Statistical Approach of High - performance Liquid Chromatography with Diode Array Detection Data from Romanian Propolis

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Forty-four propolis samples collected from various regions of Romania were assessed by a validated method of high performance liquid chromatography with diode array detection for quantifying twelve important flavonoids and polyphenolic acids. The chromatographic data coupled with statistical analysis revealed two distinct groups of samples: the group corresponding to a plain climate and the group corresponding to plateau and upper hills climate. In addition to other statistical studies based on qualitative data, we can mention the compounds which are responsible for discrimination between propolis groups.

Key words: Romanian propolis, flavonoids, HPLC-DAD, statistical analysis

Propolis is a resinous complex material produced by bees to protect the hive against microorganisms and other unwanted guests, or as a construction material for sealing openings and cracks within the hive. The hive is preserved sterile due to antibacterial, antifungal, antiprotozoal activities of propolis [1-3]. According to Simone-Finstrom and Spivak [4] the production of propolis by bees is a process that belongs to the phenomenon of social immunity at insects, respectively the individuals belonging to a particular group help to reduce transmission of disease and parasites in the colony.

Propolis has been used frequently in complementary healthcare, and the exploitation of this natural material in pharmaceutical and food preparations is increasing due to its large range of therapeutic properties including; antimicrobial, anti-oxidant, anti-inflammatory, immunomodulatory and anticancer activities [5-7]. Since propolis is considered a precious therapeutic and health-promoting agent and is often included into commercial product formulations for health benefits, chemical standardisation is a requirement to ensure quality and efficiency. Chemical complexity of propolis has been extensively studied and it was shown to be dependent on several variables such as climate, flora composition, and the harvest date, therefore, the geographic characteristics of the region are imprinted in propolis composition [8-10].

In Romania, the scientific research on the chemistry of locally produced propolis is until now incomplete, (only propolis from west zone Transylvania was studied) and the quantitative data are lacking. In a recent review about Romanian propolis research, Mãrghita' et al. [11] concluded that further works on increased nomber of propolis samples from all locations in Romania are needed for chemical standardization. Mainly, in propolis are analyzed flavonoids and phenolic acids using methods like: thin layer chromatography (TLC) [12], high performance liquid chromatography (HPLC) [13-17], gas chromatography (GC) [18-20], capillary electrophoresis (CE) [21-23]. Wu et al. [24] used Fourier transform infrared spectroscopy (FTIR) to analyze ethanolic extracts of Chinese propolis.

The statistical processing of chemical data resulted from analysys of complex and heterogeneous materials such propolis is important, especially when numerous samples from a defined territory are handled. Principal component analysis (PCA) and cluster analysis are usual projection methods, very useful for examining the relationships between items, looking for groups and trends, sorting out outliers. Regarding the statistical studies about propolis from Romania, Mo et al. [10] and Sârbu and Mo [25] performed statistical studies based on UV-vis reflectance spectra and TLC patterns of some propolis samples collected from a single region of Romania (Transilvania).

In this work, for the first time, 12 more important flavonoids and polyphenolic acids were quantified by high performance liquid chromatography with diode array detection (HPLC-DAD) validated method [17] in 44 ethanolic extracts of propolis samples from the entire Romania. The chromatographic data were coupled with statistical analysis to find possible geographical patterns of Romanian propolis.

Experimental part

Propolis samples

Propolis samples were collected as crude materials by beekeepers in various locations of Romania in the period 2012-2013. Figure 1 shows the collection site of each sample. Samples were stored at -20C°.

Chemicals

The polyphenolic standards were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Acetonitrile and methanol were purchased from Merck, Germany. Before the analysis the samples, solvents and mobile phase were filtered using 0.2 µm membranes (Millipore, Bedford, MA, USA). The dilutions and aqueous solution were prepared using Milli-Q water.

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Fig.1 Collection sites of propolis samples from Romania

Preparation of ethanolic propolis extracts (EEPs)

Ethanolic extracts of forty-four propolis samples were used throughout this work. Propolis samples collected by hand were finely grounded in a chilled mortar and subjected to extraction with 70 % ethanol (1:10 w/v), for 10 days, at 25°C, in dark. Afterwards, the samples were cooled and the waxes were removed by centrifugation. The samples were brought to a final volume of 50 mL and were concentrated in a rotary evaporator to obtain the crude extracts in paste form (EEPs).

