ROMANIAN PROPOLIS EXTRACTS: CHARACTERIZATION AND STATISTICAL ANALYSIS AND MODELLING

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Three equal mass fractions containing fine ($d < 600$ μm), medium (600 μm $< d <$ 1.25 mm), and large ($d >$ 1.25 mm) size propolis particles were subjected to extraction using demineralized water or 1:1 ethanol:water, at 150 rpm and 25° C, ensuring a 10:1 liquid:solid ratio. Extraction duration varied between 1 and 7 days. Aqueous and ethanolic extracts were evaluated in terms of polyphenols, flavonoids, and antioxidant capacity. Absorption spectra recorded in the 200 – 500 nm domain were subjected to Principal Component Analysis, Linear Discriminant Analysis, and Partial Least Squares regression. The statistical analysis enabled samples classification, mainly based on the extractant nature, and put into evidence the possibility of linking the main properties in terms of flavonoids and polyphenols content, and the antioxidant capacity to the spectral characteristics.

Keywords: Romanian propolis, polyphenols, flavonoids, antioxidant capacity, PCA, LDA, PLS

1. Introduction

Propolis, a natural material made by bees by mixing resins of certain species of trees and plants with their wax and saliva [1], is used to create an aseptic environment in the hive, for different wall repairs, and as protection against invaders [2]. Humans have been using it as a natural remedy in different medical conditions since ancient times [3]. It has been clearly proven that propolis possesses many antibacterial, antifungal, anti-inflammatory, antiviral, hepatoprotective, antiseptic, and antiparasitic activities [4, 5].

Resin, the main raw material of propolis, representing 50% of its mass, is also one of the polyphenols sources, compounds with important antioxidant properties [6, 7]. Propolis also contains wax, volatile oils, carbohydrates, amino acids, vitamins, enzymes, minerals and vegetal impurities [8].

There has been a constant interest for separating compounds responsible for the health-beneficial effects from the rest of the matrix, without affecting their performances. Solid-liquid extractions in ethanol, methanol, mixtures of the above

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with water [9, 10], CO_2 in supercritical conditions [11], and using stirring, ultrasounds or microwaves [12] as supplemental energy providers represent several approaches to separate the targeted compounds.

The aim of this study is to evaluate the Romanian propolis composition and to identify whether mild conditions, involving environmentally friendly solvents, lead to significant results. Different operational parameters were applied and their effect on the extract composition was studied, as well as the possibility of easy evaluation from *UV-Vis* spectra of the content of biological valuable compounds in the extracts.

2. Experimental

2.1. Chemicals and equipment

Ethanol (99,8 %), Folin-Ciocalteu reagent (2 M, 1,27 g/mL), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), gallic acid (ACS standard, 95,5%), $AlCl_3$ (99,99%), quercetin (95%), Trolox (95%), 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid – ABTS – (98%), $K_2S_2O_8$ (99%), $Na_2CO_3 \times 10\ H_2O$ (99,8%) were purchased from Sigma-Aldrich (Germany). The extractions were carried out in an ES 80-Grant Instruments orbital shaker**.**

A Kern 770 Analytical Balance (Germany), having a weighing accuracy of 0.0001 g was used for weighing purposes. All solutions were prepared in class A laboratory glassware. Water was purified using a TKA demineralization system (Germany), reaching a conductivity of 18.2 $M\Omega \times cm$.

A Varian Cary 50 *UV-Vis* (USA) monofascicle spectrophotometer was used to measure sample absorbances.

2.2. Propolis samples

Propolis, collected from Bihor County (Oradea) and kindly donated by dr. Roxana Spulber, Institute for Research and Development for Beekeeping, was harvested during the March-November 2016 apiculture season. It was stored at -20° C until the analyses were performed.

The frozen propolis was weighed and grinded, and the particle size distribution was measured using a Retsch AS 200 set of sieves. All resulting fractions were clustered according to the particle size into fine ($d < 600\ \mu m$), medium ($600\ \mu m < d < 1.25$ mm) and large ($d > 1.25$ mm) particles fractions.

2.3. Extract preparation

From each of the propolis fractions, 5 distinct samples of 5 g raw propolis were weighed and mixed with 50 mL solvent, either demineralized water or a 1:1 mixture with ethanol, to ensure a 10:1 liquid to solid ratio. Extractions were carried out under constant stirring (150 rpm), and temperature (25°C), using an

orbital shaker. Samples were collected after 24, 48, 72, 120, and 168 h, respectively, separated from the waxes using Filtrak No 389, Ø 12.5 cm filter paper, and stored in the freezer until analysis.

