Characterization of Romanian Honey Based on Physico-Chemical Properties and Multivariate Analysis

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Exporting 4000 - 5000 tones per year of linden, acacia, sunflower, and colza honey, Romania contributes to the European market with significant amounts of unifloral and polyfloral honey of remarkable good quality. The physico-chemical parameters of 120 samples collected in the 2011 – 2012 period were tested and chemometric techniques were used to assess variability and identify factors useful in botanical origin discrimination for the Romanian honey.

Keywords: honey, botanical origin, PCA, LDA

Honey is the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants and it consists essentially of carbohydrates, predominantly fructose and glucose, organic acids, enzymes and solid particles, mainly represented of pollen, traces of wax and variable amounts of sugar-tolerant yeast [1]. It also contains a very complex mixture of minerals, aroma compounds, flavonoids, vitamins, pigments and other phyto-chemicals [2]. In almost all honey types, fructose predominates, glucose being the second main sugar. These two account for nearly 85-95% of the honey carbohydrates [3]. The composition of honey depends on the plant species visited by the honeybees and the environmental, processing, and storing conditions [4]. As a source of energy, the beneficial characteristics of honey are the high nutritional value and the fast absorption of its carbohydrates upon consumption [5]. The progressive increase in the imported honey market, with lower prices and inferior quality, had recently led to a growing need to assess authenticity of local honeys, using a full control based on a physico-chemical, microbiological, and geographical description [6]. Honey authenticity studies are currently based on physico-chemical parameters and chemometrics to assess variability and identify factors useful in sample discrimination. The European Union issued regulations concerning the general and specific characteristics important in assessing authenticity: moisture, sugar content (fructose, glucose and sucrose), free acidity, diastase activity and hydroxymethyl furfural (HMF) content. Measurement of these parameters can provide a good information value and validate the honey quality in terms of standard regulations [7]. Multivariate statistical techniques allow identification of the natural clustering pattern and group variables on the basis of similarities between the samples, giving encouraging results in the field of food characterisation [8]. This way complexity of large data sets is reduced and a better interpretation and understanding is offered. In the last years, several chemometric techniques, such as principal component analysis (PCA) and linear discriminant analysis (LDA) were used in the field of food characterisation [2, 9-14]. PCA is a multivariate technique, permitting to reduce the dimensionality of multivariate data and provide a preview of the data structure by identifying the contribution of original variables to the definition of principal components (PC). The number of PCs generally considered is small (two, three or four); the first PC accounts for as much of the variation as possible, and the other components account successively for less. LDA approach assumes an already defined structure by allocating data samples to some groups. Discrimination relies on maximizing the between group-variance with respect to within-group variance, and define consequently new coordinates, were the data projection may reveal distinct groups.

With a very long tradition of beekeeping, Romania contributes to the European market with significant amounts of unifloral and polyfloral honey of remarkable good quality. In the recent years export production has raised to 4000 - 5000 t per year, locus, acacia and colza being the most commonly unifloral honeys produced and exported in the 2011-2012 period. Significant amounts of polyfloral honey were also produced, mostly for the internal market. However, there is little scientific research published on physico-chemical and microbiological quality of Romanian honey. In the present study, 120 acacia, linden, colza, and polyfloral honey samples, collected during 2011 - 2012 harvesting season, were processed and evaluated. Water content, HMF, sucrose, diastase activity, acidity, inverted sugars and ash were determined according to the national standards. Statistical data validation and multivariate analysis, PCA and LDA, were performed using Matlab 7.7.0 environment.

Experimental part

Samples collection

120 honey samples collected from different parts of Romania during the 2011-2012 harvesting season were provided by the beekeepers to the Beekeeping Research

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and Development Institute in Bucharest. 72 samples were unifloral honeys and 48 polyfloral. Information on the botanical origin of the samples was provided by the beekeepers and later validated by pollen spectrum. Samples were received and transferred to the laboratory in their original packages and kept at 20°C before analysis. Aliquots were homogenized by mixing with a glass rod, filtered through cheesecloth and left to stand until complete clarification, in order to eliminate the incorporated air, as recommended in SR 784-3:2009. There was no crystallized sample, therefore heating to 45°C was not necessary.