Polyphenols quantification by HPLC-DAD

The assessment of phenolic compounds was performed using a liquid chromatographic system (Agilent, USA) with a diode array detector (DAD). Separation was achieved on a 150 mm x 4.6 mm, 5 µm particle, Fortis C18 (Fortis Technologies Ltd., Cheshire, United Kingdom) column at 30°C. The mobile phase consisted in a gradient based on water and phosphoric acid at *p*H 2.5 (solvent A) and acetonitrile (solvent B). The samples were eluted as follows: Step *1*: 25 to 29% B in 0.00-20.00 min; Step *2*: 29 to 45% B in 20.00-27.00 min; Step *3*: 45 to 54% B in 27.00-40.00; Step *4*: 54 to 62% B in 40.00-45.00 min; Step *5*: 62 to 25% in 45.00-60 min. Flow rates were initially 0.8 mL min^{-1}, changed to 0.7 mL min^{-1} from 10.00 min to 20.00 min, and then 0.8 mL min^{-1}. The injection volume was 10 µL, and UV spectra were recorded between 280-400 nm at a rate of 0.8 spectrum s^{-1} and 4.0 nm resolution. Data were acquired and processed using ChemStation software (Agilent, USA). The complete separation of twelve polyphenols (considered most important and abundant from a previous study was achieved within 60 min [17]. The desired compounds were identified by their retention times, UV spectra and by addition of their standards. The polyphenols were also confirmed by MS analysis (data not show).

The HPLC-DAD method was validated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), sensitivities, specificity, precision and accuracy. All calibration curves expressed good linearity ($R^2 > 0.997$) within the test range (2.5-50 µg mL^{-1}). The LODs of polyphenols ranged between 0.06-1.29 µg mL^{-1} and the LOQs were between 0.21 and 4.25µg mL^{-1}. The method has a good intra-day precision with RSD (relative standard deviation) between 0.95 % and 2.76%. For inter-day assays, the values of RSD were less than 5%. The recovery of this method was in the range 85.13–108.48% [17].

Statistical data analysis

The statistical analyses (Principal Component Analysis) used to investigate the similarities and differences of propolis samples from various regions of Romania were carried out using XLSTAT 2014 version 5.01 software. PCA can compress the data by reducing the number of dimensions without loss of information and by defining the number of principal components. Cluster analysis was used secondarily for clustering the data.

Results and discussions

HPLC-DAD polyphenols quantification

In a study regarding the polyphenol composition of propolis from different regions of world, Gardana et al. [26] found that the most abundant compounds in the samples were flavonoids: chrysin (2–4%), pinocembrin (2–4%), pinobanksin-acetate (1.6–3%) and galangin (1–2%). Similar results were reported by Volpi and Bergonzini, Valencia et al. and Kosalek et al. [27-29]. Eight major compounds, identified as flavonoids were used by Zhou et al. [30] to establish the geographical traceability of propolis from China. It has been shown that the presence of these flavonoids and their concentration are important for determining the geographical origin of the samples. Gardana et al. [26] proposed the total flavonoid contents as a quality index, suggesting that a propolis sample with less than 11% flavonoids should be considered of low quality and samples with more than 17% could be considered as high quality.

The HPLC analysis of Romanian propolis samples (fig. 2 and Table 1) showed that they contain large amounts of flavonoids, represented by quercetin (between 1.92±0.01 and 30.26±0.14 mg g^{-1}EEP), naringenin (1.86±0.02 and 29.06±0.14 mg g^{-1}EEP), kaempferol (1.21±0.05 and 21.04±0.18 mg g^{-1}EEP), chrysin (10.57±0.15 and 92.43±1.51 mg g^{-1}EEP), galangin (3.20±0.08 and 123.69±2.31, pinocembrin (8.02±0.24 and 80.26 ± 0.16 mg g^{-1} EEP) and pinostrobin (1.81±0.03 and 20.27±0.18 mg g^{-1}EEP). These results are similar, but higher than those reported by Kumazawa et al. [31] for the propolis from other countries (e.g. China, Hungary, Ukraine, Bulgaria, United States), Medana et al. [15] for propolis samples originating from Italy, Argentina, Macedonia, Ukraine and Falcão et al. for Portuguese propolis [32].

