Anzenbacher, 1994).

4. Conclusion

FT-Raman spectroscopy was successfully applied to detect invert syrup adulteration in three different floral types of honey. The most promising finding is that FT-Raman method can also be used to discriminate between the type of adulterants, such as beet and cane inverts in honey, irrespective of its floral origin. Prediction can be improved if standard calibration and validation models are developed for each floral type of honey. Developing separate models for all the commercially available mono-floral varieties of honey can prove to be of great use in the future in establishing their authenticity whereas a combined model can be used to detect cane and beet inverts in honey of unknown or mixed floral source. For complete study, several varieties of honey from key geographical areas must be analyzed and characterized.

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