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ARTICLE

Carbon and Nitrogen Natural Stable Isotopes in Slovene Honey: Adulteration and Botanical and Geographical Aspects

Urška Kropf,*,† Terezija Golob,† Marijan Nečemer,§ Peter Kump,§ Mojca Korošec,† Jasna Bertoncelj,† and Nives Ogrinc#

†Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia, §Department of Low and Intermediate Energy Physics, and #Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia

Isotope parameters ($\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{15}N$) were determined for 271 honey samples of 7 types (black locust, multifloral, lime, chestnut, forest, spruce, and fir honeys) from 4 natural geographical regions of Slovenia. Carbon and nitrogen stable isotope ratios were measured to elucidate the applicability of this method in the identification of the botanical and geographical origin of honey and in honey adulteration. Only 2.2% of the samples were adulterated according to the internal standard carbon isotope ratio analysis method. Botanical origin did not have any major influence on the honey isotope profiles; only black locust honey showed higher $\delta^{13}C$ values. Some differences were seen across different production years, indicating that the influence of season should be further tested. Statistical and multivariate analyses demonstrated differences among honeys of various geographical origins. Those from the Alpine region had low $\delta^{13}C$ (−26.0‰) and $\delta^{15}N$ values (1.1‰); those from the Mediterranean region, high $\delta^{13}C$ (−24.6‰) and medium $\delta^{15}N$ values (2.2‰); those from the Pannonian region, medium $\delta^{13}C$ (−25.6‰) and high $\delta^{15}N$ value (3.0‰); and those from the Dinaric region, medium $\delta^{13}C$ (−25.7‰) and low $\delta^{15}N$ values (1.4‰).

KEYWORDS: Slovene honey; adulteration; botanical origin; geographical origin; isotope ratio; natural stable isotope; $\delta^{13}C$; $\delta^{15}N$; LDA; chemometry

INTRODUCTION

Honey is a natural and highly appreciated sweetener. However, honey production is time-consuming and relatively expensive, and therefore honey is often abused with different adulterants. Adulteration techniques are principally based around substitutions or extensions of the main components of honey, that is, water and sugar. One of the simplest is the addition of sugar (sucrose) to the honey, although this is easily traceable with carbohydrate analysis of honey when sucrose is added in large quantities. Sucrose should not be present above 5 g/100 g of honey according to European legislation (or above 10 g/100 g for black locust honey and for some other types) (*1*). More sophisticated adulterated products involve the use of sugar syrups, such as high-fructose corn syrup. This can also be detected by using different techniques, among which is stable carbon isotope ratio analysis (SCIRA), which was introduced by White and Doner (*2*). This was later modified to the internal standard stable carbon isotope ratio analysis (ISCIRA) method, which remains the Association of Official Analytical Chemists' (AOAC) method for honey adulteration detection (*3*−*5*). This method compares carbon isotope ratios of the honey with carbon isotope ratios of the protein fraction of the honey ($\delta^{13}C_{honey} - \delta^{13}C_{protein}$), although adulterated honeys can be detected only with the addition of sugars to > 9−10% of the whole honey mass (*3*−*7*). Nitrogen and carbon isotope ratios can also be used for detecting adulteration in royal jelly (*8*).

Honeys of different botanical origins were initially reported to have particularly uniform $\delta^{13}C$ values (*9*), but slight differences were later reported (*7*, *10*, *11*). Significant differences were shown in honeys originating from C_4 and Crassulacean acid metabolism (CAM) plants (*6*, *12*), compared to honeys from C_3 plants. Higher plants are divided into these three groups (C_3, C_4, and CAM) according to their metabolic pathways for carbon fixation in photosynthesis. C_4 and CAM plants have adaptations that enable them to survive in hot and dry areas, and they can therefore outcompete C_3 plants. This adaptation involves some additions to the basic Calvin cycle and, therefore, a few different enzymes. This results in differences in isotope ratios of different plant groups: isotope signatures of C_3 plants show higher degrees of isotope ^{13}C depletion than the C_4 plants. Therefore, products of C_4 plants have higher $\delta^{13}C$ values compared to CAM plants and, especially, C_3 plants. Most nectar sources, and also sugar beet, are C_3 plants, whereas corn and sugar cane are C_4 plants. Therefore, the addition of cane sugar or corn syrup in honey can be easily detected; in contrast, addition of beet sugar does not change the carbon isotope ratio of honey, and therefore it is difficult to detect. Differences between different types of honey are usually determined by physicochemical (*13*−*15*) and sensory (*14*, *16*) analyses and/or by elemental analysis in combination with chemometrics (*13*, *15*, *17*−*19*).

*Corresponding author (phone +386-1-3203725; fax +386-1-2566296; e-mail urska.kropf@bf.uni-lj.si).

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Table 1. Production Year and Geographical Origin Details for the Slovene Honey Types

honey type	production year (*n*)			region of geographical origin (*n*)				total (*n*)
	2004	2005	2006	Alpine	Dinaric	Mediterranean	Pannonian	
black locust (*Robinia pseudoacacia* L.)	16	17	22	0	3	30	22	55
multifloral	14	16	13	11	6	8	18	43
lime (*Tilia* spp.)	18	1	13	11	16	3	2	32
chestnut (*Castanea sativa* Mill.)	12	15	12	17	12	2	8	39
forest	15	15	10	27	13	0	0	40
spruce (*Picea abies* (L.) Karst.)	16	11	5	24	8	0	0	32
fir (*Abies alba* Mill.)	15	0	15	5	25	0	0	30

According to European legislation (*1*), the country or countries of origin where a honey is harvested have to be declared on the label. Conventional methods (chemical composition, physical characteristics, sensory parameters, pollen analysis) are used mainly for botanical classification, whereas the use of the geographical classification is nowadays almost impossible because of the lack of a national, or better still, European database of the characteristics of honeys originating from different parts of the European Union. Natural stable isotope ratios of different bioelements (C, H, N, S) in honey are now gaining increasing importance in determination of the geographical origin of honey (*20*) and other food products, including fruit (*21*−*23*), meat (*24*), and dairy products (*25*). A more common method to determine geographical origin of honey is by using elemental analysis, as the elemental profile of honey is influenced by soil, rock, weather, and environmental conditions (*15*, *26*, *27*).

