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Abstract

Pectin oligosaccharides, which can be obtained from fruit wastes, have proven their potential as plant immune-system elicitors. Although the precise size of active species is still under investigation, medium size oligosaccharides have been reported as the most active. Three defined oligogalacturonic acid (OGAs) mixtures were produced from commercial pectin, orange peel and apple pomace residues. The methodology developed involves two sequential acid treatments followed by stepwise ethanol precipitation. Without the need of chromatographic separations, three different fractions were obtained. The fractions were analyzed by high performance anion exchange chromatography (HPAEC) and were completely characterized by mass spectrometry, showing that the small size, medium size and large size fractions contained OGAs of degree of polymerization 3 to 9, 6 to 18, and 16 to 55, respectively.

Keywords

oligogalacturonic acids, pectin hydrolysis, anion exchange chromatography, mass spectrometry, waste valorization

1. Introduction

Fruit wastes are plentiful and generated through all the food supply chain. In the juice industries, a high percentage of the initial fruit is discarded as waste (40-60 % citrus fruits, 25% apple mass), amounting to about 60 million tons (Mt)/year worldwide (Putnik et al., 2017; Bhushan et al., 2008; Lobo & Dorta, 2019). These wastes can be processed to produce high-added value mixtures to be used as ingredients, chemicals and additives in the food; chemical and cosmetic industries (Barreira, Arraibi, & Ferreira, 2019; Senit et al., 2019); or as plant immune-system elicitors in green-houses and farms, reducing the need for agrochemicals (Virgen-Ortiz et al., 2020) .

Pectin is a key constituent of plant cell walls and can be obtained from different sources, the most widely used are citrus peel and apple pomace, which are wastes of juice manufacturing industries (Ciriminna, Chavarría-Hernández, Rodríguez Hernández, & Pagliaro, 2015). Pectin is mainly composed of homogalacturonan, a linear poly D-galacturonic acid with α-(1→4) linkages (HG), and a branched rhamnogalacturonan (→4)-α-D-GalA-(1→2)-α-L-Rha-(1→) containing different sugar substituents linked to rhamnose (RG-I). The backbone can carry a variable number of methyl ester acetyl groups (Round, Rigby, MacDougall, & Morris, 2010; Willats, Knox, & Mikkelsen, 2006). When a pathogen attacks a plant, fragments of the plant cell wall are released as oligogalacturonic acid mixtures (OGAs) (Ferrari et al., 2013). These oligosaccharides have been reported to enhance plant defenses against pathogens, as a consequence a promising strategy for plant protection is the use of exogenous OGAs.

The actual size of OGAs able to elicit plant defense has not been completely elucidated, as the methods reported for their preparation and/or fractionation are quite variable. Although small size OGAs of degree of polymerization (DP) 2-6 have shown to be effective in some cases such as

tomato plants, it is widely considered that medium size OGAs (DP 13-17) are the most powerful elicitors (Côté & Hahn, 1994; Ridley, O'Neill, & Mohnen, 2001; Van Cutsem & Messiaen, 1994).

Obtaining an oligosaccharide fraction of defined size is difficult, as it requires in general the chromatographic separation of different DPs. Acid hydrolysis followed by preparative HPAEC-PAD on a CarboPac PA-1 column (Hotchkiss, Lecrinier, & Hicks, 2001) has been used to obtain some mg of OGAs (DP 10-20). Nucleosil 100-5 SB column has been recently reported to achieve oligosaccharide separation at 10 mg scale (Hodroge et al., 2017). On the other hand, multistep oligosaccharide synthesis allowed to obtain pure compounds (Clausen & Madsen, 2004), but the yield of the addition of sugar units by sequential couplings strongly decreased with the growth of the oligosaccharide chain, and as a consequence the amounts of OGAs produced were very low.

Therefore, either extraction and chromatographic fractionation or organic synthesis are unable to produce in a short time sufficient amounts OGAs required for their use as elicitors. Thus, the development of new efficient methods is required to precise the actual bioactive OGAs and to envisage their application to plant protection.

In this paper, we report a simple procedure combining two consecutive acid treatments that, without the need of chromatographic separations, allowed to obtain a fraction containing exclusively medium size OGAs starting from commercial citrus peel pectin. The protocol has then been applied to dried residues of orange peel and apple pomace without the need of a pectin isolation step, so these residues could be recycled to obtain value added mixtures. The process was followed by analytical HPAEC on a DEAE column and the OGAs contained in each fraction were completely characterized by MALDI and ESI mass spectrometry.

