

Propolis extract/ β -cyclodextrin nanoparticles: synthesis, physico-chemical, and multivariate analyses

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Abstract

The paper presents a study on the propolis extraction, analysis, nanoencapsulation in β cyclodextrin, and antioxidative activity of both the extracts and micro/nanoparticles. Propolis extraction was realized in various ethanol-water solutions, at room temperature or up to ethanol reflux, and the extracts were analyzed by RP-HPLC. The bioactive compounds identified and quantified in extracts and furthermore in propolis were flavonoids (rutin, quercetin, apigenin, kaempferol, acacetin, chrysin, pinocembrine) and cinnamic acid derivatives (caffeic acid), all containing phenolic hydroxyl groups which confer antioxidative capacity. These extracts were used to obtain micro/nanoparticles by encapsulation in β -cyclodextrin (solution method) and the complexes were analyzed by SEM in order to evaluate the morphology and the dimensions of crystals, and by TG-DTG for the evaluation of the encapsulated biocompounds. Both extract and nanoparticle samples were evaluated from the antioxidative activity point of view (DPPH method), the complex revealing controlled releasing properties.

Keywords: propolis, flavonoids, β -cyclodextrin, micro and nanoparticles, encapsulation, thermogravimetry, scanning electron microscopy, antioxidant activity

1. Introduction

Propolis is a brownish, waxy product collected from buds of certain trees and exudates of the plants by bees and used by them to cement or caulk their hives. Even this product is known from ancient times, many studies in the chemical, biological and pharmacological fields are realized in the last period due to its medicinal properties [1-3].

The ethnopharmacological approach, combined with chemical and biological methods, may provide useful pharmacological leads [1].

Propolis contains more than 300 compounds and its composition depend upon the source plant and local flora. Due to this fact, the "standardization" of propolis is very difficult.

The main components of propolis are resins (benzoic and cinnamic acid derivatives and flavonoids), waxes and fatty acids, essential oils, pollen, and minerals. It is well known that the flavonoids are principally responsible for the antibacterial activity of propolis, but also for the anticancerigene and immunomodulating activity [1,3].

Due to the susceptibility of some compounds from propolis to the oxidation and reduction of the biological activity, it is indicated to protect the labile components, like flavonoids and phenolic compounds [4,5]. A convenient way to realize this desiderat is the encapsulation of propolis extracts in various biocompatible matrices, like cyclodextrins, which are cyclic oligosaccharides containing 6-8 glucopyranose units for naturally occurring α , β and γ -cyclodextrin [6-8]. They have inner hydrophobic cavities at nanometers dimensions, and many outer hydroxylic groups which confer the possibility of complexation (molecular encapsulation) of geometrically compatible more hydrophobic biomolecules in order to protect them against environmental and biological degradation factors (oxygen, humidity, light), controlled release of the bioactive compounds at the biologic target and obtaining powdery, easy handling, water soluble, and bioavailable pharmaceutical or alimentary products [6-10].

The aim of this study is to continue the series of studies of our bionanomaterials team on nanoencapsulation of biocompounds in natural matrices [11-16] with the extraction and analysis of bioactive compounds from propolis from the west side of Romania and the nanoencapsulation of them in β -cyclodextrin, evaluation of the antioxidant activity of this micro/nanoparticles in comparison with the non-encapsulated extracts, and multivariate analysis of the data obtained in order to optimize the synthesis process and obtain products with valuable properties.

2. Materials and methods

Materials

Propolis from the west side of Romania was collected in 2006 (three samples P1, P2, and P3, from Banat county). *Ethanol 96% (v/v)* used for solid-liquid extractions of flavonoids from propolis and for nanoencapsulation was purchased from Chimopar București (reagent grade). For the HPLC analysis of extracts *acetonitrile* (HPLC grade, Fluka Chemie AG) and bidistilled water were used. For the quantification of flavonoids from extracts by HPLC *rutin* (>90%, Fluka), *cinnamic acid*, *caffeic acid* (>98%, Sigma-Aldrich), *quercetin*, *apigenin*, *kaempferol*, *acacetin*, *chrysin*, and *pinocembrine* (>99%, Sigma-Aldrich) were used as etalon compounds. β -Cyclodextrin used for encapsulation was obtained from Merck&Co., Inc., New Jersey (>99% purity). *DPPH (1,1-diphenyl-2-picrylhydrazyl)* used for antioxidative activity evaluation of propolis extracts and complexes was purchased from Fluka Chemie AG (>99%).