Within the framework of the European research project "TRACE", studies have been investigating differences between 20 European regions according to the isotope ratios of C, N, H and S. Their findings have confirmed the importance of these parameters for geographical origin assignment: hydrogen and carbon isotopes were shown to be related to precipitation and climate; sulfur isotopes to the geology of the rock underlying the soil and the sea spray; and nitrogen isotopes to many influences (soil, biosynthesis pathways, habitat, fertilization), and therefore their interpretation was very difficult (*20*). The nitrogen isotope ratio in honey has already been shown to be influenced by the use of fertilizers (*28*). As a result of different kinds of soils in different parts of Brazil, differences among Brazilian regions were shown for the δ^{15}N values of honeys (*28*).

The present study is one of the largest systematic studies on stable isotope analysis of honey in Slovenia, and it included over 250 honey samples of different botanical origins collected over three consecutive years (2004−2006). The stable carbon and nitrogen isotope ratios were measured in these honeys and in the protein fractions extracted from these honeys that originated from different parts of Slovenia.

MATERIALS AND METHODS

Sampling. Samples were collected directly from beekeepers in Slovenia. The type of honey was determined for all samples by sensory analysis (*16*) and melissopalynology (*29*). A botanical classification was achieved when the pollen spectrum contained > 40% of the corresponding dominant pollen (> 80% for chestnut honey and < 40% for black locust honey). In total, 271 honey samples were included: 55 black locust samples (*Robinia pseudoacacia* L.); 43 multifloral (nectar of different flowers) samples; 32 lime (*Tilia* spp.) samples; 39 chestnut (*Castanea sativa* Mill.) samples; 40 forest (honeydew of different coniferous and latifoliae trees) samples; 32 spruce (*Picea abies* (L.) Karst.) samples; and 30 fir (*Abies alba* Mill.) samples.

The honey samples were obtained across three production years, 2004−2006, inclusive (**Table 1**), and from different natural geographical macroregions of Slovenia (**Figure 1**). The latest and most complete regionalization of Slovenia into four natural geographical regions (*30*) is primarily based on the analysis of geology, surface relief, climate, vegetation, and land use and is shown in **Figure 1**. The samples of each honey type are traditionally produced in two or more macroregions (**Table 1**). The black locust honey samples originated from the Dinaric, Mediterranean, and Pannonian regions, whereas the multifloral, lime, and chestnut samples were from all four regions. The honeydew honey samples (forest, fir, and spruce honey) originated only from the Alpine and Dinaric regions, because forests are not common in the other two Slovene regions.

Sample Preparation. All samples were liquefied at 40 °C and homogenized in an ultrasonic bath for 0.5 h prior to analysis.

Analysis. The SCIRA method was used for determination of δ^{13}C in honey and the ISCIRA method for determination of δ^{13}C in protein isolated from the honey (*3*, *5*, *31*). Honey (0.6 mg) was transferred directly into tin capsules (SerCon, U.K.), which were closed with tweezers and put into the automatic sampler of the elemental analyzer. Protein from the honey was isolated according to the AOAC Official Method 998.12. Briefly, 10 g samples of honey were placed in 50 mL centrifuge tubes, 4 mL of distilled water was added, and the samples were mixed. After 2 mL of 10% sodium tungstate solution had been mixed thoroughly with 2 mL of 0.7 N sulfuric acid, this solution was added to and mixed with the diluted honey samples in the centrifuge tubes. The tubes were agitated in a water bath at 80 °C until visible flocs were formed (5 min). The samples were then centrifuged and the supernatants removed. The precipitates were washed with distilled water and agitated, and then the precipitates were separated again. This washing was repeated at least five times, until the supernatants were clear. The precipitated protein was transferred to small (1.5 mL) Eppendorf tubes and centrifuged. Following removal of the supernatant and drying, 2−4 mg of the dried protein samples was placed into tin capsules and run separately for δ^{13}C and δ^{15}N measurements.

All of the analyses were performed on a Europa Scientific 20-20 continuous flow mass spectrometer with an automated nitrogen−carbon analyzer, solid−liquid (ANCA-SL) preparation module. The ^{13}C/^{12}C and ^{15}N/^{14}N ratios were expressed in the delta notation, δ^{13}C and δ^{15}N, as the deviation in per mil (‰) from the Vienna Pee Dee Belemnite (V-PDB) standard for carbon and the atmospheric nitrogen (AIR) standard for nitrogen. The reproducibilities of the measurements were ±0.2‰ for δ^{13}C and ±0.3‰ for δ^{15}N. The analyses were calibrated against the International Atomic Energy Agency (IAEA) standards: IAEA-NBS22 (oil), IAEA-CH-7, and IAEA-CH-6 with δ^{13}C values of −29.7 ± 0.2, −31.8 ± 0.2, and −10.4 ± 0.2‰, respectively, for carbon; and IAEA-N1 and IAEA-N2 with δ^{15}N values of +0.4 ± 0.2 and +20.3 ± 0.2‰, respectively, for nitrogen. Data quality control charts were also systematically recorded throughout the study period. In addition, to ascertain the validity and comparability of the stable isotope results, we participated in interlaboratory proficiency testing that was organized by Eurofins three times per year. The testing is named Food Analysis Using Isotopic Techniques−Proficiency Testing Scheme (FIT-PTS), and it complies with the ISO/IUPAC/AOAC International Harmonised Protocol for Proficiency Testing of analytical laboratories.

Sucrose content was determined with polarimetric method according to AOAC Official Method 920.184 (*32*).

