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Pectic Oligosaccharides from Lemon Peel Wastes: Production, Purification, and Chemical Characterization

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ABSTRACT: Lemon peel wastes were extracted with water to remove free sugars and other soluble compounds, and the insoluble solid was employed as a substrate for the manufacture of pectin-derived oligosaccharides by processing with hot, compressed water. When water-extracted lemon peel wastes were treated with water at 160 °C, the oligomer concentration reached the maximum value (31 g/L). Autohydrolysis liquors were subjected to two membrane filtration stages (diafiltration followed by concentration), yielding a refined product containing about 98 wt % of oligomers at a global yield of 14 kg/100 kg oven-dry lemon peel. The concentrate contained oligogalacturonides (with DP in the range of 2−18) and arabinooligosaccharides (with DP in the range of 2−8).

KEYWORDS: *lemon peel wastes, prebiotics, oligogalacturonides, arabinooligosaccharides, hydrothermal treatment, membrane filtration*

INTRODUCTION

The worldwide production of citrus is around 105 million metric tons/year.[1] Spain is a major producer of citrus fruits: in the case of lemons (*Citrus limon*), more than 558000 tons were produced in 2009.[2] Huge amounts of lemon peel wastes (here denoted LPW, which includes peels, seeds, and pulps) are produced in the lemon juice industries, representing about 50 wt % of the raw processed fruit.[3] Nowadays, there is increasing interest in the integral exploitation of agroindustrial wastes,[4] and the use of plant cell wall polysaccharides as sources of novel high value-added oligosaccharides has received special attention.[5]

Because of its high pectin content, LPW is a valuable feedstock for the food industry, where pectin is commonly used as a gelling, thickening, and/or stabilizing agent.[6] Three structural polymers can be found in pectins: homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). HG consists of a backbone of D-galacturonic acid residues, many of which are esterified with methyl groups and/or acetylated. The RGI backbone consists of repeating disaccharides made up of rhamnose and galacturonic acid.[7] In many cases, rhamnosyl residues present neutral sugar side chains such as arabinan, galactan, and/or arabinogalactan. The RGII sequence is composed of a main chain similar to HG associated with a variety of different and complexes oligosaccharide side chains. This polymer is one of the major plant cell wall components, and probably the most complex macromolecule in nature,[8] and can be employed as a starting material for the manufacture of galacturonic acid. This compound and its derivates can be used in the food industry as acidic agents, in the chemical industry as washing powder agents or as nonionic or anionic biodegradable surfactants, and in the pharmaceutical industry in the production of vitamin C.[9,10]

Alternatively, pectin can be employed as a source of nondigestible pectic oligosaccharides (POS), including oligogalacturonides (OGalA) and arabinooligosaccharides (AraOS), which have been proposed as a new class of prebiotics capable of exerting a number of health-promoting effects.[11] According to Gibson and Roberfroid,[12] prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (mainly *Bifidobacterium* sp. and *Lactobacillus* sp.), resulting in improved host health. Nondigestible oligosaccharides are well-known prebiotics. Although prebiotic oligosaccharides (including inulin, fructooligosaccharides (FOS), galactooligosaccharides (GalOS), and lactulose) are commercially available, the interest of consumers in healthy foods has boosted the interest in producing new prebiotic oligosaccharides with improved properties from readily available, renewable carbohydrate sources. The prebiotic potential of POS based on their ability to selectively increase the populations of beneficial bacteria (such as *Bifidobacterium* and *Eubacterium rectale*) in the human gastrointestinal tract, as well as on the intestinal production of short-chain fatty acids (SCFA, including acetic acid, propionic acid, and butyric acid) and lactic acid, has been reported in several papers.[13−15]. Additional functionalities and biological properties have been associated with pectin-derived oligosaccharides.[16] In particular, oligogalacturonides were reported to be involved in regulation mechanisms in various processes related to growing, development, ripening, and organogenesis of plants.[17] The best biological responses were observed for OGalA with DP in the range of 10−16.[5]

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To obtain POS mixtures, the pectin polymeric chains can be partially hydrolyzed by treatments with hot compressed water to give soluble products. The reaction is catalyzed by hydronium ions from water ionization and from in situ generated acids.[18] This approach has been successfully employed in previous works dealing with raw materials such as sugar beet pulp[19] or orange peel wastes.[20] Some of the advantages of this operational mode are (a) its environmentally friendly character (water and feedstock are the only reagents); (b) the avoidance of corrosion problems, because no mineral acid is added to the reaction media; (c) the ability to generate oligosaccharides in a single stage at satisfactory yields; (d) its speed, it being a faster reaction than enzymatic hydrolysis; and (e) the coproduction of spent solids enriched in cellulose suitable for further utilization.

As the aqueous hydrolysis of pectins is not selective, the resulting reaction liquors must be refined to obtain food grade saccharides. For this purpose, membrane processing has been examined in several studies,[21−23] owing to its ability to cause purification, fractionation, and/or concentration effects. In particular, diafiltration shows a great potential for applications in foods, beverages, biotechnological processes, or pharmaceutical industries.

The structure and composition of POS can affect their biological functionality, and a detailed chemical characterization is necessary to assess the structure−function interrelationships. Studies have reported the use of low-pressure anion exchange chromatography[24,25] and/or size exclusion chromatography[26,27] as separation stages prior to the application of high-performance anionic exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and/or mass spectrometry techniques.

This work deals with the manufacture of a refined POS-rich concentrate from LPW by nonisothermal aqueous processing and further purification. The final product was analyzed by low-pressure anion exchange chromatography and HPAEC-PAD to assess the hydrolysis pattern of lemon pectin during the autohydrolysis stage.

MATERIALS AND METHODS

Raw Material. LPW samples, kindly supplied by Indulleida S.A. (Lleida, Spain), were milled to a particle size below 3 mm, homogenized manually for 5 min to avoid compositional differences among aliquots, and frozen until use. Aliquots from the stored samples were subjected to moisture determination according to method ISO 638.

Aqueous Extraction of Lemon Peel Wastes. LPW samples were washed with distilled water (using 10 g of water/g of oven-dry LPW) for 15 min at room temperature in a stirred tank, and the soluble material was separated by centrifugation. Two additional washing stages were then performed using 2 g of water/g of oven-dry LPW. The liquid phases were assayed for dry weight by oven-drying at 60 °C, and their sugar contents were measured using two methods: an enzymatic kit (Boehringer Mannheim, R-Biopharm) for the determination of sucrose, D-glucose, and D-fructose and high-pressure liquid chromatography (HPLC) using an 1100 series Hewlett-Packard chromatograph fitted with a refractive index detector and a Bio-Rad Aminex HPX- 87H column eluted 0.003 M H_2SO_4 at 50 °C (flow rate = 0.6 mL/min) for the determination of galactose + xylose and arabinose. In addition, the presence of galacturonic acid, oligogalacturonides, and polygalacturonic acid was assessed according to the methods described below. The resulting solid phase (solids from hydrothermal treatment, denoted SHT) was subjected to processing with hot compressed water.

