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Characterisation and Classification of Croatian Honey by Physicochemical Parameters

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Summary

The aim of this study is to characterise 8 different monofloral and multifloral types of Croatian honey (a total of 254 samples from 2003, 2004, and 2005 harvesting seasons) based on 11 common physicochemical parameters (water mass fraction, total reducing sugar mass fraction, sucrose mass fraction, ash mass fraction, electrical conductivity, acidity, diastase and invertase activity, hydroxymethylfurfural (HMF) mass fraction, proline mass fraction and optical rotation). Differences in the above-mentioned parameters, established among the honey samples, are influenced by different factors, such as botanical origin, climate and regional circumstances. After the sample characterisation, results obtained for 2 monofloral (acacia (*Robinia pseudoacacia*) and chestnut (*Aesculus hippocastanum*)), and 2 multifloral (floral and meadow) honey types were subjected to the pattern recognition procedures. In this regard, unsupervised methods such as cluster and principal component analyses were employed, with the goal of evaluating the possibility of differentiation of Croatian honey stemming from different botanical origins, based on their physicochemical profile. Cluster analysis (CA) revealed the existence of two clusters, in the first of which is acacia honey as the best grouped, and the second corresponds to the dispersed group constituted of the remaining three honey types under investigation (chestnut, floral, and meadow). Principal component analysis (PCA), *i.e.* its first two components, stood for the average of 50.5 % of the data variance. PCA and CA showed that physicochemical parameters are able to provide enough information to allow for the classification and distinction of the types of honey originating from four botanical origins under investigation (acacia, chestnut, floral and meadow).

Key words: honey, physicochemical parameters, botanical origin, cluster analysis (CA), principal component analysis (PCA)

Introduction

According to the Codex Alimentarius: 'Honey is the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature' (*1*).

Although Croatia is a small country, differences in climate, soil and plants provide a solid base for the production of different types of honey, such as those most often produced (acacia (*Robinia pseudoacacia*), meadow, floral, sage (*Salvia officinalis*), chestnut (*Aesculus hippocastanum*), and lime (*Tilia cordata*)), but also those of a rare and specific sort, such as rosemary (*Rosmarinus officinalis*), lavender (*Lavandula officinalis*), and heather (*Calluna vulgaris*).

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Despite the long tradition of Croatian honey production, the issues of physical, chemical and sensory properties of Croatian honey have insofar been covered by only a few scientific papers (*2–5*), contrary to the large number of foreign publications dealing with these issues. Botanical origin is one of the most important properties of honey and has a great influence on its price, so that it has become very important to determine it accurately, for the benefit of both producers and consumers. Due to certain limitations of pollen analysis, which is the only method of determination of botanical origin of honey officially adopted until now, it is important to develop new methods that enable easier and more accurate determination of honey's botanical and geographical origin. In this research, pollen analysis and, in line with the aforementioned, also the classification of honey were performed by honey producers. There are a lot of studies where physicochemical parameters, sugar, flavonoids and mineral content have been utilised as a basis for such characterisation; however, dispersion and overlapping of some values obtained with samples stemming from different botanical origin sometimes reduce the efficiency of the studies in question. Recently, flame atomic absorption spectrometry (FAAS) and inductively coupled plasma-optical emission spectrometry (ICP-OES) analyses of the minority of honey components (Al, B, Ca, Cu, Mg, Mn, Ni, Zn), combined with electrolytic conductivities of 24 authentic Czech honey samples, have proven themselves as a useful tool for the differentiation between honeydew and nectar honeys (*6*).

In light of the foregoing, the aim and purpose of this study is to characterise each type of honey under research (acacia (*Robinia pseudoacacia, Fabaceae*), floral, sage (*Salvia officinalis, Lamiaceae*), chestnut (*Aesculus hippocastanum, Sapindaceae*), meadow, mountain meadow, citrus (mandarin, *i.e. Citrus reticulata, Rutaceae*), honeydew; altogether 254 samples collected during three harvesting seasons)) based on various physicochemical parameters (water mass fraction, total reducing sugar mass fraction, sucrose mass fraction, ash mass fraction, electrical conductivity, acidity, diastase and invertase activity, hydroxymethylfurfural (HMF) mass fraction, proline mass fraction, and optical rotation) and to find out whether these parameters are able to provide enough information to allow for the classification of the four honey types (acacia, chestnut, floral, meadow) by their botanical origin.

Materials and Methods

The research was conducted on 254 honey samples stemming from different botanical origin (acacia, floral, sage, chestnut, meadow, mountain meadow, citrus (mandarin), honeydew) manufactured in various parts of Croatia during three harvesting seasons (2003, 2004 and 2005). In all samples, the physicochemical parameters discussed below were determined. Water mass fraction (moisture) was measured by refractometer using the AOAC Official Method (*7*). Electrical conductivity was measured by Mettler conductivity meter, according to the method proposed by the International Honey Commission (IHC) (*8*). Total reducing sugar mass fraction, sucrose mass fraction, acidity and ash mass fraction were measured conformant to the AOAC Official Methods (*7*). Diastase and invertase activity, HMF mass fraction, proline mass fraction and optical rotation were determined using the methods proposed by the IHC (*8*). Pollen analysis was not done since all of the honey samples had already been classified by beekeepers. Before and during the analyses, samples were stored in glass containers at room temperature.

With all these analyses, the criteria laid down by the Regulations of the International Honey Commission (IHC) (*8*) were applied. The obtained results were evaluated using Croatian and international honey-profiling criteria (*9*). With each analysis, two parallel measurements were conducted at the same time, so that the results are given as mean values. Basic statistics (mean, range and standard deviation) and multivariate statistical analysis (CA, PCA) were carried out using StatSoft Statistica package (*10*).

Clustering techniques employ an unsupervised chemometric procedure that involves measurement of either the distance or the similarity between the objects to be clustered. In line with the foregoing, objects are grouped in clusters based on their nearness or similarity. The initial assumption is that the nearness of the objects in the variable p-space reflects the similarity of their properties.

PCA is a non-supervised technique mainly used to achieve a reduction of the original data matrix, fitting a j-dimension subspace into the original p-variable (p>j) space, retaining the maximum amount of variability. It allows for the establishment of relationships between variables and observations, as well as for the recognition of data structure.

Results and Discussion

Chemical composition

In order to demonstrate the variability of the data, Tables 1–3 contain mean values and standard deviations for all 11 investigated physicochemical parameters of 8 different monofloral and multifloral honey types.

