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30 **Concentration of Bioactive Compounds from Elderberry (*Sambucus nigra* L.) Juice by**
31 **Nanofiltration Membranes**

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34

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43

44 **Abstract**

45 For the first time the chemical profile, physico-chemical parameters, inhibition of carbohydrate
46 hydrolysing enzymes associated with type 2 diabetes, and radical scavenging properties of *Sambucus*
47 *nigra* L. (elderberry) juice treated by nanofiltration (NF) were investigated. Three commercial NF
48 membranes with different molecular weight cut-off (MWCO) (400 and 1000 Da) and polymeric
49 material (composite fluoro-polymer and polyethersulphone) were tested. According to HPLC
50 analyses, most part of bioactive compounds were retained by the NF membranes producing a retentate
51 fraction of interest for the production of functional foods. The NP030 membrane, a polyethersulphone
52 membrane with a MWCO of 400 Da, exhibited the highest rejection towards phenolic compounds
53 when compared with the other selected membranes. Accordingly, the produced retentate fractions
54 exhibited the highest radical scavenging activity.

55

56 **Keywords:** Sambucus nigra L. .Juice processing . Phenols . Radical scavenging activity . Type 2
57 diabetes . Nanofiltration

58

59 **Introduction**

60 Sambucus nigra L. (elderberry) is a widespread plant of the Caprifoliaceae family growing in Europe,
61 North Africa, West Asia, and USA [1]. S. nigra berries or juices are used in the processing of jellies
62 and jams, and in the preparation of pies and liqueurs. Berries have been utilised also as dietary
63 supplements worldwide. All plant parts have been used in traditional medicine for their purgative,
64 diuretic, haemostatic effects as well as for the prevention of diabetes, cardiovascular diseases, and
65 cancer [2, 3]. The health benefits of S. nigra can be attributed to its characteristic chemical profile,
66 which includes anthocyanins, flavonols, flavanones, and flavones as the main secondary metabolites
67 [4]. Elderberry constituents demonstrated interesting antioxidant, anti-bacterial, anti-inflammatory,
68 anti-viral, and anti-allergic properties in both in vitro and in vivo studies [5,6,7,8]. Several studies
69 reported the beneficial role of anthocyanins-rich plants in diabetes due to their capacity to reduce
70 hyperglycaemia-induced oxidative stress and stimulate insulin secretion [9]. A therapeutic approach
71 to treat diabetes is to decrease post-prandial hyperglycaemia by the inhibition of carbohydrates-
72 hydrolysing enzymes such as α -amylase and α -glucosidase [10].

73

74 Recently, the recovery of bioactive compounds from elderberry juice has gained a grown interest in
75 the production of functional foods based on their positive contribution to the prevention of several
76 diseases. Organic solvent extraction is the most common technique used to extract bioactive
77 compounds but it is limited by harsh conditions such as high temperatures, which could lead to
78 degradation of thermolable compounds, and the use of potentially toxic solvents for human
79 consumption. Alternative green extraction techniques, such as ultrasound assisted extraction, and
80 super critical extraction guarantee safer conditions and preservation of thermolable compounds but
81 are characterized by high capital costs, which limit their widespread use. Pressure-driven membrane

processes, such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), offer key advantages over conventional methodologies since the separation process does not involve phase changes or chemical agents; additionally, they can operate at room temperature with high efficiency, simple equipment and low energy consumption [11]. These processes are based on the principle of selective permeation of molecules through semipermeable membranes under a mechanical pressure as driving force. In particular, NF membranes are characterized by narrow pore sizes from 0.5 to 2 nm: they are able to retain lower particles when compared to MF and UF membranes, offering particular advantages for the fractionation of molecules of similar molecular weight (in the range 100–1000 Da) through the selection of membranes with suitable molecular weight cut-off (MWCO). At this purpose, NF has been widely used for the separation, fractionation and concentration of bioactive compounds from a large variety of products [12]. There are no reports in the literature about membrane fractionation of *S. nigra* juice. Therefore, following our previous studies [13, 14, 15, 16, 17, 18, 19], this work was aimed at evaluating the viability of NF in the separation and concentration of bioactive compounds from the juice in order to produce fractions to be used for the formulation of functional foods. At this purpose, the performance of three commercial polymeric membranes was compared in terms of productivity and rejection towards bioactive compounds of the juice. The antioxidant and hypoglycaemic properties of both permeate and retentate fractions were also analysed and compared to those of the untreated juice.