Autohydrolysis Processing of SHT. LPW samples (76.9 g oven-dry basis) were reacted in aqueous media was using a 3.75 L stainless steel Parr reactor, at a liquid to solid ratio (LSR) of 12 kg of water/kg of dry SHT. The reactor was stirred at 150 rpm and heated to the desired temperature (in the range of 150−180 °C). When the target temperature was attained, the reactor was rapidly cooled, and the liquid and solid phases were recovered by centrifugation, quantified, and analyzed. To facilitate the comparisons between different reaction conditions, the combined effects of time and temperature caused by the nonisothermal treatments were measured in terms of the severity factor (R_0), defined as

$$R_0 = \int_0^t e^{[T(t)-T_{ref}/w]}\, dt \quad (1)$$

where t is the time needed for achieving the desired temperature, $T(t)$, T_{ref} is the reference temperature (100 °C), and w is an empirical parameter related to the energy of activation of the reaction (for which a value of 14.75 is generally assumed).[20]

Purification of Pectin-Derived Oligosaccharides. Liquid samples from the hydrothermal treatment performed at 160 °C (corresponding to severity factor of 326 min, which led to high yields of oligogalacturonides and arabinooligosaccharides and low proportions of undesired degradation products) were purified by diafiltration and concentration, following the scheme shown in Figure 1. The

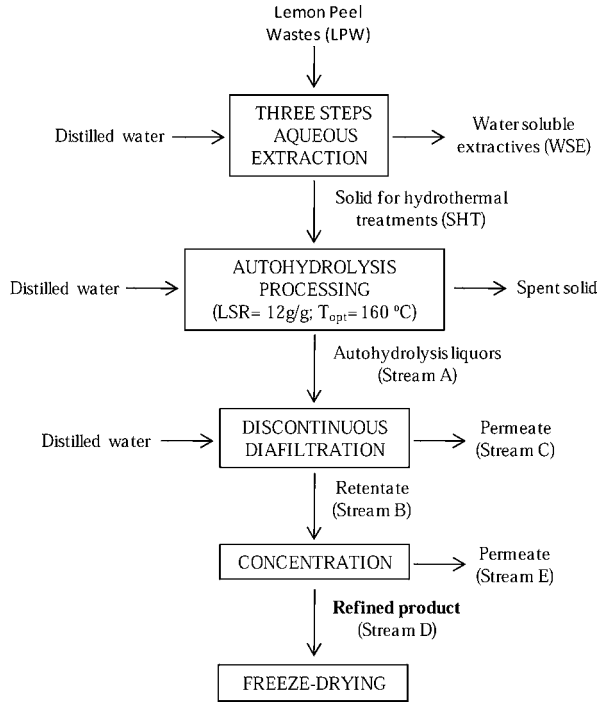

Figure 1. Scheme of the process employed in this work.

reaction liquor (50 mL) was mixed with 250 mL of distilled water, and the resulting solution was processed in an Amicon stirred cell (model 8400, Millipore) equipped with a regenerated cellulose membrane (Millipore, 1 kDa molecular weight cutoff, 41.8 cm^2 effective area, catalog code PLA07610). Pressure was provided by a compressed nitrogen gas cylinder and controlled by a regulator to keep an operating pressure of 3 bar. Dialfiltration enabled the separation of two streams: retentate (stream B, 50 mL) and permeate (stream C, 250 mL). This process was repeated six times to collect the desired volume of diafiltered liquors, which were subjected to dead-end filtration at 3 bar to achieve a volume concentration ratio (VCR) between concentrate and feed of 3.53. Streams B and D were assayed for composition as described below.

Low-Pressure Anion Exchange Chromatography. An aqueous solution of the refined product (V = 10.5 mL, product concentration =

5 g/L) was loaded into the column (30 × 2.6 cm) containing DEAE-Sepharose CL 6B pre-equilibrated with distilled water, and the column was eluted sequentially at 1 mL/min with distilled water and a NaCl stepwise gradient established in preliminary assays (0.07, 0.14, 0.2, 0.3, and 0.5 M) for different time intervals in each step (720, 450, 450, 540, 306, and 306 min, respectively). Samples of the eluate (9 mL) were collected in tubes and assayed for uronic acid content. Then, six pools were prepared by mixture of selected samples. NaCl-containing samples were desalted and concentrated using an Amicon 8400 nanofiltration disk membrane (300 Da molecular weight cutoff) before analysis.

Analysis of the SHT and Spent Solids from Autohydrolysis. Samples of SHT and spent solids from hydrothermal processing were dried at 50 °C, milled to a particle size below 0.5 mm, and subjected to quantitative acid hydrolysis with 72% sulfuric acid at 30 °C to break down the polysaccharides into oligomers, followed by a second step with 4% sulfuric acid at 121 °C to convert the oligomers into monomers (according to the TAPPI T13m method). The resulting liquid phases were assayed for rhamnose, arabinose, galactose, glucose, and xylose by HPAEC-PAD, using a Dionex (Sunnyvale, CA, USA) instrument equipped with a CarboPac PA-1 (4 mm × 250 mm) in combination with a CarboPac PA-1 guard column (4 mm × 50 mm) maintained at 30 °C. The mobile phases were degassed with helium. The gradient employed, prepared from three eluents (eluent A = deionized water; eluent B = 200 mM sodium hydroxide; and eluent C = 2 M sodium acetate in 200 mM sodium hydroxide) was as follows: 0−25 min at 88% A and 12% B; 25−30 min, from 88 to 50% A and from 12 to 50% B; 30−35 min, from 50 to 47.5% B and from 2.5% C; 35−40 min, from 47.5 to 45.5% B and from 2.5 to 4.5% C; 40−60 min, from 45.5 to 43.5% B and from 4.5 to 6.5% C; 60−65 min, from 43.5 to 24% B and from 6.5 to 26% C; and 65−95 min, from 26 to 50% C and 50% A. Acetic acid was quantified by HPLC using the same method cited above. For simplicity, the results are reported as rhamnosyl substituents, arabinan, galactan, glucan, xylan, and acetyl substituents, taking into account that some of these monosaccharides can be generated from various polysaccharides. The oven-dry weight of the solid phase from the quantitative acid hydrolysis is referred to as the acid insoluble residue. Uronic acids were determined according to the method of Blumenkrantz and Asboe-Hansen.[28] Additionally, the pectin and galacturonic acid contents of SHT were determined according to the method of Hwang et al.[29] Moisture and ash were measured according to methods ISO 638:1938 and T-244-om-93, respectively. Elemental nitrogen was determined with a Thermo Fisher Quest Flash EA 1112 analyzer, using 130 and 100 mL/min He and O_2 and an oven temperature of 50 °C. Protein was calculated from the nitrogen content (assuming the factor 6.25 g protein/g nitrogen). All determinations were made in triplicate.

