



Empowering the rapid authentication of the botanical origin of monofloral honey by coated blade spray mass spectrometry (CBS-MS)

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ABSTRACT

In the quest for a rapid and cost-effective tool for determining the botanical origin of monofloral honey, the analytical capabilities of coated blade spray mass spectrometry (CBS-MS) were investigated. To this aim, the chemical profiles of 64 honey samples from seven different botanical origins (acacia, dandelion, chestnut, rhododendron, citrus, sunflower, and linden) were captured by the absorbent of the coated blades and then analyzed by mass spectrometry. An exploratory analysis was performed by principal component analysis (PCA) to generate a graphical representation of the CBS-MS data that allows the discovery of patterns, outliers, or relations between types of honey in an unsupervised manner. Additionally, the performances of four different classification algorithms (least absolute shrinkage and selection operator (LASSO), random forest (RF), and neural network (NNET) partial least squares discriminant analysis (PLS-DA)) were built up and compared. The performances of the four classifiers were verified by a 50 times-repeated, 5-fold-cross-validation and permutation test. Although all classifiers performed well, the RF showed significantly higher performances in cross-validation (with area under the curve (AUC) of 0.99, overall accuracy 0.94, Kappa 0.93, sensitivity 0.94, and specificity 0.99). Moreover, the permutation tests showed the models were not overfitted. Finally, to determine the molecular identities of the ions that most contribute to the classification, extracts from the same honey samples were prepared, analyzed by liquid chromatography coupled to high resolution tandem mass spectrometry (LC-HRMS/MS), and the most significant features were annotated. This proof-of-principle work warrants a future large-scale study to validate and challenge this CBS-MS-based method with a greater number of honeys from different years and geographical origins.

1. Introduction

Honey is a natural supersaturated sugar solution produced by *Apis mellifera* bees, with more than 95% of its dry mass consisting of sugars and water, and which has been valued for its sweetening properties since ancient times (Manyi-Loh et al., 2011). The European Union (EU) legislation aims to preserve the quality of honey as an unprocessed raw agricultural product, prohibiting changes to its chemical composition and mislabeling ("European Union Council Directive No. 2001/110/EC," 2001, p. 110).

Honey can be classified as monofloral or multifloral. Each monofloral honey has a distinctive flavor because it is generated

predominantly from the nectar of a single plant species. Honey is categorized as multifloral if multiple pollens are present. The floral source gives honey a very small fraction of plant-derived compounds, such as terpenes, benzene derivatives norisoprenoids, polyphenols, and aromatic volatile compounds (VOCs), which affect its organoleptic and nutritional properties (Kaškonienė & Venskutonis, 2010). These compounds are highly dependent on the floral source of the nectar and pollen the bees forage to make up the honey. Because of their nutritional, therapeutic, and organoleptic properties, monofloral honeys are considered better-quality products that are highly appreciated and requested by consumers (Roman et al., 2013). Therefore, compared with multifloral honeys, monofloral honeys have higher economic value and,

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thus, are more prone to mislabeling (Soares et al., 2017).

Considering the increasing global demand for monofloral honeys with protected designation of origin (PDO) and protected geographical identification (PGI), the main concerns related to these honeys' authenticity are focused on their geographical and botanical origins. In these products, incorrect labeling claims and fraudulent practices, like admixing with multifloral honey of lower value and quality (Soares et al., 2017) and adding other substances, such as syrups or sugars, are important issues (European Commission. Joint Research Centre., 2023). Note that, while the adulteration or mislabeling of products as honey, or honey fraud, is a major concern for industry and regulators, the impact of honey fraud occurrence on consumer preferences leads to stronger preferences for locally produced honey (Gustafson et al., 2024). In order to set up new strategies for the valorization of the monofloral honey, numerous analytical spectroscopic- and chromatographic-based techniques have been developed to determine honey authenticity (Danieli & Lazzari, 2022; Jandric et al., 2021; Koulis et al., 2021; Recklies et al., 2021; Schievano et al., 2013, 2016; Tsagkaris et al., 2021; Zhang & Abdulla, 2022; Lozowicka et al., 2021).

To avoid using expensive, toxic, and unsustainable organic solvents mainly used in chromatography-based approaches, new methods have been developed to analyze the compounds in honey that codify for the discrimination of floral sources. In this context, the introduction of ambient ionization mass spectrometry (AIMS) in the mid-2000s has transformed analytical science, providing new tools for rapid and accurate analysis without pre-separation techniques (Javanshad & Venter, 2017). AIMS analysis is performed under ambient conditions (i.e., atmospheric pressure and/or room temperature) with little or no sample preparation in a nearly real-time manner. Due to its short time scales, AIMS has been extensively used in food authenticity within targeted and non-targeted approaches (Arrizabalaga-Larrañaga et al., 2021; Black et al., 2016; Damiani et al., 2021; Lu et al., 2018; Massaro et al., 2021, 2024; Tata et al., 2022). Among the AIMS techniques, rapid evaporative ionization mass spectrometry (REIMS) was used for the recognition of monofloral honey and adulterated syrup-honey samples (Wang et al., 2019). Direct analysis in real time mass spectrometry (DART-MS) was tested for its ability to discriminate two monofloral (chestnut and acacia) honeys and according to their geographical origins, i.e., Italy and Portugal for chestnut honey and Italy and China for acacia honey (Lippolis et al., 2020). The results suggest this technology could be useful in providing near real-time feedback to aid in the rapid authentication of honey's geographical origin. Moreover, proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS) coupled to chemometrics was applied in the classification of monofloral honeys (Schuhfried et al., 2016).

