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Title: Rapid quantification of honey adulteration by visible-near infrared spectroscopy combined with chemometrics

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Abstract: Honey is a pure product for which the addition of any other substance is prohibited by international regulations. Therefore, it is necessary to develop reliable analytical methods to guarantee its authenticity. Visible-near infrared spectroscopy (Vis-NIRS) combined with chemometric tools, like hierarchical cluster analysis (HCA), principal component analysis (PCA), linear discriminant analysis (LDA), has been used for the discrimination of honey adulterated with high fructose corn syrup (HFCS). Different honey samples from the Granada Protected Designation of Origin (Spain) were adulterated with HFCS at different percentages (10 - 90%). LDA was able to discriminate 100% of the samples. Partial least squares regression (PLS) was used to predict the level of adulteration. The best prediction model used 10 factors with a high coefficient of determination near 1. The developed method showed high precision (coefficient of variation below 4%). Vis-NIRS combined with chemometrics can be used for the rapid and non-destructive detection of honey adulteration. The obtained results demonstrate that the application of this technique as a screening method could be a useful tool for quality monitoring analysis in routine laboratories.

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Talanta
Editorial Office

Please find attached a revised version of our manuscript entitled **"Rapid quantification of honey adulteration by visible-near infrared spectroscopy combined with chemometrics**" by Marta Ferreiro-González, Estrella Espada-Bellido, Lucía Guillén-Cueto, Miguel Palma, Carmelo G. Barroso and Gerardo F. Barbero, which we would like to resubmit for publication in Talanta.

Reviewer´s comments have been taken into account. In the following pages are our point-by-point responses to each of the comments and suggestions. Revisions in the text are shown using yellow highlight for modifications. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript reconsidered and finally suitable for publication in Talanta. We shall look forward to hearing from you at your earliest convenience.

Kindest regards,

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List of responses to the comments of the Editor and reviewer, item by item:

Reviewer´s comments:

The following quality measures for PLS regression are usually reported and should be included in the article:

**The root-mean-square error of calibration (RMSEC), which is a measure of how well the model fits the calibration data.*

**The root-mean-square error of cross-validation (RMSECV), which is a measure of a model's ability to predict samples that were not used to build the model;*

**The root-mean-square error of prediction (RMSEP), which is a measure if the model is applied to new data.*

These values have been included and discussed in the manuscript.

Remarque: page 8 line 36-38: Figure 2S shows R2, in text is written correlation coefficient. This should be adapted by the square of the correlation coefficient … or coefficient of determination.

As reviewer suggests, correlation coefficient has been replaced by coefficient of determination.

Abstract, page 2 line 2: Honey is a pure product for which the addition of any other substance prohibited by international regulations replace by: ... is prohibited ...

This comment has been taken into account.

Page 2, line 6: replace hierchical by hierarchical

This comment has been taken into account.

Page 5 line 18: All of the samples …. Replace by All the samples …

This comment has been taken into account.

Page 6, line 18: A smoothing of the data was carried in order …., replace by A smoothing of the data was carried out in order …

This comment has been taken into account.

Which smoothing algorithm was used ? reference ?

This information has been included in the manuscript.

Page 7 line 2: … an exploratory chemometric tool (hierarchical cluster analysis (HCA) was first employed. Replace by … an exploratory chemometric tool hierarchical cluster analysis (HCA) was first employed

This change has been taken into consideration.

Page 7, line 24: the number of principal components (PCs) with eigenvalues greater than 1 was extracted. Replace by : …. were extracted

This comment has been taken into account.

Highlights

- Honey is a pure product and so the addition of any other substance is forbidden.
- High quality honey samples from the Granada P.D.O. (Spain) were used.
- Rapid discrimination of HFCS adulterated honey using Vis-NIRS was achieved.
- PLS combined with Vis-NIRS allows the quantification of the level of adulteration.
- Fast, cheap, non-destructive and easy to use for routine quality monitoring analysis.

Rapid quantification of honey adulteration by visible-near infrared spectroscopy combined with chemometrics

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Abstract

Honey is a pure product for which the addition of any other substance is prohibited by international regulations. Therefore, it is necessary to develop reliable analytical methods to guarantee its authenticity. Visible-near infrared spectroscopy (Vis-NIRS) combined with chemometric tools, like hierarchical cluster analysis (HCA), principal component analysis (PCA), linear discriminant analysis (LDA), has been used for the discrimination of honey adulterated with high fructose corn syrup (HFCS). Different honey samples from the Granada Protected Designation of Origin (Spain) were adulterated with HFCS at different percentages (10 – 90%). LDA was able to discriminate 100% of the samples. Partial least squares regression (PLS) was used to predict the level of adulteration. The best prediction model used 10 factors with a high coefficient of determination near 1. The developed method showed high precision (coefficient of variation below 4%). Vis-NIRS combined with chemometrics can be used for the rapid and non-destructive detection of honey adulteration. The obtained results demonstrate that the application of this technique as a screening method could be a useful tool for quality monitoring analysis in routine laboratories.