2.4. Total phenolic content

The total polyphenols content was determined by reaction with the Folin-Ciocalteu reagent in basic medium, using gallic acid as model compound [13, 14]. Typically, after 1:100 dilution, 1 mL extract was mixed with 5 mL Folin-Ciocalteu reagent, 10 % solution. The mixture was made up to 10 mL with Na_2CO_3, 7.5 % solution, after 5 min. The absorbance at 765 nm was measured, after 60 min rest in the dark, in 1 cm quartz cell, against water.
Gallic acid working standards, in the 10 – 80 μg/mL concentration range, were used for calibration purposes. The calibration curve was characterized by a (10.8 ± 0.06) × 10^{-3} μg/mL slope, 0.022 ± 0.003 intercept, 0.9990 determination coefficient, and a 0.008 μg/mL standard error of the response. The relative standard deviations of three repeated measurements at three concentration levels did not exceed 0.9 %, while recovery varied between 98.9 and 100.4 % for the considered concentration range. The total phenolic content in the aqueous and ethanolic extracts was expressed as mass of gallic acid (mg) contained in the mass of raw propolis (g) subjected to extraction, namely Gallic Acid Equivalent – GAE. All extracts measurements were repeated three times.

2.5. Total flavonoid content

The total flavonoid content was determined by reaction with $AlCl_3$, after 30 min reaction time [15, 16]. Quercetin was used as model compound, in the 4.8 – 25.5 μg/mL concentration range. Typically, 0.5 mL standard, and 1.5 mL $AlCl_3$ 2 % solution in ethanol, were made up to 5 mL with ethanol. Absorbance at 452 nm was measured in a 10 mm quartz cell, against ethanol. The recorded absorbance values were corrected for the quercetin absorption at 452 nm. The characteristic parameters of the calibration curve, calculated by regression, were: (7.95 ± 0.14) × 10^{-2} μg/mL slope, 0.021 ± 0.009 intercept, 0.9991 determination coefficient, and a 0.023 μg/mL standard error of the response. Repeatability did not exceed 5.4 %, while recovery varied between 97.5 and 102.1 % for the used concentration range.

The flavonoids content in the aqueous extracts was evaluated as described, but when it came to analyse ethanolic extracts, reaction mixtures were diluted 1:10 with ethanol, before measuring absorbance values at 452 nm.

The total flavonoids content in the aqueous and ethanolic extracts was expressed as mass of quercetin (mg) contained in the mass of raw propolis (g) subjected to extraction, namely Quercetin Equivalent – QE. All measurements were repeated three times.

2.6. Antioxidant activity

The antioxidant activity of extracts was tested in terms of scavenging the long-lived free radical ABTS$\bullet^+$, using Vitamin E (Trolox) as model compound [17]. The free radical was obtained from ABTS, 8×10^{-3} M aqueous solution, and $K_2S_2O_8$ (2.5×10^{-3} M aqueous solution). The reaction mixture was left in the dark, at room temperature for 24 h, then diluted with water prior use until the absorbance at 734 nm in a 10 mm cell did not exceed 0.75.

1 mL aliquots of Trolox working standards in the 0.5 – 22 μg/mL concentration domain was contacted with 3 mL free radical solution, made up to 5 mL with water, and absorbance was measured after 4 min contact time, at 734 nm wavelength, in a 10 mm cell. Calibration curve parameters were: – $(2.13 \pm 0.01) \times 10^{-2}$ μg/mL slope, $(5.43 \pm 0.02) \times 10^{-1}$ intercept, 0.9984 correlation coefficient, and 0.007 μg/mL standard error of response. The bias did not exceed 3.8 %, while recovery varied between 95.5% and 101.7 % for the investigated concentration range.

Propolis extracts were diluted either 1:100 (aqueous) or 1:1000 (ethanolic) prior reacting with the solution containing the free radicals. The antioxidant capacity was determined as equivalent concentration of Trolox as determined from the calibration curve, namely Trolox Equivalent Antioxidant Capacity (TEAC), and reported in mM Trolox / g propolis.