Obtaining the propolis extracts

The propolis extracts used for nanoencapsulation were obtained by solid-liquid extraction in 100 ml flask (1 g propolis-P1, P2, P3 and 20 ml EtOH20%, 60%, or 96%) equipped with reflux condenser (one hour at reflux or four days at room temperature in sealed flask). After extraction, the ethanolic solution is filtered and washed with 1 ml ethanol (at the same concentration like this used for extraction). These extracts were used for β -cyclodextrin micro/nanoencapsulation.

RP-HPLC analysis of extracts and standards

The reversed phase-high pressure liquid chromatography analyses (RP-HPLC) of the extracts and standard flavonoid solutions was performed on an Agilent 1100 HPLC apparatus using the following conditions: Zorbax SB-C18 column, 250 mm length, 4.6 mm i.d. and 5 μ m particle diameter, wavelength 337 nm, acetonitrile:water mobile phase at 48:52 ratio, temperature 25°C, flow 0.3 ml/min, sample volume 20 μ l.

Micro/nanoencapsulation of propolis extracts

The nanoencapsulation of the propolis extracts with β -cyclodextrin was performed by the solution method: 0.1 mmole of β -cyclodextrin (~1.34 mg) was dissolved in 6-8 ml distilled water at $50 \pm 1^\circ\text{C}$ in a thermocontrolled minireactor, equipped with reflux condenser, and then 4 ml ethanolic extract (the weight corresponding to main flavonoids : β CD molar ratio of ~1 : 1) were slowly added under continuous stirring. The solution was then stirred another 30 minutes and slowly cooled at 20°C in about 4 h. The crystallization was perfected in refrigerator at 4°C for 12 h. The complex was filtered, washed with ~1 ml 96% ethanol and dried in exicator. The filtrate was maintained again at the refrigerator for another 12 h, filtered, washed and dried. The yield of micro/nanoencapsulation was obtained using both precipitates.

Scanning electron microscopy (SEM) of the micro/nanoparticles

In order to evaluate the morphology and the approximative dimensions of the crystals the scanning electron microscopy (SEM) using a JEOL JSM 5510-LV apparatus, was performed.

Thermogravimetry (TG-DTG) analysis of micro/nanoparticles

A TG 209 NETZSCH thermogravimetric apparatus was used for the thermal analysis of the propolis extract/ β -cyclodextrin micro/nanoparticles. The temperature program was 20 to 550°C with $10^\circ\text{C}/\text{min}$. All determinations were conducted under nitrogen atmosphere. Data acquisition was performed with the TG Netzsch 209-Acquisition Soft/2000 and the data analysis was realized with the Netzsch Proteus-Thermal Analysis ver. 4.0/2000 soft.

Spectrophotometric analysis and antioxidant activity evaluation of extracts and micro/nanoparticles

Propolis extracts and micro/nanoparticles were analyzed from the antioxidative point of view using DPPH method. The reaction of DPPH was spectrophotometrically measured for 180 s at 517 nm on a CamSpec M 501 spectrophotometer, and the data were analyzed using the UV-Vis Analyst v. 4.67 acquisition&data handling soft. The crude propolis extract and 1% aqueous solution of the complex were mixed with 1 mM DPPH ethanolic solution and the decrease of the absorbance at 517 nm was measured.

3. Results and discussion

Standard flavonoids and propolis extracts analysis

For the quantification of bioactive compounds from propolis (flavonoids and cinnamic acid derivatives) standard solutions with known concenytrations were obtained (rutin, quercetin, apigenin, kaempferol, acacetin, chrysin, pinocembrine, cinnamic acid, and caffeic acid). For *rutin*, many peaks appear in the HPLC chromatogram, probably due to the partial degradation of the saccharide moiety (in the range of 6.7-7 min). The calibration curve for rutin was:

$$A_{\text{rutin}} = 365.8(\pm 47.4) \cdot c$$

In the case of *cinnamic* and *caffeic acids* none unitary peaks were obtained, but the sum of peaks conduct to the calibration curve:

$$A_{\text{acid caffeic}} = 2185.9(\pm 46.7) \cdot c$$

For the *quercetin*, two main peaks appear in the HPLC chromatogram at 12.5 and 13.1 min, which probably appear due to the enolic and ketonic tautomeric forms of the rutin aglicon. The cummulated standard curve for quercetin was:

$$A_{1 \& 2_{\text{quercetina}}} = 815.2(\pm 45.7) \cdot c$$

For *apigenin* (figure 1) and *kaempferol* unitary peaks were obtained by HPLC analysis, at 15.4 and 16.8 min, respectively, as well as for *acacetin* and *chrysin* (34.0 and 34.1, respectively). The calibration curves for these flavonoids were:

$$A_{apigenina} = 1284.2(\pm 72.7) \cdot c$$

$$A_{kaempferol} = 1612.7(\pm 49.0) \cdot c$$

$$A_{acacetina} = 1976.3(\pm 91.8) \cdot c$$

$$A_{crisina} = 766.0(\pm 26.7) \cdot c$$

Pinoembrine was separated by HPLC at 36.3 min and the calibration curve for this flavonoidic compound was:

$$A_{pinoembrina} = 348.4(\pm 8.5) \cdot c$$

According to these calibration curves and using the HPLC analysis for propolis extracts (triplicate samples), the concentration of these flavonoids and cinnamic acid derivatives could be evaluated (table 1). For example, in the case of extracts obtained at higher temperature, using water as solvent for extraction, rutin (0.3 mg/g propolis), caffeic acid (0.1 mg/g), and quercetin (0.13 mg/g) were identified by HPLC analysis (figure 2).

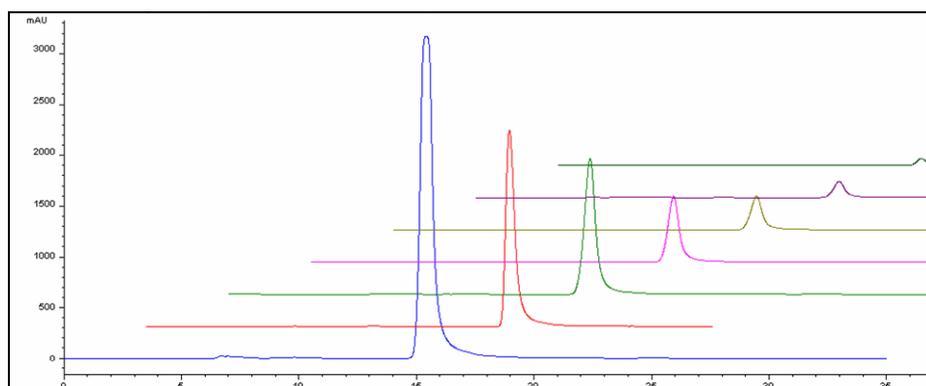


Figure 1. The superimposed HPLC chromatograms for apigenin standard solutions

Table 1. Identification and quantification of the bioactive compounds from propolis

No	Code	Rutin (mg/g)	Caffeic acid (mg/g)	Quercetin (mg/g)	Apigenin (mg/g)	Kaempferol (mg/g)	Chrysin (mg/g)
1	P1_20	1.59 ± 0.51	3.03 ± 2.98	0.63 ± 0.44	0.43 ± 0.3	0.17 ± 0.04	0.87 ± 0.21
2	P1_60	-	2.8 ± 2.3	1.7 ± 0.7	5.3 ± 2.7	2.1 ± 1.3	26 ± 11.9
3	P1_96	8.18 ± 2.82	2.45 ± 1.27	4.2 ± 3.9	5.0 ± 2.9	2.04 ± 0.8	19.8 ± 9.4
4	P2_20	0.6 ± 0.19	1.9 ± 0.58	0.74 ± 0.51	-	-	0.44 ± 0.38
5	P2_60	4.7	5.6 ± 3.5	3.1 ± 2.6	4.5 ± 3.2	3.2 ± 1.98	22.3 ± 14.1
6	P2_96	6.8	3.8 ± 1.6	1.7 ± 1.2	3.7 ± 1.8	1.44 ± 1.0	7.05
7	P3_20	1.78 ± 0.13	-	0.9 ± 0.77	0.35 ± 0.02	-	0.33
8	P3_60	4.6 ± 2.3	11.4 ± 10.1	8.8 ± 4.2	7.0 ± 3.4	-	26.0 ± 11.6
9	P3_96	16.7	4.1	7.0 ± 2.0	2.08	1.6	22.8 ± 13.9

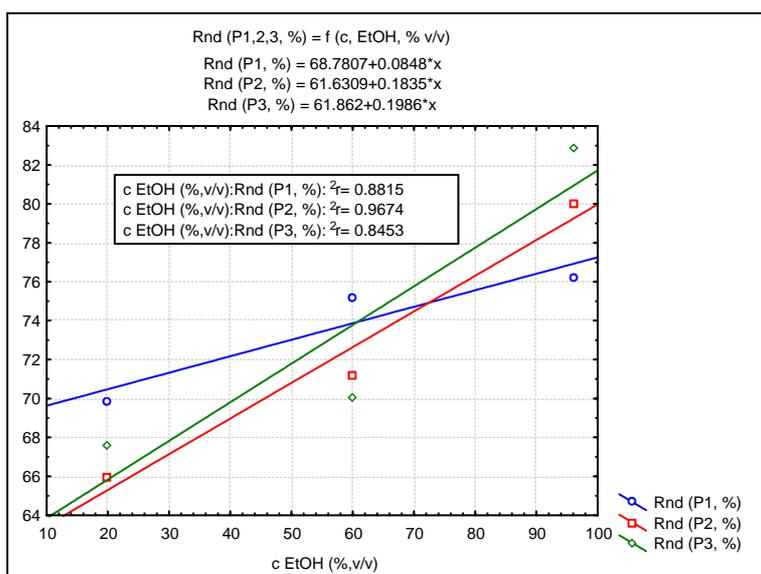


Figure 4. Yield vs ethanol concentration for the complexation of propolis extracts in β -cyclodextrin