Keywords

Adulteration; Authenticity; Chemometrics; Honey; Partial least squares regression; Visible-near infrared spectroscopy

Introduction

Food adulteration for economic gain is becoming a major issue in many countries as a consequence of growing global trade. Numerous food adulteration scandals have occurred around the world leading to international impacts [1–3]. This food fraud is not only an illegal activity but can also cause health problems in consumers. For this reason the authenticity of food is regulated by national and international legislation such as the Codex Alimentarius standards. Nowadays, honey, after olive oil [4] and milk [5], is one of the most likely food products to be a target for adulteration [6,7]. Honey is a natural sweetening agent produced by bees (*Apis mellifera*) from flower nectar and this product has been used since ancient times due to its nutritional value and health benefits. Honey is a rich source of readily available sugars (predominantly fructose and glucose) as well as many other substances such as organic acids, proteins, vitamins, enzymes, biologically active compounds, and trace minerals as minor elements [8–10]. The quality of honey is determined according to its botanical and geographical origin [11]. Regarding European Union regulations (Codex Alimentarius Comission and Council Directive 2001/110/EC of 20 December 2001 relating to honey) honey is considered to be a pure product and so the addition or removal of any other substance is prohibited.

The health benefits and the pleasant sweet taste resulting from the natural honey composition are the reasons for the high cost of this product when compared to other commonly used sweeteners and this makes it prone to falsification or adulteration [12]. The most frequent method for the adulteration of honey involves the additon of inexpesive and artifitial sweeteners, such as sugar, inverted beet syrup and maltose syrup, or fructose corn syrup [12–14]. High fructose corn syrup (HFCS) is one of the most common adulterants due to its low price and the similarity of its composition to honey [13,15].

The diversity of honey on the market and the increase in demand have made it necessary to develop reliable analytical methods to establish criteria to guarantee the authenticity of the honey [6,10]. Both consumers and producers have an interest in the proper labelling of the origin and traceability to guarantee the authenticity of the honey.

Honey from Granada (Southern Spain) was recognized as a Protected Denomination of Origin (P.D.O.) by a National regulation in 2002 (*Orden APA/3209/2002 03/12/2002 Denominación de Origen Protegida Miel de Granada)* and by the European Union in June 2005 [16] and this covers eight different types of honey. Around 70% of the bee farms in Granada are established in protected areas that confer unique characteristics and thus add special value to the honey produced there [17].

To date, several techniques have been applied to authenticate honey with HFCS and these include the C-isotope approach [18,19], gas chromatography (GC) [20], high performance liquid chromatography (HPLC) [7], and nuclear magnetic resonance (NMR) [21]. Although some of these methods have proven to be useful for the detection of adulterants in honey, they also suffer from some drawbacks since these techniques are expensive, time consuming, destructive, and they require a skilled operator. Therefore, these methods cannot be easily implemented in routine food laboratories where screening techniques for rapid analysis are more suitable.

For this reason, spectroscopic techniques like visible spectroscopy (Vis), near infrared spectroscopy (NIRS), infrared spectroscopy (IR) or Raman, combined with suitable chemometric tools have increased in importance in food quality monitoring and these can be considered as a good alternative to the more commonly used methods for food authentication [22–24]. Furthermore, by using a non-destructive technique that preserves the integrity of the sample it is possible to re-analyze if necessary with another technique. However, very few studies have focused on the identification of HFCS in honey by using spectroscopic techniques such as FT-IR [25], Raman [15] or NIRS [13,26,27]

In addition, most of the studies have focused on the identification of bands due to individual compounds, i.e. due to small number of markers. In this sense, some authors have applied NIRS in combination with aquaphotomics to describe water molecular structures in honey adulterated with HFCS [26]. However, as honey is a very complex matrix, the identification of adulterants by using only a small number of markers can be difficult and time consuming. Besides, the differences especially when detecting adulteration are usually quite subtle, so minor differences can be also important in these cases. In fact, subtle differences can be crucial to detect the adulterant. Spectra data can be used as a satisfactory fingerprint of the sample [28,29]. A method for detecting honey adulteration based on the use of not only a few markers but a fingerprint will be more difficult to elude. For this reason, the use of the whole spectrum information in combination with chemometrics can be applied as a fast screening approach for the detection and quantification of adulteration in honey samples. This approach can be a good alternative to traditional procedures which are based on the identification of single signals of independent markers. The use of chemometric tools such as HCA (hierarchical cluster analysis), LDA (linear discriminant analysis) or even PLS (partial least squares regression) to develop a predictive model can be very attractive [6,10]. To the best of our knowledge, there are only a few studies based on the detection of honey adulteration with sugar syrups using NIRS and chemometrics [10,30,31]. A recent study focused on the qualitative and quantitative detection of honey adulterated with HFCS and maltose syrup (MS) used NIRS and PLS. It was concluded that the predictive ability was satisfactory for MS-adulterated honey samples, but not for

