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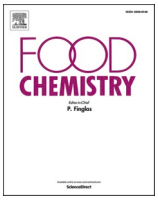

Honey fraud detection based on sugar syrup adulterations by HPLC-UV fingerprinting and chemometrics

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ABSTRACT

In recent years, honey-producing sector has faced the increasing presence of adulterated honeys, implying great economic losses and questioning the quality of this highly appreciated product by the society. Due to the high sugar content of honey, sugar syrups are among its most common adulterants, being also the most difficult to detect even with isotope ratio techniques depending on the origin of the sugar syrup plant source. In this work, a honey authentication method based on HPLC-UV fingerprinting was developed, exhibiting a 100% classification rate of honey samples against a great variety of sugar syrups (agave, corn, fiber, maple, rice, sugar cane and glucose) by partial least squares-discriminant analysis (PLS-DA). In addition, the detection and level quantitation of adulteration using syrups as adulterants (down to 15%) was accomplished by partial least squares (PLS) regression with low prediction errors by both internal and external validation (values below 12.8% and 19.7%, respectively).

1. Introduction

Honey is a traditional and natural food product, with important healing and nutritional properties, known and consumed by humans since, according to anthropologists, about 200,000 years ago. Based on the EU Directive, honey is defined as "the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plant 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 honeycombs to ripen and mature" (European Commission, 2002).

The traditionally recognized benefits of honey for human health and the wave of consumer demand for natural sweeteners lead to a market where global honey prices are at their highest levels in years. Besides, the EU market demand for honey is higher than the domestic production, leading to an important import market (European Commission, 2023b). In addition, the price of honey is much higher than the one of sugar syrups, and nowadays, the detection of honey blended with syrups is still difficult, providing attractive fraud opportunities for dishonest producers to gain illicit economic profits. This was recently highlighted by the European Commission from the *EU coordinated action "From the Hives" (Honey 2021–2022)* study, in which a significant part of honey imported into the EU – 46% based on 320 analyzed samples – was suspicious of not complying with the provisions of the EU Honey directive 2001/110/EC (European Commission, 2023a). In addition, the analytical techniques employed for honey authenticity control, such as elemental analyzer/liquid chromatography-isotope ratio mass spectrometry (EA/LC-IRMS), high-performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD), and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, provided only qualitative information (presence/absence of markers) and, therefore, it was not possible to estimate the level of exogenous syrups present in honey (Ždiniaková et al., 2023). This EU coordination action also highlighted that even stable carbon isotope ratio analysis by EA-IRMS (AOAC method 991.41), which has normally been employed to detect sugar syrups made of maize starch or sugarcane, was not effective in detecting honey samples suspicious of adulteration, being a clear indication that this kind of sugar syrups are no longer used to extend honey. Thus, the development of simple and feasible analytical methodologies to detect and quantify sugar syrup adulteration in honey

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samples is still required.

Today there are two main analytical strategies for food authentication and fraud prevention, targeted and non-targeted approaches. On the one hand, targeted strategies focus on the determination of known and specific compounds (or families of compounds), which are used as primary or secondary markers to solve authentication issues. This is the case of the previously mentioned analytical methodologies targeting saccharides and mannose markers in the detection of honey frauds based on sugar syrup adulterations. High-performance thin-layer chromatography has also been proposed as a targeted methodology to detect honey adulterations with sugar syrups to increase bulk volume (Islam et al., 2020). Phenolic acids and flavonoids are also widely employed as the markers to address honey characterization and authentication based on botanical and geographical attributes (Gašić et al., 2017; Vazquez et al., 2021). However, these methodologies often have disadvantages related, mainly, to the availability of standards and possible matrix effects (Cerdà et al., 2023). Besides, there is still a need to increase the capacity of official control laboratories to detect honey adulterated with sugar syrups, because the existing methods lack of sufficient sensitivity to detect low and intermediate levels of adulteration, even methods based on isotopic ratios do not properly work to detect honey adulteration depending on what type of sugar-based adulterants is employed (Ždiniaková et al., 2023). In addition, the fraudsters themselves adapt the level of adulteration to the analytical marker measurement capacity that is currently available.

On the other hand, non-targeted strategies (based on fingerprinting –metabolomic– approaches), which focus on detecting as many instrumental responses as possible without needing to know the sample components responsible for those signals, are emerging as feasible and powerful methodologies for solving authenticity problems (Cuadros-Rodríguez et al., 2021; Górska-Horczyczak et al., 2022; Jiménez-Carvelo et al., 2021). In general, fingerprinting approaches employ simple sample treatment procedures aiming at keeping as much chemical information from the sample during its treatment. Recording direct spectral information by near-infrared, ultraviolet–visible (UV–vis), or fluorescence spectroscopies has been proposed for honey pattern recognition to authenticate honey and to prevent fraudulent practices (de Souza et al., 2021; Hao et al., 2021; Suhandy & Yulia, 2021; Valinger et al., 2021). Liquid chromatography (LC) techniques in combination with UV-detection or coupled to low-resolution mass spectrometry (LC-MS) or high-resolution mass spectrometry (LC-HRMS) are also widely employed to address honey authenticity issues by non-targeted fingerprinting (García-Seval, Martínez-Alfaro, et al., 2022; García-Seval, Saurina, et al., 2022b, 2022a; Koulis et al., 2021; Li et al., 2017; Stanek et al., 2019).

