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INFLUENCE OF MEMBRANE SEPARATION TECHNIQUE UPON THE PHENOLIC CONTENT OF RED CORN BRAN EXTRACT

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Abstract

Different extracts from coloured corn varieties are well known for their profile of high-value phenolic compounds. In this context, the current study proposes an evaluation of the phenolic content for the hydroalcoholic extract obtained from bran of red corn, a variety grown in Romania. Therefore, the native extract, obtained by ultrasounds treatment, was separated using different membranes (regenerated cellulose and polyethersulphone), with nominal molecular weight cut-off of 5000 and 10000 KDa, by employing different working parameters. Several extract fractions were obtained and characterized in terms of their total phenolic content and total flavonoid content by means of UV-Vis spectrophotometry and phenolic acids content also, by HPLC. Total antioxidant capacity was also measured by means of UV-Vis spectrophotometry. Membrane separation has been observed to lead to extract fractions rich in phenolic compounds, especially with flavonic structures, such as rutin and quercetin, with great potential for today's modern industries (pharmaceutical, cosmetic or food industry).

Key words: *antioxidants, membrane separation, phenolics, red corn, ultrasound extraction.*

INTRODUCTION

In a world with increasing environmental issue, the plant resources, man-made or spontaneous, are some of the most valuable sources of various raw materials for bioactive molecules and for sustainable technologies also (Cantão Freitas et al., 2021; Zahmanova et al., 2023; Chaachouay & Zidane, 2024). In the last years, agricultural resources, grains and their derived byproduct became more and more explored for their potential as natural sources for bioactive molecules (Priya et al., 2023; Hazem et al., 2023). Many scientific works in this field have focused their attention on various pigmented corn species (white, yellow, red, purple, black), due to the recognized properties of their great polyphenols and anthocyanins content (Serna-Saldivar & Perez Carrillo, 2019; Colomco et al., 2021; Colombo et al., 2022; Özdemir et al., 2023; Sánchez-Nuño et al., 2024). The corn colour is given by the anthocyanin molecules (Colomco et al., 2021; Özdemir et al., 2023). Also, is well known that corn with a darker colour (black, purple, red) has a generous profile of anthocyanins, phenolic compounds and carotenoids also (Lopez-Martinez et al., 2009), thus behaving higher antioxidant activity. The oxidative stress is very important being a problematic aspect, on one hand associated to many human/animal diseases and on the other hand, for the resistance of a final product prone to oxidation (Chen et al., 2017; Herrera-Sotero et al., 2020; Hao et al., 2022). For this, phenolic compounds derived from vegetable resources have attracted their attention as very useful molecules to prevent many oxidative processes (Simukova et al., 2021; Hao et al., 2022; Jamova et al., 2022). Apart from corn kernel, high polyphenols, anthocyanins and pigments content may be found in the non-edible parts, classified as byproducts or waste, like bran, husk, cob or silk (Cristianini & Guillén Sánchez, 2020; Hao et al., 2022).

Even if there is a lot of research exploring the coloured corn species, there are also many recent papers reporting improvements in the extraction processes (Jayaprakash et al., 2023; García-Ortíz et al., 2023; Duah Boateng et al., 2023). This fact highlights that corn still represents a source to be explored and led towards various modern applications. Water, ethanol, methanol and hydroalcoholic mixtures are the common solvents involved in the extractive processes for the polyphenols and anthocyanins; additionally, hydrochloric and formic acids are used as acidifying reagents (Cevallos-Casals & Cisneros-Zevallos, 2004; Li et al., 2013; Abdel-Aal et al., 2014; Mazewski et al., 2017; Hao et al., 2022). Also, several studies focused on the extraction methods, conventional (as maceration, percolation, Soxhlet extraction and so on) or modern ones (ultrasounds treatment, microwave or enzymatic assisted extraction, supercritical fluid extraction etc.) being reported (Deepika & Gagandeep, 2014; Aourabi et al., 2020; Elsayed et al., 2022; Frosi et al., 2024). So that, using this combined research strategies, there was possible to be established the efficiency of solvents/acidifying agents or the optimal extraction methods able to conduct to some extracts derived from coloured corn rich in bioactive molecules (phenolics/anthocyanins) and great antioxidant activity.