Statistical Analysis. The statistical calculations were carried out using the StatistiXL statistical software package (1.8) and SPSS 15.0 for Windows, Evaluation version. Basic statistics included means (median, arithmetic mean), standard deviation (SD), minimum, maximum, analysis of variance (ANOVA), and Kruskal−Wallis one-way analysis of variance

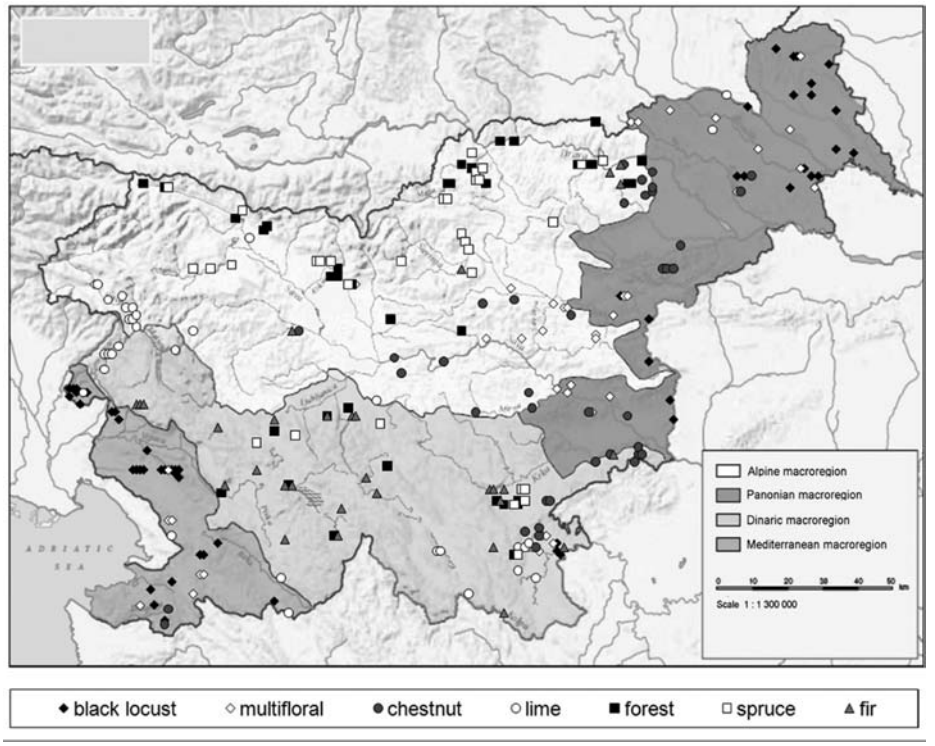

Figure 1. Map of natural geographical regions of Slovenia (after Perko (*30*)) showing geographical origins of the Slovene honey samples (as indicated).

by ranks (Kruskal−Wallis test). Multivariate analysis involved linear discriminant analysis (LDA).

RESULTS AND DISCUSSION

Altogether, 271 honey samples were analyzed. Samples were of seven botanical origins: black locust, multifloral, lime, chestnut, forest, spruce, and fir. Their sensory characteristics are described in **Table 2**. The $\delta^{13}C$ values were determined in the honey and in the honey protein fractions, and $\delta^{15}N$ values were determined only in the protein fractions, as there are not adequate quantities of nitrogen in honey for direct measurment of the N isotope ratio in honey. The isotope parameters given are means of at least two measurements.

Adulterations. Only 6 of the 271 honey samples analyzed (2.2%) were shown to have differences between the $\delta^{13}C$ value of the honey and the $\delta^{13}C$ value of the protein fraction of $> 1‰$, which indicates adulteration (*3*). The sucrose content was also measured in these samples (**Table 3**), although these were in all cases below the legislation limit (*1*). It can be concluded that sucrose was not added to these samples, but some other type of carbohydrate originating from C_4 plants might have been added. Of note, the beekeepers who participated in our study were aware that their honey would be tested for adulteration. Therefore, we believe that the possibility that they had adulterated these six honey samples is very low. Our findings are consistent with Cotte et al. (*7*), who reported suspicion of adulteration in 3 of 97 honey samples (3%) and then later indicated that this would not have been possible because of the checks that the beekeepers were under and because other honey analyses did not show any adulteration. They concluded with some reservations about the validity of the ISCIRA method, and the present study can confirm these reservations. Thus, it is important to define new methodologies for the detection of honey adulteration. Separation techniques coupled to isotope ratio mass spectrometry have been suggested to be more powerful (*33*).

Botanical Origin. The honey samples were distributed among seven honey types according to their sensory characteristics (**Table 2**). The differences in the physicochemical parameters across the different honey types have been reported previously in many studies, although only a few studies have systematically investigated the influence of botanical origin on the isotope parameters of honey. The sources of Slovene honey are all C_3 plants; therefore, no real differences in $\delta^{13}C$ values were expected due to the botanical origins. On the other hand, the N isotope composition can reflect the soil conditions of the area, as well as the botanical origin. The honey $\delta^{13}C$ values and the protein fraction $\delta^{13}C$ and $\delta^{15}N$ values for the botanical origins of these main Slovene honey types are given in **Table 4**, along with other basic statistics. These data were not normally distributed; therefore, only nonparametric tests were applied. The Kruskal−Wallis test showed statistically significant differences at $p < 0.05$ in some of the analyzed parameters across these Slovene honey types. Despite differences in mean values, there were only slight differences in the ranges, which were always overlapping. Thus, no clear differentiation between the $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, and $\delta^{15}N$ values among these Slovene honey types can be indicated.

