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Elemental and isotopic profile of honey – insights related to the nature of the exploited plant and potential environmental influences

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ABSTRACT

The goal of this work is to enhance our comprehension of the factors that influence the content of rare earth elements (REEs) in honey to have better control over their propagation from soil to plant and food. In this regard, we employed Pearson correlation to trace the possible interdependencies among REEs and several elements. A novel approach to honey classification was conducted to distinguish between two types of honey originating from herbaceous plants and from trees, assessed at both elemental and isotopic levels. The differences observed were possibly explained by the variances in plant environmental surroundings, the agricultural practices applied (including the use of pesticides and fertilizers), and the depth of root systems influencing access to soil-derived elements. Results indicate that root depth and plant type significantly influence the elemental uptake and highlight that honey quality control can serve not only to ensure product authenticity, but also as a tool to reflect environmental contamination and localized pollution affecting the plants used for honey production. Geographical influences were also assessed by comparing honey from Romania (Transylvania) with that from other EU countries and revealed that La, Ce, and Pr are markers for this classification type. Reliable recognition models for the botanical and geographical discrimination of honey were developed.

1. Introduction

Honey is a worldwide consumed commodity, valued for its enjoyable flavor and the health benefits it affords. As a result, significant amounts of honey from various botanical and geographical sources are available on the market. Europe, being one of the world's significant honey market, has substantial local production and a high level of honey consumption. In this regard, according to a recent report published by the European Commission [1], the European countries with the highest honey production are Romania, Spain, and Hungary, among others. Despite Europe's significant honey production, it only meets 60 % of its domestic demand [1]. Therefore, Europe must rely on honey imports from non-European countries to meet its remaining internal demand or consumption, with Ukraine and China being the primary sources [1]. Due to the large discrepancy in this item on the market, it is crucial to pay special attention to its composition to ensure food quality and safety.

Honey contains primarily carbohydrates, including glucose, fructose, and sucrose, along with water, as well as small quantities of other substances such as amino acids, proteins, vitamins, and trace elements [2,3]. The elemental content of honey is an important quality attribute of this product, which is naturally enriched in macroelements (Ca, K, Mg, P, Na) and essential elements (Fe, Se, Zn, etc.). Moreover, honey is considered a bio-indicator of environmental pollution, yielding information about the state of the surroundings and the toxic metal content of the soil, plants, and bees [4–9]. The pollutants that influence the bees and bee products can be of different natures, such as the metal contaminations that humans willingly or unwillingly produce in everyday activities, such as burnt gases from vehicular traffic or biomass handling, or the use of pesticides against different harmful agents in agriculture [10,11]. Thus, some of the reported toxic metals resulting from the previously mentioned pollution causes are Cu, Sb, Sn, Fe, Mn, K, Rb, Cs,

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Li, Tl, Pb, Cd, Zn, Cr, Ni, and Co [4,12–15]. Apart from these toxic elements, other emerging contaminants such as the rare earth elements group (REEs) need to be monitored. The REEs group is divided into two subcategories: light REEs (La, Ce, Pr, Nd, Sm, Eu, Gd) and heavy REEs (Tb, Dy, Ho, Er, Tm, Yb, Lu), with the addition of two other elements, namely Y and Sc. They are nonessential for living organisms but are naturally present in soils from the bedrock, with a distribution that is related to the geographical position, regional geology, rock forming, and hydrothermal processing [16,17]. They are key elements in technologies, finding various applications in high-interest domains, such as industrial technologies, device production, superconductors, solar panels, electronics, agriculture as additives, and even in medicine as contrast agents or in cancer treatment [18–24]. Because of their importance and increased global demand, there is a rising concern regarding their environmental accumulation as a result of anthropologic activities such as mining, exploitation, or uncontrolled disposal [16,25]. The understanding of how these compounds interact and how they are distributed from soil to plants and along the food chain is of particular importance for human health, as recent studies have reported possible health concerns related to REEs exposure [26] and several diseases (i.e., endomyocardial fibrosis, lung and bladder cancer, hypertension, and changes in the cognitive functions of children) [27,28]. Consequently, there is a growing recognition of the need to control these specific compounds in food commodities [29], as these elements can enter the food chain through absorption from the soil at the root level and be further dispersed to stems, flowers, and fruit [30]. Several studies proved that the concentration profile of REEs present in a given soil is consistent with that of plants and several food products, such as carrots [31] and hazelnuts [32]. Generally, based on the data reported in the literature, in the case of food and beverages that have a longer production chain, like milk [33], wine [33,34] and honey [35], an attenuated fingerprint of REEs content was observed.

In this light, the present work aims to contribute to a better understanding of the mechanism of accumulation and factors that could influence the REEs content in honey. For this purpose, the possible interdependencies among several essential trace elements and REEs were screened. For this purpose, 212 honey samples were collected from six countries, representing more than 25 botanical origins. This diverse selection ensures a high degree of generalizability in the correlations obtained between the occurrence and concentrations of REEs and other elements. To assess these interdependencies and better point out their occurrence affinities, Pearson correlation was employed using all determined variables.

Another aim of this study was to investigate how the plant growing and environmental conditions of different plants, given by their root systems, agricultural practices and possible pollution issue influence the isotopic and elemental profile of the investigated honey samples, and also to see if honey can serve as a control system and a bio-indicator of these differences. In this regard, the isotopic fingerprint and the elemental profile of honey samples derived from shallow-rooted herb (herbaceous) plants (e.g., colza, polyfloral, sunflower, raspberry, black grass, coriander, sulla, thistle, lavender, thyme) and also from deeper-rooted tree sources (e.g., acacia, honeydew, linden, chestnut, fir, orange) were compared. The motivation for investigating these two classes arises from the disparate environmental conditions regarding agricultural practices and pollution, specifically related to the fact that herb plants flourish in closer vicinity to possibly contaminated urban areas and are more prone to pollution from agricultural practices such as the use of fertilizers and pesticides, as compared to trees that thrive in regions with cleaner air, distant from anthropogenic pollution. In this regard, by analyzing honey as a control and possible bio-marker for pollution, the study seeks to understand how plants' root depth influences the uptake of elements from soil and how contaminants from environment are found in honey, offering insight into the potential of different types of honey to reflect localized environmental pollution levels.

Besides these, the variations in isotopic and elemental profiles, with a particular focus on REEs, among the honey samples having different geographical origins (horizontal distribution) was followed, by comparing the samples produced in Transylvania, Romania, with others produced in several EU countries (Italy, France, Spain, Greece and Portugal). The obtained differentiation markers for these two honey classification criteria were subsequently used as input for developing differentiation models using Partial Least Squares Discriminant Analysis (PLS-DA).

2. Experimental

2.1. Sample description

A dataset comprising 212 authentic honey samples with diverse botanical origins, which comprised more than 25 botanical varieties, sourced from various EU geographical regions including Romania, Italy, France, Spain, Greece, and Portugal, was utilized in this study. The diverse dataset essential to capture general trends was used to investigate possible correlations of isotopic and elemental determinations.