Since the introduction of the first AIMS technique, the field of analytical chemistry has exploded with dozens of novel ion sources, including coated blade spray mass spectrometry (CBS-MS). CBS-MS is a combination of AIMS and solid phase microextraction (SPME) and was first described in 2014 (Gómez-Ríos & Pawliszyn, 2014). A sorbent layer, mostly based on hydrophilic lipophilic balance (HLB) particles, is attached to a metal blade and is used to extract the compounds of interest from a matrix. Therefore, the blades can simply be dipped into the sample to extract and enrich the analytes of interest while leaving most matrix components behind. After the extraction, coated blades are positioned in front of the mass spectrometer (MS) inlet, and once the desorption solvent is added, a high voltage is applied to generate electrospray from the tip of the blade. With CBS-MS, no separate gas supply is needed, which is an advantage compared to other AIMS methods, such as DART-MS (Blokland et al., 2020). Interestingly, CBS has successfully been used for the analysis of drug residues in biological matrices such as blood, urine, and tissue (Gómez-Ríos et al., 2017, 2018; Kasperkiewicz et al., 2019; Khaled et al., 2020), classification of brain tumors (Bogusiewicz et al., 2022), and for the detection of multi-class pesticides in fruit (Kasperkiewicz & Pawliszyn, 2021a) and cannabis oil (Kasperkiewicz & Pawliszyn, 2021b). To the best of our knowledge,

CBS-MS has never been applied in the non-targeted authentication of food.

Therefore, based on the aforementioned concerns about the mislabeling of the botanical origin of monofloral honey, for sustainable and quick authentication of monofloral honey, a new path using CBS-MS in combination with statistical pattern recognition techniques was explored. Herein, this study aimed to determine whether CBS-MS, upon integration with statistics, is a viable choice for authenticating the floral source of monofloral honey. The workflow of the study is reported in Fig. 1.

2. Materials and methods

2.1. Samples

A total of 64 monofloral honeys, harvested in 2022 in Italy, were collected during the most important national competition (*Tre Gocce D'oro*), organized by the National Honey Observatory (Osservatorio Nazionale Miele), that represents beekeeping organizations at national and regional levels. The honeys originated from all the Italian regions, and therefore, the sample set can be considered representative of Italian honey production for 2022. We randomly selected and analyzed honeys of seven different botanical origins: acacia (n = 10), chestnut (n = 9), citrus (n = 9), dandelion (n = 9), linden (n = 9), rhododendron (n = 10), sunflower (n = 8). A list of the honeys with their geographical origins is provided in Table S1 of the supplementary material. The Osservatorio Nazionale Miele previously verified the botanical origin of each honey sample by the combination of sensory analysis ("European Union Council Directive No. 2001/110/EC," 2001) and physicochemical assessments (Persano Oddo & Piro, 2004).

2.2. Sample preparation

One gram of each honey was diluted in 4 mL of a solution of methanol:water (MeOH:H₂O, 20:80, v/v) (MilliQ water and methanol HPLC-grade of 99.9% purity from Sigma Aldrich, St. Louis, MO, USA), vortexed for 30 s and shaken for 25 min. The obtained sample extract was centrifuged for 5 min at 6000×g, and 1000 µL of the supernatant was transferred into a well in a 1-mL 96-well plate. To absorb each extract from the plate onto a separate blade, a sample handling unit (SHU) from Restek (Bellefonte, PA, USA) was used. This SHU enables the use of the 1-mL 96-well plates in combination with blade holders for high-throughput sample preparation. The SHU is equipped with a shaker for agitation purposes during extractions (Digital Vortex-Genie 2, Scientific Industries, Bohemia, NY, USA), and agitation speed was set to 500 rpm during each step. Coated blades were first pre-conditioned by dipping them in MeOH:H₂O (1:1, v/v) for 1 min with agitation. After drying, the blades were dipped into the extract supernatants in the 96-well plates for 10 min with agitation. Finally, the blades were washed by dipping them into milliQ water for 10 s with agitation and air-dried before analysis. For each honey sample, two replicates were acquired to check the repeatability of the developed method. The blades were dried in the open air overnight before analysis.

2.3. CBS-MS analysis

For the CBS-MS analysis, a CBS interface from Restek (Restek Corporation, Bellefonte, PA, USA) was connected to the atmospheric pressure-vacuum interface of a SCIEX QTRAP 6500 mass spectrometer (AB Sciex LLC, Framingham, MA, USA). A 10 µL volume of methanol with 12 mM ammonium acetate (methanol:H₂O, 95:5 v/v, including 12 mM ammonium acetate) was used as desorption solvent. The elution and spray times were set to 10 s. The drying and cleaning times were set to 18 and 10 s, respectively. A cleaning solution of isopropanol:methanol (1:1, v/v) was used. The instrumental parameters of the MS were set to those specified in the CBS interface manual (Restek Corporation, 2021).

