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Authentication of the geographical origin of Italian multifloral honey: multi-analytical characterization of the composition and pesticide residues to distinguish the production area

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ABSTRACT

Verifying the geographical origin of honey has become a key issue for food quality assurance, consumer safeguards and regulatory enforcement, particularly following recent European regulation mandating verifiable origin labeling. This task is particularly challenging for multifloral honey, as its chemical composition is shaped by local biodiversity, environmental factors, and agricultural activities. In this work, an integrated multi-analytical strategy was employed to characterize Italian multifloral honeys and identify indicators linked to their origin. Seventy-five multifloral honey samples collected from Northern, Central, and Southern Italy were examined using a combination of analytical techniques, i.e., the untargeted profiling of volatile organic compounds (VOCs) via dielectric barrier discharge ionization high-resolution mass spectrometry (DBDI-HRMS), quantification of sugars and amino acids by quantitative nuclear magnetic resonance (q-NMR), elemental analysis by total reflection X-ray fluorescence (TXRF), and and targeted assessment of pesticide residues. In parallel, several thousand melissopalynological analyses performed on multifloral Italian honey collected during 2015–2024 were statistically analyzed to retrieve the pollen species that characterize the Northern, Central and Southern Italian macro-areas. The statistical evaluation revealed clear regional patterns in pollen counts, VOCs, mineral composition, and specific sugars and amino acids, highlighting the influence of local flora, geological background, and environmental conditions. DBDI-HRMS exhibited a high ability to differentiate between Italian macro-regions, with cresol, santene, and acetoaminophenone having a pivotal function in demonstrating the bees' collection of chestnut, rhododendron, and linden nectars in the honey produced in Northern Italy, which is in alignment with the results of our pollen counts. Moreover, our DBDI-HRMS findings indicate that methylsyringate is a typical marker of Central and Southern honeys which had the highest counts of citrus pollen. Similarly, our melissopalynological assessment revealed that Italian Southern honey is distinguished by its high prevalence of citrus pollen. Q-NMR analysis identified distinctive trends in minor carbohydrates and amino acids. Honey from Southern Italy exhibited high proline, leucine, and isoleucine concentrations. These are quality indicators related to specific botanical origins, such as citrus and eucalyptus, which are prevalent in this region as demonstrated by our melissopalynological data. Honey samples originated from central and southern Italy presented significantly elevated bromine levels, probably due to the contribution of marine aerosol, and strontium concentrations revealed moderate increase along the north–south gradient. Within this integrated framework, pesticide residues were interpreted as indicators of environmental exposure and agricultural context rather

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than as direct geographical markers. Levels of pesticide residues, were generally minimal and largely compliant with regulatory thresholds, confirming the overall high quality of Italian honey. Overall, 62 out of 75 honeys were either completely residue-free or contained only trace amounts of pesticides. The low concentrations of acaricides, such as amitraz, fluvalinate, and coumaphos, reflects controlled use in hive treatments. The occasional detection of agricultural pesticides, such as propamocarb and chlorphenvinphos, suggests localized crop-related inputs and environmental drift consistent with regional land use. Collectively, the combination of diverse analytical datasets provides a solid approach for the geographical authentication of multifloral honey, supporting origin verification and the development of future classification models.

1. Introduction

In recent years, determining the geographical provenance of food commodities, including honey, has become a Central issue in food quality control and fraud prevention. In 2022, results from the European coordinated control plan *From the Hives* revealed that adulteration practices are widespread. Specifically, additives and colorants were used to mask the true botanical origin of honey, traceability information was falsified, and pollen was intentionally removed to conceal its authentic geographical and botanical origin [1]. In response to such findings, the European Commission (EC) has strengthened the legislation to ensure transparency and traceability [2]. For single-origin honeys, the country of origin must be specified, and any claims regarding botanical or geographical origin must be verified. Additional indications on floral, vegetal, regional, or topographical origin are permitted only when the honey originates wholly or predominantly from the stated source and meets its organoleptic, physico-chemical, and microscopic criteria. These requirements reflect the fact that the honey price is strongly influenced by both botanical and geographical origins. Consequently, the verification of the botanical and geographical origins indicated on the label is of pivotal importance.

The EC has encouraged the development of harmonized analytical methods to verify honey's authenticity and ensure compliance with quality specifications. This is particularly challenging for multifloral honeys, which are natural mixtures of nectars from diverse plant species whose composition depends on local flora, soil conditions, and climatic factors [3]. For example, multifloral honeys produced in different geographical areas express the biodiversity of their environments, and those from different regions show distinct physico-chemical profiles, especially in sugars, phenolic compounds, flavonoids, and minerals, ultimately influencing their organoleptic qualities and nutritional properties [4]. Despite its importance, the geographical origin of honey is often declared without formal analytical verification. Therefore, identifying the physico-chemical properties of Italian multifloral honey that are correlated with geography is important for determining the best analytical approaches.

Melissopalynological analysis remains the gold standard method for determining the geographical origin of honey through microscopic identification and quantification of pollen grains [5]. However, it is labor-intense, operator-dependent, and vulnerable to fraud, since pollen can be removed or manipulated [6]. These limitations have prompted the development of alternative or complementary analytical techniques in the last two decades. Physico-chemical parameters [7,8], mineral composition [9], secondary metabolites [10–13], sugars [14], amino acids [15] and volatile organic compounds (VOCs) [16–24] have been extensively investigated as potential markers of botanical and geographical origin.

Consumer perception of honey quality depends on its aroma, which is related to its composition. In addition to traditional melissopalynology, the VOC profile varies widely with geography, so has been considered as a honey authentication strategy. VOCs in honey are usually analyzed by gas chromatography–mass spectrometry, which is precise, but laborious [23,25,26]. It is worth noting that the concentration of VOCs in honey is so low that sugars must be removed before analysis. This is usually done by liquid-liquid extraction, simultaneous steam distillation extraction, or hydrodistillation [27]. Then, to avoid the use of toxic, and unsustainable organic solvents, new methods were developed to extract the VOCs from untreated samples, such as solid phase extraction (SPE) [28], headspace (HS) extraction [29], and solid phase micro-extraction (SPME) methods [30]. Moreover, innovative untargeted methods were also proposed in the last decade [31]. Among them a very sustainable, and quick approach for analysis of VOCs is dielectric barrier discharge ionization high resolution mass spectrometry (DBDI-HRMS) in combination with multivariate statistical analysis [32]. DBDI is an ambient ionization source based on the formation of a low-temperature plasma that is produced by discharge between two electrodes separated by a dielectric barrier, and which has been used for the detection of the volatiles in a variety of food matrices [33–37].

Elemental analysis of honey is necessary to assess the botanical origin and nutritional value [38]. The major elements, like potassium (K), calcium (Ca), magnesium (Mg), and phosphorus (P), are typically found in the highest concentrations, reflecting the mineral composition of the floral sources and contributing to major quality features. Trace elements, like manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn), are important for biological processes and are used to determine botanical and geographical origins [39]. Metal determination is analytically difficult due to the significant interference that occurs in this high sugar content matrix.

Numerous studies have shown that honey's sugar and amino acid composition differs significantly based on floral sources, environmental conditions, and geographical factors [15,40,41]. Quantitative nuclear magnetic resonance (qNMR) has emerged as a powerful tool for analyzing complex mixtures, offering structural and chemical characterization with minimal sample preparation [42]. Due to the proven precision of NMR in the analysis of sugar and amino acid profiles in honey [14,43–45], this technique was employed to examine these compounds our samples.

Honey is not only a food of high nutritional and commercial value, but also an important biological matrix for environmental monitoring. European legislation imposes strict controls on pesticide residues to ensure food safety: Regulation (EC) No. 396/2005 establishes maximum residue levels (MRLs) for pesticides in food products, including those of animal origin such as honey, setting a default limit of 0.01 mg/kg in the absence of specific values [46].

Honey contamination with pesticides can arise from direct exposure to substances used in beekeeping practices (i.e., acaricides applied to control Varroosis) and from indirect environmental sources linked to plant protection products applied in agriculture and to broader environmental pollution [47]. The pesticide pollution typical of intensively cultivated agricultural areas is of particular concern, as these compounds accumulate in vegetation, water, and soil, potentially harming non-target organisms, such as honey bees (*Apis mellifera* L.) and their products [48,49]. Although several compositional markers (e.g., VOCs, minerals, sugars, amino acids) have been individually proposed as indicators of geographical origin, there is still a lack of integrated, multi-analytical approaches capable of reliably discriminating the geographical provenance of multifloral honeys. This represents a critical gap, especially for Italian multifloral honey, whose diversity reflects the wide environmental heterogeneity of the country but is often not analytically verified. To address this gap, the present study applies a comprehensive, multi-platform analytical strategy to obtain a comprehensive characterization of Italian multifloral honey correlated to geography. This study combines multiple analytical approaches: VOC profiling by DBDI-HRMS, sugar and amino acid analysis by q-NMR, mineral determination by TXRF, and contaminant analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas-chromatography-tandem mass spectrometry (GC–MS/MS). Specifically, these analytical techniques were applied to honeys collected across the three Italian macro-regions, Northern, Central and Southern Italy, with the aim to investigate their potential contribution to determining the geographical origin of honey. The results were correlated to melissopalynological data. The workflow of the study is illustrated in Fig. 1.