A recent report of our team (Arlet et al., 2023) established the optimal experimental conditions to obtain a polyphenol-rich extracts from bran of red corn variety (Bloody Butcher red corn, cultivated in Romania). It was established that the mixture of equal-parts ethanol-water, under ultrasonic-assisted extraction (20 min) led to a corn extract with great biological activity as reflected on total polyphenolic content (TPC) and total antioxidant activity (AA).

Therefore, the current work is focused on the optimal red corn bran extract and its exploration for the separation of a polyphenols-rich fraction through membrane-processing as a green technology, due to the main advantages such as mild operating conditions with low energy consumption (Baptista et al., 2015). Different membranes were explored within this research and different separation/work parameters also. The obtained fractions [named concentrate (C)/permeate fraction (P)] were characterized in terms of their total phenolic content, flavonoid content, phenolic acids and antioxidant capacity. The polyphenols-rich fractions are great candidates to be tested as antioxidant ingredient for different products.

MATERIALS AND METHODS

Raw material

Dried corn grains of red corn, the raw material, represents a Bloody Butcher variety, cultivated in Romania (Brăila region), harvested in 2022. Red corn bran (RCB), obtained as a by-product from the grinding of the raw material, was used for the extraction of polyphenolic compounds.

Materials

Ethanol (Sigma Aldrich, 96% purity), purified water (Milli-Q), gallic acid (GA; Sigma Aldrich), Folin-Ciocâlteu reagent (Merck), 2,2′-Azino-bis (ABTS; 3-ethylbenzo-thiazoline-6-sulphonic acid; Sigma Aldrich), 6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox; Sigma Aldrich) and aluminium chloride (Sigma Aldrich) were used as reagents for the extraction and extract characterization. Other substances (sodium carbonate, sodium acetate, potassium persulfate, from Sigma-Aldrich) were used as received.

Acetonitrile (Sigma Aldrich, HPLC purity), ultrapure water, gallic acid (Sigma Aldrich, analytical standard), syringic acid (Sigma Aldrich, analytical standard), chlorogenic acid, (Sigma Aldrich, (≥95%), caffeic acid (Sigma Aldrich, ≥98%), p-coumaric acid (Sigma Aldrich, ≥98%), rutin (Sigma Aldrich, reference substance), quercetin (Sigma Aldrich, ≥95%) were used for HPLC determination.

Corn extract preparation

Based on previous results (Arlet et al., 2023), RCB raw material was subjected to the extraction under ultrasound treatment (30W power) for 20 min, using hydro-alcoholic solvent (1:1 (v/v) mixture of purified water and ethanol), maintaining the ratio RCB/solvent of 1/10. Several extraction phases were performed, hydro-alcoholic product being filtered (Grade 1 filter paper, Whatman), collecting ~1 L red corn extract. Considered as

reference samples, further named **P0**, the initial extract sample was stored at approx. -18°C (freezer), in brown containers, hermetically closed.

Extract processing through membrane processes. Experimental set-up

Separation experiments were performed by using a KMS Laboratory Cell CF 2 (Koch Membranen GmbH, Germany) equipment. The main characteristics of the membrane module (tangential flow) are, as follows: membrane diameter = 75 mm; effective membrane surface = 28 cm^2; maximum hold up volume = 600 mL; maximum operating pressure = 6 bars without nitrogen gas bottle; maximum operating temperature = 70°C; maximum pump capacity = 1.8 L/min at 6 bar; material = stainless steel. For the determination of ultrapure water and separation flows the following formula was used:

$$J = \frac{V}{S \times t}$$

where: J = ultrapure water flow or separation flow ($L/m^2/h$); V = permeate collected volume (L); S = effective membrane surface (m^2, in this particular case = 28 cm^2); t = time (h)

Three types of membranes were used for separation tests: 5 KDa Ultracel regenerated cellulose (Millipore, USA), Polyether sulfone 5 KDa (Sartorius, Germany) and 10 KDa K131 Polyether sulfone (Sartorius, Germany).
Ultrapure water flow as well as separation flows varied based on the membrane used due to different base polymer, cut-off values and preparation methods.

Table 1. Separation tests carried out for the corn extract

Concentration test		Working conditions	
		membrane type	pressure
1		5 KDa regenerated cellulose	5 bar
2		5 KDa polyether sulfone	5 bar
3	3.1.	10 KDa polyether sulfone	2 bar (first pass)
	3.2.		3 bar (second pass)

After each membrane separation/concentration process, resulted a concentrate fraction (referred to as concentrate, not. **C**) and a permeate fraction (referred to as permeate, not. **P**). All samples were stored in brown and hermetically close vials, at approx. -18°C.