In a previous work, a simple non-targeted high-performance liquid chromatography with UV-detection (HPLC-UV) methodology, based on a honey dilution with water as unique sample treatment and a LC separation under universal gradient elution, was developed as a novel and feasible methodology to address honey classification and authentication based on botanical origin (García-Seval, Martínez-Alfaro, et al., 2022), providing very good performance. The present contribution aimed to evaluate the feasibility of the proposed HPLC-UV fingerprinting approach to detect honey frauds based on sugar syrup adulteration, as well as to quantify the sugar syrup adulterant percentage. For that purpose, 156 honey samples, including blossom and honeydew honeys from different botanical and Spanish geographical origins, were considered. As a novelty, sugar syrups obtained from a wide variety of plant sources such as agave, corn, fiber, maple, rice and sugar cane, some of them containing honey flavours, as well as glucose-based syrups, and in some cases from different geographical origins, were employed as honey adulterants. The obtained HPLC-UV fingerprints were then used as sample chemical descriptors for sample exploration and classification by principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) multivariate chemometric methods. Finally, several honey adulteration cases with sugar syrups involving samples with similar colour attributes to simulate real-case fraudulent practices were studied by partial least squares (PLS) regression.

2. Materials and Methods

2.1. Reagents and solutions

Purified water obtained with an Elix® 3 coupled to a Milli-Q® system (Millipore Corporation, Bedford, MA, USA) and filtered through a 0.22 nm nylon membrane, and methanol (Chromosolv^{TM} for HPLC, purity higher than 99.9%) obtained from PanReac AppliChem (Barcelona, Spain) were used for sample treatment. Regarding the HPLC-UV analysis, Milli-Q purified water, acetonitrile (UHPLC supergradient ACS) from PanReac AppliChem, and formic acid (purity higher than 98%) from Sigma-Aldrich (St. Louis, MO, USA), were used for the mobile phase preparation.

2.2. Instrumentation

An Agilent 1100 Series HPLC instrument (Waldbronn, Germany) equipped with a binary pump (G1312A model), a diode-array detector (G1315B model), an automatic sample injector (WPALS G1367A model), and a PC with the Agilent Chemstation software was employed. HPLC-UV fingerprints were obtained on a Kinetex® C18 fused-core reversed-phase (100 × 4.6 mm I.D., 2.6 μm partially porous particle size) column from Phenomenex (Torrance, CA, USA), following a previously developed method (García-Seval, Martínez-Alfaro, et al., 2022). Separation gradient was attained with water (containing 0.1% formic acid) and acetonitrile (ACN) as mobile phase components at a flow rate of 400 μL min^{-1}. The elution program was as follows: 0–5 min, 3% ACN; 5–13 min, linear gradient from 3 to 95% ACN; 13–15 min, isocratic step at 95% ACN; 15–15.5 min, back to initial conditions; and 15.5–20 min, column equilibration at initial conditions (see Table S1 in supplementary material). Column was kept at room temperature and the injection volume was 5 μL. HPLC-UV fingerprints were registered at 280 nm.

2.3. Samples

A total of 156 different blossom and honeydew honeys from several botanical varieties and different Spanish geographical production regions were purchased from local markets in Spain. Two heather honeys were directly provided by Miel de Braña (León, Spain). Honey samples were labelled as follows: XX-YY-Z, where XX and YY indicate the botanical variety and the Spanish geographical origin abbreviations, respectively, and Z the sample number (for example, RO-CA-7 will correspond to the rosemary honey sample number 7 produced in Catalonia). Details of the honey samples are summarized in Table S2 (supplementary material). No melissopalynological analysis was performed to verify the botanical origin of the samples. Thus, the botanical and geographical origin of the employed honey samples is based on what is declared on the sample label.

Different sugar syrup samples (30) produced from different plant sources (agave, corn, fiber, maple, rice and sugar cane), a glucose-based syrup, and two syrup typologies containing honey flavours (fiber with honey flavour and glucose with honey flavour), and of different production regions, were employed as honey adulterants (in summary, 9 syrup types were employed). Sugar samples were labelled as follows: S-XX-Y, where S indicates syrup, XX the plant source, and Y the sample number (i.e., S-SC-2 will correspond to the syrup sample number 2 produced from sugar cane). Details of the used sugar syrups are summarized in Table S3 (supplementary material). Syrup plant source and characteristics were based on what is declared in the product label.

All the employed honey and syrups samples were stored in the dark and at room temperature.

2.4. Sample treatment

Honey, syrup and blended honey-syrup samples were prepared following the previously reported procedure (García-Seval, Martínez-Alfaro, et al., 2022). Briefly, 1 g of the sample was dissolved in 10 mL of Milli-Q water on a 15 mL PTFE centrifuge tube (Serviquimia, Barcelona, Spain) using a VibraMix Vortex (OVAN, Barcelona, Spain). After centrifugation for 5 min at 3500 × *g* (Rotina 420 Centrifuge, Hettich, Tuttlingen, Germany) to separate any non-soluble particles (bee bread, pollen, proteins, etc.), the obtained extracts were diluted with methanol in a 1:1 ratio (2 mL total final volume), filtered with 0.45 μm syringe membrane filters (FILTER-LBA, Barcelona, Spain), and stored at 4 °C until analysis. When a honey sample was crystallized (being a normal state of natural raw honeys), it was kept in a water bath at 45 °C until it melts, and then, after homogenization and cooling at room temperature, treated following the same procedure as before.

Besides, 50 μL of each diluted sample extract, belonging to the classification issue under study, were mixed to prepare a quality control (QC) composed sample that was used to evaluate the repeatability and robustness of the proposed non-targeted HPLC-UV method and to assess that chemometric results were not affected by any instrumental drifts.

2.5. Data analysis

All honey and sugar syrup sample extracts were analysed randomly to reduce the influence of instrumental drifts. An instrumental blank consisting of Milli-Q water and a QC sample were injected after every ten samples. Chromatograms were exported to an Excel spreadsheet using Unichrom software from New Analytical Systems (Minsk, Belarus). Different data matrices were built depending on the purpose: (i) classification (data dimension 205 × 3000 –samples + QCs × number of fingerprint absorbance signals–) or (ii) honey adulteration studies (data dimension 65 × 3000 –blended samples + QCs × number of fingerprint absorbance signals–). Data was autoscaled to achieve the same weight for each variable by minimizing differences in the magnitude and amplitude of their scales.