Materials and Methods

Chemicals and Reagents

All HPLC grade solvents were obtained from Carlo Erba Reagents (Milan, Italy). Astragalin, cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside, (+)-catechin, gallic acid, quercetin, protocatechic acid and p-coumaric acid were purchased by Extrasynthese (Genay, France). Acarbose was obtained from Serva (Heidelberg, Germany). Ascorbic acid, aluminium chloride hexahydrate, ferrous sulfate, sodium molybdate, sodium nitrite, sodium hydroxide, sodium carbonate, 2,2'-azino-

108 bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), o-
109 dianisidine, and peroxidase/glucose oxidase were obtained from Sigma-Aldrich (Milan, Italy).

110

111 **Preparation of Raw Juice**

112 *S. nigra* berries were collected in Southern Italy (Calabria) at the end of September 2015 near the
113 river Crati (Tarsia, Cosenza, Italy). Samples were identified by Dr. NG Passalacqua, Natural History
114 Museum of Calabria and Botanic Garden, University of Calabria. Berries were destemmed, washed
115 and then pressed by using a manual press. The extracted juice was pre-filtered through a nylon cloth
116 in order to remove solid residues and impurities, stored in a refrigerator cell at -17°C and defrosted
117 before use. The extraction procedure gave an average juice yield of 52.4% (w/w).

118

119 **Membranes and Nanofiltration Set-Up**

120 Membrane filtration tests were performed by using a high-pressure cross-flow filtration cell (model
121 HP4750) supplied by Sterlitech Corporation (Washington, USA) with a feed volume capacity of 300
122 mL, a diameter of 5.1 cm, and an effective membrane area of 20.4 cm². Experiments were performed
123 at room temperature. Before juice treatment, each membrane was conditioned in pure water for 24 h,
124 and then placed in the cell. Experiments were carried out by applying a N₂ gas transmembrane
125 pressure of 20 bar. Each membrane filtration test was conducted three times. The permeate flux (J_p)
126 through each membrane, at a given pressure, was defined as the volume permeated per unit area and
127 per unit time, according to Eq. (2):

128 **$J_p = V_p A \cdot \Delta t$**

129 where V_p (L) is the volume of permeate, A (m²) is the membrane area, and Δt (h) is the operation
130 time. The average and relative standard deviation were calculated. Three different commercial flat-
131 sheet membranes namely Etna 01PP (from Alfa Laval, Lund, Sweden), NP010 and NP030 (from
132 Mycrodin-Nadir, Wiesbaden, Germany) were used for the treatment of the juice. Their typical
133 characteristics according to the manufacturers' data sheet are reported in Table 1.

134 The rejection (R, %) of bioactive compounds was calculated by using the following Eq. (2):

135
$$R = (1 - C_p/C_f) \cdot 100$$

136 where C_f and C_p are the feed and permeate concentration, respectively.

137

138 Rejection values, expressed as average $\pm$ SD, are the means of three different experimental runs and
139 data statistically significant were evaluated according to the ANOVA test followed by Bonferroni t-
140 test ($p < 0.05$).