Analysis of Autohydrolysis Liquors, Refined Liquors, and Pools from Fractionation. Samples of reaction liquors and from membrane treatments were centrifuged, filtered through 0.45 μm membranes, and assayed for monosaccharides by using the HPAEC-PAD method explained above. Galacturonic acid, formic acid, acetic acid, hydroxymethylfurfural (HMF), and furfural were quantified by HPLC using the method indicated above. Nonvolatile compounds (NVC) were measured by oven-drying at 60 °C until constant weight, whereas the content of impurities ("other non-volatile compounds", denoted ONVC) were calculated as follows: ONVC = (NVC − monosaccharides − oligosaccharides)/NVC. To measure the oligomer content, liquors were subjected to total enzymatic posthydrolysis using a mixture of enzymatic preparations rich in endopolygalacturonase (Viscozyme L from *Aspergillus aculeatus*) and cellulases (Celluclast 1.5 L) at 37 °C for 40 h in Erlenmeyer flasks with orbital agitation (150 rpm), using an endopolygalacturonase loading of 45 U/g liquor and a cellulase loading of 5 FPU/g liquor.[30] Sodium acetate buffer (50 mM) was employed to maintain the pH at 5. Due to their low concentrations, the neutral sugar constituents in pools were measured by posthydrolysis (sulfuric acid concentration = 4%, T = 121 °C, t = 20 min) followed by sugar determination by HPAEC-PAD. The oligomer contents were calculated on the basis of the increase in each monomer concentration after posthydrolysis with respect to the original samples. All analyses were made in triplicate.

POS Identification. The identification of POS contained in pools I−VI was performed by HPAEC-PAD using the Dionex instrument described previously. Samples of pools, with a saccharide concentration ≤0.1 g/L, were filtered using 0.45 μm cellulose acetate filters. For comparison, mixtures of oligogalacturonides were prepared by hydrolysis of polygalacturonic acid (PGA) by treating a 1% w/w PGA solution at 121 °C for 40 min in media of pH adjusted to 4.4 with NaOH.[31] In this case, galacturonides were analyzed with the following eluents: eluent A = 1 M sodium hydroxide; eluent B = 1 M sodium acetate; and eluent C = deionized water. Elution was carried out with linear gradient phases of 10−50% B and 10% A (0−10 min); 50−60% B and 10% A (10−40 min); 60−70% B and 10% A (40−60 min); 0% B and 10−20% A (60−65 min) and isocratic gradient 0% B and 20% A (65−70 min). Pool I (containing mainly neutral oligomers) was analyzed using a gradient prepared from 200 mM sodium hydroxide (eluent A) and 2 M sodium acetate in 200 mM sodium hydroxide (eluent B) according to the following sequence: 0−36 min, from 0 to 21% B; 36−42 min, 50% B; and 42−57 min, 0% B.

RESULTS AND DISCUSSION

Aqueous Extraction and Composition of SHT. LPW samples were first extracted with water to remove sugars and other soluble compounds for other uses, to avoid their decomposition during autohydrolysis. The water-extracted solid (SHT) was employed as a substrate for hydrothermal processing (see Table 1 for compositional data of SHT and washing liquors). The nonvolatile solutes contained in the washing liquors (denoted water-soluble extractives, WSE) accounted for 39.5 wt % of oven-dry LPW and contained about 59.8 g of sugars/100 g of dry WSE (mainly corresponding to glucose and fructose, with minor amounts

Table 1. Composition of the Aqueous Extracts and the SHT Obtained from the Raw Material (LPW)

stream	content (wt % of oven-dry LPW)		
water-soluble extractives (WSE)	39.5		
solid for hydrothermal treatments (SHT)	60.5		
Water-Soluble Extractives (WSE)			
	content (g/100 g oven-dry WSE)		
component	washing 1 (LSR = 10)	washing 2 (LSR = 2)	washing 3 (LSR = 2)
sucrose	3.95	0.74	0.57
glucose	21.65	4.29	2.30
fructose	13.52	2.77	1.45
galactose (including xylose residues)	5.34	1.20	0.79
arabinose	0.97	0.18	0.10
Solid Phase for Hydrothermal Treatments (SHT)			
component	content (g/100 g oven-dry SHT)		
rhamnosyl substituents	1.21		
arabinan	7.24		
galactan	3.97		
glucan	23.26		
xylan	2.62		
acetyl groups	1.10		
galacturonan	23.05		
acid-insoluble residue	8.73		
ash	2.83		
protein	5.79		
pectin fraction[a]	28.60		

[a]With a galacturonic acid content of 67.5%

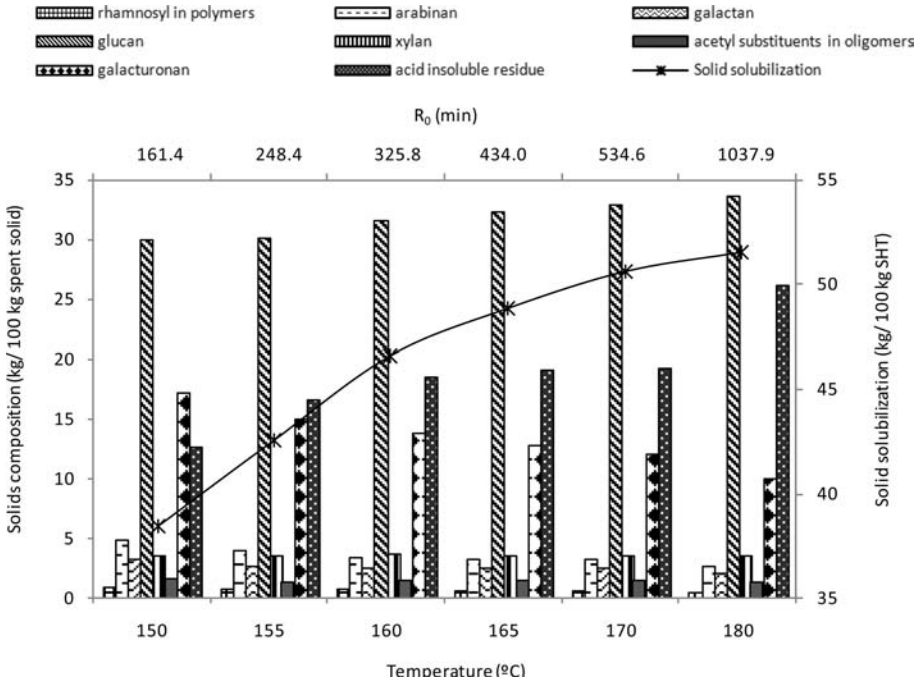

Figure 2. Percentage of solubilization and chemical composition of the spent solids obtained in autohydrolysis experiments. R_0, severity factor.