The multivariate chemometric methods used for data analysis were principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and partial least squares (PLS) regression, employing the SOLO 8.6 chemometric software (Eigenvector Research, Manson, WA, USA). The theoretical background of these chemometric tools is described elsewhere (Massart, D. L.; Vandeginste, B. G. M.; Buydens, L. M. C.; de Jong, S.; Lewi, P. J.;& Smeyers-Verbeke, 1997).

2.5.1. Sample classification studies

PCA was used as an exploratory method to mainly evaluate the distribution of the analysed samples and the QC behaviour. PLS-DA was employed as a supervised sample classificatory method to study the discrimination between honey and sugar syrup samples. The X-data matrix employed for PCA was built considering the HPLC-UV fingerprints (the absorbance value at a specific time over the entire chromatogram) recorded at 280 nm for each sample and QC. In the case of PLS-DA, the previous X-data matrix was used (without QCs), together with a Y-data matrix defining each sample class (honey or sugar syrup). The number of LVs to build the PLS-DA models was established by the first relevant minimum of the cross-validation (CV) error from the Venetian blind approach. The ellipses delimiting areas within the PCA and PLS-DA score plots were manually drawn to facilitate the visualization of the different sample clusters.

Validation of paired PLD-DA models was carried out by using 70% of the samples (randomly selected) as the calibration set, and the remaining 30% as the prediction (unknown sample) set. Then, overall accuracy and each class sensitivity (capability to detect true positives) and specificity (capability to detect true negatives) were employed for evaluating the proposed classification models (Riedl et al., 2015). Class sensitivity, specificity and accuracy were then calculated as TP/(TP + FN), TN/(TN + FP), and (TP + TN)/TS (all of them expressed as a percentage), respectively, with TP being the number of positive samples correctly assigned to the class, TN the number of negative samples correctly assigned (i.e., not belonging to the class), FN the number of false negatives incorrectly assigned as not belonging to the class, FP the number of false positives incorrectly assigned to the class, and TS the total number of samples.

2.5.2. Honey fraud adulteration cases

Three adulteration cases, as a proof of concept, were studied by PLS, consisting of honey samples belonging to the same botanical variety adulterated with different sugar syrups (selected to obtain blended honey-syrup mixtures with similar colour attributes). To begin with, we decided to work with a blossom honey (rosemary), a honeydew honey (holm oak), and with heather honey, being a blossom honey with physicochemical properties similar to that of honeydew honeys. Thus, the three adulteration cases under study were: (i) rosemary blossom honey adulterated with corn, fiber, fiber with honey flavour, and maple sugar syrups, (ii) heather blossom honey adulterated with maple and corn sugar syrups, and (iii) holm oak honeydew honey adulterated with glucose and honey-flavoured glucose syrups. Table S4 (Supplementary material) summarizes the honey and syrup sample employed for each adulteration case under study. For that purpose, the PLS calibration set comprises adulteration levels of 0, 20, 40, 60, 80, and 100%, whereas the PLS internal validation set comprises adulteration levels of 15, 25, 50, 75, and 85%. Each one of the prepared adulteration percentages was done by quintuplicate, and by employing (randomly) honey and syrup samples of different geographical origins aiming to introduce the highest variability within the same honey botanical origin and syrups with similar colour attributes. Moreover, a QC extract consisting of an additional 50% adulteration level was used. The PLS X-data matrix consisted of the HPLC-UV fingerprints of each honey-syrup blended samples and QCs, whereas the PLS Y-data matrix defines each adulterant percentage. Again, all the adulterated samples corresponding to a given adulteration case were randomly analysed with the proposed HPLC-UV methodology, and an instrumental blank (Milli-Q water) and the QC were analysed every ten samples.

Finally, the PLS external validation of each adulteration case was performed by analysing, by quintuplicate, new blended honey-syrup samples at adulteration levels of 15, 50, and 85%. For that purpose, honey samples belonging to the same botanical variety but of different producer and/or geographical origin than the ones employed in the PLS calibration sets, were used (see Table S4 for details). The proper number of LVs for building the PLS models was selected after Venetian blinds cross-validation. PLS model performance was evaluated through the root-mean square errors of calibration (RMSEC), cross-validation (RMSECV), and prediction (RMSEP), as well as the corresponding determination coefficient (R^2) values.

3. Results and discussion

3.1. HPLC-UV fingerprints of the analysed samples

As previously commented, a total of 156 honey samples of different botanical varieties and 30 sugar syrups of different plant sources, were analysed with the previously developed HPLC-UV methodology. The sample treatment consisted of a dissolution with water and 1:1 (*v*/*v*) dilution with methanol, as described by García-Seval et al. (García-Seval, Martínez-Alfaro, et al., 2022). For illustration, Fig. 1 shows the HPLC-UV fingerprints obtained for some representative samples. The analysed honey samples show similar chromatographic fingerprints comprising three main regions with abundant signals (retention time –RT– segments: 2–5 min, 7–11 min, and 11–15 min), but showing differences in the number of detected peak signals and their relative abundance according to the honey botanical variety. Mainly, two behaviours can be observed. Blossom honeys, such as, for example,