141

142 **Physico-Chemical Parameters of Elderberry Juice**

143 The total soluble solids were estimated by a digital refractometer PR-201 α (Atago, Tokyo, Japan) and
144 expressed as $^{\circ}$ Brix (sucrose percentage) at 20 $^{\circ}$ C. The pH of *S. nigra* juice was measured by a pH
145 meter (Basic Model 20, Crison). Fresh-juice colour was measured using a Konica Minolta CM-
146 700/600d spectrophotometer (Konica Minolta Sensing, Japan) at 25 $^{\circ}$ C. Data were expressed as L^*
147 (lightness/darkness in a range 0–100), a^* (greenness/redness in a range between –60 and + 60) and
148 b^* (blueness/yellowness in a range between –60 and + 60).

149

150 **High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) Analyses**

151 Analyses were carried out by using a Knauer (Asi Advanced Scientific Instruments, Berlin) system
152 equipped with two pumps (Smartiline Pump 1000), a Rheodyne injection valve, a photodiode array
153 detector uv/vis equipped with a semi-micro cell, and a Knauer RP C18 column (250 mm $\times$ 4.6 mm,
154 5 μ m). The mobile phase was water/formic acid (solvent A: 99.9:0.1, v/v) and acetonitrile/formic acid
155 (solvent B: 99.9:0.1, v/v) with the gradient profile of 0.01–20.00 min 5% B isocratic; 20.01–50 min,
156 5–40% B; 50.01–55 min, 40–95% B; 55.01–60 min 95% B isocratic. The flow rate was 1 mL/min.
157 Peaks were monitored at 280 and 350 nm. Cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside,
158 quercetin, quercetin-3-O-rutinoside, astragalin, (+)-catechin and protocatechic acid were quantified.
159 A calibration straight for each standard was obtained by analysing the standard solution diluted at

160 different concentrations. Identification and quantification were carried out based on recorded
161 retention times in comparison with authentic standards. Analyses were performed in triplicate.

162

163 **α -Amylase and α -Glucosidase Inhibitory Assay**

164 The α -amylase inhibitory test was performed as previously described [20]. Concisely, α -amylase
165 solution, starch solution, and colorimetric reagent were prepared. Control and samples (at
166 concentrations in the range 0.01–1 mg/mL) were added to starch solution and left to react with the
167 enzyme at room temperature for 5 min. The generation of maltose was quantified at 540 nm by the
168 reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. The α -glucosidase inhibition
169 was measured as previously described [21]. Both control and samples (at concentrations in the range
170 0.01–1 mg/mL) were added to maltose solution and left to equilibrate at 37 °C. The reaction was
171 started by adding the enzyme and left to incubate at 37 °C for 30 min. A perchloric acid solution was
172 used to stop the reaction. The supernatant was collected and mixed with peroxidase/glucose oxidase
173 and o-dianisidine and left to incubate for 30 min at 37 °C. The absorbance was measured at 500 nm.
174 Acarbose was the positive control in both tests.

175

176 **Radicals Scavenging Activity (ABTS and DPPH Assays)**

177 ABTS test was used to investigate the anti-radical activity of *S. nigra* samples (concentrations range
178 of 40–2.5 mg/mL) [22]. ABTS radical cation was produced by reacting 7 mM ABTS solution with
179 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at 25 °C for 12 h before
180 use. After addition of 25 μ L of sample to 2 mL of diluted ABTS solution, absorbance at 734 nm was
181 measured at 6 min. The antioxidant properties were assessed also by using DPPH assay [22]. A
182 mixture of DPPH (0.25 mM in ethanol) and samples at different concentrations (5–1000 μ g/mL) was
183 prepared and left for 30 min at room temperature. The bleaching of DPPH was measured at 517 nm.
184 The positive control was ascorbic acid.

185

186 **Results and Discussion**

187 *Physico-Chemical Parameters and Chemical Profile of S. nigra Juice*

188 S. nigra juice had a pH of 3.47 and showed a content of tartaric and citric acid of 1.04 and 19.86 g/L,
189 respectively. The content of glucose and fructose was of 27.3 and 21.1 g/L, respectively (Table 2).
190 The total soluble solids value was of 11.60 °Brix. Hunter colour values (L*, a* and b*) of the juice
191 were measured. The brightness/whiteness colour coordinate L* was of 10.94; the redness (a*) and
192 yellowness (b*) indicators were of 0.71 and 0.58, respectively (greenness/redness in a range between
193 -60 and + 60). A chroma value (C*) of 0.92 was calculated.

