Separation and Fractionation of Oligouronides by Shear-Enhanced Filtration

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Abstract: This paper investigates the separation of anionic oligosaccharides produced by thermal degradation at 120°C of polygalacturonic acids (PGA). Initially, the PGA solution was clarified by two successive centrifugations. These centrifugations were later replaced by two microfiltrations (MF) which did not modify oligosaccharides composition. A dynamic filtration module with a disk rotating at 2000 rpm near a circular organic membrane was used in all filtration steps. Several micro- and ultrafiltrations (MF and UF) in cascade (50, 20, and 10 kDa) and a nanofiltration at 1 kDa were used to separate oligomers with degrees of polymerization (Dp) ≤ 7. Permeate fluxes ranged from $400 \, \mathrm{Lh^{-1}m^{-2}}$ for the first MF to $240 \, \mathrm{Lh^{-1}m^{-2}}$ for the 10 kDa UF and 60 to $100 \, \mathrm{Lh^{-1}m^{-2}}$ for the 1 kDa NF, showing the high potential of dynamic filtration for this application.

Keywords: Oligosaccharides separation, cascade filtration, rotating disk, ultrafiltration, nanofiltration

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INTRODUCTION

Some oligosaccharides and more especially anionic oligosaccharides have been shown to present interesting biological properties on numerous organisms such as bacteria, fungi, mammalians, and plants (1). Traditionally, oligosaccharides are defined as polymers of monosaccharides with degrees of polymerization (Dp) ranging from 2 to 10 (or 3 and 10 according to the IUB-IUPAC nomenclature) but molecules with $20 < Dp < 25$ are often considered as oligosaccharides. Among these compounds, oligouronides such as oligogalacturonates (OGAs), obtained from plant pectins or bacterial oligoglucuronates (OGUs), may be considered as good models to investigate biological activities. Their structures are relatively homogeneous and they are available after pectin or glucuronan cleavage by enzymatic or other procedures (2, 3) However, the high degree of purification required for these applications is still a major obstacle for their development, as present techniques can lead to very high prices. It is thus important to develop extraction processes which are fast, effective, and suitable for large scale production at a reasonable cost.

Membrane processes such as ultrafiltration (UF) and nanofiltration (NF) are well suited for providing the initial purification steps in industrial production since they are continuous processes, easily extrapolable to large production and they generate less pollution than other processes such as solvent extraction. But membrane fouling, which often occurs with complex biological fluids, limits their output and their selectivity. At this time, these technologies have not often been applied to oligosaccharides fractionation excepted for the neutral chito-oligosaccharides (4), other fructo-oligosaccharides (5) and caprine milk oligosaccharides recovery (6) using cross-flow diafiltration with tubular ceramic membranes. In this last paper, the authors report a two–step process starting with a 50 kDa diafiltration to separate proteins from oligosaccharides, lactose and calcium in the permeate. The collected permeate was then diafiltered at 1 kDa to recover 82% of initial oligosaccharides mass in the resulting retentate, while collecting lactose and calcium in the permeate.

This paper investigates the extraction and fractionation of oligogalacturonanes with $Dp < 7$, produced by thermal degradation of polygalacturonic acid. We have used and evaluated a possible purification process consisting of a cascade of microfiltrations, ultrafiltrations, and a final nanofiltration using a shear enhanced filtration system with a rotating disk designed in our laboratory (7), described in the next section.

MATERIAL AND METHODS

Production of Oligogalacturonates

Oligogalacturonates were produced by thermal degradation of a commercial polygalacturonic acid (PGA) (Sigma) according to the method of

Simms et al. (8). A 1% PGA solution in water was first heated at 120°C for 40 min in an autoclave. After being cooled to room temperature, the solution was centrifuged at 14,000 g, and 4°C for 10 min. The supernatant (or permeate) S_1 was then adjusted to pH 2 by addition of diluted HCl in order to precipitate the high molecular weight galacturonan remained in the PGA heated solution. After a second centrifugation, the supernatant (or permeate) S_2 was collected and adjusted to pH 7 by addition of diluted NaOH. Since these successive centrifugations were time consuming because of the small capacity of our centrifuge, they were later replaced by two microfiltrations steps at 0.45 μm in cascade.

