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Extraction of biologically active compounds from propolis and concentration of extract by nanofiltration

Bartosz Tylkowski[a,*], Boryana Trusheva[b], Vassya Bankova[b], Marta Giamberini[c], Georgi Peev[a], Agnessa Nikolova[a]

[a] University of Chemical Technology and Metallurgy, Department of Chemical Engineering, 8 Kliment Ohridski blvd, 1756 Sofia, Bulgaria
[b] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[c] University Rovira i Virgili, Department of Chemical Engineering, Av. Països Catalans, 26, 43007 Tarragona, Spain

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ABSTRACT

Propolis is a natural product, rich in biologically active compounds. In the recent years the product is extensively used in food and medical applications.

This investigation reports results on the extraction of these compounds with an ethanol–water mixture and sequential concentration of the extract by nanofiltration, using two membranes: Starmem™ 122 (polyimide) and Duramem™ 200 (modified polyimide). The content of flavones, flavonols, flavanones, dihydroflavonols as well as total phenolic substances was determined spectrophotometrically in the feeds and permeates of dead-end nanofiltration experiments. Rejections of over 95% were observed and extract concentration of over three times was achieved with the Duramem™ 200 membrane. The membranes behaviour during the process was characterized by environmental scanning electron microscopy, revealing better stability and higher permeability of the modified polyimide membrane. The established conditions for extract concentration could facilitate cross-flow nanofiltration experiments regarding a technology for medicine or nutritional additive production. They could also be utilized in propolis chemistry investigations for a more reliable identification of some individual compounds.

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1. Introduction

Propolis is a resinous natural product, produced by bees (Apis mellifera), from vegetable secretions. Bees use it mainly to cover the hive interior and the breeding cells as well as to repair cracks and fissures. Propolis is composed of 45% resins, 30% waxes and fatty acids, 10% essential oils, 5% pollens and 10% organic compounds and minerals [1,2]. Wax and organic debris are removed during processing, usually by ethanolic extraction, and the propolis tincture (balsam) thus obtained, contains the bulk of propolis bioactive constituents. More than 300 compounds, among which terpenoids, steroids, sugars and amino acids have been detected in raw propolis. Their abundance is influenced by botanical and geographical factors, as well as by the collection season [3,4]. Besides, propolis-containing products have been intensely marketed by the pharmaceutical industry and health-food stores. A few bioactive compounds in propolis are flavonoids and phenolic acids as well as theirs derivatives. They have been used since ancient times as a medicine because of theirs biological properties such as antibacterial, antifungal, antiprotozoan, antiviral, immunomodulatory, anti-inflammatory, antioxidant and antitumor activity [5–10]. These compounds are obtained extracting propolis by an ethanol–water mixture [11].

There are cases in the solid–liquid extraction from natural products (herbs, fruits, vegetables, etc.) where the extracts obtained are very diluted. This can take place when the solid is in limited quantities and has a composition varying with the area wherefrom it is supplied and the time of the collection, the necessary liquid-to-solid ratio is high due to a considerable porosity of the material but the extracted products are very valuable. At these circumstances the process is carried out with a fresh solvent several times, because the use of a continuous countercurrent extractor or a battery of vessels is not justified. If the content of the extract is insufficient for its application or a dry extracted product is necessary to be finally obtained the extract needs to be concentrated.

The traditional approaches used for concentrating of biologically active compounds, extracted from natural products, include simple steam distillation and vacuum distillation, which generally require an increased temperature and high energy consumption. The first is inappropriate for heat-sensitive products. These methods may also result in a loss of compounds of low molecular weight, which can be removed together with the solvent during evaporation [12]. Another method described in literature, particularly for propolis extract concentration is lyophilization process [13]. However, this

* Corresponding author. Tel.: +359 28163301; fax: +359 28685488.
E-mail address: bartosz.tylkowski@urv.net (B. Tylkowski).

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Table 1
Specification of the membranes.

Membrane	Starmem™ 122	DURAMEM™ 200
Membrane material	Polyimide	Modified polyimide
Membrane configuration	Flat sheet membrane	Flat sheet membrane
Maximum operating pressure	60 bar	60 bar
Maximum operating temperature	50 °C	50 °C
Toluene flux at 55 bar	30 l/m^2 h	–
Molecular weight cut-off	220 Da	200 Da
Stable in solvents	Alcohols, alkanes, aromatics, ethers, ketones	Alcohols, alkanes, aromatics, ethers, ketones

method shows some of the disadvantages of the previously mentioned processes, e.g. involves a large amount of energy, comprises incubation at about 70 °C [14], etc.