194 By referring to the HPLC profile, cyanidin-3-O-sambubioside (300.7 mg/L), followed by cyanidin-
195 3-O-glucoside (147.1 mg/L) and quercetin (142.9 mg/L), were the main constituents of untreated
196 elderberry juice. These data are in agreement with those reported by Wu et al. [23] for the European
197 elderberry variety. Other studies reported significant amounts of anthocyanins in elderberry juice
198 [24,25,26]. In particular, cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside represented over
199 83% of total anthocyanins in S. nigra juice [25]. In a previous work, Lee and Finn [27] indicated also
200 quercetin-3-O-rutinoside as a major constituent of elderberry juice.

201

202 **Water Permeability of Selected Membranes**

203 Figure 1 shows the water permeability of selected membranes at 25 ± 1 °C. Among the selected
204 membranes the ETNA 01PP showed the highest water permeability of about 15.5 L/m² h. As
205 expected, the NP010 membrane presented a higher water permeability when compared to that of
206 NP030 being of the same material but with a higher molecular weight cut-off (1000 Da in comparison
207 to 400 Da).

208

209 **Evaluation of Permeate Flux**

210 The performance of membrane separation processes is usually expressed in terms of two parameters:
211 flux and rejection. Both parameters depend not only on the membrane properties but also on the

operational conditions [28]. The time evolution of the permeate flux measured in the treatment of the elderberry juice with the investigated membranes in selected operating conditions is illustrated in Fig. 2. A typical flux decay was observed due to concentration polarization and fouling phenomena until to reach a steady-state value. In particular, the J_p vs. time curve could be divided in two periods: an initial period characterized by a decrease of permeate flux, more significant for the Etna 01PP membrane; a second period in which the permeate flux reaches a steady-state value. Experimental results are in agreement with data observed for water permeability: the ETNA 01PP membrane exhibited the highest permeate flux with a steady-state value of about 6 L/m² h, followed by the NP010 and NP030 membranes with steady-state values of 4 and 2 L/m² h, respectively.

Characterization of Nanofiltered Juice

The analytical determination of organic acids (citric and tartaric acid) and sugars (glucose and fructose) in the feed and filtered juice samples indicated that these compounds are recovered in the permeate stream of the process (Table 2), as expected based on MWCO of selected membranes.

Table 3 shows the composition of the juice and permeate samples in terms of bioactive compounds determined according to HPLC measurements. All selected membranes showed high rejections towards anthocyanins (cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside), quercetin-3-O-rutinoside and astragalin (higher than 75%) and lower rejections towards protocatechic acid and catechin (between 25 and 42%). The NP030 membrane, with the lowest MWCO, showed higher rejections for all bioactive compounds in comparison to the other two NF membranes (Fig. 3). For this membrane, anthocyanins and quercetin-3-O-rutinoside were completely rejected. NF membranes with MWCO of 1000 Da exhibited a similar selectivity towards investigated compounds indicating that rejections values were not affected by the polymeric nature of the membranes. According to these results, the selected membranes displayed a good separation efficiency of sugars and organic acids from phenolic compounds. High separation factors between sugars and phenolic compounds were

also observed by Conidi et al. [29] in the treatment of clarified pomegranate juice with thin-film composite membranes having MWCO of 1000 and 2000 Da.