From this dataset, 79 samples (as outlined in Table 1) were selected for analyzing the factors that differentiate honey based on the root system of the plant used in honey production, the surrounding environment of the plant and the agricultural practices employed. In this regard, the selected honey samples were divided into two classes: *(i)* "***tree honey***" consisting of 49 samples obtained from deep-rooted (between 1 and 15 m below the ground) tree sources, such as acacia, linden, chestnut, fir, orange tree, or from bees that collected the exuded nectar of another insect present on trees (honeydew honey); and *(ii)* "***herb plant honey***" comprising 30 honey samples (Table 1) obtained from herbaceous plants, which lack persistent woody stems and typically have shorter, less dispersive root compared to tree plants [36,37]. The samples were collected from trustworthy producers which obtained the monofloral honeys by placing the hives in areas with extensive and homogeneous floral or arboreal coverage, which ensured that the nectar collected predominantly originated from the target botanical source. In the case of polyfloral honey, bees gathered the nectar from various plant species, either as a result of hive movement or the natural presence of multiple flowering plants nearby. These polyfloral honey types were classified within the herb plant honey category, as their nectar sources were exclusively herbaceous, with no contribution from tree species given the absence of such vegetation near the hives. This classification was performed to analyze how the root system of the plant used in honey production, the surrounding environment of the plant and the agricultural practices employed influence the elemental and isotopic content of

Table 1
Split classes of the investigated honey sample dataset according to the two subgroups (tree vs. herb plants honey).

Type	No.	Botanical origin
Honey from trees (variation of 1 to 15 m of root depth)	17	acacia
	12	honeydew
	11	linden
	5	chestnut
	2	fir
	2	orange
Honey derived from herb plants (<1 m root depth)	9	colza
	7	polyfloral
	5	sunflower
	2	raspberry
	2	black grass
	1	coriander
	1	sulla
	1	thistle
	1	lavender
	1	thyme

various honey types.

The same dataset was used, taking into consideration the geographical differentiation, for determining the variables that distinguish honey produced in a defined region of Romania (Transylvania), from honey produced in other EU countries (Table 2.). In this regard, to determine the factors deciding the horizontal distribution given by the geographical differentiation, the isotopic and elemental profile of 60 honey samples collected from Transylvania, Romania, were compared to 19 samples from other EU countries (8 - France, 6 - Greece, 3 - Italy, 1 -Portugal and 1 - Spain), as presented in Table 2.

2.2. Isotope ratios of light elements

The samples were analyzed for their isotopic profile, including the $\delta^{13}C$ value of honey and its corresponding protein, alongside the $\delta^{18}O$ and $\delta^{2}H$ isotope values of the water extracted from the honey samples.

To acquire the isotopic values (^{13}C, ^{2}H, and ^{18}O) of investigated honey, the first step consisted in sample preparation, using different protocols, as hereafter described:

- To obtain the ^{13}C values from bulk honey, water was removed by keeping the samples in an oven (60 °C, 48 h). Then, a combustion of 5 mg from dried honey followed (550 °C, 3 h). The resulting CO_2 was separated by other resulting gases using a cryogenic line, and then measured by Isotope Ratio Mass Spectrometry (IRMS);
- To isolate protein fraction from honey for its ^{13}C signature determination, a preparation protocol was used, in compliance with AOAC official method (AOAC official method 998.12) and presented by Magdas et al. [38];
- To acquire the isotopic values of ^{2}H and ^{18}O, the stage of water extraction without isotopic fractionation was achieved using cryogenic distillation under vacuum, previously described in our works [38,39]. The quantity of honey sample subjected to cryogenic distillation was 3 g.

According to the IUPAC protocol [40], the isotopic values are expressed in the delta scale (δ‰), versus the international standards (V-PDB for $^{13}C/^{12}C$ determinations and V-SMOW for measurements of $^{2}H/^{1}H$ and $^{18}O/^{16}O$) according to the Eq. (1):

$$\delta_{ref}\left({}^{i}E/{}^{j}E, sample\right) = \left[\frac{R\left({}^{i}E/{}^{j}E, sample\right)}{R\left({}^{i}E/{}^{j}E, ref\right)}\right] - 1 \quad (1)$$

where *ref* is the international measurement standard, sample is the analyzed sample, and ${}^{i}E/{}^{j}E$ is the isotope ratio between heavier and lighter isotopes. The delta values were multiplied by 1000 and expressed in units “per mil” (‰).

Delta V Advantage (Thermo Scientific) mass spectrometer was used, in line with a dual inlet system, to obtain the isotopic values of ^{13}C from bulk honey and its corresponding protein. Each sample was analyzed in duplicate. Each day before analyzing honey samples, one working standard was measured. This standard was calibrated using NBS-22 oil, a certified reference material provided by the IAEA (International Atomic Energy Agency), having a $\delta^{13}C_{VPDB}$ value of −30.03 ‰. The uncertainty limit for ^{13}C determinations from bulk samples and extracted honey protein samples was ±0.2 ‰.

To obtain the isotopic signatures of ^{2}H and ^{18}O from the water samples extracted from honey, the used apparatus was a liquid-water isotope analyzer (DLT – 100, Los Gatos Research). The isotopic values were calibrated using a set containing five international working standards (WS) (WS1, $\delta^{18}O = -19.57$ ‰ and $\delta^{2}H = -154.1$; WS 2, $\delta^{18}O = -15.55$ ‰ and $\delta^{2}H = -117.0$; WS3, $\delta^{18}O = -11.54$ ‰ and $\delta^{2}H = -79.0$ ‰; WS 4, $\delta^{18}O = -7.14$ ‰ and $\delta^{2}H = -43.6$ ‰; WS 5, $\delta^{18}O = -2.96$ ‰ and $\delta^{2}H = -9.8$ ‰). The uncertainty was ±0.2 ‰ for $^{18}O/^{16}O$ and ± 1 ‰ for $^{2}H/^{1}H$.

2.3. Elemental profiling

ELAN DRC-e mass spectrometer (PerkinElmer SCIEX, USA) was used for the determination of 38 elements, namely Li, B, Al, Ti, V, Cr, Mn, Co, Ni, Cu, Zn, Ga, As, Rb, Sr, Y, Zr, Nb, Mo, Pd, Sn, Sb, Ba, La, Ce, Pr, Nd, Gd, Dy, Yb, Lu, Hf, Ta, Re, Ir, Pt, Tl, Pb, while ContrAA 800D spectrophotometer (Analytik Jena, Jena, Germany) was used for the determination of the 4 macro-elements, namely: Na, Mg, K, Ca, in the honey samples, after microwave-assisted acid digestion. The analysis method was adapted from the previously described methodology of our previous work [38]. The microwave-assisted acid digestion was employed for sample pretreatment using a Speed ENTRY microwave digester (Berghof). A quantity of 0.1 g honey sample was mixtured with 3 mL of 65 % (v/v) HNO_3 and 2 mL of 40 % (v/v) HF. Following previously reported instrumental parameters and settings [38], which involves a heating process of 3 min from room temperature to 145 °C, a held for 5 min; another heating ramp from 145 °C to 170 °C in 5 min, kept for 10 min; heating to 190 °C in 2 min, held for 15 min; and finally a cooling ramp from 190 °C to 75 °C in 1 min for 10 min. Multi-element calibration standard 2 of 10 μg/mL Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sc, Sm, Tb, Th, Tm, Y, Yb (PerkinElmer Pure Plus, USA), multi-element calibration standard 3 of 10 μg/mL Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn (PerkinElmer Pure Plus, USA), and multi-element calibration standard 4 of 10 mg/L Au, Hf, Ir, Pd, Pt, Rh, Ru, Sb, Sn, Te (PerkinElmer Pure Plus, USA) were used for the standard stock solutions preparation, by dissolving the multi-element solutions with ultrapure water. For the calibration curve, the working solutions of a specific concentration and volume were prepared by diluting the stock solution. The obtained limit of

Table 2
The distribution of the investigated honey sample dataset as a function of the geographical origin (country) employed for the determination of the region-dependent factors influencing the content of honey.

