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Characterization of Italian honeys: integrating volatile and physico-chemical data

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Abstract: This article focuses on the comprehensive characterization of Italian honeys using various physico-chemical analyses and their volatile organic compounds (VOCs) fingerprint obtained through the PTR-ToF-MS technology. Honey characteristics, including pH, electrical conductivity, moisture content, hydroxymethylfurfural (HMF), and sugar content, were analyzed to assess their quality and origin. Honey samples from different flowers, including acacia, chestnut, citrus, linden, and multifloral, were collected and investigated. Furthermore, a few aged honeys were collected and analyzed and compared with the fresh ones. Physico-chemical analysis revealed that chestnut honey is characterized by high pH and EC values. Acacia honey has a higher fructose content, while aging appears to influence HMF levels, a vital indicator of honey quality, with aged samples exhibiting significant increases in HMF content. The VOC profiles have been found to vary among different honey types, suggesting that VOCs could be used as indicators of honey origin. Multivariate statistical analyses, such as partial least squares discriminant analysis (PLS-DA), have been applied to the VOCs data to differentiate honey types based on their volatile profiles. Acacia honey exhibited different physicochemical parameters but on the contrary, in the VOCs analysis, it displayed similarities with the linden honey due to their shared low emissions of volatile compounds. Citrus honey had similar chemical parameters to linden and multifloral honeys, but its distinctive VOCs emission allowed for a more accurate identification. In conclusion, the analysis performed with the PTR-ToF-MS was successful in obtaining specific volatile fingerprints of those samples and was effective for improving the characterization of honeys.

1. Introduction

Honey is a natural product known and used by humans since antiquity

(Nikhat and Fazil, 2022). The Italian legislation, transposing Directive 2001/110/EC, defines honey as "the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature". Honey characteristics, such as flavour and physico-chemical properties, can vary substantially depending on botanical and geographical origin (Zhou *et al.*, 2002; Warui *et al.*, 2019). The Italian legislation (D. lgs. 21/05/2004, n. 179) has established thresholds and values for physico-chemical criteria, including moisture, electrical conductivity, hydroxymethylfurfural (HMF), sugar, and others, to evaluate the marketability and quality of honey, which were added to the aromatic profile of honey. Nonetheless, there are roughly 320 distinct types of honey available on the market, which can be grouped into monofloral and multifloral varieties (Vîjan *et al.*, 2023). In Italy, there is a rich assortment of honeys, and this diversity is the result of the unique combination of regional production, climate conditions, and a multitude of floral sources (Castiglioni *et al.*, 2017). Monofloral honey is obtained from bees that have mainly visited a unique botanical species, these honeys are particularly valuable on the market (Schuhfried *et al.*, 2016). As reported by ISMEA, the cost of multifloral honey differs from the cost of monofloral honey (ISMEA, 2023). However, European legislation does not specify the properties of monofloral honey, so countries like Italy imposed a national regulation with a minimum percentage of pollen required for the identification as monofloral, which varied from floral origin depending on the pollen production, position and flower structure of each botanical species (Tedesco *et al.*, 2022). On the other hand, multifloral honey is produced from several types of flowers, and its characteristics and properties can differ greatly depending on the visited flowers and the geographical origin. Melissopalynological analysis is the official method for identifying the botanical and geographical origin of honey (Aronne and De Micco, 2010). However, this analysis is time-consuming and cannot be applied to filtered honey. Moreover, the execution requires palynological competence, which is a limiting factor (Mureşan *et al.*, 2022). In previous studies, PTR-ToF-MS has been used for the categorization of honey types based on their aromatic profiles, such as the monofloral classification (Kuś and van Ruth, 2015; Schuhfried *et al.*, 2016) and for the discrimination of their botanical origin (Ballabio *et al.*, 2018).

Thus, the primary objective of this study was to comprehensively characterize Italian honeys by employing a combination of volatile compound analysis, alongside conventional physico-chemical analyses. By integrating volatile profiling using PRT TOF-MS with established analytical techniques, the aim was to determine whether this analysis could serve as an additional, complementary or substitute method for discriminating different botanical origins of Italian honey.

2. Materials and Methods

Sample collection

Honey samples were gathered in 2022 from May to August directly from beekeepers from different natural geographical macro-areas (districts) of Italy to have a variety of sources that include region, province, altitude, and botanical origins. A total of 84 samples of honey were collected, 78 of these were obtained in 2022, and 6 were collected between 2020 and 2021 (aged samples). Each sample was stored in the dark in a cool and dry place. The collection focused mostly on Italian artisan-produced honey as reported in Table 1. In addition, to achieve even more powerful results, we collected 12 Italian commercial samples. The types of honey were 49 multifloral (of which 6 commercial), 16 acacia (of which 2 commercial), 11 chestnut, five citrus (of which 4 commercial) and three lindens.

Physico-chemical analysis

All the physico-chemical analysis were performed according to the guidelines of the Italian regulation DM 25/07/2003 GU number 185 (Gazzetta Ufficiale, 2003).

Determination of pH. To assess the pH, 10 g of sample was thoroughly mixed in 40 ml ultrapure distilled water (dilution 1:5) from a Millipore Milli-Q lab water system. The resulting solution was measured using a PHM 210 Standard pH Meter (MeterLab, Radiometer Copenhagen), which was previously calibrated with standard pH 4 and pH 7 solutions.

