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Honey botanical origin and honey-specific protein pattern: Characterization of some European honeys

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ABSTRACT

Honey adulteration generates low quality products on the market. The study aimed to find a simple, specific and less time-consuming method than standard melissopalynology only, for monitoring honey botanical and geographical origin. In this study 42 honey samples from different sources were examined for their botanical origin by using melissopalynology and their specific protein patterns by electrophoresis on SDS polyacrylamide gels (SDS-PAGE). The melissopalynological analysis consisted of counting all melliferous pollen, non-melliferous pollen and honeydew elements to identify the species of pollen present in each sample. The honey samples had predominant pollen from Apiaceae, Boraginaceae, Brassicaceae, Compositae H, Fabaceae (*Trifolium* sp.) and Fagaceae family. From the Fagaceae family, the most important species was *Castanea sativa* Mill., while the Boraginaceae was represented by *Echium* sp. and *Myosotis* sp. SDS-PAGE showed that the different origin honeys shared protein bands between 45 and 85 kDa (animal origin proteins, i.e. major royal jelly proteins, enzymes) and that specific proteins (presumably plant origin proteins) can be attributed to individual honey types. This study shows that the combination of melissopalynology and SDS-PAGE is a useful tool for modern discrimination between different kinds of honey, even without performing specific protein identification.

1. Introduction

The Food and Agriculture Organization (FAO) estimated worldwide honey production at 1.9 million tons in 2018 (FAOSTAT, http://faostat.fao.org). The 2001/110/EC defined honey as a natural sweet substance made by honey bees by combining plant nectar or honeydew to which they add their own secretions (EC, 2001). Monofloral honey is based on a single major plant nectar that is processed into honey, while multifloral honey is a combination of several different nectar types that are combined by honey bees while converting stored nectar into honey. Melliferous plants are divided into: nectariferous, polleniferous and mixed plants, from which nectariferous plants are the ones with under-represented pollen, and polleniferous plants are plants with over-represented pollen. The number of pollen grains in honey is influenced by flower morphology and physiology (Louveaux, Maurizio, & Vorwohl, 1978; Todd & Vansell, 1942), the behavior of foraging bees (Molan, 1998), and contamination in the hive. Melissopalynological analysis allows the detection of its botanical and geographical origin based on the vegetation types that form the honey matrix (von der Ohe, Oddo, Piana, Morlot, & Martin, 2004). Relative percentage of the main pollen types in various unifloral honeys, such as *Citrus* (10–20%), *Lavandula* (10–20%), *Medicago* (20–30%), *Robinia* (20–30%), *Rosmarinus* (10–20%), *Salvia* (10–20%) and *Tilia* (20–30%), are honey types with under-represented pollen, whereas *Castanea sativa* (>90%) and *Myosotis* sp. honey are derived from highly melliferous plants with over-represented pollen (von der Ohe, Oddo, Piana, Morlot, & Martin, 2004).

Honeydew honey is a specific type of honey, which derives from plant secretions (mostly Coniferous and Latifoliae trees) or excretions of plant-sucking insects (aphids and scale insects) (Directive 2014/63/EU and Council Directive 2001/110/EC relating to honey, 2014). The main Coniferous honeydew honeys are produced from *Abies* sp. (fir), *Picea* sp. (spruce) and *Pinus* sp. (pine) trees. They are marketed with specific denominations and are highly valuable in different European countries.

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Most of Latifoliae honeydew honeys are not characterized, being marketed as "forest" honey and mixed with honeydew honeys from Coniferous trees. Nonetheless, besides the melissopalynological analysis, careful attention should be given when selecting monofloral honeys, as other under-represented pollens may affect their classification as monofloral. The honey composition and nutritional quality depend on environmental conditions, plant source, geographic location and bee species (Kaškonienė & Venskutonis, 2010).

Due to its health benefits, honey has been consumed by humans since ancient times. Multiple recent studies have shown its antimicrobial, anti-inflammatory, anti-carcinogenic and antioxidant effects, as well as its analgesic activity in the human organism (Nguyen, Panyoyai, Kasapis, Pang, & Mantri., 2019; (Pasupuleti, Sammugam, Ramesh, & Gan., 2017) Siddiqui, Musharraf, & Choudhary, 2017). Since adulterated honey is commonly reported worldwide, one of the main reasons for analysing honey and its properties is the authenticity (Osés et al., 2017). The high nutritional value and distinct flavors of honey determine its monetary value to be usually higher than those of other sweeteners, thus attracting counterfeiter's attention. In multiple countries, honey is adulterated with sugar, sweeteners derived from beet or corn (glucose), invert sugar, saccharose syrups and high fructose corn syrup (HFCS) (Fakhlaei et al., 2020). Currently, honey authenticity is detected by tracing its botanical and/or geographical origin, and by the detection of certain compounds (carbohydrates, proteins, organic acids, amino acids, minerals, polyphenols, vitamins and volatile compounds) (Chin & Sowndhararajan, 2020, pp. 1–33; Nayik, Suhag, Majid, & Nanda, 2018). The widely known standard method for honey authenticity is melissopalynology (i.e. the analysis of pollen grains contained in honey sediments by light microscopy) (Louveaux et al., 1978). Many factors can influence the results of melissopalynological analysis: plant morphology, contamination of the hive with pollen unrelated to the nectar source, and contamination during uncapping and processing. Many pollen types are over-represented (e.g., chestnut, eucalyptus) or under-represented (e.g., citrus, asphodel) in different types of nectar (Corvucci, Nobili, Melucci, & Grillenzoni, 2015). This method has the disadvantages that it is time-consuming, requires experts with adequate skills and experience, and cannot be applied in filtered honeys or honey adulterated by pollen addition. In contrast, melissopalynology is important for identifying unifloral honeys with a high percentage of identical pollen and for allowing suitable species identification and differentiation (von der Ohe et al., 2004).

Nowadays, experts are seeking new techniques with low difficulty and fast analysis, compared to the melissopalynological method. In their extensive reviews, Puścion-Jakubik, Borawska, and Socha (2020) and Balkanska, Stefanova, & Stoikova–Grigorova (2020) summarized the latest methods for assessing the quality of natural bee honey. The newest approaches used to distinguish between honey samples are the electric tongue in combination with optical spectroscopy, Ultraviolet–Visible-Near Infrared (UV-VIS-NIR), DNA-metabarcoding and several statistical analysis: cluster analysis and principal component analysis (PCA) (Geana & Ciucure, 2020). The combination of these techniques resulted in a more precise classification of honey samples. PCA is one of the methods most used for performing multivariate analysis (Corbella & Cozzolino, 2006; Corvucci et al., 2015; di Bella et al., 2015; Pauliuc, Dranca, & Oroian, 2020). Cluster analysis is another statistical method performed in few studies for determining the geographical (de Alda-Garcilope, Gallego-Picó, Bravo-Yagüe, Garcinuño-Martínez, & Fernández-Hernando, 2012) and botanical origin of honey (Ansari et al., 2018; de Alda-Garcilope et al., 2012; Gok, Severcan, Goormaghtigh, Kandemir, & Severcan, 2015).

Proteins are minor constituents in honey and were first shown to be present in honey by colour tests (Moreau, 1911). White and Kushnir (1967b) discovered by starch-gel electrophoresis that the protein pattern from honey of different floral origin included four to seven proteins from which four were believed to be of bee origin. In the following decades, more sensitive methods were developed for studying honey protein patterns: two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), immunoblotting, etc. (for references see Supplementary Table S1). Recently, Erban, Shcherbachenko, Talacko, & Harant (2021) demonstrated a proteomics approach employing LC-MS/MS that is useful for proving honey adulteration and assessing honey quality.

The most relevant genuine proteins in honey are the ones belonging to the Major Royal Jelly Protein family that is composed of nine homologous members (MRJP1-9) secreted by the hypopharyngeal glands of nurse bees taking care of the brood in the hive (Buttstedt, Moritz, & Erler., 2014). MRJP1-5 have a theoretical molecular weight (MW) between 46 and 68 kDa (Buttstedt, Moritz, & Erler, 2014). The major honey protein is MRJP1 (Chua, Lee, & Chan, 2015), which in honey is found in N-glycosilated or glycated form with different MW of 56, respectively 59 kDa, depending on the number of N-linked oligosaccharides characteristic to the bee species: *Apis cerana*, respectively *Apis mellifera* (Won, Lee, Ko, Kim, & Rhee, 2008).