Oligogalacturonates (OGA) Analysis

These OGAs were quantified by microscale colorimetric assays using *meta*-hydroxydiphenyl (mHBP) (9). The colorimetric assays were achieved on a microtitration plate using D-galacturonic acid as standard. A microplate spectrophotometer (Opsys MR, Dynex technologies, VA, USA) was used for absorbance measurements.

The molecular weight profile of galacturonan obtained by PGA heating was controlled by ion exchange chromatography using a Cyclobond I 2000 connected to a RI detector (Melz). The eluent was a sodium phosphate buffer (100 nM, pH5).

Oligogalacturonates (OGAs) separation was obtained by size exclusion chromatography. 100 mg in 10 ml of a 50 mM ammonium formate solution were loaded on an Amersham Bioscience column (2.6 × 100 cm) packed with a Biogel P6 fine (Biorad), the elution was performed with the ammonium formate solution at a flow rate of 0.8 ml/min (10). Detection was achieved with a RI detector (Melz). Dps were evaluated by comparison with purified oligogalacturonates used as standards.

Table 1 lists the massic distribution per degrees of polymerisation for $3 < Dp < 10$ obtained by molecular sieving on a column of Biogel for 1 g of S_2 solution. Their total mass was 541.4 mg.

Filtration Systems

The rotating disk filtration system used at all filtration steps (MF, UF and NF) was designed in our laboratory. It consisted of a cylindrical housing of inner

Table 1. Massic repartition per degree of polymerisation obtained for 1 g of S_2 solution resulting from 3 g of PGA

Degrees of polymerization	10	9	8	7	6	5	4	3
Mass (mg)	71.6	134.1	82.0	70.8	50.1	45.2	42.4	45.2

radius R = 0.0775 m, machined from stainless steel and featured an aluminum disk equipped with 6 mm-high vanes of radius $R_d = 0.0725$ m rotating at adjustable speeds up to 3000 rpm around a horizontal shaft (Fig. 1). A 190 cm^2 organic fixed membrane of appropriate pore size or cut-off, supported by a 0.3 mm thick polypropylene grid, was mounted on the flat front end of the housing, while the solution was fed through the back plate on the other side of the disk. The fluid was then centrifuged by the disk over the rim to the membrane side and recirculated between the membrane and disk, before being evacuated through the hollow shaft. The gap (e) between the membrane and the disk was 15 mm. The permeate was collected from a tap located at the top of the front plate. The permeate flow rate was measured by collecting the permeate in a beaker continuously weighted on an electronic scale (Sartorius, Germany) connected to a micro-computer calculating the derivative of the collected volume with respect to time and providing a value of flux every ten seconds if needed. The peripheral pressure was measured at the top of the cylindrical housing by a Validyne DP15 pressure transducer. The test fluid was fed from a 10 L-thermostated reservoir by a volumetric membrane pump at a flow rate of 240 L h^{-1} in all filtration tests in order to be in excess of the highest permeate flow rate encountered. Circuit internal volume was 1 L. In concentration tests, the fluid was recirculated to the reservoir while the permeate was collected in another tank.

In the microfiltration steps, a MSD pilot built by Wesfalia Separator Membraflow (Aalen, Germany) and described in (11) was used for comparison. It consisted of two parallel hollow shafts rotating at the same speed, each one bearing 6 ceramic membrane disks with a central hole for passage of the shaft (pore size 0.2 μm, skin thickness 10 μm) of 45 mm outer radius (R_1) and 20.5 mm inner radius (R_2). The total membrane area was 0.121 m^2 (100.8 cm^2 for each disk), and the shafts and disks were enclosed in a stainless steel housing. Disks from each shaft overlap each over for 15.5%
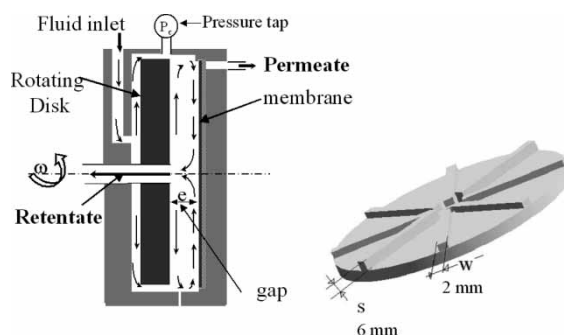
Figure 1. Schematic of rotating disk-fixed organic membrane module with a disk bearing 8 radial vanes.
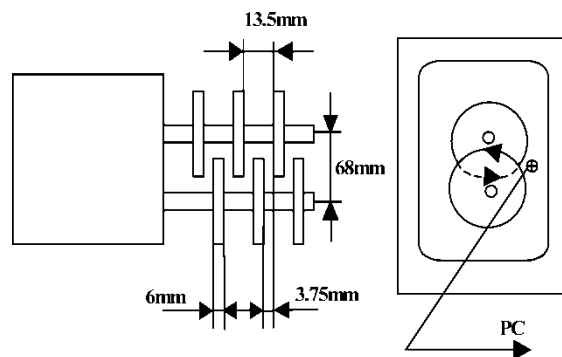
*Figure 2.* Schematic of the MSD laboratory pilot, showing only 6 ceramic membrane disks instead of 12.