of arabinose). Negligible amounts of galacturonic acid or oligogalacturonides were found in the extractives. On the other hand, SHT accounted for 60.5 wt % of oven-dry LPW and was mainly made up of glucan and galacturonan (23.3 and 23.1 wt % of oven-dry SHT, respectively), followed by arabinan (7.2 wt %) and galactan (4 wt %). Because of the limitations of the analytical method (based on total hydrolysis), glucan stands for the glucose from cellulose (and probably from xyloglucan), whereas arabinose and galactose can be generated from polymers such as arabinan, galactan, or arabinogalactan. Minor contents of xylan (2.6 wt %) and rhamnosyl units (1.2 wt %) were also found. The substitution by residual acetyl groups was of minor importance. To obtain further compositional information, SHT was subjected to acidic extraction of pectin,[29] which showed the content listed in Table 1 (28.6 wt %). The galacturonic acid content of pectin (67.5%) was below the results found by Gullón et al.[32] for apple pomace (73.5−79%), but in the range of other reported data.[28,33,34] Other nonsaccharide fractions of minor importance for the purposes of this study were ash and protein (which accounted for 2.8 and 5.8 wt % of oven-dry SHT, respectively).

Effects of Hydrothermal Processing. To obtain liquors with high oligomer concentrations, SHT was subjected to hydrothermal processing under nonisothermal conditions to reach 150, 155, 160, 165, 170, or 180 °C, corresponding to R_0 values of 161, 248, 326, 434, 535, and 1038 min. In accordance with Figure 2, the percentage of SHT solubilization increased with temperature, with a less pronounced temperature dependence under harsh conditions. The severest experiment led to 51.5% SHT solubilization, whereas 46.6% SHT solubilization was achieved in the experiment considered as optimal (performed at 160 °C, R_0 = 326 min). The solubilization percentages observed in this work were rather lower than that reported (about 70%) for orange peel wastes.[20]

Effects of the Hydrothermal Treatments on the Composition of Spent Solids. The temperature range explored in this work covered the optimal experimental domain for producing soluble pectin-derived products. Under the conditions tested, both cellulose and AIR were expected to remain in the exhausted solids,[35,36] making their further utililization possible.[37−39] This idea was confirmed by the experimental data: in comparison with SHT, the spent solids resulting from the hydrothermal stage showed increased proportions of cellulose and AIR.

Figure 2 shows that the relative content of acid-insoluble residue in the spent solids increased to 26.2 wt %, as a result of the selective solubilization of susceptible fractions. A similar behavior was observed for glucan, which was the most abundant component of these treated solids. In experiments carried out within the temperature range of 160−180 °C, glucan accounted for about 33 wt % of the spent solids (corresponding to 80% glucan recovery). This result compares well with the results reported in related studies.[19,20,40]

The major effect of hydrothermal processing was the hydrolytic breakdown of hemicelluloses and polygalacturonic acid,[41] leading to a number of reaction products (from low molecular weight polymer to oligomers, sugars, and decomposition products). Owing to the susceptibility of galactan, arabinan, and galacturonan to depolymerization, their content in spent solids decreased upon hydrothermal processing of increasing severity. In comparative terms, this variation pattern was more pronounced for arabinan and galacturonan. Under the selected operational conditions, nearly 75% of arabinan and 70% of galacturonan were solubilized, in comparison with about 65% for galactan. This finding is in agreement with literature results.[19,20,42−44] Although similar solubilization percentages were found for these polymers, the oligomers of higher DP were obtained from galacturonan (see below). On the other hand, the xylan content of spent solids varied in the range of 3.5−3.7%, whereas the acetyl group content remained almost constant along different experiments, following a reaction pattern similar to that reported for orange peel wastes.[20]

Effects of the Operational Conditions on the Composition of Liquors. Depending on the severity of

hydrothermal processing, both pectin and hemicelluloses can be converted into high molecular weight compounds, oligomers (with or without substituents), monomers, or decomposition products.

Oligomers. Figure 3 presents the temperature dependence of the autohydrolysis liquor composition. OGalA were the major

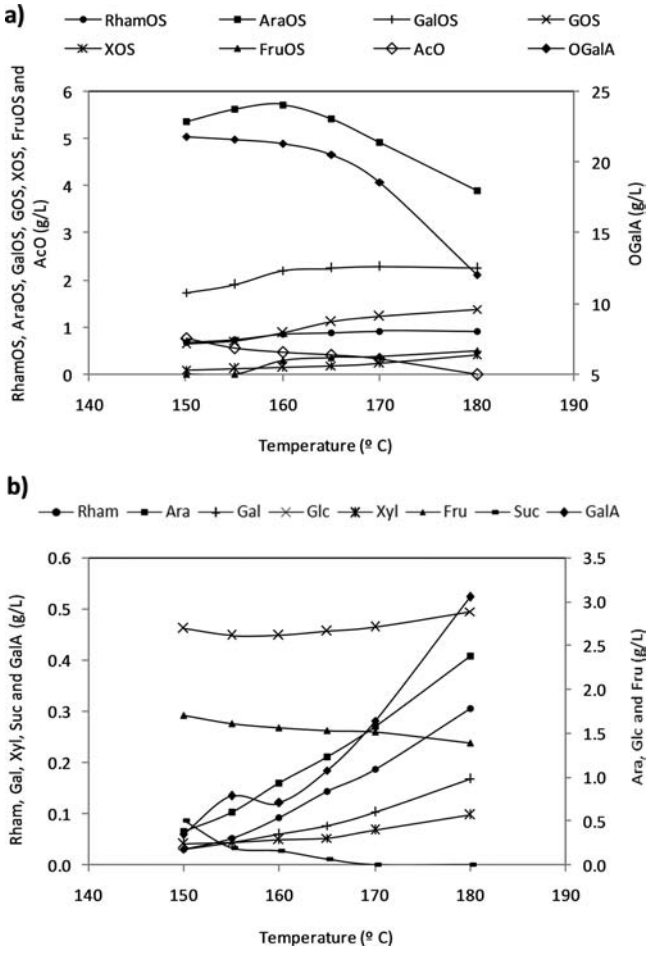

Figure 3. Effect of temperature on the composition of autohydrolysis liquors: (a) oligomers expressed in g/L; (b) monomers expressed in g/L. Ara, arabinose; AraOS, arabinooligosaccharides; AcO, acetyl substituents in oligomers; Fru, fructose; FruOS, fructosyl moieties in oligomers; Gal, galactose; GalOS, galactooligosaccharides; GalA, galacturonic acid; Glc, glucose; GOS, glucooligosaccharides; Rham, rhamnose glucooligosaccharides; Suc, sucrose; Rham, rhamnose; RhamOS, rhamnosyl moieties in oligomers; Xyl, xylose; XOS, xylooligosaccharides.