240

241 **In vitro Antioxidant and Hypoglycaemic Activity**

242 Negative impact of radicals on humans and animals is responsible for growing research studies in
243 antioxidant properties of compounds that could protect living organisms from the damaging influence
244 of these reactive species. Herein, the elderberry untreated juice as well as retentate and permeate
245 samples obtained with selected NF membranes were tested for their antioxidant potential by using
246 ABTS and DPPH assays. Retentate fractions showed a higher radical scavenging potential in
247 comparison to the untreated juice and permeate fractions (Table 4). In DPPH test, the retentate
248 obtained by membrane NP030 was the most active with an IC₅₀ value of 0.3 mg/mL, followed by
249 retentates of ETNA01PP and NP010 membranes, with IC₅₀ values of 0.5 mg/mL. The same trend
250 was observed in ABTS test: the retentate of NP030 membrane showed an IC₅₀ value of 0.5 mg/mL,
251 followed by retentates of ETNA01PP and NP010 membranes with IC₅₀ values of 0.7 and 0.8 mg/mL,
252 respectively

253 The potential hypoglycaemic properties were analysed by means of the inhibition of α -amylase and
254 α -glucosidase assays (Table 4). The most promising activity was found against α -glucosidase. In
255 particular, the permeate of NP010 membrane was the most active with an IC₅₀ value of 0.21 mg/mL,
256 followed by NP030 permeate (IC₅₀ of 0.3 mg/mL). Both permeates were more active than the
257 untreated juice (IC₅₀ of 0.4 mg/mL) and retentates (IC₅₀ of 0.7 and 0.6 mg/mL, respectively). The
258 best α -amylase inhibitory activity was observed for the NP030 permeate with an IC₅₀ value of 0.5
259 mg/mL. A variety of phenolic compounds, identified in elderberry, are able to inhibit the activity of
260 α -amylase and α -glucosidase [30, 31]. Among them, cyanidin and its glycosides are considered
261 promising candidates for the use in the prevention of type 2 diabetes. Structure-activity relationship
262 of cyanidin and its glycosides against α -amylase and α -glucosidase evidenced that the presence of
263 glucose moiety at the 3-O-position of cyanidin increases the potency of pancreatic α -amylase

264 inhibition with IC₅₀ values of 0.4 and 0.3 μ M, respectively. Moreover, when cyanidin or cyanidin-
265 3-O-glucoside at concentration of 1 μ M were added to acarbose (3.12 μ M) a synergistic effect against
266 α -amylase activity was observed. A good activity was observed also for quercetin with IC₅₀ values
267 of 0.5 mM and 8 μ M for α -amylase and α -glucosidase, respectively [31, 32]. These compounds are
268 the most representative of our samples.

269

270 **Conclusions**

271 The radical scavenging activity and the carbohydrate hydrolysing enzymes inhibitory properties of
272 elderberry juice treated by NF were investigated in relation to the phytochemical content. For this
273 purpose, fractions obtained with three commercial NF membranes were characterized and compared
274 with the untreated juice. All selected membranes showed high rejections towards anthocyanins,
275 quercetin-3-O-rutinoside and astragalin and lower rejections towards protocatechuic acid and
276 catechin. Retentate fractions exhibited a higher antioxidant activity in comparison to the untreated
277 juice and permeate fractions with the NP030 membrane, characterized by the lowest MWCO, the
278 most promising one. Permeate samples from the NP030 membrane exhibited also a higher
279 hypoglycaemic activity in comparison to that of the untreated juice, as demonstrated by α -amylase
280 and α -glucosidase inhibitory assays.

281

282 **Abbreviations**

283 ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

284 DPPH: 2,2-Diphenyl-1-picrylhydrazyl

285 IC₅₀ : Half maximal inhibitory concentration

286 MWCO: Molecular weight cut-off

287 NF: Nanofiltration

288

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Table 1 Characteristics of selected flat-sheet membranes for the treatment of *S. nigra* juice

Membrane type	Etna 01 PP	NP010	NP030
Manufacturer	Alfa Laval	Mycrodin-Nadir	Mycrodin-Nadir
Membrane material	Composite fluoropolymer	Polyethersulphone	Polyethersulphone
Molecular weight cut-off (Da)	1000	1000	400
pH operating range	1–11	0–14	0–14
Max. operating temperature (°C)	60	95	70