of their surface. The axial gap between the two overlapping disks was 3.75 mm and the disk thickness 6 mm (Fig. 2). The inter axes distance "d" was 68 mm. The permeate was collected inside each disk by 14 flat hollow channels, 1.0 mm high and 4 to 9 mm wide and was evacuated through the hollow shafts. The inlet flow was also 240 Lh^{-1}.

RESULTS

Clarification of Heated PGA Solutions by Microfiltration (MF)

After heating, the PGA solution was filtered on the single rotating disk system with a 0.45 μm Nylon membrane, using a disk equipped with 6 mm vanes rotating at 2000 rpm, at a temperature of 23°C and a pressure of 200 kPa, without permeate recycling. The variation of permeate flux of this first MF producing a permeate S_1, with the volume reduction ratio (VRR) is displayed in Fig. 3. The same solution was also microfiltered with the MSD pilot using 0.2 μm ceramic disk membranes rotating at 1500 rpm and a pressure of 170 kPa and temperature of 21°C. The corresponding variation of flux with VRR is also shown in Fig. 3. If, at low VRR, the MSD permeate flux was higher than that of the single disk system, it dropped faster with increasing VRR and became lower than the other flux at a VRR of 3. This effect may be due in part to the fact that the bulk oligomer concentration in the MSD housing was about 40% higher than the feed one, as the permeate flow rate was around 70 Lh^{-1} against a feed flow of 240 Lh^{-1}. In the single disk system, permeate flow rate was very small in comparison with feed flow rate, so housing concentration was nearly equal to the feed one.

The previous permeate S_1, brought to pH 2, was then filtered on a 0.45 μm nylon membrane as in the 1st MF in order to collect a permeate
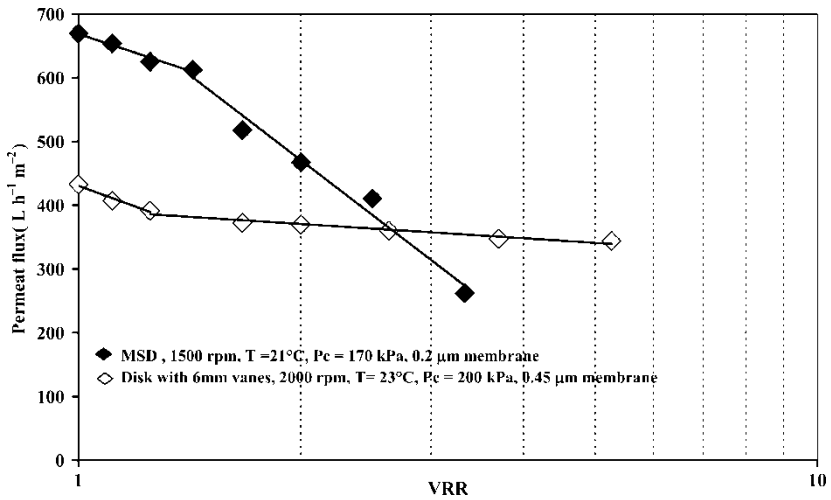
Figure 3. Comparison of variations of permeate fluxes during the 1st MF (permeate S_1) with volume reduction ratio (VRR) for the MSD pilot using 0.2 μm ceramic membranes and the rotating disk-fixed membrane module using a 0.45 μm Nylon membrane.

(S_2) containing only low molecular weight galacturonans. The corresponding variation of flux with VRR is shown in Fig. 4. The fluxes were much lower than for the first MF, but after an initial decay as in Fig. 3, the flux remains almost independent of VRR.