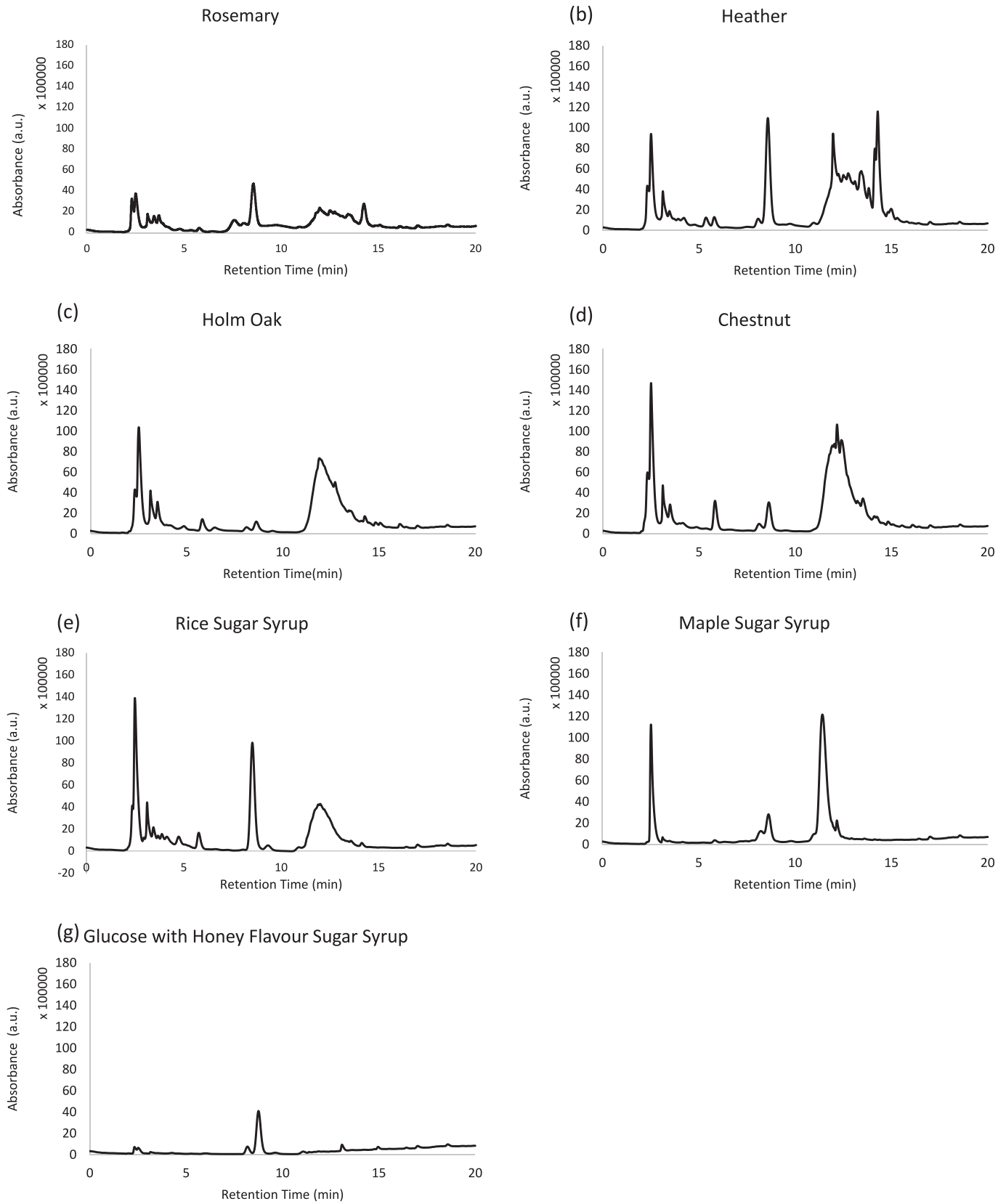

Fig. 1. HPLC-UV fingerprints (at 280 nm) of selected samples: (a) rosemary blossom honey (sample RO-E-12), (b) heather blossom honey (sample HE-CN-7), (c) holm oak honeydew honey (sample HO-AR-1), (d) chestnut honeydew honey (sample CH-E-2), (e) rice sugar syrup (sample S-*R*-3), (f) maple sugar syrup (sample S-M−3), and (g) glucose with honey flavour sugar syrup (sample S-GH-1).

rosemary (Fig. 1a) and heather (Fig. 1b) samples, are characterized by a more intense peak signal at a RT around 8 min, and a broad absorbance signal within the 11–15 min RT segment with the presence of noticeable peak signals. In contrast, honeydew honeys, such as, for example, holm oak (Fig. 1c) and chestnut (Fig. 1d), are characterized by the absence of the abundant peak signal at RT of 8 min, and the presence of the broad absorbance signal at 11–15 min RT segment with no other conspicuous signals. Regarding sugar syrups, the obtained fingerprints tend to be simpler than those observed for honeys, such as, for instance, maple sugar (Fig. 1f) and glucose with honey flavour (Fig. 1g), with fingerprints characterized by the presence of several specific signals, with very low signal intensity in the case of glucose-based syrups. Some exceptions were observed, such as in the case of rice sugar syrups (Fig. 1e), depicting chromatograms that resemble those of honey samples, although with different relative absorbance abundances. This fact could be cause of the difficulty of several honey authentication methods to detect sugar adulterations depending on the type of sugar-based adulterant employed (Ždiniaková et al., 2023). Chromatographic fingerprints of the other sugar syrups under study are provided in Fig. S1 of the supplementary material. It must be highlighted the important signal abundance observed on corn (Fig. S1b), fiber (Fig. S1c), fiber with honey flavour (Fig. S1d) and sugar cane (Fig. S1e) sugar syrups in comparison to other syrups and honey samples. For a more comprehensive comparison, an Excel data sheet containing the chromatographic fingerprinting signal obtained for the 156 honey and the 30 syrup samples analysed is provided in the supplementary material (analysed_samples.xlsx file).

Because of the observed fingerprint differences among samples, and the fact that the fingerprints tend to be consistent within the same sample group (except for those observed for multifloral honeys), they will be evaluated as sample chemical descriptors to address honey authenticity issues based on syrup adulteration by means of chemometrics.