HFCS-adulterated honeys [13]. As a consequence, NIRS requires further study to demonstrate its potential for the detection of such adulterants in honey [10].

In the present work, Vis-NIRS combined with multivariate analysis such as HCA, PCA, LDA, and PLS has been studied in detail in order to evaluate the capacity of this technique as a rapid screening method to detect the percentage of adulteration with HFCS from 10% to 90% in high quality honey samples from the Granada P.D.O.

Materials and methods

Samples

A total of 33 pure multi-floral honey samples (n = 33) from different providers were supplied directly from the Regulation Council of Granada Protected Designation of Origin (P.D.O.) (Lanjarón, Granada, Spain). All the samples were from the harvest of 2016. The samples were stored in plastic bottles in darkness at room temperature prior to analysis.

Adulterant

High fructose corn syrup (HFCS), specifically 81% dry solid fructose corn syrup with 8.5% fructose, was used as the adulterant (Cargill S.L.U., Martorell, Barcelona, Spain). HFCS was stored in darkness at room temperature prior to analysis.

Honey adulteration

Multi-floral honey, which is the most common type of honey, was selected for adulteration. Mixtures were prepared using the 33 pure P.D.O. honey samples and HFCS. Firstly, a mixture that consisted of all pure honey samples (22 g of each sample) was prepared in order to cover the greatest heterogeneity. Pure model mixtures were then prepared by adding HFCS to honey samples at ratios of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% by weight. Honey samples free of adulteration (0%) and pure HFCS syrup samples were also prepared (100%). All adulterated samples were carried out in duplicate, so a final set of 22 samples was used in this study. In addition, pure P.D.O adulterated samples for external calibration were prepared (in duplicate) at different adulteration rates not included in the above list, i.e., 5%, 15%, 25%, and 45%. After adding the HFCS all samples were kept in an oven at 30 °C for at least 24 h and manually stirred to ensure homogeneity prior to analysis.

Spectra collection

Spectra were recorded on a FOSS XDS Rapid Content™ Analyzer with XDS near infrared technology (FOSS Analytical, Hilleroed, Denmark). All samples were analyzed in the range of 400 nm to 2500 nm with a spectral resolution of 0.5 nm, which means that the spectra contain data from the Vis-NIR regions.

The aquisition of the spectra was performed with ISIscanTM Routine Analysis Software (Foss, Denmark). All samples were analyzed in duplicate and the average spectrum for each sample was used for the multivariate analyses.

Data analysis

A moving average smoothing of the data was carried out in order to remove random variation from the data set . Recorded spectra were finally arranged in a D_{mxn} data matrix where *m* is the number of absorbance values (m = 247) and *n* the number of honey samples (n = 22).

Multivariate analysis of the data included the use of non-supervised or exploratory techniques, such as hierarchical cluster analysis (HCA) and principal component analysis (PCA), and supervised techniques, such as linear discriminant analysis (LDA). The statistical computer package IBM SPSS Statistics 22 (Armonk, NY, USA) was used for all chemometric analyses. A multivariate regression model based on partial least squares (PLS) was also applied using the software Statgraphics Centurion XVI (Warrenton, VA, USA). The optimum number of factors for the calibration was selected on the basis of the predicted residual sum of square (PRESS), which should be minimized. The model prediction capability was tested by checking both the root-mean-square error of calibration (RMSEC) and the root-mean-square error of prediction (RMSEP).

Results and discussion

Exploratory chemometric study

The average spectra (raw data) obtained for all the samples adulterated with HFCS at each level of adulteration are presented in Fig. 1. Non–adulterated honey samples (0%) and syrup samples (100%) were also represented. Visual inspection of the spectra shows some areas in the ranges 400–600 nm and 1500–2000 nm that have differences in the intensity of the absorbance regarding the percentage of adulteration. However, these differences are not clear enough to neither draw any firm conclusion nor quantify the level of adulteration. As a consequence, the application of suitable chemometric tools that allow the extraction of useful information contained in the spectral range, which is related to the level of the adulteration, is of great interest in this field.