Electrical conductivity. The EC of honey was obtained from the same diluted solution used to assess the pH. The measurement was done using a conductometer (Conductimeter GLP 31 CRISON) cali-

Table 1 - Description of the traits of the samples analyzed, considering the different botanical origin, geographical area, and year of production

Botanical Origin	Region	Province	Production source	Harvest year	No. of samples
Acacia	Tuscany	Firenze	Beekeeper	2022	5
Acacia	Tuscany	Livorno	Beekeeper	2022	1
Acacia	Tuscany	Arezzo	Beekeeper	2022	1
Acacia	Abruzzo	Pescara	Beekeeper	2022	1
Acacia	Tuscany	-	Commercial	2022	1
Acacia	Italy	-	Commercial	2022	1
Acacia	Lombardy	Cremona	Beekeeper	2022	1
Acacia	Abruzzo	Teramo	Beekeeper	2022	1
Acacia	Tuscany	Prato	Beekeeper	2022	1
Acacia	Piedmont	Torino	Beekeeper	2022	1
Acacia	Tuscany	Pisa	Beekeeper	2022	1
Acacia	Emilia-Romagna	Forlì	Beekeeper	2022	1
Chestnut	Tuscany	Firenze	Beekeeper	2022	4
Chestnut	Tuscany	Livorno	Beekeeper	2022	1
Chestnut	Tuscany	Arezzo	Beekeeper	2022	1
Chestnut	Lombardy	Cremona	Beekeeper	2022	1
Chestnut	Piedmont	Torino	Beekeeper	2022	1
Chestnut	Tuscany	Pisa	Beekeeper	2022	1
Chestnut	Emilia-	Forlì	Beekeeper	2022	1
Chestnut	Campania	Salerno	Beekeeper	2022	1
Citrus	Calabria	-	Commercial	2021	1
Citrus	Italy	-	Commercial	2021	1
Citrus	Italy	-	Commercial	2022	2
Citrus	Sicily	Ragusa	Beekeeper	2022	1
Linden	Tuscany	Firenze	Beekeeper	2022	1
Linden	Lombardy	Cremona	Beekeeper	2022	1
Linden	Emilia-	Forlì	Beekeeper	2022	1
Multifloral	Tuscany	Firenze	Beekeeper	2020	1
Multifloral	Tuscany	Firenze	Beekeeper	2021	1
Multifloral	Tuscany	Firenze	Beekeeper	2022	21
Multifloral	Tuscany	Prato	Beekeeper	2021	1
Multifloral	Tuscany	Livorno	Beekeeper	2022	1
Multifloral	Tuscany	Arezzo	Beekeeper	2022	3
Multifloral	Abruzzo	Pescara	Beekeeper	2022	1
Multifloral	Italy	-	Commercial	2021	1
Multifloral	Italy	-	Commercial	2022	5
Multifloral	Lombardy	Cremona	Beekeeper	2022	4
Multifloral	Abruzzo	Teramo	Beekeeper	2022	1
Multifloral	Sicily	Ragusa	Beekeeper	2022	3
Multifloral	Piedmont	Torino	Beekeeper	2022	1
Multifloral	Umbria	Todi	Beekeeper	2022	1
Multifloral	Tuscany	Pisa	Beekeeper	2022	1
Multifloral	Lazio	Roma	Beekeeper	2022	2
Multifloral	Campania	Salerno	Beekeeper	2022	1
Total					84

brated with appropriate standard solutions. The results were expressed in mS/cm. The maximum EC for honey is 0.8 mS/cm according to Italian law (Directive 2001/110/EC), while for the honeydew, multifloral/mixed, and chestnut honey the EC values must be greater than 0.8 mS/cm.

Moisture content. The water content of the honey samples was determined with a handheld refractometer (HHTEC) with automatic temperature compensation. The samples were measured as-is, and the results are expressed as moisture content percent. The legal threshold for selling honey is 20%, but in competitions for premium/quality honeys, the limit is usually lowered to 18%.

Hydroxymethylfurfural (HMF) and furfural (F) quantification by HPLC. The HMF and F were quantified following the HPLC method, which had been previously described in other studies with a few adjustments in accordance with Italian legislation guidelines (Fallico *et al.*, 2004; Truzzi *et al.*, 2012). Briefly, 5 g of honey was diluted with ultrapure distilled water (1:5) and mixed. Then, within 12h, samples have been filtered on a 0.45 µm syringe filter and 20 µl were injected into the HPLC system (Azura, Knauer, Berlin, Germany) coupled to a UV detector (Analytical UV Flow Cell detector UVD 2.1S, Knauer). The chromatographic column was Eurospher II 100-5 C18 150 x 4 mm, and the analysis conditions were: isocratic mobile phase, water-methanol 90:10 v/v; flow rate 0.6 mL/min; column temperature 30°C. The detector wavelength was fixed at 285nm, the identification of HMF and F was done by comparing the retention time of standard solution, and the quantification was done using a calibration curve specific for each molecule (Fig. 1 A). The calibration curve for HMF was made with five solutions at different concentrations (0.0005, 0.005, 0.01, 0.05, 0.1 mg/ml), while the F calibration curve was 0.0006, 0.001, 0.002, 0.006, 0.01 mg/ml. According to the law, the results were expressed in mg/kg, and the legal limit for HMF in commercial honey is 40 mg/kg.

Sugars determination

Brix determination. Brix degrees of the honey samples was measured with the same refractometer of moisture content measurement. Brix degrees represent the percentage of sugar content in honey by weight, with 1 Brix degree equivalent to 1 g of sucrose in 100 g of solution (Geană *et al.*, 2020).

