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Application of Total Reflection X-ray Spectrometry in Combination with Chemometric Methods for Determination of the Botanical Origin of Slovenian Honey

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This work on the botanical origin of various types of honey produced in Slovenia and based on the mineral content analyses by the total reflection X-ray spectrometry (TXRF) is a continuation of this group's preliminary work (Golob, T.; Doberšek, U.; Kump, P.; Nečemer, M. *Food Chem.* **2005**, *91*, 593–600), which introduced the analytical methodology and employed only a simple statistical evaluation and which examined the possibility to determine the botanical origin of honey samples via elemental content. A much more comprehensive study on a total of 264 major types of honey samples harvested in 2004, 2005, and 2006 and interpreting the results with up to date chemometric methods was performed in this work. Slovenia is a small country by surface area, but it is pedologically and climatically diverse, therefore offering interesting possibilities for studying the influence of these diversities on the elemental content of natural products. By employing principal component analysis (PCA) and regularized discriminant analysis (RDA) it was established that from all of the measured elements only the four characteristic key elements Cl, K, Mn, and Rb could be used to best discriminate the types of honey. It was established that the employed combination of a simple, fast, and inexpensive multielement TXRF analytical approach and the evaluation of data by chemometric methods has the potential to discriminate the botanical origins of various types of honey.

KEYWORDS: Slovenian honey; botanical origin; TXRF; PCA; RDA; chemometry

INTRODUCTION

Honey, a naturally sweet substance produced by honeybees, contains mainly simple sugars or monosaccharides. Fructose and glucose are the main components (65%) together with approximately 18% water. Proteins, flavonoids, phenolic compounds, free amino acids, organic and inorganic acids, and vitamins constitute the minor components of honey, which contain also all of the essential minerals. Therefore, honey is considered to be one of the most complex natural nutrients, but certainly the only sweetening agent that can be used by humans without processing.

Honeybees collect nectar and honeydew and then, in a complex process involving the addition of their own substances such as enzymes, store it to ripen and mature in the honeycomb, where it is transformed into honey.

Characterization of honey as a commercial product is a demanding task, which was initiated in the European Union (EU) in response to consumer demands. Besides the quality, determination of the geographical and botanical origins was also demanded, which initiated the establishment of corresponding

regulations in a number of member countries. Honey regulation in the EU (2) states that the botanical and geographical origins of the product must be printed on the label. The control of commercially sold honey therefore requires the determination of parameters that unambiguously establish the origin and also the quality of honey.

Investigations in this direction have been performed in many countries in Europe, based mainly on the determination of the biological properties (melissopalynology) and physicochemical parameters of honey, such as water content, ash, electrical conductivity, pH value, free lactic and total acidity, specific rotation, sugar content, and elemental content (3–7). The traditional determination of the botanical origin of honey was based on microscopic identification of the characteristic pollen present in honey (melissopalynology) (8) but also relied on sensory analysis performed by trained experts. An improvement in the determination of the botanical origin can obviously be achieved only by the application of the multivariate analytical approach combined with statistical methods of pattern recognition. The application of chemometric methods in food chemistry for the characterization and classification of products according to their origin, quality, and variety is not only useful but in many cases also necessary (9, 10). Several such applications of honey

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classification, including the elemental content, have been reported (11–16). Statistical methods of principal component analysis (PCA) (11, 14–16), *K*-nearest neighbor (KNN) (11), soft independent modeling of class analogy (SIMCA) (11), cluster analysis (CA) (14–16), linear discriminant analysis (LDA) (11–14, 16), and correspondence factor analysis (CFA) (11, 13, 15) were used in evaluating the measured physicochemical parameters of honey.

The elemental or mineral content of honey is one such set of parameters and has been determined by many authors applying different instrumental techniques such as atomic absorption spectrometry (AAS) (16–21), inductively coupled plasma atomic emission spectrometry (ICP-AES) (15–23), ion chromatography and voltammetry (24), and also TXRF (25).