2. Materials and methods

2.1. Chemicals

Pectin from citrus peel (P9135 – Degree of Methylation DM = 60%) (Luzio & Cameron, 2013) was purchased from Sigma-Aldrich (Saint Louis, MO, USA), ammonium formate 99% was bought from Acros Organics and TFA (peptide synthesis) from Biosolve Chimie SARL (Dieuze, France). Water used for HPAEC experiments was of very low conductivity (MilliQ grade, 18 MΩ, Millipore, Bedford, MA). For MS, matrices and calibrating chemicals were purchased from Sigma-Aldrich.

2.2. Hydrolysis

2.2.1. Pectin hydrolysis with H_2O_2

The H_2O_2 treatment was performed as previously reported (Zhang, Hu, Wang, Liu, & Pan, 2018). Briefly, a solution of pectin from citrus peel (1 g) in water (100 mL) at pH 9-10 was heated at 90 °C for 10 min, then H_2O_2 (30%, 10 mL) was added and the solution was stirred at 90 °C for 6 h. After workup, a white solid was obtained.

2.2.2. Pectin hydrolysis with HCl

A mixture of pectin from citrus peel (2 g) in water (200 mL) and HCl (37%, 2 mL) was heated at 100 °C for 18 h. After cooling at room temperature, the pH was adjusted to 8-9 with 4 mol/L NaOH, and 96% EtOH (600 mL) was added. After 4 h, the mixture was centrifuged for 20 min at 9500 rpm to obtain a brownish pellet that was dissolved in water and lyophilized to afford the OGAs (**Solid 1**, 1.76 g).

2.2.3. Hydrolysis of ***Solid 1*** *with TFA and separation of OGAs families*

Solid 1 (1 g) was dissolved in water (90.8 mL). Trifluoroacetic acid (9.2 mL) was added and the solution was heated at 85 °C for 2.5 h. After cooling to room temperature, the mixture was

centrifuged for 20 min at 9500 rpm to obtain a pellet (**Solid 2**, 586 mg) and a supernatant, to which 96% EtOH (800 mL) was added. After 3 days at 4 °C, the precipitate was recovered by centrifugation and dried under vacuum at 40 °C (**Solid 3**, 160 mg) (Zhang et al., 2018). The supernatant was concentrated until 100 mL of this solution remains in the flask. Then, 4 mol/L NaOH was added to adjust the pH to 8-9. The resulting suspension was cooled to 4 °C and after 1 h it was centrifuged to obtain the last solid (**Solid 4**, 131 mg).

2.2.4. Hydrolysis of orange and apple wastes

Orange peel (FRUSA, Albal, Valencia, Spain) and apple pomace (COVILLASA, La Almuña de Doña Godina, Zaragoza, Spain) were collected in the fruit juice factories as a fresh residue that was immediately frozen. Afterwards, they were subjected to coarse milling till a particle diameter of 2-3 mm and dried in a convection oven for 72 h at 50 °C. Samples of 5 g of the dried residues were grinded separately in water (500 mL) with a disperser (T 18 digital ULTRA-TURRAX®, IKA) for 15 min. Mixtures were treated with 37% HCl (5 mL) as in section 2.2.2. After adjusting the pH with NaOH 4 mol/L, the residues were filtered. 96% EtOH was added to the mother liquor and then the suspensions were treated as mentioned above. OGAs from orange peel (**O-Solid 1**, 793 mg) and apple pomace waste (**A-Solid 1**, 614 mg) were obtained. The hydrolysis with TFA was performed using the same protocol detailed in sections 2.2.3 on 500 mg of **O-** and **A-Solid 1**.

2.3. High performance anion exchange chromatography (HPAEC) analysis

HPAEC analysis were performed on a Waters autopurification system (Waters, France) equipped with a 1525 binary pump coupled to a SEDEX LT-ELSD LC detector (Sedere, France) set at 60 °C and a gain of 7. The run was performed at room temperature, the compounds were loaded on a TSKgel DEAE 5PW column (10 µm particle size, 75 mm x 7.5 mm) and the sample injection volume was 20 µl (aqueous solutions of compounds at 10 mg/mL). The mobile phase consisted of 1 mmol/L

ammonium formate (solvent A) and 1 mol/L ammonium formate (solvent B). The composition of the mobile phase varied during the run as follows:

Condition 1: A:B: 0-80 min (85:15 to 62:38 v/v), 80-90 min (62:38 to 0:100 v/v), 90-100 min (0:100 v/v) at a flow rate of 1 mL/min.