Physico-chemical analyses

Physico-chemical parameters were analyzed according to the national standard SR 784-3:2009 [15]. Hydroxymethylfurfural was determined using the Winkler method, based on the reaction with barbituric acid in the presence of *p*-toluidine. Moisture was evaluated with an Abbe Ziess refractometer equipped with temperature control. All measurements were carried out at 20°C and the samples refractive index was correlated with moisture using Chataway charts. Free acidity was determined by titration with a standardized solution of NaOH in aqueous honey solution (10 g in 50 mL distilled water). Pollen spectrum was examined microscopically, in terms of density and shape of granules. Diastase activity was determined following Gothe method, based on buffered solutions of soluble starch and 1 g of honey heated at 45°C for 1 h. Inverted sugar was quantified by the Elser method, determining the amount of reducing sugar present in the aqueous honey solution (3 g in 200 mL water) before and after acidic hydrolysis by titration with iodine standard solution. Sucrose was determined as the reducing sugar present in the sample before and after acidic hydrolysis calculated in sucrose equivalents.

Statistical analysis

In the first stage of statistical analysis, the measured data were investigated using descriptive statistic tools and one way ANOVA factor analysis. Mean, variance, and skewness were calculated for all honey types. PCA was performed for the whole data set, including polyfloral honey, considering the 6 physico-chemical characteristics investigated (HMF, acidity, diastasic index, water content, inverted sugar, and sucrose). A data matrix (6 columns and 120 rows) was defined for this analysis. Further analysis (PCA and LDA) were developed for the discrimination of unifloral honeys.

Results and discussions

Physico-chemical parameters

The summarized parameters of descriptive statistics on the physico-chemical parameters of the 120 samples are collected in table 1.

Variable	Honey type	N	Average	St. dev	Skewness	Min	Max
HMF, mg/100 g	acacia	32	0.83	0.77	1.08	0.04	2.95
	colza	10	0.14	0.14	1.19	0.00	0.40
	linden	25	0.43	0.35	1.38	0.06	1.27
	sunflower	5	0.61	0.39	-0.69	0.06	1.04
	polyfloral	48	1.20	1.11	2.90	0.12	6.58
	overall	**120**	**0.83**	**0.90**	**3.00**	**0.00**	**6.58**
Acidity, mL NaOH 1 N	acacia	32	1.44	0.26	0.61	0.95	2.10
	colza	10	1.44	0.22	2.17	1.30	2.00
	linden	25	2.18	0.63	-0.15	1.00	3.20
	sunflower	5	2.24	0.05	0.61	2.20	2.30
	polyfloral	48	2.48	0.86	0.13	0.80	4.60
	overall	**120**	**2.04**	**0.78**	**0.79**	**0.80**	**4.60**
Moisture, %	acacia	32	16.67	0.83	0.40	15.40	18.60
	colza	10	17.49	1.17	0.17	15.60	19.60
	linden	25	16.74	0.96	-0.21	14.50	18.80
	sunflower	5	17.32	0.23	-0.40	17.00	17.60
	polyfloral	48	17.07	0.85	0.62	15.40	19.10
	overall	**120**	**16.94**	**0.91**	**0.30**	**14.50**	**19.60**
Inverted sugar, %	acacia	32	73.89	1.36	0.19	71.18	77.00
	colza	10	72.20	1.21	0.40	70.58	74.12
	linden	25	73.90	1.53	-0.10	70.04	77.08
	sunflower	5	77.77	1.00	0.51	76.88	78.98
	polyfloral	48	75.18	2.98	-0.01	68.50	81.65
	overall	**120**	**74.43**	**2.42**	**0.48**	**68.50**	**81.65**
Sucrose, %	acacia	32	2.98	1.19	-0.03	1.14	4.99
	colza	10	4.23	0.65	-1.13	2.85	4.87
	linden	25	2.73	0.98	-0.12	1.11	4.73
	sunflower	5	2.02	0.41	-0.18	1.56	2.45
	polyfloral	48	2.54	0.96	0.64	1.11	4.71
	overall	**120**	**2.82**	**1.09**	**0.25**	**1.11**	**4.99**
Diastase activity, Gothe units/g	acacia	32	15.05	5.35	0.85	6.50	29.40
	colza	10	21.66	5.05	-2.63	8.30	23.80
	linden	25	25.02	4.49	-0.46	17.90	29.40
	sunflower	5	26.04	3.07	0.61	23.80	29.40
	polyfloral	48	27.52	8.54	0.90	13.90	50.00
	overall	**120**	**23.12**	**8.32**	**0.58**	**6.50**	**50.00**
Pollen	acacia	32	22.4	14.9	2.2	5	71
	colza	10	68.2	14.11	-0.4	47	84
	linden	25	46.5	12.0	0.2	26	71
	sunflower	5	54.2	8.4	-0.3	44	63