2.7. Spectral characteristics

The absorption spectra were recorded in the 200 – 500 nm domain, after 1:100 dilution of extracts with ethanol, using a Cary 50 monofascicle spectrophotometer, in a 1 mm quartz cell, against ethanol. Spectra recording in the 500 – 900 nm was carried out with aqueous and ethanolic extracts in 10 mm cell, against the appropriate solvent.

2.8 Statistical analysis and modelling

Multivariate statistical analysis of the spectral data was carried out using Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA). Since 300 data sets of spectra in the 200 – 500 nm domain were to be analysed, PCA seemed a good instrument for variable reduction, providing unsupervised samples grouping. This algorithm transforms the observations into a set of linearly uncorrelated new variables, the principal components (PCs).

The PCs are calculated by a linear transformation of original variables (absorbances at each wavelength in the studied domain, characteristic for samples) of the form:

$$Y = W^T \cdot X \tag{1}$$

where W is a weights matrix for the linear relation, which corresponds to the eigenvectors of the covariance matrix of the initial data set (X), while the

variances of *Y* (calculated PCs) represent the eigenvalues of the covariance matrix. By this transformation the PCs encompass, in decreasing order, the variability of the original data set [18].

LDA is a supervised statistical method which assumes that the classes exist and enhances the differences between them by maximizing the *J(W)* function, defined by the ratio between classes (S_B) and within classes variances (S_W)

$$J(W) = \frac{W^T \cdot S_B \cdot W}{W^T \cdot S_w \cdot W} \tag{2}$$

which finally also reduces to the solution of an eigen-value problem:

$$S_w^{-1} \cdot S_B \cdot W = \lambda \cdot W \tag{3}$$

As this method assumes inversion of within-classes variance, impossible to be done for 300 variables (number of wavelengths considered), LDA was performed using the first 7 PCs obtained in PCA, applying the so-called PCA-LDA method. The new coordinates of data projections are the eigenvectors of the $S_w^{-1} \cdot S_B$ matrix, while the eigenvalues give the relative importance of data separation on these directions [19].

PLS regression was used as modelling tool to obtain the correlations between the spectral information and the physico-chemical properties of the extracts. PLS applies a PCA–type variable reduction and uses these new variables for the least square regression. The values of the regression coefficients are subsequently distributed over the initial variables, the wavelengths characterizing the samples, using the relation between the initial variables and the principal components. It thus allows making some assumption upon the relative importance of spectral characteristics in the correlation function of given physical properties. Unlike PCA, PLS defines the components by considering the combined covariance structures in the space of independent variables and the space of properties. PLS is the only possibility for polynomial correlation when the number of variables is larger than the number of observations, as in the case of spectral analysis [18]. The statistical data analysis was performed in the frame of Matlab15 implementation (The MathWorks, Inc., Natick, MA).

3. Results and Discussions

Propolis extraction with water or water:ethanol (1:1) was carried out using different contact times, and particle sizes. The effects of changing these operational parameters were monitored in terms of total content of polyphenols and flavonoids, antioxidant capacity in the presence of ABTS, and spectral characteristics in the 200 – 900 nm domain. The data obtained at different

extraction times allowed the analysis of the process dynamics for each specific operation condition.

Larger levels of polyphenolic compounds and flavonoids, together with higher antioxidant capacities were recorded for ethanolic extracts than the corresponding levels in aqueous extracts.

Total phenolic content measured in the ethanolic extracts from each propolis fraction after 168 h contact time varied from 86.2 to 94.5 mg GAE/g propolis with a mean of 88.9 mg/g. In the aqueous extracts the amount of phenolics varied in the 9.8 – 16.98 mg GAE/g propolis range. When processing large particles, the phenolics levels in the ethanolic extracts after 168 h were 9 times larger than those measured in the aqueous extracts.

Stoia *et al.* [20] also studied the phenolics content of Romanian propolis from Brasov county. After a 190 min extraction in methanol, at 25°C, they found out that raw propolis contained 971±79.6 mg GAE/100 g propolis, value comparable only with the polyphenols extracted in water after 168 h. The 1:1 water:ethanol mixture allowed separation of 4 to 8 times more polyphenols than the value reported by Stoia group. The result is not unexpected, since longer contact times, and constant stirring were employed in the present study. Brazilian propolis originating from Paraná contained 48 - 87 mg GAE/g extract [21], making it difficult to compare with the present study. São Paulo samples analysed by Mello *et al.* [22] fall within the 49 – 100 mg GAE/g propolis, domain comparable to that obtained in the present study, where the total polyphenols level in ethanolic mixtures after the first day of contact varied between 38 and 95 mg GAE/g propolis.