Concerning the correlations between concentrations in flavonoids and the location of the samples, it could be noticed that samples from plain zones (South and West) contain the most abundant concentrations of naringenin, chrysin, galangin, pinocembrin and pinostrobin. The samples from central zone (Transilvania and Western Moldavia) presented, generally, the highest concentrations

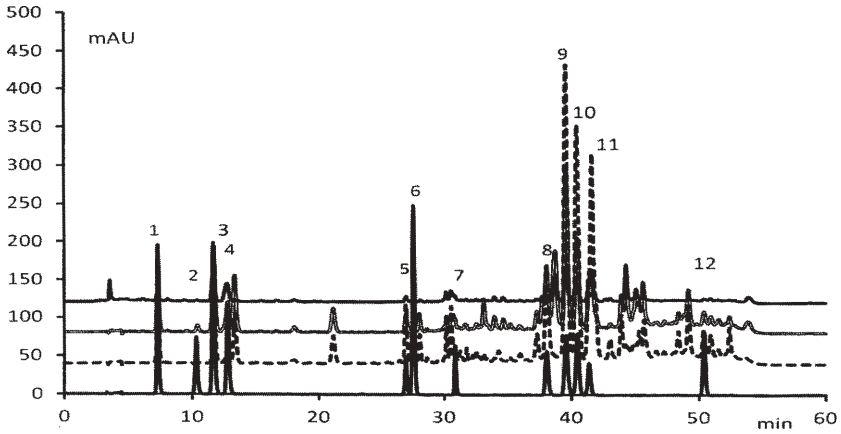

Fig.2 Chromatograms: (a) standards 1- Caffeic acid; 2 - Coumaric acid; 3 -Ferulic acid; 4 - Quercetin; 5 - Cinnamic acid; 6 - Naringenin; 7 - Kaempferol; 8 - CAPE (Caffeic acid 2-phenylethyl ester); 9 - Chrysin; 10 - Pinocembrin; 11 - Galangin; 12 - Pinostrobin; (b) Sample W42, (c) Sample E18, (d) Sample M29

Table 1

LOCATIONS CORRELATED WITH SAMPLES ID AND QUANTITATIVE DATA RESULTED FROM HPLC-DAD ANALYSIS (mg g^{-1} EEP)