In order to verify that the replacement of these two centrifugations by two microfiltrations at 0.45 μm had not altered the composition of S_2, we have compared in Fig. 5 chromatographic profiles of S_2 produced by each process. The molecular weight distribution of galacturonans in S_2 reflected that high, medium, and low molecular weight molecules fractionated by SEC and eluted respectively during the first 60 ml, from 120 to 220 ml and after 220 ml of elution were present in each S_2 samples collected either by centrifugation or microfiltration on the nylon membrane.

Extraction of Oligosaccharides of Dp < 25

Two filtration procedures were tested. The first included an UF at 50 kDa followed by a NF at 1 kDa on PES membranes. The second one corresponded to two UF in cascade at 20, and 10 kDa before the NF at 1 kDa in order to retain more of the large solutes. The variations with VRR of permeate fluxes resulting from these UF are displayed in Fig. 6. As expected, the permeate flux was larger at 50 kDa than at 20 kDa, but it dropped faster with increasing VRR and became smaller at VRR > 5. However, all these
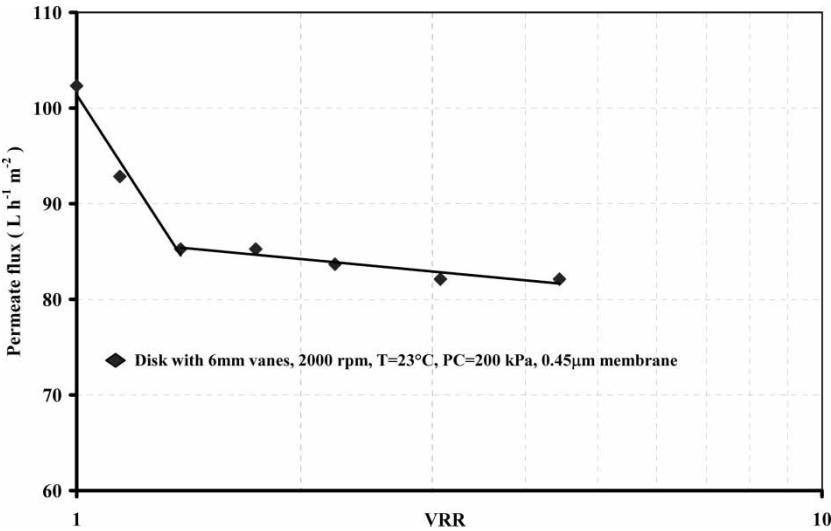
Figure 4. Variation of permeate flux during the 2nd MF (permeate S2) with VRR for the rotating disk- organic membrane module.