2. Materials and methods

2.1. Honeys

A total of 75 multifloral honeys, harvested in 2024 in Italy and equally distributed among the three Italian macro-areas (Central, Northern, Southern Italy), were characterized in terms of VOCs sugar, and mineral profiles. Moreover, the same honeys were analyzed to evaluate the presence of pesticides. The list of honeys, including macro-area, region of production and altitude above sea level, is reported in the supplementary material (**Table S1**).

The multifloral honeys were collected during the most important national competition ("Tre Gocce D'oro"), organized by the Osservatorio Nazionale Miele, the national support body for the beekeeping sector that associates beekeeping organizations at national and regional levels. Note that the sample set partially relies on a broader melissopalynological dataset extending beyond the subset subjected to chemical analyses.

2.2. Melissopalynological profile

Moreover, we received from the private laboratory "Piana Ricerca e Consulenza" a summary table with the melissopalynology data for.

5932 multifloral honeys analyzed between 2015 and 2024. The number of samples in the locations of interest were distributed as follows: 1542 samples originated from the Northern regions, 2924 samples were produced in the center of Italy, and 1466 samples were sourced from the south. The melissopalynology dataset contained the species-specific pollen counts for each honey. The melissopalynology assessment was carried out by microscopy as described earlier [50]. Further details about the sample preparation can be found elsewhere [51].

2.3. Volatile profile by DBDI-HRMS

A 2 g amount of each honey sample was placed in a glass tube, warmed to 40 °C for 20 min in a thermostat and then analyzed using a SICRIT® source (Plasmion GmbH, Augsburg, Germany) coupled to an Exactive Orbitrap (Thermo Fisher Scientific, Waltham, USA). After warming, the honey samples were analyzed in duplicate by placing two open containers of honey directly in front of the sampling source for ten seconds each, within an overall acquisition duration of 30 s for the background removal. The mass range was set to 75–300 Da in positive ion mode. The mass spectrometry parameters were described previously [19,52]. The *m/z* values were tentatively assigned by consulting the online FooDB library (mass error ≤ 5 ppm) with a level of identification of confidence equal to 4 (combination of exact mass and isotope ratios) as proposed by Schymanski et al. [53]. Note that we could not provide an unequivocal identification but only a putative annotation of the VOCs. Prior to preprocessing, the repeatability of the two spectral repetitions for each honey was checked using cosine similarity. The isotopes were removed and the signals aligned using internally developed codes. The data were normalized according to the relative intensity of the most

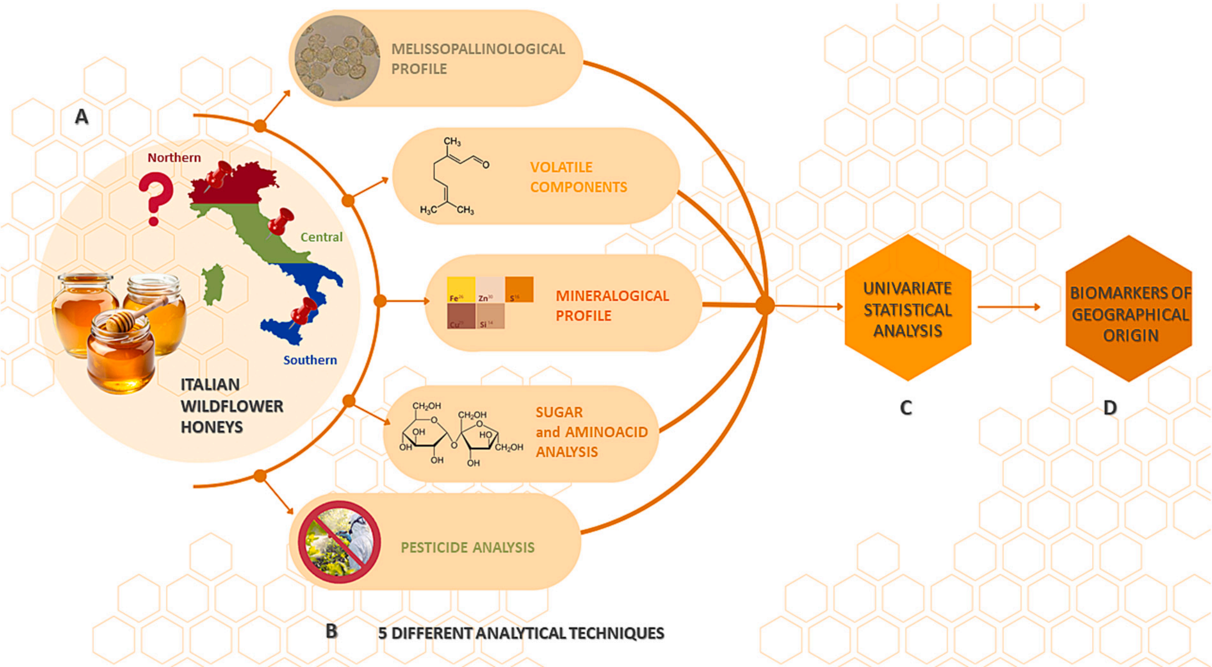

Fig. 1. Workflow of the study. A) Multi-floral honeys collected from Northern, Central, and Southern Italian regions were characterized by a variety of analytical techniques. The objective of this analysis was to identify biomarkers that would indicate the geographical origin of honey. B) Dielectric barrier discharge ionization high-resolution mass spectrometry (DBDI-HRMS) was used to analyze the molecules responsible for honey aroma. Total reflection X-ray fluorescence (TXRF) was used to determine the macro, micro and trace elements. Nuclear magnetic resonance was used to determine the sugar and amino acid concentrations. Liquid chromatography and gas chromatography coupled to mass spectrometry was used to investigate the presence of pesticides. In parallel, several thousand melissopalynological analyses performed on Italian multifloral honeys collected from 2015 to 2024 were evaluated statistically. C) The data were statistically analyzed using univariate methods. D) Finally, the biomarkers associated with geographical origin were defined.

intense signal in each spectrum.

2.4. Mineral analysis

Initially, 0.25 g of each honey was mixed with 1 mL of ultrapure water and shaken until complete dissolution. After, 450 μL of solution was mixed with 50 μL of a Gallium standard solution, achieve a final Ga concentration of 1 mg/L and homogenized. Due to the high sugar content of the honeys, a further dilution step with Milli-Q water was necessary. Finally, 10 μL of each diluted sample was deposited onto a silicon-quartz reflector and dried on a heating plate at around 50 °C [54]. TXRF analysis of prepared samples was performed using a commercial benchtop TXRF spectrometer equipped with Mo low-power X-rays tube (HORIZON, GNR srl). The spectrometer was equipped with air-cooled low-power X-ray tubes operating at 15 mA and 40 kV and a cooled silicon drift detector (SDD) of 20 mm^2 and thus, no cooling media or gas consumption were required. Both blank reflectors and sample deposits were irradiated for 600 s in real time. Spectral analysis and quantification of net peak areas were conducted using the instrument's dedicated software.

2.5. Sugar and amino acid analysis by q-NMR

NMR samples were prepared by dissolving ~240 mg of each honey in D_2O, maintaining a precise honey-to-buffer ratio of 240 mg/ml. Spectra were acquired using a Bruker Avance Neo 600.13 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a Prodigy cryoprobe. Samples were thermally equilibrated at 298.1 K for at least five minutes prior to analysis.

Amino acid quantification was performed on conventional proton NMR spectra (128 scans, 2 s relaxation delay, 6000 Hz spectral width, 64 k points) using the internal standard method. Metabolite concentrations were determined by comparing signal integrals with that of trimethylsilylpropanoic acid (TSP) at a known concentration.

Due to signal overlapping in the sugar region, sugar quantification was performed using the CSSF-TOCSY method proposed recently [55]. CSSF-TOCSY parameters were: 2–16 scans × 14 increments; 16 dummy scans; 11,904 Hz spectral width (64 k points); 5 s relaxation delay; 50–62.5 ms filter duration; 30–80 ms Gaussian pulse duration; 50–200 ms DIPSI-2 mixing scheme. Acquisition time varied from 2 to 15 min depending on analyte concentration. Spectra were acquired using IconNMR software for full automation. Sugar concentrations were extracted from CSSF-TOCSY signal intensities using calibration curves and converted to g/100 g honey. All spectra were processed using ACDLab v.12.5 (zero filling to 32 k, exponential multiplication with 0.3 Hz line broadening). Automated phase and baseline corrections were applied. Spectra were referenced to the anomeric α-glucopyranose chemical shift at 5.230 ppm.

2.6. Statistical analysis

The melissopalynological, VOC, mineral, sugar and amino acid data were statistically analyzed using Rstudio 4.3.2 software and the MetaboAnalyst 6.0 web portal (www.metaboanalyst.ca). Before performing the statistical analysis, the data obtained from the various techniques were normalized, specifically: for the melissopalynology data, the raw counts were used; for the VOCs, the absolute intensities of each spectrum were normalized by the relative intensity of the most intense m/z value for each spectrum; for minerals, the original quantification of the data was retained; and finally, for sugars and amino acids, the data were normalized using Pareto scaling. Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were performed on all datasets as exploratory multivariate tools using the MetaboAnalyst 6.0 web portal. Additionally, to assess the statistical significance of the differences between groups and to retrieve the most informative variables, a non-parametric ANOVA (using the Kruskal-Wallis test) was performed. The False Discovery Rate (FDR) method was used to adjust the p-value for multiple comparisons, with a significance threshold set at $\text{p-value}_{\text{adj}} \leq 0.05$. This approach allowed for detection of significant differences while controlling for false positives in the analysis. Post-hoc pairwise comparisons were not performed, as the primary objective was to identify variables significantly differing across groups as a whole. The box plots were used to visualize the distribution of the most informative variables across groups. The metabolites retrieved by non-parametric ANOVA were annotated by consulting the metabolomics libraries www.foodb.ca.