Locality	County	Zone	ID	Caffeic acid	Coumaric acid	Ferulic acid	Quercetine	Naringenin	Cinnamic acid	Kaempherol	CAPE	Chrysin	Pinocembrin	Galangin	Pinostrobin
Godineşti	Mehedinţi	S	**S01**	4.89	0.00	10.29	14.90	5.45	1.18	5.81	**13.09**	**39.48**	**32.37**	**11.39**	**10.36**
Piscu Vechi	Dolj	S	**S02**	**9.21**	0.94	3.47	1.92	**26.97**	2.00	7.41	**45.40**	**55.55**	**59.44**	**42.05**	**9.45**
Craiova	Dolj	S	**S03**	**9.79**	0.00	5.61	2.90	**15.73**	2.53	3.09	**32.74**	**34.80**	**30.37**	**18.40**	**11.98**
Drăgăşani	Râmnicu-Vâlcea	S	**S04**	**12.73**	0.00	9.03	11.19	**17.29**	1.59	9.08	**29.19**	**54.37**	**40.32**	**26.08**	**8.43**
Drăgăneşti-Olt	Giurgiu	S	**S05**	**9.19**	0.94	4.80	3.19	**20.71**	2.13	4.85	**33.92**	**49.38**	**40.50**	**22.76**	**12.89**
Turnu Măgurele	Teleorman	S	**S06**	**11.42**	0.00	5.48	3.75	**21.95**	4.96	3.34	**35.18**	**53.52**	**43.19**	**96.27**	**18.03**
Alexandria	Teleorman	S	**S07**	**8.25**	0.00	3.62	4.24	**25.70**	1.88	3.67	**35.69**	**52.28**	**35.81**	**21.89**	**11.81**
Găeşti	Dâmboviţa	S	**S08**	**8.88**	1.50	8.71	7.58	**16.34**	3.30	5.99	**43.27**	**47.05**	**51.40**	**25.03**	**13.36**
Târgovişte	Dâmboviţa	S	**S09**	4.37	0.00	3.19	3.61	9.93	1.08	3.25	**20.68**	**28.29**	**25.61**	**48.76**	**5.54**
Butimanu	Dâmboviţa	S	**S10**	5.76	0.00	**12.60**	**20.94**	**13.87**	2.75	4.01	**20.58**	**40.80**	**35.37**	**20.02**	**13.92**
Băicoi	Prahova	S	**S11**	6.54	0.00	13.32	16.91	8.49	1.52	4.45	**19.44**	**32.23**	**25.40**	**14.91**	**12.81**
Plopeni	Prahova	S	**S12**	5.98	26.36	20.80	0.00	3.99	1.26	4.24	**11.84**	**55.60**	**20.41**	8.50	3.11
Berceni	Prahova	S	**S13**	**9.33**	0.94	4.23	2.55	**25.12**	2.00	4.78	**27.51**	**54.14**	**50.56**	**27.02**	**10.10**
Călăraşi	Călăraşi	SE	**SE14**	**9.00**	0.00	3.34	2.79	**21.16**	5.21	4.18	**38.90**	**43.68**	**56.17**	**24.28**	**12.63**
Gura Ialomiţei	Ialomiţa	SE	**SE15**	**9.70**	0.00	4.05	3.11	**29.06**	1.71	6.99	**53.57**	**79.44**	**71.00**	**117.01**	**13.97**
Cumpăna	Constanţa	SE	**SE16**	1.79	3.46	9.55	10.72	0.33	0.00	2.15	3.80	20.03	8.02	5.20	1.59
Constanţa	Constanţa	SE	**SE17**	**8.72**	0.00	2.49	2.26	**18.49**	1.10	4.57	**18.38**	**51.02**	**39.06**	**19.68**	7.22
Măluşteni	Vaslui	E	**E18**	5.45	0.00	4.84	6.45	16.19	1.66	3.82	**24.93**	**44.45**	**32.70**	18.84	8.10
Gura Buştii	Vaslui	E	**E19**	6.68	0.00	5.78	4.13	14.30	1.63	3.64	**26.74**	**40.17**	**32.75**	17.24	7.44
Negreşti	Vaslui	E	**E20**	1.34	2.02	3.66	4.89	1.86	0.00	1.21	2.60	11.10	6.62	6.17	0.00
Bârnova	Iaşi	E	**E21**	12.55	23.72	0.00	3.78	20.74	3.04	4.65	**50.65**	**50.59**	**44.07**	**34.49**	**20.27**