Table 1
DESCRIPTIVE STATISTICS FOR HONEY SAMPLES

The HMF content, indicative of freshness and/or overheating should not exceed 1.5 g/100 g unifloral honey for all honey types [15]. As for polyfloral honey marketed in glass containers, the maximum allowed concentration is 4 mg HMF/100 g honey, value also accepted internationally [16]. The HMF content varies in the 0 – 6.58 mg/100 g honey, with an average value of 0.83 ±0.90 mg/100 g honey. The colza samples display the lowest mean HMF of 0.14 mg/100 g. Linden, sunflower, and acacia samples show mean HMF values of 0.43, 0.61, and 0.83 mg/100 g respectively, similar to those reported for Portugal [5], and honey Hatay [8]. Two polyfloral samples presented for analysis in November 2011 and March 2012 exceeded the 4 mg HMF /100 g product limit and raised freshness questions.

More than 20 % moisture [15, 16] signals irregularities concerning the level of maturity reached in the hive, manufacturing and storage conditions, climatic conditions [2], and adulteration attempts. With an average of 16.94 %, the entire data set fulfils the quality requirements. Dispersion between honey groups is rather low, the largest value for water content, 19.60 %, being registered for a colza sample. The average moisture content is similar to the 16.88 % average reported for the lighter type Spanish honeys [17], the 16.3 % value in German and Swiss samples [18], and 16.6 % (citrus), 16.5 % (eucalyptus) and 16.9 % (wildflower) of [19]. It differs significantly from the 22.1 - 32.2 % moisture content of the polyfloral honey produced in the eastern, central, and northern Tanzania [20]. Argentinean honeys had 18.4 % water [3], while in Estonian summer honeys from ranged from 16.1 to 18.9 % [1].

The free acidity varied significantly among the four botanical types investigated. The lowest average value was determined for acacia and colza honey samples, 1.44 mL. Linden, sunflower, and polyfloral honey samples needed in average 2.18, 2.24, and 2.48 mL of NaOH, 1 N solution, to neutralize the free acidity. Two polyfloral samples, collected in August and September 2011 displayed higher acidities, signalling the debut of acetic fermentation. Except for the two polyfloral samples, the studied honey complied with the Romanian quality acidity requirements. Acacia and colza samples were more acidic (14.4 meq/kg) than the Serbian honeys values (11.20 and 13 meq/kg) [2], less acidic than the Spanish values (22.93 to 35.66 meq/kg, with the exception of rosemary honey characterised by a 15.87 meq/kg value) of Nalda [17]. The Nigerian samples [21] displayed acidity in the 22.3 - 37.5 meq/kg range, dependent on the harvesting method. The Italian samples showed acidities varying with the botanic origin from 13.3 to 38.3 meq/kg [19], the acacia values being rather similar to the studied Romanian acacia honey. Turkish honey from the Hatay region varied in the 18.06 – 34.88 meq/kg range [8], while Portugal honey from seems more acidic, 29.8 meq/kg as given in [5]. Romanian honey samples display free acidities of similar order of magnitude to other European honeys, complying with the national and European regulations [15,16].

The diastase activity for the Romanian acacia honey varies from 6.5 to 29.4 Gothe units, with an average of 15.1, and a standard deviation of 5.4. Colza samples display between 8.3 and 23.8 Gothe units, with an average of 21.6, and a standard deviation of 5.1 units. The polyfloral honey display a larger variation range, between 13.9 and 50.0 Gothe units, with a 27.5 average and a standard deviation of 8.5 Gothe units. The sunflower honeys show the lowest standard deviation, of 3.1 units. Acacia Romanian honey is similar just to the Rosemary honey from the Spanish Soria province, 14.04 units, the other lighter or darker types studied ranging from 32.23 to 49.24 Gothe units [17]. Pinus, flower, capparis, caluna, eucalyptus, and citrus honeys from province Hatay have lower enzymatic activity, 11.58 Gothe units in average [8, 21] has demonstrated that usage of heat during the traditional harvesting of Nigerian honey is accompanied by lower diastase activity.