A membrane separation process can be utilized as an alternative of the approaches mentioned above. In the cases of substances with a molecular mass less than 1000 Da nanofiltration has received a great attention as a process with reduced operation cost, carried out at ordinary temperature [15–17]. The last is very important because most of the species extracted from the natural products are very unstable at elevated temperature. A possible use of the permeate as an extraction solvent allows to decrease the required rejection of the membranes.

The low content of bioactive compounds, its dependence on the area and season of propolis collection as well as the high solvent to solid ratio used for their removal [11] require the extract concentration. Being realized at ordinary temperature the latter will allow not only a better marketing of the product but also a more reliable identification of the individual compounds in the propolis which is important for its chemistry. For this reason the aim of the present work is to investigate the effect of nanofiltration on the concentration of the valuable bioactive constituents extracted from propolis.

2. Experimental

2.1. Materials

Propolis was provided by the Centre of Phytochemistry of the Institute of Organic Chemistry, BAS (Bulgaria); Ethanol (99.9%) was supplied by Valerus (Bulgaria); Aluminium chloride anhydrous, sodium carbonate and sulfuric acid (96%) were supplied by Fluka; Folin–Ciocalteu's phenolic reagent, methanol (99.8%), 2,4-dinitrophenylhydrazine (DNP) and potasium hydroxide were supplied by Merck; pinocembrin was supplied by Extrasynthese (France) and galangin was supplied by Sigma–Aldrich.

Two nanofiltration membranes were used: Starmem™ 122 and Duramem™ 200 supplied by Membrane Extraction Technology Ltd., UK. Table 1 provides the specification of the membranes used as given by the manufactures.

2.2. Extraction

Propolis (cooled at 5 °C) was ground before extraction. The real average size of the propolis particles was calculated using a "Image-ProPlus 5®" software and the environmental scanning electron microscopy. The diameter of 314 particles and 15 agglomerations were measured and were considered as 100%.

On the base of a preliminary information about extract content [11] two successive extractions of a propolis portion were performed with 70% EtOH for 24 h at room temperature and liquid-to-solid ratio 10 ml per gram. The mixture of two extracts each one filtered with paper filter was used as a feed for nanofiltration experiments. This mixture was analyzed for the determination of the respective substance content.

2.3. Membrane stirred cell

A 270 ml stirred cell (METcell), Membrane Extraction Technology Ltd., UK was used to conduct the dead-end nanofiltration experiments. The effective membrane area was 54 cm^2. The maximum operating pressure for this cell was 1000 psig (69 bar).

2.4. Experimental set-up and procedure

Fig. 1 shows the experimental set-up. The cell was pressurized using compressed high purity nitrogen (99.996%). The pressure in the permeate side was approximately atmospheric under all conditions. The stirring rate was kept constant at 350 rpm, in order to minimize concentration polarization [18]. The membrane conditioning was realized by initial filtration of 300 ml of ethanol at 30 bar as to avoid a compression effect in the later stage of experiment and to remove the oil which was used to protect the active membrane layer. For nanofiltration of the extract the cell was emptied, filled in with 50 ml of feed solution and then pressurized at the operating pressure (30 or 50 bar). Permeate from the bottom of the cell was continuously collected and its volume was measured. Permeate flux was calculated from the slope of the function $V_i = f(At_i)$ where V_i is the cumulative volume, t_i is the time for its collection and A is the membrane area. Plotting this function straight lines with coefficients of linear correlation more than 0.99 were obtained. This is an indication that up to the final operating feed to permeate ratio of 1.43 a membrane fouling has not taken place. The nanofiltration experiments were conducted at room temperature. The feed, retentates and permeates were analyzed for the content of total flavones, flavonols, flavanones, dihydroflavonols and phenolic substances. The rejection R of these compounds was calculated using definition 1, where C_f and C_p are the feed and