Condition 2: A:B: 0-20 min (100:0 to 85:15 v/v), 20-60 min (85:15 to 62:38 v/v), 60-70 min (0:100 v/v) at a flow rate of 1 mL/min.

Condition 3: A:B: 0-80 min (83:17 to 65:35 v/v), 80-90 min (65:35 v/v), 90-100 min (65:35 to 0:100 v/v), 100-110 min (0:100 v/v) at a flow rate of 1 mL/min.

Data acquisition and processing were performed with assLynx V4.1 software.

2.3.1. Preparative HPAEC: Preparation of pure OGAs of DP3, 4 and 5.

OGAs of DP 3, 4 and 5 were obtained by a preparative HPAEC chromatography of **Solid 1**.

The separation was performed in the same equipment at room temperature on TSKgel DEAE 5PW column (10 µm particle size, 200 mm x 50 mm) using a sample injection volume of 900 µL (water solutions at 120 mg/mL for the sample). The effluent was flow-split via a peek tee with 1/5 of the flow directed toward the ELSD instrument. The mobile phase consisted of 1 mmol/L ammonium formate (solvent A) and 1 mol/L ammonium formate (solvent B). The composition of the mobile phase varied during the run as follows:

Condition prep: A:B: 0-15 min (100:0 to 85:15 v/v), 15-50 min (85:15 to 62:38 v/v), 50-65 min (0:100 v/v) at a flow rate of 30 mL/min.

Detection was performed with ELSD detector and data acquisition and processing were performed with MassLynx V4.1 software.

2.4. Mass Spectrometry

2.4.1. Matrix assisted laser desorption ionization - time of flight - mass spectrometry (MALDI-TOF-MS)

MALDI-TOF HRMS experiments were performed using a SYNAPT G2-Si hybrid quadrupole time-of-flight instrument (Q-TOF) equipped with an intermediate pressure (IP) MALDI ionization source (Waters, Manchester, UK). The source was operated with a 2.5 KHz solid state UV laser system (λ = 355 nm). HRMS data were recorded in the negative ion mode. Mass calibration from 50 to 4000 Da was carried out using 2-[(2E)-3-(4-tert-butylphenyl)-2-methylprop-2-enylidene] malononitrile (DCTB) matrix mixed with CsI_3 in a 1:2 molar ratio in THF. The scan range was m/z 50-3000 at 1 s/scan. The TOF was operated in the sensitivity mode, providing an average resolving power of 20,000 (FWHM). The source parameters were as follow: sample plate, 30 V; extraction, 30 V; hexapole, 20 V and aperture, 5V. The HMRS spectra were recorded in the continuum mode. Data acquisition was performed with MassLynx software (V4.1, Waters).

The ionic liquid matrix (ILM) of HABA/TMG_2 used in this study was prepared as previously reported (Armstrong, Zhang, He, & Gross, 2001; Przybylski et al., 2009). HABA (30 mg) was mixed with TMG (31.2 µL) in methanol (300 µL), and the resulting solution was sonicated for 15 min at 40 °C and dried under vacuum overnight. ILM was then prepared at a concentration of 70-90 mg/mL in methanol for use as a matrix, without further purification.

For the preparation of the samples, 1-2 mg of the oligosaccharide fraction were dissolved in 500 µL of water and Dowex 50X8-400 ion exchange resin was added. Equal volumes of this solution

and ionic liquid matrix were mixed. An amount of 1.3 µL of the mixture was deposited on a mirror polished stainless steel MALDI target (Waters 405010856, 8 cm x 12 cm, 96 well) and was air-dried at room temperature and atmospheric pressure for 2 h.

2.4.2. Electrospray ionization – mass spectrometry (ESI-MS)

2.4.2.1. Ultra performance size-exclusion chromatography (UP-SEC)

Samples were prepared in water at a concentration of 1 mg/mL. Chromatographic separations were performed on an ACQUITY UPLC H-Class system (Waters, Milford, MA, USA) using an ACQUITY UPLC Protein BEH SEC column (125Å, 1.7 µm, 4.6 mm x 300 mm). Elution was conducted in 50 mmol/L ammonium formate, formic acid 0.1% at a flow rate of 400 µL/min and a column oven temperature of 40 °C. The injection volume was set to 2 µL.

