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Concentration of flavonoids in ethanolic extracts from tobacco leaves through nanofiltration

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Bioactive compounds (BAC) such as soluble polyphenols and flavonoids, extracted from plant materials, are successfully treated by membrane operations, in view of their separation or concentration. In the present study nanofiltration of ethanolic extracts from tobacco leaves is performed, focusing on concentrating the content of polyphenols and flavonoids (mainly rutin). Membranes Duramem^{TM} 300 and Starmem 240 with molecular weight cut off (MWCO) 300 and 400Da have been used. The obtained rejections with both membranes are about 88%, close to the measured value for the model system rutin-ethanol (92%), and tend slightly to decrease during operation. Observed average flux for real extracts are close to the values for the model system rutin-ethanol: 4.5 – 5.5 vs. 5.3 L/(m^2.h) for Duramem 300 and somewhat lower for Starmem 240 membrane. The flux vs time evolution for both membranes shows a similar initial decrease and tends to stabilize during longer operation time. The results prove that the two membranes are suitable for concentrating (volume concentration factor 2.5-3.5) extracts from tobacco leaves in terms of flavonoids.

Keywords: nanofiltration, solid-liquid extraction, rutin, tobacco leaves.

INTRODUCTION

Bioactive compounds (BAC) as polyphenols and flavonoids in plant materials, extracted by appropriate solvent and further treated by membrane operations, are promising and intensively investigated area of scientific research in view of BAC separation or concentration. A large number of potential applications are focused on organic solvent nanofiltration (OSN) coupled with solid-liquid extraction of valuable compounds from plant material [1].

The leaves of *Nicotiana tabacum* are not only the most important row material for the tobacco industry, but also an interesting source of bioactive natural compounds, among which the group of flavonoids is increasingly studied [2-5]. Rutin ($C_{27}H_{30}O_{16}$, quercetin-3-rutinoside) is one of the major polyphenol components of tobacco leaves with a number of pharmacological activities [6]. Rutin is a low solubility compound (0.125mg/ml in water [6, 7]), which further motivates the search of an optimum extraction method regarding yield and reasonable cost.

A comprehensive overview of the methods applied for rutin extraction from plant materials is shown in [6, 8, 9]. Either a HPLC component analysis, or total phenolics (TP) and total flavonoids (TF) characterization is used. In the latter case TP are usually referred to the concentration of chlorogenic acid, being highest among the polyphenol compounds in tobacco leaves. Different solvents are reported: water and organic solvents such as methanol, ethanol, acetone, N,N-dimethylformamide etc [10], higher content of polyphenols being obtained with increase in polarity of the solvent. The addition of water (EtOH-H_2O, MeOH-H_2O etc.) usually results in higher polyphenols content than in the pure solvent [10]. Largely varying liquid-solid ratios (10:1 to 90:1) and contact times (most often less than 1h) are reported. Some of the literature data about rutin extraction from tobacco leaves and waste are summarized in Table 1.

Concerning membrane techniques application, concentration of the extract from tobacco leaves has been realized by electrodialysis in combination with filtration through membrane with MWCO of 500 Da, as well as by ion-exchange membranes such as AM-2 and AM-4 [15] (especially for chlorogenic acid, scopoletin and rutin). Concentration or separation of the valuable bioactive components by nanofiltration has not been studied, though OSN has been increasingly investigated in view of treating plant extracts, including separation/concentration of polyphenols

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Table. 1. Details of the extraction of flavonoids from milled tobacco leaves/ wastes.