Other honey bee-added honey proteins are specific enzymes: diastases (α- and β-amylase), invertases (sucrase, saccharase or glucosidase), glucose oxidase (oxido-reductase), acid phosphatase, catalase, transglucosylase, phosphorylase, SOD1 and proteolytic enzymes (Alonso-Torre et al., 2006; Gillette, 1931; Rossano et al., 2012; Schepartz, 1965; Schepartz & Subers, 1966; White & Kushnir, 1967a,b). The honey proteases activity is associated with honey type and they were identified as serine enzymes, which can degrade MRJPs, especially MRJP1, found in honey (Rossano et al., 2012) or the original specific pollen proteins (Baroni, Chiabrando, Costa, & Wunderlin, 2002). These proteolytic enzymes can be of bee, nectar or pollen origin and are different forms of trypsin and chymotrypsin (Rossano et al., 2012).

Most of the previous studies (for references see Supplementary Table S1) investigated the honey proteome to differentiate the floral origin or honey bee species origin, and were realized mainly on honey, some on nectar and pollen, while a few analysed and discussed the correlation of individual honey proteins with its floral diversity (Baroni et al., 2002; Baroni, Chiabrando, Costa, Fagúndez, & Wunderlin, 2004; Bauer et al., 1996; Bilikova & Simuth, 2010; Lewkowski, Mureş;;an, Dobritzsch, Fuszard, & Erler, 2019). Still, each approach has its advantages and disadvantages. Although mass spectrometry-based proteomics is a powerful method, it has limitations in terms of costs, sample processing (protein extraction, digestion and fractionation), protein detection and/or identification, and demand for highly specialized experts. As for immunoblotting, the disadvantages would be the time-consuming character, the risk of false-positive results, its high costs (for antibodies, equipment) and demand for skilled analysts. Also, the load ability, the membrane and hydrophobic proteins' fractionation are challenging when using 2-DE analysis. Therefore, compared to these methods, SDS-PAGE is a reliable, simple and cost-effective method preferred by most scientists for analyzing honey proteins.

The aim of the study was to examine diverse botanical origin honey samples in terms of their protein patterns by using SDS-PAGE, in combination with melissopalynology, and their authenticity by multivariate analysis. This new approach is needed to offer a simple, inexpensive method for honey source identification and provides new opportunities to identify honey adulteration.

2. Material and methods

2.1. Honey samples

Forty-two honey samples from five European countries (Belgium, France, Italy, Romania and Spain), produced by local beekeepers were assessed. Each sample was taken directly from the apiaries of the beekeeper (Table 1).

Table 1
The origin, year of sampling and beekeeper labeling of the honey samples. Italicized labels were not confirmed.

Sample ID	Country	Bee species	Harvesting	Year	Labeling	Geographical origin
H1	Belgium	*Apis mellifera*	summer	2018	*Canola*	Villers-le-Gambon
H2	Belgium	*A. mellifera*	summer	2018	*Canola*	
H3	Belgium	*A. mellifera*	summer	2018	Raspberry	Liege (Rue de Sendrogne)
H4	Spain	*A. mellifera iberica*	in the year	2019	Multifloral	
H5	Spain	*A. mellifera*	in the year	2019	*Sunflower*	
H6	Spain	*A. mellifera*	in the year	2019	Multifloral	
H7	Spain	*A. mellifera*	in the year	2019	*Multifloral*	
H8	Spain	*A. mellifera iberica*	in the year	2019	*Multifloral*	
H9	Spain	*A. mellifera*	in the year	2019	*Eucalyptus*	
H10	Spain	*A. mellifera*	in the year	2019	Coriander	
H11	Spain	*A. mellifera*	in the year	2019	Coriander	
H12	Spain	n.i.	in the year	2019	Multifloral	
H13	Spain	n.i.	in the year	2019	Eucalyptus	
H14	Spain	n.i.	in the year	2019	Eucalyptus	
H15	Spain	n.i.	in the year	2019	Multifloral	
H16	France	*A. mellifera*	summer	2018	Multifloral	Sainte Marguerite sur mer
H17	Belgium	*A. mellifera*	spring	2018	*Canola*	Uccle
H18	Romania	*A. mellifera*	summer	2018	Canola	Bucov
H19	France	n.i.	summer	2018	Chestnut	Issoire
H20	France	n.i.	summer	2018	Chestnut	Saint-Nom-la-Bretèche
H21	France	n.i.	summer	2018	*Lime tree*	Saint-Nom-la-Bretèche
H22	France	n.i.	summer	2018	Chestnut	Brest
H23	France	n.i.	in the year	2019	Multifloral	Combourtille
H24	France	n.i.	in the year	2019	*Multifloral*	Combourtille
H25	France	n.i.	in the year	2019	*Sunflower*	Combourtille
H26	Italy	*A. mellifera ligustica*	in the year	2019	*Black locust*	
H27	France	*A. mellifera*	in the year	2019	Multifloral	Provence
H28	France	*A. mellifera*	in the year	2019	Citrus	Provence
H29	France	*A. mellifera*	in the year	2019	Coriander	Arboussols
H30	France	*A. mellifera*	in the year	2019	Multifloral	Arboussols
H31	France	n.i.	in the year	2019	*Clover*	Marvejols
H32	France	n.i.	in the year	2019	Multifloral	Montadet
H33	France	n.i.	in the year	2019	Alder buckthorn	Andernos les bains
H34	France	n.i.	in the year	2019	Multifloral	Ortaffa
H35	France	n.i.	in the year	2019	Multifloral	Chatearoux les Alpes
H36	France	n.i.	in the year	2019	Chestnut	Plemy
H37	France	n.i.	in the year	2019	Oak	Alpes de Haute Provence
H38	France	n.i.	summer	2018	*Buckwheat*	Benassay
H39	France	n.i.	summer	2018	Chestnut	Scrignac 29,640
H40	France	n.i.	spring	2018	Multifloral	Toulon sur Arroux
H41	Italy	n.i.	in the year	2019	Honeydew	
H42	Romania	*A. mellifera*	in the year	2019	Honeydew	Sibiu

n.i., no information, but presumably *A. mellifera* sp.

2.2. Melissopalynological analysis

For the melissopalynological analysis the method of Louveaux et al. (1978) was used without acetolysis. A sample of 10 g honey, from each sample, was prepared by dissolving the honey in 20 mL H_2SO_4 (5 mL/L) followed by centrifugation for 15 min (4500×*g*). The supernatant was discarded and the method was repeated with bidistilled water, for the complete elimination of honey sugars. The remaining pellet was mounted on a slide using Kaiser's glycerol gelatine (Merck KGaA, Dramstadt, Germany)and a few drops of fuchsin (optional) for permanent fixation. Slide examination was performed using an Olympus BX51 optical microscope at 1000 × magnification (for identification) and 400 × magnification for counting. Five hundred pollen grains were counted from every slide and percentages of different botanical species were calculated. The appearance frequency of the different pollen types was divided into the following four classes: predominant pollen (>45% of the total pollen detected in honey); secondary pollen (16–45%); minor important pollen (3–15%); minor pollen (<3%) (Louveaux et al., 1978) (Supplementary Table S2).

2.3. Colorimetric protein quantification by the Bradford method

The protein content of each honey sample was estimated by using the Quick Start™ Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, California, USA) in a microplate assay. A quantity of 0.5 g of each honey sample was mixed with 0.5 mL distilled water and vortexed for 5 min at 50 °C. A calibration curve was obtained by serial dilution of the protein standard solution (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg/mL), bovine serum albumin (BSA), as described by the manufacturer. From each sample a volume of 10 μL was mixed with 200 μL of Bradford reagent. The absorbance was measured at 595 nm on a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA) after incubation in darkness for 5 min.

2.4. Honey protein separation by SDS polyacrylamide gel electrophoresis (SDS-PAGE)

A volume of 10 μL from each diluted with water honey sample was mixed with 5 μL Laemmli sample buffer and incubated at 70 °C for 10 min (Lewkowski, Mureşan, Dobritzsch, Fuszard, & Erler, 2019). Following centrifugation at 12,000×*g* for 1 min, the supernatant was loaded on 12% SDS-PA gels and the proteins were separated by SDS-PAGE (Laemmli, 1970). The molecular weight marker used was the BLUEye Prestained Protein Ladder (range 10–245 kDa, GeneDireX, Inc.). Electrophoresis was performed at 90 V for 10 min for protein concentration and after that at a constant voltage of 175 V for 60 min for protein migration. Protein gels were stained either with Colloidal Coomassie Brilliant Blue G-250 according to Neuhoff, Arold, Taube, and Ehrhardt (1988) or with Silver Stain kit (Bio-Rad Laboratories Inc., Hercules, California, USA) according to the manufacturer instructions.