fluxes were very large, even when the VRR exceeded 5 and reached $300 \, \mathrm{Lh^{-1}m^{-2}}$ at 20 kDa and $235 \, \mathrm{Lh^{-1}m^{-2}}$ at 10 kDa. Chromatographic profiles of supernatant S_2 after the 2nd centrifugation, retentate of UF at 50 kDa and retentate of NF at 1 kDa obtained with a second Biogel P6 column are shown in Fig. 7. The molecular weight distribution of galacturonans in S_2, compared to those obtained in retentates collected after UF at 50 and NF at 1 kDa membranes, revealed that the high molecular weight molecules fractionated from 120 to 220 ml of elution were completely
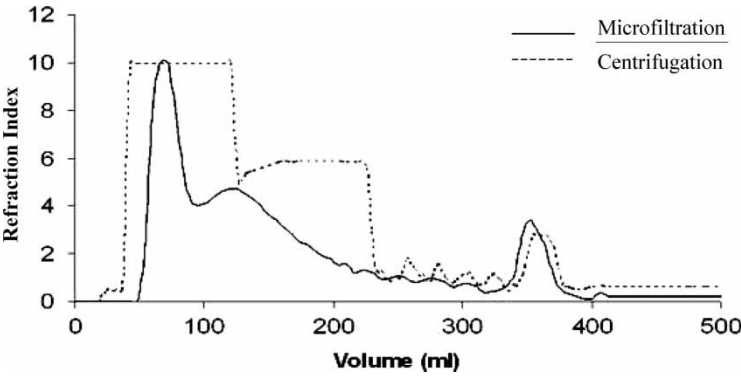
Figure 5. Size exclusion chromatography- on Biogel P6- of S_2 fractions obtained by centrifugation (solid line) and MF (dashed line).
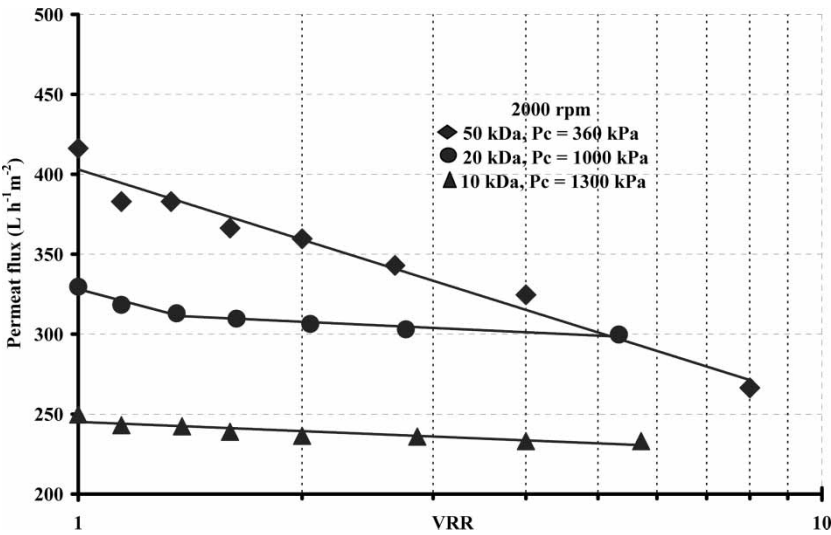
Figure 6. Comparison of variations of permeate fluxes with VRR for a single UF at 50 kDa and for two UF in cascade at 20 and 10 kDa successively.

eliminated after filtration on the 50 kDa membrane, while the content of the low molecular weight galacturonan increased. So, the procedure used was considered to be adequate for the purification of medium and low molecular weight galacturonans.