Time [min]	Extracting solvent	Analysis of the chemical composition	Liquid to solid ratio	Extraction mode	Ref. and raw material
15	C_2H_5OH-H_2O 8:2 (v/v)	TP**: UV-vis, Folin–Ciocaulteu, ref. compound:. chlorogenic acid	10:1	ultrasound assisted	[11] tobacco leaves
-	CH_3OH-H_2O 7:3 (v/v)	Apigenin, quercetin, rutin :UV-vis, NMR, HPLC	-	conventional	[2] waste tobacco leaves
1	$(CH_3)_2CO$-H_2O, (v/v) 0:1; 3:7; 4:6	Chlorogenic acid UV-vis	40:1	microwaves assisted	[12] tobacco leaves and waste
90 (25)* 120(60)*	H_2O C_2H_5OH (96%)	Total yield, weight analysis.	15:1 -40:1	conventional	[13, 14] tobacco leaves
30	EtOAc-CH_3OH 1:1; CH_3OH	HPLC	10:1 to 50:1	ultrasound assisted	[5] tobacco leaves and waste
60	C_2H_5OH (85% in water)	UV-vis: TP (Folin–Ciocaulteu , gallic acid), TF** ($AlCl_3$)	10:1	heat reflux	[4] tobacco leaves
10-90 (30*)	CH_3OH	HPLC	15:1 to 90:1 (45:1)	ultrasound assisted	[7] cigarettes tobacco

* to reach the plateau of the kinetic curve

** TP- total polyphenols; TF – total flavonoids

and flavonoids [16-26]. The method has advantages for multicomponent systems, containing sensitive to elevated temperatures components and allows for the regeneration of the solvent. The technology is especially promising when dealing with extracts from cheap and widely available materials, containing bioactive components that can be concentrated by nanofiltration. The content of some valuable flavonoids (such as rutin) in the tobacco waste in low concentrations makes them a suitable object for concentration by this process.

This study concerns the recovery of rutin from tobacco leaves and waste by solid-liquid extraction and subsequent concentration of the extract by nanofiltration. The molecular weight of most of the important polyphenols components in the extract from tobacco leaves [5] is over 300 Da - chlorogenic acid ($C_{16}H_{18}O_9$, 354 Da) and its isomers neochlorogenicacid (5-O-caffeoylquinic acid) and 4-O-caffeoylquinic acid, and highest for the flavonoids rutin (quercetin-3-O-rutinoside, MW 610 Da) and kaempferol-3-rutinoside (MW 594). It is expected that nanofiltration using organic solvent resistant (OSR) membranes with MWCO ≥300 Da will allow the successful concentration of the extract in terms of flavonoids. In the present study two OSR membranes were used - Duramem^{TM} (modified polyimide) and Starmem 240 (polyimide) with MWCO 300 and 400 Da respectively.

EXPERIMENTAL

◦ *Extraction*

The plant material for extraction was provided by Bulgartabac Sofia - tobacco leaves with defined origin (the region of Petrich) and moisture content 11%. All samples were grounded to powder and then subjected to extraction with ethanol. After batch extraction in a stirred vessel at room temperature 20±1 ◦C and intensive mixing (stirring speed 300 rpm) the extract was filtered and analyzed. If not additionally treated, the ethanolic extract from tobacco leaves has slightly acidic pH. In our study pH of the extract was 5.93, obtained with liquid/solid ratio 10:1 and 70% ethanol (where highest content of polyphenols is observed), the deviation from neutral being less pronounced for the extracts, obtained with 96% ethanol. The latter were further used in the membrane separation runs with two organic solvent resistant membranes.

The extraction kinetics with 96% ethanol was followed during 12h; the obtained results indicate a contact time of 3h as needed to reach the plateau of the kinetic curve. The recovered amount of flavonoids was 0.8 mg/(g solids) at liquid to solid ratio 10:1. Increasing the latter up to 30:1 allowed evaluating the maximum extractable flavonoids concentration as 1.108 mg/g solid. Further increase of the liquid volume has practically negligible effect on the amount of the extracted target component.

º *Spectrophotometric analysis*

For the spectrophotometric determination of total flavonoids concentration a color reaction with aluminum trichloride was used. According to Ordonez [27] 0.5 ml of the sample was added to 0.5 ml AlCl3 (2% solution in ethanol). After 1 hour in the dark the absorbance was measured at a wavelength of 425 nm. Three measurements were performed for each sample. The concentrations in the extract (C_f), retentate (C_r) and permeate (C_p) were calculated as rutin equivalent, according eq. (1):

$$Abs = 10.953\ C\ (R^2=0.997) \quad (1)$$

where C [mg/ml] is the concentration of total flavonoids (up to 0.07 mg/ml) and Abs is the measured absorbance. The calibration curve was obtained with model solution of rutin-hidrate in ethanol. Ethanol (96%) was supplied by Valerus (Bulgaria); Aluminium chloride anhydrous and rutin (as rutin hydrate ≥ 94%) was supplied by Sigma-Aldrich.