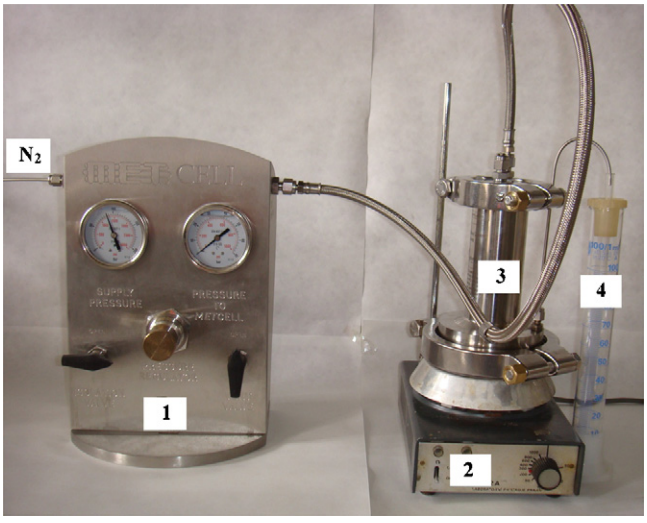


Fig. 1. Experimental set-up: (1) pressure regulator, (2) magnetic stirrer, (3) nanofiltration METcell, and (4) measuring cylinder.

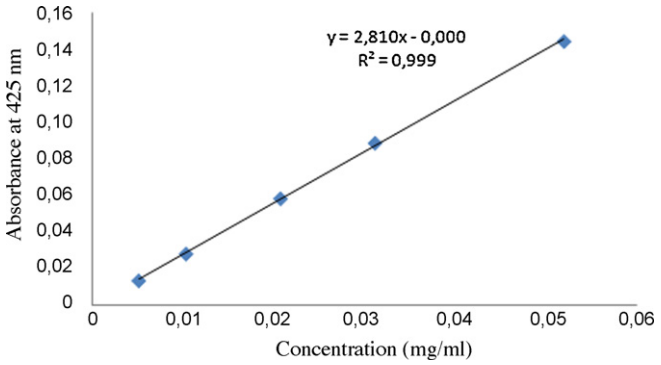


Fig. 2. ESEM micrographs of: (a) propolis particles; (b) a single agglomeration of the propolis particles.

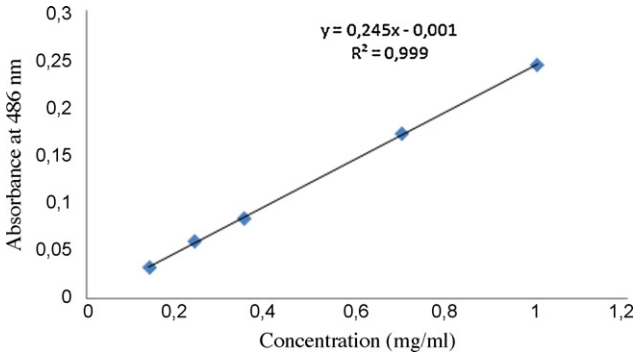


Fig. 3. Characteristic size distribution, as measured on 314 propolis particles.

permeate concentrations of the respective biologically active compound extracted from propolis

$$R = \frac{C_f - C_p}{C_f} \times 100\% \quad (1)$$

2.5. Characterization

2.5.1. Surface and cross-section morphology of the membranes

The surface and cross-section morphology of membranes Duramem™ 200 and Starmem™ 122 before and after nanofiltration were characterized by environmental scanning electron microscopy [ESEM (Quanta 600, FEI)]. The cross-sections were prepared by fracturing the membranes in liquid nitrogen. The details of this method can be found elsewhere [19,20]. The real sizes of the activate layers of the membranes (10 measures) and the diameter of the particles distributed on the membrane surfaces (400 measures) were calculated using a "Image-ProPlus 5®" software and the ESEM micrographs.

2.5.2. Flavone and flavonol content

Total flavone and flavonol content were measured by spectrophotometric assay based on aluminum chloride complex formation [21]. Ten millilitres of methanol and 0.5 ml of 5% $AlCl_3$ were added to 1 ml of the test solution and the volume was made up to 25 ml (volumetric flask). After 30 min, the absorbance was measured at 425 nm. Blank: 1 ml methanol instead of test solution. Flavone and flavonol content was estimated using a calibration curve of galangin, concentration range of 0.0052–0.052 mg/ml, shown in Fig. 2.