components, followed by AraOS. The same figure justifies the criterion followed for selecting the optimal temperature. Even if the OGalA concentrations were higher under mildest conditions assayed (Figure 3a), the maximum yield (18.28 kg OGalA/100 kg SHT) was obtained at 160 °C due to the high liquor recovery achieved at this temperature (see Table 2). The recovery increased continuously with the severity of the treatment due to both increased solid solubilization and decreased pectin content of spent solids (resulting in a lower water retention capacity). Operating at 160 °C, the amount of total oligosaccharides (OS) without glucooligosaccharides (GOS) was 26.62 kg/100 kg SHT. These conditions are milder than the ones leading to optimal yields of xylooligosaccharides (XOS) from a number of xylan-containing substrates.[22,43,45,46] Higher severities (temperatures in the range of 170−180 °C, R_0 in the range of 535−1038 min) resulted in reduced oligomer generation, as a consequence of hydrolysis and decomposition reactions. Similar variation patterns have been observed for sugar beet pulp and orange peel wastes.[19,20] The maximum AraOS concentration reached in this study (5.7 g/L) was lower than the one achieved with sugar beet pulp (15.1 g/L) and close to the one obtained with orange peel wastes (6.6 g/L) using the same liquor to solid ratio. In comparison with the same feedstocks, SHT from LPW provided the highest concentration of OGalA (>20 g/L). The concentration profiles of both GalOS and XOS were defined by a slight and steady increase with temperature to reach maximum concentrations of about 2.3 and 0.42 g/L, respectively. GOS were also found at low concentrations (<1 g/L at the optimal temperature) due to the low susceptibility of cellulose to the hydrothermal treatment. The content of acetyl groups bound to oligomers (denoted AcO) decreased with the severity of treatments, with a pronounced drop in the harshest treatment (180 °C). Neither LPW nor orange peel wastes[20] were rich in acetyl substituents, oppositely to sugar beet pulp.[19] Rhamnosyl and fructosyl moieties were also detected, but at low concentrations (<1 g/L).

Monosaccharides. Glucose, arabinose, and fructose were the most abundant monosaccharides in liquors (Figure 3b). The concentrations of arabinose (Ara), galacturonic acid (GalA), and rhamnose (Rham) increased with temperature, as a consequence of the hydrolytic reactions, reaching 2.4, 0.52, and 0.30 g/L at 180 °C respectively, whereas the glucose concentration remained fairly constant. On the other hand, the concentrations of xylose (Xyl) and galactose (Gal) increased continuously, reaching 0.1−0.2 g/L under the harshest conditions assayed.

Nonsaccharide Compounds in Liquors. Due to the side processes taking place during hydrothermal treatments, undesired nonsaccharide compounds (protein, lignin-derived products, sugar decomposition products, and inorganic salts) appear in the reaction media, making the refining of media necessary. Figure 4 shows the temperature dependence of the concentrations of formic acid, acetic acid, HMF, and furfural. The most remarkable finding was the marked increase in acetic acid concentration (from 0.3 to 0.6 g/L) observed at temperatures in the range of 170−180 °C. A close correspondence between the decrease in AcO and the increase in the acetic acid concentration can be observed.

The content of NVC (expressed as kg of NVC/kg of liquor), including saccharides and nonsaccharides, was quantified by oven-drying of liquors. Figure 5 shows that NVC first increased with temperature to reach a maximum at 170 °C (0.0473 kg/kg of liquor) and then decreased in the experiment of 180 °C (0.0458 kg/kg of liquor), whereas the ONVC concentration increased with temperature during the experimental domain. At 160 °C (R_0 = 326 min) the results obtained for both NVC and ONVC were 0.0458 kg/kg liquor and 18.3 kg/100 kg NVC, respectively. Both the variation pattern observed for ONVC and their concentration at 160 °C were similar to the results reported for orange peel wastes[20] and sugar beet pulp[19] and compared favorably with the results reported for other agro-industrial feedstocks such as industrial barley wastes.[47]

Material Balances. Table 2a lists the amount of autohydrolysis liquors recovered and the yields of the different reaction products (expressed as kg/100 kg of oven-dried SHT). The mass of liquid phase increased with the severity of

Table 2. Material Balances and Conversion Yields

(a) Reaction Liquors (Data Based on 100 kg of Oven-Dried SHT)

T (°C)	recovered liquors (kg)	Rham	Ara	Gal	Glc	Xyl	Fru	GalA	RhamOS[a]	AraOS	GalOS	GOS	XOS	FruOS[a]	AcO	OGalA	OS[b]
150	669.23	0.02	0.26	0.02	1.80	0.03	1.14	0.04	0.46	3.60	1.17	0.43	0.07	0.00	0.51	14.62	20.43
155	744.77	0.04	0.44	0.03	1.94	0.03	1.20	0.10	0.56	4.19	1.41	0.54	0.09	0.00	0.42	16.10	22.77
160	856.76	0.08	0.80	0.05	2.24	0.04	1.34	0.10	0.73	4.91	1.90	0.76	0.14	0.26	0.40	18.28	26.62
165	865.48	0.12	1.07	0.07	2.30	0.05	1.32	0.16	0.76	4.60	1.96	0.96	0.17	0.32	0.37	17.77	25.95
170	875.55	0.16	1.39	0.09	2.37	0.06	1.32	0.25	0.81	4.31	2.00	1.07	0.21	0.33	0.30	16.26	24.22
180	953.59	0.29	2.26	0.16	2.75	0.09	1.32	0.50	0.88	3.72	2.15	1.33	0.40	0.48	0.00	11.49	19.12

(b) Conversion Yields (Expressed as Grams of Monomer Equivalents/100 g of Polymer in SHT)

	150 °C	155 °C	160 °C	165 °C	170 °C	180 °C
rhamnosyl in polymers conversion into Rham	1.71	3.24	6.46	10.19	13.41	24.08
arabinan conversion into Ara	3.53	6.13	11.08	14.70	19.13	31.21
galactan conversion into Gal	0.49	0.80	1.27	1.68	2.25	4.01
glucan conversion into Glc	7.75	8.35	9.64	9.89	10.19	11.84
xylan conversion into Xyl	1.04	1.25	1.57	1.76	2.29	3.53
galacturonan conversion into GalA	0.17	0.43	0.45	0.69	1.07	2.16
rhamnosyl in polymers into RhamOS	38.31	46.32	60.24	62.87	67.25	72.34
arabinan conversion into AraOS	49.67	57.83	67.75	63.62	59.50	51.34
galactan conversion into GalOS	29.41	35.59	47.78	49.18	50.37	54.16
glucan conversion into GOS	1.84	2.31	3.26	4.13	4.62	5.70
xylan conversion into XOS	2.68	3.51	5.16	6.43	7.95	15.43
acetyl groups conversion into AcO	46.84	38.15	36.77	37.14	27.24	0.00
galacturonan conversion into OGalA	63.42	69.86	79.32	77.10	70.57	49.85

[a]Rhamnosyl or fructosyl moieties bonded to other sugars in oligomeric compounds. [b]Total oligomers (without GOS).