º *Nanofiltration*

Batch nanofiltration in dead-end mode was performed on a laboratory cell (METcell, Evonic MET LTD, UK) with effective surface area of 54 cm^2 at transmembrane pressure of 20 bar and working volume up-to 200 ml. During nanofiltration flow and rejection evolution over the time of filtration was measured. The concentration of equivalent rutin was determined after each 20 ml permeate.

RESULTS AND DISCUSSION

The permeate flux and rejection evolution during nanofiltration with Duramem 300 (MWCO 300Da) and Starmem 240 (MWCO 400Da) are illustrated in Figs.1 and 2 for similar feed concentrations of rutin (0.022, 0.025 mg/ml respectively, see Fig.2). An initial pronounced flux decrease within the first 3 hours is observed with both membranes. The flux vs time data tend to stabilize within longer operation time (6h), the final flux being in the range of 2 - 3 l/(m^2.h).

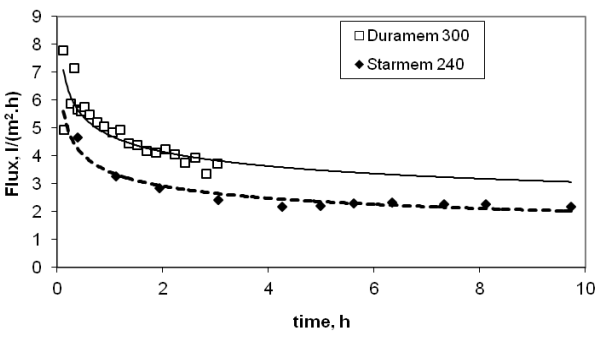

Fig. 1. Measured flux versus time of filtration

A similar tendency is observed in the time evolution of observed rejections, shown in Fig.2. An initial increase is better observed with Duramem 300, corresponding to the more pronounced flux decrease in this period and associated with an increasing membrane resistance. After that the rejection values tend to stabilize and even a slight tendency to decrease can be observed. The two membranes show similar rejections values.

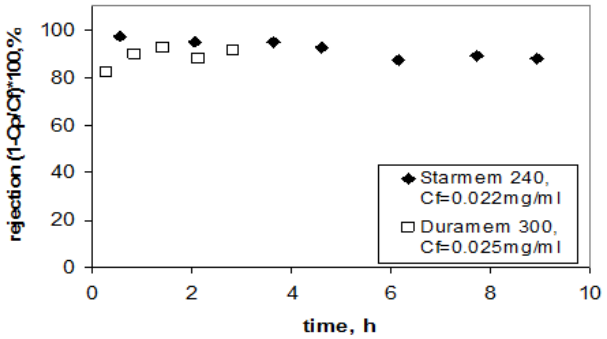

Fig. 2. Measured rejections versus time of filtration

The additional membrane resistance is usually attributed to fouling and related with different phenomena such as concentration polarization, cake layer formation, adsorption of solute molecules inside the pores or pore blocking when the pore size is similar to the molecular dimensions [28].

The four kinetic models commonly used for systems showing flux decline are given in Table 2 together with the calculation results for the first 6 hours of filtration. As can be seen from Table 2, the cake layer formation model gives best correlation, but a statistically good description of the flux decline is also obtained with the rest of the tested models, which rather suggests that fouling phenomenon is not very pronounced under the working conditions (range of feed concentrations in term of flavonoids 0.012 to 0.042 mg/ml).

Similar observations were already reported with nanofiltration of natural extracts containing polyphenols and flavonoids [29, 30], based on the original model, proposed by Hermia to describe the permeate flux decline during constant-pressure filtration [31].