Table 2 below summarizes the membrane concentration experiments and the sample code that were given to each obtained fraction.

Table 2. The membrane concentration experiments and the samples code

Experiment No.	Sample code	Sample description
1.	P0	Initial extract
2.	P1-C	Concentrate fraction (RC[a], 5 KDa, 5 bar)
3.	P1-P	Permeate fraction (RC[a], 5 KDa, 5 bar)
4.	P2-C	Concentrate fraction (PES[b], 5 KDa, 5 bar)
5.	P2-P	Permeate fraction (PES[b], 5 KDa, 5 bar)
6.	P3-C1	Concentrate fraction (PES[b], 10 KDa, 2 bar, first pass)
7.	P3-P1	Permeate fraction (PES[b], 10 KDa, first pass)
8.	P3-C2	Concentrate fraction (PES[b], 10 KDa, 3 bar, second pass)
9.	P3-P2	Permeate fraction (PES[b], 10 KDa, 3 bar, second pass)

[a]*RC - regenerated cellulose membrane*
[b]*PES - polyethersulfone membrane*

Processing of the resulted fractions

Each fraction (P1-P3, concentrate or permeate fraction), as well as the initial extract (P0) were conditioned in two steps: first, ethanol was eliminated by reduced pressure evaporation. Then, they were frozen (-55°C) and lyophilized (Freeze Dryer, D-37520; Osterode am Harz, Germany) for 24h, to remove also the water (and traces of ethanol). The dry extracts obtained were stored in hermetically closed vials, protected from light at approx. -18 °C.
For their evaluation, the lyophilized samples were resolubilized in the extraction solvent (mixture ethanol:water 1:1, v/v), at a final concentration of 10 mg DM/mL.

Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method (ISO 14502-1:2005(E); Munteanu & Apetri, 2021). 0.01 mL corn extract (concentration of 10 mg/mL) were diluted with purified water (0.99 mL), then being added 5 mL of Folin-Ciocâlteu reagent (sol., 10%). After 4 min, 4 mL Na_2CO_3 solution (7.5%) and resulting mixtures were incubated in dark place for 1h (at room temperature).
Absorbance was registered at 750 nm using an ultraviolet-visible spectrophotometer (Helios

Beta UV-Vis, Thermo Electron Corporation, Waltham, MA, SUA; Thermo Scientific™ VISION pro™ software).
The TPC was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DM), based on the standard curve of gallic acid (0.5 to 50 mg/mL).

Determination of total flavonoid content
Total flavonoid content (TFC) was determined using an aluminium chloride complexation assay (Giurescu et al., 2023). The protocol proposed by Pękal & Pyrzynska (2014) was modified as follows: dilluted samples were mixed with sodium acetate (1:1) and after filtration, 2.5 ml sample was mixed with 1 ml aluminium chloride solution (2.5%) and 1.5 ml distilled water. After 45 minutes incubation period, the optical densities of the samples were measured at 420 nm. A calibration curve was obtained with rutin solution with various concentrations (10-160 µg/ml). Total flavonoid content was expressed as mg of rutin equivalent per gram dry weight (mg RE/g).

Determination of phenolic compounds by HPLC method
HPLC analysis was performed using a Waters 2695 Alliance system equipped with a quaternary pump, autosampler and UV/Vis detector. Separation was achieved by reversed-phase with 5 µm SunFire Column (3.9 x 150 mm). A binary elution gradient consisting in 0.5% orthophosphoric acid in water (A) and acetonitrile (B) was used according to the following gradient: 90/10 as initial condition, changed to 75/35 at 25 min, changed to 10/90 at 40 min, held 10/90 for 5 min, changed to 90/10 at 45.10 min, held 90/10 for 10 min. The column temperature was controlled at 40°C and the samples temperature at 20°C. Chromatograms were acquired at 280 and 300 nm wavelength.
For separation the samples were appropriate diluted, filtered by 0.22 nylon syringe filters and injected (5 µL).
The identification of phenolic compounds was done according to their retention times compared to those obtained by injecting of the standard solutions (4.35 min for gallic acid, 9.85 min for chlorogenic acid, 12.15 min for caffeic acid, 12.90 min for syringic acid, 17.30 min for 4-coumaric acid, 19.40 min for rutin and 24.58 min for quercetin). For analytes quantification sample peak area was processed using the calibration curves obtained for the corresponding standards.