Table 2 Analytical determination of sugars and organic acids in filtered samples

Compounds (g/L)	Feed	Permeate		387
		NP010	NP030	ETNA01PP
Tartaric acid	1.1 ± 0.1	2.1 ± 2.2	1.8 ± 0.6	2.3 ± 9.3
Citric acid	19.9 ± 1.1	19.1 ± 1.8	18.3 ± 1.1	19.7 ± 2.5
Glucose	27.3 ± 2.1	26.6 ± 2.3	25.1 ± 2.1	27.1 ± 2.8
Fructose	21.1 ± 2.0	20.1 ± 1.9	19.8 ± 2.0	20.9 ± 2.6

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Table 3 HPLC profile of permeate samples from NF processing of elderberry juice

Compounds (mg/L)	Feed	Permeate		
		NP010	NP030	ETNA01PP
Cyanidin-3-O-sambubioside	300.7 ± 2.6	24.9 ± 0.8	ND	35.5 ± 0.8
Cyanidin-3-O-glucoside	147.1 ± 1.4	9.8 ± 0.9	ND	30.9 ± 0.7
Quercetin-3-O-rutinoside	18.2 ± 0.4	1.2 ± 0.2	ND	2.1 ± 0.1
Quercetin	142.9 ± 2.5	61.3 ± 1.5	47.3 ± 2.1	81.1 ± 2.7
Astragalin	6.47 ± 0.2	1.0 ± 0.1	0.5 ± 0.2	1.5 ± 0.2
(+)-Catechin	19.3 ± 0.9	12.0 ± 1.1	11.1 ± 0.3	14.6 ± 0.8
Protocatechic acid	38.0 ± 1.0	24.7 ± 1.4	21.4 ± 0.6	28.6 ± 0.9

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Table 4. Antioxidant properties and inhibition of carbohydrates-hydrolysing enzymes of elderberry juice

S. nigra	ABTS test	DPPH test	α -Amylase inhibitory test	α -Glucosidase inhibitory test
Untreated juice	> 0.5	2.9 ± 0.8	0.6 ± 0.8	0.4 ± 0.8
R-ETNA01PP	0.7 ± 0.06	0.5 ± 0.06	NA	0.6 ± 0.7
R-NP010	0.8 ± 0.08	0.5 ± 0.05	0.9 ± 0.8	0.7 ± 0.9
R-NP030	0.5 ± 0.09	0.3 ± 0.02	NA	0.6 ± 0.8
P-ETNA01PP	> 0.5	> 1	0.8 ± 0.8	0.8 ± 0.5
P-NP010	> 0.5	> 1	NA	0.2 ± 0.2
P-NP030	> 0.5	> 1	0.5 ± 0.8	0.3 ± 0.3