Sugar quantification by HPLC. HPLC coupled to a refractive index detector was used for the qualitative
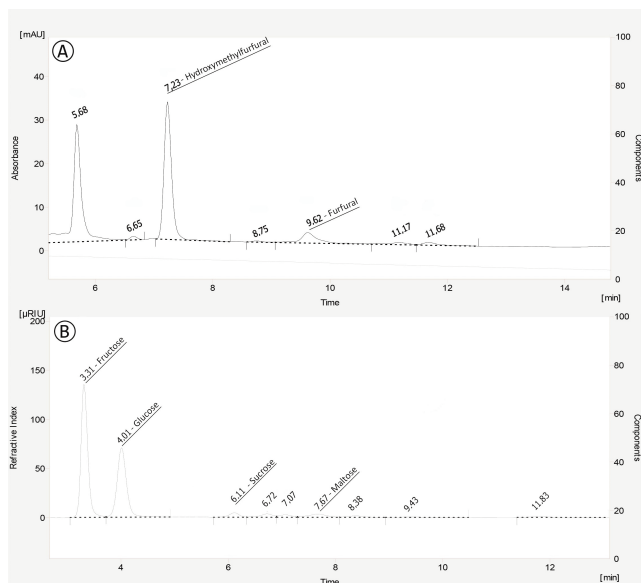
Fig. 1 - Chromatographic profiles of analyzed compounds (A) Chromatogram representing the analysis of hydroxymethylfurfural (HMF) and furfural (F) content in honey samples. The retention times and identified compound names are indicated. (B) Chromatogram illustrating the sugar analysis in honey samples. Retention times and identified sugar names are provided.

and quantitative analysis of sugars (AZURA RID 2.1L, Knauer, Berlin, Germany). The chromatographic column was Eurospher II 100-3 NH2 150 x 4 mm, employing a mobile phase 80:20 of acetonitrile-water and an isocratic flow rate of 1.5 ml/min at 35°C. Honey samples were prepared by placing 0.5 g in 10 mL of H_2O (1:20), mixing for 12-24h, then filtering and diluting 1:1 using the same solution as the mobile phase. Calibration curves were prepared using fructose, glucose, sucrose, and maltose standards for quantification, and retention times were used for identification (Fig. 1 B). Six distinct solutions, each with a different concentration, were employed to construct the calibration curve. The concentrations used were 0, 2.5, 3.75, 5, 7.5, 10, and 15 mg/ml.

Defect identification by sensory analysis

Before to test, each samples were homogenized by mixing with a glass rod, filtered and left until completely clear, after which they were subjected to organoleptic analysis (consistency, color, smell and taste) according to the national standard SR 784-3:2009 (Council European Union, 2001). Particular attention was directed towards identifying any potential defects in the honey samples, with a specific focus on the detection of fermentation. To confirm

the conformance of honeys and eventually exclude samples with imperfections, the odour, colour, taste, and texture of honey were assessed. All the samples were found to be conforming and free from defects based on that assessment.

PTR-ToF-MS measurements and data analysis

Using a PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) with $H3O^+$ as the reagent ion and over the mass range of m/z 20-250, volatile fingerprints of 84 samples were acquired. The benefits of the PTR-MS technology are fully and completely described in a previous study (Blake *et al.*, 2009).

Volatile headspace from each sample was analyzed follow the setup previously proposed by Schuhfried *et al*. (2016) with some modification. In short, 5 g of honey (±0.1 g) were placed into a 250 ml glass jar with two Teflon septa on the cap's opposing sides for the VOCs analysis. Then, each jar has been sealed and fluxed with clean air for 60 seconds before the incubation time, in order to remove all the VOCs accumulated during the sample preparation. Subsequently, the samples have been incubated at 37°C for 30 min in order to allow VOCs to fill the head-space. Finally, the volatile compounds were analyzed using the PTR-ToF-MS in its standard configuration. The zero air-generator (Peak Scientific Instruments) supplied clean air at a flow rate of 0.5 lpm (lpm = liter per minute) to the entry of the sampling device during all analyses, and the same flow rate was set for the PTR-MS inlet flow. To prevent the systematic memory effect, clean air was fluxed for five minutes in the tool apparatus between measurements. For each sample run, 120 s worth of mass spectra were captured. The instrument's settings of 2.20 mbar for the drift-tube pressure, 60°C for the drift temperature, and 550 V for the drift voltage produced an electric field strength to number density ratio (E/N) of 120 Td. Every sample was examined twice. Internal calibration of ToF spectra was performed off-line after dead time correction in order to achieve high mass resolution (Cappellin *et al.*, 2011).

The PTR-ToF-MS's better resolution offers a sum formula and a rough identification of each mass peak found. The TofDaq programme (Tofwerk AG, Thun, Switzerland) was used to collect, record, and analyze the data. Data were expressed in ppbv using a process outlined by Lindigner and Jordan (Lindinger and Jordan, 1998). Finally, all the VOC data were filtered using a threshold of 0.50 ppbv and by eliminating any signals that may be attributed to the chem-

istry of the water or to interfering ions, which are thought to be challenging to precisely quantify. Statistics were applied to the filtered data.

Statistical analysis

Multivariate partial least square-discriminant analysis (PLSDA) (supervised method) was applied to the spectra obtained from 84 honey samples produced by different genotypes, comprehensive of 38 protonated masses, for exploring the possibility of correctly classifying the botanical origin of the honeys (acacia, chestnut, citrus fruits, linden, and wildflower, this last coming from a mix of species). As a pre-processing step, data were submitted to logarithmic transformation and auto-scaling. The whole data set was split into training and validation subset, optimally chosen with the Euclidean distances based on the algorithm of Kennard and Stone (1969). The training set consisted of about 85% of the samples, used for selection of the optimal number of latent variables (LVs), model calibration and cross validation (internal validation). The test set, used to predict the class membership (external validation), included 15% of samples removed from the data set. The training set was used to build a model based on venetian blinds cross validation procedures, evaluated by the number of correct predictions and the root-mean-square error of cross-validation (RMSECV), subsequently validated with the removed samples (external validation set). External validation of the model was quantified by the root-mean-square error of prediction (RMSEP). The optimal number of LVs was selected as those associated to the minimum error and misclassification rate of the calibration dataset. Confusion matrices were used to study the reliability of the models. The threshold to assign a sample to a class was chosen minimizing the number of false positives and false negatives (Bayes theorem). Variable Importance in Projection (VIP) scores (p = 0.01) were also calculated.