Only two out of 254 samples did not meet the demands imposed by the regulations concerning the water mass fraction (moisture); namely, water mass fraction established in these samples exceeded 20 %. These two samples were the honeydew harvested during 2004 season and the floral honey harvested during 2005 season, their water mass fraction being 20.2 and 20.6 %, respectively. The mean values obtained with different honey types were very similar, and ranged from 15.4 % for the mountain meadow honey harvested during 2003 season, to 17.5 % for the floral honey harvested during 2004 season. As apparent from Tables 1–3, the widest water mass fraction range was obtained with the samples of the floral honey harvested during 2005 season (14.6–20.6 %), chestnut honey harvested during 2004 season (13.9–19.4 %), and honeydew harvested during 2004 season (15.0–20.2 %), while the narrowest water mass fraction range was identified in the sage honey harvested during the season 2003 (14.6–16.8 %), and the floral honey harvested during the season 2004 (16.4–18.6 %). By comparing these results to the results of other researchers, it can be noticed that our acacia honey samples contained

Table 1. Physicochemical parameters of the honey samples harvested in the season 2003

Honey type	No. of samples		*w*(water) / %	Electrical conductivity / mS/cm	*w*(total reducing sugars) / %	*w*(sucrose) / %	Acidity / mmol/kg	*w*(ash) / %	Diastase activity / DN	Invertase activity / IN	*w*(HMF) / mg/kg	*w*(proline) / mg/kg	Optical rotation / degree
		Mean	15.4	0.20	71.5	4.3	8.4	0.08	12.2	12.4	7.2	254.9	–2.4
Acacia	23	S.D.	0.881	0.059	2.424	1.725	1.424	0.026	3.425	4.608	10.958	68.820	0.269
		Range	14.2–17.7	0.13–0.38	66.7–75.7	1.9–7.3	6.1–12.0	0.04–0.16	7.4–22.1	2.2–20.5	0.4–52.4	167.4–452.8	(–3.0)–(–2.0)
		Mean	16.8	0.60	72.5	3.8	18.0	0.22	26.0	14.8	3.5	602.1	–1.8
Floral	5	S.D.	1.297	0.301	3.539	1.358	4.8	0.121	3.200	5.456	1.471	203.228	1.078
		Range	15.5–18.6	0.27–0.88	66.6–75.8	2.0–5.2	14.1–26.1	0.07–0.32	23.4–29.6	9.6–22.9	1.3–4.9	393.9–907.2	(–3.2)–(–0.5)
		Mean	15.8	0.57	71.8	2.1	18.5	0.22	26.6	15.3	8.2	590.5	–1.6
Sage	6	S.D.	0.774	0.301	2.643	1.516	5.999	0.114	6.211	8.070	9.841	290.191	0.774
		Range	14.6–16.8	0.28–1.08	68.3–74.9	0.7–5.0	11.1–25.5	0.44–1.13	15.8–34.7	6.7–25.9	2.6–27.9	233.1–1020.8	(–2.3)–(–0.1)
		Mean	16.9	1.27	73.1	3.1	12.4	0.46	29.9	17.8	4.8	568.7	–2.7
Chestnut	7	S.D.	1.024	0.317	2.489	0.703	4.727	0.087	3.638	2.762	4.532	105.582	0.727
		Range	15.4–18.6	0.81–1.62	70.2–76.3	2.4–4.5	8.0–21.7	0.33–0.58	24.2–33.0	12.4–20.8	0.7–11.7	435.1–749.6	(–3.5)–(–1.8)
		Mean	16.0	0.61	71.2	2.4	21.4	0.23	19.4	8.7	6.7	688.5	–2.2
Meadow	9	S.D.	1.607	0.207	3.942	1.273	6.401	0.088	5.314	6.148	8.279	245.063	0.508
		Range	14.1–18.2	0.38–0.97	62.1–76.1	0.2–4.5	13.9–35.0	0.14–0.38	12.95–28.04	0.1–18.2	0.6–27.3	375.8–1009.9	(–3.0)–(–1.6)
		Mean	15.4	0.66	70.5	4.4	17.5	0.26	21.7	13.5	8.0	631.0	–1.2
Mountain meadow	9	S.D.	0.763	0.23	3.082	3.082	5.737	0.096	3.497	6.218	8.077	204.923	0.830
		Range	14.2–16.8	0.35–0.92	66.7–77.0	1.5–10.7	10.0–25.1	0.38–1.12	16.0–27.0	6.6–23.0	0.4–25.8	383.7–949.9	(–2.2)–(–0.1)

S.D.=standard deviation

Table 2. Physicochemical parameters of the honey samples harvested in the season 2004

Honey type	No. of samples		*w*(water) / %	Electrical conductivity / mS/cm	*w*(total reducing sugars) / %	*w*(sucrose) / %	Acidity / mmol/kg	*w*(ash) / %	Diastase activity / DN	Invertase activity / IN	*w*(HMF) / mg/kg	*w*(proline) / mg/kg	Optical rotation / degree
		Mean	16.3	0.17	69.6	4.9	7.3	0.06	14.4	8.6	4.7	191.7	–2.7
Acacia	45	S.D.	0.957	0.045	2.474	2.197	1.915	0.026	4.198	4.374	3.568	83.356	0.408
		Range	14.0–18.2	0.11–0.32	65.2–73.9	1.4–9.7	5.0–15.1	0.01–0.15	8.0–28.4	1.3–21.1	0.4–18.1	24.0–378.8	(–3.6)–(–1.9)
		Mean	17.5	0.40	71.6	3.5	14.7	0.18	20.6	12.3	10.2	446.5	–2.5
Floral	7	S.D.	0.906	0.138	2.606	1.048	4.199	0.076	7.307	6.759	9.286	132.577	0.431
		Range	16.4–18.6	0.19–0.53	67.4–75.1	1.8–4.8	9.1–21.5	0.07–0.27	10.0–29.3	2.1–18.6	1.9–27.4	281.6–606.0	(–2.9)–(–1.8)
		Mean	16.5	0.51	70.1	4.2	15.6	0.19	24.5	15.8	5.6	423.4	–2.2
Sage	7	S.D.	0.520	0.247	2.567	2.088	3.015	0.110	9.416	10.908	3.573	117.599	1.014
		Range	15.7–17.2	0.31–1.01	67.7–75.0	2.1–7.7	10.0–19.2	0.07–0.41	9.8–35.2	0.8–26.1	1.9–11.5	282.6–573.0	(–3.1)–(–0.6)
		Mean	16.6	1.04	71.7	3.7	11.6	0.47	20.7	13.0	3.5	556.7	–3.0
Chestnut	7	S.D.	1.812	0.296	4.051	1.693	5.279	0.214	4.251	4.813	2.845	98.009	0.190
		Range	13.9–19.4	0.58–1.38	66.0–77.0	1.6–5.5	6.1–18.1	0.19–0.78	16.9–29.2	8.4–19.6	0.8–8.8	391.7–660.5	(–3.3)–(–2.7)
		Mean	16.8	0.47	69.7	3.7	18.5	0.18	25.5	13.9	6.6	585.2	–2.4
Meadow	17	S.D.	1.387	0.152	2.607	0.948	6.629	0.068	8.041	7.215	5.802	161.341	0.591
		Range	14.8–19.6	0.19–0.73	65.3–74.7	2.1–4.9	5.0–28.2	0.07–0.35	11.9–38.8	2.1–27.9	0.8–22.7	394.4–885.5	(–3.3)–(–0.9)
		Mean	16.2	0.17	69.5	3.2	9.3	0.05	12.2	2.8	6.7	215.6	–2.2
Citrus	6	S.D.	1.615	0.033	2.019	1.274	1.020	0.016	2.867	0.914	4.855	60.046	0.686
		Range	14.5–18.2	0.15–0.22	67.4–72.3	2.0–4.8	8.1–11.2	0.02–0.06	9.1–15.7	1.9–4.3	0.4–15.4	125.1–282.0	(–3.0)–(–1.5)
		Mean	16.4	1.03	68.4	8.7	15.9	0.56	22.0	15.3	1.7	404.0	2.4
Honey-dew	5	S.D.	2.150	0.339	4.547	1.457	3.910	0.227	8.134	3.814	0.343	99.013	1.316
		Range	15.0–20.2	0.68–1.45	61.0–72.9	7.1–10.5	9.0–19.0	0.33–0.90	12.8–35.0	11.6–20.7	1.3–2.1	229.9–466.2	0.9–3.9