Horizontal split classes (geographical origins)											
Romania (Transylvania)		Other EU countries									
		France		Greece		Italy		Portugal		Spain	
No.	Variety	No.	Variety	No.	Variety	No.	Variety	No.	Variety	No.	Variety
16	acacia	2	polyfloral	2	orange	1	chestnut	1	polyflower	1	honeydew
10	honeydew	1	acacia	1	fir	1	sulla				
10	linden	1	fir	1	chestnut	1	thistle				
9	colza	1	honeydew	1	black grass						
5	sunflower	1	chestnut	1	thyme						
4	polyfloral	1	linden								
2	raspberry	1	lavender								
2	chestnut										
1	coriander										
1	black grass										

quantification (LOQ) are: Li 0.018 μg/L, B 0.016 μg/L, Al 5.101 μg/L, Ti 0.069 μg/L, V 0.058 μg/L, Cr 0.006 μg/L, Mn 0.174 μg/L, Co 0.138 μg/L, Ni 0.063 μg/L, Cu 0.059 μg/L, Zn 0.867 μg/L, Ga 0.001 μg/L, As 0.069 μg/L, Rb 0.126 μg/L, Sr 0.186 μg/L, Y 0.001 μg/L, Zr 0.017 μg/L, Nb 0.001 μg/L, Mo 0.026 μg/L, Pd 0.033 μg/L, Sn 0.036 μg/L, Sb 0.034 μg/L, Ba 0.040 μg/L, La 0.028 μg/L, Ce 0.061 μg/L, Pr 0.001 μg/L, Nd 0.057 μg/L, Gd 0.003 μg/L, Dy 0.006 μg/L, Yb 0.003 μg/L, Lu 0.001 μg/L, Hf 0.001 μg/L, Ta 0.001 μg/L, Re 0.001 μg/L, Ir 0.036 μg/L, Pt 0.077 μg/L, Tl 0.001 μg/L, Pb 0.076 μg/L, Na 1.9 μg/L, Mg 1.3 μg/L, K μg/L, Ca 9.3 μg/L.

2.4. Data processing

The acquired dataset, comprising 46 attributes (42 elements: Li, B, Na, Mg, Al, K, Ca, Ti, V, Cr, Mn, Co, Ni, Cu, Zn, Ga, As, Rb, Sr, Y, Zr, Nb, Mo, Pd, Sn, Sb, Ba, La, Ce, Pr, Nd, Gd, Dy, Yb, Lu, Hf, Ta, Re, Ir, Pt, Tl, Pb, and 4 isotopic ratios: $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{2}H$, $\delta^{18}O$) illustrated the isotopic and elemental fingerprints of the honey samples. First, the dataset underwent exploratory data analysis using Pearson's correlation test to assess the degree of linear relationship between specific variables. The Pearson's correlation coefficient, often denoted as *r*, can range from −1 to 1. A higher positive value indicates a positive linear relationship (i.e. a similar trend of increase/decrease), while a lower negative one corresponds to negative linear relationships (i.e. as one variable increases, the other one decreases or vice versa). Values close to 0 suggest that the two variables are not linearly related [41].

2.4.1. Markers identification

To identify the most relevant isotope or elemental content for discriminating between different classifications (either honey produced from trees versus herb plants, or honey originating from Transylvania versus that produced in other EU countries), two supervised techniques were employed: a feature selection method based on partial least squares (PLS) and analysis of variance (ANOVA). The first technique, using PLS regression, involves iteratively identifying relevant variables based on their importance in projection and selectivity ratio scores obtained through PLS modeling. This method was applied using the Solo 8.9 software (R8.9.1) from [42], Manson, WA, USA. The second technique, based on ANOVA, assesses the differences in the mean values recorded for a variable across different sample groups and assigns each variable an F-score proportional to its discrimination power. Variables with higher F-scores are considered important differentiation markers, while those with lower scores are deemed less significant [41]. Both techniques provide advantages for the model development: PLS provides insight into which variables are most influential for prediction, while ANOVA provides insight into which variables show statistically significant differences between groups and is suitable even for datasets with imbalanced class distribution [43]. Together, they offer a more comprehensive understanding of both predictive power and statistical significance, adding robustness to the analysis.

2.4.2. Classification model development

The relevant variables identified from the isotope and elemental profiles of the honey samples underwent Partial Least Squares Discriminant Analysis (PLS-DA) modeling to assess their potential for distinguishing honey samples based on botanical source and geographical origin. Before model development, the data matrix was preprocessed using autoscale, which involves standardizing each variable (isotope/elemental concentration) by subtracting its mean value and dividing by its standard deviation. To evaluate the performance of the PLS-DA models, 10-fold cross-validation was employed, a method chosen due to its reliability with limited data instances. Unlike other evaluation methods that may compromise sample integrity, cross-validation ensures a more robust assessment of the models' prediction capability [44].

3. Results and discussions

3.1. Correlations between the elemental content of honey.

The first aim of the study was to contribute to a better understanding of the factors that can influence the REEs content in honey. For this purpose, an exploratory data analysis was conducted with the aim of identifying possible relationships among the contents of REEs (La, Ce, Pr, Nd, Gd, Dy, Yb, Lu, Y), their cumulative concentration (∑REEs) and other elements whose concentrations were measured (Li, B, Na, Mg, Al, K, Ca, Ti, V, Cr, Mn, Co, Ni, Cu, Zn, Ga, As, Rb, Sr, Zr, Nb, Mo, Pd, Sn, Sb, Ba, Ta, Hf, Re, Ir, Pt, Tl, Pb). Some elements among which Mn, Co, Cr, Sr, and Ni were previously reported as being positively correlated to REEs in carrot samples [31]. The identification of possible interconnections was achieved by applying Pearson's correlation test on a dataset consisting of 212 honey samples and evaluating the obtained coefficients with respect to each pair of elements. In the present work, the relevant correlations were considered the ones having an absolute value for *r* above 0.65, according to the threshold proposed by Asuero et al. [45]. The strongest correlations observed above this threshold are shown in Fig. 1, with moderate and high positive correlations corresponding to Yb and Lu, Zr and Pr, Dy and Y, ∑REEs and Dy, ∑REEs and Y, La and Ce, La and Sr. Given the high heterogeneity of the honey sample set induced by the presence of more than 25 varietal sources and more than 5 countries of origin, observations with a high degree of generalizability aimed to be identified in the current study.

In this regard, very strong positive correlations were observed between Yb and Lu ($r = 1$), Zr and Pr ($r = 0.9$), while strong correlations were obtained between Dy and Y ($r = 0.74$). Also, the sum of REEs concentrations was strongly positively correlated with Dy and Y ($r = 0.85$ in both cases). The correlations between Yb, Lu, Dy and Y could be explained by their similar physicochemical properties and ionic radius, being classified as heavy REEs [46,47]. The average concentrations of these elements found in the honey samples were ten times smaller than the concentrations of the so-called light REEs (La, Ce, Pr, Nd, Gd). This is in agreement with the previously studies that suggest that light REEs are more mobile and are easier absorbed by the plant through the soil [16,30].