Besides, the application of these chemometric tools provides automatic and objective data interpretation that allows the rapid identification of the adulterant.

In order to study the trends of the honey samples to be grouped according to the level of adulteration by HFCS, an exploratory chemometric tool hierarchical cluster analysis (HCA) was first employed. HCA was applied to the raw spectra (400–2500 nm) of all the samples and these were arranged in a D_{247x22} data matrix. The Ward method was selected for cluster preparation and square Euclidean distance to measure distances between clusters. The results of the HCA are represented graphically in the dendrogram in Fig. 2. As can be observed, there is a tendency for the samples to be grouped according to the level of adulteration, since honey samples with adulterations equal and higher than 70% are included in one cluster (cluster A) and samples with adulterations lower than 70% are present in another group (cluster B). The results suggest some kind of classification based on the level of adulteration (high and low levels) but a full classification was not achieved. This finding indicates that the spectra contain useful data related to the adulteration of honey. For this reason, another exploratory technique was applied to the data, namely principal component analysis (PCA), which is the main linear technique for dimensionality reduction. PCA was applied to the same D_{247x22} data matrix. As an initial criterion the number of principal components (PCs) with eigenvalues greater than 1 were extracted. The score plot for all the samples according to the first three PCs is shown in Fig. 3. It can be seen that PC1 accounts for 57.09% of the variance of the Vis-NIR data and that the first three factors account for more than 96% of the total variance. In order to study the wavelengths that had the most influence on these three PCs, the loading values were represented (Supplementary Fig. 1S). As depicted in Fig. 3, the PC1 that seemed to be most related to the level of adulteration showed high loading values (higher than 0.7) for almost all the wavelengths, so a specific spectral region was not selected for further study. For this reason, a supervised technique such as linear discriminant analysis (LDA) was carried out.

Discriminant analysis

LDA was applied using the whole D_{247x22} data matrix to obtain a discrimination model. Eleven classes, one for each level of adulteration, were established for the LDA. A leave-one-out cross validation method was selected to obtain a robust discrimination. A stepwise discriminant analysis was applied for the identification of those specific wavelengths in the spectroscopic data that are more significant than others when classifying the honey according to the level of adulteration. The resulting discriminant functions allowed a full discrimination between the eleven groups of samples. The coefficients obtained for the resulting Fisher's linear discriminant functions

are provided in Supplementary Table 1S. The following wavelengths were selected to develop the discrimination functions: λ = 411.5 nm, λ = 444.5 nm, λ = 472.5 nm, λ = 1093.0 nm, and λ = 1462.0 nm. The wavelengths in the visible region are related to those compounds in the honey that absorbe in the blue-violet range, and so giving the characteristic orange-amber colour of the honey. As HFCS colour is pale yellow, the intensity of these wavelength decreases as the adulteration with the syrup increase. As far as NIR region is concerned, the wavelength at 1462.0 nm is related to the first overtone of the vibrational mode of O-H stretch of alcohol groups. It can be observed that signals from both regions, i.e., the Vis-NIR spectra, are needed making the method more difficult to elude. At low ratios of adulteration (≤ 20%) the coefficient of the wavelength = 444.5 nm is high and negative, and as the adulteration increases this coefficient becomes positive and greater. Similar behaviour was observed with wavelength = 1462 nm. In contrast, the coefficient of wavelength = 472.5 nm becomes increasingly negative as the adulteration becomes greater.

The distribution of the honey samples according to the first three canonical discrimination functions is represented in Fig. 4. As can be observed, high scores in the first discriminant function correspond to high levels of adulteration and *vice versa*. Scores from FC2 and FC3 are required to discriminate mainly between non–adulterated samples and 10% ratio of adulteration. These results show the potential of Vis-NIRS for the quantification of HFCS adulterant in honey samples. For this reason, a regression study was then carried out.

Regression study

Based on the results discussed above, partial least squares regression (PLS) was applied for calibration and to develop a predictive model for the ratio of adulteration in honey samples. PLS with a cross validation method was performed on the D_{247x22} data matrix. The best predictive model employed 10 factors with a high coefficient of determination of 0.9990 for the calibration set and 0.9855 for the prediction model. The RMSEC and RMSEP values were 3.05 and 4.71 respectively. These low error values indicate that the regression model is robust, and therefore reliable when applied to samples outside the model.

The results of the calibration model and cross validation obtained in the PLS are shown in Supplementary Fig. 2S. In addition, an external validation using a different set of adulterated samples at ratios not included before (5%, 15%, 25%, and 45%) was performed in order to study the predictive capacity of the developed regression method. A pure honey sample from Granada P.D.O. was also included in the prediction study. The prediction

values obtained for this set of samples are given in Table 1. These results demonstrate the accuracy of the developed model. The error was below 1% for all honey samples adulterated below 45%.