For the evaluation of the morphology of the propolis extracts/ β -cyclodextrin crystals the optical microscopy and further the scanning electron microscopy analyses were used. Interestingly, the crystals

corresponding to the 20% EtOH extracts have parallelepipedic forms, and those obtained using 60% and 96% EtOH as solvent for extraction have romboidal forms (figure 5).

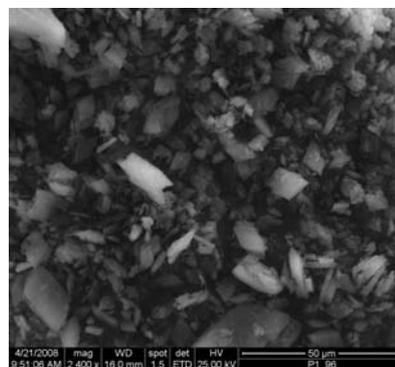
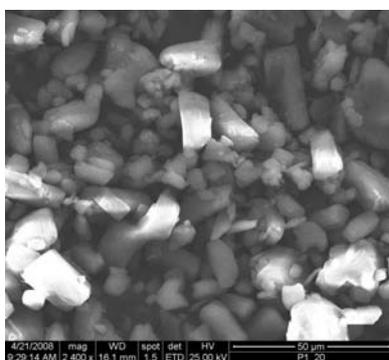


Figure 5. SEM micrographs for the 20% EtOH (left) and 96% EtOH (right) propolis P1 extracts/ β -cyclodextrin micro/nanoparticles (2400 \times magnitude)

It can be observed that the dimensions of crystals in the case of 20% EtOH extract complex are in the range of hundred of nanometers to micrometers, while in the case of 96% EtOH these are much smaller

(submicronic dimensions). For example, the main dimensions for a medium crystal from the 20% EtOH P3 extract/ β -cyclodextrin complex, are (figure 6):

$$L \times l \times h = 91 \times 47 \times 24 \text{ } \mu\text{m}^3$$

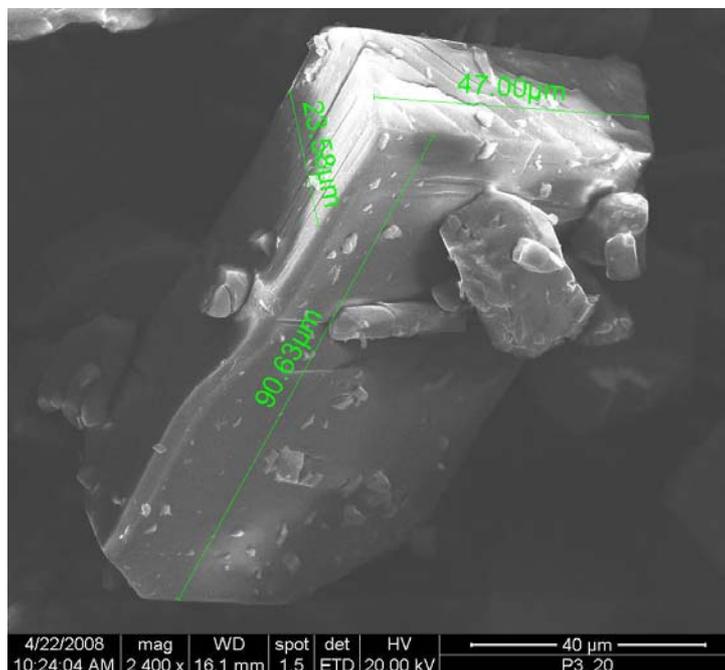


Figure 6. SEM micrograph for a crystal from 20% EtOH propolis P3 extract/ β -cyclodextrin complex

From the thermogravimetric analysis (TG-DTG) a significant difference between the TG profiles of the propolis extracts/ β -cyclodextrin micro/nanoparticles and pure β -cyclodextrin can be observed (figure 7). In the case of P1 propolis extracts/ β -cyclodextrin nanoparticles the TG-DTG analysis revealed that the mass loss was 5.2-9.4% in the range 20-100°C, and 1.6%, 2.9%, and 2.1% for the range of 100-225°C in the case of 20%, 60%, and 96% EtOH P1 extract nanoparticles. The optimum ethanolic concentration seems to be 60%, where the concentration of encapsulated flavonoids was close to 3% (figure 8).