Each honey protein sample was stained with both methods seperatly.

2.5. Gel imaging and analysis

Electrophoresis gels were scanned using a GS 800 calibrated imaging densitometer (Bio-Rad Laboratories Inc., Hercules, California, USA) with dimensions of 10,200 × 14,031 pixels (width × height), a 1200 dpi resolution, 24 bit depth, RGB and saved as TIFF images. All images were further analysed with ImageJ 1.48v and GelAnalyzer 2010a as described in Mureş;an, Schierhorn & Buttstedt (2018). The GelAnalyzer software was used to estimate the raw volume for the most abundant protein bands of each sample (Supplementary Fig. S1).

2.6. Statistics

Samples were analysed in triplicate for protein quantification and in duplicate for electrophoresis gel densitometry. Statistical significance was tested by one-way analysis of variance (ANOVA) using the Kruskal-Wallis test using GraphPad Prism 8.2.1.441. Results were given as average ± standard deviation. To reduce the pollen dataset to a lower number of dimensions, cluster analyses were performed using the Paleontological Statistics (PAST) software (Hammer, Harper, & Ryan, 2001). Cluster analyses were performed on a Bray-Curtis similarity matrix using complete linkage. Heatmap and dendrograms were generated using the Euclidean distance based on Ward's algorithm for clustering (Ward, 1963).

3. Results

3.1. Melissopalynological analysis

Across all honey samples 66 pollen types were identified. Pollen type diversity ranged from 5 to 25 per sample. The highest diversity was observed for sample H35 (multifloral) and the lowest for sample H19, with *Castanea* sp. as predominant pollen. The pollen assemblages of the 42 honey samples are shown as a pollen percentage diagram arranged in a sequence determined by their sample ID and geographic origin (Fig. 1). The distribution of pollen types showed that the majority of the honey samples were palynologically dominated by Apiaceae, Boraginaceae, Compositae, and Fagaceae, followed by Fabaceae and Myrtaceae, being observed in almost all tested samples. According to the palynological analysis, nine plant families were found in the 42 honey samples as predominant pollen constituents (>45%): Apiaceae, Boraginaceae (*Echium* sp., *Myosostis* sp.), Brassicaceae (*Brassica* sp.), Compositae (*Helianthus annus*), Fabaceae (*Trifolium* sp.), Fagaceae (*Castanea* sp.and *Quercus* sp.), Myrtaceae (*Eucalyptus* sp.), Rhamnaceae (*Rhamnus* sp.) and Rosaceae (*Rubus* sp.) (Fig. 1, Supplementary Table S2). The Fagaceae family was found to be predominant (>45%) in eight samples (e.g. H16, H19, H20, H22, H27) with *Castanea* sp. and in H37 with *Quercus* sp. With the exception of H16 and H27, which were classified as multifloral, the other samples corresponded to *Castanea* sp. labeling. Another twelve families were found as secondary pollens (Fig. 1, Supplementary Table S2). The Fagaceae family was also present as secondary pollen (16–45%) for another six samples (H12, H30, H31, H34, H38 and H41, respectively), whereas the Apiaceae family was predominant in three samples (H10, H11, H29), and secondary in another three (H4, H5, H15). Samples H1, H3, H18 proved to be multifloral, not corresponding to the canola and raspberry labeling. The same was noticed for samples H5, H9, H26, H31, and H38, labeled as sunflower, eucalyptus, black locust, clover and buckwheat, respectively. The Boraginaceae family was predominant with *Echium* sp. in samples H7, H8 and H24, and secondary in H5, H9, H12, which was not consistent with the multifloral labeling. In the case of Rosaceae family, *Rubus* sp. was found predominant in sample H3 and secondary in four samples (H1, H16, H31, H39). From the analysed honey samples, 13 samples (H1, H2, H5, H7-9, H17, H21, H24-26, H31, H38) were not labeled correctly for their botanical origin, suggesting that the melissopalynolgy analysis proved to be significant for accurate labeling. Among the reasons for the nonconformity of the beekeeper's declaration in comparison with the results of the melissopalynology analysis might be incorrect labeling, secondary contaminated honey in honeycombs and/or accidental pollen addition during harvesting of the final product.

A cluster analysis was performed to differentiate the floral origin of honeys based on specific pollen types and compositionally similar samples (Fig. 2, $r = 0.82$). The hierarchical clustering organized the samples in two main clusters except samples H25 and H26, which are considered 'outliers', not being grouped with the others, which might be based on the predominant pollen in Compositae H (*Helianthus* sp.), secondary in Brassicaceae (*Brassica* sp.) and only few minor pollen for H25. The same was noticed for sample H26, which is mainly multifloral.

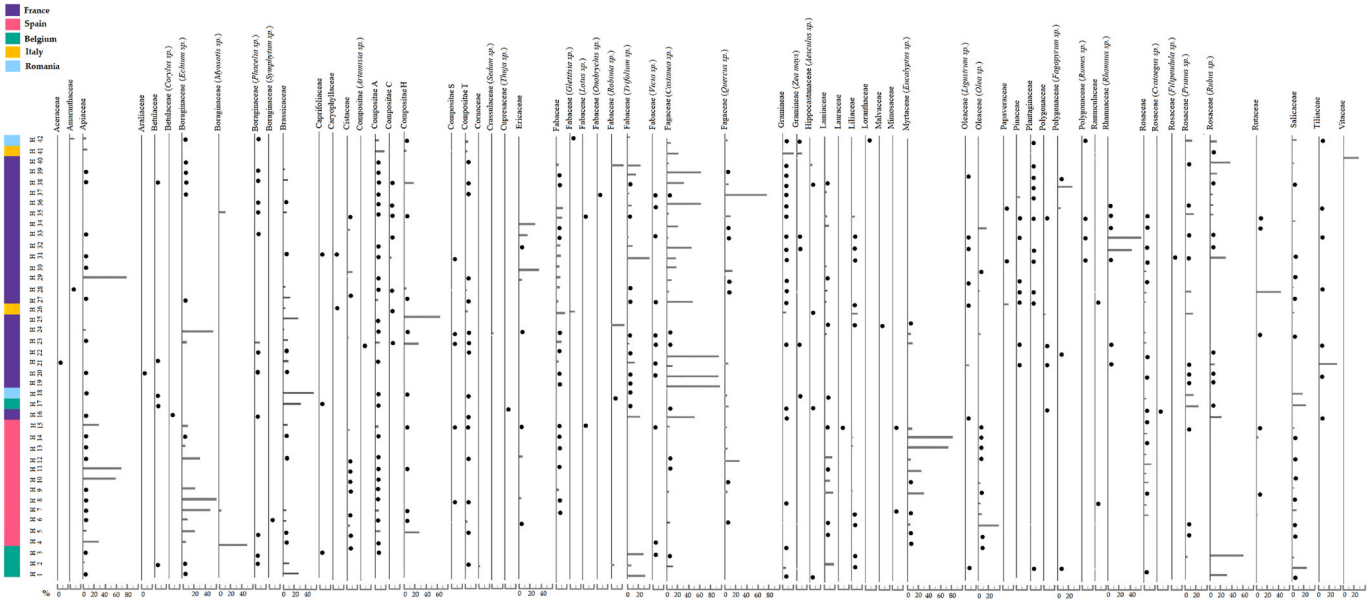

Fig. 1. Percentage pollen diagram, illustrating the pollen content of the honey samples (bottom line). Dots represent minor pollen (<3%). Colored bars indicate sample geographic origin.