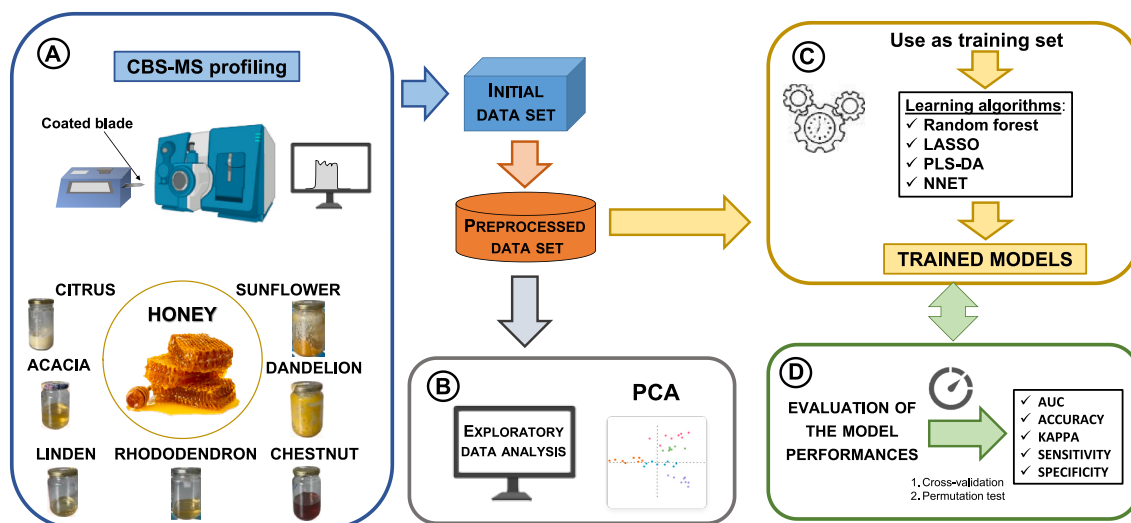


Fig. 1. Workflow of the study. A) A total of 64 samples from seven types of monofloral honey were analyzed by coated blade spray mass spectrometry (CBS-MS). B) The dataset was preprocessed and submitted to an exploratory principal component analysis (PCA) that graphically evaluated the discrimination capabilities of the CBS-MS with regard to the honeys' botanical origins. C) The preprocessed dataset was used to build up four different classifiers. D) The performances of the four classifiers (least absolute shrinkage and selection operator (LASSO), random forest (RF), and neural network (NNET) partial least squares discriminant analysis (PLS-DA)) were evaluated by cross-validation and permutation tests. The calculated indicators of performances were the area under the curve (AUC) of the receiving operating characteristic curve (ROC), accuracy, Kappa-statistic, sensitivity, specificity.

Briefly, the curtain gas was set to 5, ion source gas 1 to 10, and ion source gas 2 to 0 (arbitrary units, a.u.). The spray voltage was set to 4000 V, the interface heater temperature to 150 °C, and the collision gas to "low". The instrument was used in unit resolution mode. The MS was operated using the Analyst version 1.7 software package in full scan mode and in negative ion mode. The dwell time was set to 50 ms, and the pause between mass ranges was set to 5 ms. For data processing, Analyst version 1.6.2 software was used.

2.4. Statistical analysis

The spectral data were pre-processed and statistically analyzed using the R Statistical Software (v4.0.2; (R Core Team, 2020) software with the *MALDIquant* (Gibb & Strimmer, 2012) and *caret* packages (Kuhn, 2008). In each spectrum (that is the m/z ratios of the ions present in a sample plotted against their signal intensities), the ions with signal-to-noise-ratio lower than 5 were removed, and the m/z signals were binned with a tolerance of 0.105 Da with the subsequent generation of a single data matrix comprising all the spectral data. The data matrix was composed of 359 features, each of them consisting of a combination of m/z and intensity. The variables without assigned absolute intensity were not removed. Instead, we replaced the missing intensity value with 1/5 of the lowest recorded absolute intensity. With respect to whether the mass spectra of two independent replicates of each honey (the duplicates) were repeatable, we investigated the repeatability of the pre-processed files by using the cosine similarity function (Stein & Scott, 1994). Assessing the repeatability between two analyses of the same bulk sample is an essential step in non-targeted approaches and even more so when AIMS methods are applied (Abbassi-Ghadi et al., 2015; Dill et al., 2011; Woolman et al., 2017). Cosine similarity measures how much one mass spectrum overlaps with its duplicate. If the two mass spectra of the two independent replicates of each extract have near-perfect overlap, then the cosine similarity is high (>95%). See some examples in the supporting info (Figs. S1–S3 of the supplementary material).