2.7. Contaminant analysis

2.7.1. Chemicals and reagents

Analytical grade (98–99.9% purity) multiresidue pesticide standard kits were obtained from Restek (Bellefonte, PA, USA). The complete list of pesticide compounds targeted by the analytical kits employed in this study is provided in **Table S2**. Each kit comprised validated multiresidue panels designed for high-throughput screening using LC-MS/MS and GC–MS methodologies, ensuring compliance with EU regulatory requirements for pesticide residue analysis. Internal standards were Imidacloprid-d_4 PESTANAL® (Supelco, Bellefonte, PA, USA), PCB-28-^{13}C (50 μg/mL in nonane and toluene, 10%) and PCB-180-^{13}C (50 μg/mL in nonane and toluene, 10%), both obtained from Wellington Laboratories (Ontario, CA, USA). For the determination of glyphosate, aminomethylphosphonic acid (AMPA), and glufosinate, the following internal standards were used: AMPA (100.0% purity, glufosinate-ammonium (99.5%) and glyphosate solution 1000 μg/mL in water (99.0%), purchased from Supelco® (Merck KGaA, Darmstadt, Germany). As internal standards, glyphosate-2-13C,15 N (99.9%) was purchased from Supelco®, while 13C-15 N-AMPA solution 100 μg/mL in water (100.0%) and glufosinate-D3 solution 100 μg/mL in water (96.5%) were from HPC Standards GmbH (Borsdorf, Germany).

Methanol, acetonitrile (HPLC gradient grade), heptane, formic acid, ammonium formate (reagent grade), acetic acid (glacial ReagentPlus®, 99%), potassium hydroxide (KOH) ACS reagent (≥ 85%) pellets, sodium carbonate powder (Na_2CO_3) ACS (American chemical society) reagent, dimethyl sulfoxide (DMSO) ACS grade, and fluorenylmethylchloroformate (FMOC-Cl, 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Q-sep Extr. Salt Packets (4 g $MgSO_4$; 1 g NaCl; 1 g sodium citrate TSDC; 0.5 g Disodium hydrogen citrate DHS) and Q-sep® QuEChERS Q373 (Quick, Easy, Cheap, Effective, Rugged, Safe), 15 mL centrifuge tubes (900 mg $MgSO_4$, 150 mg Primary Secondary Amine-PSA, 150 mg Octadecylsilane -C18), used to extract and purify honey samples, were obtained from Restek (Bellefonte, PA, USA). Dichloromethane (DCM) was purchased from Carlo Erba Reagents S.r.l. (Milan, Italy). Supel™ Swift HLB SPE Tubes (60 mg) were obtained from Supelco®. High purity water was prepared using a Milli-Q water purification system (Millipore, Burlington, MA, USA).

2.7.2. Honey sample preparation for pesticide residue analysis

The samples were prepared following the procedure described by [56]. Honey samples were processed in duplicate for subsequent analysis using both LC and GC techniques. Pesticides extraction and purification was performed using the QuEChERS method. After accurate homogenization, 5 g of honey were weighed and the surrogate standard clothianidin-D3 (0.050 mg/kg) was added to the LC samples and allowed to equilibrate for 10 min. Honey was then dissolved in 5 mL of water and vortexed for 10 min. Subsequently, 10 mL of acetonitrile were added, and the mixture was shaken for another 10 min. QuEChERS extraction salts were then added, and the sample was vigorously agitated for 1 min, followed by centrifugation at 4724 ×g for 10 min. The supernatant (8 mL) was transferred into tubes containing QuEChERS dispersive purification reagents, shaken for 1 min, and centrifuged for 5 min to obtain purified extract. For UHPLC–MS/MS 8040 analysis, 0.5 mL of the purified extract was transferred into auto-filtering vials equipped with 0.2 μm PTFE filters. The internal standard imidacloprid-d4 (50 μg/kg) was automatically injected by the LC autosampler during each analytical run. For GC–MS/MS TQ 8040 analysis, 3 mL of purified extract were transferred into 15 mL tubes, evaporated to dryness under vacuum at 45 °C, and reconstituted in 0.5 mL of heptane. The final solution was filtered with 0.2 μm PTFE filters and transferred into amber vials.

2.7.3. LC-MS/MS analysis for pesticide residue analysis

The analysis was performed on a Shimadzu LCMS-8045, a liquid chromatograph equipped with a tandem quadrupole analyzer in multiple reaction monitoring (MRM) mode, using an electron spray ionization source in both positive and negative ionization mode. The chromatography was performed on a Ascentiss® Express RP-Amide (Supelco, 10 cm × 2.1 mm, 2.7 μm), thermostated at 30 °C. *mobile* phase (A) consisted of 2 mM ammonium formate in water with 0.2% formic acid and (B) with 2 mM ammonium formate in methanol with 0.2% formic acid. The mobile phase B gradient was programmed as follows: starting at 2% B, held until 0.50 min. The percentage was then increased to 10% at 1.00 min and to 55% at 3.00 min. The gradient continued to 98% at 10.50 min, maintained until 12.00 min. At 12.10 min, mobile phase B was returned to 2%, and this condition was held until 18.00 min for column re-equilibration. The flow rate was 0.4 mL/min, and the injection volume was 3 μL. The nebulizing gas flow was set at 2 L/min, while the drying gas flow was maintained at 15 L/min. The desolvation line (DL) temperature was adjusted to 250 °C, and the heat block temperature was set at 400 °C. Quantitative and qualitative analyses were performed with LabSolution Insight software based on the two most intensive fragment ion transitions. A matrix-matched calibration was used for quantification.

2.7.4. GC–MS/MS analysis for pesticide residue analysis

The analysis was performed on a Shimadzu gas-chromatography mass spectrometer (GCMS-TQ8040) equipped with a ZB-semivolatile column (Phenomenex, 30 m × 0.25 mm ID, 0.25 μm). The sample volume of 1 μL was injected in splitless mode at an injector temperature of 95 °C. The GC temperature was programmed as follows: 60 °C (2 min) increased by 70 °C/min to 200 °C, then increased by 6 °C/min to 300 °C (2 min), for a total analysis run time of 23 min. The ion source and interface temperature were held at 200 °C and 280 °C, respectively. Quantitative and qualitative analyses were performed with LabSolution Insight software, based on the two most intensive fragment ion transitions. Matrix-matched calibration was used for quantification.

2.7.5. Honey sample preparation for glyphosate AMPA and glufosinate residue analysis

Briefly, 2.00 g of homogenized honey were dissolved in 5 mL of 1% formic acid solution, sonicated, and vortexed. The pH was adjusted to 7.0 ± 0.5 with potassium hydroxide (KOH). A 0.5 mL aliquot was derivatized with 0.5 mL of sodium carbonate (0.1 M) and 0.2 mL of fluorenylmethyloxycarbonyl chloride (Fmoc-Cl, 50 mg/mL in acetonitrile), followed by 1 h shaking in the dark. The reaction was quenched with 0.32 mL of 1% formic acid solution and centrifuged (10,000 ×*g*, 10 min). The supernatant was purified on HLB SPE cartridges (methanol and acidified water conditioning), washed with double-distilled water and dichloromethane, eluted with methanol, evaporated at 45 °C, and reconstituted in 0.5 mL of mobile phase mixture (50:50, A:B) for LC-MS/MS analysis.

2.7.6. LC-MS/MS analysis for glyphosate AMPA and glufosinate residue analysis

Instrumental analysis was carried out using a Shimadzu LC-MS-8060NX equipped with a triple quadrupole system coupled to a LC-40D XR pump, SIL-30 AC autosampler, and CTO-20 AC column oven. Chromatographic separation was performed on an Accucore C18 column (2.1 × 100 mm, 2.6 μm) maintained at 35 °C. *mobile* phase A consisted of water/methanol (95:5, *v*/v) with 5 mM ammonium formate and 0.1% formic acid; mobile phase B was methanol/acetonitrile (50:50, v/v). The flow rate was set at 0.3 mL/min with a 10 μL injection volume under a gradient program (5% B to 90% B in 11.5 min; total run time 15.5 min). Mass spectrometric detection was carried out in ESI negative mode with optimized source conditions (interface 350 °C; desolvation line 180 °C; heat block 300 °C; drying gas 10 L/min). Quantification and confirmation were based on MRM transitions, with primary transitions for quantification and secondary transitions for identity confirmation.

2.7.7. Statistical analysis of contaminant data

Each of the 75 honeys was analyzed for the presence of 293 pesticides (listed in **Table S2**) as well as the highly polar herbicides glyphosate, its metabolite AMPA, and glufosinate. Values below the limit of quantification (LOQ = 0.010 mg/kg) were treated as non-detects (0 mg/kg). Data analysis and visualization were performed using R software (version 4.3.1) [57]. For each analyte and macro-area, the following descriptive statistics were calculated: percentage of positive samples, mean, median, standard deviation, and maximum concentrations (including zero values). The distribution of co-occurrence of pesticides across macro-areas was investigated. Differences among macro-areas were assessed using the Kruskal-Wallis non-parametric test applied to each individual analyte and to the overall detected analytes per sample.