In the case of P2 propolis extract, the TG-DTG analysis in the range of 100-225°C is similar to that of P1 extract, the mass loss for the 20% EtOH extract complex was 1.5%, comparatively with the other two cases, where this mass loss was almost identical (2.2%). In the case of P3 propolis extract, the concentrations of encapsulated flavonoids were relatively different comparatively with the first two cases: 2.4%, 1.7%, and 2.2% for the 20%, 60%, and 96% EtOH propolis P3 extract complexes, respectively (in the range of 100-225°C).

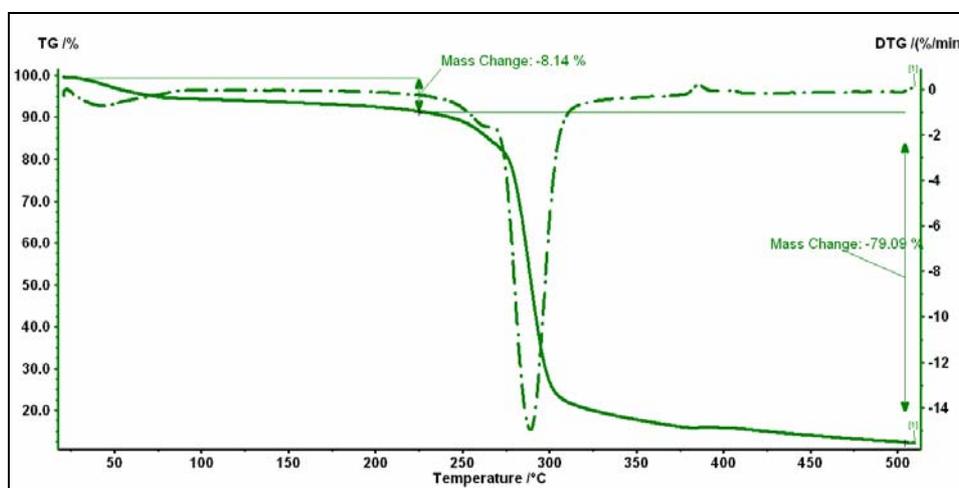
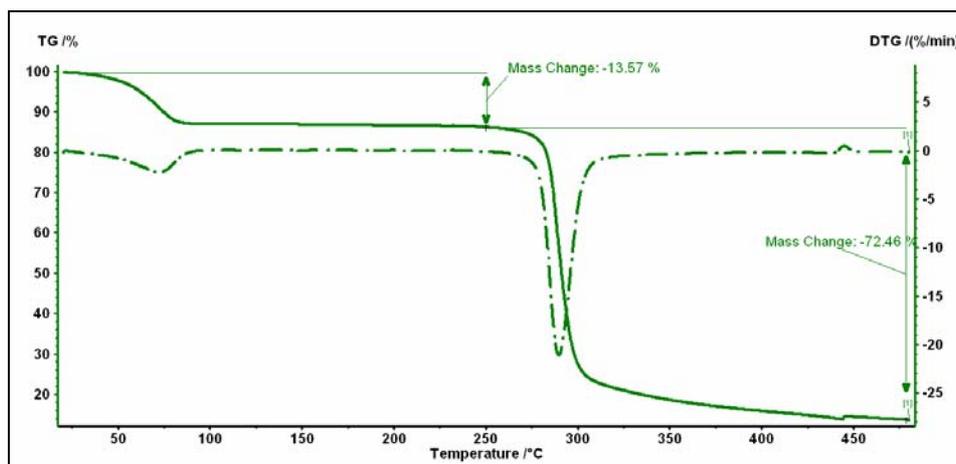


Figure 7. TG-DTG analysis of the pure β -cyclodextrin (up) and 60% EtOH P1 extract/ β -cyclodextrin micro/nanoparticles (down) (the range of 20-520°C)