Investigation of the elemental content of honey in Slovenia was first performed by Golob et al. (1) by the TXRF technique. Beekeepers in Slovenia produce many types of unifloral honey from nectar (acacia) or honeydew (spruce and fir) as well as honeys of mixed origin (lime and chestnut). Common types of honey in Slovenia also include mixed floral honey, which is of nectar origin, and forest honey that is a mixture of various types of honeydew. This preliminary study was intended to establish the quality of Slovenian honey and compare the results on elemental content with similar studies in other countries and regions. We also tried to determine the botanical origin by the simple statistical method of Duncan's test following the analysis of variance (ANOVA) (26). Improvement in establishing the botanical origin of honey obviously requires a much larger number of samples collected all over Slovenia and also a more comprehensive chemometric approach. Therefore, systematic research on the physicochemical parameters in various types of Slovenian honey has been carried out since 2004. Analysis of water content, electric conductivity, pH value, free and total acidity, content of lactones, sugars, diastase activity, etc., was performed. Additionally, the basic carbon isotope parameters, such as $\delta^{13}\text{C}$ in bulk honey and in extracted proteins, were also determined. In this study seven types of the most popular and frequently produced honeys in Slovenia were selected. According to their predominant botanical origin, these were classified as acacia, floral, lime, chestnut, spruce, fir, and forest honey. The honey type was generally divided into nectar or honeydew according to the origin of the bee foraging and whether nectar of the plants or honeydew from secretions of living parts of plants and excretions of plant-sucking insects on the living parts of plants was collected. In this way, spruce, fir, and forest honeys were classified as honeydew honey and acacia and floral honeys as nectar honey, whereas lime and chestnut honeys were classified as mixed nectar and honeydew honeys. Honeydew honey was distinguished from nectar honey by higher values of physicochemical parameters such as acidity, ash content, and electrical conductivity, a darker color, and a higher elemental content as well (1, 7). From the 2004 to 2006 a total of 264 samples of various types of honeys were collected and analyzed, and a data bank was created. These data were statistically processed with the main purpose of determining the botanical and geographical origins and estimating the quality of Slovene honey to check the authenticity and possible adulteration of honey available on the market. In this work only a part of the current investigations is reported, presenting the possibility of determination of the botanical origin of honey simply by TXRF analysis of the elemental content in combination with chemometric methods, as a fast, simple, and therefore cheap approach, which does not require any additional and lengthy analysis of numerous physicochemical parameters.

MATERIALS AND METHODS

Sample Preparation. In our study honey samples from the three different production years 2004, 2005, and 2006 were collected to investigate also the influence of different climatic conditions in a particular year on the quality and different parameters. In 2004 and 2006 all of the different types were available, whereas in 2005 we had no samples of lime and fir honeys. Samples were collected from beekeepers in Slovenia. All 264 samples of honey were classified according to their type using pollen analysis (26). The following seven types of honey were identified: acacia (*Robinia pseudoacacia*), floral (nectar of different flowers), lime (*Tilia* spp.), chestnut (*Castanea sativa*), spruce (*Picea abies*), fir (*Abies alba*), and forest (mixed honeydew type of honey). A botanical classification was considered to be achieved when the pollen spectrum contained >40% (>80% for chestnut honey and even less than 40% for acacia honey) of the corresponding dominant pollen. Classification by the sensory method performed by experts was also applied.

Approximately 0.3–0.7 g of honey was weighed in a 30 mL plastic tube (Sarstedt); 1 mL of a solution of Ga (0.01 g/L) was added as an internal standard and diluted to 10 mL total volume by double-distilled water. The solution was shaken until all of the honey was dissolved, and then the solution was homogenized in an ultrasonic bath for 1 h. By ultrasonic homogenization of the samples, the homogeneity test, which is the main factor contributing to the repeatability of the results, has been essentially improved compared to our previous work (1). Ten microliters of the obtained solution was transferred onto a quartz (reflector) substrate and dried in the desiccator overnight, and the sample as a very thin honey residue on the reflector was then measured by TXRF. The analytical results on metal content were determined on a honey samples basis. The uncertainty due to the variation of moisture content in honey samples was included and also represents just a minor contribution to the total uncertainty of the analytical procedure.