Determination of total antioxidant capacity
Total antioxidant activity (AA) of the colored corn extracts was evaluated using **TEAC method** (Trolox equivalent antioxidant capacity (Kim et al., 2023; Tociu et al., 2023). ABTS salt (7.0 mM) and potassium persulfate solution (2.45 mM) were mixed and maintain in dark place at room temperature for 16 h. Then, the $ABTS^{\bullet+}$ stock solution was normalized (by ethanol dilution) to a final absorbance of 0.68 ± 0.02 at 734 nm.
The calibration curve was done by using 0.25-1.25 mM Trolox-ethanol solutions. For this, 990 µL $ABTS^{\bullet+}$ solution and 10 µL Trolox standard solution were well mixed; after exactly 1 min, the absorbance was registered at 734 nm, against a suitable blank solution, the percentage of inhibition being calculated for each Trolox solution, then plotted as function of concentration. The same procedure was performed for the test samples (the different fractions of coloured corn extract), the percentage of inhibition of absorbance being calculated for each sample based on the calibration curve.
AA of the corn extracts (of 10 mg/mL concentration) were expressed as mg Trolox equivalents/100 g dry matter (mg TE/g DM).

RESULTS AND DISCUSSIONS

In attempt to fractionate the complex mixture of polyphenolic components contained by the RCB hydroalcoholic extract, ultrafiltration experiments were considered. The impact of membrane separation process upon the phenolic composition of the resulted fraction was investigated.

Concentration by membrane processes
Comparison of ultrapure water and separation flows obtained when using the same working pressure (5 bar) proved that much higher values are obtained when using regenerated cellulose as base polymer. On the other hand, different cut-off values for the same base polymer

(polyether sulfone) led as expected to higher ultrapure water and separation flows for higher cut-off values. The experimental results are presented in Table 3 and Figure 1.

Table 3. Experimental results for the flow rate of membrane separation process

Results	Concentration test			
	Test 1	Test 2	Test 3	
			First pass	Second pass
Jw initial[a] ($L/m^2/h$)	75.140	53.493	221.785	81.225
Js[b], (mL/min)	1.101	0.125	1.898	1.586
Jw final[c] ($L/m^2/h$)	67.798	47.408	71.410	71.666

a - initial ultrapure water flow ($L/m^2/h$);
b - separation flow (mL/min);
c - ultrapure water flow after separation ($L/m^2/h$).

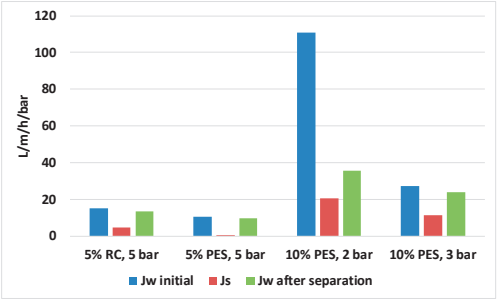

Figure 1. Representation of the separation flows for the different membrane separation tests

TPC assays

The products resulted following each separation experiment (C and P fractions) and the initial corn extract (P0), adjusted to a concentration of 10 mg DM/mL, were explored in terms of their TPC by means of Folin-Ciocalteu determination.

Based on the obtained results, as shown in Figure 2, the higher TPC was registered for the P1-C extract fraction, obtained by using RC membrane, greater also than other studied corn species (Zhang et al., 2017).

Also, it may be observed that the different membrane separation procedure (membrane type, RC/PES; membrane cut-off, 5/10 KDa; the working pressure, 2/3/5 bar) led to different concentrate/permeate fractions in terms of their phenolic content. The observed differences are related to the cut-off of each used membrane, which will separate the polyphenols present in the P0 extract comparatively to their average molecular weight.