Figure 8 compares the variations with VRR of two permeates resulting from a NF at 1 kDa, the first corresponding to a feed solution S_3 obtained from UF at 50 kDa and the second to a feed S'_3 provided by a cascade of two filtrations at 20 and 10 kDa. It can be seen that the feed issued from the
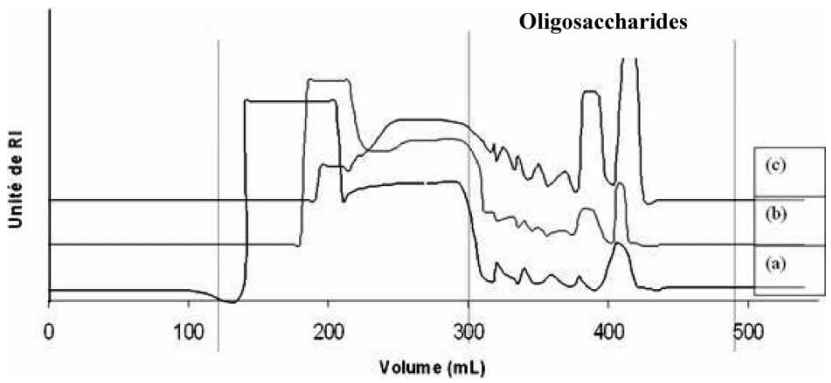
Figure 7. Chromatographic profiles of fractions obtained on fine Biogel P6. for; (a): supernatant S_2 after 2nd centrifugation (b): 50 kDa retentate; and (c): 1 kDa retentate.
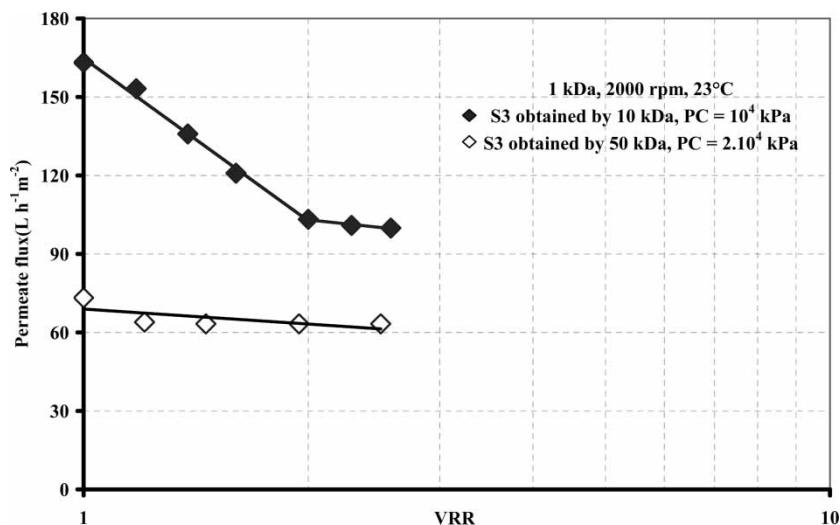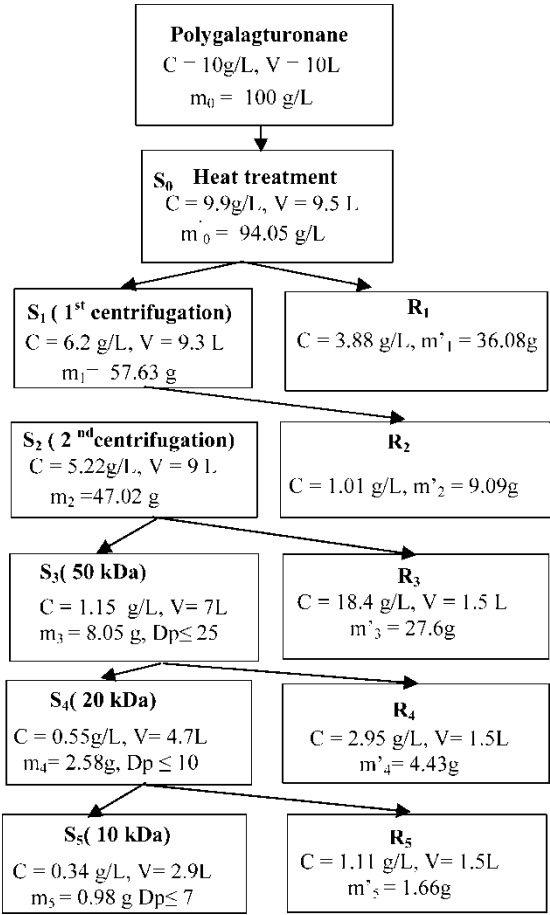
Figure 8. Comparison of variations of permeate fluxes S_4 with VRR for two NF at 1 kDa, using as feed, 1) a permeate at 50 kDa, 2) a permeate produced by two successive UF at 20, and 10 kDa.

cascade filtration led to a 1 kDa permeate flux much larger than that obtained after a single UF at 50 kDa, because there was less dry matter in the 10 kDa permeate than in the 50 kDa one, due to its higher retention.

Extraction Process Combining Centrifugations and UF

This section presents the results of another process, schematized in Fig. 9, in which the two initial clarifications were carried out by centrifugations and the separation of oligosaccharides was achieved by a cascade of three UF at 50, 20, and 10 kDa. In this test, oligosaccharides concentrations and masses were measured at each stage of the process by colorimetric assay. At the beginning of the oligosaccharides production and purification procedure, a solution of polygalacturonan (100 g in 100 L) was degraded by heating at 120°C. All the polymer was not degraded into oligomers. The medium and low molecular weight galacturonan mass, remaining in solution S_1 after the first centrifugation, was 57.6 g and that collected in S_2 was found to be 47.0 g. The mass of oligomers of $Dp \leq 25$ in permeate S_3 obtained after UF at 50 kDa was 8.05 g and that of oligomers of $Dp \leq 10$ in permeate S_4, obtained after UF at 20 kDa was 2.58 g. Finally, the oligomers mass of $Dp \leq 7$ in S_5 (10 kDa permeate) was only 0.98 g. The apparent mass loss of permeate and retentate combined between two consecutive filtrations is due to the removal of samples for analysis.