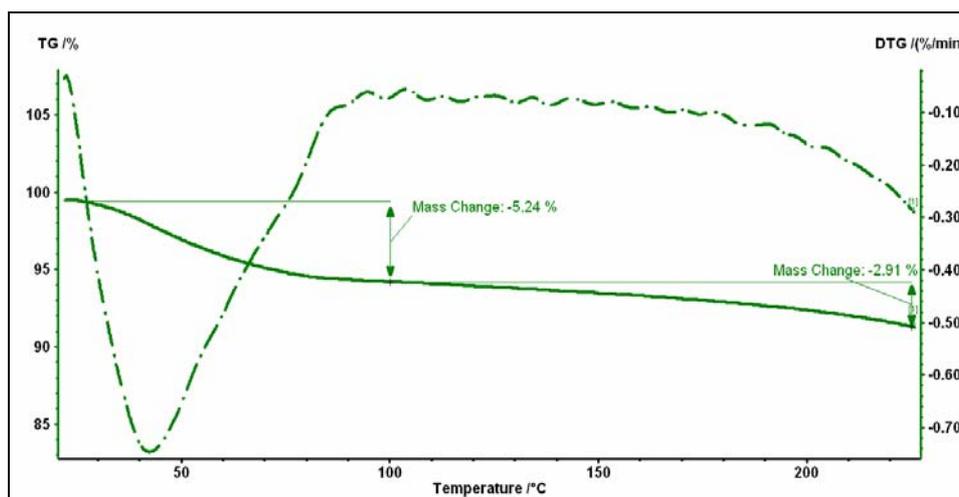


Figure 8. TG-DTG analysis of the 60% EtOH P1 extract/ β -cyclodextrin micro/nanoparticles (the range of 20-225°C)

Multivariate analysis of the yields and TG-DTG data obtained for these micro/nanoparticles containing propolis bioactive compounds indicates a good classification in two classes: nanoparticles containing diluted EtOH extracts and concentrated EtOH extracts. Thus, the principal component analysis of these data classifies the samples in two main groups: 20% EtOH propolis extract nanoparticles – P*bCD_20, and 60% or 96% EtOH

propolis extract nanoparticles – P*bCD_60, P*bCD_96. The explained variance for the first principal component was 59%, for the second 31%, and for the third 9% (the first two principal components explain 90% from the data variance) (figure 9). The variables responsible for this classifications are especially the mass loss in the range of 225-520°C and the complexation yield (figure 10).

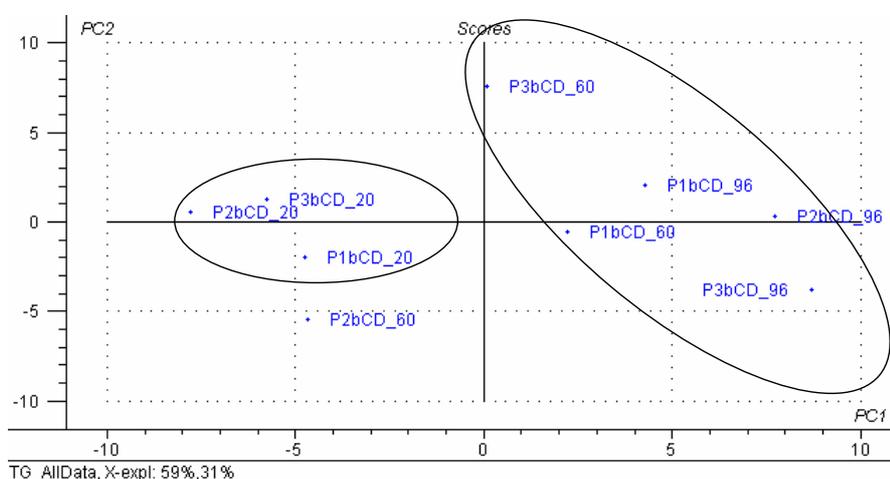


Figure 9. Scores from the PCA analysis of the obtaining and analysis of the propolis extracts/ β -cyclodextrin micro/nanoparticles (yields and TG data)

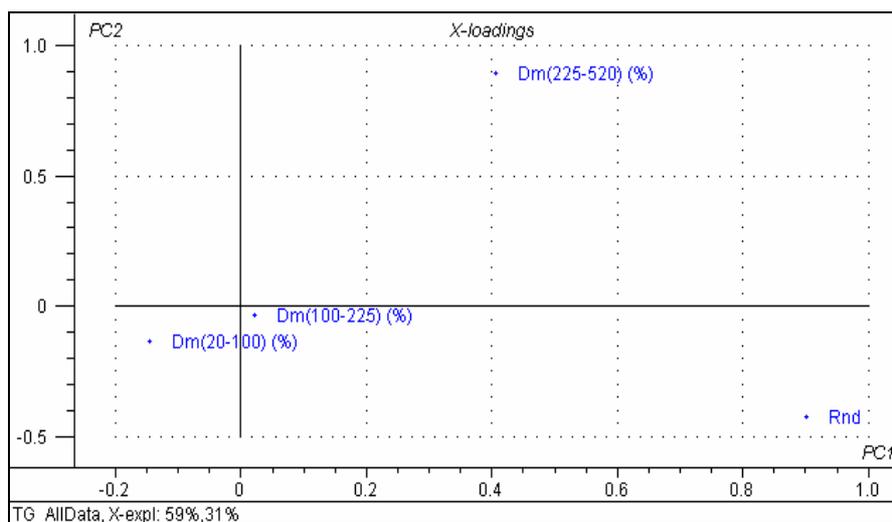


Figure 10. Loadings from the PCA analysis of the obtaining and analysis of the propolis extracts/ β -cyclodextrin micro/nanoparticles (yields and TG data)