Flavonoids levels extracted in 1:1 ethanol:water varied in time, and with particles size. After 168 h they reached 3.1 - 3.3 mg QE/g raw propolis. The flavonoids found in aqueous extracts after 168 h varied in the 0.055 – 0.088 mg QE/g raw propolis range, being 47 times less than those in the ethanolic extracts.

Kosalec *et al.* [23] analysed Croatian propolis, reporting flavonoids in the 13.6 – 21.6 % range. The difference is related to the extraction conditions (crude propolis extracted with 25 mL ethanol for 24 h at 37 °C) and the model compound used for calibration purposes, namely naringenin.

Extraction of polyphenolics and flavonoids is expected to tend towards saturation concentrations in time, therefore experimental data for all three granulometric classes at varying extraction durations were correlated using the general accepted saturation model:

$$C(\tau) = C_{\max} \cdot \frac{\tau}{K + \tau} \tag{4}$$

where $C(\tau)$ is the concentration in the extract at time τ, C_{max} the maximum concentration reachable in the given experimental conditions, and K the extraction kinetic constant. The least squares sum, as in equation (5), was minimized:

$$F = \sum_{i=1}^{n} (C_{\exp,i} - C_{comp,i})^2 \quad (5)$$

where C_{exp} is the experimental value, C_{comp} the computed value of the composition in each experimental point i, and n the number of experiments. Minimization of F was carried out using the Matlab build-in function *fminunc* and quasi-Newton algorithm. Results are presented in Table 1, with Err standing for the absolute relative error between experimental and computed values.

Table 1

Solid – liquid extraction parameters

Parameter	Polyphenols					
	Ethanolic extracts			Water extracts		
	Small particles	Medium particles	Large particles	Small particles	Medium particles	Large particles
C_{max}, mg/g	92.7	90.1	89.7	15.1	6.80	Inadequate model
K, d	0.25	0.11	0.32	1.68	0.68	
Err, %	5.1	5.4	2.8	27	17	
Parameter	**Flavonoids**					
	Ethanolic extracts			Water extracts		
	Small particles	Medium particles	Large particles	Small particles	Medium particles	Large particles
C_{max}, mg/g	3.09	4.099	4.156	0.317	0.33	0.167
K, d	0.049	0.32	1.105	14.77	14.75	14.98
Err, %	3.00	12.6	17.5	26.2	27.6	37.13

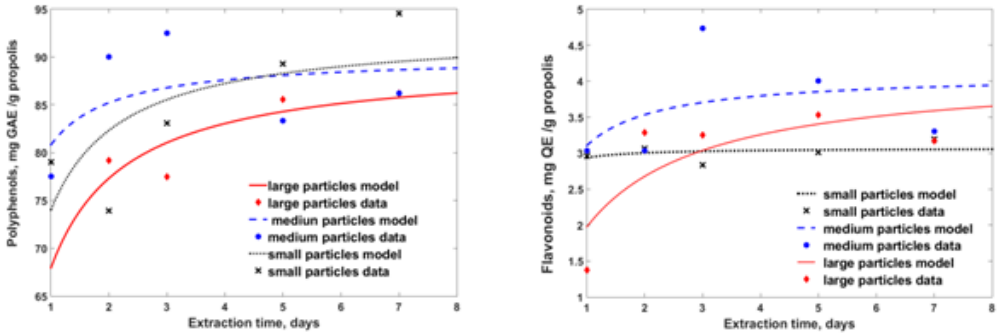


Fig. 1. Polyphenols and flavonoids in ethanolic extracts according to the saturation kinetic model.

The experimental levels of polyphenols in aqueous extracts for large particles could not be correlated with a saturation model; practically, the measured

values were defining a linear model, showing that the process was in its first stages (*Table 1*). Fig. 1 gives the estimated model predictions for ethanolic extracts as regards the total extracted polyphenols and flavonoids.

Trolox equivalent antioxidant capacity differed very much with the type of extracting agent. The ethanolic extracts showed a scavenging ability for ABTS●$^{+}$ equivalent to 5.11 – 10.7 mM Trolox/g propolis, with an average of 9.7 mM Trolox/g propolis after 7 days of extraction.