We autoscaled the absolute intensities in each mass spectrum of the resultant data matrix by log₁₀ scaling. Afterward, we removed the sparse variables by using the *NearZeroVar* function from *caret* package

(Kuhn, 2008). After running the *NearZeroVar* the data matrix was composed of 252 features, each of them consisting of a combination of m/z and intensity. We kept the mass spectral data from the individual repetitions of each honey extract for further processing. The pre-processed data were then submitted to principal component analysis (PCA) for quick visualization of the discrimination capability of this CBS-MS method by using the *MetaboAnalyst 5.0* web portal (<https://www.metaboanalyst.ca/>). Using *caret* package, the pre-processed spectral data were then submitted to 25 times-repeated, 5-fold cross-validation recursive feature elimination (RFE), which is a feature selection technique that iteratively eliminates less important features with the aim of teasing out the relevant molecular features (Granitto et al., 2006). Reducing the features can allow the subsequent machine learning algorithms to run more efficiently (less space or time complexity). Note that less important features can mislead some machine learning algorithms, resulting in poor predictive performance. Taking this into account, the resulting most significant variables, optimized for all the following classification algorithms, were used to build up three different classifiers by using *caret* package: a random forest (RF) model classifier (using 86 variables), a neural network (NNET) classifier (using 78 variables) and a partial least squared discriminant analysis (PLS-DA) classifier (using 89 variables). Moreover, an absolute shrinkage and selection operator (LASSO) was built up. Unlike the aforementioned classification algorithms, LASSO shrinks and selects the best variables with the subsequent generation of a "sparse" model that involves only a subset of the variables/predictors (Tibshirani, 1996) without the need for an initial data reduction. Therefore, no RFE function was applied prior to LASSO modelling.

Each model was cross-validated by 50 times-repeated, 5-fold cross-validation with simultaneous optimization of the hyperparameter. While running the cross-validation, we kept the duplicates of each sample together in the training or test set (group split). Finally, to check for overfitting, a permutation test was performed on each model (500 iterations) by randomly permuting the class labels of the samples in the training set. Using *caret* package, the performances of the classification models for each test were evaluated by calculating the contingency table-derived parameters of accuracy, Kappa-statistic, sensitivity and specificity (Ellison & Fearn, 2005). Moreover, the area under the curve

(AUC) of the receiving operating characteristic curve (ROC) was calculated (López et al., 2014). Note that the AUC, which is a probabilistic-based method, can be considered the main performance parameter (Hossin & Sulaiman, 2019). For the permutation test, we reported the highest values of performance parameters obtained from the 500-iterated permuted models.

2.5. HPLC-HRMS/MS analysis

After statistical evaluation and selection of significant ions (the significance of which were confirmed by non-parametric analysis of variance (ANOVA) with false discovery rate (FDR) adjustment ($p_{adj} \leq 0.05$)), honey extracts were prepared very similarly to the method described above. The only difference was that 1 g of honey was diluted in 9 mL of methanol:water (20:80, v/v). The extracts were transferred to a plastic HPLC vial and subjected to LC-HRMS/MS by using an Ultimate 3000 UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Q-Exactive (quadrupole-Orbitrap) mass spectrometer (Thermo Fisher Scientific, MA, USA) operating in parallel reaction monitoring (PRM) mode. The UHPLC system was equipped with a reversed phase C₁₈ column (Hypersil Gold, 100 mm × 2.1 mm, 1.9 μm, Thermo Scientific, USA) kept at 35 °C. Chromatographic separation of target ions was

obtained over 25 min by mixing mobile phase A (water containing formic acid, 0.1% (v/v)) and mobile phase B (acetonitrile containing formic acid, 0.1% (v/v)) as follows: 95/5 (A/B) from 0 to 2.5 min, then linearly increased to 0/100 (A/B) at 15.5 min, kept at 0/100 (A/B) until 20 min, brought to 95/5 (A/B) at 20.5 min and kept unchanged until 25 min to re-equilibrate the system. The flow rate was 0.3 mL/min and the injection volume was 3 μL.

Honey extracts were analyzed in full scan mode at 70,000 resolution (full width at half maximum (FWHM)) to gather accurate measurements of precursor ions and in PRM mode in negative electrospray polarity to obtain information about product ions deriving from selected precursors. Targeted HRMS/MS fragmentation spectra were acquired using HCD fragmentation at a resolution of 17,500 FWHM with a normalized collision energy value optimized for each precursor and an isolation window of 1.0 Th. Source parameters were set as follows: electrospray voltage of 3000 V, sheath gas flow rate 40 a.u., auxiliary gas flow rate 10 a.u., capillary temperature 325 °C, heater temperature 325 °C, and S-lens voltage 50 V.