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┌─────────────────────────────┐
│      Polygalagturonane       │
│      C − 10g/L, V − 10L      │
│        m₀ =  100 g/L         │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│  S₀   Heat treatment         │
│   C = 9.9g/L, V = 9.5 L      │
│     m'₀ =  94.05 g/L         │
└─────────────────────────────┘
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S_0 Heat treatment: $C = 9.9$ g/L, $V = 9.5$ L, $m'_0 = 94.05$ g/L

S_1 (1st centrifugation): $C = 6.2$ g/L, $V = 9.3$ L, $m_1 - 57.63$ g

R_1: $C = 3.88$ g/L, $m'_1 = 36.08$ g

S_2 (2nd centrifugation): $C = 5.22$ g/L, $V = 9$ L, $m_2 = 47.02$ g

R_2: $C = 1.01$ g/L, $m'_2 = 9.09$ g

S_3 (50 kDa): $C = 1.15$ g/L, $V = 7$ L, $m_3 = 8.05$ g, $Dp \leq 25$

R_3: $C = 18.4$ g/L, $V = 1.5$ L, $m'_3 = 27.6$ g

S_4 (20 kDa): $C = 0.55$ g/L, $V = 4.7$ L, $m_4 = 2.58$ g, $Dp \leq 10$

R_4: $C = 2.95$ g/L, $V = 1.5$ L, $m'_4 = 4.43$ g

S_5 (10 kDa): $C = 0.34$ g/L, $V = 2.9$ L, $m_5 = 0.98$ g $Dp \leq 7$

R_5: $C = 1.11$ g/L, $V = 1.5$ L, $m'_5 = 1.66$ g

Figure 9. Schematic of a production process combining two centrifugations and 3 UF in cascade at 50, 20, and 10 kDa.

The variations of permeate flux and oligosaccharides permeate concentration are plotted in Fig. 10 versus ln(VRR) for the 50 kDa and 20 kDa membranes at a pressure of 550 kPa and a temperature of 35°C. The permeate fluxes at 20 kDa were lower than those shown in Fig. 6 for the same membrane, although the temperature was higher (35°C instead of 22°C), but this was due to the lower pressure, 550 kPa instead of 1000 kPa in Fig. 6. The permeate flux was highest for the 20 kDa membrane because the initial concentration was smaller than with the first UF at 50 kDa, but it dropped faster when the concentration increased and became lower than that at 50 kDa when VRR > 3. A similar graph for the 10 kDa membrane is presented in Fig. 11. As with the 20 kDa membrane, the permeate flux decayed relatively little with increasing VRR and remained larger than 180 $Lh^{-1}m^{-2}$, which is a high value for a 10 kDa membrane.
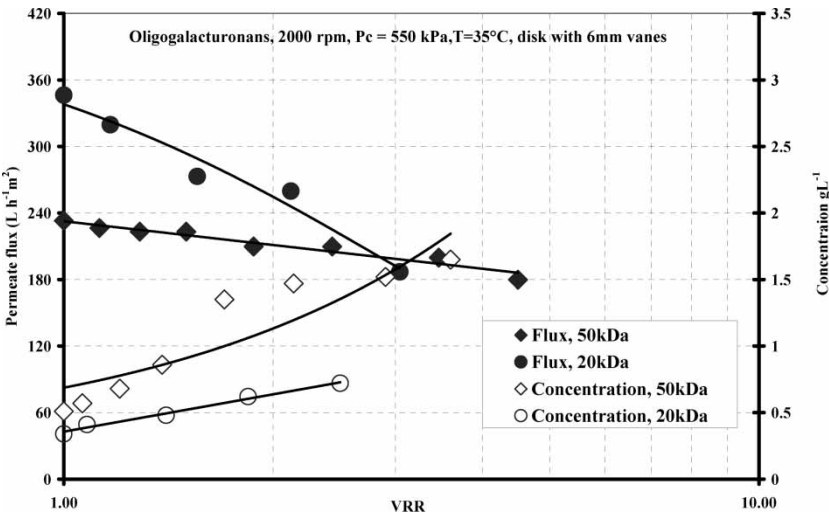
Figure 10. Variation of permeate fluxes and oligomers concentrations with VRR for two UF at 50 and 20 kDa.