No significant relationships were identified between the determined concentrations of REEs and Ni, Cr, or Mo despite being previously reported as co-dependent in carrots [31]. This discrepancy may arise from differences in uptake and translocation mechanisms between root vegetables and the floral or honeydew sources of honey. While root vegetables accumulate elements directly from the soil, honey is derived from nectar-producing plants or from honeydew excreted by insects feeding on tree phloem sap. In particular, REEs are known to accumulate preferentially in roots due to their low mobility, whereas the transfer of these elements within plant vascular systems and their subsequent transfer into nectar or phloem is limited. In contrast, transition metals such as Ni, Cr, and Mo are more readily transported into the aerial parts of plants. Further targeted studies would be required to determine the translocation mechanism of these elements in soil-plant systems and their transfer to plant parts and subsequently to honey. A moderate correlation was observed in this case of honey between La and Sr, which was also observed in carrots [31]. This can be explained by the fact that in the case of the previously conducted studies, the analyzed matrix directly reflects the elemental distribution in the soil and the transfer of elements in the plant, whereas in the case of honey, the production chain is considerably longer (i.e. from soil to nectar/insects, to bees and to the final product) and the reflection of the soil composition is highly attenuated or even might be lost. The low efficiency of the REEs profile for the processed food matrix (e.g. wine) compared to vegetables, fruits, and meat due to the fractionation of REEs during the making process was previously reported and discussed [48,49].

Nonetheless, significant positive correlations were also observed between La and Ce ($r = 0.67$) and La and Sr ($r = 0.66$). La and Ce have

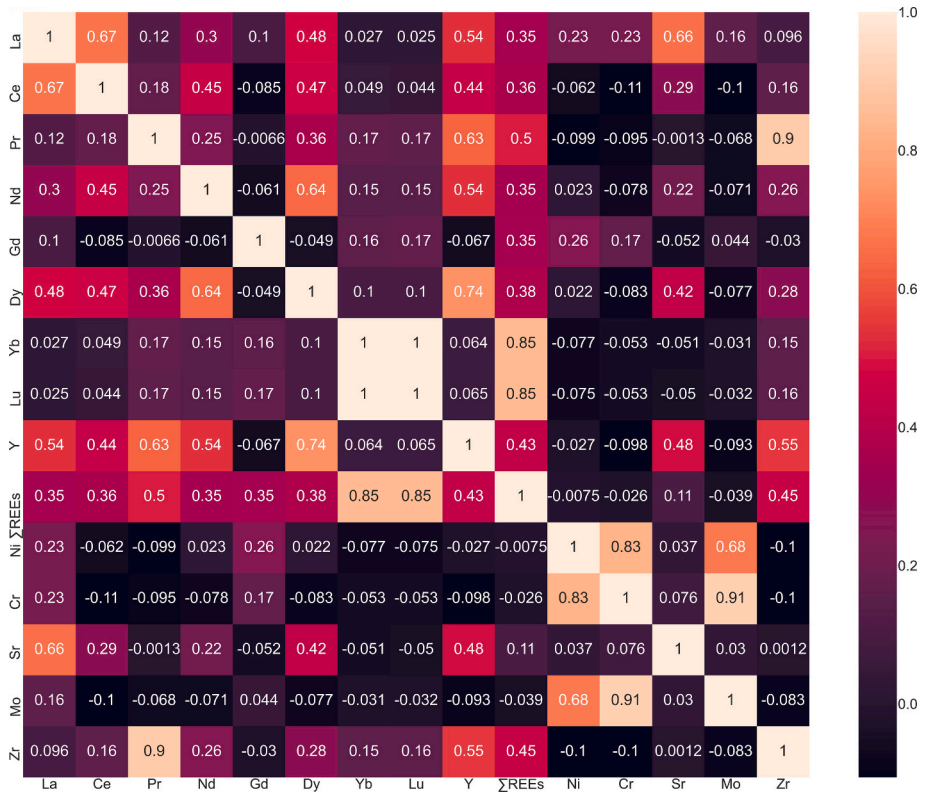

Fig. 1. Pearson's correlation coefficients between REEs, sum of REEs, Zr, Mo, Sr, Cr and Ni in the investigated honey sample set.

been extensively studied in previously published works ever since reports from China expressed the increase in plant growth, crop yield, and quality when these two REEs elements were used in fertilizers [50–52]. However, recent studies showed that high concentrations of La and Ce in soil or water can have a negative impact on plants [18]. Their positive correlation might be explained by their increased presence in soils as a result of natural abundance or anthropogenic pollution, due to their extensive use in technological applications (e.g. devices, computers), the automobile industry, as well as medicine [22,24]. Besides their similar soil abundance, the positive correlation between La and Ce concentrations in honey can be explained by the high mobility they possess as compared to heavier REEs, a fact that directly influences the affinity of plants to absorb light REEs such as La and Ce [30]. Moreover, Ca^{2+}, an important regulatory ion, has a similar radius to that of La^{3+} and Ce^{3+}, and thus the latter can become cofactors in many biological processes [53] and may even substitute calcium ions in plants ([54];).

Other positive dependencies were displayed between Mo and Ni ($r = 0.68$), Ni and Cr ($r = 0.83$), and Mo and Cr ($r = 0.91$). Mo and Ni are essential micronutrients for plants and animals, while Cr is considered to be a micronutrient in the trivalent form Cr(III) or can be hazardous in Cr (IV) state, inhibiting plant growth [55]. It may enter the plant because of the fertilizers used [55]. Mo role in plants assures nitrogen fixation, transforming nitrogen to amino acid and was shown to be present in less acidic soils, thus an increased concentration of Mo in plants leads to greater yields [56]. Ni also plays an important role in plant growth and development as it is essential for urease to transform proteins into amino acids and to mobilize nitrogen compounds [57].

No relevant negative correlations were identified between element concentrations in the investigated sample set. This suggests that by analyzing a honey sample set with high variability in terms of botanical and geographical origins, no element inhibited the presence or abundance of another one.

3.2. General aspects of the honey classification studies

Another aim of this study was to investigate how different plants, through their root systems and distinct environmental surroundings, might lead to differences in terms of isotopic and elemental profiles (including REEs contents) of the analyzed honey produced from these plants, offering insight into the potential of different honey types to reflect possible localized pollution levels. Moreover, the most significant attributes from the elemental and isotopic fingerprint, capable of differentiating honey produced in Transylvania, Romania, from that originated from other EU countries (Italy, France, Spain, Greece, and Portugal) were determined to assess the markers that reflect the distinct pedological influences and the horizontal distribution of elements in the earth soil.

The concentrations of REEs were previously determined and widely used in food authentication and traceability studies [58,48,49,59], as their content in a given food sample (i.e. chestnuts, hazelnuts, pumpkin seeds, milk) proved to be an important marker for the geographical area of production. Moreover, REEs content ensured the traceability validation of foodstuffs, as these elements are absorbed by the plant from soil and water and distributed along roots, leaves, fruit, and flowers [58]. The possibility of guaranteeing traceability along the production chain is dependent on the length of the chain – the shorter the chain, the easier to prove [58], as was previously reported in the case of truffles. If the gap between soil and product is wide, as for wine, milk, and honey, the link between authentication and the REEs concentration must be verified case by case [58]. In the case of honey, a traceability study conducted by Gulino et al. [35] proved that honey's elemental composition (including REEs) is driven by soil composition. This is achieved as the bees' digestion processes do not interfere with the elemental profile, and thus, their distribution seems to be relatively unaltered from soil to honey.