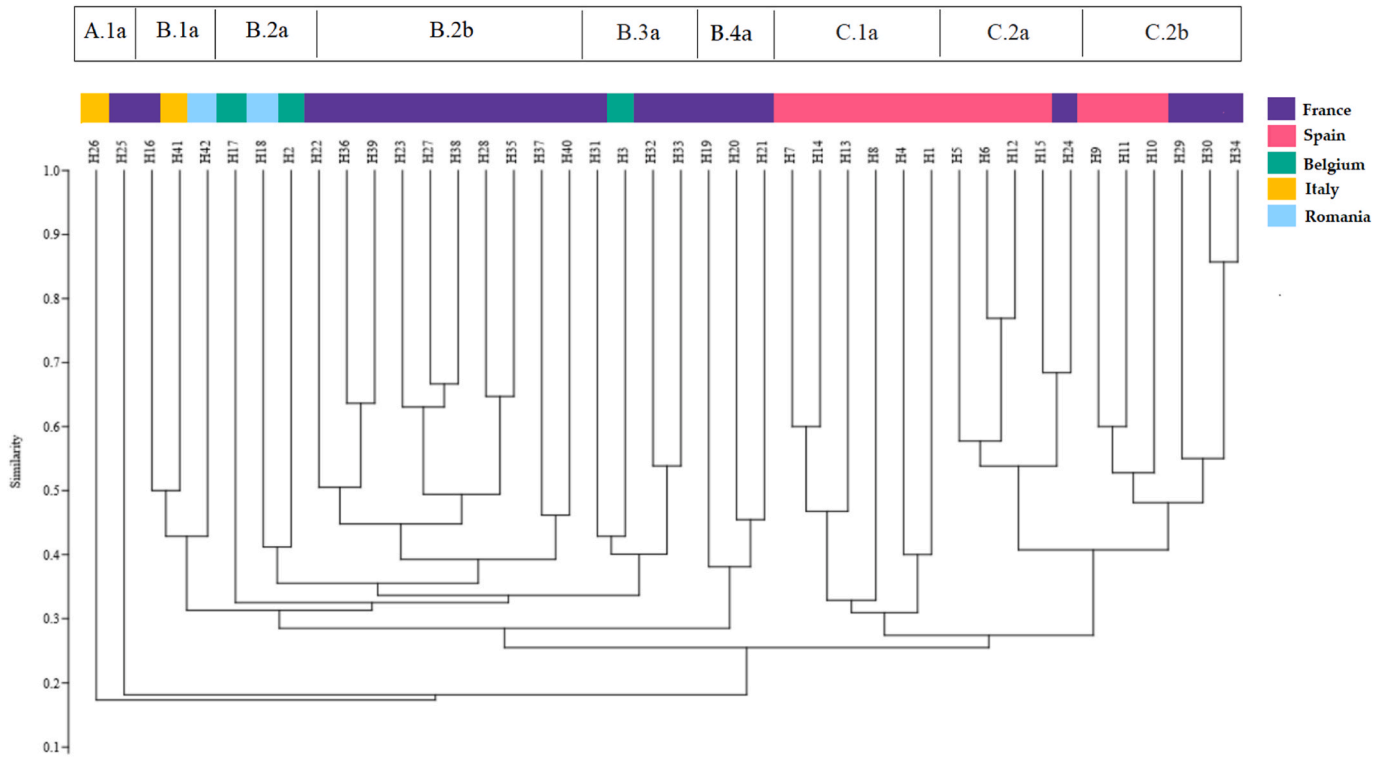

Fig. 2. Hierarchical clustering of the 42 honey samples (H1-H42) based on melissopalynological analysis (Bray-Curtis similarity, $r = 0.82$). Colored bars indicate sample geographic origin.

The first major branch of the dendrogram includes five sub-clusters. The first and second sub-clusters comprise the samples from Belgium and Romania, as well as a sample from Italy (H41) and one from France (H16). Noticeable, in the third sub-cluster, the polyfloral samples H17, H18 and H2 are closely related which may be due to their similar plant families in Brassicaceae, Rosaceae (*Prunus* sp.) and Salicaceae. The following sub-clusters (B.2b, B.3a, B.4a) mainly group the samples from France. Cluster B2.b comprises the Western French samples (atlantic climate, fairly wet and cool), followed by B.3.a which grouped the Southern samples (H31, H32 and H33) from Marvejols, Montadet and Andernos les bains, respectively. The second major branch, including three sub-clusters (C.1a, C.2a, C.2b), consists of Spanish honey samples (from H4 to H15) and few Southern French (mediterranean climate) samples (H29, H30, H34) from Arboussols and Ortaffa, respectively. These groups are based on similar plant families, particularly in the secondary pollen constituents. According to the pollen diagram (Fig. 1), these samples showed similar predominant plant families, as follows: Brassicaceae (*Brassica* sp.), Boraginaceae (*Echium* sp.) and Fagaceae (*Castanea* sp.). This shows that the hierarchical cluster analysis can discriminate the honey samples based on their geographic and botanical origin, even though few samples (from Italy and Romania) were not clustered correctly. This might be explained by the botanical origin of the samples that in these cases are more important than the geographical origin, as seen by the mixed colours per cluster (Fig. 2). Multiple samples are needed to better discriminate honeys based on their geographic and botanical origin. One of the study's purpose was to show that based on melissopalynological analysis the geographic origin can be discovered. This is important when analyzing honey samples of unknown provenance. With this, the study demonstrated that honeys from France and Spain can be discriminated by the combination of melissopalynology and cluster analysis.

Investigating the botanical and geographical origin is a crucial step to ensure the authenticity of honey, which is a natural product commonly targeted for adulteration due to its high market value in comparison to other sweeteners. Furthermore, there are countries that produce honey with lower prices, which is afterward sold with a different provenance and a higher price (like honeys labeled as: EU provenance, mixed honey from EU and non-EU countries without a specific geographical origin). Multivariate data analysis and machine learning techniques proved to be efficient tools for the botanical and geographical origin determination of honey samples, presenting high discrimination accuracy when applied to various descriptive parameters of honey.

In order to declare specific honeys as monofloral, the national legislations in different European countries have established minimal limits for specific pollen (Bobiş et al., 2020; Oddo, Piazza, Sabatini, & Accorti, 1995; Oddo et al., 2004a,b; Thrasyvoulou et al., 2018). Using only the monofloral honey samples (n = 19) and their specific pollen spectra, the hierarchical cluster analysis revealed two main clusters corresponding to Spain (including two french samples) and France (mixed with Italy, Belgium and Romania) (Supplementary Fig. S2).

3.2. Protein quantity

Some honey samples had low protein quantities (H14, H23, H25, H27, H28, H37, H40) and a few had high protein quantities (H10, H22, H36, H38, H39). The protein content ranged from 0.16 (H25) to 2.15 mg/mL (H22) (Supplementary Table S2). As expected from the variance in pollen quantities and floral origin, the protein content differed significantly among samples (Kruskal-Wallis ANOVA, H = 113.4, dF = 41, n = 126, $p < 0.0001$).

3.3. SDS-PAGE

The SDS-PAGE analysis showed typical protein bands known for different honey types, with clearly detectable protein bands in the range 45–85 kDa (Supplementary Fig. S1). These results are in accordance with Bilikova, Kristof Krakova, Yamaguchi, and Yamaguchi (2015) and Lewkowski et al. (2019). The most intensive protein band was always a protein product in the range 50–60 kDa (denominated as 57 kDa in

Supplementary Fig. S1). A further common protein band was the protein product between 15 and 20 kDa (denominated 18 kDa in Supplementary Fig. S1). These proteins could be of bee-origin (50–60 kDa, namely MRJPs; Lewkowski et al., 2019), however the lower one (15–20 kDa) is of unknown function (XP_397512.1; Lewkowski et al., 2019). The honeys from France, H32 and H33, which have abundant pollen from Rhamnaceae, had higher molecular weight (MW) protein products. H37 with predominant Fagaceae pollen and H42, a honeydew honey, had high MW protein bands of around 130 kDa. Generally, the honeys containing Fagaceae pollen had high MW protein products (Supplementary Fig. S1).

A clustering analysis based on the complete protein spectra (bee and plant proteins) of all honeys revealed that the samples were grouped into several clusters including subclusters, which are mostly not based on their geographic origin. The 1st cluster (A1.a.) comprises the samples that mainly had bee-derived proteins, mostly multifloral honeys, except H7 (Boraginaceae, *Echium* type), H10 and H11 (Apiaceae) and H39 (*Castanea* sp.) (Supplementary Fig. S3). The 2nd cluster (B.1a) comprises the honeydew samples (H41, H42), followed by the Fabaceae with *Robinia* sp. (H26, H40) and Fagaceae with *Quercus* sp. (H37). The last major cluster (C) comprises the Oleaceae with *Olea* sp. (H6), Fagaceae with *Castanea* sp. (H19, H22), Ericaceae (H30, H34) and Tiliaceae (H21) (C1.a), followed by mainly multifloral samples (C2.a) and lastly (C2.b) with Compositae H (H5, H23, H25), Brassicaceae (H18) and Fagaceae (*Castanea* sp.) (H16, H36) (Supplementary Fig. S3). This shows that the floral origin and its associated proteins can be used as a method for honey identification. Comparing the cophenetic correlation coefficients of HCA dendrograms for the plant-derived proteins ($r = 0.91$) vs. the overall protein pattern ($r = 0.81$), showed that the plant-derived proteins have higher explanatory power. Therefore, in order to avoid inconsistent results, the bee-originated proteins were removed for further analysis.