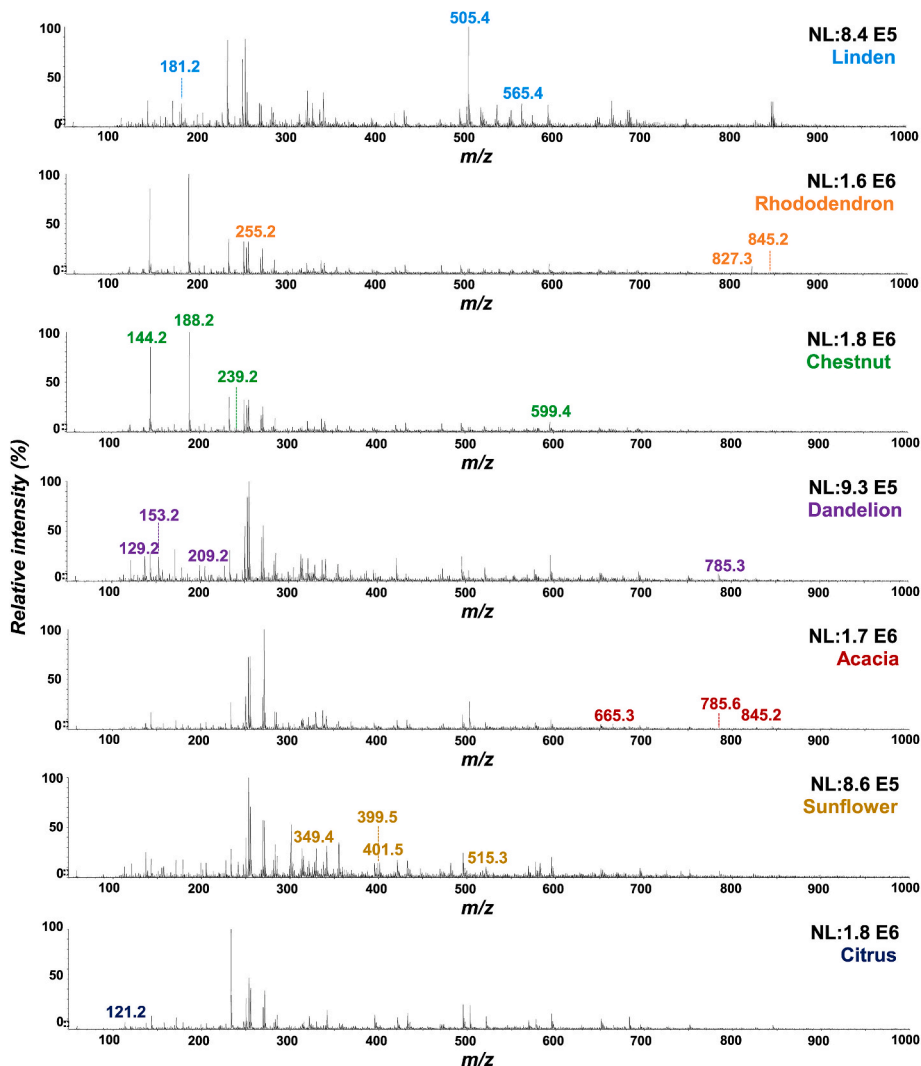


Fig. 2. Representative coated blade spray mass spectrometry (CBS-MS) raw spectra of seven types of monofloral honey. The CBS-MS spectra are shown with the relevant classifying m/z signals, determined by statistical analysis, and which identified the honey-floral sources. These informative m/z values were further identified by high-performance liquid chromatography and tandem mass spectrometry. Note that we have only highlighted the m/z values of interest that were visible on these representative spectra. For a comprehensive assessment of all the informative m/z values, see Table 2.

3. Results

3.1. Evaluation of the CBS-MS analysis

After data acquisition, the average spectra of the first 10 s after the high-voltage was applied to the blade were retrieved. These spectra were first visually inspected and compared between the honeys of different botanical origins. Fig. 2 shows representative average mass spectra of honeys from different botanical origins acquired by CBS-MS. Very distinct volatile profiles for each different botanical source can be observed. For example, when the spectrum of linden is compared with rhododendron, it is clear that much higher m/z are present in the spectra of the linden honey samples.

Although a simple comparison of the CBS-MS profiles enabled the visual discrimination of the floral source of the honey (Fig. 2) and indicated that CBS-MS is indeed a viable choice for botanical origin authentication, the spectral data were further interrogated by statistical analysis.

The repeatability of the two CBS-MS spectral repetitions was assessed by cosine similarity. We observed that only 8 out of 64 honeys produced spectra that were not repeatable using a threshold of 95% similarity (Table S2). As the repeated spectra for these eight honeys presented similarities ranging between 88% and 93%, we decided to include all the readings in the classifiers. The decent repeatability of the duplicated spectra alleviated the need for multiple instrumental repetitions and for invoking “majority rule” decision-making in the case of discordant outcomes of the classifiers resulting from the predictions of the duplicates of the spectra.

3.2. Exploratory statistical analysis

Exploratory unsupervised data analysis was performed by PCA analysis of the CBS-MS data to determine if the spectral differences could be used for classification analysis. The plot of the resultant PCA three-dimensional (3D) scores provides graphical representations that allow the discovery of patterns, outliers, or relations between groups. The PCA 3D scores plot exhibited good clustering of the chestnut, linden, sunflower, and dandelion honeys, but a high degree of overlap between acacia, citrus and rhododendron honeys (Fig. 3). The PCA scores plot demonstrated that there were enough spectral differences between the groups that they can be discriminated by more powerful statistical models.