Therefore, the REEs content along with the elemental fingerprint of some pollutants (i.e. Cu, Sb, Sn, Fe, Mn, K, Rb, Cs, Li, Tl, Pb, Cd, Zn, Cr, Ni, Co) can act as a fingerprint of soil, as honey can be considered a bio-

indicator [10,11,35]. Moreover, the concentrations of essential elements and potential pollutants in the plants used for honey production can be impacted by two factors related to the depth distribution of these elements in soil and the environmental surroundings that the plant encounters. In this regard, the elemental profile that the plants possess is influenced by variations in the depths from where the plant roots began to where they can reach (Fig. 2). Therefore, on one hand, tree roots are capable of deeper entering the earth layers and possibly encounter distinct concentration distribution of some elements belonging to the REEs group, all of which will be reflected in the honey concentration. On the other hand, herb (herbaceous) plants have a distinct elemental fingerprint, as their roots and steam are closer to the ground and, thus the nutrients are extracted from a narrower soil layer. Furthermore, herb plants are usually cultivated near cities or human-populated places often rely on pesticides and fertilizers to sustain crop productivity. Therefore, their environment may encounter different pollutants, both acknowledged and emerging, which will result in soil or air contamination with various elements (i.e. Cr, Pb, REEs). Thus, not only the soil will be polluted, but also the nectar of the plant that will be collected by bees. Because of this, the isotopic and elemental compositions of the herb plants honey and tree honey are expected to exhibit distinct characteristics, which are being discussed in the present study. A particular attention will be paid to the investigation of the hypothesis that the root depth and possible environmental variation of these two types of plants has a distinct elemental profile that will be uncovered by the honey elemental profile analysis (Fig. 2).

In this regard, based on Pearson's correlation results and the observations made, the present study aims to further discuss how the root system of the plant used in honey production, the surrounding parameters and the agricultural practices influence the elemental content of various honey types, and to establish the attributes that are affected by the geographical origin of honey. This was achieved by identifying the relevant markers from the isotopic and elemental fingerprint of honey samples for differentiating *i*) honey produced from trees and from herb (herbaceous) plants, and *ii*) honey produced in a narrow region (Transylvania, Romania) from that originating from other EU countries (Italy, France, Spain, Greece, and Portugal). The elements analyzed for this purpose were: Li, B, Na, Mg, Al, K, Ca, Ti, V, Cr, Mn, Co, Ni, Cu, Zn, Ga, As, Rb, Sr, Y, Zr, Nb, Mo, Pd, Sn, Sb, Ba, La, Ce, Pr, Nd, Gd, Dy, Yb, Lu, Ta, Re, Ir, Pt, Tl, Pb, along with the isotopic ratios of light elements: carbon from bulk honey and its corresponding protein ($\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$) and the isotopic ratios of the water that was extracted, without isotopic fractionation, from honey ($\delta^{2}H$, $\delta^{18}O$). To obtain relevant markers for each classification, two supervised feature selection methods were applied, namely a PLS-based selection algorithm and the ANOVA test. The resulting markers were subsequently used for developing classification models capable to discriminate between the botanical and geographical origin of honey.

3.3. *Screening of the isotopic and elemental uptake as a function of root depth and environmental variations of the honey plant*

The variables corresponding to the isotopic ratios, concentrations of nutrients, and pollutants (including REEs) that have the highest differentiation power between the two classification groups, namely honey obtained from the collection of nectar from trees (acacia, linden, honeydew, chestnut, fir, and orange) or from herbal plants (colza, raspberry, black grass, sunflower, sulla, thyme, lavender, coriander, polyflower, and thistle) were highlighted. In this regard, the application of an automatic feature selection method based on PLS regression allowed the identification of the following markers for this root depth: $\delta^{13}C_{honey}$, $\delta^{2}H$, $\delta^{18}O$, B, Cr, Mn, Rb, Sb (Fig. 3a). These results are in good agreement with the ones obtained through the employment of ANOVA (Fig. 4a), where the variables presenting the highest f scores and corresponding to p values smaller than 0.05, and, thus being the most representative for allowing this classification were: B, $\delta^{13}C_{protein}$, Sb, Cr,

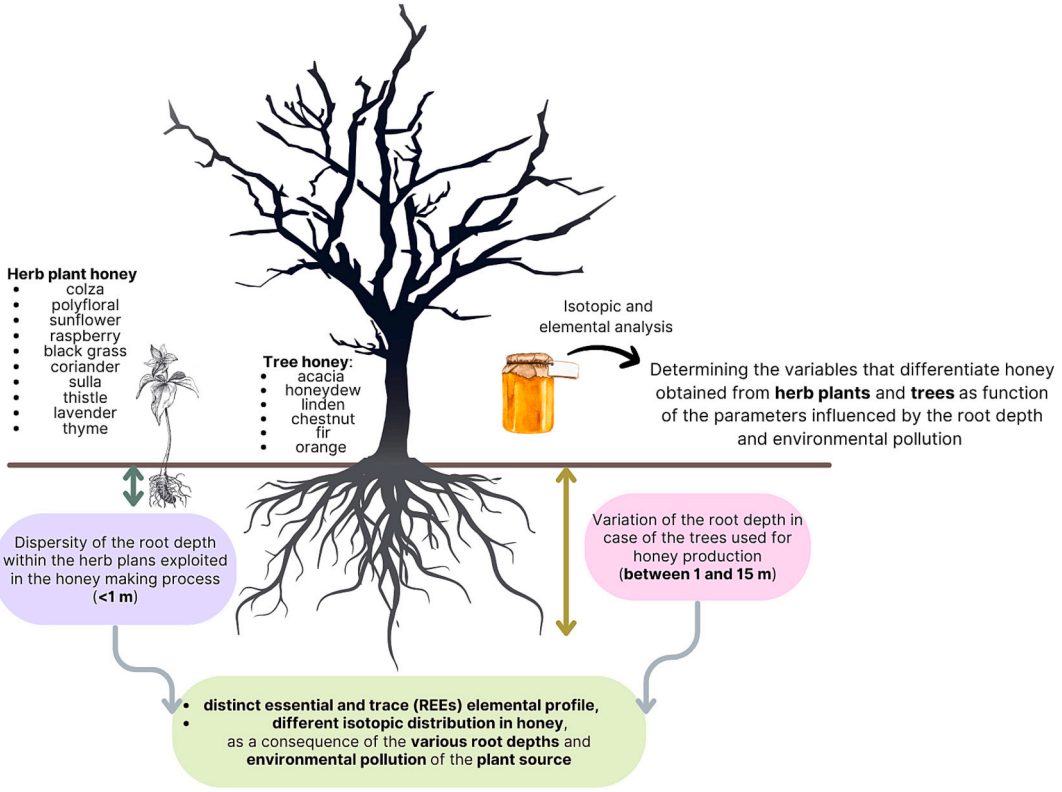

Fig. 2. Graphical representation of the novel honey classification approach employed to examine the influences of the source plant's root system and environment on the absorption of different essential and trace elements.