Analysis. The TXRF system was composed of the total reflection module, the X-ray spectrometer, and the X-ray tube excitation system, and its characteristics were well described in our previous paper (1). In this work the X-ray spectrometer was based on the Si drift detector (SDD) from KETEK, with a resolution of about 140 eV at 5.9 keV. By replacing the standard Si(Li) detector used in our previous work, the sensitivity for the analyses of light elements has been improved. The analysis of complex X-ray spectra was performed by the AXIL (27) spectral analysis program. The evaluated uncertainty of this procedure included the statistical uncertainty of the measured intensities and the uncertainty of the mathematical fitting procedure. The overall uncertainty of spectral measurement and analysis was in most cases better than 1%. Quantitative analysis based on the internal standard (Ga) of known concentration was straightforward because the sample was thin and matrix corrections could be neglected (28).

Statistical Analysis. The statistical calculations were performed using the Origin statistical software package (29). PCA and RDA were performed using the SCAN software package (30).

RESULTS AND DISCUSSION

The results of univariate statistical analysis of a set of honey samples by the most significant data on elemental content of Cl, K, Mn, and Rb are presented in Tables 1–3. Each sample was characterized as a pure type determined by sensory and pollen discrimination among the different types of honeys. In general, the mean values for all four elements are comparable in the years 2004 and 2005, but they differ from those in year 2006. This fact is especially clearly seen in chlorine and potassium contents, which were relatively lower in 2004 and 2005 in comparison with 2006 (Tables 1–3). The main reasons are most probably the different climatic conditions in these three years. In June and July of 2006, which are months of maximum honey production, the average temperature and solar irradiation were higher, but precipitation was lower. These facts (average temperature between May and September, rainfall, sunshine hours) influenced the higher content of chlorine and potassium in honey, because the nectar and honeydew were probably more concentrated.

Table 1. Number of Samples (*n*), Mean, Standard Deviation (SD), and Minimum and Maximum Concentrations of the Four Elements That Best Discriminate Samples in Production Year 2004

type of honey	<i>n</i>	Cl (mg/kg)				K (mg/kg)				Mn (mg/kg)				Rb (mg/kg)			
		mean	SD	min	max	mean	SD	min	max	mean	SD	min	max	mean	SD	min	max
acacia	15	67.6	20	44.4	106	313	69	230	434	3.42	7.6	2.40	5.2	1.01	0.23	0.60	1.5
floral	15	240	66	104	322	1120	341	380	1610	3.79	7.5	2.60	5.8	3.11	1.8	1.40	8.2
lime	15	273	32	116	324	1540	253	2240	1030	4.42	1.3	2.4	7.8	5.27	2.9	2.2	10.0
chestnut	12	205	34	140	256	3900	674	3190	5520	24.2	6.5	10.6	35.0	19.8	7.9	10.1	33.2
forest	15	304	72	147	404	2800	315	2160	3310	7.58	2.1	4.7	12.1	12.8	5.5	6.8	26.3
fir	14	287	51	187	373	2960	414	2200	3640	7.81	1.9	4.0	10.7	13.4	4.3	7.3	19.1
spruce	15	243	32	198	323	2820	514	1960	3430	4.57	2.0	2.80	9.4	19.0	5.8	7.8	25.0

Table 2. Number of Samples (*n*), Mean, Standard Deviation (SD), and Minimum and Maximum Concentrations of the Four Elements That Best Discriminate Samples in Production Year 2005

type of honey	<i>n</i>	Cl (mg/kg)				K (mg/kg)				Mn (mg/kg)				Rb (mg/kg)			
		mean	SD	min	max	mean	SD	min	max	mean	SD	min	max	mean	SD	min	max
acacia	17	84.5	31	34.7	147	285	77	175	457	8.40	1.71	5.28	11.7	1.18	0.37	0.82	1.99
floral	16	224	77	108	375	1020	373	343	1750	9.96	3.46	5.09	18.9	4.37	1.9	1.35	0.73
chestnut	14	146	31	103	204	2510	855	790	3680	18.5	4.2	9.50	25.7	17.7	9.0	3.81	34.1
forest	11	288	39	235	364	2590	401	2030	3340	12.5	2.7	7.43	16.8	16.5	10.4	7.82	35.7
spruce	15	335	126	206	709	2570	601	1490	3610	10.5	3.7	7.09	21.0	13.8	6.4	6.65	28.0