PLS-DA analysis was performed using PLS-Toolbox v. 8.0.2 (Eigenvector Research Inc., West Eaglerock Drive, Wenatchee, WA) for MATLAB R2015b (Mathworks Inc., Natick, MA, USA).

In addition, to study the relationships between the different samples as a function of different physico-chemical variables, a Factor Analysis (FA) was applied, considering as factors the content of the different analyzed sugars (glucose, fructose, sucrose, maltose), pH, electrical conductivity (mS/cm), and HMF (5-hydroxymethylfurfuraldehyde) level. This last parameter is essential to evaluate the compliance of honey with current legislation. The level of HMF is used as an indicator of the heating or high temperature storage of the honey. In fact, it is generally not present in fresh honey, while its content increases during conditioning and storage (Zappalà *et al.*, 2005). Furthermore, it is inversely proportional to the fructose content and the fructose/glucose ratio (Kesić *et al.*, 2014).

Factor Analysis (FA) allows to visualize variables and samples simultaneously in a two or three-dimensional space and to study the relationships between the observations (honey samples) and the variables (Greenacre, 1984; Escofier and Pagès, 1992). Computations were performed by XLSTAT Version 2014.5.03.

3. Results and Discussion

pH

The pH values of the examined honey samples fell within the acidic range, varying between 3.5 and 6, reported in Table 2 as the mean and the standard deviation (mean ± SD). Acacia honey exhibited the lowest average pH of 3.77 ± 0.13, closely followed by citrus honey with an average pH of 3.90 ± 0.51.

Table 2 - Physico-chemical honey characteristics. The table reports the value of pH, electrical conductivity (EC), moisture content, hydroxymethylfurfural (HMF), and furfural (F)

	pH	EC (mS/cm)	Moisture content (%)	HMF (mg/kg)	F (mg/kg)
Acacia	3.77 ± 0.13	0.27 ± 0.10	16.44 ± 0.74	0.95 ± 0.45	0.95 ± 0.54
Chestnut	5.19 ± 0.49	1.58 ± 0.33	16.60 ± 0.73	1.78 ± 0.48	2.21 ± 1.15
Citrus	3.90 ± 0.51	0.57 ± 0.54	17.20 ± 0.86	3.50 ± 1.64	2.74 ± 1.38
Linden	4.16 ± 0.06	0.92 ± 0.12	15.33 ± 1.15	2.40 ± 0.20	3.70 ± 1.53
Multifloral	4.12 ± 0.36	0.80 ± 0.42	16.16 ± 1.12	1.67 ± 0.56	1.53 ± 1.30
Aged	4.00 ± 0.19	0.70 ± 0.51	16.48 ± 1.85	6.82 ± 3.04	2.96 ± 1.17

Data are reported as the mean ± standard deviation.

Linden and multifloral honeys demonstrated slightly higher average pH values of 4.16 ± 0.06 and 4.12 ± 0.36, respectively. As expected, multifloral honeys displayed a considerable range of pH values (3.44 to 5.05), reflective of their inherent compositional diversity. In contrast, chestnut honey exhibited the highest pH value of 5.19 ± 0.49 among the tested varieties. Notably, the pH of aged honey, at 4.00 ± 0.19, aligned closely with the pH values of other honeys of corresponding botanical origins, such as citrus and multifloral. The acidic nature of the honey samples has implications for their antimicrobial activity (Acquarone *et al.*, 2007). The observed pH variations align with previous findings, with acacia and citrus honeys consistently displaying lower pH levels while chestnut the highest (Bertoncelj *et al.*, 2011; Živkov-Baloš *et al.*, 2018). The near 4 pH level observed in other honey types is consistent with results reported in earlier studies (Truzzi *et al.*, 2014).

Electrical conductivity (EC)

The EC of honey samples ranged between 0.1 and 2 mS/cm, depending by the botanical origin (Table 2). Acacia honeys were characterized by a considerably low EC (with an average value of 0.27 ± 0.10 mS/cm); in contrast, chestnut honeys had high EC values, with an average of 1.58 ± 0.33 mS/cm and a maximum value of 1.96 mS/cm. Citrus showed a mean of 0.57 ± 0.54 mS/cm, and linden of 0.92 ± 0.12 mS/cm. On the other hand, multifloral honeys exhibited a wide range of values, from 0.21 mS/cm to 1.86 mS/cm, the overall mean was 0.80 ± 0.42 mS/cm. Additionally, aged honey samples did not show significant differences when compared to honey of the same botanical origin (0.69 ± 0.51 mS/cm). The EC values of most honey samples fell within the standard limit (Table 2), with the exception of two honeys. Notably, linden honey exhibited an average electrical conductivity (EC) that exceeded the established threshold. However, when considering a limit of 0.8 mS/cm, the EC values for linden honey (0.92 mS/cm) remained acceptable, thanks to an exemption stated in D.lgs. 179/04, which allows EC levels above 0.8 mS/cm. Nevertheless, EC is considered a reliable indicator of the botanical origin of honey. Chestnut honey is characterised by a high EC value, followed by linden honey, which also displayed a relatively high value, as reported in other studies (Truzzi *et al.*, 2014; Živkov-Baloš *et al.*, 2018). Excluding the outlier value of citrus honey, the average EC of the samples was similar to findings in other studies (0.24 mS/cm) (Di Marco *et al.*, 2017; Di Rosa *et al.*, 2019). Conversely, multifloral honeys showed a wide range of EC values, ranging from 0.21 mS/cm to 1.86 mS/cm, reflecting the variation in floral sources visited by the bees.