SR EN 784/2:2009 regulates the minimum allowed inverted sugar to 70 % in the flower honey, and to 60 % in the honeydew honey. As for sucrose, the standard sets the limits to maximum 5 and 10 % in the unifloral honey and honeydew honey respectively. All verified samples fulfil the inverted sugar condition, leading to an average value of 74.43 ± 2.42 %. Two polyfloral honey sample, presented for analysis in August 2011 and March 2012, had 69.3 %, and 68.5 % inverted sugar, respectively. The largest content of inverted sugar was found in the sunflower honey samples, characterised by an average of 77.77 %. The reducing sugars in the lighter honeys harvested from the Spanish Soria province varied in the 65.54 - 68.94 % range, while the darker, dryer honeys contained more inverted sugar, 69.09 to 71.12 % [17].

Sucrose values in the studied Romanian honeys do not exceed the 5 % national limit, varying between 1.11 and 4.99 %. The sucrose average value was 2.82 ± 1.09 %, with a variation pattern dependent on the type of honey in the order:

sunflower < polyfloral < linden < acacia < colza

Statistical analysis

The data in table 1 show the specific distribution parameters, such as mean, standard deviations, and skewness of all measured variables, for each honey group and all over the 120 samples. The HMF skewness proves that the experimental values spread out more to the right compared to the mean, fact attributed to several samples with very high HMF content, as mentioned above. Colza honey has significant positive or negative skewness values for acidity, diastase activity, and sucrose. As skewness reflects the data asymmetry around the sample mean, a better visualization of outliers is given by a box plot representation, which is centred on the median value and represents the main domain defined by the 25th and 75th percentiles, with outliers plotted individually. As shown in figure 1, HMF has significant outlier values.

The influence of the botanic origin on the physico-chemical properties was studied by one-way ANOVA using as single factor the honey type (table 2). As pollen content is not determined for polyfloral honey, this was not considered in ANOVA.

The acidity and diastase activity prove to be essentially influenced by the honey types, with very low *p* values (4.4E-10 and 1.75E-11). All other properties differ to an acceptable extent in the studied honey types. Therefore botanic origin discrimination might be carried out based on this set of physico-chemical properties.

Multivariate analysis, performed to reduce the problem dimensionality and possible classification for honey botanical origin gave more complex information. As the original variables (HMF, acidity, water content, inverted sugar, sucrose and diastase activity) have different units, the standardized data matrix was used in PCA. The standardization was made by dividing each variable to its standard deviation in the whole data set. The data set considered consisted of all 120 honey samples. The first three eigenvalues are larger than 1, thus the first three PCs explain more variability in the data set than the variables

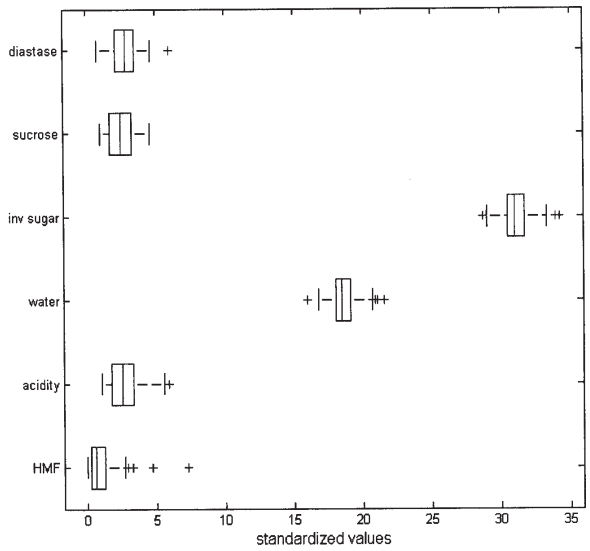

Fig. 1. Boxplot representation of experimental data

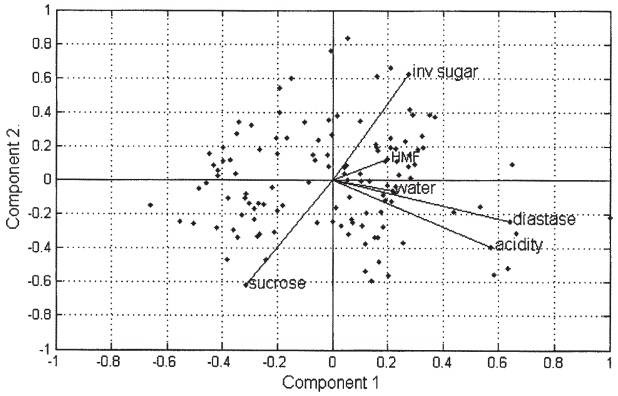

Fig. 2. Biplot representation in PCA

Variable	HMF	Acidity	Moisture	Inverted sugar	Sucrose	Diastase acidity
F	5.6217	15.4089	2.5003	7.8061	5.9261	17.9649
F_{crit}	2.4506	2.4506	2.4506	2.4506	2.4506	2.4506
p-value	3.61E-04	4.4E-10	0.0463	1.33E-05	2.34E-04	1.75E-11