Repeatability and intermediate precision

In order to study the precision of the developed method, the honey samples adulterated at 40% were selected. The repeatability and intermediate precision were determined by analysis of this sample on three different days: nine replicates on the first day and three on the two following days. The intra-day and inter-day coefficient of variation (C.V.) for the analysis was calculated. To do so, the average of C.V. obtained in the whole body of spectra was calculated. The C.V. obtained for the repeatability was 3.90% and for intermediate precision it was 3.63%, meaning that the measurements had high precision.

Conclusions

The applicability of visible-near infrared spectroscopy combined with chemometrics for a rapid quantification of honey adulteration has been studied. The obtained results suggested that non-supervised techniques such as HCA and PCA showed a tendency for the adulterated honey samples to be grouped according to the percentage of adulteration. However, a full differentiation was not possible on using this technique. The full discrimination between the nine levels of adulteration (in the range 10–90%) was achieved by applying supervised LDA. Useful wavelengths were selected from both the visible and NIR spectroscopic regions. A high correlation coefficient was obtained in the PLS regression analysis, and the maximum error in the external prediction model was below 1% for samples adulterated below 45%. Besides the accuracy, the developed method proved to be precise (coefficient of variation lower than 4%).

The results demonstrate that the application of this screening method could be a promising tool for quality monitoring analysis in routine laboratories, since the method is cheap, non-destructive and easy to use. Furthermore, it would be interesting to study other adulterants and/or other high quality honey samples to increase the scope of this approach.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

Graphical Abstract

Fig. 1 Vis-NIR spectra (400 - 2500 nm) for all the studied samples (n = 22).

Fig. 2 Dendrogram obtained in the HCA for all the samples by Vis-NIRS (n = 22).

Fig. 3 Score plot PC1 vs PC2 Vs PC3 for all the samples (n = 22).

Fig. 4 Territorial map obtained in the LDA using Vis-NIR data for all the honey samples (n = 22).

Supplementary Fig. 1S Loading values for each variable obtained for the first three PCs in the PCA.

Supplementary Fig. 2S PLS regression model for all the honey samples (n = 22).

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Real value (%)	Prediction value
0	0.69
5	5.27
15	14.85
25	24.31
45	42.63

Rapid quantification of honey adulteration by visible-near infrared spectroscopy combined with chemometrics

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Abstract

Honey is a pure product for which the addition of any other substance is prohibited by international regulations. Therefore, it is necessary to develop reliable analytical methods to guarantee its authenticity. Visible-near infrared spectroscopy (Vis-NIRS) combined with chemometric tools, like hierarchical cluster analysis (HCA), principal component analysis (PCA), linear discriminant analysis (LDA), has been used for the discrimination of honey adulterated with high fructose corn syrup (HFCS). Different honey samples from the Granada Protected Designation of Origin (Spain) were adulterated with HFCS at different percentages (10 – 90%). LDA was able to discriminate 100% of the samples. Partial least squares regression (PLS) was used to predict the level of adulteration. The best prediction model used 10 factors with a high coefficient of determination near 1. The developed method showed high precision (coefficient of variation below 4%). Vis-NIRS combined with chemometrics can be used for the rapid and non-destructive detection of honey adulteration. The obtained results demonstrate that the application of this technique as a screening method could be a useful tool for quality monitoring analysis in routine laboratories.

Keywords

Adulteration; Authenticity; Chemometrics; Honey; Partial least squares regression; Visible-near infrared spectroscopy

Introduction

Food adulteration for economic gain is becoming a major issue in many countries as a consequence of growing global trade. Numerous food adulteration scandals have occurred around the world leading to international impacts [1–3]. This food fraud is not only an illegal activity but can also cause health problems in consumers. For this reason the authenticity of food is regulated by national and international legislation such as the Codex Alimentarius standards. Nowadays, honey, after olive oil [4] and milk [5], is one of the most likely food products to be a target for adulteration [6,7]. Honey is a natural sweetening agent produced by bees (*Apis mellifera*) from flower nectar and this product has been used since ancient times due to its nutritional value and health benefits. Honey is a rich source of readily available sugars (predominantly fructose and glucose) as well as many other substances such as organic acids, proteins, vitamins, enzymes, biologically active compounds, and trace minerals as minor elements [8–10]. The quality of honey is determined according to its botanical and geographical origin [11]. Regarding European Union regulations (Codex Alimentarius Comission and Council Directive 2001/110/EC of 20 December 2001 relating to honey) honey is considered to be a pure product and so the addition or removal of any other substance is prohibited.