3. Results and discussion

The application of non-parametric ANOVA to the pollen data of multifloral honeys allowed us to observe pollen species characteristic of multifloral honey from each Italian macro-area (Fig. 2). Pollens from the species *Tilia* and *Ericaceae* were more abundant in honeys from the north, while pollens from *Rhus* and *Citrus* were typical of multifloral honeys from the south. *Lavandula stoechas* pollen was more frequent in multifloral honeys collected from Central Italy. *Acer* pollen was more abundant in multifloral honey produced in Northern and Central Italy. *Trifolium pratensis* pollen was present in small quantities in honeys from Northern Italy but was much more abundant in those from Central and Southern Italy. *Echium* and *Hedysarum* pollens were typical of honeys from Central and Southern Italy. *Aesculus* and *Potentilla* pollens were characteristic of honeys from Central and Northern Italy. **Fig. S1** illustrates pictures of the pollen species that characterize the multifloral honey from each Italian macro-area.

DBDI-HRMS is a plasma-based technique that allowed the solventless, direct, and rapid analysis of the volatile compounds released in just a few seconds after opening the lid of a jar of thermally treated honey. Fig. 3 shows representative DBDI-HRMS spectra of honeys with different geographical origins. Very distinct volatile profiles for each different botanical origin can be observed.

The simple comparison of the DBDI-HRMS profiles enabled the visual discrimination of the geographical source of the honey samples (Fig. 3, **S4-S7**). Table 1 reports the list of observed ions. Through detection of high-resolution masses of protonated volatile compounds, this method rapidly interrogated the important and distinctive compounds in the aroma of Italian honeys, which allowed them to be geographically distinguished. The statistical analysis confirmed our visual observations (Fig. 4).

The DBDI-HRMS revealed the presence of high concentrations of cresol (*m/z* 137.0960, also known as trimethylphenol) in multifloral honey from Northern and Central Italy. Cresol has been identified as a significant marker of linden (also known as lime tree) honey [62,71]. This compound contributes to the phenolic and musty odor characteristics of honey. The distinctive characteristics of Northern honeys are attributed to the presence of aminoacetophenone, a chemical compound that plays a crucial role in differentiating this geographical variety of honey. High concentrations of aminoacetophenone have been previously reported as markers of chestnut nectars as a floral source of monofloral chestnut honey [22,23,58]. Chestnut trees are distributed

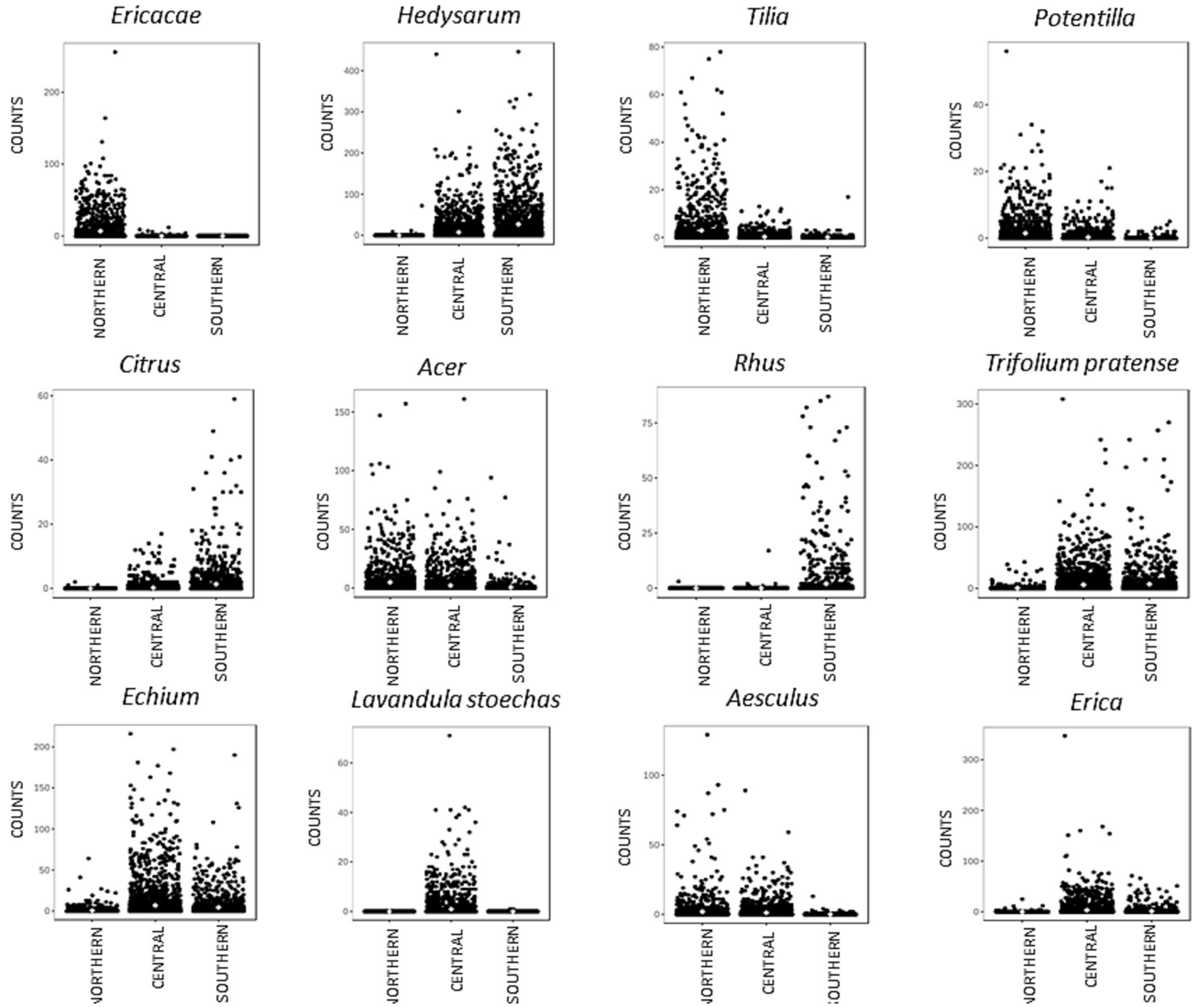

Fig. 2. Box plots showing the statistically significant differences (by non-parametric ANOVA, *p*-value $_{\text{adj}} \leq 0.05$) in species-specific pollen counts in honeys from Northern, Central, and Southern Italy. The y-axis of the box-plots represents the pollen counts. The x axis shows the geographical origin.

extensively throughout Italy, particularly in the hilly and mountainous regions of the Apennines and Alps. Significant concentrations of chestnut trees can be found in the Northern regions of Emilia-Romagna, Piedmont, and Veneto, as well as in the Central regions of Lazio and Tuscany. These regions collectively cover approximately 8% of Italy's total forest area [72].

In addition, the volatile compound tentatively assigned as santene (*m/z* 123.1169) has been previously observed in honey, particularly in highland [60] and rhododendron [59] honey. Our data accord with Guyot et al. (1988), who observed a high level of pyridinecarboxaldehyde in linden honey. Linden (*Tilia* spp.) and rhododendron (*Rhododendron* spp., from *Ericaceae* family) are archetypal specimens of arboreal flora indigenous to Northern Italy. Rhododendron and multifloral honey of the Alps are both rich in linalool-oxide [68], as our current study confirms. Our melissopalynology data shows *Acer* pollen is highly abundant in multifloral honey from Northern Italy. Similarly, we found that linalool oxide is prevalent in multifloral honey produced in Northern Italy and is the most concentrated volatile organic compound (VOC) in *Acer* honey [67].

The relative abundance of octanoic acid in multifloral honey decreased from Northern to Southern Italy. Octanoic acid has been previously detected in honey [21,63]. As Schievano et al. have suggested, the floral origin modulates the content of lipid markers in honey [73]. Specifically, plants rich in seeds or oil provide a high content of lipid markers compared to other common honeys. For example, dandelion and sunflower honey present high levels of lipids [73].

Heptanal is one of the compounds that define the specific aroma and flavor of honey, contributing to a "green" or sometimes pungent note. This VOC was present in significant quantities in the honeys from Northern and Central Italy. In accordance with the melissopalynological data indicating elevated counts of *Lavandula stoechas* in multifloral honey collected in Central Italy, heptanal emerged as a substantial marker for lavender honey, particularly for honeys derived from *Lavandula stoechas* and *Lavandula angustifolia*. [59]

Heptadienal, a significant marker we found in Northern multifloral honey, has been previously identified as a volatile compound in the leaves of some *Ericaceae* species [74]. This compound has been identified in the essential oils of *Rhododendron* spp. and other *Ericaceae* plants [74].