Moisture content

The moisture content of all the honey types ranged from 13.4% to 19.6%, and all the samples were under the maximum limit of 20% (Table 2). Moreover, 94% of the samples meet the criteria for quality competitions (value ≤ 18%). The highest average value was 17.20 ± 0.86% of citrus honey, while the lower was 15.33 ± 1.15% of linden honey. Acacia, chestnut, multifloral, and aged honeys had similar values of 16.44 ± 0.74%, 16.60 ± 0.73%, 16.16 ± 1.12%, and 16.48 ± 1.85%, respectively. Honey moisture content is an important factor and a parameter used to evaluate the product's quality. Values that are too low can cause processing problems, while values that are too high could lead to the onset of fermentation processes, altering its quality, shelf life, taste, and composition (El Sohaimy *et al.,* 2015). There was no discernible difference between the water content of various varieties of honey when the samples were compared, despite the significant variety and botanical origin of the samples. Indeed, there is a relationship between moisture content and honey maturation, production season, ventilation of the beehive, meteorological conditions and work processes (Kirs *et al.*, 2011; Escuredo *et al.*, 2014; De Sousa *et al.*, 2016; Lazarević *et al.*, 2017).

Hydroxymethylfurfural (HMF) and furfural (F)

The HPLC quantification of HMF (hydroxymethylfurfural) showed that all samples had HMF content within the standard thresholds. Acacia honey exhibited an average HMF content of 0.95±0.45 mg/kg, while chestnut honey showed a higher value of 1.78 ±0.48 mg/kg of HMF. Citrus honey recorded an even higher content, with 3.50±1.68 mg/kg of HMF with the higher value represented from the aged commercial sample. Samples of linden honey displayed an average HMF content of 2.40±0.20 mg/kg, while multifloral honey showed a mean value of 1.67±0.56 mg/kg of HMF. However, the main difference was highlighted between fresh and aged honey. Indeed, samples of aged honey showed a considerable rise in HMF content, with an average of 6.82 ± 3.04 mg/kg. All honey harvested in 2022 had values from 0.5 to 3 mg/kg, while aged honey had significantly higher values from 4.6 to 12.12 mg/kg (Table 2).

These results clearly indicate that ageing process can significantly influence HMF levels, which serve as

an important indicator of honey quality and freshness. Indeed, as reported in previous studies, these compounds are related to the heating practices and preservation conditions of honey and derive from the degradation of fructose (Aronne and De micco, 2010; Tedesco *et al.*, 2022).

In the same chromatographic run of HMF, furfural (F) data was also obtained. Acacia honey showed a mean of 0.95 ± 0.54 mg/kg, representing the honey with the lowest average F content, ranging from 0 to 1.89 mg/kg. Multifloral honey followed with a content of 1.53-1.30 mg/kg. Chestnut honey exhibited an average F content of 2.21 ± 1.15 mg/kg. Both citrus and aged honey displayed similar values of F: 2.74 ± 1.38 mg/kg and 2.96 ± 1.17 mg/kg, respectively. The highest mean value was found in linden honey (3.70 ± 1.53 mg/kg); however, the sample with the highest F content was multifloral honey with 6.74 mg/kg. Also, if neither restrictions nor indications are reported for furfural in the legislation, it is related to storage and of honey, since both F and HMF are usually produced by the Maillard reaction (Zhang *et al.*, 2009). However, the average furfural content in the different honey types was in line with other studies (Gaspar and Lopes, 2009; Apriceno *et al.*, 2018; Tedesco *et al.*, 2022).

Brix

The degree Brix analysis, representing the total sugar content in honey, revealed that all honey samples exhibited values ranging from 78.8% to 85.5%. Among the varieties, citrus honey displayed the lowest mean value (81.10±0.84%), while linden honey showcased the highest (82.93 ± 1.10%) (Table 3). Similarly, in line with previous studies, our Brix values were found to be comparable to those reported, reaffirming the absence of significant distinctions in sugar content among different honey botanical origins.

However, similar to what was reported in earlier studies that also found similar brix values, the study of sugars using the refractometer did not reveal any appreciable differences between different types of honey (Oroian and Ropciuc, 2017; Geană *et al.*, 2020).

Sugar quantification

Quantitative analysis of fructose, glucose, sucrose, and maltose was conducted using HPLC, with results expressed as percentages (g/g). Comprehensive data, including the sum of fructose and glucose, as well as individual sugar levels, are presented in Table 3. Acacia honey exhibited the highest fructose content at 49.08 ± 1.71%, while aged honey displayed the lowest fructose content (39.18 ± 3.50%). The fructose content across all samples ranged from 33.1% to 52.8%. Glucose content ranged from 22% to 40.2%, with chestnut honey demonstrating the lowest average value (27.24 ± 2.93%) and aged honey the highest (33.71 ± 4.76%). The range of maltose concentration was 0.9% to 4.8%, with chestnut honey having the highest level and linden honey the lowest (Table 3). Additionally, according to Council Directive 2001/110/CE of December 20, 2001, in unadulterated honeys, the sum of glucose and fructose should not fall below 60 g/100g for nectar honey, while sucrose must not exceed 5 g/100g. More specifically, 5g/100g for Acacia (*Robinia pseudoacacia*), Lucerne (*Medicago sativa*), Banksia (*Banksia menziesii*), Sulla (*Hedysarum coronarium*), Eucalyptus (*Eucalyptus camaldulensis*), not more than 10 g/100 g for Citrus (*Citrus* spp.) honey, and no more than 15 g/100 g for Lavender (*Lavandula* spp.) and Borage (*Borago officinalis*). In reference to these criteria, all 84 samples were unadulterated, in accordance with the legislation. The sum of glucose and fructose ranged from 63.80 to 84.90%, affirming the high quality of the honey samples, while sucrose was always lower than

Table 3 - The table reports the value (average and standard deviation) of degree Brix of honey (%) that represents the total sugar content and Fructose, Glucose, and Maltose expressed as percentages (g/g). In the table it is also reported the sum of Glucose and Fructose (G+F) and the Fructose Glucose ratio (F/G)