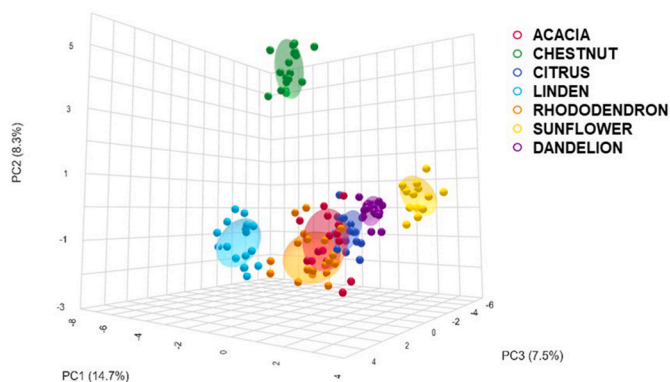


Fig. 3. Exploratory principal component analysis (PCA) three-dimensional (3D) scores plotting the honey analyzed by coated spray blade mass spectrometry (CBS-MS). The PCA 3D scores plot shows good clusterization of linden, chestnut, sunflower, and dandelion honeys, but a clear overlap of acacia, citrus, and rhododendron honeys. The highlighted area for each group corresponds to a confidence interval of 95%.

3.3. Generation and validation of statistical classifiers

To investigate the possibility of authenticating the botanical origin of the honeys only on the basis of the chemical signatures captured by CBS-MS, we built-up the machine learning classifiers. To this aim, we applied four classification algorithms, LASSO, RF, NNET, and PLS-DA. Afterward, we performed a variety of control experiments to ensure that the models' performances were not artifacts of our statistical analysis, and thus, we estimated their error probabilities.

As shown in Table 1, the four classifiers were cross-validated. The hyperparameter optimization plots of each model are shown in the supplementary material (Figs. S4–S7) together with the PLS-DA score plot (Fig. S8). We observed that the classifications of the honey were robust against cross-validation, with the RF achieving the highest classification performances (AUC 0.99, accuracy 0.94, Kappa 0.93, sensitivity 0.94, and specificity 0.99) and the LASSO reaching the lowest performances (AUC 0.97, accuracy 0.85, Kappa 0.82, sensitivity 0.85, and specificity 0.97). The contingency tables of each classifier are reported in Tables S3–S6.

Permutation tests, which are based on random relabeling of the data and on repeating the modelling multiple times, were carried out to ensure the models were not over-fitted. The low performance indicators achieved by the permutation tests showed that the classification performances of each model were not likely due to coincidence or over-fitting (Table 1).

3.4. Annotation of the most significant molecular features

Considering the good performances of the classifiers built on the m/z values retrieved by RF-RFE (and verified by ANOVA test), we explored the possibility of determining these ions' molecular identities. To this aim, we prepared suitable extracts from the same honey samples and subjected them to LC-HRMS/MS. Tentative annotations for 20 of the most informative m/z values were established (Table 2). Fig. S9 of the supplementary material illustrates the HPLC-HRMS/MS of the annotated molecular features.

Table 2 shows the list of the most distinguishing m/z , alongside the observed m/z of precursor ions from HPLC-HRMS and associated mass shifts from theoretical m/z , ion forms, predicted molecular formulas, and hits from the library. We have included the observed diagnostic fragments from LC-HRMS/MS characterization. A literature search further confirmed these assignments, as discussed in the text.

4. Discussion

The described study evaluates the capabilities of CBS-MS as a rapid screening method to ascertain monofloral honey's botanical origin. We demonstrated that the CBS-MS spectra can capture the chemical signatures that differentiate the botanical origin of the seven studied monofloral honey types (Fig. 2). We showed that CBS-MS unveils various molecular features that distinctly characterize the floral source of the honey. Generally, AIMS analyses outcomes largely depend on the matrix effect, whether on food or biomedical samples, resulting in limited quantification, high detection limits, and poor repeatability (Gross, 2014). However, unlike other AIMS techniques, CBS-MS is a solid-phase microextraction (SPME)-based technique that provides a number of advantages over other AIMS techniques: i) it guarantees an efficient sample clean-up and enrichment, ii) it is fast, and iii) it has proven to be an appealing alternative tool for the fast screening of target analytes in complex matrices. These benefits led us to the successful first application of CBS-MS to food authentication in a non-targeted interrogation of the samples without a priori knowledge of the chemical profile. Our statistical analysis of the chemical profiles confirmed that different monofloral honey types, produced by honeybees capturing nectars and pollens of different plant species, can be categorized by different classifiers to a clear extent. All the classifiers achieved highly satisfactory

Table 1

Classification performance indicators (area under the curve-AUC, accuracy, Kappa statistics, sensitivity and specificity) for the evaluations of the Least Absolute Shrinkage and Selection Operator (LASSO), random forest (RF), neural network (NNET) and partial least squared discriminant analysis (PLS-DA) classifiers. Classification performances of each model were evaluated by repeated cross-validation and permutation tests.