The ions of *m/z* 151.1114, *m/z* 153.1271, *m/z* 167.1063, and *m/z*

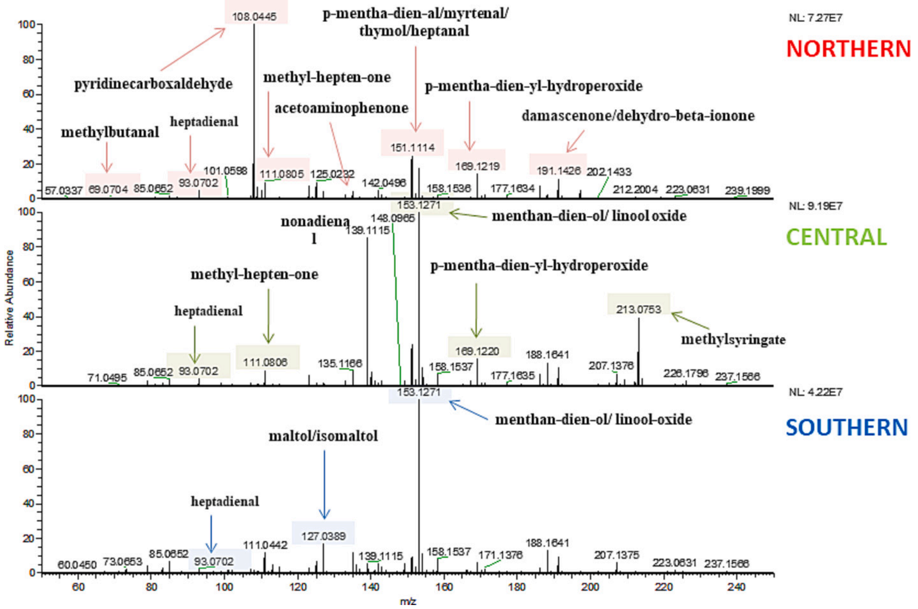

Fig. 3. Representative spectra of multifloral honey acquired by dielectric barrier discharge ionization high resolution mass spectrometry (DBDI-HRMS). The volatile molecules reported in bold are those that presented a p-value $_{adj}$ ≤ 0.05 by non-parametric ANOVA.

Table 1
Volatile organic compounds observed in Italian multifloral honeys. The observed *m/z*, the theoretical *m/z*, error (ppm), elemental formula, type of ion, putative annotation, *p*-value adjusted and literature references are reported.

m/z	Theor *m/z*	Error ppm	Ion type	Formula	Putative annotation	P_{adj}	References
69.0704	69.0705	−1.44	$[M + H\text{-}H_2O]^+$	$C_5H_{10}O$	Methylbutanal		[22]
80.0499	80.0501	−2.50	$[M + H\text{-}H_2O]^+$	C_5H_7NO	Dimethyloxazole	3.36×10^{-8}	
85.0652	85.0648	4.70	$[M + H]^+$	C_5H_8O	Pentenal		[19]
93.0702	93.0705	−3.22	$[M + H\text{-}H_2O]^+$	$C_7H_{10}O$	Heptadienal	1.98×10^{-2}	
108.0445	108.0444	0.93	$[M + H]^+$	C_6H_5NO	Pyridinecarboxaldehyde	5.23×10^{-16}	[58]
110.0629	110.0634	−4.54	$[M + NH_4]^+$	C_3H_8OS	Methyltioethanol	5.71×10^{-9}	
111.0805	111.0804	0.90	$[M + H]^+$	$C_7H_{10}O$	Heptadienal/ Methyl-hepten-one	9.91×10^{-3}	[22,59]
121.1011	121.1012	−0.83	$[M + H]^+$	C_9H_{12}	Trimethylbenzene	1.34×10^{-4}	
123.1168	123.1168	0.00	$[M + H]^+$	C_9H_{14}	Santene	2.17×10^{-2}	[60]
125.0597	125.0597	0.00	$[M + H]^+$	$C_7H_8O_2$	Guaiacol		[58]
127.0388	127.0390	−1.57	$[M + H]^+$	$C_6H_6O_3$	Maltol/Isomaltol	1.23×10^{-3}	
135.1166	135.1168	−1.48	$[M + H]^+$	$C_{10}H_{14}$	p-Cymene		[61]
136.0759	136.0757	1.47	$[M + H]^+$	C_8H_9NO	Aminoacetophenone	5.68×10^{-3}	[23]
137.0960	137.0961	−0.73	$[M + H]^+$	$C_9H_{12}O$	Trimethylphenol/Cresol	8.83×10^{-7}	[62]
139.1115	139.1117	−1.43	$[M + H]^+$	$C_9H_{14}O$	Nonadienal		[63]
143.1064	143.1067	−2.10	$[M + H]^+$	$C_8H_{14}O_2$	Octanoic acid	1.33×10^{-2}	[21,63]
151.1114	151.1117	−1.99	$[M + H]^+$	$C_{10}H_{14}O$	p-Mentha-dien-al/Myrtenal/Thymol/Heptanal	8.70×10^{-3}	[21–23,58]
153.0543	153.0546	−1.96	$[M + H]^+$	$C_8H_8O_3$	Vanillin		[63]
153.1271	153.1274	2	$[M + H]^+$	$C_{10}H_{16}O$	p-Mentha-dien-ol/Linalool oxide		[23,64]
155.1427	155.1430	−1.93	$[M + H]^+$	$C_{10}H_{18}O$	Geraniol/Rose oxide/Myrcenol		[65]
165.0907	165.0910	-1.82	$[M + H]^+$	$C_{10}H_{12}O_2$	Eugenol		[66]
165.1271	165.1274	−1.82	$[M + H]^+$	$C_{11}H_{16}O$	Methyl-phenyl-butanol	2.52×10^{-4}	[7]
167.1063	167.1067	−2.39	$[M + H]^+$	$C_{10}H_{14}O_2$	Epoxy mentha-diene/Hydroxy-p-mentha-1,8-dien-6-one	6.14×10^{-7}	
169.1219	169.1223	−2.37	$[M + H]^+$	$C_{10}H_{16}O_2$	p-mentha-dien-yl-hydroperoxide	1.53×10^{-2}	[23]
171.1376	171.1380	−2.33	$[M + H]^+$	$C_{10}H_{18}O_2$	Linalool oxide		[25,63,67]
184.1332	184.1332	0.00	$[M + NH_4]^+$	$C_{10}H_{14}O_2$	Hydroxy-p-mentha-dien-one	1.55×10^{-3}	[19]
191.1426	191.1430	−2.09	$[M + H]^+$	$C_{13}H_{18}O$	Damascenone/Dehydro-beta-Ionone	1.65×10^{-7}	[63,68,69]
213.0753	213.0757	−1.87	$[M + H]^+$	$C_{10}H_{12}O_5$	Methyl syringate	4.71×10^{-3}	[70]

169.1219, tentatively assigned as menthan terpenoids (mentha-dien-al, mentha-dien-ol, epoxy mentha-diene/hydroxy-p-mentha-1,8-dien-6-one hydroxy-p-mentha-dien-yl-hydroperoxide), were highly abundant in Northern Italy honeys. Menthan terpenoids were well documented in Italian honey [30]. Our findings accord with Bonometti et al. (2022) [74], who reported a high concentration of menthan terpenoids in rhododendron honey. In agreement with our melissopalynology data, pollen from the *Ericaceae* family, which includes Rhododendron, was prevalent in honey collected in Northern Italy. Pyridinecarboxaldehyde, a characteristic marker of Northern Italian multifloral honey, was previously identified in honey samples by Mădaş et al. (2019) [18]. These researchers demonstrated that the presence of pyridinecarboxaldehyde in honey is indicative of its production in mountainous regions of Transylvania. Our melissopalynology data revealed the presence of *Rhus* species in the multifloral honey produced in Southern Italy. In a similar vein, *Rhus* spp. honey demonstrated elevated concentrations of (*E*)-β-damascenone, a chemical compound that we identified as a constituent of Southern multifloral honey through our aroma analysis [69]. This