	Brix	Fructose	Glucose	Maltose	G + F	F/G
Acacia	81.90 ± 0.72	49.08 ± 1.71	30.05 ± 2.08	3.12 ± 0.45	79.12 ± 2.68	1.64 ± 0.13
Chestnut	81.71 ± 0.77	45.34 ± 2.43	27.24 ± 2.93	3.36 ± 0.82	72.58 ± 4.76	1.68 ± 0.15
Citrus	81.10 ± 0.84	42.65 ± 2.57	32.79 ± 5.24	2.90 ± 1.02	75.44 ± 6.74	1.32 ± 0.17
Linden	82.93 ± 1.10	43.28 ± 2.02	28.39 ± 8.25	2.48 ± 0.80	71.66 ± 8.22	1.61 ± 0.42
Multifloral	82.19 ± 1.16	43.85 ± 3.71	30.26 ± 3.79	2.98 ± 0.87	74.11 ± 5.25	1.47 ± 0.23
Aged	82.24 ± 1.89	39.18 ± 3.50	33.71 ± 4.76	2.74 ± 0.62	72.89 ± 7.81	1.17 ± 0.10

1.8%, detected in only 15 samples of the total, with a maximum value of 1.8% (7 acacia, 1 citrus and 7 multifloral). Moreover, the Fructose/Glucose (F/G) ratio was calculated. The F/G ratio determines whether honey may crystallise; therefore, a ratio higher than 1 suggests a fluid honey, whereas a ratio lower than 1 indicates honey crystallizing more quickly (Geană *et al.*, 2020). The highest values were found in acacia, chestnut, and linden honeys (1.64, 1.67, and 1.60, respectively), indicating honey's ability to remain liquid for a longer amount of time. Citrus, multifloral flowers, and aged honeys, on the other hand, showed lower ratios (1.32, 1.47, and 1.17, respectively). Additionally, no samples had a value lower than 1, however aged honey with an F/G ratio of 1.17 is most likely to have crystallized. In the current study, the fructose and glucose values found across different honey samples are, on average, higher compared to those reported in other studies. However, the F/G ratio for acacia, citrus, and multifloral honeys remains consistent with literature values (Oddo and Piro, 2004; Geană *et al.*, 2020).

Factor analysis (FA)

In figure 2, the FA biplot simultaneously represents the relationship between the different sugars analyzed (glucose, fructose, sucrose, maltose), the level of HMF, the pH and the electrical conductivity, highlighting the relative distances among the 84 honey samples. The first axis explains 37.18% of the total variability in the spectral data, the second axis 18.01%. From the FA graph, some groups of samples emerge which seem to be related to a compound or
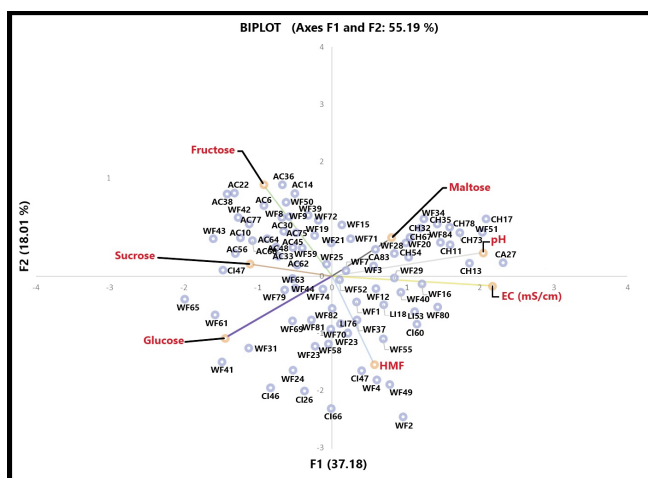

Fig. 2 - Biplot from Factor analysis. Relationships between 84 honey samples and different physicochemical parameters. AC = acacia, CI = citrus fruits, CH = chestnut, WF = wildflower, LI = linden.

to a specific physico-chemical characteristic of the honey. In particular, the wildflowers are concentrated on the HMF vector, and, in a diametrically opposite position, the samples of acacia honey are grouped very close along the fructose axis. This is in accordance with the fact that HMF is formed through the degradation of fructose, thus establishing a negative correlation between these two parameters. Citrus and Linden honeys are situated in the lower region of the graph, indicating slightly higher glucose values for citrus honey and higher HMF values for both honeys. The pH values are also significantly higher in chestnut samples, although maltose seems to somehow influence its distribution.

PTR-ToF-MS results

Data on the emissions of volatile organic compounds (VOCs) from the five honey groups - Multifloral, Acacia, Chestnut, Citrus, and Linden- are presented in Table 4. All signals have been separated according to their respective molecular weights and are expressed as the mean concentration in parts per billion by volume (ppbV). The Table shown a subset of 33 compounds obtained upon filtering the data (were eliminated all signals with an average concentration below to 1 ppbV). From our study on 84 different honey samples a total of 37 different compounds with an average value higher than 1ppbv were found. Among these, the peaks with the higher emission were detected at 33.034, 45.033, 47.010, 59.049, which corresponding to the following compounds methanol, acetaldehyde, formic acid and acetone. Similar results were obtained from other studies on honey samples from different botanical origins (Kuś and van Ruth, 2015; Schuhfried *et al.*, 2016). The average of total emission recorded for each honey botanical origin varies from a minimum value of 265.2 ppbv for Acacia and 336.1 for Linden honey to a maximum value of 1971.8 for citrus honey (Table 4). Acacia and Linden honey showed a rather similar volatile profile characterized by both a lower level of emission and a lower number of signals (30 and 28 respectively) compared to the other botanical origins. Citrus honey emerged both for a higher emission of methanol, acetaldehyde and acetone compounds as well for a higher emission of terpene compounds (mz 111.101, 121.101, 135.116, 137.132) compared to the other botanical origins. Chestnut samples are characterized by large signals of compounds detected at m/z: 69.033 (C4H5O+, Tentatively Identified as Furan), 83.086 (C6H11+, TI as C6 compounds) and 105.069 ($C_5H_{13}S^+$, TI pen-