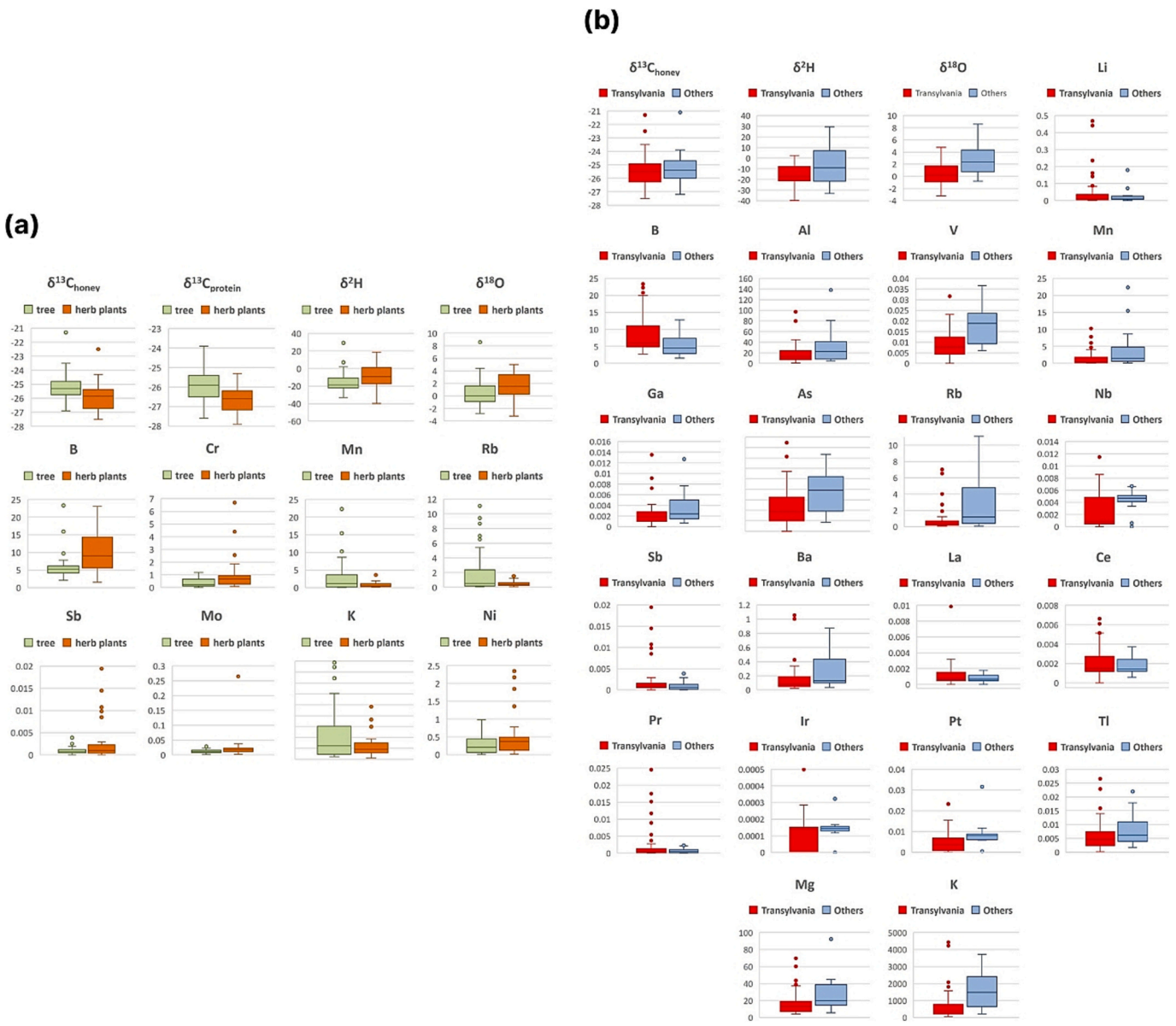

Fig. 3. (a) Boxplots of the measured concentrations (mg/kg) of the identified markers for the botanical differentiation model. (b) Boxplot displaying the concentration data (mg/kg) of the horizontal differentiation markers. The interquartile range of the box corresponds to the lower 25 % and upper 75 % quantiles are depicted as vertical lines; the median value appears as a horizontal line in the box and outlier points are present outside the box.

$\delta^{13}C_{honey}$, Rb, Mn, $\delta^{18}O$, $\delta^{2}H$, Ni, K, Mo (Fig. 3a). As can be seen, among these identified markers, no REEs element was found. These results suggest that REEs are not generally significant discriminators for from plants characterized by distinct root morphologies and growth forms, such as deeper-rooted arboreal species and shallower-rooted herbaceous plants [36,37].

Boxplots were created to visualize the data distribution of the identified markers for the botanical differentiation model, with separate plots for tree and herb plant-derived honey groups (Fig. 3a). In the investigated sample set, chromium (Cr) concentrations in samples produced from tree plants ranged between 0.0037 and 1.1782 mg/kg, with a mean of 0.3838 mg/kg and a median of 0.2295 mg/kg. Samples from herb plants exhibited concentrations ranging from 0.0839 to 6.6846 mg/kg, with an average of 1.0003 mg/kg and a median of 0.6839 mg/kg. Interestingly, Cr values in Romanian samples were higher than those reported in honey from Turkey (0.088 mg/kg; [60]) but lower than those from Hungary (4.0 to 36 mg/kg; [61]) and Croatia (2.69 to 49.90 mg/kg; [62,63]). The difference in Cr content in honey is related to variations in environmental conditions [64]. This study revealed that honey derived from herb plants exhibits a higher concentration of Cr, possibly due to the elevated pollution levels these plants encounter from being cultivated in the city proximities.

Manganese (Mn) has been previously reported as a marker for botanical differentiation of several food commodities, including honey [38,48,49,64]. This element can be present in soil because of industrial and traffic pollution [5]. The obtained values in this study (between 0.8 and 2.9 mg/kg) are comparable to those previously reported in the literature for Romanian honey having various origins (acacia, tilia, sunflower, and polyfloral) (mean values between 0.8 and 2.5 mg/kg) [64] and Turkish honey (0.32–4.56 mg/kg) [65]. Moreover, bees and bee products proved the ability to accumulate Mn from beehives' surrounding environment and showed their great aptitude for monitoring purposes [5].

Boron (B) is an essential micronutrient that facilitates cell growth and is typically used as fertilizer for increasing crop production [66,67]. Thus, it may be a marker for differentiating honey that is obtained from