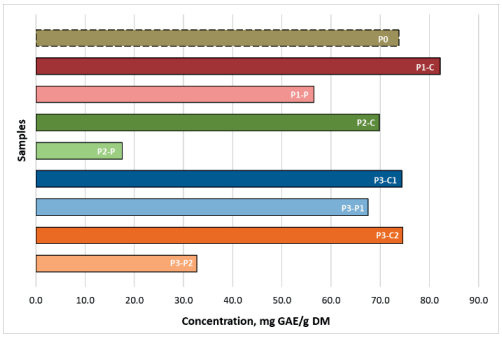

Figure 2. TPC of the initial corn extracts (P0) and resulted concentrate/permeate fractions resulted by membrane separation experiments

The polyphenols content is clearly higher for all the concentrate fractions (P1-C, P2-C, P3-C1, P3-C2) when compared the TPC values with those obtained for the permeate fractions (P1-P, P2-P, P3-P1, P3-P2), as observed from the graphical representation depicted in Figure 2.

Of the two membranes tested in this study, the RC type (cut-off 5 KDa) leads to a concentrate fraction richer in polyphenols, with a TPC value around 82 mg GAE/g DM (P1-C sample). At same working pressure (5 bar) but using PES membrane (cut-off 5 KDa), the obtained concentrate fraction P2-C registered a TPC around 70 mg GAE/g DM. The same way, permeate fractions resulted by these two experiments indicate very different TPC, near 57 mg GAE/g DM for P1-P and 18 mg GAE/g DM for P2-P.

Therefore, a higher retention of polyphenols may be reached by using RC membrane, that will promote the enrichment of the extract in valuable phenolic molecules.

Testing another type of PES membrane (cut-off 10 KDa), looks like the higher cut-off promotes the improvement of polyphenol content for the concentrate fraction, probably allowing the suspension in hydroalcoholic media of those compounds with higher molecular weight, avoiding their deposition on the membrane surface (losses) or their entrapment in the membrane pores (and, again, their loss). A possible conjugation of small phenolic species with higher glycoside fragments, poly- and

monosaccharides may be considered, being known the tendency of these entities to join in different molecular arrangements (Balasundram et al., 2006; Alara et al., 2012).

Total flavonoid content (TFC)

Natural flavonoids from different sources have been widely used due to their biological activity, such as antioxidant, antidiabetic, and antihyperlipidemic (Li et al., 2023). However, a great correlation of the corn extracts with potential biological activities was not established yet.

For the corn extract and derived C or P fractions, TFC was determined, being established the influence of the membrane separation process and membrane type upon the flavonoid bioactive components within each collected fraction.

According to Figure 3, the resulted values of TFC for P1-C, P3-C1 and P3-C2 fractions, were higher than the one obtained for P0 extract. The results showed that regenerated cellulose membrane separation (5 KDa, 3 bar working pressure) lead to the flavonoid-richest fraction (P1-C, 37.411 RE/g DM). However, the most efficient separation of the flavonoids was recorded when using PES membrane at the second pass, because less active compounds remained in the permeate fraction whose volume was also very small (20 ml). A strong correlation of TPC and TFC was registered (r = 0.928).

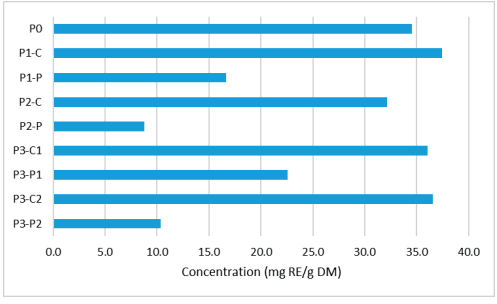

Figure 3. Total flavonoids content (TFC) of the initial corn extracts (P0) and different C/P fractions resulted by membrane separation

Quantification of individual phenolic acids, rutin and quercetin

Dominant group of cereals phenolic species, phenolic acids (PAs) are very important for their biologic activity (Kasprzak et al., 2018; Horvat et al., 2020). Thus, corn extracts, due to the PAs content became a valuable source of natural antioxidants.

Separation by HPLC of the obtained fractionated corn extract, resulted in a great content of syringic acid, as shown in Figure 4. The results are consistent with those reported by Kapcum et al. (2016) who found 31-200 mg/100 g syringic acid in red corn cob.

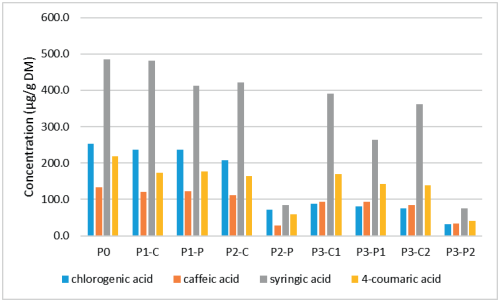

Figure 4. Phenolic acids quantification of the initial corn extracts (P0) and the resulted separation fractions

Initial P0 extract indicates the greatest content and concentrated fractions indicate significant syringic acid contents, a promising phenolic derivative with a wide range of pharmacological properties, including antioxidant, hepato-, cardio-, neuro-protective, antidiabetic or antimicrobial properties (Srinivasulu et al., 2018; Mirza et al., 2022; Sahari et al., 2024).