3.2. Exploratory principal component analysis

An exploratory non-supervised study by principal component analysis was performed by employing the HPLC-UV fingerprints of all the honey and sugar syrup samples and QCs. Three principal components, providing a cumulative variance contribution rate of 59.95%, were employed to build the PCA model. The contribution of the next principal component (PC4) was lower than 5%, and then decreasing at higher PCs, not being necessary to take them into consideration as no relevant information among the analysed samples was provided. For illustration, the score plot of PC1 *vs*. PC3 and the 3D PCA plot are shown in Fig. S2 (supplementary material). As can be seen in Fig. S2a, QCs appeared in a very compacted group close to the central area of the plot, confirming the reproducibility of the proposed non-targeted HPLC-UV method and the robustness of the chemometric results, and demonstrating that no remarkable instrumental drifts during the sample sequence analysis are affecting the results.

Regarding sample distribution, they tend to be grouped according to the sample class, with the analysed honeys located mainly at negative PC1 or PC3 values, while sugar syrup samples tend to exhibit mainly positive PC1 and PC3 values (the distribution of the different sugar syrup types under study have also been included in Fig. S2a). Besides, it seems that higher sample discrimination is accomplished within the sugar syrup sample group, which is related to the higher diversity in the chromatographic fingerprints (clearly related to the variability on the sugar syrup plant source and the countries of origin). For example, sugar syrups based on the same plant source but coming from different countries tend to be separated, as can be observed, for example, with maple and rice sugar syrups.

3.3. Sample classification by partial least squares-discriminant analysis (PLS-DA)

Honey and sugar syrup samples were also evaluated by the supervised PLS-DA using the proposed HPLC-UV fingerprints to stablish classification models. For method simplification, QCs were not considered. Fig. 2a depicts the PLS-DA scores plot of LV1 *vs*. LV2 *vs*. LV3. As can be seen, very good sample discrimination between honey and sugar syrup samples was accomplished. Cross-validation of the PLS-DA multiclass model predictions for the training set was also performed, and the sensitivity (capability to detect true positives), specificity (capability to detect true negatives), and accuracy (expressed as classification errors) values are summarized in Table 1. Overall, results were very satisfactory, with sensitivity and specificity values higher than 98.7% and 98.1%, respectively, and with very low classification errors (lower than 0.9%).

The PLS-DA model was validated to evaluate the classification capacity of the proposed methodology. For that purpose, 70% of the samples, randomly selected, were employed as a calibration set to build the model, and the remaining 30% of the samples were used as unknown samples for prediction purposes. As can be seen in Fig. 2b, 100% classification rates for both calibration and prediction were attained, showing the exceptional potential of the proposed fingerprinting methodology to address honey adulteration issues with sugar syrups.

3.4. Honey adulteration case studies by partial least squares (PLS) regression

The feasibility of the proposed fingerprinting methodology for the detection and quantitation of honey frauds based on adulteration with sugar syrups was evaluated by PLS regression. For that purpose, all the analysed syrup samples were first evaluated by PLS-DA to find groups of samples with similar chromatographic features; the PLS-DA score plot of LV1 *vs*. LV2 is shown in Fig. 3. As observed, samples are clearly grouped according to the sugar plant source, and based on their chromatographic similarities (by sample group category and plot proximity) they can be re-grouped into three main syrup-adulterant groups: (i) rice, sugar cane, and some maple syrups –located at the top-centre area of the plot–, (ii) some maple, agave, corn, fiber and fiber with honey flavour syrups –mainly located at the bottom-right area of the plot–, and (iii) glucose and glucose with honey flavour syrups –located at the left area of the plot–. Considering this information (similarity among the chromatographic fingerprints), as well as employing sugar syrups adulterants with similar colours to the pure honeys being adulterated to simulate a more realistic honey fraud situation, three honey fraud adulteration cases were defined: (i) rosemary blossom honeys adulterated with fiber, fiber with honey flavour, corn and maple syrups, (ii) heather blossom honeys adulterated with maple and rice syrups, and (iii) holm oak honeydew honeys adulterated with glucose and glucose with honey flavour syrups. See Fig. S3 (supplementary material) for the visual comparison of three pure honey samples and the corresponding honey adulterated at a 50% level with a sugar syrup.

For each honey adulteration under study, two sets of samples at different adulterant percentages were considered for calibration and internal validation, as described in section 2.5.2. All the adulteration percentages were prepared in quintuplicate (see Table S4 in the supplementary material for details) blending randomly the selected samples. With this, each adulteration level involved different honey and syrup samples, introducing this way a high variability within the PLS regression models. In addition, external validation was also performed using a new set of samples at three honey-blended adulteration levels (15%, 50%, and 85%) prepared with different honey samples than those employed in calibration and internal validation.

Fig. 4 shows (a) the PCA score plot of PC1 *vs*. PC2 showing the distribution of the blended honey-syrup samples for calibration and internal validation, (b) the PLS calibration model with the internal validation