2.5.3. Flavanone and dihydroflavonol content

For flavanones and dihydroflavonols determination, the colorimetric method from DAB9 was modified for propolis [22,23]. Half millilitre of test solution and 1 ml of DNP solution (1 g DNP in 2 ml 96% sulfuric acid, diluted to 100 ml with methanol) were heated at 50 °C for 50 min. After cooling to room temperature, the mixture was diluted to 10 ml with 10% solution of KOH in methanol (w/v). One millilitre of the resulting solution was added to 10 ml methanol and diluted to 25 ml with methanol. Absorbance was measured at 486 nm. Blank: 0.5 ml methanol instead of test solution was used in analogous procedure. Flavanone and dihydroflavonol content was estimated using calibration curve of pinocembrin, concentration range of 0.14–1.0 mg/ml, shown in Fig. 3.

2.5.4. Total phenolic substances

For quantification of total phenolics, the Folin–Ciocalteu's method [24] was used. Half millilitre of the test solution was transferred into a 25 ml volumetric flask, containing 7.5 ml distilled water, and 2 ml of the Folin–Ciocalteu's reagent and 3 ml of a 20% sodium carbonate solution were added. The volume was made up with distilled water to 25 ml. After 2 h, the absorbance was measured at 760 nm. Blank solution: 0.5 ml methanol instead of test solution was used in analogous procedure. Total phenolics content was estimated using calibration curve of standard mixture pinocembrin–galangin 2:1, concentration range 0.025–0.3 mg/ml, shown in Fig. 4.

The UV–vis studies were performed on a Helios Gamma UV-spectrophotometer. Every assay was carried out in triplicate.

3. Results and discussion

Fig. 5a gives an illustration of the propolis particles, used as extraction material. Most of the particles appear separated, but a trend towards the formation of large agglomerates is also observed. Fig. 5b shows one of these agglomerations. By means of "Image-ProPlus 5®" software, we analyzed the ESEM micrographs and obtained the characteristic distribution of the particles size, which is shown in Fig. 6. It demonstrates that the particles, used for extraction, had diameter fluctuating between 10 and 80 μm. The resulting mean number-based diameter was 32 μm, with 90% of the particles being in the range of 15–52 μm. The mean number-based size of the particles agglomerations was 344 μm. The maximum absolute error of these measurements was 2 μm.

In order to evaluate the rejection of flavones, flavonols, flavanones, dihydroflavonols and phenolic substances, extracted from propolis, their concentrations in the feed and permeate were analyzed. The results are given in Table 2. The comparison of calculated retentate concentrations with those analytically found has demonstrated a difference of less than 5%, which may be devoted to some losses in the membrane and outflowing tube. Both of the used membranes show very good rejections. Only around 11% of the total flavonoids and 12% of the total phenolics compounds have passed through the dense active layer of the polyimide membrane

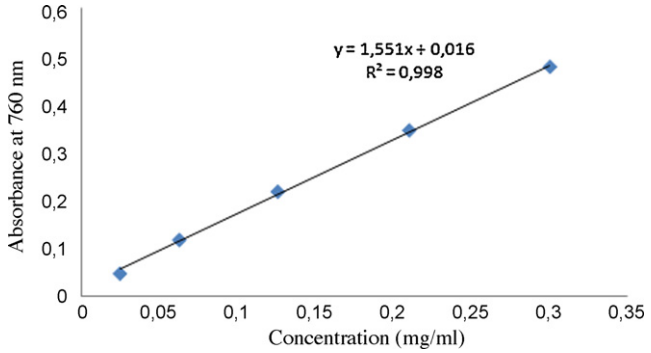


Fig. 4. Calibration curve of galangin.