Bucecea	Botoşani	E	**E22**	4.66	0.00	11.59	**26.16**	10.96	1.72	3.89	**17.02**	**33.59**	**22.78**	**19.98**	9.73
Racovăţ	Botoşani	E	**E23**	1.99	0.00	8.64	**25.84**	0.00	1.82	2.28	1.61	10.57	9.10	3.20	2.29
Ştefan cel Mare	Piatra Neamţ	M	**M24**	2.84	7.48	**14.08**	**22.86**	3.80	1.16	**12.00**	8.02	**54.40**	26.74	6.99	6.02
Piatra Neamţ	Piatra Neamţ	M	**M25**	2.79	2.99	**16.82**	**21.51**	3.28	1.23	**18.23**	5.10	**29.45**	18.81	5.68	7.29
Bodeşti	Piatra Neamţ	M	**M26**	**15.48**	10.02	8.88	**17.76**	2.58	1.24	4.87	**27.00**	**56.98**	20.13	4.50	4.45
Bicaz	Piatra Neamţ	M	**M27**	4.08	1.06	8.00	**24.40**	3.49	1.69	3.85	13.63	23.33	22.52	28.12	16.46
Bicaz-Chei	Piatra Neamţ	M	**M28**	2.49	1.06	**15.76**	**25.33**	2.56	1.66	**10.86**	5.68	19.46	12.99	11.42	2.00
Burdujeni	Suceava	M	**M29**	3.87	4.71	9.11	**20.45**	6.10	1.46	4.70	10.82	**36.19**	20.55	10.80	7.12
Siret	Suceava	M	**M30**	2.62	1.34	7.05	11.21	5.97	1.03	5.37	6.19	16.40	13.67	**44.09**	1.91
Dealu	Harghita	C	**C31**	5.02	0.00	**14.15**	**30.26**	9.30	2.23	4.75	14.18	**34.73**	**29.79**	15.08	6.67
Corund	Harghita	C	**C32**	3.33	0.00	**12.69**	**21.18**	2.60	1.09	**14.39**	6.56	**26.93**	**17.08**	4.02	1.81
Ibănesti	Mureş	C	**C33**	8.69	2.59	9.64	7.51	8.38	1.24	4.57	**26.05**	**42.28**	**28.98**	**17.47**	**9.70**
Livezeni	Mureş	C	**C34**	3.37	5.56	**19.43**	**25.17**	1.93	1.35	4.55	**31.92**	**42.09**	**17.98**	3.83	2.48
Cluj	Cluj Napoca	C	**C35**	4.45	1.33	**20.02**	**29.70**	7.41	2.24	**16.73**	10.59	**29.85**	26.00	8.00	6.53
Gilău	Cluj	C	**C36**	4.06	1.57	**15.63**	**18.70**	5.21	1.98	**11.86**	8.70	**24.47**	16.09	**27.16**	3.80
Zalău	Sălaj	C	**C37**	6.63	1.34	**20.11**	**27.19**	5.40	1.97	**21.04**	**17.98**	**34.93**	**24.68**	**13.61**	**16.91**
Fersig	Maramureş	W	**W38**	**10.86**	0.94	9.40	6.42	**18.94**	2.63	5.02	**32.93**	**48.03**	**45.19**	**24.99**	**12.43**
Satu Mare	Satu Mare	W	**W39**	**12.87**	0.00	8.50	5.01	**19.59**	2.53	4.89	**30.66**	**57.81**	**40.77**	**27.46**	**17.02**
Oradea	Bihor	W	**W40**	**8.85**	1.61	7.32	6.00	**12.84**	2.48	5.84	**31.52**	**39.75**	**31.78**	**19.84**	**10.73**
Şimand	Arad	W	**W41**	**8.62**	0.00	7.03	5.41	**23.40**	1.22	5.98	**42.17**	**63.30**	**54.01**	**36.71**	**15.27**
Livada	Arad	W	**W42**	**11.62**	0.00	7.50	3.33	**26.89**	1.34	6.30	**43.87**	**74.85**	**64.41**	**101.73**	**8.73**
Izvin	Timiş	W	**W43**	**8.16**	0.00	3.03	2.24	**22.93**	2.09	5.68	**44.03**	**73.65**	**65.52**	**103.08**	**10.68**
Caraşova	CaraşSeverin	W	**W44**	**9.29**	1.20	5.20	7.15	**29.78**	0.00	8.55	**45.96**	**81.36**	**68.11**	**123.69**	**10.75**