Classifier	Classifier evaluation	AUC	Accuracy	Kappa	Sensitivity	Specificity
LASSO	Repeated cross validation	0.97	0.85	0.82	0.85	0.97
	Permutation test (n = 500)	0.59	0.17	0.04	0.18	0.86
RANDOM FOREST	Repeated cross validation	0.99	0.94	0.93	0.94	0.99
	Permutation test (n = 500)	0.57	0.15	0.02	0.17	0.86
NNET	Repeated cross validation	0.98	0.93	0.92	0.94	0.98
	Permutation test (n = 500)	0.58	0.18	0.06	0.20	0.86
PLS-DA	Repeated cross validation	0.98	0.90	0.88	0.91	0.98
	Permutation test (n = 500)	0.60	0.19	0.07	0.20	0.87

performances, with the RF classifier being the most powerful and having the highest values of the key indicators in cross-validation. The high AUC obtained, 0.99, illustrates the high discriminative ability of the RF model, since a good non-targeted method should have an AUC-value close to 1 (USP Pharmacopeia, 2018). In order to exclude overfitting issues, we performed permutation tests, making use of the proof of contradiction. The lowest permutation outcomes were obtained by the RF classifier, demonstrating its reliability and the statistical significance of its performances.

Having addressed the recommended crucial points for a robust validation of our RF model, we are confident with the performance of this non-targeted method that saves time and resources. Unfortunately, an agreed, harmonized, and 'official' workflow for developing and validating non-targeted methods has not yet been published. However, careful late-stage validation is still needed according to Alewijn et al. (Alewijn et al., 2016), the guidelines of the United States (US) Pharmacopoeia (USP Pharmacopeia, 2018), and the recommendations on developing and validating non-targeted methods described in recent reviews (Cavanna et al., 2018; McGrath et al., 2018).

While correctly applying a feature reduction before classification may result in classifiers being less susceptible to population noise compared with wide mass range models, the correct assignment of the molecular features (m/z values) can strengthen their statistical association with the authenticity. However, statistical association as the sole criterion for the use of diagnostic m/z values in a non-targeted approach can be misleading if not supported by additional verification of m/z identity by LC-HRMS/MS analysis in conjunction with additional separation (ion mobility and/or chromatographic) methods (Hanash, 2011; Katz et al., 2021). Therefore, our significant molecular features were tentatively assigned by LC-HRMS/MS.

CBS-MS revealed high levels of kynurenic acid (m/z 188.1), and its potential in-source fragment of m/z 144.1, in chestnut honey. In accordance with Combarros-Fuertes et al., the honeys in which chestnut pollen predominated presented higher amounts kynurenic acid, which suggests the relationship between these compounds and a chestnut source (Combarros-Fuertes et al., 2019; Turski et al., 2016). The high relative abundance of pinocembrin mainly codifies for the differentiation of rhododendron honey. This flavonoid, present in honeys from various botanical origins, has various antioxidant, antimicrobial, and anti-inflammatory properties (Cianciosi et al., 2018). While Kečkeš et al. (Kečkeš et al., 2013) claimed that a large amount of dicaffeoylquinic acid was detected in honey samples derived from annual plants, such as sunflower, the same secondary metabolite allowed differentiation of the geographical origin of multifloral honey (Gasić et al., 2014). Stachyose is an ubiquitous and abundant oligosaccharide of plants, and it has already been detected in Italian acacia honey by ion-chromatography mass spectrometry (Tedesco et al., 2020). Malto-oligosaccharides, such as maltopentaose, are common oligosaccharides in honey (Shinde & Vamkudoth, 2022). Maltopentaose usually characterizes immature honey (Ji et al., 2019).

While our CBS-MS-based approach cannot be a replacement for confirmatory tests (Persano Oddo & Piro, 2004), it provides a

complementary method for the rapid authentication needed by beekeepers and for industrial quality checks. This method could be used by the competent authority prior to launching confirmatory tests on suspicious samples, thereby accomplishing time and resource savings and enabling the more efficient deployment of laboratory instrumentation.

5. Conclusions

This study presents the combination of coated blade enrichment signatures and multivariate statistical analysis to demonstrate the potential of CBS-MS as a rapid authentication platform of high sensitivity and specificity for the differentiation of monofloral honey. The RF algorithm generated the most powerful classifier, based on our multiple tests. The most significant m/z values were annotated by LC-HRMS/MS. This study shows encouraging results that could open a new avenue for the rapid and accurate authentication of monofloral honey. The careful late-stage validation of all non-targeted methods, including controls on the consistency of measurements and classification outcomes over time and with different operators and samples from various geographical origins and years, is mandatory to move these non-targeted methods to official controls and quality checks for accredited certifications. For this reason, future challenges to this CBS-MS method will be made to evaluate its possible adoption in routine analysis. We envision that, compared with the current situation, greater numbers of honey samples could be screened by incorporating CBS-MS into the quality control explorations of the food companies or other analytical laboratories. On the maturity of this CBS-MS method, technicians will be able to perform faster analysis than is currently possible, and at the same time, laborious melissopalinalogy analyses will be performed only on suspicious samples.

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CRedit authorship contribution statement

Alessandra Tata: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation. **Ane Arrizabalaga-Larrañaga:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Andrea Massaro:** Writing – original draft, Formal analysis. **Roberto Stella:** Writing – original draft, Investigation. **Roberto Piro:** Supervision, Project administration, Conceptualization. **Martin Alewijn:** Writing – review & editing, Formal analysis. **Marco Blokland:** Supervision, Resources, Project administration.