Table 3. Number of Samples (*n*), Mean, Standard Deviation (SD), and Minimum and Maximum Concentrations of the Four Elements That Best Discriminate Samples in Production Year 2006

type of honey	<i>n</i>	Cl (mg/kg)				K (mg/kg)				Mn (mg/kg)				Rb (mg/kg)			
		mean	SD	min	max	mean	SD	min	max	mean	SD	min	max	mean	SD	min	max
acacia	22	100.0	95	24.2	43.4	255	143	174	862	0.93	0.31	0.47	1.78	0.62	0.52	0.25	2.17
floral	13	369	165	162	721	1160	628	467	2850	2.23	1.11	1.19	4.97	2.54	1.94	0.64	7.08
lime	13	562	142	443	927	2070	274	1630	2560	3.97	2.99	1.40	10	5.26	3.31	1.81	11.4
chestnut	12	326	162	144	612	4040	601	3110	5230	28.13	12.1	8.66	44	15.87	6.73	7.92	31.9
forest	10	467	294	181	1170	3010	915	778	3930	6.46	3.31	1.19	10.8	23.01	13.7	1.36	41.6
fir	5	460	207	224	714	2670	4049	2310	3320	5.42	1.92	3.6	8.62	17.2	6.19	6.98	22.6
spruce	15	553	275	240	1190	3520	341	2770	3930	7.00	3.49	3.37	14.00	24.15	7.02	12.6	34.1

Table 4. Loadings Values of the Variables Associated with the First Principal Component Calculated Using Analytical Data for Samples from 2004, 2005, and 2006

variable	PC1	variable	PC1
Cl	−0.268	Ni	−0.241
K	−0.467	Cu	−0.349
Ca	−0.395	Zn	−0.052
Cr	0.193	Pb	0.064
Mn	−0.373	Br	−0.103
Fe	0.117	Rb	−0.408

Chemometric Analysis. Fourteen different elements were measured by TXRF (P, S, Cl, K, Ca, Cr, Mn, Fe, Ni, Cu, Zn, Pb, Br, and Rb). In the first step PCA was used to find which parameters are crucial for the optimal separation of the different types of honey and which of them contribute only to some evenly distributed noise. The calculations showed that only Cl, K, Mn, and Rb are the most important parameters for discriminating the types of honey. The loading values of the variables associated with the first principal component are reported in **Table 4** and show the contribution of the variables to the most significant principal component PC1. According to these values, the most significant variables are Cl, K, Ca, Cr, Mn, Ni, Cu, and Rb, but on this point results should be evaluated carefully. There are several reasons all of the important elements could not be used or they do not contribute

significantly to better separation. Some of the elements could be present as contaminants in the processing equipment and stainless steel containers. Acidic foods such as honey that are in contact with a stainless steel surface during harvesting, processing, and/or packaging could be contaminated by a rather high content of Fe, Ni, Cu, and Cr (12). Contamination by Zn via galvanized steel equipment (12) is also possible. For these reasons quite a number of measured elements are not directly related to botanical origin and were therefore excluded from statistical data evaluation. Calcium, for instance, could be used for botanical discrimination of honey types, but its variance between samples is very similar to that of chlorine, and therefore its contribution to the final discrimination is minimal as compared to Cl. This was the reason that in further calculations Ca was not considered. It was also one of the main purposes of this work to find the most critical elements for discrimination of the botanical origin of honey and consequently to simplify the statistical evaluation.

Figure 1A represents a projection of the first two principal components representing 91.6% of the total variance (PC1 = 61.2%, PC2 = 23.8%) of the data for all three production years. A good separation of chestnut and acacia honey samples was found, whereas the separation between lime and floral honey samples showed that the harvested honey was mixed. The same was true for the forest, spruce, and fir honeys. The main reason for the rather indistinct separation was the determination of the