Table 4 - Number of signals detected, chemical formula, tentative identification, VIP score and average amount (ppbV) of each compound detected from different honey samples

N° of compounds	mz	Chemical Compound	Tentative Identification*	Multifloral (n=49)	Acacia (n=16)	Chestnut (n=11)	Citrus (n=5)	Linden (n=3)
1	27022	C2H3+	Acetylene	33.49	10.80	30.91	99.50	11.85
2	33033	CH5O+	Methanol	189.78	61.30	61.15	243.54	49.04
3	41038	C3H5+	Alkyl fragment	22.58	5.39	11.16	50.39	19.03
4	43018	C2H3O+	aceton or acetate fragments	44.33	14.86	47.49	87.52	23.39
5	45033	C2H5O+	Acetaldehyde**	369.53	88.24	506.01	884.92	106.64
6	47010	CH3O2+	Formic acid/formats**	25.21	7.05	41.97	180.30	7.90
7	49011	CH5S+	S compound (Methanethiol)	3.23	2.10	2.93	8.96	2.00
8	53038	C4H5+	Cyclobutadiene	2.48	2.03	2.21	2.79	2.00
9	55054	C4H7+	Alkyl fragment	6.43	4.09	5.93	18.59	3.68
10	57069	C4H9+	Alcohol fragments	13.25	5.02	3.02	6.33	2.59
11	59049	C3H7O+	Acetone**	119.70	42.56	210.57	284.23	100.21
12	61028	C2H5O2+	Acetic acid	12.25	7.25	11.14	15.98	5.94
13	63033	C2H7S+	S compound (Dimethyl sulfide)	13.07	4.07	17.29	25.27	2.15
14	65.00	C5H5+	S compound	2.59	Tr	2.35	3.63	Tr
15	67050	C5H7+	3-Penten-1-yne/Terpene fragment	Tr	Tr	Tr	2.65	Tr
16	69033	C4H5O+	Furan	5.33	2.93	16.02	5.84	Tr
17	71086	C5H11+	Alcohol compounds	2.24	2.77	2.24	1.10	1.10
18	73054	C4H9O+	Butan-2-one	13.60	5.54	10.70	20.21	3.48
19	75044	C3H7O2+	Isobutanol	2.67	2.71	2.58	2.64	2.33
20	77038	C6H5+	Alkyl fragment	2.11	2.03	2.06	2.14	1.01
21	79049	C6H7+	Benzene/terpene fragment	2.88	2.72	2.52	3.34	0.00
22	83086	C6H11+	C6 compounds	2.35	2.02	6.92	2.87	1.10
23	85059	C5H9O+	(E)-2-Pentenal	3.14	2.17	2.14	3.21	0.00
24	87044	C4H7O2+	2,3-Butanedione	7.59	3.65	10.10	15.91	3.81
25	93069	C7H9+	Terpene fragments	Tr	Tr	Tr	3.23	Tr
26	95011	C2H7O2S+	dimethyl sulfone	7.34	4.53	3.52	6.44	3.82
27	97033	C5H5O2+	Furfural	6.09	3.26	4.30	8.49	2.63
28	99044	C5H7O2+	Furfuryl alcohol	Tr	Tr	Tr	Tr	Tr
29	101060	C5H9O2+	Dihydro-methyl-furanone	Tr	Tr	0.00	0.00	0.00
30	103080	C5H11O2+	2-/3-methylbutyric acid	Tr	0.00	0.00	2.71	0.00
31	105069	C5H13S+	Pentanethiol**	Tr	0.00	4.26	1.89	Tr
32	107086	C8H1 1+	1,3-Dimethylbenzene/Terpenes fragments	Tr	Tr	1.87	1.58	0.00
33	109070	C7H9O+	Benzyl alcohol	Tr	0.00	Tr	1.12	1.41
34	111101	C8H15+	Terpenes fragments**	Tr	0.00	Tr	1.88	0.00
35	121101	C9H1 3+	Terpenes fragments	Tr	0.00	1.12	2.54	0.00
36	135116	C10H15+	Terpenes**	Tr	0.00	Tr	2.10	0.00
37	137132	C10H17+	Terpenes	Tr	0.00	0.00	2.08	0.00
			Total emission average	889.30	265.28	996.51	1971.86	332.26
			Total signals detected	37	30	34	36	28

* Each value has been tentatively assigned to compounds, based on PTR-honey literature data (Kuś and van Ruth, 2015; Schuhfried *et al.*, 2016; Ballabio *et al.*, 2018).

** compounds with the highest VIP score.

Tr means trace and these compounds have been identified in at least 1 sample per group but with an overall average value below 1 ppbV.

tanethiol) in agreement with Ballabbio *et al.* (2018). As can be seen from the data shown in the table 4, the average volatile profile of multifloral honey showed an average emission for many signals and trace compounds in large numbers (identified only in some samples).

No significant differences were observed between the volatile organic compound (VOC) emissions of commercial honey and those produced by beekeepers.

PLS-DA analysis

With the aim to get an overview of the VOC data collected, a PLS-DA analysis was applied on the whole dataset obtained from 37 different VOCs data collected from 84 samples. It emerges that the honey samples distance themselves from each other according to their botanical origin. Multifloral honey seem to show a variable trend probably linked to its botanical origin. To provide a more detailed characterization of the VOCs emitted by different honey samples, VIP scores higher than 1 and their possible identification on the basis of literature data were reported in Table 4 (marked by two asterisks). The volatile compounds with higher VIP value could be good candidates for the honey species identification. In particular, the chemical species with the higher significance were detected at mz 45.033(TI Acetaldehyde), 47.01 (TI Formic acid/formates), 59.049 (TI Acetone), 105.069 (TI Pentanethiol), 111.10 (TI Terpenes fragments),

135.116 (TI Terpenes).