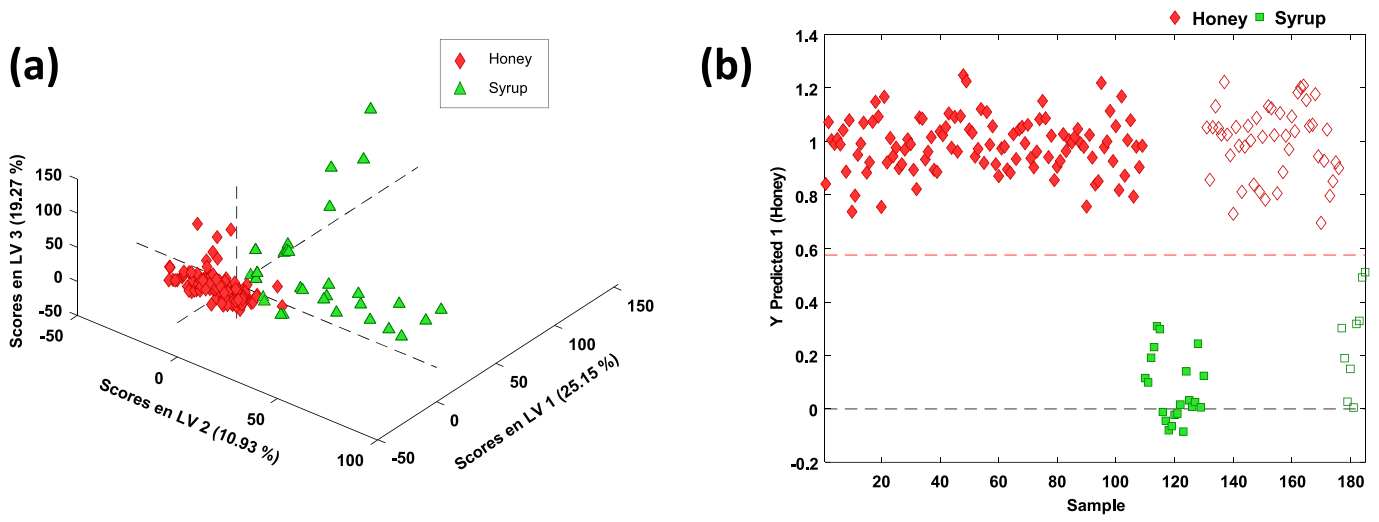

Fig. 2. PLS-DA score plot of (a) LV1 *vs.* LV2 *vs.* LV3 for all the analysed samples when using HPLC-UV fingerprints as sample chemical descriptors (5 LVs were employed to build the PLS-DA model). (b) Classification of the paired PLS-DA model of all honey samples versus syrup samples. Red line indicates the separation threshold between classes. Filled and empty symbols correspond to the calibration and prediction sets, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Cross-validated multiclass PLS-DA model predictions using 5 LVs for the set of samples analysed.

Sample class	Sensitivity (%)	Specificity (%)	Classification error (%)
Honey	98.7	100	0.6
Sugar syrup	100	98.1	0.9

study, and (c) the PLS calibration model with the external validation study in the case of adulterated rosemary blossom honey samples. Similar information is provided in Fig. S4 and S5 (supplementary material) for the other two honey fraud adulteration cases involving heather blossom and holm oak honeydew honey samples, respectively. In addition, Table 2 summarizes the PLS performance results. As can be observed in the PCA plots, samples tend to be grouped according to the adulterant level. Although complete discrimination between the different blended adulteration levels is not clearly accomplished due to the higher variability included in the study (each adulteration percentage quintuplicate was prepared using different syrup sources, as previously commented), blended honey-syrup samples tend to be in between pure honey and pure syrup samples (right and left areas, respectively, in the PCA plot shown, as an example, in Fig. 4a). Besides, lower adulterated samples tend to be close to the pure honey sample while higher adulterated samples are distributed close to the pure syrup samples, as expected. Regarding the PLS performance, excellent results were accomplished, with R^2 values higher than 0.879, and calibration and cross-validation errors below 11.9% and 15.0%, respectively. Good results were also accomplished in both internal and external validations, with prediction errors below 12.8% and 19.7%, respectively. Despite the high sample variability intentionally employed when blending honey samples with the syrup adulterants (see Table S3 in supplementary

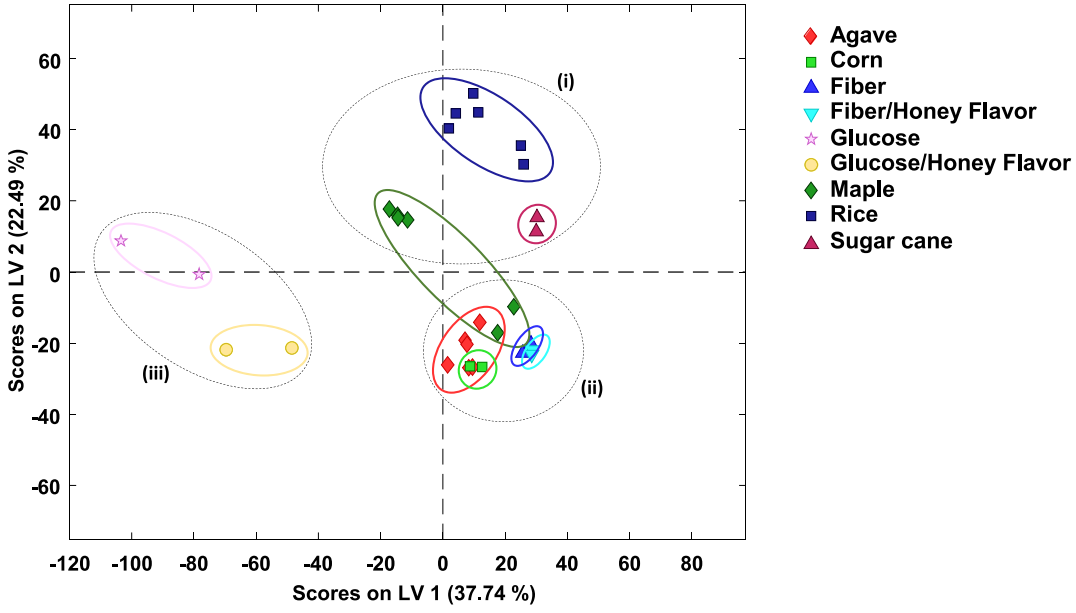

Fig. 3. PLS-DA score plot of LV1 *vs.* LV2 for all the sugar syrup samples when using non-targeted HPLC-UV fingerprints as sample chemical descriptors. (i), (ii), and (iii) indicates the three main syrup-adulterant groups based on fingerprints' similarity.