Table 2

List of discriminant compounds, retrieved by random forest recursive feature elimination (RF-RFE), and used to build up the RF classifier. The table reports for each discriminant compound: the botanical origin of honey to which the ion contributes to distinguish, the m/z observed by coated blade spray mass spectrometry (CBS-MS), the m/z observed by liquid chromatography-high resolution mass spectrometry (LC-HRMS), the theoretical m/z , the ion adduct, the retention time, the ion assignment, the predicted molecular formula, mass shift (Δ ppm), and the distinguishing MS/MS fragments (product ion 1, product ion 2, product ion 3), the tentative assignment, and the adjusted p-value (p_{adj}) that demonstrates the statistical significance.

BOTANICAL ORIGIN	m/z observed by CBS-MS	m/z observed by LC-HRMS	Theoretical m/z	Retention Time (min)	Ion Assignment	Predicted molecular formula	Δ ppm	Product 1	Product 2	Product 3	Annotation	p_{adj} value
Acacia	665.3	665.2154	665.2140	0.8	[M-H] ⁻	C ₂₄ H ₄₂ O ₂₁	2.04	383.1200	179.0550	161.0450	stachyose oligosaccharide	3.5E-18
Chestnut	144.2	144.0443	144.0449	4.65 and 6.29	[M-H] ⁻	C ₉ H ₇ NO	-4.03	114.0290	86.0310	72.0157	in source fragmentation of kynurenic acid	8.0E-11
Chestnut	188.2	188.0343	188.0348	4.65 and 6.29	[M-H] ⁻	C ₁₀ H ₇ NO ₃	-2.66	144.0440			kynurenic acid	1.2E-14
Chestnut	239.2	239.0822	239.0821	6.28	[M-H] ⁻	C ₁₄ H ₁₂ N ₂ O ₂	0.46	179.0680	102.9600		-	9.0E-10
Chestnut	599.4	599.3194	599.3196	13.5	[M-H] ⁻	C ₂₇ H ₅₃ O ₁₂ P	-0.27	531.2290	283.2700	152.9950	1-stearoylglycerophosphoinositol	3.1E-15
Citrus	121.2	-	-	-	-	-	-	-	-	-	-	9.0E-15
Dandelion	129.2	128.9585	-	0.8	[M-H] ⁻	-	-	-	-	-	-	5.2E-10
Dandelion	153.2	-	-	-	-	-	-	-	-	-	-	8.4E-13
Dandelion	209.2	209.0787	209.0774	7 and 9.5	[M-H] ⁻	C ₆ H ₁₄ N ₂ O ₆	6.41	181.0840			-	3.8E-18
Dandelion and Acacia	785.6	785.3550	785.3596	8.21	[M-H] ⁻	C ₄₆ H ₅₀ N ₄ O ₈	-1.27	665.2990	545.2400	145.0290	N1,N5,N10,N14-tetra-trans- <i>p</i> -coumaroylspermine	5.2E-20
Linden	181.2	181.0861	181.0865	5.5	[M-H] ⁻	C ₁₀ H ₁₄ O ₃	-2.32	163.0760	136.9800	112.9840	In source fragmentation of the ion of m/z 505.4	2.7E-15
Linden	505.4	505.1932	505.1935	5.5	[M-H] ⁻	C ₂₃ H ₃₀ N ₄ O ₉	-0.61	323.0980	221.0700	181.0860	-	2.5E-16
Linden	565.4	565.2136	-	6.2	-	-	-	-	-	-	-	1.2E-10
Rhododendron	255.2	255.0661	255.0657	9.8	[M-H] ⁻	C ₁₅ H ₁₂ O ₄	1.53	213.0650	151.0000	107.0130	pinocembrin	1.4E-12
Rhododendron	827.3	827.2676	827.2669	0.8	[M-H] ⁻	C ₃₀ H ₅₂ O ₂₆	0.85	707.2270	545.1700	383.1210	maltopentaose	4.3E-14
Rhododendron and Acacia	845.2	845.2782	-	0.8	-	-	-	-	-	-	-	2.1E-18
Sunflower	349.4	349.1867	349.1862	6.86	[M-H] ⁻	C ₁₆ H ₃₀ O ₈	1.46	331.1770	187.1330		(1S,2R,4R,8S)- <i>p</i> -menthane-2,8,9-triol 9-glucoside	2.5E-11
Sunflower	399.5	-	-	-	-	-	-	-	-	-	-	5.7E-15
Sunflower	401.4	401.1424	401.1448	14.25	[M-H] ⁻	C ₁₈ H ₂₆ O ₁₀	-6.08	265.1480	115.9200		benzyl O-[arabinofuranosyl-(1->6)-glucoside]	2.7E-11
Sunflower	515.3	515.1201	515.1190	6.55	[M-H] ⁻	C ₂₅ H ₂₄ O ₁₂	2.08	450.8310	353.0900	242.9430	dicafeoylquinic acid	5.5E-16

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 data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2024.110738>.

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