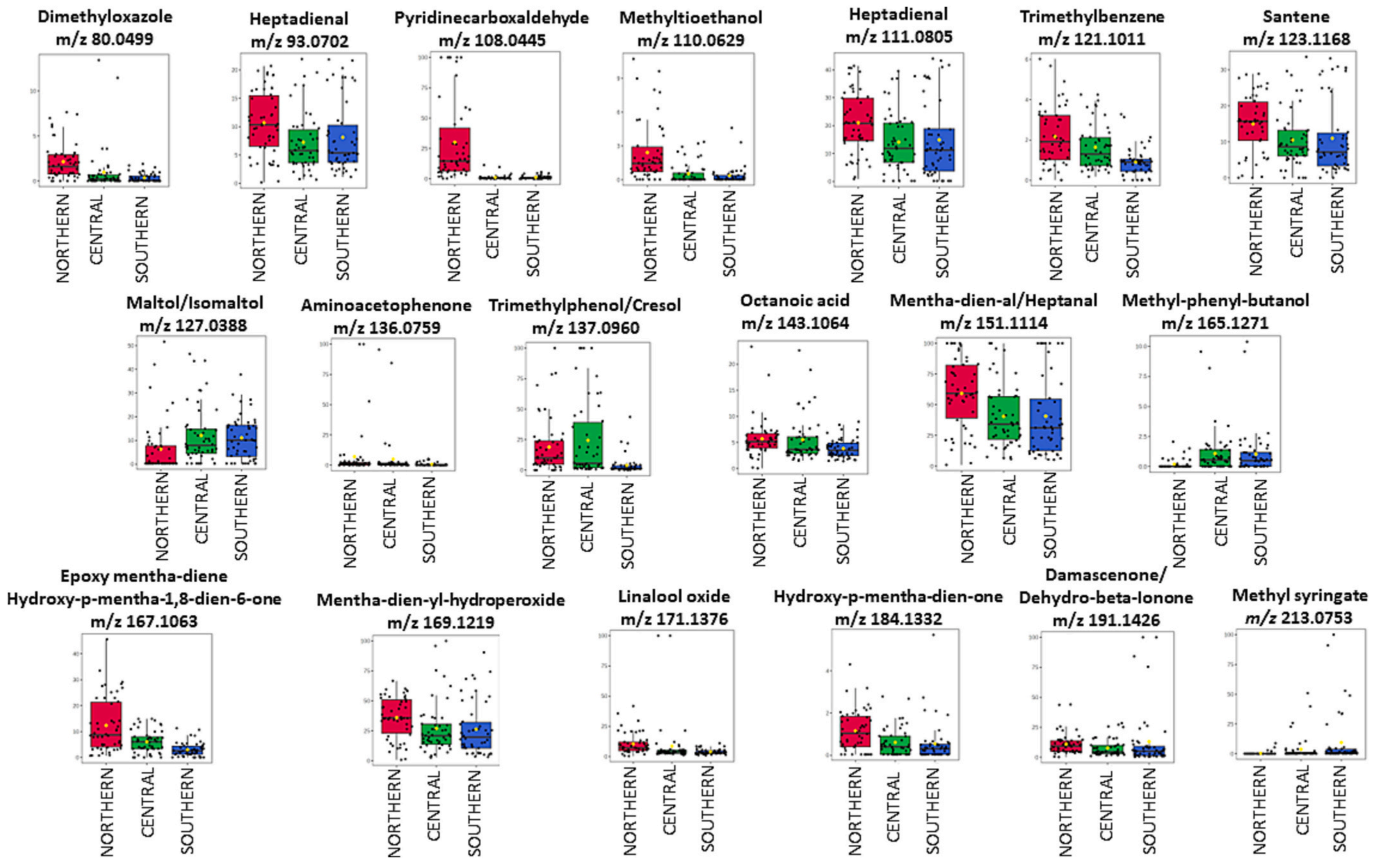

Fig. 4. Characteristic volatile organic compounds acquired by dielectric barrier discharge high resolution mass spectrometry (DBDI-HRMS) in positive ion mode, and that were found to be statistically significant by non-parametric ANOVA (p-value $_{adj} \leq 0.05$) in Northern, Central and Southern Italy. The bottom and top of the boxes represent the first and third quartiles (25th and 75th percentiles), respectively. The horizontal line indicates the median, while the yellow diamond represents the mean value. Three repetitions for each honey were used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

component is responsible for the fruity aroma. Our DBDI-HRMS-based findings indicate that methyl-syringate is a typical marker of Central and Southern honey where the highest counts of *Citrus* pollen were found. Methyl-syringate was previously found in citrus honey [29] Similarly, our melissopalynological assessment revealed that Italian multifloral honey was distinguished by its high prevalence of citrus pollen. Methyl-syringate was also identified in asphodel honey from Sardinia [70].

TXRF analysis enabled the identification in honey samples of a broad range of elements, including K, Ca, Ba, Mn, Fe, Ni, Cu, Zn, Br, Rb, Sr (**Fig. S2-S3)**. Besides them, cobalt was detected only in two samples, high mountain multifloral honeys from the Alps (sample ID 606 and ID 610). The most abundant detected elements were K and Ca, with concentrations ranging from 30 to 400 mg/kg and 10–52 mg/kg, respectively. These two elements are used as indicators to assess honey quality and its botanical origin. The statistical results indicated that calcium levels were significantly higher in Central Italy, with slightly lower levels observed in Southern Italy (p-value $_{adj} = 9.13 \times 10^{-4}$, Fig. 5). Statistical assessment also revealed significantly greater K abundance in honeys from Central than in those from Southern and Northern Italy, where the lowest concentrations of this mineral were observed (p-value $_{adj} = 2.16 \times 10^{-4}$), in a good agreement with geological pattern of these areas [75]]. Rubidium was also detected in all honeys, with concentrations spanning 0.1–8 mg/kg; although not an essential element, Rb correlates with specific plant sources, thereby contributing to geographical or botanical differentiation [54]. The non parametric ANOVA revealed that Rb was similarly abundant in Northern and Central Italy, but significantly less so in Southern areas (p-value $_{adj} = 8.05 \times 10^{-4}$, Fig. 5). The observed distribution of Rb aligns with known geochemical patterns in Italy. Elevated Rb levels have been reported in the Alpine areas (near the Austrian-Italian border) and in the Roman and Neapolitan magmatic provinces related to felsic igneous rocks and the associated sedimentary/metamorphic formations. Rb is similar to K and substitutes in silicate minerals [76] Barium concentrations in the honeys ranged from 0.1 to 0.6 mg/kg, levels comparable to those previously reported [77]. Iron and Zn contents were mostly 0.05–0.7 mg/kg. The multifloral and high-mountain multifloral honeys from the Alps (Piemonte and Lombardy, Northern Italy) had higher Mn concentrations of about 4 mg/kg and 1.7 mg/kg, respectively, a statistically significant difference (p-value $_{adj} = 6.14 \times 10^{-7}$). The Fe content in honey samples of Northern Italy was lower and slightly increased from Central to Southern Italy (p-value $_{adj} = 5.19 \times 10^{-5}$). Copper and Ni were found at only low levels in the honeys, with no statistically significant differences among Italian areas. Ni in honey could derive from common apiary management techniques and/or pedo-geochemical backgrounds [78], but anthropogenic contributions are likely negligible [79]. Most honeys exhibited Br concentrations in the range of 0.02–0.10 mg/kg. However, multifloral honeys from Central and Southern Italy contained significantly higher Br levels, between 0.3 and 0.4 mg/kg (p-value $_{adj} = 6.19 \times 10^{-9}$, Fig. 5). It is likely that the elevated levels of bromine are a result of marine aerosol contribution [80]. The concentration of Sr in honey exhibited a significant linear increase, albeit to a limited extent, from geographic north to south (p-value $_{adj} = 1.87 \times 10^{-2}$, Fig. 5).

Sugar and amino acid compositions in honey depend primarily on botanical and geographical origins and are influenced by climate, processing, and storage conditions [81]. The detection and quantification of

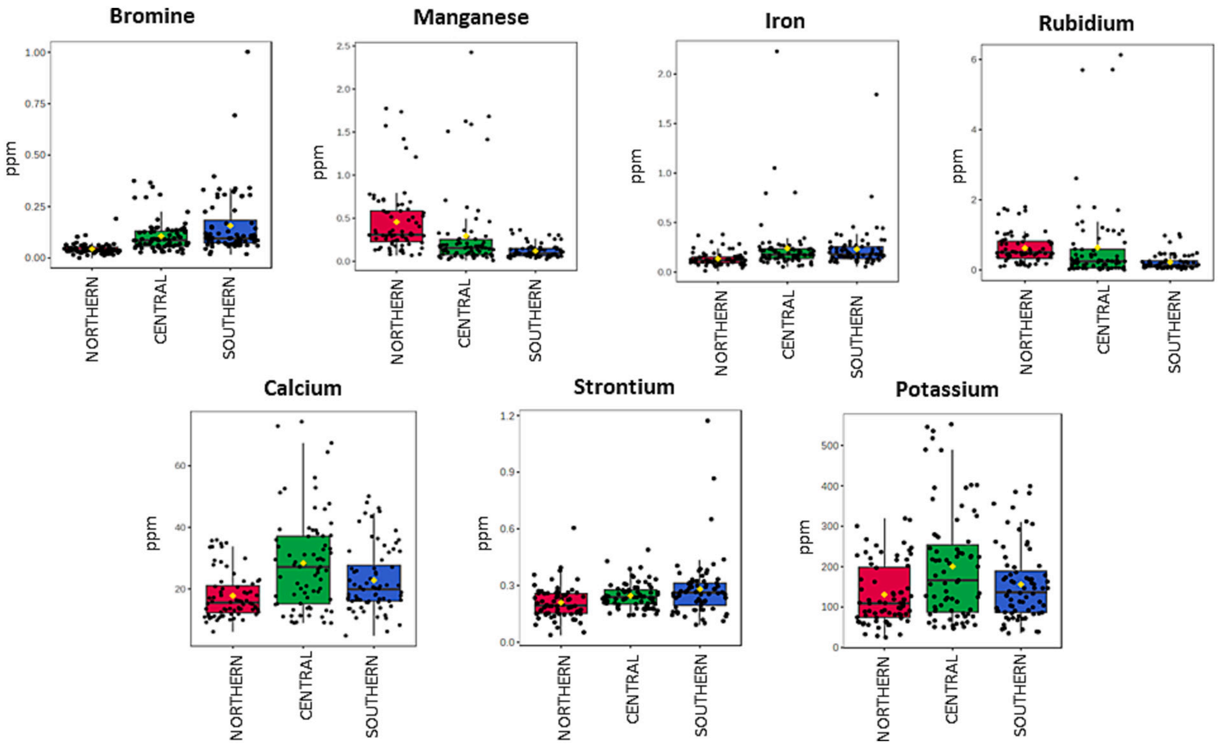

Fig. 5. Box plots showing the characteristic elements, acquired by total reflection X-ray fluorescence (TXRF) spectrometer, that were found to be statistically significant by non-parametric ANOVA (p-value $_{\text{adj}} \leq 0.05$) in Northern, Central and Southern Italy. The bottom and top of the boxes represent the first and third quartiles (25th and 75th percentiles), respectively. The horizontal line indicates the median, while the yellow diamond represents the mean value. Three repetitions for each sample were used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sugars and minor amino acids in honey were successfully performed without separation using quantitative NMR measurements. Nineteen carbohydrates, including three monosaccharides, one cyclitol, ten disaccharides, and five trisaccharides, were determined in honey samples using CSSF-TOCSY NMR spectral analysis. Six amino acids were quantified directly by 1D NMR conventional spectra. The resulting data matrix was subjected to non-parametric ANOVA.