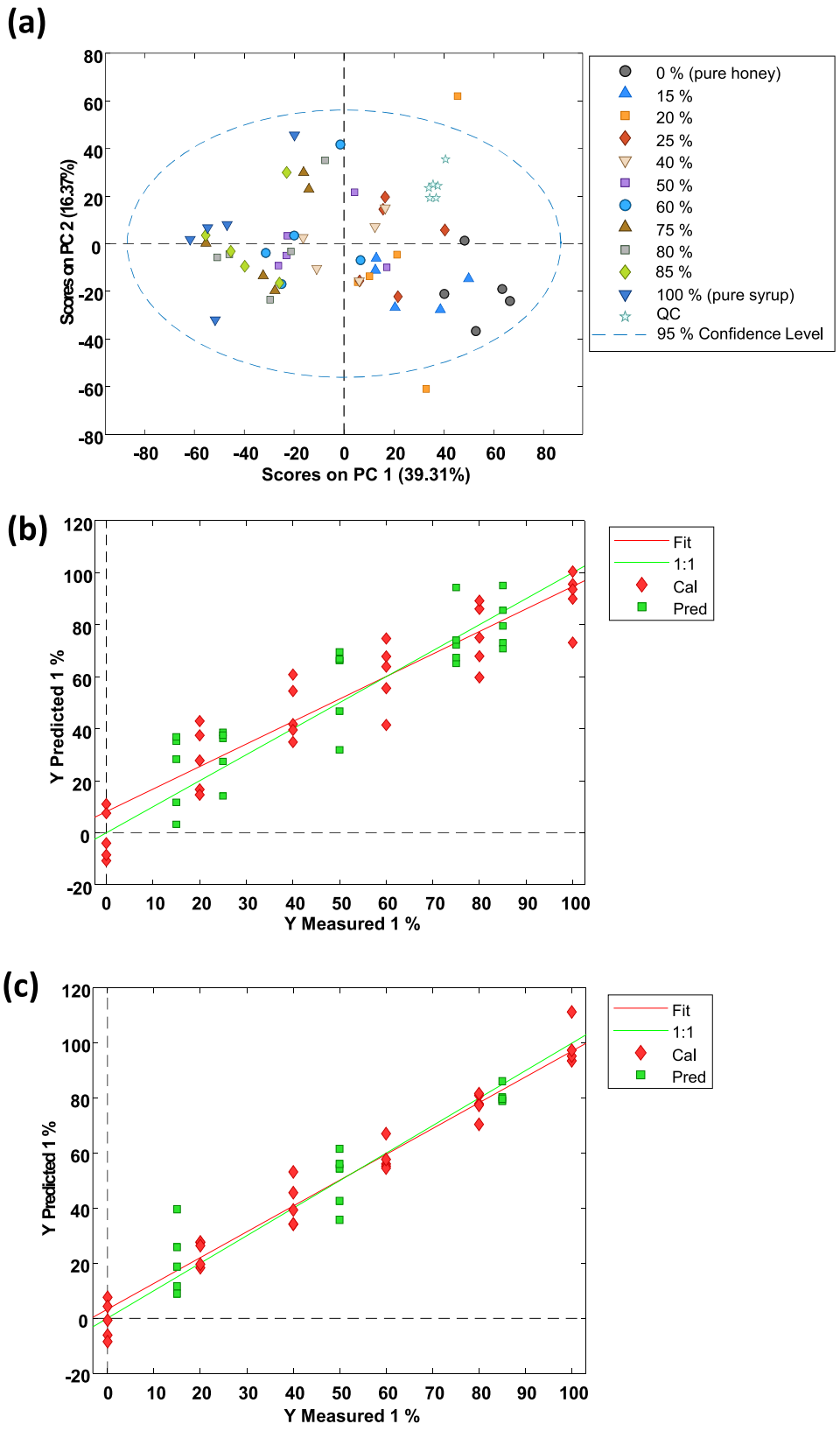

Fig. 4. Results of rosemary blossom honey adulteration case. (a) PCA score plot of PC1 *vs*. PC2 depicting the distribution of the blended honey-syrup sample adulteration levels used for PLS calibration and internal validation; (b) PLS calibration model with the internal validation study; and (c) PLS calibration model with the external validation study.

Table 2
Evaluation of the honey adulteration cases by PLS using HPLC-UV fingerprints as chemical descriptors.

Adulteration case	LVs	Linearity (R^2)	Calibration error (%)	Cross-validation error (%)	Prediction			
Internal validation					Prediction error (%)	Average Bias (%)	Ratio predicted deviation	Range error ratio (RER)
Rosemary honey	1	0.879	11.9	15.0	12.8	10.9	1.1	2.8
Heather honey	4	0.987	3.9	14.8	12.3	9.4	1.2	2.9
Holm oak honey	7	0.999	1.3	10.6	8.1	6.2	1.0	2.6
External validation								
Rosemary honey	3	0.968	6.0	12.0	9.5	9.5	0.9	2.2
Heather honey	7	0.987	3.7	10.4	19.7	19.5	0.9	2.1
Holm oak honey	7	0.999	1.3	10.6	14.2	10.3	1.2	2.8

material), the obtained prediction relative errors demonstrate a very acceptable accuracy when predicting the adulterant proportion in the analysed honey samples. Other error estimators, such as average bias or range error ratio, also provided acceptable values in the different cases under study.

Below, the possibilities of our approach are compared and discussed concerning other options reported in the scientific literature. The adulteration of honey with syrups from different sources is a very relevant topic scientifically and socially, as can be deduced from the dozens of papers published in recent years. A recent review dealing with this issue has been published (Siddiqui et al., 2017). Currently, vibrational (infrared and Raman) spectroscopies continue to be one of the most popular options. The resulting fingerprints are useful for a preliminary evaluation of the samples, the detection of adulterations, and the quantification of adulterant percentages. A recent representative application of IR spectroscopy within the MIR range by Limm and coworkers proposed the rapid screening of honey adulterated with corn or rice syrup (Limm et al., 2023). The spectra recorded by FTIR were treated with SIMCA, achieving prediction rates above 94% for syrup percentages higher than 7%. The NIR range is also valuable, such as in the application by Bodor et al. to authenticate Hungarian honey from various botanical varieties adulterated with fructose, glucose, and rice syrups (Bodor et al., 2023). In addition to the identification of adulterated samples by LDA, the syrup percentage was quantified satisfactorily. Another NIR example consisted of the study of acacia honey adulterated with glucose syrup, in which adulterant levels were determined by PLS and ANN (Benkovic et al., 2022). Wu and coworkers carried out a qualitative and quantitative study on acacia, litchi, and linden honey adulterated with sugar and rice syrups by Raman spectroscopy. Samples were analyzed by neural networks, obtaining prediction errors better than 5% (Wu et al., 2022).