Chemometric methods offer mathematical models for seeking differences in physicochemical parameters. LDA of the honey isotope parameters revealed that only the black locust honey differed significantly from the other Slovene honey types (**Figure 2**). Black locust samples have higher values according to function 1 and are therefore placed more on the right side of the graph. Other samples are placed mixed, because their values according to functions 1 and 2 are very similar. Specific group width is 3−4 units. Function 1 comprised all three parameters ($\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{15}N$), whereas the main effect on function 2 was from the $\delta^{13}C_{honey}$ value. The classification data from the LDA are given in **Table 5**, and they show that 80% of the black locust honey samples were correctly classified using only $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, and $\delta^{15}N$ values. The other honey samples were misclassified in $> 50\%$ of cases, except for the multifloral honey samples.

This more precise differentiation of black locust honey was expected: first, because black locust honey is known to have

Table 2. Sensory Characteristics of Slovene Honey Types

	sensory property			
honey type	appearance	odor	taste	aroma
black locust (*Robinia pseudoacacia* L.)	color: almost colorless to pale yellow clarity: always clear crystallization: does not crystallize	very weak, inexpressive beeswax, black locust flowers, floral (rose, violet), fruity (apple, pear), vanilla, cream candies, fresh butter, crumpled leaf, straw	medium to strong persistence sweetness: medium to strong acidity: weak	very weak intensity weak to medium persistence waxy, black locust flowers, floral (roses, violet) fruity (apple, pear), vanilla, cream candies, fresh butter, straw
multifloral	color: very colorful, from yellow to brown; depending on the plant species and the presence of honeydew clarity: rarely clear crystallization: quick crystallization, small or big and rough crystals	medium to strong intensity, fruity, floral (violet, dandelion, alpine flora), meadowy, waxy, sugar, sometimes woody	medium to strong persistence, sweetness: medium to strong, well-defined acidity: weak to medium, sometimes refreshing	medium to strong intensity, medium to strong persistence, floral (violet, meadow plants, herbs, clover), fresh fruit (apple, pear, peach, muscat grapes), cooked fruit, caramel or milk candies, brown sugar, molasses, green (grass, crumpled leaf)
lime (*Tilia* spp.)	color: white to cream or ivory, with a yellow or green tinge clarity: not always clear crystallization: quick	medium to strong intensity, fresh, lime flowers, menthol, lemon peel, medicinal, chemical	medium to strong persistence, sweetness: medium to strong, acidity: weak to medium, bitterness: weak to medium refreshing aftertaste	medium to strong intensity, medium to strong persistence, fresh, menthol, fresh walnut, herbs, lime tea
chestnut (*Castanea sativa* Mill.)	color: amber, more or less dark, with a reddish tinge clarity: always clear crystallization: usually does not crystallize	strong intensity bitter, pungent, chestnut flowers	strong persistence sweetness: medium acidity: weak bitterness: medium to very strong	strong to very strong intensity, extremely long lasting persistence with bitter aftertaste sharp, bitter, burned sugar, smoke, herbal, absinthe
forest	color: light to dark amber, with reddish or green tinge clarity: clear or muddy crystallization: may crystallize	weak to medium intensity, beeswax, caramel, milk powder, resin, smoke, molasses, humus, stuffy, gingerbread	medium to strong persistence sweetness: medium to strong acidity: weak to medium bitterness: none to weak	medium intensity medium to strong persistence walnut, hazelnut, milk powder, herbs, brown sugar, caramel, molasses, absinthe, dried fruit
spruce (*Picea abies* (L.) Karst.)	color: medium to dark amber, reddish tinge, clear, glittering surface clarity: always clear crystallization: usually does not crystallize	medium to strong intensity resinous, medical syrup, herbal bonbons	short to medium persistence sweetness: medium acidity: weak to medium	medium intensity short to medium persistence resinous, medical syrup, herbal bonbons
fir (*Abies alba* Mill.)	color: dark gray-brown, with a dark green tinge clarity: muddy crystallization: sometimes crystallizes	medium intensity resinous, milk powder, brandy	medium persistence sweetness: medium to strong acidity: weak	weak to medium intensity medium to strong persistence pleasant, malty, milk powder, caramel, brandy

Table 3. ISCIRA Results and Sucrose Content in the Samples in Which the Difference between $\delta^{13}C_{protein}$ and $\delta^{13}C_{honey}$ Values Was >1‰

sample	honey type	$\delta^{13}C_{honey}$ (‰)	$\delta^{13}C_{protein}$ (‰)	difference ($\delta^{13}C_{honey} - \delta^{13}C_{protein}$) (‰)	calculated adulteration (%)	sucrose (g/100 g)
A39	black locust	−21.4	−23.7	2.3	16.8	3.29
L20	lime	−26.2	−27.8	1.6	8.9	0.57
K15	chestnut	−22.7	−26.2	3.5	20.9	1.57
G20	forest	−24.3	−25.5	1.2	7.9	3.15
H4	fir	−23.7	−25.4	1.7	10.8	4.49
H22	fir	−23.9	−25.1	1.2	7.9	2.43

higher $\delta^{13}C$ values compared to dark honeys (*7*, *11*) and, second, because the black locust is a member of the Fabaceae plant family. These plants have root nodules where *Rhizobia* bacteria live in symbiosis with the plant. These *Rhizobia* fix nitrogen from the air, and the plant also exploits this source of nitrogen, as well as nitrogen organically fixed in the topsoil. Therefore, the $\delta^{15}N$ value of black locust products should be around zero, as for other N_2-fixing plants (*34*). Here, the black locust honey had the highest mean value of $\delta^{15}N$ (2.9‰) of all of the analyzed honey types, although the ranges of these $\delta^{15}N$ values overlapped with those of the other honey types. There can be several reasons for this mismatch, such as precipitation, water stress, and, primarily, the type of soil (loamy soil retains soluble nitrogen compounds better than sandy soil) (*20*), although these possibilities need to be

Table 4. Basic Statistics of $\delta^{13}C$ and $\delta^{15}N$ in Slovene Honeys of Different Botanical Origins