S.D.=standard deviation

Table 3. Physicochemical parameters of the honey samples harvested in the season 2005

Honey type	No. of samples		*w*(water) / %	Electrical conductivity / mS/cm	*w*(total reducing sugars) / %	*w*(sucrose) / %	Acidity / mmol/kg	*w*(ash) / %	Diastase activity / DN	Invertase activity / IN	*w*(HMF) / mg/kg	*w*(proline) / mg/kg	Optical rotation / degree
		Mean	16.1	0.15	67.4	2.4	7.6	0.05	9.7	6.4	36.5	305.4	–1.8
Acacia	41	S.D.	1.022	0.032	1.721	1.986	1.733	0.022	2.684	4.413	30.785	175.192	0.201
		Range	14.3–19.4	0.11–0.23	65.0–70.6	0.0–9.9	5.0–13.1	0.01–0.10	6.2–18.9	0.9–21.5	1.5–89.9	84.5–782.3	(–2.2)–(–1.4)
		Mean	17.0	0.45	68.7	1.5	12.1	0.14	18.1	10.7	45.5	469.2	–1.5
Floral	14	S.D.	1.986	0.394	2.175	1.674	4.715	0.126	6.031	6.121	29.031	235.230	0.312
		Range	14.6–20.6	0.15–1.33	63.9–70.6	0.0–4.4	5.1–20.4	0.03–0.41	9.6–30.2	2.1–19.7	6.7–93.7	183.7–850.3	(–2.0)–(–1.0)
		Mean	16.6	0.47	69.1	1.8	18.1	0.20	28.0	19.6	27.6	356.8	–1.5
Sage	5	S.D.	1.409	0.217	1.965	1.683	5.829	0.085	4.472	6.818	25.159	162.901	0.291
		Range	15.5–19.0	0.31–0.85	66.4–70.6	0.0–3.5	10.0–24.1	0.11–0.33	21.7–33.7	11.7–26.7	4.8–67.2	153.4–598.9	(–1.9)–(–1.2)
		Mean	16.6	1.18	69.6	1.1	11.8	0.50	24.5	18.8	29.5	480.5	–1.7
Chestnut	14	S.D.	1.381	0.233	1.483	1.425	4.335	0.243	5.350	8.503	31.895	221.874	0.214
		Range	14.7–19.0	0.92–1.50	66.5–71.5	0.0–4.7	6.0–18.0	0.19–0.93	18.5–36.6	8.6–34.7	3.1–99.8	134.8–769.9	(–2.2)–(–1.5)
		Mean	16.7	0.47	68.2	1.8	21.0	0.22	20.3	13.2	26.5	439.2	–1.5
Meadow	21	S.D.	1.445	0.145	1.763	1.240	8.331	0.125	4.084	5.169	22.960	190.039	0.404
		Range	14.8–19.1	0.23–0.68	65.5–70.6	0.0–4.4	7.0–37.7	0.09–0.60	14.9–29.0	6.0–27.3	2.3–90.2	131.1–762.8	(–2.1)–(–0.6)
		Mean	17.1	0.60	67.3	2.0	20.8	0.23	31.5	20.0	12.8	416.0	–1.0
Mountain meadow	6	S.D.	1.323	0.185	2.725	1.649	4.291	0.093	7.433	6.253	7.072	173.773	0.643
		Range	15.7–19.1	0.39–0.82	63.7–70.6	0.0–4.0	15.8–27.0	0.12–0.34	23.5–42.3	9.4–27.0	3.8–20.0	237.1–661.3	(–1.7)–(–0.2)

S.D.=standard deviation

slightly less water than those employed in the research of Popek (*11*), and Golob and Plestenjak (*12*), while all other samples showed quite similar values. Similar results were also obtained in one Spanish (*13*) and one Argentinean (*14*) research. De Rodriguez *et al*. (*15*) analysed multifloral honey samples from one Venezuelan region, collected during rainy (November–June) and dry season (July–October), and concluded that honey moisture mass fraction depends on the harvest season and the degree of maturity reached in the hive. It is interesting that, as concerns our research, weather conditions had no influence on the honey water mass fraction whatsoever, since the samples harvested during 2004 season, which had witnessed considerably more rain than the season before, failed to exhibit higher water mass fraction values as compared to the honey samples harvested during 2003 season.

Electrical conductivity represents a parameter increasingly used in routine honey quality control, and can be considered as a valid criterion for the determination of honey's botanical origin or, more specifically, for the differentiation between nectar honey and honeydew (*8*). Because of its specific chemical composition (a higher content of mineral compounds), honeydew shows higher values of electrical conductivity than other nectar honey types, except for the chestnut honey, which is, as concerns this parameter, closer to honeydew than to other honey types. Because of that, Croatian and European regulations require that electrical conductivity in honeydew and chestnut honey be higher than 0.80 mS/cm, while in other nectar honey types (in case they aspire to be declared as such), this value has to be lower than 0.80 mS/cm (*1,7*). As apparent from Tables 1–3, the highest electrical conductivity values were measured in chestnut honey (range in the samples harvested during all three study seasons was 0.58–1.62 mS/cm), and honeydew samples (0.68–1.45 mS/cm), while the lowest values were measured in the acacia honey sample (0.11–0.38 mS/cm). As for the acacia honey, similar values were also obtained by Golob and Plestenjak (*12*), and Popek (*11*). Altogether, seventeen samples of the honey harvested in all three seasons failed to meet the demands provided by the regulations, out of which 5 mountain meadow, 3 floral, 3 sage, 2 meadow, 2 chestnut, and 2 honeydew samples.