Table 2
ANOVA RESULTS FOR INVESTIGATED HONEY TYPES

themselves. The first three principal components considered explain 67.38 % of the variability (PC1 25.91 %, PC2 24.54 %, and PC3 16.93 %). The loadings in the first PC have high values for acidity and diastase activity (0.573 and 0.639), signalling that these two variables account for most of the variability in the data set, conclusion in line with the factor analysis results. In terms of PC2, inverted sugar, sucrose, have significant loadings (0.623 and 0.617), standing for the assumption that these three variables are next relevant in discriminating the botanical origin of the honey samples. HMF and water content have very small loadings in PC1 and PC2, but higher loadings in PC3 (0.905 and 0.339), revealing a small contribution in samples variability. The bi-plot representation (fig. 2) simultaneously shows the variables represented as vectors and the points corresponding to all samples in the data set. The biplot allows visualisation of the magnitude and sign of each variable contribution in the first two PCs.

Sugar and inverted sugar have opposite signs loading, indicating that PC2 distinguishes between samples with low sucrose content and high inverted sugar content, and vice versa. The projection of samples in the first two principal components space is presented in figure 3. The ellipses cover about 95 % of the population of specific honey types.

As figure 3 shows, acacia and linden honeys are separated on PC1 direction, where acidity and diastase activity present the highest loadings. These two characteristics are able to differentiate between the two honey types. Colza honey is well separated on PC2 direction, so sugar and inverted sugar content distinguishes colza from the other honeys. Sunflower honey samples are practically overlapped by linden samples, but they are well separated from acacia on PC1, and from colza on PC2. The polyfloral honey samples (specific Romanian product) have a large variability of characteristics and can hardly be considered a group, being randomly distributed in figure 3. This is not unexpected since polyfloral honey has various botanic origins.

A more advanced discrimination was further considered only for unifloral types according to their predefined botanical origin by LDA. Only the physico-chemical

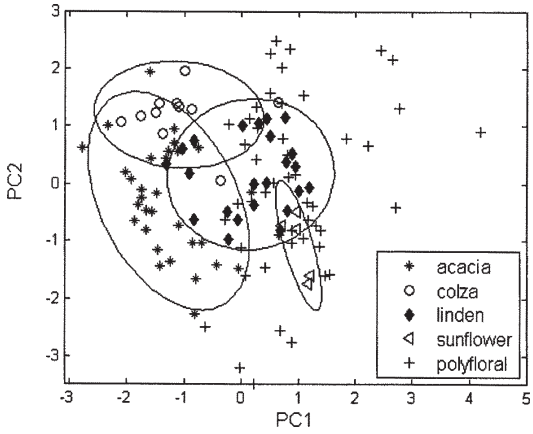

Fig. 3. Data projection (scores) in the first two PCs space

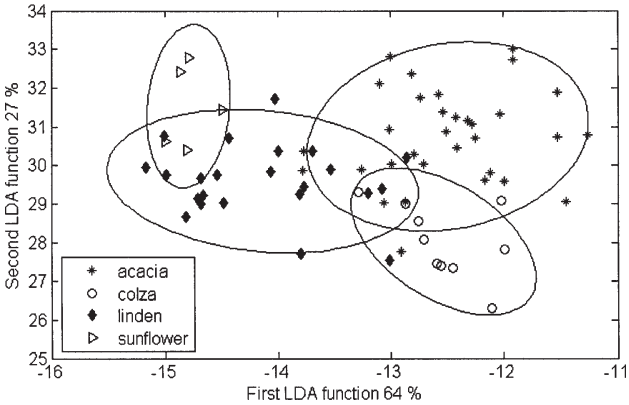

Fig. 4. Data discrimination along the first and second LDA functions

properties were used, the pollen content being overlooked. Four groups were defined: acacia (32 samples), colza (10 samples), linden (25 samples), and sunflower (5 samples). The first two LDA functions explained about 91% of the between classes variability (LDA1 64%, and LDA2 27%). The defined classes are presented in figure 4. The classification gave relatively good results: linden and acacia are separated along the direction given by the first LDA function, while colza honey samples are separated from

linden on the direction of second LDA function, and from acacia on both first and second functions.