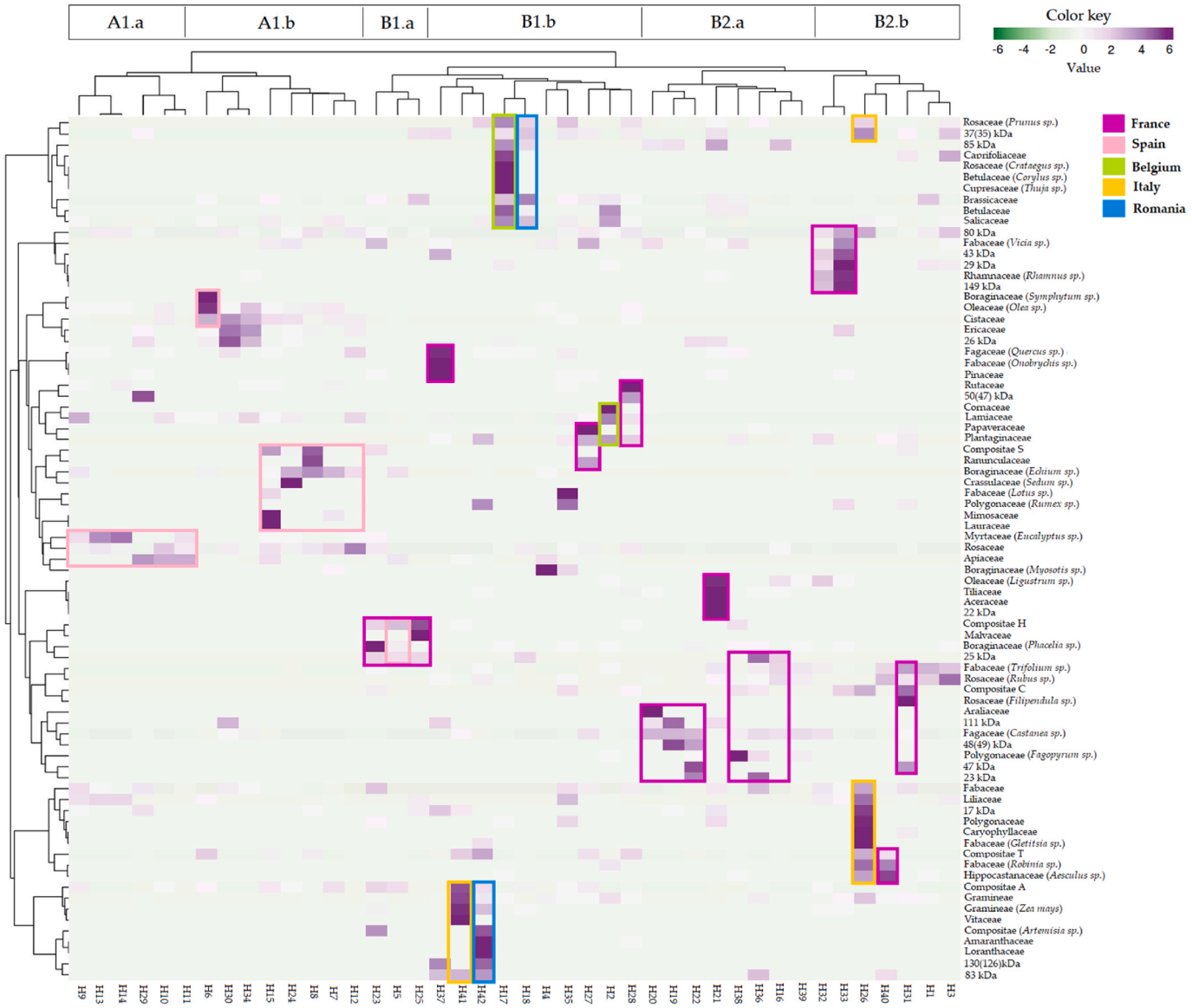

Fig. 3. Hierarchical clustering and heatmap visualization of the 42 honey samples based on their protein and melissopalynological profiles. Columns indicate the honey samples and rows the protein pattern and melisopalynological analysis. Cells are colored based on the quantity in each honey sample, where purple represents a strong positive correlation and green a strongly negative correlation. The row dendrogram resulted from the correlation between the protein pattern and pollen content; the column dendrogram showed the correlation between honey samples. Strong positive correlations, based on the honey's geographical origin, are highlighted in colored frames (France-violet; Spain-light pink; Belgium-light green; Italy-yellow; Romania-blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Relationship between the melissopalynological analysis and the protein pattern

Hierarchical clustering was used to visualize similarities and differences between protein composition and melissopalynological analysis (Fig. 3). The 1st and 2nd sub-clusters (A1.a and b) mainly contain honey samples from Spain (highlighted in light pink), along with samples H24, H29, H30 and H34, respectively. Following the importance score, sample H28 predominant in Rutaceae and sample H29 predominant in Apiaceae are strongly correlated to the 50(47) kDa protein band. The protein band with a MW of 26 kDa was found to correspond to the honey samples containing as major pollen the families of Oleaceae (*Olea* sp.), Cistaceae and Boraginaceae (*Symphytum* sp.), for example H30. The 3rd and 4th sub-clusters (B1.a and b) contain several samples from France, Belgium (H2, H17) and Romania (H18, H42). Several higher molecular weight protein bands corresponded to the honeydew honeys (H41 and H42). Sample H41 with secondary and important minor pollen in Compositae A, Gramineae, Gramineae (*Zea mays*) and Vitaceae grouped with the protein band of 83 kDa, whereas sample H42 with secondary pollen in Amaranthaceae, Compositae (*Artemisia* sp.) and Loranthaceae corresponded to the 130(126) kDa protein band. The protein band of 25 kDa corresponded to the honey samples H5, H23 and H25 (B1.a) containing as major pollen Compositae H (*Helianthus* sp.), and important minor pollen in Malvaceae and Boraginaceae (*Phacelia* sp.). Samples H17 and H18 corresponded to the 85 kDa protein band, whereas Rosaceae (*Prunus* sp.) grouped with a protein band of 37(35) kDa, found as secondary and important minor pollen in samples H17 and H18. Honey samples H2, H4, H27, H28, and H35, grouping in the same cluster, might have similar protein patterns and pollen families. Interestingly, following the importance score, these samples contained secondary and minor pollen from Lamiaceae, Papaveraceae and Plantaginaceae, highlighting the importance of minor pollen in influencing clustering levels. The 5th sub-cluster (B2.a) comprises samples from France collected in summer (H16, H19-22, H38, 39) with the Aceraceae, Oleaceae (*Olea* sp.) and Tiliaceae family as predominant families in sample H21, corresponding to the 22 kDa protein band. Samples H19-22 predominant in Fabaceae (*Castanea* sp.) grouped with the 47, 48(49) kDa and 111 kDa protein band. The 6th sub-cluster (B2.b) comprises the Fabaceae, Liliaceae, Polygonaceae and Caryophyllaceae, which grouped with a 17 kDa protein band (sample H26), whereas the Fabaceae (*Trifolium* sp.), Rosaceae (*Filipendula* sp. and *Rubus* sp.) and Compositae C families (H1, H3, H31) were found to be similar to samples H16, H36, H38 and H39 from sub-cluster B2.a. Most protein bands were detected for H32 and H33 with 29, 43, 80 and 149 kDa, grouping together with Fabaceae (*Vicia* sp.) and Rhamnaceae (*Rhamnus* sp.). Further details can be seen in Fig. 3. To reduce heterogeneity of the results, further investigation on these families found in monofloral honeys is required to group them to species-specific proteins.

The protein profile can be associated with the palynological analysis. This relationship might help estimate the protein pattern of a pollen collection if the palynological analysis has been done. This means that the palynological analysis is still an important step in the botanical and geographical origin determination of honey. Further steps are required to optimize this new method as a mean to assist in identifying adulterated honey. Adulteration by adding foreign pollen or diluting honey with artificial sweeteners might nevertheless be detectable with the method proposed here.