Fig. 6 illustrates the content of five metabolites showing statistically significant differences across the three Italian areas: three amino acids (proline, isoleucine, and leucine) and two sugars (erlose and meso-inositol). Proline is the predominant amino acid in honey, while isoleucine, and leucine are minor amino acids. Erlose is a trisaccharide formed via α-glucosidase action by glycosyl transferase reaction in which one glucose moiety binds with sucrose. Meso-inositol, a cyclitol, is a secondary metabolite, naturally occurring in plant material, playing an important role in plant self-defense against unfavorable environmental conditions [82].

The mean concentration of proline was 591 ± 223 mg/kg, 1054 ± 293 mg/kg, and 1227 ± 470 mg/kg in Northern, Central, and Southern Italy, respectively. Statistically, honeys from the north contained less proline than did those from the center and south, which did not differ significantly from each other (*p*-value adj $= 4.42 \times 10^{-6}$). Honey from Southern Italy generally exhibited high proline concentrations. Proline is a quality indicator and can be related to specific flowering trees, such as citrus and eucalyptus, that are prevalent in this macro-area [83,84].

A similar trend was observed for leucine and isoleucine levels: the mean concentrations of leucine were 10 ± 4 mg/kg, 20 ± 10 mg/kg, and 20 ± 8 mg/kg for Northern, Central, and Southern multifloral honey, respectively (*p*-value adj = 0.02). The mean isoleucine content of the honeys was 6 ± 2 mg/kg, 13 ± 7 mg/kg, and 13 ± 7 mg/kg for Northern, Central, and Southern honey, respectively (p-value adj $= 3.52 \times 10^{-4}$). High amounts of leucine and isoleucine in citrus and eucalyptus honey from Calabria, Southern Italy, were reported earlier [83].

Conversely, erlose and meso-inositol show higher mean levels in Northern honeys than in those collected from other Italian regions. Specifically, the mean erlose concentration was 2.2 ± 1.2 g/100 g, 0.9 ± 0.5 g/100 g, and 1.0 ± 0.6 g/100 g, while that of meso-inositol was 0.10 ± 0.03 g/100 g, 0.06 ± 0.04 g/100 g, and 0.05 ± 0.03 g/100 g for Northern, Central, and Southern honey, respectively. The decrease of these two sugars in honeys when moving from north to south was confirmed by non-parametric ANOVA (p-value adj $= 4.40 \times 10^{-6}$ and 2.25×10^{-5} for erlose and meso-inositol, respectively). The content of these two sugars was similar in honeys from Central and Southern Italy. The high concentration of erlose in honey from the Northern Italian mountains is attributable to a significant contribution from rhododendron, a typical flowering plant native to this region [85,86].

The PCA and PLS-DA were also performed on all datasets as exploratory multivariate tools; however, the resulting score plots did not reveal clear clustering among the geographic groups of study (Supplementary **Figs. S8-S11**).

Within the framework of geographical authentication, the qualitative compositional traits of multifloral honey and the pesticide residue patterns should be interpreted as complementary layers of information, jointly reflecting the interplay between local flora, environmental conditions, and region-specific agricultural practices that characterize the production area.

Out of the 292 pesticides evaluated in this study, three analytes exceeded the LOQ: acetamiprid, amitraz, and glyphosate (Table 2 and Fig. 7). Furthermore, eight analytes (acetamiprid, amitraz, coumaphos, chlorphenvinfos, etofenprox, fluvalinate, glyphosate, and propamocarb) were detected in trace amounts (below the LOQ, <0.010 mg). A total of 13 honeys contained at least one analyte above the LOQ. The complete analytic results are provided in Table S3 of the supplementary material. However, one honey from Southern Italy exhibited the presence of two analytes, namely amitraz and glyphosate, at concentrations that exceeded the LOQ (0.012 mg/kg amitraz and 12.4 mg/kg glyphosate). All

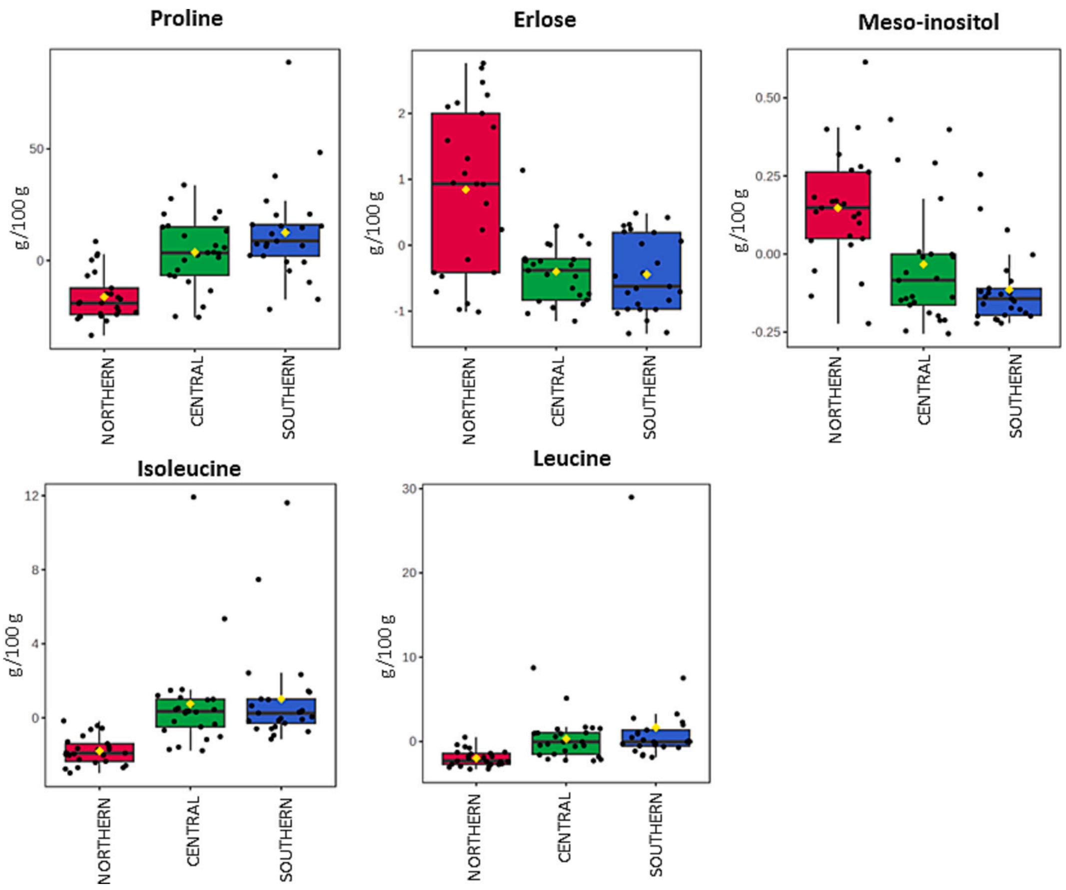

Fig. 6. Box plots showing the characteristic metabolites, quantified by nuclear magnetic resonance (q-NMR), that were found to be statistically significant by non-parametric ANOVA ($p\text{-value}_{adj} \leq 0.05$) in the Norther, Central and Southern Italy. The bottom and top of the boxes represent the first and third quartiles (25th and 75th percentiles), respectively. The horizontal line indicates the median, while the yellow diamond represents the mean value. Three repetitions for each sample were used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Number of positive samples (> limit of quantification, LOQ = 0.010 mg/kg) for each Italian macro-area, mean and maximum pesticide concentration detected in honey samples (mg/kg).

	Northern Italy			Central Italy			Southern Italy			Total	
	Positive samples	Mean (mg/kg)	Max (mg/kg)	Positive samples	Mean (mg/kg)	Max (mg/kg)	Positive samples	Mean (mg/kg)	Max (mg/kg)	Positive samples	Mean (mg/kg)
Glyphosate	n.d	–	–	5	0.028	0.052	3	0.014	0.019	8	0.023
Amitraz	1	0.020	0.020	1	0.016	0.016	3	0.015	0.021	5	0.016
Acetamiprid	n.d	–	–	n.d	–	–	1	0.010	0.010	1	0.010
Total	1			6			6*			13*	

Note: *co-occurrence of amitraz and glyphosate was detected in a honey from Southern Italy.

detected residues complied with the EU MRLs, with the exception of one glyphosate-positive honey (0.052 mg/kg), which exceeded the permitted threshold. The study revealed that 61.3% (46/75) of the honeys exhibited no detectable pesticide residues or traces of them. According to national studies, glyphosate and other plant protection products have been documented in Italian honey [87,56]. These studies have generally found the presence of these compounds to be within legal limits. However, occasional exceedances have been documented for compounds such as fosetyl-aluminum, acetamiprid, and glyphosate [87,88].