As expected, the increase in antioxidant capacity for samples corresponding to higher extraction duration follows a time limitation tendency, as this property is mainly due to polyphenols and flavonoids present in the extract (Fig. 2). Since significantly less phenolics and flavonoids were extracted in water, the antioxidant activity is correspondingly smaller with 1.51 mM Trolox/g propolis, on average for aqueous extracts after 7 days.

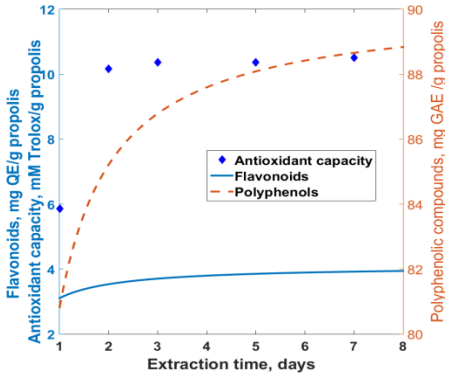


Fig. 2. Time evolution of the antioxidant capacity in ethanolic extracts of medium size propolis particles

The spectral study concentrated in the 200 – 500 nm range. There were differences in time, and with particles size (Fig. 3). Aqueous extracts presented two overlapping bands of different intensities, centred at 320 and 280 nm, together with a shoulder in the 220 – 230 nm region. The extracts yielded by the large and small particles showed only hyperchromic changes in time, with no bathochromic shifts (Fig. 3a). Medium size particles extracts display unexpected spectral changes in the 280 – 300 nm domain, with at least two visible isosbestic points (Fig. 3b). The 1:1 water:ethanol extraction medium lead to different spectral features (Fig. 3c). There are two equal intensities overlapping broad bands, centred on 290 and 330 nm, and a 230 nm shoulder. There are only hyperchromic changes in time, with no shifts to longer or shorter wavelengths.

PCA applied to the absorption spectra of all extracts in the 200 - 500 nm domain (300 wavelengths) revealed that the first 7 PCs reflect over 99.9 % of data variability. In the PC1 - PC2 space (Fig. 4) aqueous and ethanolic extracts form two different classes. The aqueous samples are less differentiated by the granulometric characteristics than the ethanol-water extracts, where the dimension of propolis particle seems to influence the extract properties reflected by the absorption spectra.

LDA was carried out using the first 7 PCs for samples characterization. It gave clearer samples separation in terms of extraction solvent and granulometric

fraction used (Fig. 5). The main differentiation is on LDA1, proving that the use of aqueous *versus* ethanolic solutions can lead to different extract properties. The three granulometric classes used for ethanolic extraction appear as separate groups, mainly on the direction of LDA2 function, proving that propolis granulation is a factor likely to influence the extract content, as also shown in Figs. 1-2.

Regression analysis by PLS was carried out separately for ethanolic and aqueous extracts for the main properties measured: polyphenols, flavonoids, and antioxidant capacity. The minimum number of PCs, reflecting over 95% of data variability, was used.