2.4.2.2. Electrospray ionization high-resolution mass spectrometry (ESI-HRMS)

MS-detection was performed with the SYNAPT G2-Si instrument hyphenated with the ACQUITY UPLC H-Class system. The ESI source was operating in the negative ionization mode using a capillary voltage of -2.5 kV and the following conditions: cone voltage, 120 V; source offset, 20 V; source temperature, 120 °C; desolvation gas temperature, 450 °C; desolvation gas flow, 800 L/h, and cone gas flow, 50 L/h. Nitrogen (> 99.5%) was employed as the desolvation gas. Mass calibration was carried out using a sodium formate solution (10 mmol/L NaOH in isopropanol/water/formic acid 49.9:49.9:0.2, v/v/v) and a lock mass correction was applied for accurate mass measurements using the $[M-H]^-$ ion (m/z 554.2615) obtained from a Leu-enkephalin solution (1 ng/µL in H_2O/CH_3CN/formic acid 50:49.9:0.1, v/v/v). The scan range was m/z 50-2500 at 0.25 s/scan. The TOF was operated in the sensitivity mode, providing an average resolving

power of 20,000 (FWHM). All spectra were recorded in the continuum mode. Data acquisition was performed with MassLynx software (V4.1, Waters).

3. Results and discussion

3.1. Hydrolysis

3.1.1. Pectin hydrolysis with H_2O_2

It has been reported that H_2O_2 degradation of pectin produced oligosaccharide mixtures of different main molecular weight varying H_2O_2 concentration (Zhang et al., 2018). However, in our hands this alkaline treatment was hardly repeatable from one run to another as revealed by HPAEC analysis (data not shown).

3.1.2. Pectin hydrolysis with TFA

Direct TFA treatment of pectin (in similar conditions to those described in 2.2.3) produced partial degradation and gave mixtures containing medium to large size oligosaccharides, from which it was not possible to obtain clear cut fractions. Probably, under these conditions the neutral sugars and/or the methyl and acetyl groups on galacturonic acids are not completely eliminated, contributing to the high heterogeneity of the samples.

3.1.3. Pectin hydrolysis with HCl

Pectin from citrus peel was treated with 0.1 mol/L HCl. Under these conditions the acid easily cleaved the glycosidic linkages at the branches, and partially degraded the galacturonic acid backbone of the polysaccharide (Thibault, Renard, Axelos, Roger, & Crépeau, 1993). It has been shown (Round et al., 2010) that upon HCl hydrolysis the neutral sugars of RG-I polymers (containing arabinose, galactose and rhamnose) are degraded, and only the homogalacturonic

regions remain. In contrast to enzymatic degradation using pectin lyases and/or endo-polygalacturonases (Ognyanov et al., 2016), the acid treatment of pectin cleaves acetyl and methyl ester groups.

Therefore, mainly OGAs of different degree of polymerization were obtained. To analyze the product (**Solid 1**), anion exchange chromatography HPAEC-DEAE was performed using *condition 1* as described in section 2.3. The chromatogram showed the distinct OGAs with very good resolution (Fig. 1). From this elution profile, up to DP 21 can be clearly identified.