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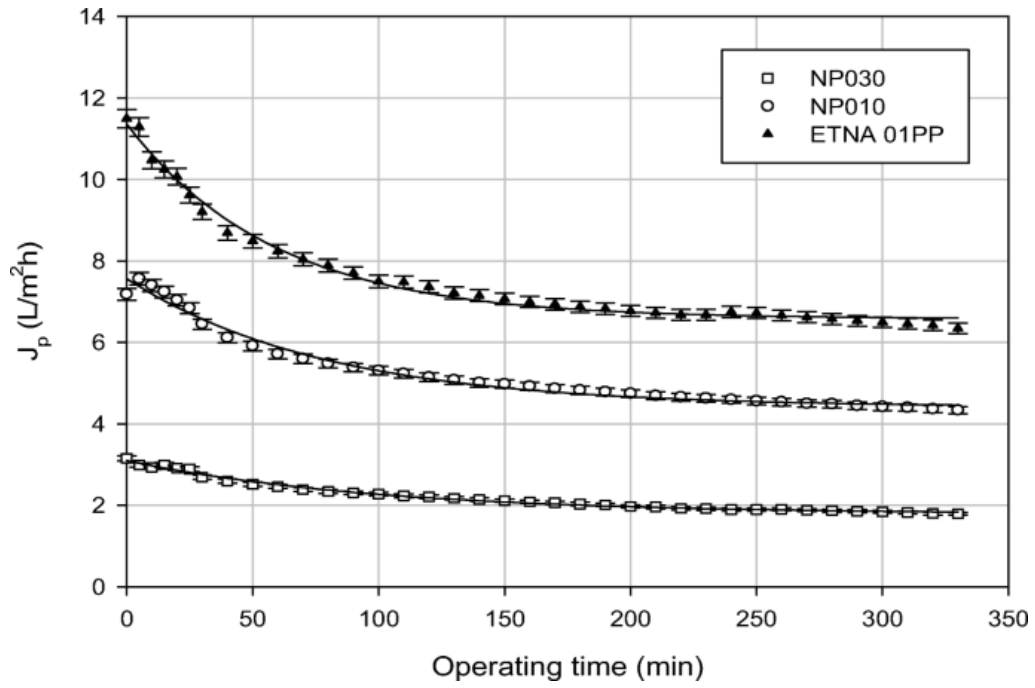


Fig. 1. Water permeability of selected membranes at 25 ± 1 °C

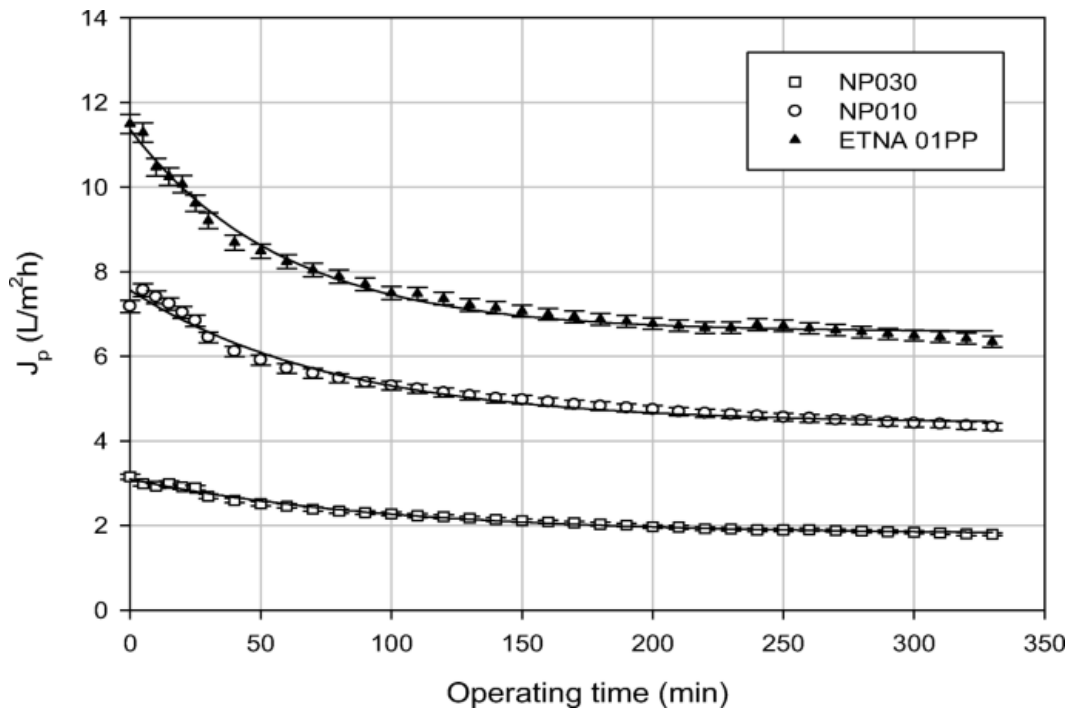


Fig. 2. Treatment of *S. nigra* juice with selected membranes. Permeate flux as a function of operating time