The mean values of total reducing sugar mass fraction ranged from 67.3 % in the mountain meadow honey harvested during 2005 season to 73.1 % in the chestnut honey samples harvested during 2003 season. The highest mass fraction of 77.0 % was measured in one of the chestnut honey samples harvested in 2004 season, while the lowest mass fraction of 61.0 % was recovered from the honeydew sample harvested during 2004 season. In their study, Merin *et al.* (*16*) obtained somewhat larger total reducing sugar mass fraction than the one found in Croatian honey samples, and the results that were much closer to ours were those of Azeredo *et al.* (*17*). Besides, reducing sugar mass fraction recovered from the honeydew was lower than in the nectar honey. Looking at all three seasons, altogether 4 samples (floral, meadow, mountain meadow and honeydew) failed to meet the demands imposed by the regulations regarding the reducing sugar mass fraction (*8*).

The mean values of sucrose mass fraction ranged from 1.1 % in the chestnut honey samples harvested during 2005 season, to 8.7 % in the honeydew samples harvested during 2004 season (Tables 1–3). Several samples harvested during the season 2005, including 3 acacia, 2 floral, 2 sage, 2 chestnut, 1 meadow and 1 mountain meadow honey, were sucrose-free. The largest sucrose mass fraction was measured in one mountain meadow sample harvested in the season 2003 (10.7 %), and one honeydew sample harvested during the season 2004 (10.5 %). These values are a little bit higher than those obtained by Esti *et al.* (*18*), and more like the values obtained by Merin *et al.* (*16*). Regulative demands which require sucrose mass fraction to be lower than 5.0 % (except for sage honey and honeydew, which have to have less than 8.0 and 10.0 % of sucrose, respectively), were not met by 1 floral, 2 chestnut, 3 mountain meadow and 1 honeydew samples.

The highest average acidity values, equal to 21.4, 21.0, and 20.8 mmol/kg, were measured in the meadow honey samples harvested during the seasons 2003 and 2005, and the mountain meadow honey harvested during the season 2005, respectively. Honey samples characterised by the lowest average acidity were the acacia honeys harvested during all three study seasons, with the values of 7.3 mmol/kg for the honey harvested in 2004, 7.6 mmol/kg for the honey harvested in 2005, and 8.4 mmol/kg for the honey harvested in 2003 season. These acidity values are considerably lower than those obtained by Golob and Plestenjak (*12*). All of the investigated samples met the demands imposed by the regulations, which require that the acidity should not exceed 40.0 mmol/kg. With its acidity of 37.7 mmol/kg, only one meadow honey sample discussed herein almost obtained this limit value.

Although nowadays the determination of ash mass fraction in routine honey quality control settings has been replaced with electrical conductivity analyses, due to its simplicity the ash mass fraction is still deemed a useful parameter in determining botanical origin of honey and differentiating between nectar honey and honeydew (*19*). In this research, the highest ash mass fraction was hosted by honeydew, with the average value of 0.56 % and the range from 0.33 to 0.90 %. Fairly high average ash mass fraction of 0.48 %, ranging from 0.19 to 0.93 %, was also recovered from chestnut honey (harvested in all three study seasons). Other honey types possessed lower ash mass fraction, with average values of 0.24 % (mountain meadow) and 0.21 % (meadow) obtained in all the samples. The lowest average value of 0.05 % was measured in the acacia honey samples harvested during 2005 season. The average values obtained for the acacia honey harvested during all three study seasons (0.06 %) are similar to those published by Golob and Plestenjak (*12*), and Popek (*11*). According to the Croatian and European regulations (*8,9*), ash mass fraction should not be higher than 0.60 %, except for honeydew, which can contain up to 1.20 % ash. Those criteria were not met by three chestnut honey samples harvested during the season 2004, nor by four chestnut and one meadow honey sample harvested during the season 2005, the ash mass fraction of which exceeded 0.60 %.

Diastase activity is one of the main parameters utilised in the determination of the intensity of heating to which honey is exposed during processing and storage (*20–22*). During heating, diastase activity decreases. The highest average value for diastase activity (Tables 1–3) was measured in the mountain meadow honey sample harvested during the season 2005, equal to 31.5 DN (diastase number). The widest diastase activity range was measured in the meadow and sage honey harvested during the season 2004 (11.9 to 38.8 and 9.8 to 35.2 DN, respectively). The narrowest diastase activity range was measured in the floral honey harvested during the season 2003 (23.4 to 29.6 DN). The average diastase activity value obtained with all of the honey samples was 21.3 DN. Comparing our results to those obtained by Costa *et al.* (*22*), it can be noticed that diastase activity in Brazilian honey is lower than in Croatian, probably due to the warmer climate and higher environmental temperatures. The diastase activity obtained for chestnut honey in the research by Marini *et al.* (*23*) was almost identical to our results obtained with the same honey type. According to the regulations, diastase activity has to be higher than 8.0 DN, and if lower, the HMF mass fraction should not exceed 15.0 mg/kg. In this research, 5 acacia honey samples harvested during the seasons 2003 and 2004 had diastase activity lower than 8.0 DN, but as the HMF mass fraction in these samples was also lower than 15.0 mg/kg, they managed to meet the regulatory demands.

Invertase number (IN) is used to express invertase activity, and represents the number of grams of sucrose decomposed within an hour due to the activity of invertase present in 100 g of honey (*19,24*). According to the results obtained for invertase activity, the highest average value was recovered in the mountain meadow honey harvested during the season 2005, having the IN of 20.0, and the lowest one in the citrus honey harvested during the season 2004, having the IN of 2.8. As many as 5 out of 6 citrus honey samples showed invertase activity lower than 4.0 IN. Such low values might arise as the result of a lower invertase activity level, or due to the complete enzyme destruction that might occur as a result of high temperature or honey ageing (*25*). Since prior to the analyses all samples had been stored under the same storage conditions, causes of such low values are probably relative to the age of honey samples and naturally low enzyme activity typical of citrus honeys (*26*). The lowest level of invertase activity was established in one meadow honey sample harvested during the season 2003 (0.1 IN), and the highest in the chestnut honey harvested in the season 2005 (34.7 IN) (Tables 1–3). Compared to the results obtained by Persano Oddo *et al.* (*27*), our values for the same honey types are a bit higher, which might be attributable to the relative age of the honey samples. The average invertase activity in the chestnut honey, levelled a little bit higher than ours, and the one in the acacia honey, levelled somewhat lower than ours, was established by Serra Bonvehí *et al.* (*21*). Those results show that, apart from plant origin, the degree of nectar processing and nectar excretion, the activity of invertase recovered from honey also depends on climate conditions and harvesting season (*28,29*). Additional criteria proposed by the International Honey Commission (IHC) (*8*), according to which the invertase activity should be higher than 4 IN, were not met by altogether 35 of our honey samples: 1 acacia and 3 mountain meadow honey samples harvested during 2003 season; 7 acacia, 5 citrus, 2 sage and 1 meadow honey harvested during 2004 season; and 14 acacia and 2 floral samples harvested during 2005 season. As the samples in question represent 14.7 % of the total number of analyzed samples, and since the deviations were noted in the samples harvested during all three study seasons, these deviations are probably attributable to honey adulteration. Namely, from the beginning of the honey processing to the time of physicochemical analyses, a few months had elapsed. Croatian regulations (*9*) do not provide for invertase activity levels.