In the case of the multivariate analysis of the TG data only, the explained variance for the first two principal components was 98% (80% for PC₁ and 18% for PC₂), and better classifications were obtained (figure 11). The antioxidant activity of the propolis extracts and for the micro/nanoparticles was evaluated using the DPPH method. A detailed study on this subject will be presented in a further publication, but it can

be seen that the propolis extract have excellent antioxidative properties (remnant relative absorbance <10%), with a relatively higher rate of reaction, comparatively with the case of micro/nanoparticles, which have a lower rate of DPPH consumption, probably due to the lower release of the flavonoids from β -cyclodextrin cavity (figure 12).

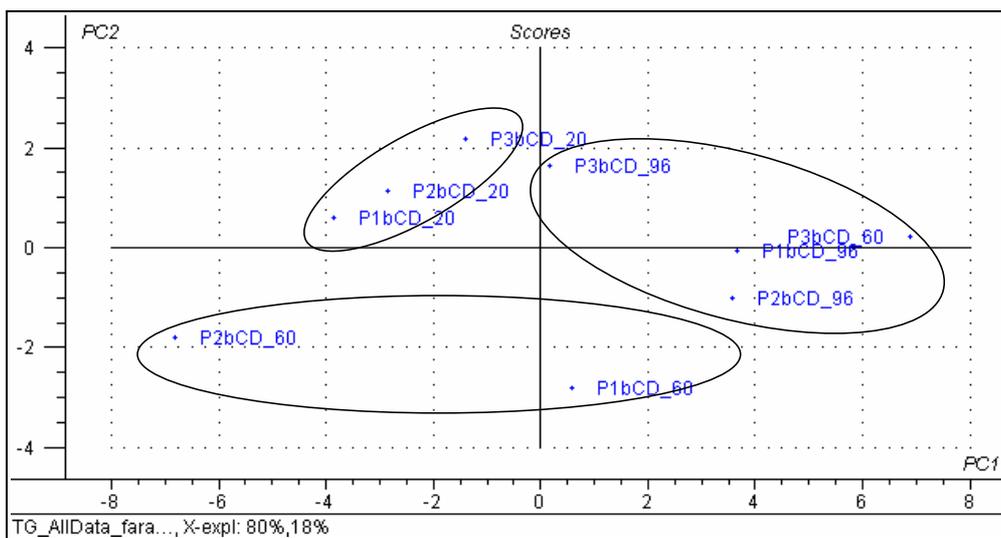


Figure 11. Scores from the PCA analysis of the obtaining and analysis of the propolis extracts/ β -cyclodextrin micro/nanoparticles (TG data only)

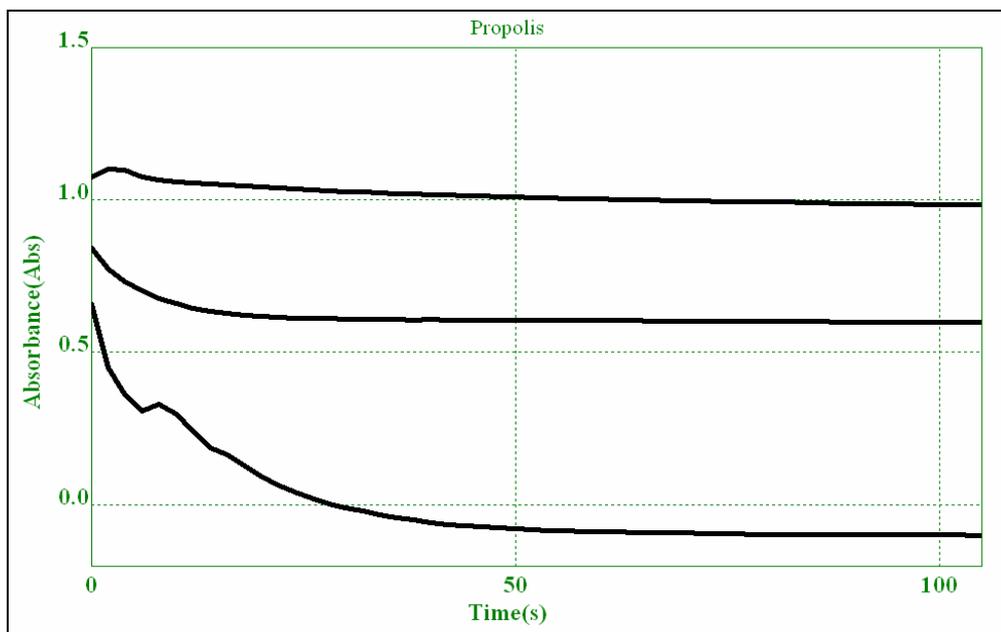


Figure 12. Time scan analysis for the diluted propolis extract (P2 propolis extract in 60% EtOH, down), and for the corresponding micro/nanoparticles (initial solution-middle, and 1:10 dilution-up)