The health benefits and the pleasant sweet taste resulting from the natural honey composition are the reasons for the high cost of this product when compared to other commonly used sweeteners and this makes it prone to falsification or adulteration [12]. The most frequent method for the adulteration of honey involves the additon of inexpesive and artifitial sweeteners, such as sugar, inverted beet syrup and maltose syrup, or fructose corn syrup [12–14]. High fructose corn syrup (HFCS) is one of the most common adulterants due to its low price and the similarity of its composition to honey [13,15].

The diversity of honey on the market and the increase in demand have made it necessary to develop reliable analytical methods to establish criteria to guarantee the authenticity of the honey [6,10]. Both consumers and producers have an interest in the proper labelling of the origin and traceability to guarantee the authenticity of the honey.

Honey from Granada (Southern Spain) was recognized as a Protected Denomination of Origin (P.D.O.) by a National regulation in 2002 (*Orden APA/3209/2002 03/12/2002 Denominación de Origen Protegida Miel de Granada)* and by the European Union in June 2005 [16] and this covers eight different types of honey. Around 70% of the bee farms in Granada are established in protected areas that confer unique characteristics and thus add special value to the honey produced there [17].

To date, several techniques have been applied to authenticate honey with HFCS and these include the C-isotope approach [18,19], gas chromatography (GC) [20], high performance liquid chromatography (HPLC) [7], and nuclear magnetic resonance (NMR) [21]. Although some of these methods have proven to be useful for the detection of adulterants in honey, they also suffer from some drawbacks since these techniques are expensive, time consuming, destructive, and they require a skilled operator. Therefore, these methods cannot be easily implemented in routine food laboratories where screening techniques for rapid analysis are more suitable.

For this reason, spectroscopic techniques like visible spectroscopy (Vis), near infrared spectroscopy (NIRS), infrared spectroscopy (IR) or Raman, combined with suitable chemometric tools have increased in importance in food quality monitoring and these can be considered as a good alternative to the more commonly used methods for food authentication [22–24]. Furthermore, by using a non-destructive technique that preserves the integrity of the sample it is possible to re-analyze if necessary with another technique. However, very few studies have focused on the identification of HFCS in honey by using spectroscopic techniques such as FT-IR [25], Raman [15] or NIRS [13,26,27]

In addition, most of the studies have focused on the identification of bands due to individual compounds, i.e. due to small number of markers. In this sense, some authors have applied NIRS in combination with aquaphotomics to describe water molecular structures in honey adulterated with HFCS [26]. However, as honey is a very complex matrix, the identification of adulterants by using only a small number of markers can be difficult and time consuming. Besides, the differences especially when detecting adulteration are usually quite subtle, so minor differences can be also important in these cases. In fact, subtle differences can be crucial to detect the adulterant. Spectra data can be used as a satisfactory fingerprint of the sample [28,29]. A method for detecting honey adulteration based on the use of not only a few markers but a fingerprint will be more difficult to elude. For this reason, the use of the whole spectrum information in combination with chemometrics can be applied as a fast screening approach for the detection and quantification of adulteration in honey samples. This approach can be a good alternative to traditional procedures which are based on the identification of single signals of independent markers. The use of chemometric tools such as HCA (hierarchical cluster analysis), LDA (linear discriminant analysis) or even PLS (partial least squares regression) to develop a predictive model can be very attractive [6,10]. To the best of our knowledge, there are only a few studies based on the detection of honey adulteration with sugar syrups using NIRS and chemometrics [10,30,31]. A recent study focused on the qualitative and quantitative detection of honey adulterated with HFCS and maltose syrup (MS) used NIRS and PLS. It was concluded that the predictive ability was satisfactory for MS-adulterated honey samples, but not for

HFCS-adulterated honeys [13]. As a consequence, NIRS requires further study to demonstrate its potential for the detection of such adulterants in honey [10].

In the present work, Vis-NIRS combined with multivariate analysis such as HCA, PCA, LDA, and PLS has been studied in detail in order to evaluate the capacity of this technique as a rapid screening method to detect the percentage of adulteration with HFCS from 10% to 90% in high quality honey samples from the Granada P.D.O.

Materials and methods

Samples

A total of 33 pure multi-floral honey samples (n = 33) from different providers were supplied directly from the Regulation Council of Granada Protected Designation of Origin (P.D.O.) (Lanjarón, Granada, Spain). All the samples were from the harvest of 2016. The samples were stored in plastic bottles in darkness at room temperature prior to analysis.

Adulterant

High fructose corn syrup (HFCS), specifically 81% dry solid fructose corn syrup with 8.5% fructose, was used as the adulterant (Cargill S.L.U., Martorell, Barcelona, Spain). HFCS was stored in darkness at room temperature prior to analysis.