The highest average value of HMF mass fraction was measured in the floral honey harvested during the season 2005 (45.5 mg/kg), and the lowest in the honeydew harvested during the season 2004 (1.7 mg/kg). In general, the honey samples harvested during the season 2005 had considerably higher HMF values than those harvested during the previous two seasons. The average HMF value obtained with all honey samples harvested during 2005 season was 29.7 mg/kg, while that of 2004 samples was 7.0, and that of 2003 samples 6.4 mg/kg. Except for 2005 samples, the average HMF values established in chestnut and floral honey were somewhat higher than those obtained by Golob and Plestenjak (*12*). On the other hand, our average HMF value found in the honeydew was lower than that obtained by Vorlová and Čelechovská (*30*). The reason for so high HMF mass fraction of 2005 samples may be the prolonged period of heating employed within the honey processing, combined with too long and improper storage, and adulteration emerging from invert sugar syrup addition (*31–33*). However, adulteration is not likely to be the cause, since the number of samples having a high HMF mass fraction is fairly large. Croatian regulations allow the honey HMF mass fraction to rise up to 40.0 mg/kg. Out of 254 analyzed samples, as many as 26 failed to meet this criterion, but it has to be noted that 25 out of these 26 samples were harvested during 2005, as well as that all of the samples harvested during the season 2004 met the criterion laid down in the referent regulations. As regards the honey type, provisions of the regulations currently in effect were not met by 1 acacia sample harvested during the season 2003, and 14 acacia, 4 floral, 3 chestnut, 3 meadow, and 1 sage honey harvested during the season 2005. Again, the reason why is most probably honey adulteration, *i.e.* too long storage before the time of physicochemical analyses.

Average values of proline mass fraction in the analysed honey samples ranged from 191.7 mg/kg in acacia honey harvested during the season 2004, to 688.5 mg/kg in the meadow honey harvested during the season 2003. The mountain meadow and floral honey samples harvested in 2003 comprised the average proline mass fraction of 631.0 and 602.1 mg/kg, respectively. It can be noticed that multifloral honey samples host the highest amount of proline. Some researchers believe that the main source of proline in honey is the pollen, and multi-

floral honeys are known to have high pollen mass fraction (*34*). The highest proline mass fraction was measured in one sage honey sample harvested in 2003 (1020.8 mg/kg), and the lowest in the acacia honey harvested in 2004 (the mass fraction in reference was as low as 24.0 mg/kg). When it comes to sunflower and eucalyptus honey samples, Meda *et al.* (*35*), and Tsigouri and Passaloglou-Katrali (*36*) established the average values of proline mass fraction higher than ours, while those of Singh and Bath (*37*) were lower. The average value of proline mass fraction recovered from the honeydew during the course of our research (404.0 mg/kg) is substantially lower than the correspondent value obtained in the above-mentioned research, while the average results for meadow and mountain meadow honey samples (571.0 and 523.5 mg/kg, respectively) are comparable (to a certain extent) to the values obtained with multifloral honey. Additional criterion proposed by the IHC (proline content equal to or higher than 180.0 mg/kg) was not met by altogether 39 samples; 3 acacia honeys harvested in 2003, 20 acacia and 1 citrus honey harvested in 2004; and 12 acacia, 1 sage, 1 chestnut and 1 meadow honey harvested in 2005. Out of those, as many as 35 are samples of the acacia honey, which is not surprising, while acacia honey is characterised by a naturally low proline content (*21*). There is also the possibility that these samples were stored at higher temperatures (>30 °C) after processing, *i.e.* under the conditions that go in favour of the decrease of proline content (*38*).

Aqueous honey solution is optically active, *i.e.* capable of rotating the polarized light angle. Because of the higher fructose mass fraction, nectar honey rotates the polarized light angle to the left, *i.e.* it has negative optical activity. On the other hand, because of its higher oligosaccharide mass fraction (mainly melecitose and elose), honeydew rotates the polarized light angle to the right, *i.e.* it has positive optical activity. It has to be noted that neither Croatian nor international regulations define the exact values of the rotation angle concerned; however, in certain countries (Greece, Italy, UK) this rationale is employed with honey/honeydew differentiation (*39*). In this research, optical rotation angles ranged from –3.6 to –0.1 ° for nectar honey samples, and from 0.9 to 3.9 ° for honeydew samples (Tables 1–3). The widest optical rotation angle range was measured in the honeydew samples harvested in the season 2004 (0.9 to 3.9 °), and the narrowest one in the chestnut honey samples harvested during the same season (–3.3 to –2.7 °). Similar values were obtained by Přidal and Vorlová (*40*), while in the research by Marini *et al.* (*23*) the average specific rotation angles attributable to chestnut honey and honeydew were a bit wider than ours. Since all nectar honey samples had negative specific rotation angles, and all honeydew samples had positive, it can be concluded that this parameter can be used as one of the criteria for distinguishing nectar honey from honeydew.

Multivariate analysis

A study of the data structure using cluster analysis and principal component analysis (PCA) was carried out to establish whether acacia, flower, meadow and chestnut honey samples harvested during each study season constitute distinctive, well-defined groups. Because of its unsupervised nature, cluster analysis is frequently used to screen data intended to be clustered. Cluster analysis that includes all of the investigated physicochemical parameters (11 variables) in form of columns describes the overall nearness of the honey samples in the form of rows.

The Euclidean distance was used to calculate the sample similarities and as complete linkage clustering. The results obtained are shown in Figs. 1a–c in form of dendrograms. The authors regret to state that, due to the abundance of data shown, Fig. 1c might not be as apparent as the remaining graphics.

In all three study seasons, the samples were clustered in two groups. All of the acacia samples are aggregated in one cluster (on the right), at the linkage distance of around 200. The remaining meadow, floral and chestnut honey samples agglomerated in the second cluster (on the left), at the linkage distance of around 400, lacking the apparent, 'clear cut' class structure. The cluster composition indicates that the data pertinent to physicochemical parameters of the analysed honey can confer the information that might aid in distinguishing the acacia samples (monofloral honey) from the other samples (multifloral honey samples).