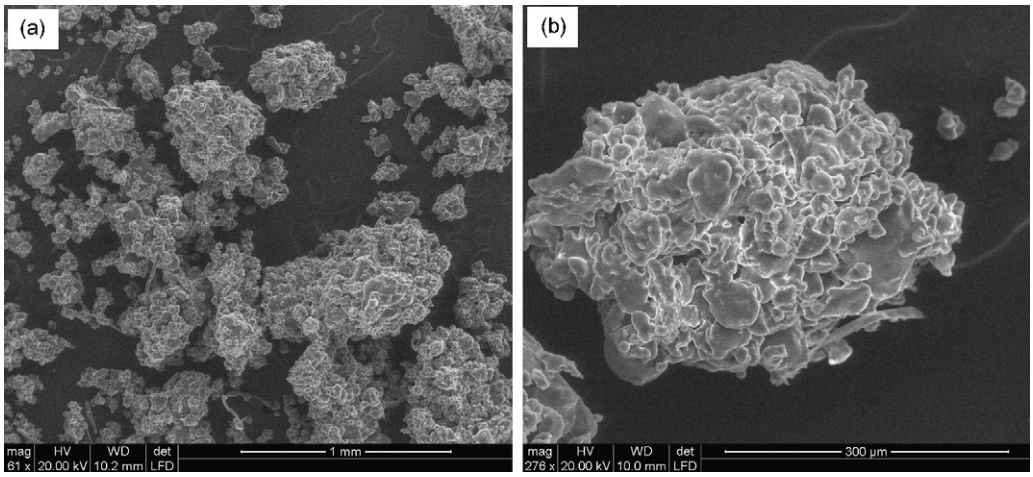


Fig. 5. Calibration curve of pinocembrin.

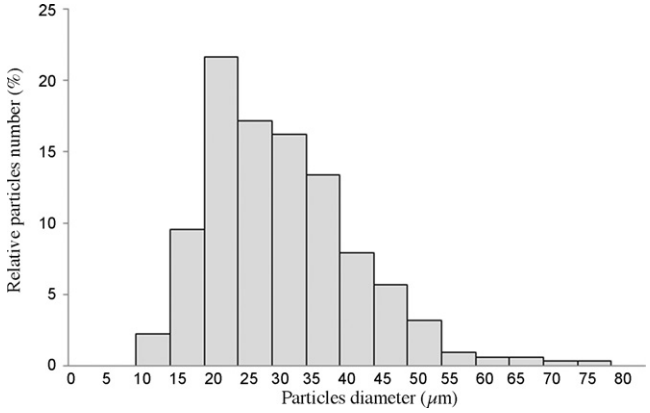


Fig. 6. Calibration curve of standard mixture pinocembrin–galangin 2:1.

Starmem™ 122. In the case of modified polyimide membrane Duramem™ 200 these values are much lower—3% and 5%, respectively.

The higher rejection of the Duramem™ 200 membrane (approximately 7%) as compared to Starmem™ 122 membrane can be interpreted at least in three different ways:

(1) The molecular weight cut-off (MWCO) of Duramem™ 200 membrane as specified by the supplied is 20 Da lower. It could be expected that the organic compounds with molecular weight between 200 and 220 Da will be rejected only by the Duramem™ 200 membrane.
(2) The Duramem™ 200 membrane, contrary to the Starmem™ 122, is prepared from modified polyimide (cross-linked). Accordingly, the mechanism of transport of the organic compounds through the active layer of the membrane could be different, due to membrane–solvent interaction. This interaction is very important in the mechanism that governs solvent transport in a particular system [25]. This fact can affect the membrane rejection. A deeper investigation on the mechanism transport through the modified and non-modified membrane should be performed and it will be the object of a forthcoming paper.
(3) By comparing the results from Tables 2 and 3, as well as the ESEM micrographs of the cross-sections of the membranes before and after nanofiltrations (Figs. 7 and 8) it seems most likely that the Duramem™ 200 membrane (Fig. 8) shows higher stability than Starmem™ 122 (Fig. 7). After nanofiltration with Starmem™ 122 more than 30% decrease of the active layer thickness is noted. This phenomenon is not observed in the case of Duramem™ 200 membrane. Therefore, the decrease of the active layer thickness can also affect the rejection results.

Table 2
Quantitative analysis of propolis extracts obtained by nanofiltration.

Membrane	Applied pressure [bar]	Compound	Feed concentration [mg/ml] ± sd[a]	Permeate concentration [mg/ml] ± sd[a]	Rejection [%]	Retentate concentration[b] [mg/ml]
Starmem™ 122 (MWCO 220 Da)	30	Flavones and flavonols	2.99 ± 0.059	0.284 ± 0.002	90	9.29
		Flavonones and dihydroflavonols	5.12 ± 0.16	0.596 ± 0.008	88	15.7
		Total phenols	19.9 ± 0.92	2.41 ± 0.033	88	60.6
	50	Flavones and flavonols	2.99 ± 0.059	0.245 ± 0.004	92	9.38
		Flavonones and dihydroflavonols	5.12 ± 0.16	0.552 ± 0.015	89	15.8
		Total phenols	19.9 ± 0.92	2.20 ± 0.021	89	61.1
Duramem™ 200 (MWCO 200 Da)	30	Flavones and flavonols	2.99 ± 0.059	0.070 ± 0.005	97	9.79
		Flavonones and dihydroflavonols	5.12 ± 0.16	0.248 ± 0.008	95	16.5
		Total phenols	19.9 ± 0.92	1.02 ± 0.040	95	63.8
	50	Flavones and flavonols	2.99 ± 0.059	0.089 ± 0.007	97	9.75
		Flavonones and dihydroflavonols	5.12 ± 0.16	0.228 ± 0.020	95	16.5
		Total phenols	19.9 ± 0.92	1.16 ± 0.004	95	65.8