4. Discussion

Melissopalynology has been extensively used to determine the purity, geographical and floral origin of honey. It is also used to assess correlations with *in situ* climatic parameters such as rainfall and temperature (Bilisik, Cakmak, Bicakci, & Malyer, 2008; Jato, Iglesias, & Rodriguez-Gracia, 1994), important external factors influencing pollinators and pollination networks (Abrol, 2013; Vicens & Bosch, 2000). Newly developed pollen identification methods, based on high-throughput multispectral imaging flow cytometry, might help reducing time for pollen identification (Dunker et al., 2021) to get an even more precise picture on what pollen profiles can tell us about the honey's origin. Statistical analyses, mainly ordination (PCA, cluster analysis), have been conducted on melissopalynological data in quantitative studies to obtain more robust characterization of honey in terms of their geographic and botanical origin (Aronne & De Micco, 2010; Corbella & Cozzolino, 2008; Herrero, María Valencia-Barrera, San Martín, & Pando, 2002; Oroian, Amariei, Rosu, & Gutt, 2015). Such pollen-frequency dependent analysis is still the most commonly used multivariate method and can now be extended by including protein profiles. Future approaches can also try to include polyphenolic spectra data describing the olfactory and gustatory bouquet of honey.

The results of the pollen diagram and the following cluster analysis clearly showed the geographical origin of the tested honey samples, with some exceptions that are probably based on similar botanical origin species in different geographical regions (Figs. 1 and 2). As for the future studies, an increasing number of samples from the same country is mandatory for a better honey discrimination based on their geographic origin, especially for Italy and Romania. Endemic plant species, present as minor pollen, might help in discriminting the samples based on their geographic origin, which can be demonstrated by cluster analysis. In this aspect, Sniderman, Matley, Haberle, and Cantrill (2018) analysed the pollen content of 173 unblended honey samples sourced from Western and South-Eastern regions of Australia. Based on their cluster analysis, the Western and Southern honey types could not be discriminated by palynological analysis, mainly due to the presence of secondary and important minor pollen species. They further showed that Myrtaceae morphotypes, as endemic or highly specific pollen, may be a feasible criterion for authenticating the origin of Australian honeys.

The seven plant families in the French honey samples (Apiaceae, Boraginaceae (*Echium* sp.), Compositae H, Fagaceae (*Castanea* sp. and *Quercus* sp.), Rosaceae (*Prunus* sp. and *Rubus* sp.), Rhamnaceae and Rutaceae) are very well known for typical honey from France (Piroux et al., 2014). The latter examined the correlation between pollen diversity and landscape features by palynological analyses of honey collected from sixteen apiaries in Western France. Their results showed that the dominant plant families were Asteraceae, Brassicaceae, Fabaceae, Fagaceae, Poaceae, and Rosaceae, respectively. In the samples from Spain (H4-H15), the predominant families were Apiaceae, Boraginaceae (*Echium* sp.and *Myosotis* sp.), Myrtaceae (*Eucalyptus* sp.), Oleaceae (*Olea* sp.) and Rosaceae (*Rubus* sp.). Interestingly, except Myrtaceae and Oleaceae families, the others strongly correspond to the predominant families found in the samples from France, but resulted in a separate major cluster for Spain (Fig. 2, Supplementary Fig. S2). The composition and not only the most dominant plant families contributed to the differences between honey samples of the two countries. The same effect can be seen in Fig. S3, which means that the method proposed here showed that honeys with similar plant families can be differentiated based on their protein pattern. However, future work is needed on protein identification. For instance, samples H5, H23, and H25 with Compositae H (*Helianthus* type) as predominant and secondary plant family corresponded to a 25 kDa protein band. Previous studies showed contradictory results for sunflower honey. Baroni et al. (2002) reported that sunflower pollen in honey is characteristic to 33–36 kDa proteins, whereas Marshall and Williams (1987) revealed that honey protein constituents mainly originate from bees and that sunflower corresponds to a 17 kDa protein band. Lewkowski et al. (2019) identified sunflower proteins of 13.5 and 27–29 kDa in a honey sample. Such discrepancies will be solved with the availability of plant specific protein profiles.

Belgian honeys were predominant in Rosaceae (*Prunus* sp.), also present in other samples, corresponding to 37(35) kDa, along with Brassicaceae, Fabaceae (*Trifolium* sp.), Lamiaceae, Rosaceae (*Rubus* sp.) and Salicaceae, which grouped with an 85 kDa protein band. As there are no published studies regarding the palynological and/or protein

pattern of honey samples from Belgium, these results extend our understanding of honey identification from this particular region.

Generally, protein content of the studied honeys varied strongly among honey types, with having a range of two orders of magnitude variance. Honey bees processing nectar to honey seem to add constant quantities and quality of bee-specific proteins into the final product (Lewkowski et al., 2019). This means that the variance observed here is mainly driven by proteins of floral origin, added from pollen or secreted in nectar. Conti et al. (2018) showed that the sample's (corbicular pollen) palynological traits, for the identification of foraging season, are characterized by different protein content in pollen. An overview on variance in protein content of pollen may extent the protein profiling of honey. Even though it is presently incomplete, Conti et al. (2016, 2018) and Roulston, Cane, and Buchmann (2000) are constructing such a database to estimate the protein content of a pollen stock, if the family and pollen type are known.

According to the heatmap and hierarchical clustering, the botanical origin of pollen samples was correlated with the protein profile. However, plant protein databases are currently lacking protein profiles of many melliferous plant genera and species, therefore single protein identification is the major task of future studies. Only a hand full of studies identified monofloral honey protein profiles, such as buckwheat, eucalyptus, sunflower and canola honey (Baroni et al., 2002; Borutinskaitė et al., 2017; Lewkowski et al., 2019). Improvement in plant genome sequencing will fill this knowledge gap, which might help for a clear identification of each protein band that was associated with specific, plant genera or families. Nevertheless, the analysis showed that protein profiles and even specific products are useful candidates for future studies to improve honey identification.

This study is an example of monitoring research where the relationship between the palynological spectra and protein pattern in honey samples was explored. This approach attempts to apply the palynological analysis of honey samples as a tool for their separation in homogenous groups in terms of protein profiles. It is also a first step toward developing a protein-plant database for honey. Further methodological research for honey quality assessment should focus on developing techniques for rapid evaluation of the botanical origin of honey (like high-throughput pollen identification; Dunker et al., 2021) since the principal method currently used, the melissopalynological method, is highly demanding and time consuming, requires considerable experience and botanical knowledge. The pollen analyst's experience occasionally generates inconclusive results that can be difficult to interpret. Moreover, it is necessary to devise quick, reliable, and inexpensive methods for detecting honey adulteration.

5. Conclusions

The combination of two widely established reliable methods, melissopalynology and SDS-PAGE, is a useful tool for discriminating between different kinds of honey, even without performing advanced protein identification. The honey protein distribution analysis by SDS-PAGE is a simple and less time-consuming method than MS, which could be useful for monitoring honey authenticity and adulteration together with melissopalynology. The 2D heatmap demonstrates the relationship between melissopalynological analysis and protein pattern for a better authentication of honey. The proposed two-step workflow enables (i) comparison of protein profiles by SDS-PAGE, an easy and fast method, along with melissopalynolgical analysis, and (ii) clustering and visualization of the results as a pollen-protein diagram and a 2D heatmap for data interpretation. Further research is required on honey and pollen protein patterns, and on bee-origin proteins, to get a better understanding of behavior, development and role of honey bees while processing nectar into honey.

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CRediT authorship contribution statement

Carmen Ioana Mureșan: Conceptualization, Supervision, Investigation, Data curation, Formal analysis, Methodology, Validation, Funding acquisition, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **Mihaiela Cornea-Cipcigan:** Investigation, Data curation, Formal analysis, Methodology, Validation, Project administration, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Ramona Suharoschi:** Conceptualization, Supervision, Investigation, Data curation, Formal analysis, Methodology, Validation, Funding acquisition, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **Silvio Erler:** Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Rodica Mărgăoan:** Formal analysis, Methodology, Validation, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112883.