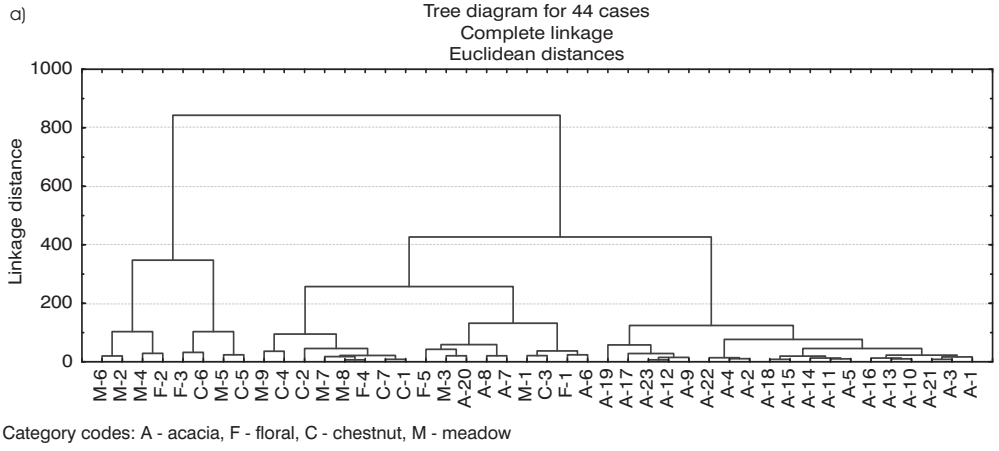

Fig. 1. Dendrogram of the cluster analysis pertinent to the three study seasons: a) season 2003, b) season 2004, c) season 2005

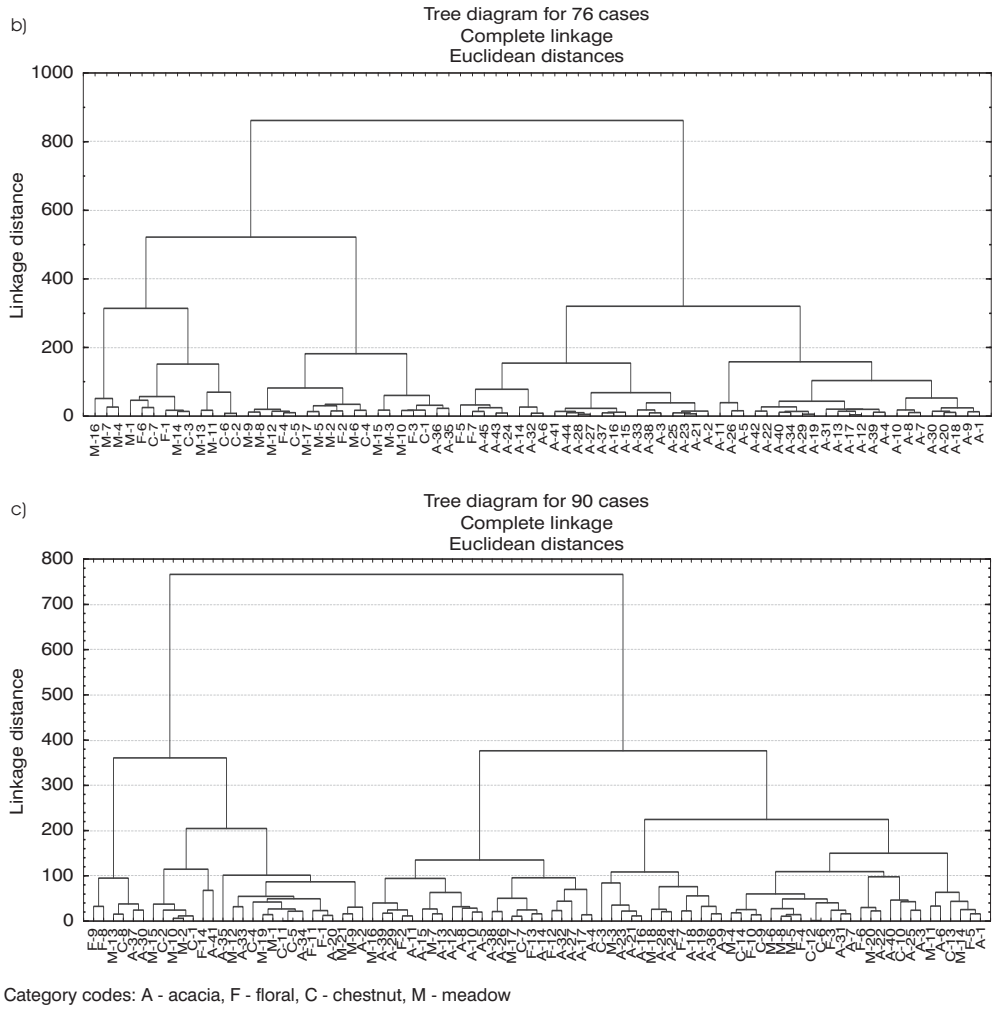

Fig. 1. Dendrogram of the cluster analysis pertinent to the three study seasons: a) season 2003, b) season 2004, c) season 2005

On the other hand, the results of the cluster analysis revealed the physicochemical parameters of the acacia honey to be different from those of other honey types. Cluster analyses of chemical data pertinent to honey analyses have been employed in several studies (*41–43*).

The data matrix containing all physicochemical parameters was subjected to the principal component analysis, separately for each season, with the goal of showing the differences between the 2 monofloral and the 2 multifloral honey types (acacia/chestnut; floral/meadow). Table 4 shows the factor-variable correlations (factor loadings) obtained for the eigenvalues of the 4 factors (PCs), and the percentage of variance and cumulative variance they account for.

The results of the PCA came out in the form of 6 graphs (projections of variables, loading plots and cases – score plots), but also in form of eigenvalues of correlation matrix, as well as factor-variable correlation (factor loadings), and case contributions, which are shown here (Figs. 2 and 3).

As for the season 2003 (Figs. 2a and 3a), the first two factors (PC1 and PC2) represent 52.39 % of the initial data variability. This result is satisfactory, however some information still might be hidden behind the next factors (the third factor, PC3, 13.38 %, and the fourth factor, PC4, 10.63 %), which are not presented herein, although the eigenvalues of the fourth factor were greater than 1 (1.17). Fig. 2a represents visually the differences between the four honey types; all of the acacia samples are positioned on the left side of the PC1, and other samples are positioned on its right side, with chestnut samples situated in the down-right, and the meadow samples in the up-right position.

The first principal component (PC1) stands for 37.18 % of the total variance, and was positively correlated with conductivity, ash mass fraction, diastase number and proline mass fraction. The second principal component (PC2) stands for 15.20 % of the variance, and was positively correlated with reducing sugar mass fraction and acidity, and negatively correlated with invertase number and sucrose mass fraction.