		$\delta^{13}C_{honey}$ (‰)				$\delta^{13}C_{protein}$ (‰)				$\delta^{15}N$ (‰)			
honey type	*n*	mean	SD	min	max	mean	SD	min	max	mean	SD	min	max
black locust	55	−24.8	0.7	−26.5	−23.4	−24.0	0.8	−26.0	−22.9	2.9	1.5	−0.2	6.4
multifloral	43	−25.9	0.8	−27.4	−24.4	−24.8	0.7	−26.5	−23.5	2.1	1.1	−0.4	4.8
lime	32	−25.6	0.7	−26.6	−24.0	−24.9	0.4	−25.7	−24.0	1.8	1.8	−1.7	5.8
chestnut	39	−25.8	0.3	−26.6	−25.2	−25.2	0.4	−26.2	−24.3	1.7	1.0	−0.4	3.7
forest	40	−26.1	0.5	−27.0	−24.8	−25.2	0.6	−26.6	−23.9	1.2	1.3	−1.3	4.3
spruce	32	−26.0	0.5	−26.9	−25.0	−25.3	0.4	−26.2	−24.2	1.1	2.0	−3.6	3.8
fir	30	−25.3	0.9	−26.8	−23.7	−24.9	0.7	−26.1	−23.4	1.2	1.4	−1.7	3.8

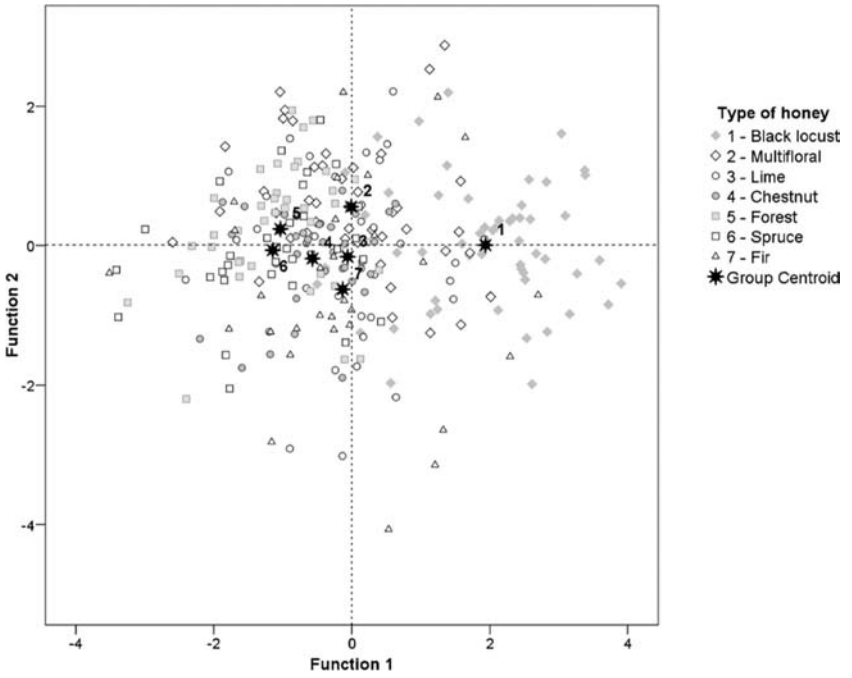

Figure 2. LDA showing cross-plot of the first two functions for the honey stable isotope data ($\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{13}N$) for the botanical origins of the Slovene honey samples (as indicated).

Table 5. Classification of Slovene Honey Samples According to Botanical Origin

	predicted honey type (*n*)								
original honey type	black locust	multifloral	lime	chestnut	forest	spruce	fir	total (*n*)	correctly classified (%)
black locust	44	5	1	1	0	0	4	55	80.0
multifloral	8	22	0	3	6	1	3	43	51.2
lime	4	11	1	2	4	1	9	32	3.1
chestnut	0	10	2	13	11	2	1	39	33.3
forest	0	11	0	4	19	3	3	40	47.5
spruce	0	7	1	7	11	4	2	32	12.5
fir	5	6	0	5	2	3	9	30	30.0

investigated in future studies of black locust honey and the soil conditions where black locust plants grow. Our results are based on the determination of $\delta^{15}N$ in protein fraction; however it is known that honeys contain a colloidal material enriched in N of nonprotein origin. In addition, the differences in the pollen content should also be considered in these honeys. Thus, elimination of these two sources of nitrogen might help in obtaining data that better reflect the true values of $\delta^{15}N$ protein fractions in honeys of different botanical origins. This research would be considered in the future.

Influence of the Year. Overall, the box-plot diagrams presented in **Figure 3a−c** show similarities in the $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, and $\delta^{15}N$ values across the production years of these honeys: no real differences can be seen between the years 2004, 2005, and 2006. These similarities agree with the findings of Schellenberg et al. (*26*), who also showed no statistically significant differences between honeys from 2005 and 2006. However, closer inspection revealed some differences.

When these analyzed isotope parameters were compared across the production years with regard to honey type (**Figure 3d−f**), the year 2004 was seen to be an exception for the $\delta^{13}C_{protein}$ values of black locust and multifloral honey: these had higher means and narrower ranges in 2004 compared to 2005 and 2006 (**Figure 3e**). In 2004, the average temperature and solar intensity in Slovenia were lower than usual (*35*). Indeed, our conclusions here are not in agreement with findings for isotope parameters in Slovene wines,