References

Abrol, D. P. (2013). Environmental factors influencing pollination activity of *Apis mellifera* on *Brassica campestris*. *Journal of the Indian Institute of Science, 68*(1&2), 49.

de Alda-Garcilope, C., Gallego-Picó, A., Bravo-Yagüe, J. C., Garcinuño-Martínez, R. M., & Fernández-Hernando, P. (2012). Characterization of Spanish honeys with protected designation of origin "Miel de Granada" according to their mineral content. *Food Chemistry, 135*(3), 1785–1788. https://doi.org/10.1016/j.foodchem.2012.06.057

Alonso-Torre, S. R., Cavia, M. M., Fernández-Muiño, M. A., Moreno, G., Huidobro, J. F., Sancho, M. T., et al. (2006). Evolution of acid phosphatase activity of honeys from different climates. *Food Chemistry, 97*(4), 750–755. https://doi.org/10.1016/j.foodchem.2005.06.010

Ansari, M. J., Al-Ghamdi, A., Khan, K. A., Adgaba, N., El-Ahmady, S. H., Gad, H. A., et al. (2018). Validation of botanical origins and geographical sources of some Saudi honeys using ultraviolet spectroscopy and chemometric analysis. *Saudi Journal of Biological Sciences, 25*(2), 377–382. https://doi.org/10.1016/j.sjbs.2017.09.005

Aronne, G., & De Micco, V. (2010). Traditional melissopalynology integrated by multivariate analysis and sampling methods to improve botanical and geographical characterization of honeys. *Plant Biosystems, 144*(4), 833–840. https://doi.org/10.1080/11263504.2010.514125

Balkanska, R., Stefanova, K., & Stoikova–Grigorova, R. (2020). Main honey botanical components and techniques for identification: A review. *Journal of Apicultural Research, 59*(5), 852–861. https://doi.org/10.1080/00218839.2020.1765481

Baroni, M. V., Chiabrando, G. A., Costa, C., Fagúndez, G. A., & Wunderlin, D. A. (2004). Development of a competitive ELISA for the evaluation of sunflower pollen in honey samples. *Journal of Agricultural and Food Chemistry, 52*(24), 7222–7226. https://doi.org/10.1021/jf049068e

Baroni, M. V., Chiabrando, G. A., Costa, C., & Wunderlin, D. A. (2002). Assessment of the floral origin of honey by SDS-page immunoblot techniques. *Journal of Agricultural and Food Chemistry, 50*(6), 1362–1367. https://doi.org/10.1021/jf011214i

Bauer, L., Kohlich, A., Hirschwehr, R., Siemanna, U., Ebner, H., Scheiner, O., et al. (1996). Food allergy to honey: Pollen or bee products?: Characterization of allergenic proteins in honey by means of immunoblotting. *The Journal of Allergy and*

Clinical Immunology, 97(1), 65–73. https://doi.org/10.1016/S0091-6749(96)70284-1

di Bella, G., Turco, V. L., Potortì, A. G., Bua, G. D., Fede, M. R., et al. (2015). Geographical discrimination of Italian honey by multi-element analysis with a chemometric approach. *Journal of Food Composition and Analysis, 44*, 25–35. https://doi.org/10.1016/j.jfca.2015.05.003

Bilikova, K., Kristof Krakova, T., Yamaguchi, K., & Yamaguchi, Y. (2015). Major royal jelly proteins as markers of authenticity and quality of honey. *Arhiv Za Higijenu Rada I Toksikologiju, 66*, 259–267. https://doi.org/10.1515/aiht-2015-66-2653

Bilikova, K., & Simuth, J. (2010). New criterion for evaluation of honey: Quantification of royal jelly protein apalbumin 1 in honey by ELISA. *Journal of Agricultural and Food Chemistry, 58*(15), 8776–8781. https://doi.org/10.1021/jf101583s

Bilisik, A., Cakmak, I., Bicakci, A., & Malyer, H. (2008). Seasonal variation of collected pollen loads of honeybees (*Apis mellifera* L. *anatoliaca*). *Grana, 47*(1), 70–77. https://doi.org/10.1080/00173130801923976

Bobiş, O., Dezmirean, D. S., Bonta, V., Urcan, A. C., Moise, A. R., & Mărgăoan, R. (2020). Fungal diversity and over-represented non-nectariferous plants pollen in honey. Case study on acacia honey authenticity, analyzed in APHIS laboratory. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca - Animal Science and Biotechnologies, 77*(2), 54–61. https://doi.org/10.15835/buasvmcn-asb:2020.0009

Borutinskaitė, V., Treigytė, G., Matuzevičius, D., Zaikova, I., Čeksterytė, V., Navakauskas, D., et al. (2017). Proteomic analysis of pollen and blossom honey from rape seed *Brassica napus* L. *Journal of Apicultural Science, 61*(1), 73–92. https://doi.org/10.1515/jas-2017-0006

Buttstedt, A., Moritz, R. F. A., & Erler, S. (2014). Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. *Biological Reviews, 89*(2), 255–269. https://doi.org/10.1111/brv.12052

Chin, N. L., & Sowndhararajan, K. (2020). *A review on analytical methods for honey classification, identification and authentication in Honey Analysis: New Advances and Challenges*. IntechOpen.

Chua, L. S., Lee, J. Y., & Chan, G. F. (2015). Characterization of the proteins in honey. *Analytical Letters, 48*(4), 697–709. https://doi.org/10.1080/00032719.2014.952374

Conti, I., Medrzycki, P., Grillenzoni, F. V., Corvucci, F., Tosi, S., Malagnini, V., et al. (2016). Floral diversity of pollen collected by honey bees (*Apis mellifera* L.)–Validation of the chromatic assessment method. *Journal of Apicultural Science, 60*(2), 209–220. https://doi.org/10.1515/jas-2016-0028

Conti, I., Medrzycki, P., Iannone, A., Grillenzoni, F. V., Corvucci, F., Dagnino, D., et al. (2018). Preliminary survey of the nutritional and palynological traits of honey bee-foraged pollen from Liguria (Italy). *Journal of Apicultural Research, 57*(4), 572–579. https://doi.org/10.1080/00218839.2018.1460910

Corbella, E., & Cozzolino, D. (2006). Classification of the floral origin of Uruguayan honeys by chemical and physical characteristics combined with chemometrics. *LWT-Food Science and Technology, 39*(5), 534–539. https://doi.org/10.1016/j.lwt.2005.03.011

Corbella, E., & Cozzolino, D. (2008). Combining multivariate analysis and pollen count to classify honey samples accordingly to different botanical origins. *Chilean Journal of Agricultural Research, 68*(1), 102–107. https://doi.org/10.4067/S0718-58392008000100010

Corvucci, F., Nobili, L., Melucci, D., & Grillenzoni, F. V. (2015). The discrimination of honey origin using melissopalynology and Raman spectroscopy techniques coupled with multivariate analysis. *Food Chemistry, 169*, 297–304. https://doi.org/10.1016/j.foodchem.2014.07.122

Dunker, S., Motivans, E., Rakosy, D., Boho, D., Maeder, P., Hornick, T., et al. (2021). Pollen analysis using multispectral imaging flow cytometry and deep learning. *New Phytologist, 229*(1), 593–606. https://doi.org/10.1111/nph.16882

EC. (2001). Council directive 2001/110/EC of 20 December 2001 relating honey. *Official Journal of the European Communities*, 12.1.2002 L10/47-52 . (Accessed 16 December 2020).

EC. (2014). Directive 2014/63/EU of the European parliament and of the Council. *Amending Council Directive 2001/110/EC Relating to Honey Official Journal of the European Communities L164/1*. . (Accessed 16 December 2020).

Erban, T., Shcherbachenko, E., Talacko, P., & Harant, K. (2021). A single honey proteome dataset for identifying adulteration by foreign amylases and mining various protein markers natural to honey. *Journal of Proteomics, 239*, 104157. https://doi.org/10.1016/j.jprot.2021.104157

Fakhlaei, R., Selamat, J., Khatib, A., Razis, A. F. A., Sukor, R., Ahmad, S., et al. (2020). The toxic impact of honey adulteration: A review. *Foods, 9*(11), 1538. https://doi.org/10.3390/foods9111538

Geana, E. I., & Ciucure, C. T. (2020). Establishing authenticity of honey via comprehensive Romanian honey analysis. *Food Chemistry, 306*, 125595. https://doi.org/10.1016/j.foodchem.2019.125595

Gillette, C. C. (1931). Honey catalase. *Journal of Economic Entomology, 24*(3), 605–606. https://doi.org/10.1093/jee/24.3.605

Gok, S., Severcan, M., Goormaghtigh, E., Kandemir, I., & Severcan, F. (2015). Differentiation of Anatolian honey samples from different botanical origins by ATR-FTIR spectroscopy using multivariate analysis. *Food Chemistry, 170*, 234–240. https://doi.org/10.1016/j.foodchem.2014.08.040

Hammer, Ø., Harper, D. A., & Ryan, P. D. (2001). Past: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica, 4*(1), 1–9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm. (Accessed 16 January 2020).