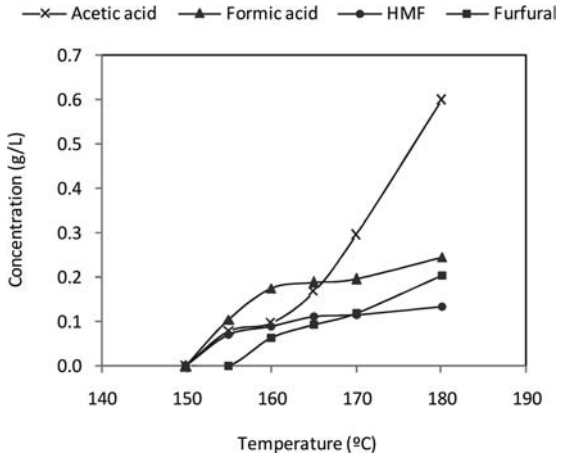

Figure 4. Dependence of the concentrations of organic acids and furans in autohydrolysis liquors on maximum treatment temperature. HMF, hydroxymethylfurfural.

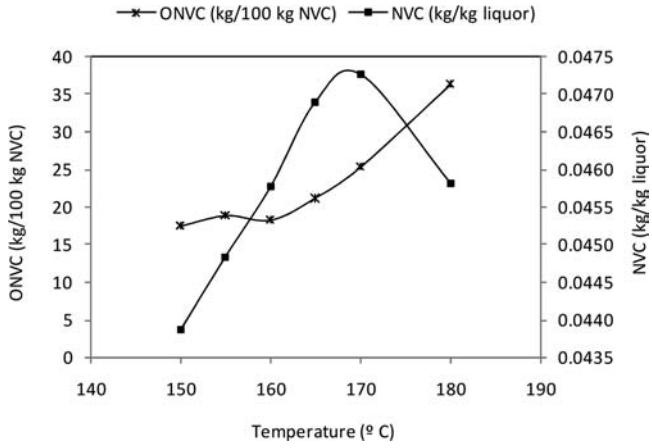

Figure 5. Temperature dependence of the concentrations of NVC and ONVC in reaction liquors (NVC, nonvolatile compounds; ONVC, other nonvolatile compounds or impurities).

treatments, owing to the partial solubilization of SHT. In the experiment performed at the highest temperature considered, the amount of liquid phase recovered was maximal (953.6 kg/100 kg SHT), but the yield in the target products AraOS and OGalA decreased, due to hydrolysis. The maximal oligomer yield (26.6 kg of non-GOS oligomers per 100 kg of dry SHT) was achieved at 160 °C (R_0 = 326 min), together with 856.8 kg of liquor. Figure 5 also shows that the reaction liquors contained limited proportions of undesired compounds (in the range of the ones reported for sugar beet pulp[19] and orange peel wastes[20]). Table 2b lists the conversions calculated for the various products. The results confirmed that arabinan and galacturonan were the polymers most susceptible to hydrothermal treatments, reaching satisfactory conversions into the oligomers (67.7 and 79.3%, respectively) at 160 °C.

Membrane Processing of Liquors. To obtain food grade products, the reaction media must be refined to remove undesired compounds. Among the various techniques applicable for purification and concentration, membrane technologies offer an interesting alternative, due to their simplicity, energy efficiency, operation without harmful organic solvents, and the small space required.[48] In this work, two sequential steps of membrane filtration (discontinuous diafiltration and concentration) were employed for refining and concentrating the autohydrolysis liquors obtained at 160 °C (stream A in Figure 1).

A sample of autohydrolysis liquors (50 mL) was diluted with distilled water (250 mL), and the resulting solution was subjected to membrane diafiltration to reach the desired retentate volume (in this case, 50 mL). Operation was carried out with a transmembrane pressure of 3 bar and repeated six

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times to obtain a refined diafiltered solution (stream B in Figure 1). After this stage, the diafiltered solution (stream B) was further processed by nanofiltration in concentration mode, to increase the proportion of the target products in retentate and to decrease the amount of unwanted compounds, leading to stream D in Figure 1. The degree of concentration was measured in terms of the volume concentration ratio (VCR), defined as[49]

$$\mathrm{VCR} = \frac{V_F}{V_F - V_P} \quad (2)$$

where V_F is the volume of feed subjected to filtration and V_P is the volume of permeate (see Figure 1).

The recovery yield of a given component i in retentate after diafiltration or concentration stages (Y_{Ri}, expressed as a percentage respect to its amount in the feed stream) can be calculated using the equation[49]

$$Y_{Ri} = \frac{V_R C_{Ri}}{V_F C_{Fi}} \quad (3)$$

where C_{Ri} is the final concentration of component i in the retentate, C_{Fi} is the concentration of component i in the feed stream, V_F is the volume of feed subjected to filtration, and V_R is the final volume of retentate.

Concentration was performed to reach a VCR = 3.53. Table 3 shows compositional data concerning the autohydrolysis liquors (stream A), the diafiltered solution (stream B), and the concentrated product (stream D), as well as the recovery yields calculated for the various products respect to the respective amounts present in the feed. Membrane processing resulted in purification effects (including the removal of monosaccharides and ONVC): material balances showed that just 15% of the initial ONVC remained in diafiltered liquors and that similar recoveries were achieved for monosaccharides. In comparison, higher recovery yields were obtained for the target products, especially GalOS, XOS, and OGalA, confirming the suitability of membrane processing for the objectives of this study. The major target compound (OGalA) reached the highest proportion of 0.740 kg/kg NVC in the concentrate, followed by AraOS (0.122 kg/kg NVC) and GalOS (0.063 kg/kg NVC). The total oligosaccharide content (excluding GOS and fructosyl moieties) of the concentrate stream was 0.955 kg/kg NVC. Considering that the same compounds accounted for 0.672 kg/kg NVC in the autohydrolysis liquor, the purification effects achieved with membrane processing are remarkable. In more detail, the recoveries of the target products (taking into account both stages of membrane filtration) were 79.8% for GalOS, 59.3% for AraOS, and 96.1% for OGalA, in comparison with 0−6% for monosaccharides. The ONVC content of the concentrate was just 0.013 kg/kg ONVC (a value in the range reported for commercial oligosaccharides),[50] but it has to be considered that this fraction is underestimated due to the correction for hydration (both oligomers and polymers are measured as "monomer equivalents", according to the analytical methodology employed in this work). If both oligomers and polymers were measured as "polymer equivalents", the ONVC content would increase to 0.10 kg/kg NVC, whereas the "oligomer equivalent" would be intermediate between these two figures and dependent on the average molecular weight. The protein content of the refined product (based on the determination of elemental nitrogen), which also contributes to the ONVC fraction, was 0.028 kg/kg NVC. On the basis of these data, it can be concluded that the purity of the concentrate lies in the range reported for commercial prebiotics.[50,51]