The third and the fourth factor (PC3 and PC4) were not presented graphically, despite the fact that they stand for the cumulative 76.40 % of the initial data variability. Physicochemical parameters, optical rotation and acidity were strongly negatively correlated with PC3, while the reducing sugar mass fraction was strongly positively correlated with PC3. HMF mass fraction was strongly

Table 4. Factor-variable correlations (factor loadings), eigenvalues, and percentages of variance and cumulative variance that can be explained by the first 4 PCs

Variable	Factor											
	Season 2003				Season 2004				Season 2005			
	PC1	PC2	PC3	PC4	PC1	PC2	PC3	PC4	PC1	PC2	PC3	PC4
Moisture	0.60	–0.37	–0.20	0.15	–0.39	0.55	0.39	0.28	0.42	–0.04	–0.71	–0.16
Conductivity	0.88	–0.19	0.23	–0.001	–0.83	0.16	–0,23	–0.34	0.84	–0.13	–0.23	–0.15
Reducing sugars	0.07	0.52	0.65	0.16	–0.30	0.56	0.10	0.09	0.41	0.69	0.39	–0.01
Sucrose	–0.44	–0.51	–0.07	–0.48	0.36	0.09	0.16	0.53	–0.39	–0.79	–0.17	0.06
Acidity	0.66	0.46	–0.45	–0.22	–0.74	–0.24	0.47	0.15	0.44	–0.13	–0.04	0.54
Ash	0.88	–0.18	0.16	0.005	–0.76	0.17	–0.31	–0.40	0.76	–0.19	–0.02	–0.30
DN	0.88	–0.13	0.10	0.02	–0.81	–0.18	–0.02	0.40	0.90	–0.16	0.18	0.01
IN	0.34	–0.70	0.14	0.25	–0.69	–0.07	–0.31	0.37	0.83	–0.17	0.17	–0.21
HMF	–0.13	0.14	–0.16	0.83	–0.01	–0.24	0.77	–0.41	–0.18	–0.01	0.29	–0.62
Proline	0.81	0.43	–0.17	–0.22	–0.86	–0.13	0.23	–0.08	0.36	0.34	–0.30	0.50
Optical rotation	0.03	–0.04	–0.80	0.18	–0.16	–0.84	–0.13	0.13	0.23	–0.46	0.68	0.37
Eigenvalue	4.090	1.673	1.472	1.169	4.096	1.569	1.318	1.150	3.683	1.543	1.428	1.253
Variance/%	37.18	15.20	13.38	10.63	37.24	14.27	11.27	10.45	33.49	14.03	12.98	11.39
Cumulative/%	37.18	52.39	65.77	76.40	37.24	51.51	63.48	73.93	33.49	47.52	60.50	71.89

positively correlated with PC4, while sucrose mass fraction exhibited a strong negative correlation with PC4.

In an attempt to determine the parameters characterising certain honey types harvested in the year 2004, another PCA was run. Again, the first two factors (PC1 and PC2) shown in Figs. 2b and 3b represent 51.51 % of the initial data variability. The first principal component (PC1) accounted for 37.24 % of the data variance, and was strongly negatively correlated with the proline mass fraction, conductivity, diastase number, ash mass fraction, acidity and invertase number. Moisture and reducing sugar mass fraction substantially contributed to the second component (PC2), which accounts for 14.27 % of the data variance, while optical rotation exhibited negative correlation with the component in question. All of the acacia samples were situated on the right side of the PC1, and can be linked to the sucrose mass fraction.

Some information about the next two factors (the third factor, PC3, 11.27 %, and the fourth factor, PC4, 10.45 %), which are not presented graphically, but stand for cumulative 73.93 % of the initial data variability, is also worth mentioning. HMF mass fraction, acidity and moisture positively correlated with PC3, while the invertase number and conductivity were negatively correlated with it. Positive correlation with PC4 was revealed for sucrose, diastase and invertase number, while the HMF, ash mass fraction and conductivity were negatively correlated with it. Ash mass fraction and conductivity were used to characterise the chestnut honey.

As for the season 2005 (Figs. 2c and 3c), the first two factors (PC1 and PC2) stand for only 47.52 % of the initial data variability. PC1 stands for 33.49 % of the data variance, and positively correlates with diastase and invertase numbers, ash mass fraction and conductivity. Sucrose and reducing sugar mass fractions offered the most remarkable negative/positive contribution to the second component (PC2), which accounts for 14.03 % of the data variance. Again, all of the acacia samples were positioned on the left side of PC1, while other types of honey were positioned on its right side, with chestnut samples situated in the up-right, and meadow samples in the down-right position of the coordinate system. Although omitted in graphics, based on their eigenvalues, PC3 and PC4 may be of importance (cumulative 71.89 % of the initial data variability). Optical rotation, reducing sugar and HMF mass fractions were all positively correlated with PC3, while moisture and proline mass fractions were negatively correlated with it. Negative correlation with PC4 was established also for the HMF and ash mass fraction, and the positive one for acidity, proline mass fraction and optical rotation.

According to the factor loading matrix (Table 4), it can also be noted that in all study seasons PC1 was basically in function of conductivity, ash and proline mass fractions, diastase and invertase numbers, while PC2 was in function of reducing sugar and sucrose mass fractions. Figs. 2a–c (three study seasons in separate) show that honey samples are split into four loading clusters, corresponding to specific honey types. All of the acacia honey samples are clearly isolated and form a condensed cluster, while, as regards other honey types, PCA yielded a fairly solid ground for their separation based on physicochemical parameters.

The classical manner of recognising the variables responsible for the formation of four clusters presented in Figs. 2a–c is the inspection of the correlation circle (Figs. 3a–c). Thus, in Figs. 3a–c HMF mass fraction is located near the origin of PC1/PC2, and has no influence on the formation of the clusters presented in Figs. 2a–c. Conductivity, ash mass fraction and diastase number account for the formation of the chestnut cluster. Although acacia honey formed a clearly condensed cluster, this could not be attributed to any of the physicochemical parameters studied.