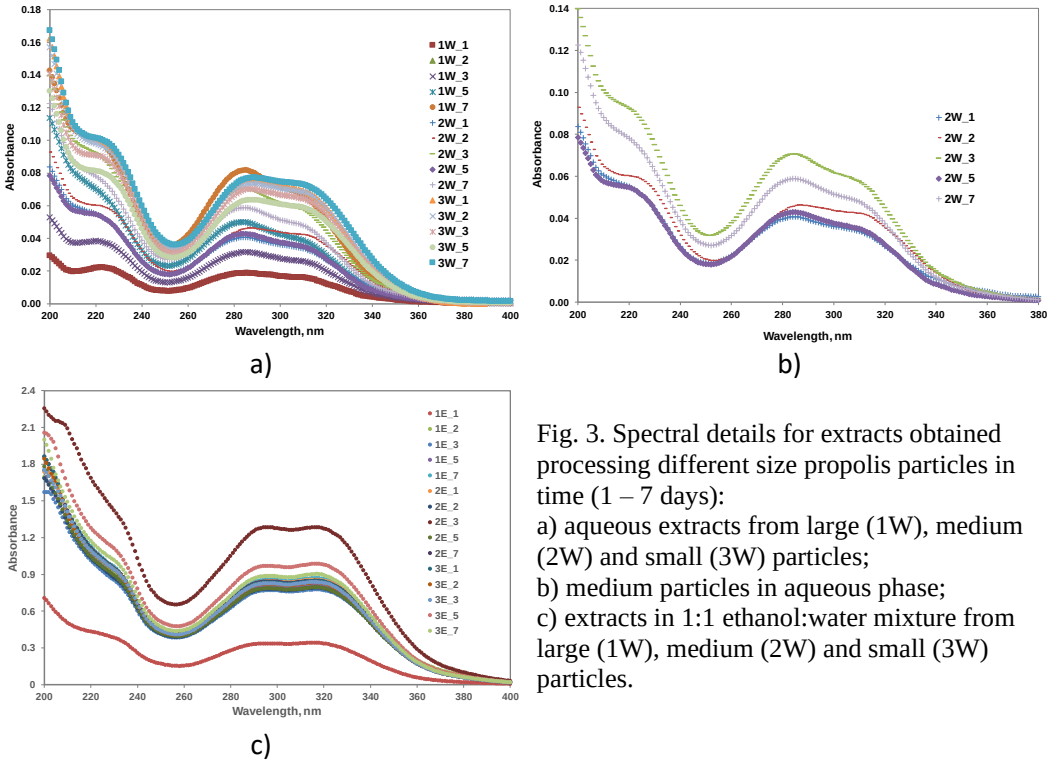


Fig. 3. Spectral details for extracts obtained processing different size propolis particles in time (1 – 7 days):
a) aqueous extracts from large (1W), medium (2W) and small (3W) particles;
b) medium particles in aqueous phase;
c) extracts in 1:1 ethanol:water mixture from large (1W), medium (2W) and small (3W) particles.

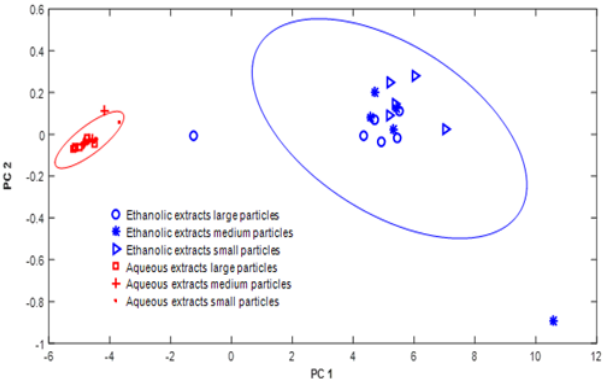


Fig. 4. Water and ethanolic extracts represented in PC1-PC2 coordinates

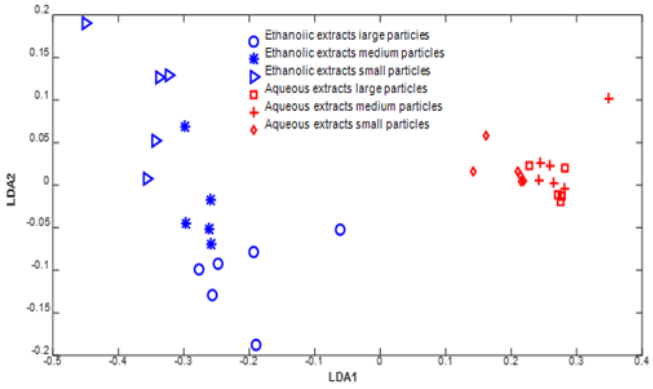


Fig. 5. Samples representation in LDA functions coordinates

In each case, available data were divided in a training set and test set, and, for each case the relative error was calculated with the relation:

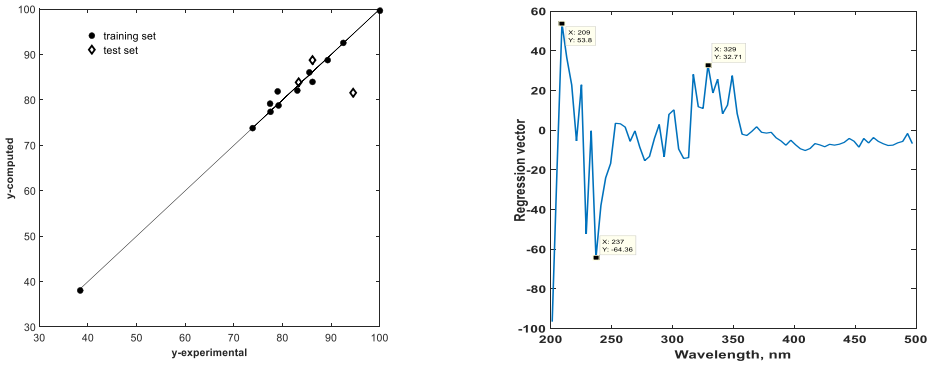
$$Err = \frac{1}{n}\sum_{i=1}^{n}\frac{abs\left(y_{\exp} - y_{comp}\right)}{y_{\exp}} * 100 \quad (4)$$

where n is the number of samples in the training and test set, respectively, and y represents the value of the property considered (experimental, y_{exp} and computed, y_{comp}). The determination coefficient, R^2, was calculated for the linear regression line obtained in the training step. Results are synthetized in *Table 2*.