[a] Average concentration of three measurements ± standard deviation.
[b] Calculated from the process material balance.

Table 3
Flux and permeability coefficient of the permeate through the membrane and decrease of the active layer thickness after nanofiltrations.

Membrane	Applied pressure [bar]	Flux of the permeate through the membrane [l/m^2 h]	Active layer thickness before nanofiltrations[a] [μm]	Active layer thickness after nanofiltrations[a] [μm]	Permeability coefficient × 10^6 [l/m h bar]
Starmem™ 122 (MWCO 220 Da)	30 50	1.70 2.05	38	26	1.5 1.1
Duramem™ 200 (MWCO 200 Da)	30 50	2.66 4.05	72	70	6.2 5.7

[a] Maximum absolute error is 2 μm.

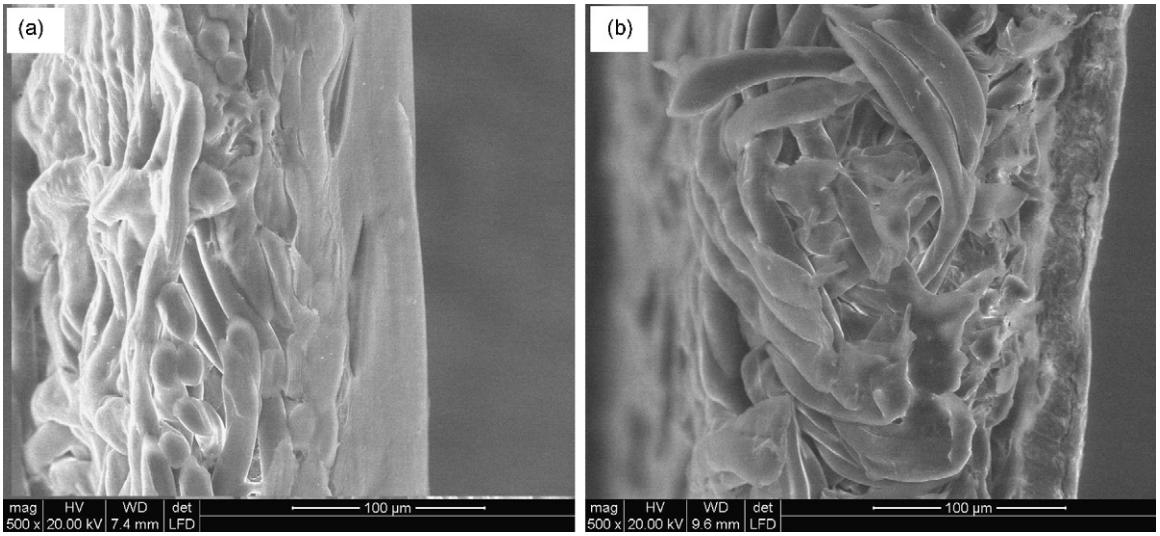


Fig. 7. ESEM micrographs of cross-sections of the Starmem™ 122 membranes after fracturing in liquid nitrogen: (a) before nanofiltration; (b) after nanofiltration.

Furthermore, as it is shown in Table 2, increasing the pressure from 30 to 50 bar leads to a slight increase of the rejection in case of Starmem™ 122. This result is probably due to membrane compressing. The combined effect of increased pressure and decreased thickness of the active layer could modify the MWCO value of the membrane.

By increasing the pressure from 30 to 50 bar no change in the active layer thickness, and rejection was observed with Duramem™ 200 membrane. As a result of its stability the flux increases with a higher pressure gradient and the permeability as seen in Table 3 remains approximately constant while with Starmem™ 122 it considerably decreases.