Table 2 lists initial oligomers concentrations in permeate (C_{p0}) and in retentate (C_{r0}) and the corresponding rejection coefficient $R_0 = 1-C_{p0}/C_{r0}$ for the three membranes. We have also indicated the volume reduction ratio (VRR_f) corresponding to the last concentration measurements, the last
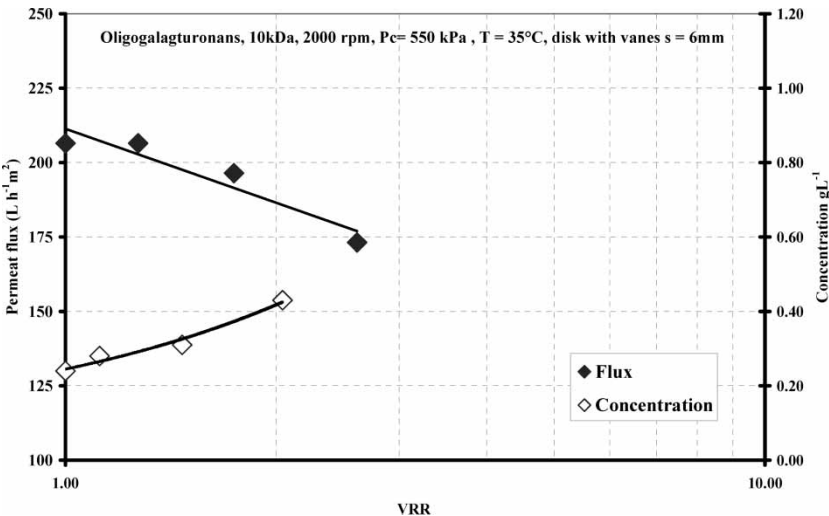

Figure 11. Variation of permeate flux and oligomers concentration with VRR for a UF at 10 kDa.

Table 2. Initial values of oligomers concentrations in permeate and retentate and rejection coefficient. Also final permeate concentrations and concentration factor in permeate for 50, 20, and 10 kDa membranes

Membrane cut-off kDa	C_{p0} gL^{-1}	$C_{r0,}$ g L^{-1}	$R_0,\%$	VRR$_f$	$C_{pf,}$ g L^{-1}	FC$_p$
50	0.51	1.65	69	3.60	1.65	3.24
20	0.34	1.15	70	2.50	0.72	2.12
10	0.24	0.55	56	2.04	0.43	1.79

measured permeate concentration (C_{pf}), and the concentration factor of oligomers in permeate during filtration ($FC_p = C_{p0}/C_{pf}$). The rejection coefficients were highest for the 50 kDa and 20 kDa membranes due to the higher proportion of high Dp in the feed solution than for the 10 kDa membranes.

CONCLUSION

This work confirms that our rotating disk filtration system is well suited to the fractionation of oligosaccharides as was found earlier (12). It provides high permeate fluxes both in MF at 0.2 or 0.45 μm and in UF at 50, 20, and 10 kDa as well as good oligomers transmission. These permeate fluxes declined relatively little with increasing concentration or volume reduction ratio. The replacement of centrifugations at 14,000 g by two MF at 0.45 μm did not modify the composition of oligomers solutions and the filtration process is both faster and more economical on a large scale than centrifugation.

In the process of Fig. 9, the oligomers mass of Dp < 7, in permeate S$_5$ at 10 kDa was relatively small (0.98 g). This mass represented only 2.1% of the initial oligomers mass present in solution S$_2$, according to Fig. 9. In contrast, according to Table 1, the mass of oligomers of Dp ≤ 7, present in solution S$_2$ was around 25% of the total oligomers mass. This result indicates that a large fraction of small Dp oligomeres was lost in the various retentates or by adsorption inside the membranes during the cascade filtration process. Thus it would be important to try to reduce the number of UF steps. Of course the final mass should have been larger if the various retentates had represented a smaller percentage of the initial volume, as it would be the case in large scale production.

These results obtained on a small pilot can certainly be scaled up to large industrial units of dynamic filtration. Large scale rotary disks systems, upto at least 30 m^2 of membrane area are commercialized by Spintek in the US, Westfalia Separator Membraflow in Germany and Raisio Flootek in Sweden for MF and UF. For the NF step of the process, vibrating systems such as the VSEP (New Logic Research Inc, Emeryville, Ca, USA) or the PallSep which can stand pressures up to 4000 kPa and are available in large sizes would also be suitable. We have indeed verified, in treatment of dairy

effluents by NF and RO (13, 14) that the VSEP, which can generate shear rates of more than $10^5 s^{-1}$ at the membranes yield similar performances as our rotating disk prototype.

ACKNOWLEDGMENTS

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