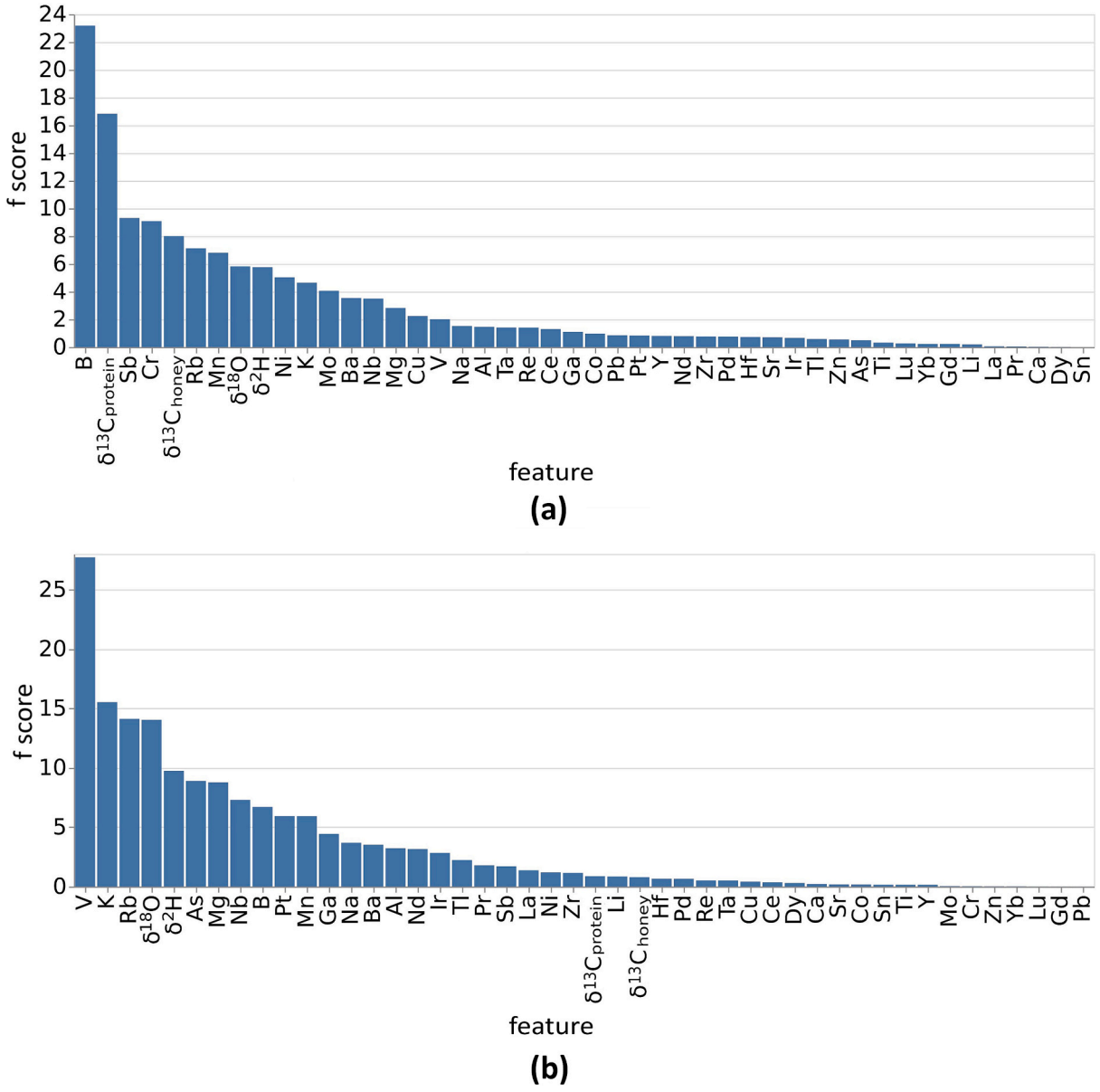

Fig. 4. F scores of the isotope and elemental determinations of honey after the application of ANOVA test on the investigated (a) botanical and (b) geographical honey groups.

herb plants grown in fertilized crop fields from the tree-derived honey obtained in wilder areas where traces of fertilizers are harder to find. This is consistent with the higher B concentrations found in herb plant honey as compared to tree honey (Fig. 3a). Moreover, B and Mn in crop plants have been previously reported to be dependent on the genotypic variation along the roots [68,69].

Rubidium (Rb) concentrations have been observed to decrease in the order: exotic trees > larger trees > smaller trees > shrubs [70]. Moreover, Nyholm & Tyler [71] demonstrated that there is an increased Rb^{+} concentration in trees due to the acid soils of the forest ecosystems that allow the preference intake of Rb. According to them, this imbalance may be propagated in the food products (e.g. honey), and it can be seen that in the present study, the Rb concentrations of tree honey are higher than the ones of herb plant honey (Fig. 3a).

Antimony (Sb) is a non-essential element for plants and is mostly present due to the anthropogenic inputs from vehicle emissions, mining, and industrial activities. Although Sb is usually absorbed through the roots and smaller amounts are found in the aerial parts [72,73], herb plants that grow on sites near highways or in urban regions present a risk of aerial contamination by air dust. Thus, the nectar of herb plants may contain a higher amount of Sb than the nectar from trees, and this can be passed to honey, as can be seen in Fig. 3a.

The $\delta^{13}C_{honey}$ and $\delta^{13}C_{protein}$ markers might be related to the different harvesting periods, while $\delta^{2}H$ and $\delta^{18}O$ reflect the signature of precipitations, temperature, and atmospheric humidity and are region-specific as herb plants are usually cultivated at lower altitudes, where the meteorological factors are significantly different from where trees grow. Thus, these varying values can be reflected in the honey isotope concentration [74].

Potassium (K) content was previously reported as a botanical differentiation marker when discriminating acacia, honeydew, sunflower, and linden honey [38]. It can be observed that higher K concentrations were observed for tree honey, which may derive from the available K content of the originated tree. It has been reported that K has a key role in wood formation [75], and thus higher amounts of K may be found in the tree nectar used for honey production. As it was previously mentioned, Mo and Ni are essential micronutrients for crop growth and yield and an imbalance or deficiency of these elements in plants may

have negative impacts on production [56,57]. As herb plants have usually other applications and are not only used for honey production, farmers may fertilize the fields and plants to avoid the negative impact on production. Usually, those fertilizers contain Mo and Ni and thus, herb plants may have a higher concentration of these elements that can be transferred to honey.

The application of PLS-DA modeling on the identified marker set (i.e. $\delta^{13}C_{honey}$, $\delta^{2}H$, $\delta^{18}O$, B, Cr, Mn, Rb, Sb) has allowed the correct classification of 81 % of the honey samples belonging to the two compared groups, following a 10-fold cross-validation procedure. Therefore, the developed supervised model correctly predicted 41 out of 49 samples produced from tree plants and 23 out of 30 samples belonging to the group of honey produced from herb plants. Among the 15 misclassified samples, 12 samples were produced in Transylvania and 3 belonged to the group of honey produced in other EU countries. Based on this consideration, the discrimination ability of the developed model is indeed related to the investigated criterion and the geographical distribution does not influence the decision function, as 16 out of 19 honey samples from other countries (i.e. France, Greece, Italy, Spain or Portugal) were correctly assessed by the PLS-DA model. Nonetheless, the generalizability of the developed classifier is illustrated by the fact that despite having a high variability in terms of geographical origin, the accuracy of the model is high (over 80 %).

The obtained results have highlighted that in the case of the investigated honey sample set, the root depth distribution of the sample caused by the plant profile (i.e. herb plants versus trees) did not illustrate significant differences in the REEs content. It was observed that the root depth and environmental surroundings of the plant exploited for honey production influence the isotopic and elemental profile of honey. In this regard, honey from herb plants can accumulate increased amounts of pollutants (i.e. B, Cr, Mn, Sb, Mo, Ni) compared to tree honey that is more abundant in essential elements (i.e. K). This can possibly be correlated to the environment of the exploited plant, namely herbaceous plants are usually grown closer to anthropologic pollution such as cities and are generally cultivated in agricultural regions characterized by the prevalent use of fertilizers and pesticides. While, honeys derived from arboreal species are typically found in more remote regions with diminished direct human influence. The results suggest the potential of monitoring honey quality to detect possible environmental changes and pollution issues.

3.4. *Geographical differentiation – screening of the horizontal influence*

To assess the geographical influence on the honey sample set, samples from the Transylvania region (Romania) were compared to those from other EU countries. When applying the ANOVA test to identify relevant variables for classifying the two honey sample groups based on isotope and elemental concentrations, markers with a p-value <0.05 included: V, K, Rb, $\delta^{18}O$, $\delta^{2}H$, As, Mg, Nb, B, Pt, Mn, and Ga (Fig. 4b). All these variables were also identified as significant markers using the PLS-based selection procedure, which found the following markers relevant to this geographical criterion: $\delta^{13}C_{honey}$, $\delta^{2}H$, $\delta^{18}O$, Li, B, Al, V, Mn, Ga, As, Rb, Nb, Sb, Ba, La, Ce, Pr, Ir, Pt, Tl, Mg and K (Fig. 3b). The markers identified for the geographical differentiation of honey samples reflect both environmental and geological influences. Isotopic signatures such as $\delta^{2}H$ and $\delta^{18}O$ are primarily linked to climatic conditions, including altitude, latitude, and precipitation regimes, which strongly influence the isotopic composition of plant water and, consequently, honey. The $\delta^{13}C$ values of honey provide additional information on vegetation type and photosynthetic pathways, further supporting differentiation. Elemental markers such as K, Mg, B, V, Al, and Mn, are nutrients whose concentrations vary with soil mineralogy and agricultural regime that are expected to be dependent on the geographical source. The REEs proved to be markers for this honey classification which may be related to the different pedological influence and distribution of these elements in the earth soil.