Figs. 9 and 10 show the images of the surfaces of Starmem™ 122 and Duramem™ 200 membranes, respectively, before (a) and after (b) nanofiltration. As can be seen from the micrographs, smooth membrane surfaces before nanofiltrations and surface heterogeneity after nanofiltration is noted. The deposition of particles, with mean size of 0.3 μm, on the membrane surface after nanofiltration could be attributed to the presence of waxes, fatty acids, essential oils, pollens and minerals extracted from the propolis [1,2]. The presence of this sediment may affect the multiple use of the mem-

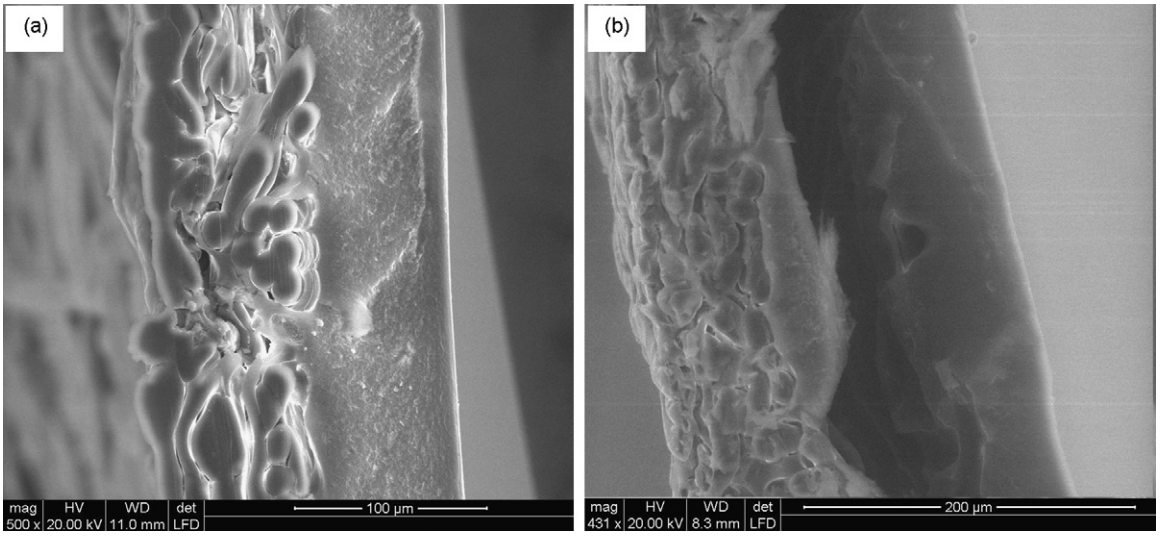


Fig. 8. ESEM micrographs of cross-sections of the Duramem™ 200 membranes after fracturing in liquid nitrogen: (a) before nanofiltration; (b) after nanofiltration.