When testing the LDA classification capability, it gave a 12.5% error. All colza and sunflower samples were correctly assigned, while 5 out of 32 acacia samples and 4 out of 25 linden samples were misclassified. The results obtained may be a basis for classifying unifloral Romanian honey only considering their physical properties, which needs further the validation by a pollen content evaluation.

Conclusions

Water content, hydroxymethyl furfural, diastase activity, acidity, inverted sugar, and sucrose were evaluated in 120 Romanian honey samples according to the national standards. The experimental data set was tested with uni- and multivariate analysis instruments to establish the chances for discriminating the botanical origin based on the compulsory quality tests for honey in the Romanian legislation. PCA explained 67 % or more of the variance with the first three PCs. The variables with higher discrimination power were acidity and diastase activity. Sucrose and inverted sugar are the next important variables in defining the variability in the data samples. LDA proved that unifloral Romania honeys samples can be discriminated based on their physical properties.

References

1. KIRS, E., PALL, R., MARTVERK, K., LAOS, K., Procedia Food Science, 1, 2011, p. 616
2. LAZAREVIĆ, K.B., ANDRIĆ, F., TRIFKOVIĆ, J., TEŠIC, Z., MILOJKOVIĆ-OPSENICA, D., Food Chem., 132, 2012, p. 2060
3. FINOLA, M.S., LASAGNO, M.C., MARIOLI, J.M., Food Chem., 100, 2007, p. 1649
4. YÜCEL, Y., & SULTANO LU, P., 2013, http://dx.doi.org /10.1016/ j.fbio.2013.02.001.
5. FEÁS, X., PIRES, J., IGLESIAS, A., ESTEVINHO, M. L., Food Chem. Toxicol., 48, 2010, p. 3462
6. ESTEVINHO, L.M., FEÁS, X., SEIJAS, J.A., VÁZQUEZ-TATO, M.P., Food Chem. Toxicol., 50, 2012, p. 258
7. DIACU, E., TANTAVEANU, L., REV. CHIM. (Bucharest), **58** , no. 12 , 2007
8. HERNÁNDEZ, O.M., FRAGA, J.M.G., JIMÉNEZ, A.I., JIMÉNEZ, F., ARIAS, J.J., Food Chem., 93, 2005, p. 449
9. MAJOR, N., MARKOVIĆ, K., KRPAN, M., ŠARIĆ, G., HRUŠKAR, M., VAHČIĆ, N., Talanta, 85, 2011, p. 569
10. ERDAL, D., EN KÖKTAS, N., BALEANU, D., Rev. Chim. (Bucharest), **60**, no. 7, 2009, p. 662
11. PLESA, C. M., HADARUGA, D. I., HADARUGA, N. G., BRANIC, A. G., ARDELEAN, A., LUPEA, A. X., Rev. Chim. (Bucharest), **62**, no. 9, 2011, p. 941
12. ANTOCE, O., NAMOLOSANU, D., Rev. Chim. (Bucharest), **62**, no. 6, 2011, p. 503
13. CHIRA, N. A., TODASCA, M. C., PAUNESCU, G., DAVID, I., IONESCU, N., STANCIU, M., ROSCA, S., Rev. Chim. (Bucharest), **62**, no. 4, 2011, p. 391
14. *** SR 784/2:2009 Honey. Part 1. Requirements for producers; Part 2: Quality requirements at sale; Part 3: Analysis methods
15. *** Council Directive 2001/110/EC and Revised Codex Standard for Honey, Codex STAN 12-1981.
16. NOZAL NALDA, M. J., BERNAL YAGÜE, J. L., DIEGO CALVA, J. C., MARTIN GOMEZ, M. T., Anal. Bioanal. Chem., 382, 2005, p. 318
17. RUOFF, K., LUGINBÜHL, W., BOGDANOV, S., BOSSET, J.O., ESTERMANN, B., ZIOLKO, T., KHERADMANDAN, S., AMAD , R., Eur. J. Tes. Technol., 225, 2007. p. 415
18. ZAPPAL , M., FALLICO, B., ARENA, E., VERZERA, A., Food Control 16, 2005, p. 272
19. GIDAMIS, A., CHIVE, B., SHAYO, N., NNKO. S., BANGU, N., Plant Food. Hum. Nutr., 59, 2004, p. 129
20. BABARINDE, G. O., BABARINDE, S.A., ADEGBOLA, D.O., AJAYEOBA, S., J. Food Sci. Technol., 48, No. 5, 2011, p. 628

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