Previous studies also reported the variables K, V, Cr, As, Nb, $\delta^{2}H$, Ce, and $\delta^{13}C_{honey}$ as being significant for differentiating Romanian honey from the French honey samples [38]. Romanian honey proved to have a lower K concentration (679.70 mg/kg), than the honey collected from other EU countries (France, Greece, Spain, and Portugal) (1579.74 mg/kg).

Squadrone et al. [76] reported that metal concentrations in honey samples, mainly Al, Cd, Co, Cu, Zn, Fe, Mn, Ni, Se, V, and Zn (according to PCA analysis), are strongly influenced by geographical origin. Moreover, they can affect plants and enter the food chain, posing risks to humans. Contaminated pollen from metal-polluted areas of origin can cause high levels of these elements in honey [77]. Specifically, the concentration of arsenic (As) in Romanian honey (mean value of 4.7 μg/kg) was lower than in samples from other countries (mean value of 7.5 μg/kg), while vanadium (V) concentrations in honey from other EU countries (mean value of 18.5 μg/kg) exceeded those in Romanian honey (8.6 μg/kg) and other reported values in the literature for regions such as Kazakhstan, South America, Argentina, and Brazil [76].

Lithium (Li) concentrations in honey can be influenced by various factors, including soil pollution from sources such as Li-ion batteries. The aluminum (Al) concentrations in Transylvanian honey samples (mean value of 18.97 mg/kg) were found to be lower than those in honey from other countries (mean value of 29.88 mg/kg). Aluminum Al^{3+} is released in acidic soils worldwide and can enter into the roots [78]. Additionally, increased concentrations of rubidium (Rb) are also dependent on high soil pH levels. Boxplots of these elements (Fig. 3b) indicate elevated Rb values in certain honey samples, although further analysis is needed to understand the implication of these findings. All this can indicate a lower alkalinity of the Transylvanian soil on which the plants for honey production grew.

To study the REEs pattern, the average concentration data for La, Ce, Pr, Nd, and Gd corresponding to the classes for geographical origin were normalized so that to avoid zig-zag patterns and to smooth the distribution [16]. For this, the PAAS (Post Archean Australian Shales) values were used for the normalization [79–81], which characterizes the average upper continental crust. The PAAS normalized patterns were similar (Fig. 5), with La and Ce concentrations in the cases of honey from other EU countries slightly lower than the ones presented in Transylvanian honey. Ce negative anomaly in other countries honey is consistent with previously reported studies [76,82]. Significant Pr negative anomaly was observed in foreign honey, which can be linked to the specific lithology, Pr being the fourth-most abundant lanthanide (behind Ce, La, and Nd), and usually found in minerals along with Ce and La. The markers and the differences in the concentrations of each element based on geographical origin observed in Fig. 5 may be explained by environmental and geological factors influencing the bioavailability of REEs. Transylvania is characterized by a complex lithological background, including volcanic and metamorphic rocks, which are naturally enriched in light REEs such as La, Ce, and Pr [83,84]. The weathering of these substrates, coupled with local soil pH and organic matter content,

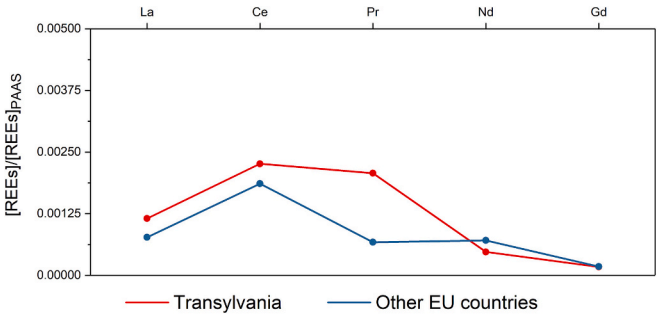

Fig. 5. PAAS normalized patterns of several mean value of the REEs concentrations present in honey samples.

may facilitate higher mobilization of REEs into the soil–plant system, thereby leading to their elevated levels in honey from Transylvania region. In contrast, honeys from other European countries often originate from areas dominated by sedimentary lithologies, where REE concentrations in soils are generally lower and mobility is more restricted. As a result, the REE patterns observed when analyzing honey samples mirror the underlying geochemical signatures of the local environment. The ∑REEs (La, Ce, Pr, Nd, Gd, Dy, Yb, Lu, and Y) in Transylvania honey had a mean value of 10.0 μg/kg and 8.2 μg/kg for other EU countries, which is similar to that reported by Squadrone et al. [76]. The PAAS-normalized REEs patterns provide a powerful tool for assessing the influence of geological substrates on the elemental composition of honey and for supporting geographical differentiation.

The discrimination potential of the geographical markers, including $\delta^{13}C_{honey}$, $\delta^{2}H$, $\delta^{18}O$, Li, B, Al, V, Mn, Ga, As, Rb, Nb, Sb, Ba, La, Ce, Pr, Ir, Pt, Tl, Mg, and K, was assessed using PLS-DA modeling. The classification model accurately identified the geographical origin of the honey samples with an overall accuracy of 87 %. The true positive rates for the Transylvania and foreign groups were 90 % and 78.9 %, respectively. Despite the highly diverse sample set, encompassing 16 classes based on botanical origin, the model successfully distinguished between honey samples produced in Transylvania and other countries, achieving high sensitivity scores (>78.9 %), despite class imbalance (60 Transylvanian samples versus 19 produced in foreign European countries).

4. Conclusions

The present study used the analysis in terms of elemental and isotopic fingerprint determination of 212 honey samples having a diverse botanical and geographical origin to observe possible correlations between these variables and to enhance our comprehension of the factors that influence the content of rare earth elements (REEs) in honey. Exploratory data analysis using Pearson's correlation revealed strong positive relationships between La and Ce, and La and Sr, indicating that the composition of honey reflects the elemental characteristics of the soils in which the source plants grow. The concentration of these elements in honey is closely linked to their levels in soil; thus, it is crucial to monitor the concentrations of La and Ce in soil that can be due to natural factors or anthropogenic pollution, as these elements are increasingly utilized as critical technical components in various industrial applications, yet have been shown to have negative health effects.

Furthermore, this study discussed how the root system of the plant used in honey production, the surrounding environment of the plant and the agricultural practices employed influence the elemental and isotopic content of various honey types. The markers capable to best differentiate between two honey classes namely, honey originated from herbaceous plants and honey from tree sources reflects the environmental context of the source plants, suggesting that honey quality control can also serve as a means to monitor soil and environmental pollution. Overall, these findings support the potential of honey not only as a valuable natural product for quality assurance but also as a promising bio-indicator for localized pollution assessment and environmental monitoring, which could be further exploited in future studies designed at a regional scale. In addition, the markers that differentiated Transylvanian honeys from those of other EU countries revealed the combined effect of environmental and geological factors, with La and Ce emerging as key discriminators linked to regional lithology. Correlating the isotope and elemental profiles using PLS-DA modeling enabled the development of reliable models with accuracies exceeding 81 %, for differentiating honey samples based on botanical (tree versus herb plants) and geographical (Transylvania versus EU countries) factors. These accuracies were achieved after a feature selection step, which identified the most important differentiation markers for each classification.

CRediT authorship contribution statement

Maria David: Writing – review & editing, Writing – original draft, Validation, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Ariana Raluca Hategan:** Writing – review & editing, Writing – original draft, Validation, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Adriana Dehelean:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Romulus Puscas:** Writing – original draft, Validation, Formal analysis, Data curation. **Gabriela Cristea:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Nastasia Belc:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis. **Gabriel Mustatea:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Dana Alina Magdas:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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