The average cumulative variance related to the first two factors equals 50.47 %, which is, viewed on the ave-

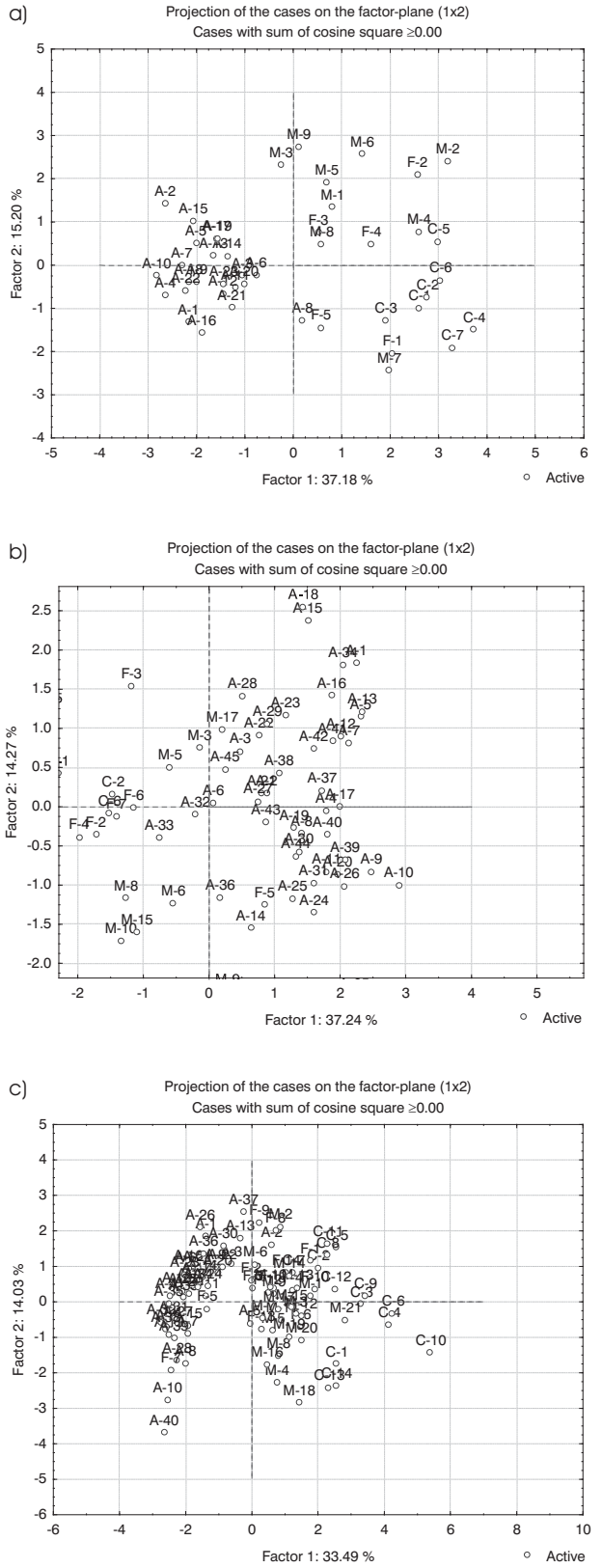

Fig. 2. Projections of the cases on the factor plane for the three study seasons: a) season 2003, b) season 2004, c) season 2005

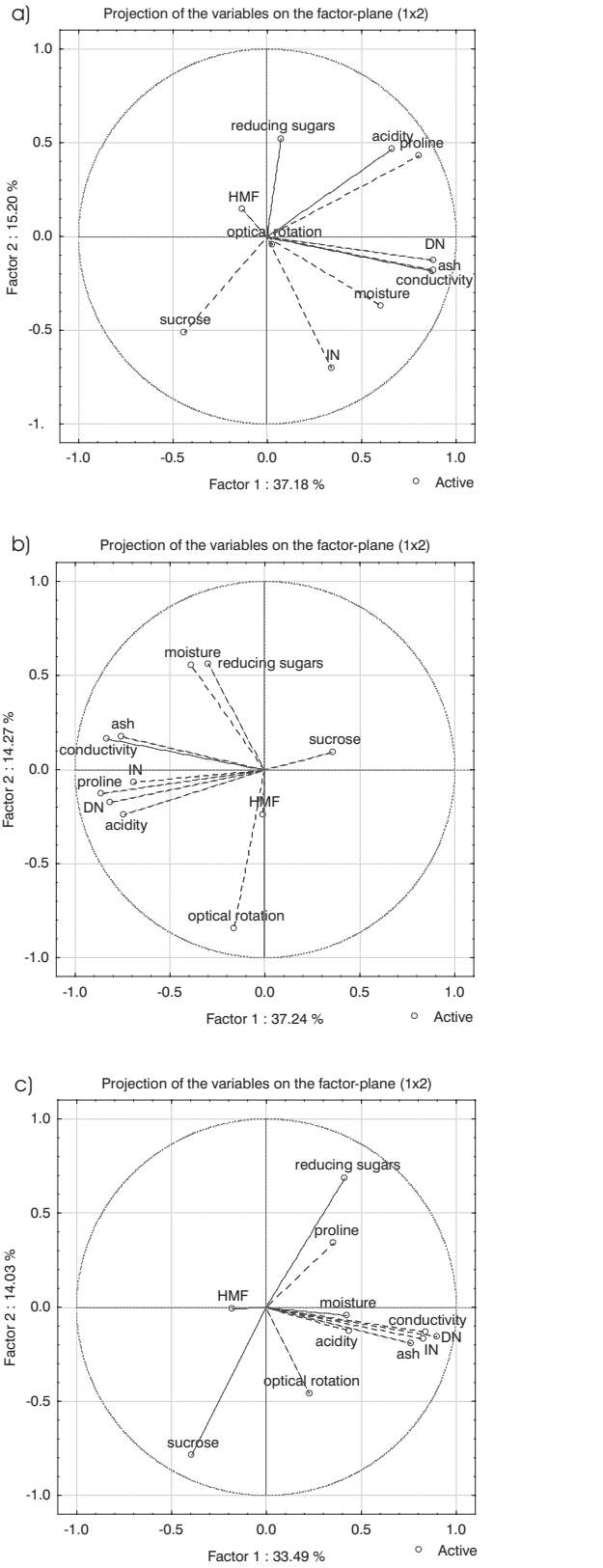

Fig. 3. Projections of the variables on the factor plane for the three study sesons: a) season 2003, b) season 2004, c) season 2005

rage, the value that is a little bit lower than those reported in the literature (*44–46*). This shows that two monofloral honey types (acacia and chestnut) can be well-distinguished based on their physicochemical properties, while multifloral honey types cannot, although some trends in this regard had been observed.

Conclusions

This research gives the complete physicochemical profile of honey samples originating from all parts of Croatia. All of the parameters under evaluation are prerequisites and tools utilised for the purpose of characterisation and differentiation of various honeys. Results are comparable, and similar (to a certain extent) to those of other researchers.

Out of the great number of honey samples studied, altogether 4.6 %, harvested during all three study seasons, failed to meet the demands imposed by Croatian regulations (*9*) and the Regulations issued by the International Honey Commission (IHC) (*8*). Ten out of eleven study parameters were taken into consideration with all elaborations, while optical rotation was not taken in because no provisions regarding this parameter have been laid down until now.

Basic and multivariate statistical evaluation confirmed the validity of physicochemical analysis as a tool to be employed with the characterisation and classification of honey samples based on their botanical origin.

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