Table 3. Composition and Recovery Yields of the Streams in Figure 1 (Stream A, Autohydrolysis Liquors; Stream B, Retentate from Diafiltration; Stream D, Final Refined Product)[a]

	mass fraction (kg/kg NVC)			recovery yield (%)	
component	stream A	stream B	stream D	stream B (Y_{Ri})	stream D (Y'_{Ri})
Rha	0.002	0.000	0.000	15.6	26.1
Ara	0.020	0.005	0.002	17.5	25.0
Gal	0.001	0.001	0.000	29.2	13.8
Glc	0.057	0.009	0.001	10.6	12.5
Xyl	0.001	0.000	0.000	9.0	0.0
Fru	0.034	0.007	0.002	13.2	24.9
GalA	0.003	0.003	0.000	90.2	6.7
RhamOS	0.019	0.015	0.015	56.7	89.4
AraOS	0.125	0.141	0.122	78.1	75.9
GalOS	0.048	0.063	0.063	90.5	88.2
GOS	0.019	0.018	0.023	61.7	∼100
XOS	0.003	0.006	0.006	∼100	96.2
fructosyl moieties	0.007	0.003	0.004	28.3	∼100
AcO	0.010	0.009	0.008	61.6	76.7
OGalA	0.466	0.681	0.740	100	96.1
OCNV	0.183	0.039	0.013	14.9	30.2

[a]Amount of NVC in stream A, 0.046 kg NVC/kg liquor; amount of NVC in stream B, 0.035 kg NVC/kg liquor; amount of NVC in stream D, 0.111 kg NVC/kg liquor.

Fractionation by Low Pressure Anion-Exchange Chromatography and Chemical analysis. The detailed analysis of the refined product was carried out to assess the effects of the hydrothermal treatment on the hydrolysis products of lemon pectin. To achieve this objective, a freeze-dried sample of the refined product was subjected to semipreparative chromatography in a DEAE-Sepharose CL-6B column, and the resulting fractions were analyzed for galacturonic acid to obtain the elution profile shown in Figure 6. Six pools were obtained by the combination of selected eluate samples, corresponding to the major peaks. Samples from the various pools were subjected to acid posthydrolysis, and the resulting hydrolysates were analyzed for structural units as described under Materials and Methods. The results are listed in Table 4, which also shows data of the yields. As expected, pool I was mainly made up of neutral sugars (92%) and accounted for 16.5 wt % of the sample, whereas the rest of the pools had an acidic character and were obtained at yields corresponding to 14.6, 36.2, 22.3, 7.0, and 2.1 wt % of the initial sample. Related elution patterns were reported by Zhang et al.[25] Arabinose was the major component in pool I (63.5 g/100 g saccharides in pool I), followed by galactose (13.3%), glucose (12.2%), xylose (1.9%), and rhamnose (0.8%). Galacturonic acid was also present (8.4% of pool I).

This neutral pool contained 86 and 35% of the arabinose and galactose, respectively, fed into the column, in comparison with 1.9% recovery for GalA. High Ara/Rha and Ara/GalA molar ratios were observed, indicating that this pool contained mainly fragments from RGI branches and minor amounts of the pectin backbone. Figure 7 shows a HPAEC-PAD chromatogram of pool I. A comparison with chromatograms obtained for linear

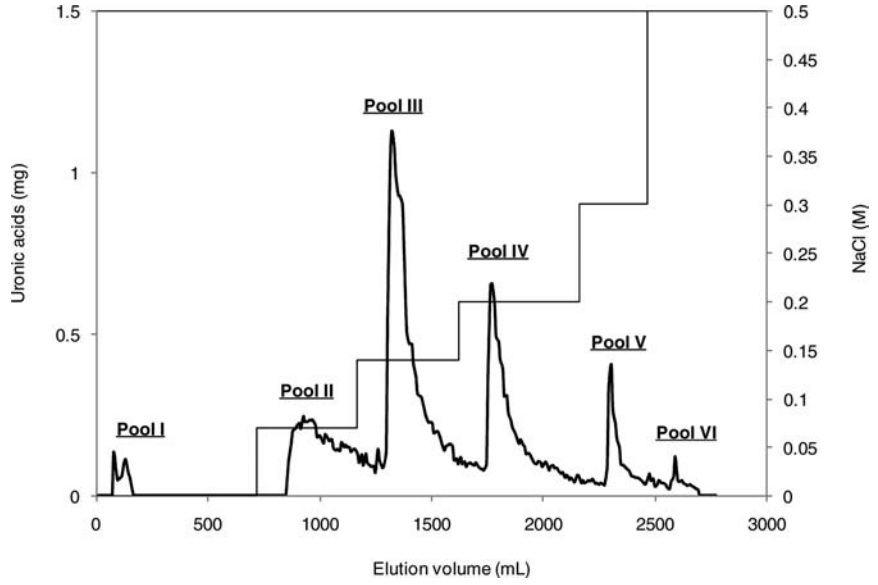

Figure 6. Elution profile of galacturonic acid from freeze-dried sample on DEAE-Sepharose CL-6B, eluted by a stepwise gradient of NaCl. Peaks: eluted with distilled water (neutral pool I), eluted with 0.07 M NaCl (pool II), eluted with 0.14 M NaCl (pool III), eluted with 0.2 M NaCl (pool IV), eluted with 0.3 M NaCl (pool V), and eluted with 0.5 M NaCl (pool VI).

Table 4. Yields and Sugar Compositions of Pools

	pool I	pool II	pool III	pool IV	pool V	pool VI
yield[a] (%)	16.5	14.6	36.2	22.3	7.0	2.1
monomer composition (%)						
rhamnose	0.8	2.1	0.7	2.1	5.8	1.3
arabinose	63.5	4.4	0.9	2.1	3.4	1.0
galactose	13.3	6.0	2.4	6.3	7.3	2.0
glucose	12.2	3.4	1.2	1.0	0.0	0.0
xylose	1.9	2.2	0.8	1.4	0.4	0.8
acetic acid	0.0	0.0	6.5	1.8	0.4	0.0
galacturonic acid	8.4	81.9	87.5	85.3	82.8	94.8

[a]Yield (mg saccharides/100 mg refined product applied to column).