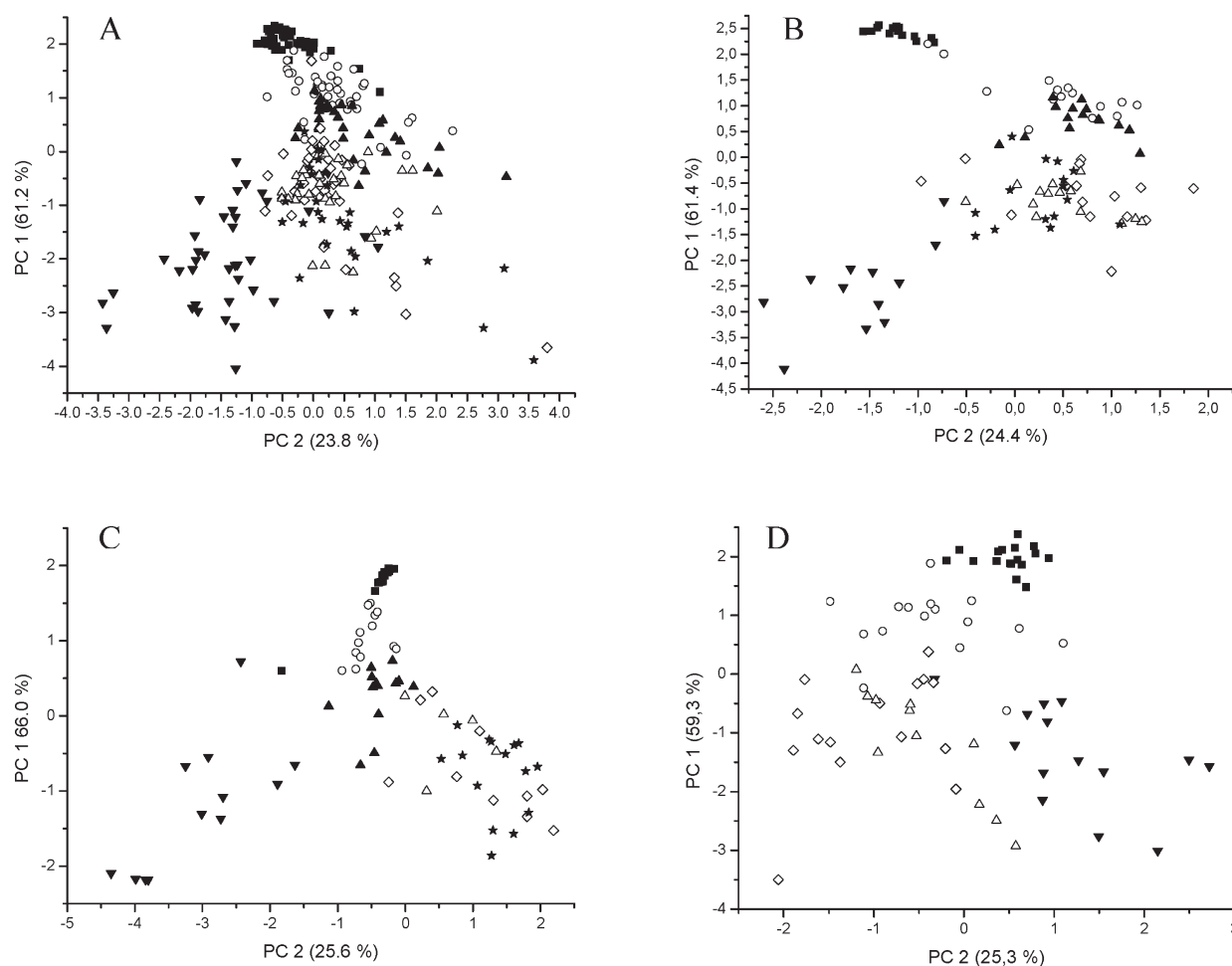


Figure 1. PCA performed using concentrations of Cl, K, Mn, and Rb for the production years 2004, 2005, and 2006 from 264 honey samples corresponding to 7 different types of honey (A), for the production year 2004 with 101 honey samples corresponding to 7 different types of honey (B), for the production year 2006 with 90 honey samples corresponding to 7 different types of honey (C), and for the production year 2005 with 73 honey samples corresponding to 5 different types of honey (D): (■) acacia; (○) floral; (▲) lime; (▼) chestnut; (◇) forest; (△) spruce; (★) fir. The planes are constituted by the first two principal components PC1/PC2 representing 91.6% of the total variance for the four variables (A), 85.8% (B), 91.6% (C), and 84.6% (D).

botanical origin of the honey samples based on sensory and pollen discrimination among the different types of honey, characterizing each sample as a pure type. However, the elemental content shows more details, because honeybees harvest from different nectar sources and especially from honeydew sources at the same time. This leads to a mixture of different types of honey in particular honey samples, which depends on the time and also climatic and other environmental conditions during the period of harvesting. These conclusions suggest that it would be useful to perform statistical evaluation on each year's data bank separately. It is also possible to conclude that analysis based on elemental content offers more information regarding the botanical origin of honey samples than the classical sensory or pollen analysis.