Honey adulteration

Multi-floral honey, which is the most common type of honey, was selected for adulteration. Mixtures were prepared using the 33 pure P.D.O. honey samples and HFCS. Firstly, a mixture that consisted of all pure honey samples (22 g of each sample) was prepared in order to cover the greatest heterogeneity. Pure model mixtures were then prepared by adding HFCS to honey samples at ratios of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% by weight. Honey samples free of adulteration (0%) and pure HFCS syrup samples were also prepared (100%). All adulterated samples were carried out in duplicate, so a final set of 22 samples was used in this study. In addition, pure P.D.O adulterated samples for external calibration were prepared (in duplicate) at different adulteration rates not included in the above list, i.e., 5%, 15%, 25%, and 45%. After adding the HFCS all samples were kept in an oven at 30 °C for at least 24 h and manually stirred to ensure homogeneity prior to analysis.

Spectra collection

Spectra were recorded on a FOSS XDS Rapid Content™ Analyzer with XDS near infrared technology (FOSS Analytical, Hilleroed, Denmark). All samples were analyzed in the range of 400 nm to 2500 nm with a spectral resolution of 0.5 nm, which means that the spectra contain data from the Vis-NIR regions.

The aquisition of the spectra was performed with ISIscanTM Routine Analysis Software (Foss, Denmark). All samples were analyzed in duplicate and the average spectrum for each sample was used for the multivariate analyses.

Data analysis

A moving average smoothing of the data was carried out in order to remove random variation from the data set . Recorded spectra were finally arranged in a D_{mxn} data matrix where *m* is the number of absorbance values (m = 247) and *n* the number of honey samples (n = 22).

Multivariate analysis of the data included the use of non-supervised or exploratory techniques, such as hierarchical cluster analysis (HCA) and principal component analysis (PCA), and supervised techniques, such as linear discriminant analysis (LDA). The statistical computer package IBM SPSS Statistics 22 (Armonk, NY, USA) was used for all chemometric analyses. A multivariate regression model based on partial least squares (PLS) was also applied using the software Statgraphics Centurion XVI (Warrenton, VA, USA). The optimum number of factors for the calibration was selected on the basis of the predicted residual sum of square (PRESS), which should be minimized. The model prediction capability was tested by checking both the root-mean-square error of calibration (RMSEC) and the root-mean-square error of prediction (RMSEP).

Results and discussion

Exploratory chemometric study

The average spectra (raw data) obtained for all the samples adulterated with HFCS at each level of adulteration are presented in Fig. 1. Non–adulterated honey samples (0%) and syrup samples (100%) were also represented. Visual inspection of the spectra shows some areas in the ranges 400–600 nm and 1500–2000 nm that have differences in the intensity of the absorbance regarding the percentage of adulteration. However, these differences are not clear enough to neither draw any firm conclusion nor quantify the level of adulteration. As a consequence, the application of suitable chemometric tools that allow the extraction of useful information contained in the spectral range, which is related to the level of the adulteration, is of great interest in this field.

Besides, the application of these chemometric tools provides automatic and objective data interpretation that allows the rapid identification of the adulterant.

In order to study the trends of the honey samples to be grouped according to the level of adulteration by HFCS, an exploratory chemometric tool hierarchical cluster analysis (HCA) was first employed. HCA was applied to the raw spectra (400–2500 nm) of all the samples and these were arranged in a D_{247x22} data matrix. The Ward method was selected for cluster preparation and square Euclidean distance to measure distances between clusters. The results of the HCA are represented graphically in the dendrogram in Fig. 2. As can be observed, there is a tendency for the samples to be grouped according to the level of adulteration, since honey samples with adulterations equal and higher than 70% are included in one cluster (cluster A) and samples with adulterations lower than 70% are present in another group (cluster B). The results suggest some kind of classification based on the level of adulteration (high and low levels) but a full classification was not achieved. This finding indicates that the spectra contain useful data related to the adulteration of honey. For this reason, another exploratory technique was applied to the data, namely principal component analysis (PCA), which is the main linear technique for dimensionality reduction. PCA was applied to the same D_{247x22} data matrix. As an initial criterion the number of principal components (PCs) with eigenvalues greater than 1 were extracted. The score plot for all the samples according to the first three PCs is shown in Fig. 3. It can be seen that PC1 accounts for 57.09% of the variance of the Vis-NIR data and that the first three factors account for more than 96% of the total variance. In order to study the wavelengths that had the most influence on these three PCs, the loading values were represented (Supplementary Fig. 1S). As depicted in Fig. 3, the PC1 that seemed to be most related to the level of adulteration showed high loading values (higher than 0.7) for almost all the wavelengths, so a specific spectral region was not selected for further study. For this reason, a supervised technique such as linear discriminant analysis (LDA) was carried out.