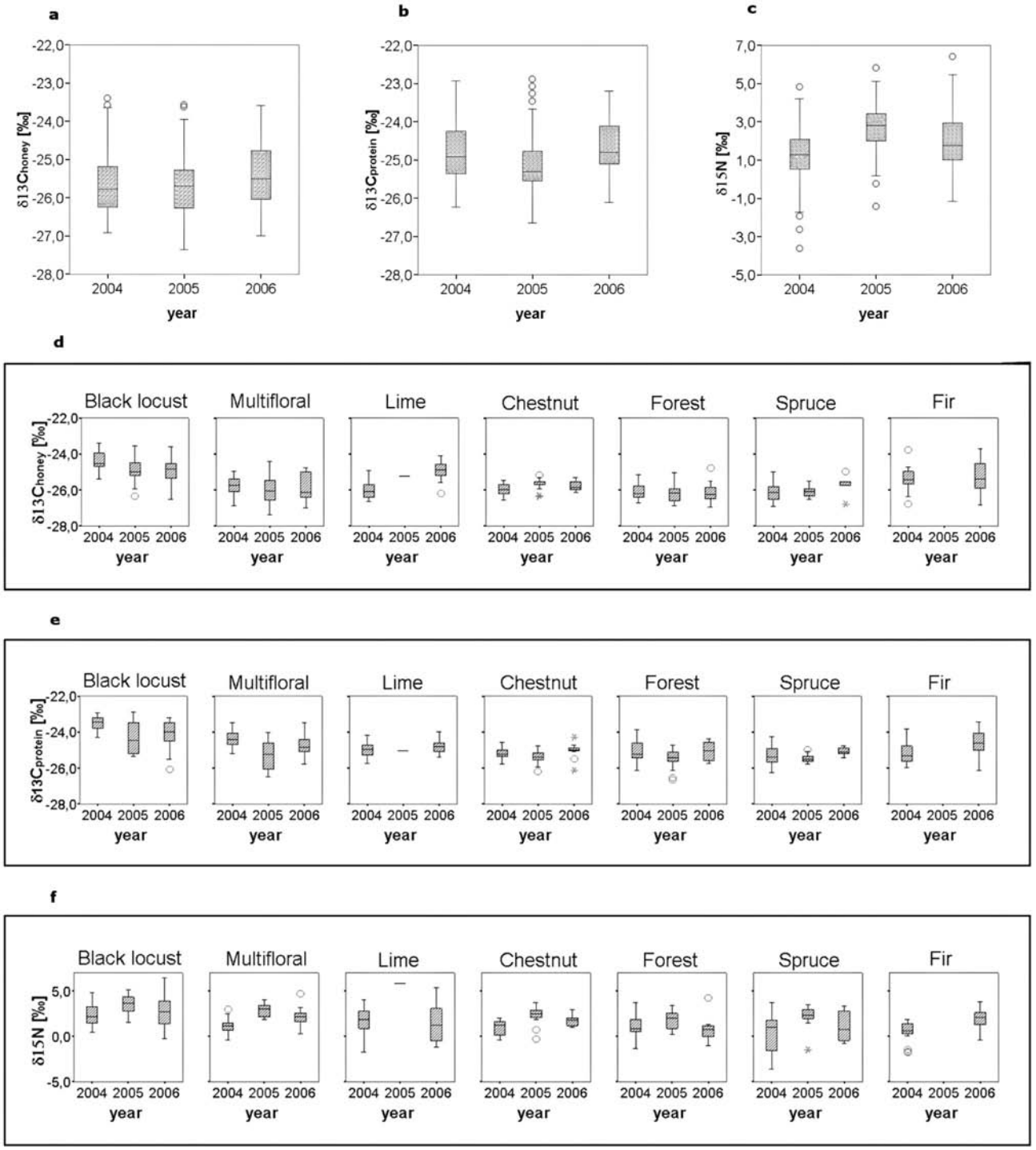

Figure 3. Box plots for influence of year of collection (2004−2006) on isotope parameters of the Slovene honey samples: (**a**−**c**) full-year data sets for $\delta^{13}C_{honey}$ (**a**), $\delta^{13}C_{protein}$ (**b**), and $\delta^{13}N$ (**c**); (**d**−**f**) individual year data sets according to honey sample type, as indicated.

where higher temperature and solar intensity result in higher $\delta^{13}C$ values in wines (*36*). These variations might thus be explained by the different climatic conditions across small distances in the sampling areas and by the different harvesting times of the individual honeys.

The influence of the year was also tested on the $\delta^{15}N$ values (**Figure 3f**). Some differences were seen for the multifloral, chestnut, and spruce honeys, with the ranges of the $\delta^{15}N$ values much narrower in 2005 compared to 2004 and 2006. Different ranges indicate that production year has an influence also on this parameter, but this needs to be investigated in further research.

Geographical Origins. Slovenia is divided into four macroregions. The honey samples originated from across all of these regions, but their distributions were not the same. The majority of samples (66%) were from the Alpine and Dinaric regions, 95 and 83, respectively, and the rest (34%) was from the Mediterranean and Pannonian regions, 43 and 50, respectively. The $\delta^{13}C$ and $\delta^{15}N$ values were normally distributed with regard to geographical origin; therefore, parametric tests can be applied. The mean values and basic statistics of these isotope values of the honey and protein from these Slovene regions are given in **Table 6**. When the ranges of the $\delta^{13}C$ values among these honeys from the four Slovene regions are compared, no significant differences

Table 6. Basic Statistics of $\delta^{13}C$ and $\delta^{15}N$ in Slovene Honeys of Different Geographical Origins[a]

		$\delta^{13}C_{honey}$ (‰)				$\delta^{13}C_{protein}$ (‰)				$\delta^{15}N$ (‰)			
geographical origin	*n*	mean	SD	min	max	mean	SD	min	max	mean	SD	min	max
Alpine	95	−26.0 a	0.6	−27.0	−24.1	−25.2 a	0.6	−26.6	−23.9	1.1 a	1.5	−3.6	4.0
Dinaric	83	−25.7 b	0.7	−27.0	−23.7	−24.9 b	0.6	−26.5	−23.4	1.8 b	1.4	−1.7	5.8
Mediterranean	43	−24.6 c	0.6	−26.0	−23.4	−23.8 c	0.7	−25.8	−22.9	2.2 b	1.2	−0.4	5.1
Pannonian	50	−25.6 b	0.7	−27.4	−24.4	−24.8 b	0.7	−26.3	−23.3	3.0 c	1.5	−0.2	6.4

[a] Values in columns with different letters are statistically significantly different at $p < 0.05$.

can be seen. The honeys from the Mediterranean region, which is the warmest of these regions, with the most sun irradiation and the least precipitation, had the highest mean values of $\delta^{13}C$. The honeys from the Alpine region, which is the coldest of these regions, had the lowest $\delta^{13}C$ values, whereas the honeys from the other two regions were in between. ANOVA showed that for the $\delta^{13}C$ of both the honey and protein samples, the honey from the Alpine region had statistically significant lower values, and the honeys from the Mediterranean region had statistically significant higher values, compared with the other regions (**Table 6**).