The flux dependence on feed concentration is shown in Fig.3. These results concern average flux values, obtained with Duramem 300 during the initial 3h of filtration, where the flux decline is most pronounced. The volume ratio permeate to feed was kept in the range of 0.6 to 0.7, which defines the limits of the achieved degree of concentration. The feed concentration affects the measured flux, but the latter remains close to the measured value for the model system rutin-ethanol: 4.5 – 5.5 vs. 5.3 L/m2.h (model system, R^2=0.998).

Table 2. Kinetic models to evaluate flux decline data

Fouling mechanism	Model equation (linearized) [29]	Calculated flux vs time data	R^2
total pore blocking	$\ln(J) = \ln(J_0) - k_1 t$	$\ln(J)$= 1.4344-0.0737·t	0.9043
standard pore blocking model	$J^{-1} = J_0^{-1} + k_2 \cdot t$	J^{-1} = 0.2354+0.0215·t	0.9384
intermediate pore blocking	$J^{-1/2} = J_{0/2}^{-1} + k_3 \cdot t$	$J^{-0.5}$=0.4868+0.0199·t	0.9224
cake layer formation	$J^{-2} = J_0^{-2} + k_4 \cdot t$	J^{-2} = 0.0534+0.0128·t	0.9639

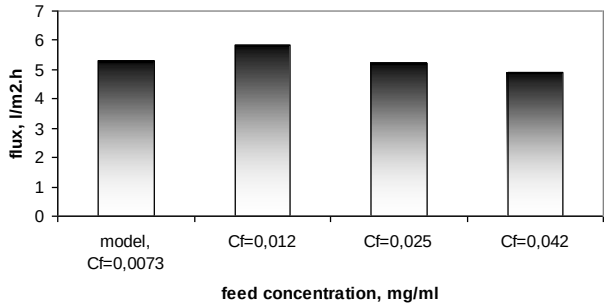

Fig.3. Measured average flux vs feed concentration

The average rejections, obtained with different flavonoids concentrations in the feed are shown on Fig.4. The increase of the feed concentration has a slight effect, resulting in decreasing rejection, whose average value is about 88%. The observed rejections are close to the measured ones for the model system rutin-ethanol (92%). This fact together with the high and approximately constant rejections proves the suitability of the membranes Duramem 300 and Starmem 240 for concentrating natural extracts from tobacco in terms of flavonoids. Both membranes were previousely used for nanofiltration of ethanolic extracts from Sideritis [16], where comparable, though higher rejections were observed and the possible reuse of the permeate as extracting agent was proven.

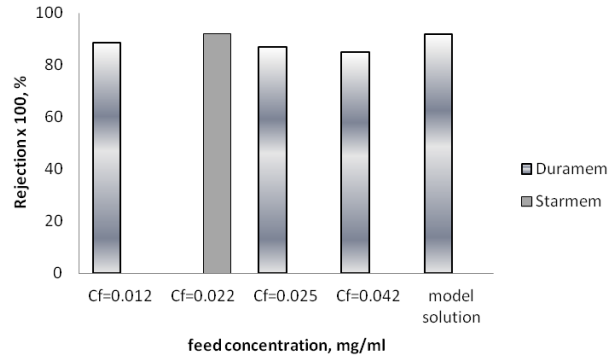

Fig.4. Measured average rejections vs feed concentration

The concentration effect on the rejection is strongly dependent on the mass transfer characteristics of the system [32], which explains the variety of observations, obtained in the OSN literature – practically constant [24, 33, 34], increasing [35] or decreasing [32] rejections. There are also different reasons for these observations. The constancy is viewed as indication for low membrane solute interaction and stable membrane behavior towards both solute and solvent [23, 36]. Membrane compaction could lead to increasing rejections. Such effect is observed in nanofiltration of ethanolic extracts from *Sideritis* with DuramemTM 500 when the transmembrane pressure increased from 30 to 50 bar [16]. In [28] a decrease of rejection is observed and predicted with the solution-diffusion model. For complex multicomponent solutions (as natural extracts) the analysis of the rejection vs concentration profile is even more difficult to allow for definite conclusions. In order to check the concentration effect nanofiltration was performed at high degree of concentration (permeate to feed ratio up to V_p/V_f= 0.85), as well as with model system rutin hydrate - ethanol with maximum feed concentration (determined experimentally 1.49 mg/ml, close to the solubility data found for rutin-3 hydrate in ethanol [39]). In case of pronounced effect of concentration polarization the solute concentration at the membrane surface is expected to be different from the one in the bulk retentate (C_r) and this fact should be taken into account [37]. Otherwise the calculated rejections by eq. (2) and (3) are expected to differ essentially, eq.(3) giving lower rejections than expected from the mass balance [24].