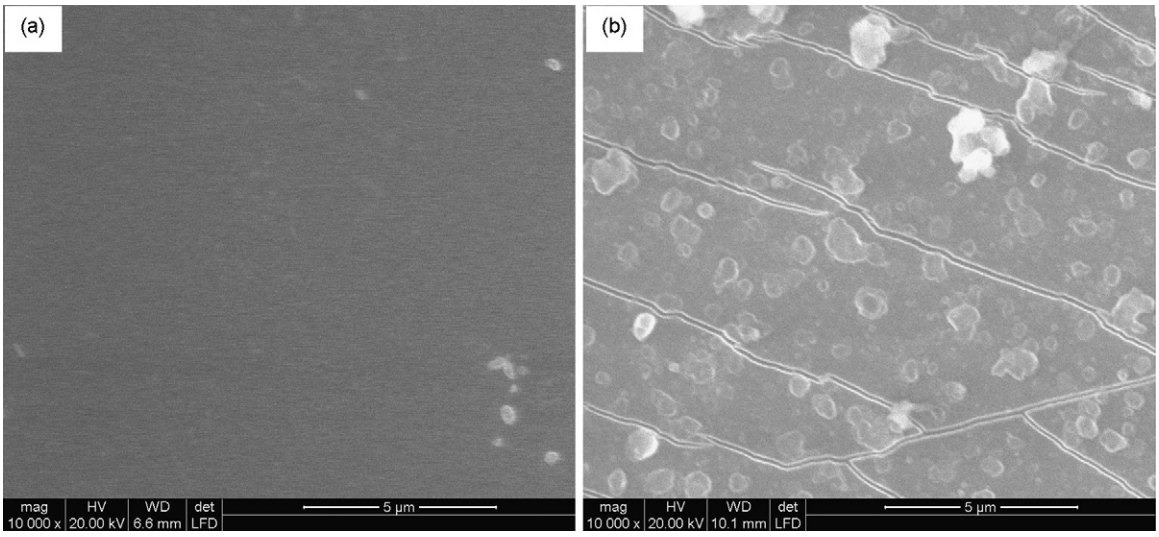


Fig. 9. ESEM micrographs of surfaces of the Starmem™ 122 membranes after fracturing in liquid nitrogen: (a) before nanofiltration; (b) after nanofiltration.

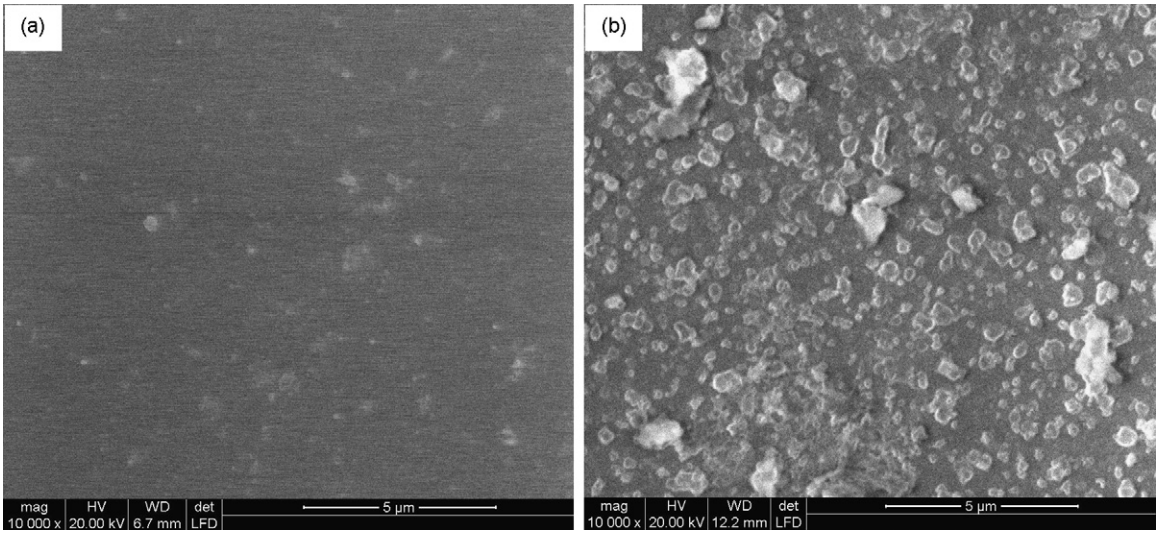


Fig. 10. ESEM micrographs of surfaces of the Duramem™ 200 membranes: (a) before nanofiltration; (b) after nanofiltration.

branes if it is not considered. The protective actions against a cake formation are discussed in the literature [26].

On the base of the extract concentrations given in Table 2 and the solvent to solid ratio used, the following contents of the bioactive compounds in propolis investigated were found: flavones and flavonols 5.97 wt%, flavonones and dihydroflavonols 1.02 wt% and total phenolic substances 39.7 wt% which are in good agreement with literature data [27].

4. Conclusions

The removal of biologically active compounds from propolis by an ethanol–water mixture results in very diluted extract due to their relatively low content and the liquid-to-solid ratio imposed. It can be concentrated more than three times by dead-end nanofiltration using the modified (cross-linked) polyimide membrane Duramem™ 200 with molecular weight cut-off 200 Da with a rejection of more than 95% for each kind of analyzed components (flavones, flavonols, flavanones, dihydroflavonols and phenolic). The membrane is stable under the applied pressure of 30 and 50 bar and keeps its flux at an approximately constant value of 2.66 and 4.05 l/m^2 h, respectively. Sedimentation of some very small particles (diameter of less than 0.3 μm) on the membrane surface has been found to take place. Although not affecting the flux this phenomenon should be accounted for regarding the multiple membrane use. Cross-flow nanofiltration experiments utilizing the information obtained can be recommended regarding a technology for medicine or nutritional additive production based on propolis extracts.

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