The $\delta^{15}N$ values of the honey protein were highest in honeys from the Pannonian region, which was expected as this region is known to have dark brown to black, very fertile soil, as the residue of the Pannonian Sea. The honey samples from the Alpine and Dinaric regions had lower average $\delta^{15}N$ values, which is in agreement with the poorer soil of these mountainous landscapes. ANOVA showed that the honey from the Alpine region had a statistically significant lower $\delta^{15}N$ value, whereas the honey from the Pannonian region had a statistically significant higher $\delta^{15}N$ value, compared with the other regions (**Table 6**). When the ranges of the $\delta^{15}N$ values are compared, small differences can also be seen: the Alpine region showed the smallest ranges, the Dinaric region was intermediate, and the Mediterranean and Pannonian regions showed the largest ranges.

The LDAs of the $\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, and $\delta^{15}N$ values of all of the analyzed honey samples independent of their botanical origin showed that there were some differences in the isotope profiles of those from the different Slovene regions. Overall, 55.0% of the samples were correctly classified according to the four regions and these three analyzed parameters. Schellenberg et al. (*20*) indicated that they achieved 60.2% correctly classified samples using four parameters (δD, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$). The honey from the Alpine and Mediterranean regions was successfully classified in 63.2 and 79.1% of cases, respectively (**Table 7**). This was, however, much lower for the Dinaric and Pannonian honeys. We believe that this is a result of nonspecific weather and soil influences on the honey isotope profiles, especially for the Dinaric region. Indeed, the LDA illustrated in **Figure 4** shows that the samples from the Alpine region are positioned to the left (negative function 1), the samples from the Mediterranean region are positioned to the right (positive function 1), and the samples from the Dinaric and Pannonian regions are positioned in the middle, although the Pannonian samples are placed slightly higher (more positive function 2), which is the result of the higher $\delta^{15}N$ values in this honey. The $\delta^{13}C_{honey}$ and $\delta^{13}C_{protein}$ values had the highest impact on function 1, and the $\delta^{15}N$ values on function 2. **Figure 4** also indicates the need to present this kind of graphical analysis with all of the samples, not just showing only the group centroids as Schellenberg et al. (*20*) did. Indeed, if only the group centroids were shown in **Figure 4**, it could be incorrectly concluded that there are large differences among these four regions for the isotope profiles, where in particular the Pannonian and Mediterranean centroids stand out.

Table 7. Classification of Slovene Honey Samples According to Geographical Origin

	predicted geographical origins (*n*)					
geographical origin	Alpine	Dinaric	Mediterannean	Pannonian	total (*n*)	correctly classified (%)
Alpine	67	27	0	1	95	70.5
Dinaric	35	29	8	11	83	34.9
Mediterannean	1	7	34	1	43	79.1
Pannonian	10	15	6	19	50	38.0

Differences among the regions were sought also inside individual honey types. Here, the statistical analysis was performed only on the selected groups that contained more than five honey samples. Across all of the analyzed honey types except the multifloral, only two groups were formed: black locust honey from the Mediterranean and Pannonian regions, and lime, chestnut, forest, spruce, and fir honey from the Alpine and Dinaric regions. Significant differences were seen between the regions for black locust, lime, and multifloral honeys (**Table 8**): black locust honey from the Mediterranean region was significantly different from that from the Pannonian region; lime honey from the Alpine region was significantly different from that from the Dinaric region; and multifloral honey from the Mediterranean and Dinaric regions was significantly different from that from other regions. The classification of the honey samples within an individual honey type into a predicted group regarding the geographical origin was very successful (> 55% correctly classified samples in cross-validation tests) for all of the Slovene honey types except multifloral. The results of this classification are given in **Table 9**. The classification that took into account the botanical origin of the honey was more successful than that which neglected the honey type.

Schellenberg et al. (*20*) reported large differences among honeys from different parts of Europe, with one of the most discriminating factors being sulfur. This was shown not to be influenced by the yearly changing climate conditions or the botanical origin of the honey, but was instead shown to be strongly related to the soil isotope ratio, which is related to the geological composition of the soil.

Findings. Slovenia is known to be a country with thousands of beekeepers who have fewer than 50 hives each. We believe that there was no intentional forgery of the honeys among these small beekeepers who participated in this study in Slovenia. We have strong reason to believe honey samples were not adulterated, whereas the ISCIRA method showed that 6 of the 271 samples analyzed were adulterated. Similar findings were published by Cotte et al. (*7*). Further studies are needed to explain the reason for observed deviations.