$$\boldsymbol{R} = \left(1 - \frac{\boldsymbol{C_p}}{\boldsymbol{C_f}}\right) \cdot 100,\% \qquad (2)$$

$$\boldsymbol{R} = \left(\frac{\ln(\boldsymbol{C_r}/\boldsymbol{C_f})}{\ln(\boldsymbol{V_f}/\boldsymbol{V_r})}\right) \cdot 100,\% \qquad (3)$$

Here V_f and V_r stand for the respective volumes of the feed and retentate.

Calculations according to eq. (2) and (3) showed comparable rejections close to 80% (77.8% and 79.8% respectively), which supports the absence of an essential concentration effect at the membrane surface. For higher feed concentrations the solubility limit was exceeded in the retentate, the fact being already pointed out in the literature for rutin extraction [38].

CONCLUSION

Extraction with ethanol of tobacco leaves at room temperature shows total flavonoids content 1.1 mg/(g solid) rutin equivalent. About 90% of the flavonoids are extracted during the first 3h, so this time can be considered as sufficient for practical applications.

The nanofiltration of the extracts was studied with OSR membranes Duramem 300 and Starmem 240. Average rejections show small variation with concentration, difficult to separate from the experimental error during the measurements, in view of the solution (natural extract, multicomponent) and the accuracy of the chemical analysis (group analysis, spectrophotometric).

The concentration effect is better seen from the rejection vs time plot. Both membranes show rejections about 88% and tendency to decrease with increasing degree of concentration. This corresponds to observed and predicted rejections in the OSN literature [31].

Permeate flow decreases with increasing concentration, the effect being important in the beginning (the first 3h of operation). Then the flux decline is much less pronounced, tending to stabilize at flux values between 2 and 3 l/m2.h. Flux and rejections with real extracts are close to the measured with the model system. The results prove that the two membranes are suitable for concentrating extracts from tobacco leaves in terms of flavonoids.

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КОНЦЕНТРИРАНЕ НА ФЛАВОНОИДИ В ЕТАНОЛОВ ЕКСТРАКТ ОТ ТЮТЮНЕВИ ЛИСТА ЧРЕЗ НАНОФИЛТРУВАНЕ

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(Резюме)

Мембранни процеси на разделяне се прилагат успешно към биоактивни вещества като разтворими полифеноли и флавоноиди, извлечени от растителни материали, с оглед на тяхното концентриране или разделяне. В настоящото изследване е проведено нанофилтруване на етанолови екстракти от тютюневи листа с цел концентриране на съдържанието на полифеноли и флавоноиди (главно рутин). Използвани са мембрани Duramem^{TM} 300 и Starmem 240 с праг на разделяне съответно 300 и 400Da. Наблюдавано е 88% задържане по общи флавоноиди, близко до измерената стойност за моделна система рутин-етанол (92%), и с тенденция за леко намаляване във времето. Наблюдаваните средни стойности за потока пермеат при реални екстракти са близки до тези за моделната система рутин-етанол: 4.5-5.5 vs. 5.3 L / (m^2.h) за Duramem 300 и малко по-ниска за Starmem 240. Развитието на потока във времето за двете мембрани показва подобен ход: първоначално намаляване с тенденция към стабилизиране при по-големи времена на филтруване. Резултатите доказват, че двете мембрани са подходящи за концентриране на флавоноиди от екстракти на тютюневи листа в изследваните обемни съотношения захранване спрямо ретентат (2.5-3.5).