4. Conclusion

The following conclusion on the extraction and analysis of flavonoidic compounds from propolis and the complexation of these in β -cyclodextrin can be drawn. In the propolis extracts significantly concentrations of flavonoidic and cinnamic derivatives could be identified, especially hydrophyllic ones (like rutin and cinnamic acid derivatives) in the case of aqueous and diluted ethanol extracts, and more hydrophobic flavonoids in the case of extracts using more concentrated ethanol.

The optimum extraction of the bioactive compounds from propolis was realized with 60% ethanol.

The separation of biocompounds by reversed phase-high pressure liquid chromatography conduct to relatively small retention times (RTs) for the more polar compounds, and higher ones in the case of more hydrophobic flavonoids. An inverse dependence of the RT with the logP (logarithm of the octanol/water partition coefficient) can be observed (table 2).

Table 2. LogP values (calculated and experimental) for the main bioactive compounds identified in propolis

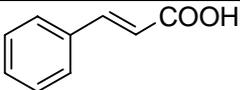
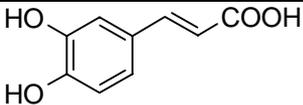
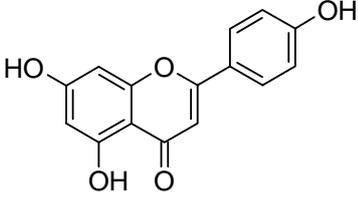
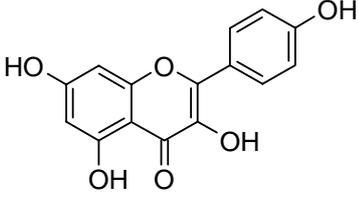
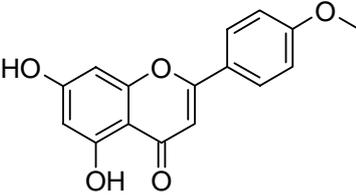
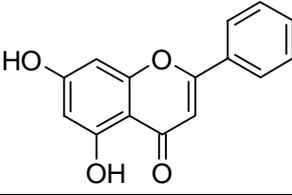
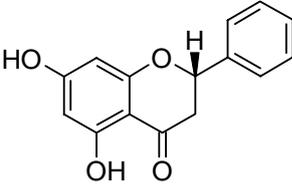
No	Name	Structure	RT (min) ^a	logP ^b
1	Rutin		6.7-7.0	-1.61
2	Cinnamic acid		-	2.15
3	Caffeic acid		10.4	1.58
4	Quercetin		12.5; 13.1	0.28
5	Apigenin		15.4	1.46 (3.02)
6	Kaempferol		16.8	0.56

Table 2. LogP values (calculated and experimental) for the main bioactive compounds identified in propolis (continuare)

No	Name	Structure	RT (min) ^a	logP ^b
7	Acacetin		34.0	1.50
8	Chrysin		34.1	1.75 (3.52)
9	Pinocembrine		36.3	2.27

^a Retention time from the RP-HPLC analysis of the standard compounds

^b Logarithm of the octanol/water partition coefficient: calculated with the QSAR Properties program from the HyperChem 4.5 package and the experimental logP values (in paranthesis)

The micro/nanoencapsulation yields increase with the ethanol concentration used for propolis extraction. This fact is supported by the higher content of more hydrophobic flavonoids of the propolis extracts obtained with concentrated ethanol, compounds which conduct to the better interactions with the β -cyclodextrin cavity and further to a higher yield in the obtaining of micro/nanoparticles which have lower solubility in ethanol-water system. This theory is demonstrated by the TG-DTG analysis of the nanoparticles, the higher mass loss being obtained for the case of 60% and 96% EtOH propolis extracts (>2% in the range corresponding

to the release of bioactive compounds, >150°C).

The presence of the bioactive compounds with phenolic OH groups, which are susceptible to oxidation in the propolis extracts (especially flavonoids and cinnamic acid derivatives) confer to the extracts significant antioxidant activity (determined by DPPH method), as well as for the propolis extracts/ β -cyclodextrin micro/nanoparticles. Furthermore, it can be observed a retard effect in the case of complexes, therefore a longer antioxidative activity, due to the slower release of the flavonoids from the complex.

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