Botanical origin had only minor effects on the isotope profile of this Slovene honey, with the only exception being the black locust honey, with higher $\delta^{13}C$ values compared to the other Slovene honey types here. However, this might be an influence of geographical origin, because most of the black locust samples

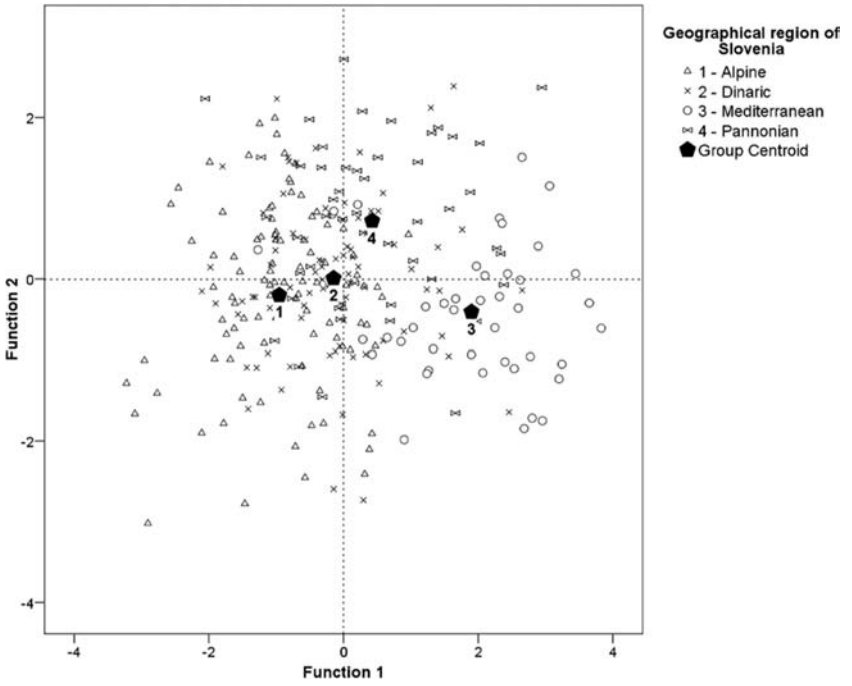

Figure 4. LDA showing cross-plot of the first two functions for the honey stable isotope data ($\delta^{13}C_{honey}$, $\delta^{13}C_{protein}$, $\delta^{13}N$) for the geographical origins of the Slovene honey samples (as indicated).

Table 8. $\delta^{13}C$ and $\delta^{15}N$ Parameters of the Different Honey Groups (Type versus Geographical Origin)[a]

		value by geographical origin (‰)				
original honey type	parameter	Alpine	Dinaric	Mediterannean	Pannonian	*t* test/ANOVA significance[b]
black locust	$\delta^{13}C_{honey}$			−24.3	−25.3	0.000
	$\delta^{13}C_{protein}$			−23.5	−24.6	0.000
	$\delta^{15}N$			2.4	3.6	0.002
multifloral	$\delta^{13}C_{honey}$	−26.4 a	−25.5 b	−24.9 c	−26.1 a	0.000
	$\delta^{13}C_{protein}$	−25.2 a	−24.4 b	−24.3 b	−24.9 a,b	0.030
	$\delta^{15}N$	1.9	2.0	1.8	2.5	
lime	$\delta^{13}C_{honey}$	−25.4	−25.8			
	$\delta^{13}C_{protein}$	−25.0	−24.9			
	$\delta^{15}N$	0.1	2.8			0.000
chestnut	$\delta^{13}C_{honey}$	−25.9	−25.8			
	$\delta^{13}C_{protein}$	−25.3	−25.1			
	$\delta^{15}N$	1.4	1.7			
forest	$\delta^{13}C_{honey}$	−26.1	−26.1			
	$\delta^{13}C_{protein}$	−25.1	−25.3			
	$\delta^{15}N$	1.3	1.2			
spruce	$\delta^{13}C_{honey}$	−26.1	−26.0			
	$\delta^{13}C_{protein}$	−25.3	−25.3			
	$\delta^{15}N$	0.8	2.0			
fir	$\delta^{13}C_{honey}$	−25.9	−25.2			
	$\delta^{13}C_{protein}$	−25.3	−27.8			
	$\delta^{15}N$	1.1	1.3			

[a] Values in rows with different letters are statistically significantly different at $p < 0.05$. [b] Only significances lower than 0.05 are presented.

originated from the Mediterranean region, which is known to have more sun irradiation and higher average temperatures than the other Slovene regions. Differences in $\delta^{15}N$ values were also expected, because the black locust plant is known to have the ability for nitrogen fixation from the air. This would increase the $\delta^{15}N$ values of products from this plant, although for the Slovene black locust honey this effect was not dominant, and so no clear differentiation was seen among these tested honey types from the different regions for the $\delta^{15}N$ values.

The stability of the honey isotope profiles was examined across three production years: 2004, 2005, and 2006. Some differences were seen for the 2004 production year, when higher $\delta^{13}C_{protein}$

Table 9. Classification of Slovene Honey According to Botanical Type versus Geographical origin

	correct classification to natural geographical region (%)	
original honey type	correctly classified	correctly classified with cross-validation
black locust	84.6	78.8
multifloral	58.1	48.8
lime	88.9	88.9
chestnut	65.5	55.2
forest	65.0	55.0
spruce	62.5	53.1
fir	66.7	60.0

values were detected in the black locust and the multifloral honeys. Normally, this would be connected to greater solar intensity and higher temperatures, but in Slovenia the year 2004 had precisely the opposite features. Therefore, the influence of the year on the honey isotope profiles needs to be tested over longer periods, such as at least five continuous years.

Significant differences were seen among the honeys from different natural geographical Slovene regions. On average, honeys from the Alpine region had low $\delta^{13}C$ (−26.0‰) and $\delta^{15}N$ values (1.1‰); those from the Dinaric region, medium $\delta^{13}C$ (−25.7‰) and low $\delta^{15}N$ values (1.4‰); those from the Pannonian region, medium $\delta^{13}C$ (−25.6‰) and high $\delta^{15}N$ values (3.0‰); and those from the Mediterranean region, high $\delta^{13}C$ (−24.6‰) and medium $\delta^{15}N$ values (2.2‰). Using the LDA classification and independent of the honey botanical origin, 55.0% of the samples were correctly classified. When the honey type was taken into consideration, this rate was increased to 84.6 and 88.9% for black locust and lime honeys, respectively.

These isotope parameters are thus shown to be very useful for determination of the geographical origin of a honey, although, in contrast, there were no clear differences among the honeys of the different botanical types, possibly because the honey in Slovenia is almost always a mixture of nectars and/or honeydews of different C_3 plants.

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