PLS-DA approach was applied to find VOCs able to discriminate among species. By applying the model developed by the PLS-DA on honey samples of different botanical origin, a correct distinction of the taxonomic category of two/five different groups was achieved. Indeed, the multifloral honey could be obtained by honeybees from the nectar of different flowers. Score plot from the PLS-DA model is shown in figure 3. The global quality of the model, evaluated by its performances indicators (Table 5), resulted robust enough to discriminate the botanical origin of the citrus and chestnut samples compared to the others in the calibration/validation data set, and in the independent test set. Indeed, the PLS-DA three-component model successfully classified 100% of
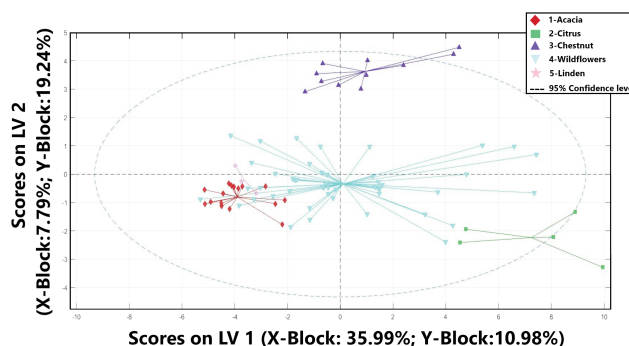

Fig. 3 - Score plot (LV1, LV2) of the PLS-DA model. Samples of different botanical origin are highlighted. Red = acacia; green = citrus fruits; blue = chestnut; light blue = wildflower; lilac = linden.

Table 5 - PLS-DA statistics for the honey samples for the five botanical origins: 1 = acacia, 2 = citrus fruits, 3 = chestnut, 4 = wildflower, 5 = linden. Sensitivity (SE), Specificity (SP), Class. Error, RMSEC, RMSECV, RMSEP, for Calibration (Cal), Cross Validation (CV), and Prediction (Pred), respectively

| Statistics | Y-BLOCKS | | | | |
	Class 1 - acacia	Class 2 – citrus fruits	Class 3 - chestnut	Class 4 - wildflower	Class 5 - linden
Sensitivity (SE) (Cal)	1.000	1.000	1.000	0.721	1.000
Specificity (SP) (Cal)	0.800	1.000	1.000	0.724	0.643
Sensitivity (SE) (CV)	0.833	1.000	1.000	0.744	1.000
Specificity (SP) (CV)	0.800	0.970	1.000	0.552	0.643
Sensitivity (SE) (P)	1.000	0.000	0.000	0.000	1.000
Specificity (SP) (P)	0.875	1.000	0.833	0.182	0.583
Class. error (Cal)	0.100	0.000	0.000	0.277	0.178
Class. error (CV)	0.183	0.015	0.000	0.352	0.178
Class. error (Pred)	0.062	0.500	0.583	0.909	0.208
RMSEC	0.319	0.104	0.169	0.414	0.161
RMSECV	0.328	0.159	0.199	0.464	0.166
RMSEP	0.387	0.296	0.719	0.674	0.044

honey samples from classes 2-chestnut and 3-citrus into their respective taxonomic categories during fitting, cross-validation (internal validation), and prediction (external validation), while the acacia and linden honey samples are confused with the multifloral samples.

4. Conclusions

In this study, a comprehensive analysis of various physico-chemical properties and volatile organic compounds (VOCs) present in Italian honeys was conducted. It was revealed that the quality of the honey sold is excellent, as legal limits were adhered to for all samples (except for few EC values). The electrical conductivity (EC) values demonstrated significant variability, with chestnut and linden honeys standing out due to their high and relatively high EC values, respectively. The observed pH variations among different honey types were consistent with their botanical origins. Furthermore, parameters such as sucrose content and the fructose-to-glucose ratio, indicative of potential adulterations, remained within legal limits for both commercial and beekeeper honey.

Moreover, within this context, the honey varieties were discerned based on their distinctive characteristics.

Aged honey, as expected, was characterized by a high HMF level; however, it remained within legal thresholds. Acacia honey, characterized by its high fructose content, exhibited low pH and EC values. Interestingly, in VOC analysis, it displayed similarities with linden honey due to their shared low emissions of volatile compounds. Chestnut honey, which had high pH and EC values, was easy to differentiate from other types of honey using both conventional metrics and PTR-ToF-MS-based VOC analyses. Its distinctive profile made it simple to classify. Citrus honey displayed physicochemical characteristics similar to linden and multifloral honeys, but its distinctive VOC emissions allowed for a more accurate identification. Conversely, linden and multifloral honeys shared close resemblances in chemical and physical analyses, and the significant variability in the multifloral variety's composition due to its diverse floral sources hindered differentiation through VOCs.

Factor Analysis provided insights into the relationships between different sugars, HMF, pH, and electrical conductivity, highlighting distinct groupings of honey samples. Finally, VOCs analysis revealed a diverse range of compounds, with noticeable variations attributed to the botanical origin of the honey. Partial Least Squares-Discriminant Analysis (PLS-DA) facilitated discrimination among different honey types based on their VOC profiles highlighting the potential for this method in distinguishing honey types based on VOC profiles. The analysis of VOCs has the advantage of being a faster alternative to pollen analysis, providing an efficient means of differentiating between honey samples with varying botanical sources. For this reason, PTR-ToF-MS-based VOC analysis serves as a valuable tool to complement or even replace melissopalynological analysis, as demonstrated by the effective combination of VOC analysis and PLS-DA for distinguishing honeys of different botanical origins.

In conclusion, the collective application of physico-chemical and VOC analyses yielded a comprehensive means of effectively characterizing honey varieties. This integrated approach underscores the robustness of employing multiple techniques for a thorough understanding of honey attributes.

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