S = South, Wallachia

SE = South-East Dobroudja, assimilated with southern zone (Plain zone)

E = Eastern Moldavia, east of Siret River

M = Western Moldavia, west of Siret River

C = Centre, Transylvania

W = Western Plain (Banat and Crişana)

of quercetin and kaemferol, small amounts of naringenin and mean values from the the rest of flavonoids.

The content of phenolic acids in the analyzed samples (table 1) showed that CAPE was the most abundant compound (between 2.60±0.09 and 53.57±0.09 mg g^{-1}EEP) followed by ferulic acid (from 3.19±0.1 to 20.11 ± 0.12 mg g^{-1} EEP), caffeic acid (from 1.79±0.03 to15.48±0.08 mg g^{-1} EEP), coumaric acid (0.94±0.001 and 26.36±0.12 mg g^{-1} EEP) and cinnamic acid (1.08±0.01 and 5.21±0.01 mg g^{-1} EEP). These values are similar, but higher that those reported by Pellati et al. (2013) in a study about Italian propolis [33]. The presence of CAPE in samples represents a supplementary guarantee regarding the pharmacological properties of Romanian propolis.

The highest concentrations of CAPE are found in samples from the plain zones (South and West), and the same samples contain the highest concentrations of caffeic acid.

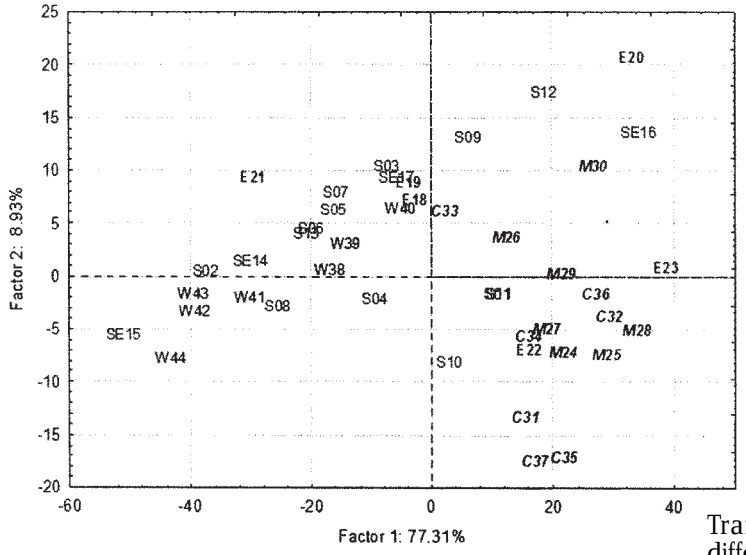

Fig.3. Principal Component (Factor) scores PC1/ PC2 plot for EEPs from different regions of Romania (obtained by processing the HPLC-DAD data. Principal Components and Classification Analysis: analysis based on covariance as SS/(N-1). Samples named according to table 1

The samples from central zones are abundant in ferulic acid. According to Murtaza et al. [34], CAPE is a very active compound effective in chemotherapy and with good properties for diminishing chemotherapy-induced toxicities. Demestre et al. [35] demonstrated that CAPE completely suppressed the growth of a human NF1 and supported the almost complete regression of human NF2 tumor.

Moldavia) presented, generally, the highest concentrations of quercetin and kaempferol, small amounts of naringenin and mean values from the rest of flavonoids.

Statistical data analysis

Chemometric analysis was performed in order to classify the propolis samples according to their geographical origin. The data used in chemometric processing were those obtained by HPLC-DAD (polyphenols composition – namely the concentration of flavonoids and phenolic acids from EEP).

Principal component analysis (PCA) was made via the covariance matrix, and it was used to establish general relationships between samples. Figure 3 shows the scores scatter plots on the two first principal components (PC1 and PC2) representing the similarities and differences among the 44 propolis samples. For the reason that galangin and chrysin presented a great variability in samples and caused perturbation in analysis they were removed from the variables. PC1 and PC2 represent more than 86% from the results range and are influenced mainly by CAPE and pinocembrin for PC1 and by quercetin for PC2.

Romania is located in southeastern Europe continent and has a temperate-continental climate. In the graphical representation of figure 3 the propolis samples presents a tendency to group according to their geographical position and climate characteristics in two main categories: the group of plain climate (South-Wallachia, South-East-Dobroudja, Eastern Moldavia and Western plain of Banat and Cri'ana), and the group of plateau climate or high hills (Transylvania and Western Moldavia) respectively. The groups are well defined with some exceptions, especially the Eastern Moldavia Group, characterized by harsh winters and arid summers, which is overlapping the central group with 2 samples from North (it is known that increasing latitude has the same effect with increasing altitude).

In cluster analysis one can find the same two clusters discussed above (data not shown).

About the comparison with other literature data, we can say that our results are in accordance with Sarbu and Mot [25] regarding the two categories of propolis from Transylvania and about the fact that is a significant difference in chemical composition of the propolis samples related with their geographical origin and local flora.

Conclusions

Forty four propolis samples from entire Romanian territory were analyzed and twelve polyphenolic compounds were quantified by a validated method of HPLC-DAD. The samples showed very good qualities, high levels for important compounds such as CAPE, quercetin, galangin, kaempferol, chrysin, naringenin and pinocembrin (high content of flavonoids).

The chromatographic data were subjected to statistical analysis and both PCA and cluster analysis showed two distinct groups of the samples: the group corresponding to a plain climate and the group corresponding to plateau and upper hills climate. In addition to other statistical studies based mainly on qualitative data, in our study we can mention the compounds which are responsible for discrimination between propolis groups. The number of propolis samples studied may be too small for generalization, but in the same time they can be a start for the authentication of propolis according to its geographical origin.

Acknowledgments: The work was supported by the National Research and Development Agency of Romania, the National Program Nucleu PN-09-360106/2009.

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Manusacript received: 9.05. 2015