Table 2

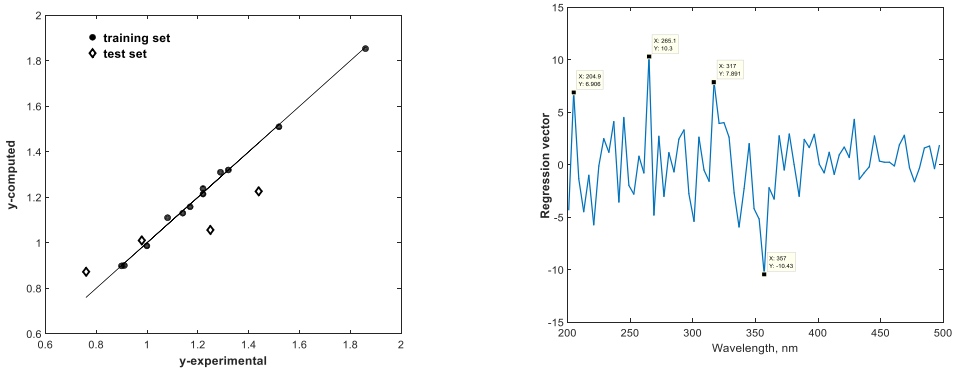
Results of the PLS regression analysis

Property	Ethanolic extracts				Aqueous extracts			
	PCs	R^2	Training error, %	Testing error, %	PCs	R^2	Training error, %	Testing error, %
Polyphenols	6	0.992	1.53	6.50	4	0.995	3.17	15.8
Flavonoids	6	0.993	1.48	8.62	5	0.993	5.08	14.2
Antioxidant capacity	7	0.978	2.00	6.64	6	0.997	0.99	12.0



a) b)

Fig. 6. Polyphenols PLS correlation results for ethanolic extracts
a) correlation data, b) relative importance of wavelengths in the correlation model



a) b)

Fig. 7. Polyphenols PLS correlation results for aqueous extracts
a) correlation data, b) relative importance of wavelengths in polyphenols correlation model

Figs. 6 and 7 present the correlation results and relative importance of original variables (the wavelengths) in the build-up of the regression model. As Figs. 6b and 7b point out, the main contribution for establishing a correlation between the spectral data and polyphenols content are represented by wavelength around 220 nm, 250 nm, and 320 nm.

6. Conclusions

Three granulometric fractions of propolis originating from the Bihor county in Romania were subjected to solid – liquid extraction in demineralized water and 50 % (vol) ethanol to verify how the particle size, time, and solvent influence the composition of the collected extracts as regards the polyphenols, flavonoids, and antioxidant capacity. The ethanol:water 1:1 mixture was more

efficient in extracting polyphenols and flavonoids, the extracted amounts being less affected by the size of the propolis particles. By itself, water was able to extract less beneficial compounds from the complex natural mixture, and the levels extracted were inversely proportional with the particles' dimensions. The time evolution of the extracts allowed the acceptance of a saturation-type process model. The model parameters gave very long contact times for a complete extraction, therefore different types of process intensification techniques should be applied, like microwaves, ultrasound or supercritical fluid extraction.

The multivariate statistical analysis proved its efficiency in data analysis and gave new possibilities to establish good extraction conditions for valuable components. Spectral properties in the *UV–Vis* region allowed preliminary classification of the extracts according to the solvent nature, by PCA analysis. Better discrimination was obtained when applying a PC-LDA routine. PLS regression could correlate spectral information with the evaluated physico-chemical properties. As spectral investigation is very rapid, straightforward and cheap, it becomes, in combination with PLS, a good candidate for the quick on-site evaluation of new propolis samples. The direct correlation of the spectral characteristics with the antioxidant capacity, a property given by a complex composition, stands for recommending the usage of *UV–Vis* spectra for the rapid evaluation of propolis extracts.

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