NMR spectroscopy is another powerful option for authentication through both non-targeted and targeted analysis since some markers of adulterations are eventually elucidated (Cagliani et al., 2022). The non-targeted approach explores the whole NMR spectrum as a fingerprint, while the targeted counterpart measures specific signals associated with compounds, such as β-maltose, inulin, fructose, or inverted sugar that provide information about adulteration compounds. Some recent works proposed the detection of honey adulteration using benchtop 1H NMR spectroscopy, a simple and cheap technique that does not require expert personnel to use. Despite their capacity and limited spectral resolution, the generated fingerprints allow for detecting adulteration with corn, glucose, and wheat syrups at concentrations starting at 5% (Rhee et al., 2023).

UV–visible spectroscopies are mainly used in molecular fluorescence mode, especially employing excitation-emission data. For example, excitation-emission spectra were used to detect adulterations in four types of honey (tilia, sunflower, acacia, and rape) with various syrups (agave, maple, inverted sugar, corn, and rice) added at percentages between 5 and 20%. The recognition of adulterated samples was satisfactory with support vector machine (SVM) that provided superior performance to PLS-DA (Ropciuc et al., 2023). In other examples, Hao et al. have also used fluorescence for a qualitative study of adulterations using PCA (Hao et al., 2023; Hao et al., 2021). Alternatively, UV–vis spectra recorded between 220 and 550 nm provide generic information on the content of phytochemicals, mainly phenolic acids and flavonoids. Hence, some authors explored this information for a qualitative study of adulterations (Dimakopoulou-Papazoglou et al., 2023; de Souza et al., 2021). Atomic spectroscopies also differentiate adulterated from pure honey. For instance, the composition of 12 elements determined by ICP-OES correctly discriminated honey from 6 botanical origins, three syrup types, and several adulterations (Liu et al., 2021).

In line with their progressive introduction of electronic tongues into food analysis, these devices may detect adulterations and fraud in honey. In the application by Ciursa and coworkers (Ciursa et al., 2021), an e-tongue composed of five working electrodes (gold, silver, copper, platinum, and glass) to record cyclic voltammograms of the honey samples was assessed. Finally, general physicochemical parameters, such as total phenolic content, antioxidant capacity, antiradical activity, hydroxymethylfurfural concentration, and diastase activity, can be applied to honey authentication since they are affected by the presence of syrups, resulting in a decrease in the overall content of phytochemicals (Brar et al., 2023).

Excellent descriptors or markers of adulteration conditions can be obtained with chromatographic techniques. For instance, high-performance thin-layer chromatography was applied to sugar profiling for honey adulteration (Islam et al., 2020). The non-targeted metabolomic approach by LC-HRMS was used in a preliminary study of honey adulteration with sugar syrups or honey from overfeeding honeybees. The authors indicate that samples with percentages higher than 5% were recognized as adulterated (Martinello et al., 2022).

Our paper proposes a new way to resolve the authentication of honey potentially adulterated with syrups of diverse origins. Liquid chromatography with UV detection has been seldom used to study varieties, qualities, and adulterations of honey. Most papers are non-chromatographic, just attempting to identify adulterated samples with spectroscopic and electrochemical techniques. In contrast, the quantification of percentages of adulterants through multivariate calibration, such as PLS, has been barely addressed. It has been proved that UV chromatographic fingerprints are extraordinarily rich in features distinctive from genuine varieties or adulterants. Furthermore, the method is simple, cheap, relatively fast, and does not require specialized personnel. Therefore, we consider that is a good option for dealing with quality control issues in large sample sets. Overall, the excellent performance obtained confirms that HPLC-UV fingerprints are good and feasible sample chemical descriptors to assess honey characterization and classification, detect honey adulterations with sugar syrups, and quantify the adulterant percentages down to 15% within a wide range of sugar syrup plant sources.

4. Conclusions

This study demonstrates the suitability of non-targeted HPLC-UV method to assess honey authentication when adulterated with sugar syrups from different plant sources. In this line, an excellent classification accuracy by PLS-DA was accomplished when classifying more than 155 honey samples of different botanical varieties and geographical origins against 30 sugar syrups of different plant source origin, with a 100% classification rate.

Besides, the capability of the proposed methodology to detect honey fraud and quantify syrup adulteration levels down to 15% by PLS regression was assessed by studying three honey adulteration cases. In each case, honey-syrup blended levels were prepared using honey samples of the same botanical variety but different geographical origin and sugar syrups of different plant sources, while keeping similar colour attributes to simulate real fraudulent practices. Despite the high variability intentionally introduced in the PLS models, very good results were accomplished with calibration and cross-validation errors below 11.9% and 15.0%, respectively. Very acceptable prediction errors were also attained by both internal and external validations (values below 12.8% and 19.7%, respectively).

Therefore, the non-targeted HPLC-UV methodology can be proposed as a reliable and straightforward method to prevent honey fraud adulteration practices based on sugar syrup adulterations.

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CRediT authorship contribution statement

Carla Egido: Methodology, Formal analysis, Investigation, Writing – original draft. **Javier Saurina:** Formal analysis, Writing – review & editing. **Sònia Sentellas:** Formal analysis, Conceptualization, Writing – review & editing. **Oscar Núñez:** Formal analysis, Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137758.

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