Glyphosate exhibited the highest detection rate and concentration range among the pesticides, particularly in Central Italy (5/25 positive honeys, 20%; maximum concentration 0.052 mg/kg). Southern Italy showed a moderate detection rate (3/25; maximum glyphosate concentration 0.019 mg/kg), whereas all honeys from Northern Italy were below the LOQ. AMPA and glufosinate were not detected in any of the honeys. These findings are consistent with recent European studies reporting glyphosate detection rates between 10 and 12%, typically below regulatory limits, but occasional exceedances [89,90]. In contrast, higher contamination levels have been documented in locations with intensive herbicide use, such as Brazil and Hawaii (United States of America), where glyphosate prevalence reached 27–33% and concentrations exceeded 0.3 mg/kg [91,92].

Low concentrations of the acaricide amitraz were found in 6.7% of our honeys. The detection of acetamiprid, a neonicotinoid of particular concern for pollinator health, although at low levels, remains noteworthy. Neonicotinoids are frequently reported in honey worldwide, with prevalences from 45% to over 70% depending on agricultural intensity [93]. For example, Polish studies reported acetamiprid in 52% of honeys, with 21% exceeding the MRL, highlighting regional variations

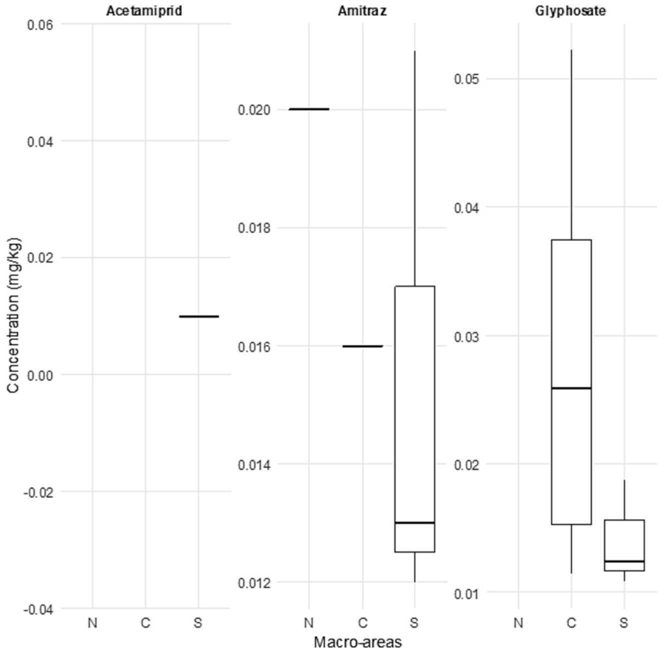

Fig. 7. Boxplot of concentrations (>limit of quantification, LOQ) of Acetamiprid, Amitraz and Glyphosate in honey samples divided per macro-area (N = Northern, C = Central, S = Southern).

driven by crop protection practices [94].

Other acaricides, such as fluvalinate (two honeys, Northern Italy) and coumaphos (five honeys, Central and Southern Italy), were detected only below the LOQ, reflecting controlled use in beekeeping and limited environmental persistence. The agricultural fungicide propamocarb appeared below the LOQ in three Central Italy honeys, indicating low-level environmental exposure. Chlorphenvinphos and etofenprox were also detected in trace amounts (in four and one honeys, respectively), all below the LOQ. The presence of acaricides, such as amitraz, fluvalinate, and coumaphos, at low concentrations reflects controlled use in hive treatments and aligns with previous studies reporting that these substances rarely exceed safety thresholds represented by MRLs [95]. Likewise, the occasional detection of agricultural pesticides, such as propamocarb and chlorphenvinphos, suggests environmental drift or indirect exposure, a pattern commonly observed in European contexts [96].

Overall, 62 out of 75 honeys were either completely pesticide residue-free or contained only trace amounts of pesticides, demonstrating the high quality of Italian honey. This is consistent with European monitoring reports, where most honey samples are residue-free or show pesticide concentrations well below safety limits [97].

The comparison of overall pesticide occurrence across macro-areas indicated a trend toward geographical differences, but this was not statistically significant (Kruskal-Wallis $\chi^2 \approx 5.25$, df = 2, p ≈ 0.07). The geographical pesticide occurrence, therefore, suggests a qualitative gradient only, likely due to the low number of positive samples and the high prevalence of non-detects.

Previous studies on agricultural products have demonstrated that pesticide residue profiles exhibit significant regional variability driven by crop distribution, land use, and local agricultural practices, supporting their interpretation as indicators of environmental and agronomic context rather than compound-specific markers of origin [98,99].

The geographical origin of honey has been proposed as a relevant factor influencing its contamination profile, particularly because countries and regions differ markedly in their pesticide regulations and agricultural practices [100]. Even within a single country, honey collected from different localities can reflect distinct contamination patterns driven by surrounding land use, industrial activities, urbanization, and local agronomic inputs [101]. For example, strong associations between glyphosate incidence and agricultural land use were shown, with higher concentrations observed in honey from areas adjacent to intensively maintained golf courses or major highways [91].

Local environmental context can significantly shape residue profiles in hive products, confirming honey bee and beehive matrices as appropriate sentinels for monitoring contamination in the environment [102]. In this respect, the lack of statistically significant associations between macro-area and glyphosate contamination observed in the present study should not be interpreted as evidence of uniform exposure. Rather, it suggests that finer geographical resolution, coupled with multi-component analytical approaches, might be needed to capture subtle environmental patterns. Indeed, multi-residue and multi-matrix assessments, integrated with modern multivariate or machine learning methods, have proven powerful in disentangling complex contamination sources and in strengthening environmental interpretation authentication [103].

Pesticides identified in this study relate to both hive treatments and agricultural practices, underscoring the complex interaction between beekeeping and the environment. To further enhance the discriminatory power of contaminant-based approaches, the integration of land-use information is a promising strategy. Incorporating data on the types of crops surrounding apiaries and, consequently, the specific plant protection protocols typically associated with each crop could improve the interpretation of contamination patterns. Since many pesticides are applied in a crop-dependent and seasonally predictable manner, aligning contaminant profiles with local agricultural landscapes could help refine geographical attribution and support the use of residues in honey as indicators of regional agricultural practices.

Agricultural practices, crop distribution, and land-use intensity differ markedly among Northern, Central, and Southern Italy and may indirectly influence the type and occurrence of pesticide residues in honey; however, given the generally low incidence and trace-level concentrations observed in the present dataset, a robust correlation between specific pesticides and regional crop patterns could not be established. Nevertheless, these findings do not exclude the potential use of pesticide residue analysis, when combined with qualitative compositional markers of honey, as an additional layer of information contributing to geographical origin authentication within a multi-analytical framework.

Overall, these results indicate that honey can serve as a reliable biomonitor for pesticide exposure, reflecting local agricultural practices and general environmental conditions of the production area, supporting honey's use in environmental surveillance and food safety assessment. This study presented a multi-analytical framework for characterizing Italian multifloral honey based on its geographical origin by integrating chemical data on pollen, volatile compounds, minerals, sugars, amino acids, and contaminants. Our findings show that these markers collectively reflect the interaction between local flora, soil composition, geology, and environmental factors. We established that while individual chemical parameters could be sufficient for verifying origin, combining the parameters creates a robust geographical fingerprint. By leveraging multivariate statistical tools to identify a limited number of discriminative markers, a reliable chemometric classification model, with merged variables [104,105], could be built-up. This approach maintains high predictive accuracy while streamlining analytical workflows and reducing operational costs.

4. Conclusions

In this study, a multi-analytical characterization of honey was performed. The use of multiple analytical techniques allowed the simultaneous evaluation of complementary physico-chemical parameters, i.e., pollen signatures, VOCs, minerals, sugar and amino acid profiles, and pesticide presence. Moreover, we demonstrated that the retrieved markers are relatable to the local flora, soil, geology and environmental pollution. Therefore, our multi-analytical approach was able to capture this complexity. We demonstrated that no single analytical parameter is sufficient to reliably link multifloral Italian honey to a specific area, but combining multiple chemical markers creates a robust geographical fingerprint. However, the application of a full multi-analytical approach is often not feasible for routine control, due to high costs, long analysis times, and the need for specialized instrumentation and expertise. To overcome these limitations, a tiered and targeted analytical strategy can be adopted. In this context, the study that we performed can be considered a screening step. Furthermore, the development of chemometric models based on a reduced set of our most informative markers could enable reliable geographical classification while minimizing analytical complexity. Marker selection can be guided by multivariate statistical tools to identify parameters with the highest discriminative ability.

CRediT authorship contribution statement

Francesca Asson: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Maria Christina Chotzara:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Carmela Zacometti:** Writing – review & editing, Writing – original draft, Investigation. **Andrea Massaro:** Writing – review & editing, Writing – original draft, Formal analysis. **Fabjola Bilo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Marianna Martinello:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Chiara Manzinello:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Michela Bertola:** Writing – review & editing, Writing – original draft, Formal analysis. **Greta Morbin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Roberto Piro:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Lucia Piana:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Franco Mutinelli:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition. **Laura Borgese:** Writing – review & editing, Writing – original draft, Supervision, Resources. **Elisabetta Schievano:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Alessandra Tata:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Franco Mutinelli reports financial support was provided by the Ministry of Agriculture, Food Sovereignty and Forests. Lucia Piana is founder and owner of the “Piana Ricerca e Consulenza” consulting center that provided the melissopalynological data. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2026.118662.

Data availability

Data will be made available on request.

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