Discriminant analysis

LDA was applied using the whole D_{247x22} data matrix to obtain a discrimination model. Eleven classes, one for each level of adulteration, were established for the LDA. A leave-one-out cross validation method was selected to obtain a robust discrimination. A stepwise discriminant analysis was applied for the identification of those specific wavelengths in the spectroscopic data that are more significant than others when classifying the honey according to the level of adulteration. The resulting discriminant functions allowed a full discrimination between the eleven groups of samples. The coefficients obtained for the resulting Fisher's linear discriminant functions

are provided in Supplementary Table 1S. The following wavelengths were selected to develop the discrimination functions: λ = 411.5 nm, λ = 444.5 nm, λ = 472.5 nm, λ = 1093.0 nm, and λ = 1462.0 nm. The wavelengths in the visible region are related to those compounds in the honey that absorbe in the blue-violet range, and so giving the characteristic orange-amber colour of the honey. As HFCS colour is pale yellow, the intensity of these wavelength decreases as the adulteration with the syrup increase. As far as NIR region is concerned, the wavelength at 1462.0 nm is related to the first overtone of the vibrational mode of O-H stretch of alcohol groups. It can be observed that signals from both regions, i.e., the Vis-NIR spectra, are needed making the method more difficult to elude. At low ratios of adulteration (≤ 20%) the coefficient of the wavelength = 444.5 nm is high and negative, and as the adulteration increases this coefficient becomes positive and greater. Similar behaviour was observed with wavelength = 1462 nm. In contrast, the coefficient of wavelength = 472.5 nm becomes increasingly negative as the adulteration becomes greater.

The distribution of the honey samples according to the first three canonical discrimination functions is represented in Fig. 4. As can be observed, high scores in the first discriminant function correspond to high levels of adulteration and *vice versa*. Scores from FC2 and FC3 are required to discriminate mainly between non–adulterated samples and 10% ratio of adulteration. These results show the potential of Vis-NIRS for the quantification of HFCS adulterant in honey samples. For this reason, a regression study was then carried out.

Regression study

Based on the results discussed above, partial least squares regression (PLS) was applied for calibration and to develop a predictive model for the ratio of adulteration in honey samples. PLS with a cross validation method was performed on the D_{247x22} data matrix. The best predictive model employed 10 factors with a high coefficient of determination of 0.9990 for the calibration set and 0.9855 for the prediction model. The RMSEC and RMSEP values were 3.05 and 4.71 respectively. These low error values indicate that the regression model is robust, and therefore reliable when applied to samples outside the model.

The results of the calibration model and cross validation obtained in the PLS are shown in Supplementary Fig. 2S. In addition, an external validation using a different set of adulterated samples at ratios not included before (5%, 15%, 25%, and 45%) was performed in order to study the predictive capacity of the developed regression method. A pure honey sample from Granada P.D.O. was also included in the prediction study. The prediction

values obtained for this set of samples are given in Table 1. These results demonstrate the accuracy of the developed model. The error was below 1% for all honey samples adulterated below 45%.

Repeatability and intermediate precision

In order to study the precision of the developed method, the honey samples adulterated at 40% were selected. The repeatability and intermediate precision were determined by analysis of this sample on three different days: nine replicates on the first day and three on the two following days. The intra-day and inter-day coefficient of variation (C.V.) for the analysis was calculated. To do so, the average of C.V. obtained in the whole body of spectra was calculated. The C.V. obtained for the repeatability was 3.90% and for intermediate precision it was 3.63%, meaning that the measurements had high precision.

Conclusions

The applicability of visible-near infrared spectroscopy combined with chemometrics for a rapid quantification of honey adulteration has been studied. The obtained results suggested that non-supervised techniques such as HCA and PCA showed a tendency for the adulterated honey samples to be grouped according to the percentage of adulteration. However, a full differentiation was not possible on using this technique. The full discrimination between the nine levels of adulteration (in the range 10–90%) was achieved by applying supervised LDA. Useful wavelengths were selected from both the visible and NIR spectroscopic regions. A high correlation coefficient was obtained in the PLS regression analysis, and the maximum error in the external prediction model was below 1% for samples adulterated below 45%. Besides the accuracy, the developed method proved to be precise (coefficient of variation lower than 4%).

The results demonstrate that the application of this screening method could be a promising tool for quality monitoring analysis in routine laboratories, since the method is cheap, non-destructive and easy to use. Furthermore, it would be interesting to study other adulterants and/or other high quality honey samples to increase the scope of this approach.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

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*Graphical Abstract (for review)

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