Herrero, B., María Valencia-Barrera, R., San Martín, R., & Pando, V. (2002). Characterization of honeys by melissopalynology and statistical analysis. *Canadian Journal of Plant Science, 82*(1), 75–82. https://doi.org/10.4141/P00-187

Jato, M. V., Iglesias, M. I., & Rodriguez-Gracia, V. (1994). A contribution to the environmental relationship of the pollen spectra of honeys from Ourense (NW Spain). *Grana, 33*(4–5), 260–267. https://doi.org/10.1080/00173139409429008

Kaškonienė, V., & Venskutonis, P. R. (2010). Floral markers in honey of various botanical and geographic origins: A review. *Comprehensive Reviews in Food Science and Food Safety, 9*(6), 620–634. https://doi.org/10.1111/j.1541-4337.2010.00130.x

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685. https://doi.org/10.1038/227680a0

Lewkowski, O., Mureşan, C. I., Dobritzsch, D., Fuszard, M., & Erler, S. (2019). The effect of diet on the composition and stability of proteins secreted by honey bees in honey. *Insects, 10*(9), 282. https://doi.org/10.3390/insects10090282

Louveaux, J., Maurizio, A., & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World, 59*(4), 139–157. https://doi.org/10.1080/0005772X.1978.11097714

Marshall, T., & Williams, K. M. (1987). Electrophoresis of honey: Characterization of trace proteins from a complex biological matrix by silver staining. *Analytical Biochemistry, 167*(2), 301–303. https://doi.org/10.1016/0003-2697(87)90168-0

Molan, P. C. (1998). The limitations of the methods of identifying the floral source of honeys. *Bee World, 79*(2), 59–68. https://doi.org/10.1080/0005772X.1998.11099381

Moreau, E. (1911). Identification et dosage des substances protéiques dans les miels. *Annales des Falsifications et des Fraudes, 4*, 36–41.

Mureşan, C. I., Schierhorn, A., & Buttstedt, A. (2018). The fate of major royal jelly proteins during proteolytic digestion in the human gastrointestinal tract. *Journal of Agricultural and Food Chemistry, 66*(16), 4164–4170. https://doi.org/10.1021/acs.jafc.8b00961

Nayik, G. A., Suhag, Y., Majid, I., & Nanda, V. (2018). Discrimination of high altitude Indian honey by chemometric approach according to their antioxidant properties and macro minerals. *Journal of the Saudi Society of Agricultural Sciences, 17*(2), 200–207. https://doi.org/10.1016/j.jssas.2016.04.004

Neuhoff, V., Arold, N., Taube, D., & Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis, 9*(6), 255–262. https://doi.org/10.1002/elps.1150090603

Nguyen, H. T. L., Panyoyai, N., Kasapis, S., Pang, E., & Mantri, N. (2019). Honey and its role in relieving multiple facets of atherosclerosis. *Nutrients, 11*(1), 167. https://doi.org/10.3390/nu11010167

Oddo, L. P., Piana, L., Bogdanov, S., Bentabol, A., Gotsiou, P., Kerkvliet, J., et al. (2004b). Botanical species giving unifloral honey in Europe. *Apidologie, 35*(Suppl. 1), S82–S93.

Oddo, L. P., Piazza, M. G., Sabatini, A. G., & Accorti, M. (1995). Characterization of unifloral honeys. *Apidologie, 26*(6), 453–465. https://doi.org/10.1051/apido:19950602

von der Ohe, W., Oddo, L. P., Piana, M. L., Morlot, M., & Martin, P. (2004). Harmonized methods of melissopalynology. *Apidologie, 35*(Suppl. 1), S18–S25. https://doi.org/10.1051/apido:2004050

Oddo, L. P., Piro, R., Bruneau, É., Guyot-Declerck, C., Ivanov, T., Piskulová, J., et al. (2004a). Main European unifloral honeys: Descriptive sheets. *Apidologie, 35*(Suppl. 1), S38–S81, 10.1051/apido:2004049.

Oroian, M., Amariei, S., Rosu, A., & Gutt, G. (2015). Classification of unifloral honeys using multivariate analysis. *Journal of Essential Oil Research, 27*(6), 533–544. https://doi.org/10.1080/10412905.2015.1073183

Osés, S. M., Ruiz, M. O., Pascual-Maté, A., Bocos, A., Fernández-Muiño, M.Á., & Sancho, M. T. (2017). Ling heather honey authentication by thixotropic parameters. *Food and Bioprocess Technology, 10*(5), 973–979. https://doi.org/10.1007/s11947-017-1875-6

Pasupuleti, V. R., Sammugam, L., Ramesh, N., & Gan, S. H. (2017). Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxidative Medicine and Cellular longevity*, 1259510. https://doi.org/10.1155/2017/1259510, 2017.

Pauliuc, D., Dranca, F., & Oroian, M. (2020). Antioxidant activity, total phenolic content, individual phenolics and physicochemical parameters suitability for Romanian honey authentication. *Foods, 9*(3), 306. https://doi.org/10.3390/foods9030306

Piroux, M., Lambert, O., Puyo, S., Farrera, I., Thorin, C., L'Hostis, M., et al. (2014). Correlating the pollens gathered by *Apis mellifera* with the landscape features in western France. *Applied Ecology and Environmental Research, 12*(2), 423–439. https://doi.org/10.15666/AEER/1202_423439

Puścion-Jakubik, A., Borawska, M. H., & Socha, K. (2020). Modern methods for assessing the quality of bee honey and botanical origin identification. *Foods, 9*(8), 1028. https://doi.org/10.3390/foods9081028

Rossano, R., Larocca, M., Polito, T., Perna, A. M., Padula, M. C., Martelli, G., et al. (2012). What are the proteolytic enzymes of honey and what they do tell us? A fingerprint analysis by 2-D zymography of unifloral honeys. *PLoS One, 7*(11), Article e49164. https://doi.org/10.1371/journal.pone.0049164

Roulston, T. A. H., Cane, J. H., & Buchmann, S. L. (2000). What governs protein content of pollen: pollinator preferences, pollen–pistil interactions, or phylogeny? *Ecological Monographs, 70*(4), 617–643. https://doi.org/10.1890/0012-9615(2000)070[0617:WGPCOP]2.0.CO;2.

Schepartz, A. I. (1965). The glucose oxidase of honey II. Stereochemical substrate specificity. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis, 96*(2), 334–336. https://doi.org/10.1016/0005-2787(65)90597-6.

Schepartz, A. I., & Subers, M. H. (1966). Catalase in honey. *Journal of Apicultural Research, 5*(1), 37–43. https://doi.org/10.1080/00218839.1966.11100130

Siddiqui, A. J., Musharraf, S. G., & Choudhary, M. I. (2017). Application of analytical methods in authentication and adulteration of honey. *Food Chemistry, 217*, 687–698. https://doi.org/10.1016/j.foodchem.2016.09.001

Sniderman, J. K., Matley, K. A., Haberle, S. G., & Cantrill, D. J. (2018). Pollen analysis of Australian honey. *PLoS One, 13*(5), Article e0197545. https://doi.org/10.1371/journal.pone.0197545

Thrasyvoulou, A., Tananaki, C., Goras, G., Karazafiris, E., Dimou, M., Liolios, V., et al. (2018). Legislation of honey criteria and standards. *Journal of Apicultural Research, 57*(1), 88–96. https://doi.org/10.1080/00218839.2017.1411181

Todd, F. E., & Vansell, G. H. (1942). Pollen grains in nectar and honey. *Journal of Economic Entomology, 35*(5), 728–731. https://doi.org/10.1093/jee/35.5.728

Vicens, N., & Bosch, J. (2000). Weather-dependent pollinator activity in an apple orchard, with special reference to *Osmia cornuta* and *Apis mellifera* (Hymenoptera: Megachilidae and Apidae). *Environmental Entomology, 29*(3), 413–420. https://doi.org/10.1603/0046-225X-29.3.413

Ward, J. H., Jr. (1963). Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association, 58*(301), 236–244.

White, J. W., Jr., & Kushnir, I. (1967a). Composition of honey. VII. Proteins. *Journal of Apicultural Research, 6*(3), 163–178. https://doi.org/10.1080/00218839.1967.11100177

White, J. W., Jr., & Kushnir, I. (1967b). The enzymes of honey: Examination by ionexchange chromatography, gel filtration, and starch-gel electrophoresis. *Journal of Apicultural Research, 6*(2), 69–89. https://doi.org/10.1080/00218839.1967.11100163

Won, S. R., Lee, D. C., Ko, S. H., Kim, J. W., & Rhee, H. I. (2008). Honey major protein characterization and its application to adulteration detection. *Food Research International, 41*(10), 952–956. https://doi.org/10.1016/j.foodres.2008.07.014