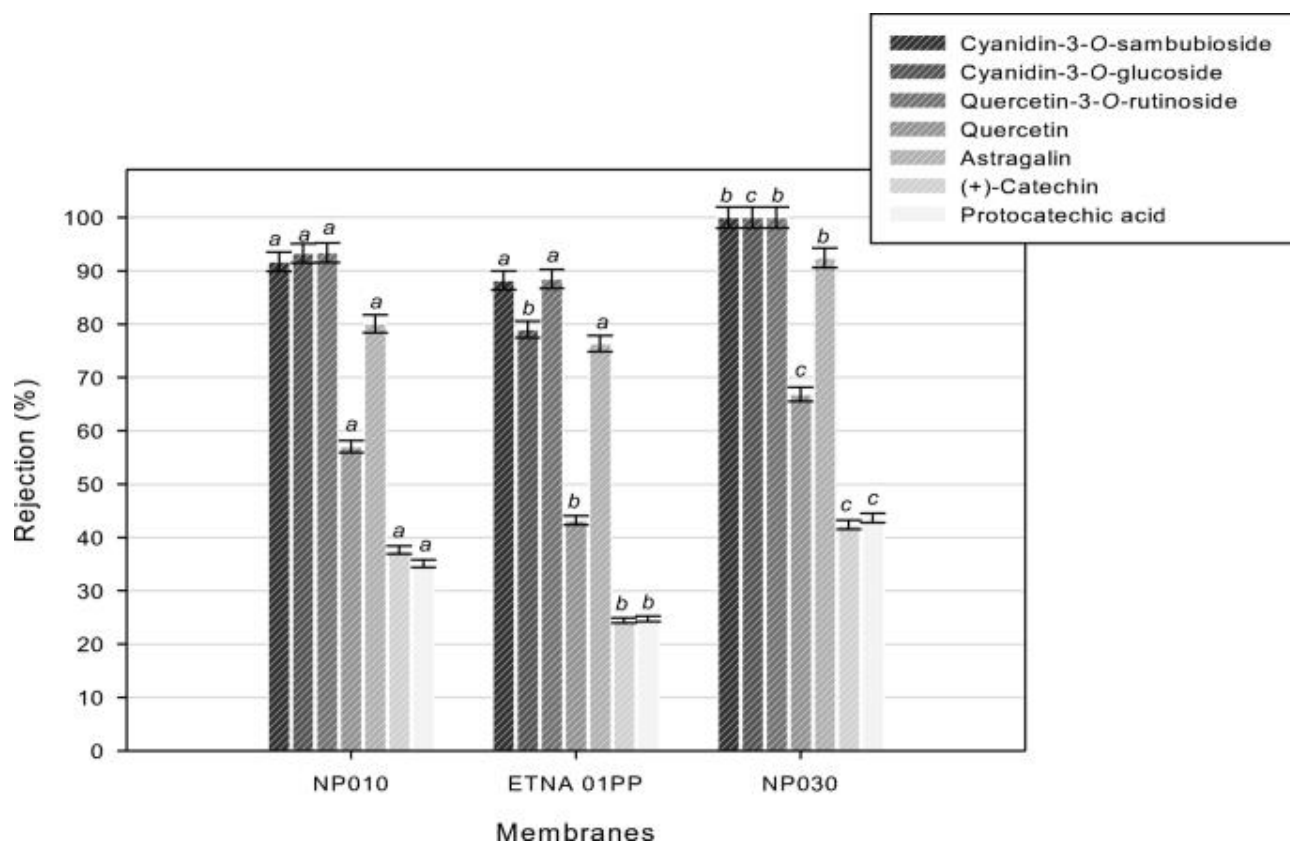


Fig. 3. Rejection of NF membranes towards bioactive compounds of *S. nigra* juice. Data are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). For each specific compound, values for different membranes with different letters are significantly different at $p < 0.05$