The projection of PC1 (61.4%) and PC2 (24.4%) values for honey samples from production year 2004 is shown in **Figure 1B**. As in the previous case a good separation of acacia and chestnut honeys is evident. Honeydew honeys (fir, spruce, and forest) form a separate group, as well as the lime and floral types. In the case of fir, spruce, and forest honeys, mixing is logical because these honeys are usually harvested at the same location. In Slovenia it is hard to find forest that consists only of one botanical species, either spruce or fir. However, spruce and fir honeys are produced at slightly different times of summer and not in the same geographical locations. Normally they are mixed with other types

of honeydew. Fir or spruce honeys should be labeled as pure only when they consist of one prevailing type of honeydew, fir or spruce, and have specific sensory characteristics. Otherwise, the label forest honey should be used, where the latter is a mixture of different types of honeydew with none prevailing.

The purity of lime and floral honeys depends very much on the climatic conditions. When the harvesting of floral honey is finished prior to the period of lime blooming, these two honeys are not mixed. On the contrary, mixing can be considerable in unsuitable climatic conditions when floral harvesting extends to the period of lime blooming. If the differentiation of honeys in 2004 and 2006 (**Figure 1B,C**) is inspected together with the climatic conditions, it is possible to observe that in 2004 separation between lime and floral honeys is not clear, whereas in the case of 2006 the two groups are well separated. The reason is most probably the lower average temperature and solar intensity in 2004, which caused prolongation of the blooming time of flowers for floral honey and consequently the mixing of the two harvesting periods.

Figure 1D represents the score plot of honey samples from 2005 defined by PC1/PC2 that represents 84.6% of the total variance (PC1 = 59.3%, PC2 = 25.3%). Well-separated acacia, floral, chestnut, and forest/spruce honey groups are clearly seen, with only a few samples in the wrong place.

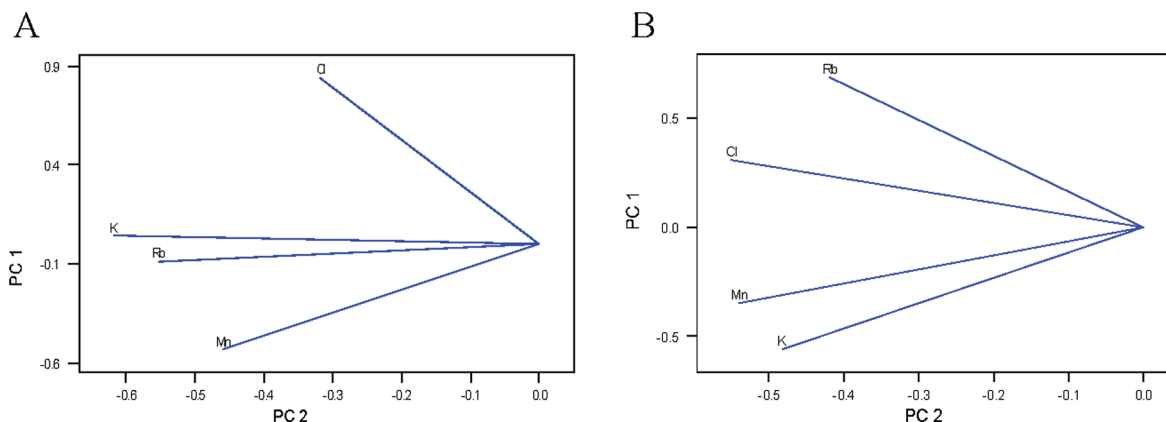


Figure 2. Principal components loading plot for the four variables Cl, K, Mn, and Rb for the production years 2004 (A) and 2006 (B).