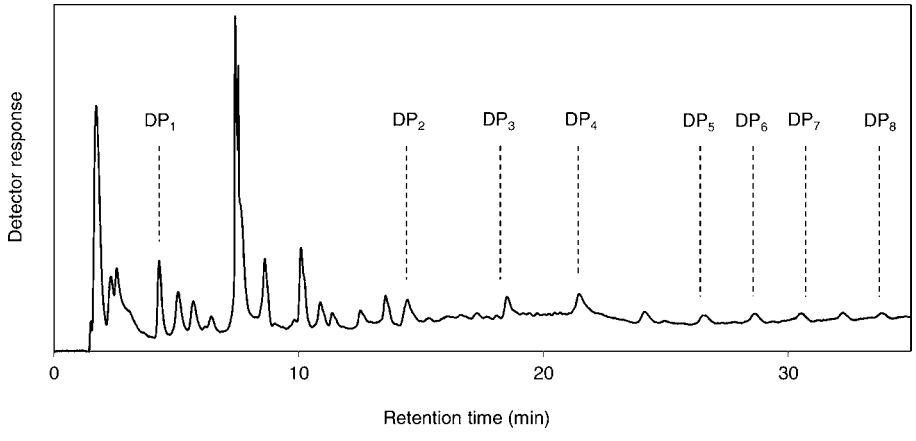

Figure 7. HPAEC-PAD elution of neutral pool with indication of standard linear arabinooligosaccharides.

AraOS standards (DP 2−8) confirmed the presence of at least linear oligomers made up of arabinose units as well as other peaks that could attributed to the presence of other neutral oligosaccharides (for example, GalOS, branched AraOS, or arabinogalactooligosaccharides) in the refined product (see composition in Table 4). Figure 7 also confirms the partial retention of low DP oligomers in the final product.

On the other hand, the predominance of galacturonic acid structural units is clear in pools II, III, IV, V, and VI, accounting for 81.9−94.8% of the total.

Pool III contains up to 36.2% of the saccharides of the sample, and it is mainly made up of GalA (which accounts for 87.5% of the pool). The GalA/Rha and GalA/Ara molar ratios in pool III were 113 and 72, respectively, so that it can be stated

that this pool contains mainly HG fragments. A similar composition was observed for pool VI (94.8% of the pool is GalA), although it is a minor component, accounting for 2% of the refined product.

On the other hand, pools II, IV, and V contain significant amounts of neutral sugars (13−18% of pool) including rhamnose (2.1−5.8%). Pool V shows the lower GalA/Rha molar ratio (12 mol/mol). With these date taken into account, it can be deduced that these pools contain RGI backbone fragments and branches made up of Ara and/or Gal.

Figure 8 shows the chromatograms of pools II−VI as well as the one belonging to a PGA hydrolysate with indication of commercial standards (DP1, DP2, and DP3). As it can be seen, similar peaks (but with different response areas) were found in the chromatograms belonging to pools II−V. The explanation can be found in their degree of methylation (DM). Due to the high pH (13) of the eluent, all ester groups are removed and the oligogalacturonides are eluted according to their polymerization degree.[24] As observed by Ralet et al.,[24] when DEAE-Sepharose CL-6B is employed to separate OGalA, the elution is governed by the overall negative charges and the DP. For example, according their observations, pool III includes OGalA with DP up to 18 (from this size, the peaks are less appreciable) but with higher DM (lower overall charge) than observed for pools IV and V. On the other hand, pool II and V mainly contain oligomers with DP >6 and DP <6, respectively.

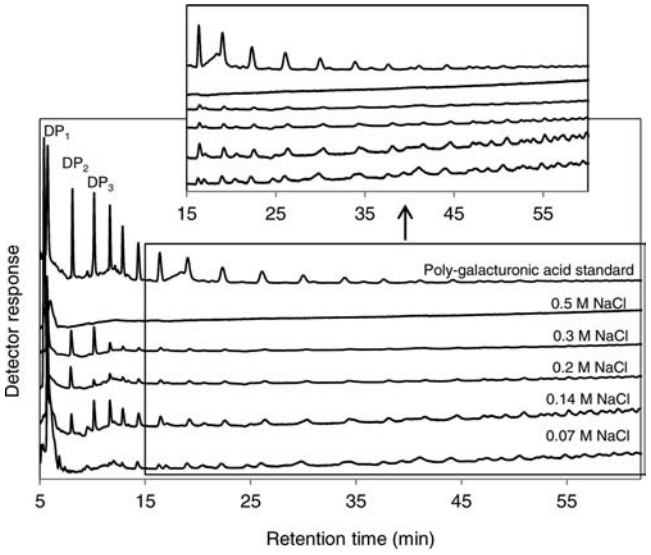

Figure 8. HPAEC-PAD chromatograms of acidic pools and the PGA hydrolysate with indication of commercial standards. PGA, polygalacturonic acid.

The data in Figure 8 and the recovery yields (Table 3) confirm that the membrane employed was able to retain all of the OGalA.

In consequence, it can be confirmed that hydrothermal processing can break the pectin structures, generating a mixture of neutral and acidic oligomers (these last ones showing variable DP and DM) that can be purified by membrane technology and fractionated by preparative chromatography to obtain a variety of products suitable to be compared in prebiotic activity assays.

Conclusions. Lemon peel wastes were employed as a substrate for obtaining refined mixtures of pectin-derived oligosaccharides by means of a process involving water extraction of free sugars, autohydrolysis of the remaining solid, and membrane processing of the liquors. When water-extracted lemon peel wastes were treated under nonisothermal conditions at 160 °C (R_0 = 326 min), the oligomer concentration reached a value of 31 g/L. When liquors were subjected to several stages of diafiltration and concentration, the purity of the target products increased significantly (up to 98 g oligomers/100 g dry product), at a global yield of 14 kg/100 kg of oven-dry lemon peel wastes. A detailed analysis of the refined product (based on samples obtained by semipreparative chromatography and HPAEC-PAD) showed that the major reaction products detected in the final product were arabinooligosaccharides with DP 2−8 and oligogalacturonides with DP in the range of 2−18 and variable degree of methylation.

The comparative evaluation of its prebiotic ability is being carried out in our laboratory.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AcO, acetyl substituents in oligomers; Ara, arabinose; AraOS, arabinooligosaccharides; DM, degree of methylation; DP, degree of polimerization; Fru, fructose; FruOS, fructosyl moieties in oligomers; Gal, galactose; GalOS, galactooligosaccharides; GalA, galacturonic acid; Glc, glucose; GOS, glucooligosaccharides; HG, homogalacturonan; HMF, hydroxymethylfurfural; HPAEC, high-performance anionic exclusion chromatography; HPLC, high-pressure liquid chromatography; LPW, lemon peel wastes; LSR, liquid to solid ratio; NVC, nonvolatile compounds; OGalA, oligogalacturonides; ONVC, other nonvolatile compounds; OS, oligosaccharides; PAD, pulsed amperometric detector; PGA, polygalacturonic acid; POS, pectic oligosaccharides; RGI, rhamnogalacturonan I; RGII, rhamnogalacturonan II; Rham, rhamnose; RhamOS, rhamnosyl moieties in oligomers; SHT, solid for hydrothermal treatment; VCR, volume concentration ratio; WSE, water-soluble extractives; Xyl, xylose; XOS, xylooligosaccharides

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