No less significant is the content of chlorogenic and 4-coumaric acids, well known for their therapeutic effects (Kaur, J., Kaur, R., 2022; Tehami et al., 2023; Huang et al., 2023; Nguyen et al., 2024).

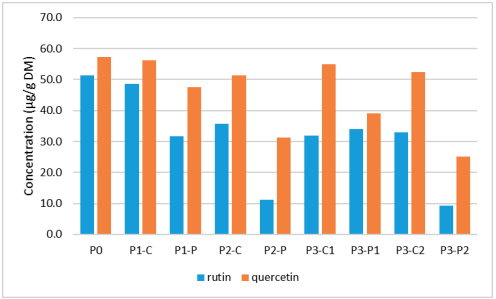

Figure 5. Rutin and quercetin quantification of the initial corn extracts (P0) and the resulted separation fractions

Rutin and quercetin are two important flavonols with potential applications in the pharmaceutical industry as well as that of food supplements. The values determined for these compounds in the analysed samples (Figure 5) were rather low, but together with the other polyphenols may contribute to the whole biological activity of the obtained extract.

Antioxidant activity

The total antioxidant activity was evaluated for C and P extract fractions and initial extract, P0, the obtained results being graphically represented in Figure 6. They are in good agreement with the TPC results, also to other report (Bani et al., 2023).

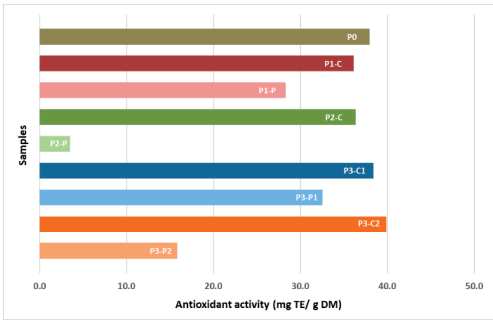

Figure 6. Graphic representation of the antioxidant activity for the initial corn extracts (P0) and different C/P fractions resulted by membrane separation

Higher antioxidant activity values are well correlated with the TPC values. When fractionate, P0 extract conduce to concentrated fractions with AA ranging from 36 near to 40 mg TE/ g DM.

As Figure 6 presents, the higher antioxidant activity is exerted by P3-C2 extract fraction, resulted when PES membrane was used for the separation, on the working pressure of 3 bar (P2-C3, AA of almost 40 mg TE/ g DM), well correlated to other studied corn species (Zhang et al., 2017).

Also, permeate fractions registered lowest AA values, P2-P barely reaching a value of 3.5 mg TE/ g DM. Conversely, the concentrate fraction resulted from this test indicates great antioxidant capacity (P2-C, 36.330 mg TE/g DM). Corroborating these experimental data, it can be concluded that the PES 5 KDa membrane is efficient for the concentration of corn extract in biological compounds with notable antioxidant activity.

CONCLUSIONS

A byproduct derived from coloured corn (husk of a Bloody Butcher red corn variety) was used to obtain a polyphenol-rich extract using mild extraction condition, with ethanol-water (1/1) by ultrasound treatment for 20 minutes.

By means of membrane separation several extract fractions were obtained with different concentration of phenolic compounds, starting from the investigated initial corn extracts.

According to obtained results, both regenerated cellulose membrane and polyethersulfone membrane can lead to efficient separation process. Higher cut-off of the used polyethersulfone membrane leads to higher ultrapure water and separation flows.

All the obtained fractions after separation process were characterized in terms of total phenolic content and antioxidant activity but also related to their flavonoid content and phenolic acids also. Total phenolic content and antioxidant capacity of the studied extract fractions were found to be comparative with other reports (around 80 mg GAE/g DM and respectively 40 mg TE/ g DM).

Important results were obtained for the concentrated fractions, well reflected both in the total content of phenols and antioxidant activity.

Also, significant flavonoid content and phenolic acids were identified also for the concentrated fractions derived by the corn extract.

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