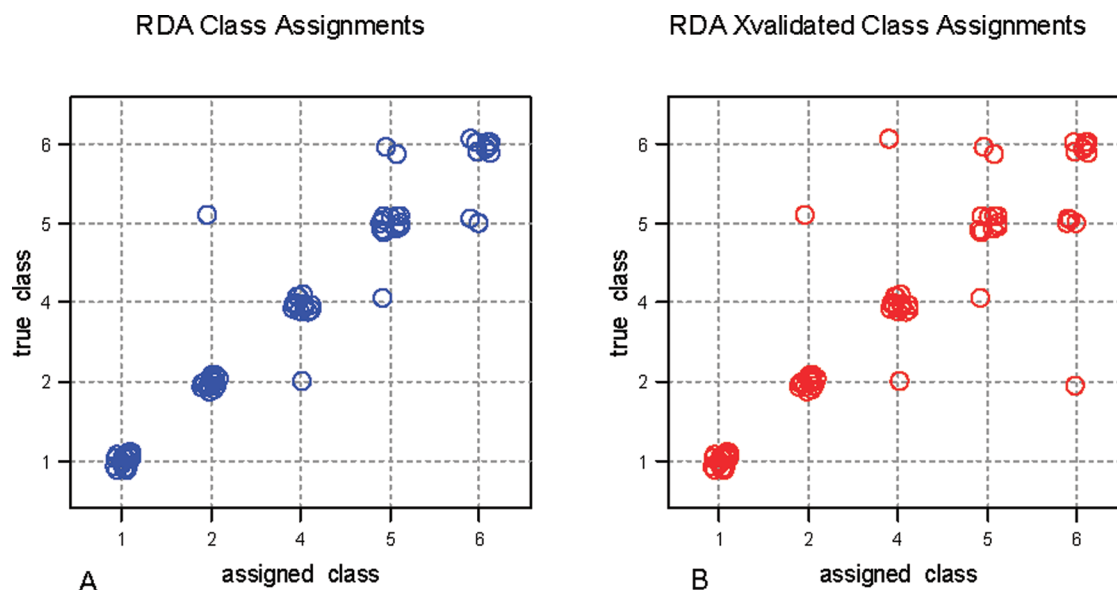


Figure 3. Classification of honey samples on the basis of the calculated model with RDA (A) and the validation test results obtained by the cross-validation method (B) for the samples produced in 2005.

In 2005 using sensory analysis none of the samples were found to be pure lime or fir honey; therefore, samples of these two types of honey are missing in the analysis.

In **Figure 2** the loading plots for the four variables Cl, K, Mn, and Rb concentrations are presented for 2004 (**Figure 2A**) and 2006 (**Figure 2B**). The loadings in both years differ in the correlations of new parameters with the previous ones and are not completely the same. This suggests that it would be better to perform the statistical evaluation for both years (2004 and 2006) separately.

The same results were also obtained when we used the PCA scores of all four calculated PCAs as input parameters in the chemometric method of RDA. The model gave 90.4% of classification ability with and without cross-validation. The prediction ability of the model, determined by the test set method, was again 90.4% (**Figure 3**). We used the RDA method on all sets of samples from different production years and obtained comparable results, with a prediction ability of the model of approximately 90%.

In conclusion, we can say that simple, fast, and inexpensive TXRF multielemental analysis of honey samples in combination with PCA and RDA statistical evaluation of the measured data offers a fast screening method for determination of the botanical origin of honey.

The proposed approach enables discrimination of the botanical origin of Slovenian nectar honeys such as floral and acacia and

of mixed nectar and honeydew types such as lime and chestnut honey, whereas the honeydew honey types such as forest, spruce, and fir honeys cannot be distinguished from each other. The reason is the simultaneous harvesting of different types of honeydew by honeybees, resulting therefore in a mixture of these types of honey. Forest honey is a mixture of different types of honeydew including spruce, fir, and many other types of honeydew. Spruce and fir honeys are also mixtures of several honeydews, but one type of honeydew, for example, spruce, prevails, which is expressed in its sensory characteristics as well.

It seems that the above-mentioned analytical approach using only TXRF is promising for applications in multivariate statistical analysis of other different food products such as nutrients, food supplements, and additives. By such an approach quality control and determination of the geographical origin, production location, and authenticity could be established.

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