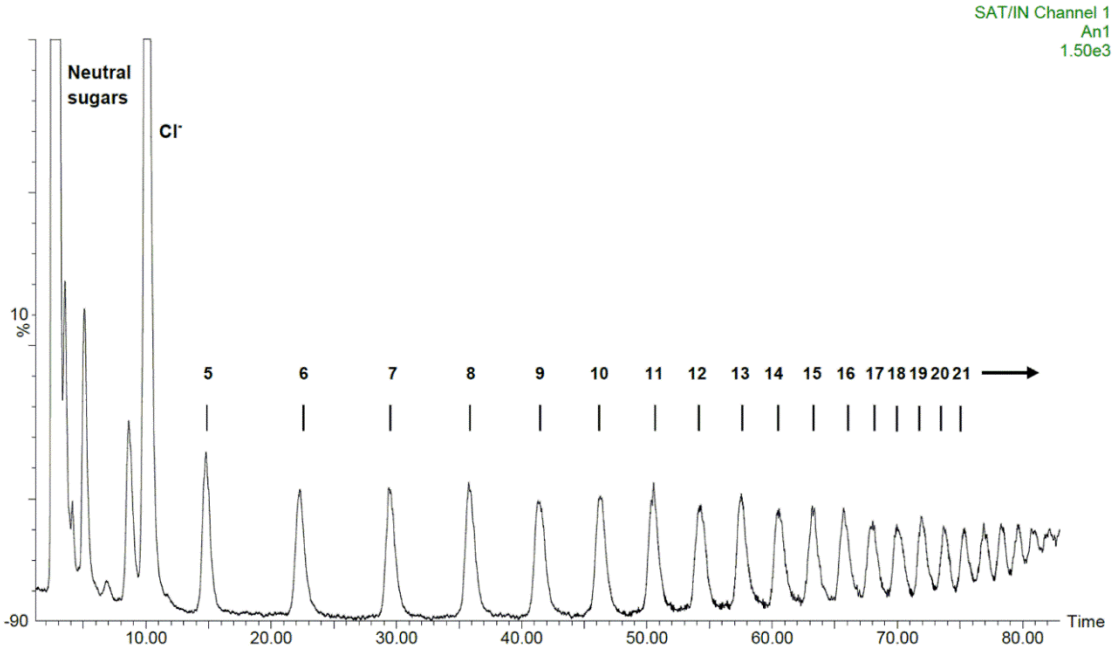

Fig. 1: HPAEC chromatogram of **Solid 1** in *conditions 1*.

When a separation of different families of OGAs was attempted by ethanol precipitation and centrifugation, the fractionation failed, as the main component of the mixture were the large size OGAs, and a second hydrolysis was necessary.

3.2. Pectin depolymerization followed by TFA hydrolysis: production of different size families of OGAs

To obtain and separate the families of different degree of polymerization, TFA treatment was then performed on the mixture resulting from the hydrolysis with HCl. These two subsequent steps were performed as described in section 2.2.2 and 2.2.3. Fig. 2 summarizes the protocol applied.

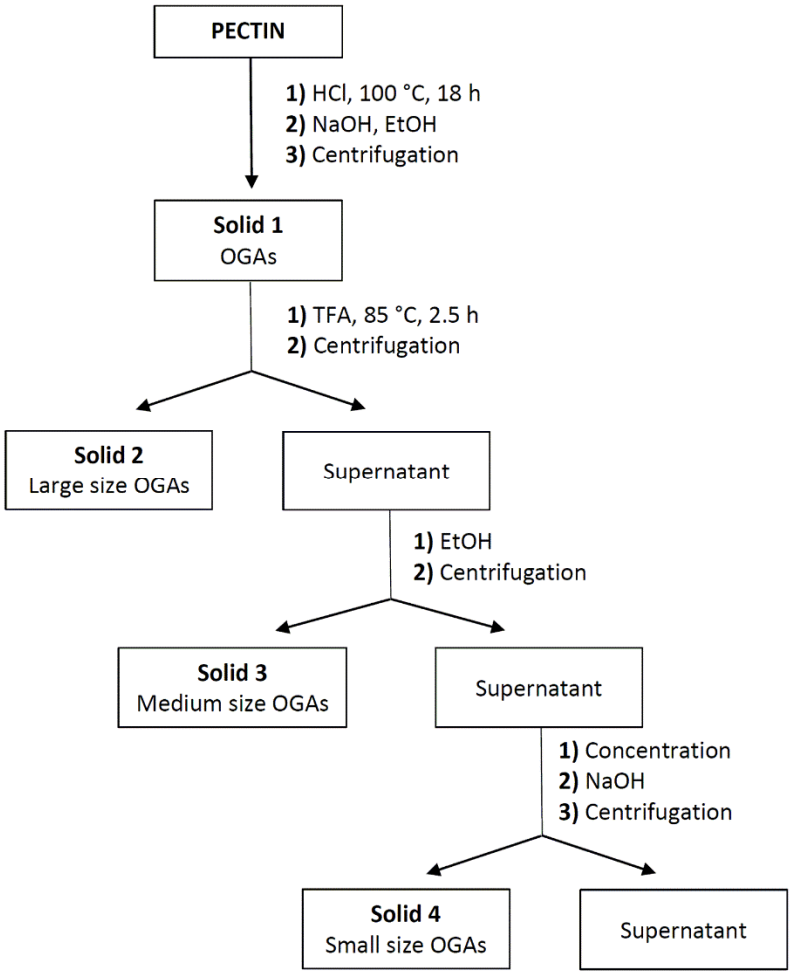

Fig. 2: Scheme of the hydrolysis and separation of OGAs families.

This protocol allowed to fractionate the OGAs in three families: **Solid 2** was obtained as a pellet in the first centrifugation after the TFA treatment and contained large size OGAs. Addition of ethanol to the supernatant followed by a second centrifugation afforded **Solid 3**, which contained medium size OGAs. The second supernatant was concentrated, the pH was adjusted to 8-9 and after centrifugation **Solid 4** was obtained, containing small OGAs. Starting from 1 g of **Solid 1**, 586 mg of

Solid 2, 160 mg of **Solid 3** and 131 mg of **Solid 4** were obtained. All these fractions were analyzed using HPAEC (Fig. 3).

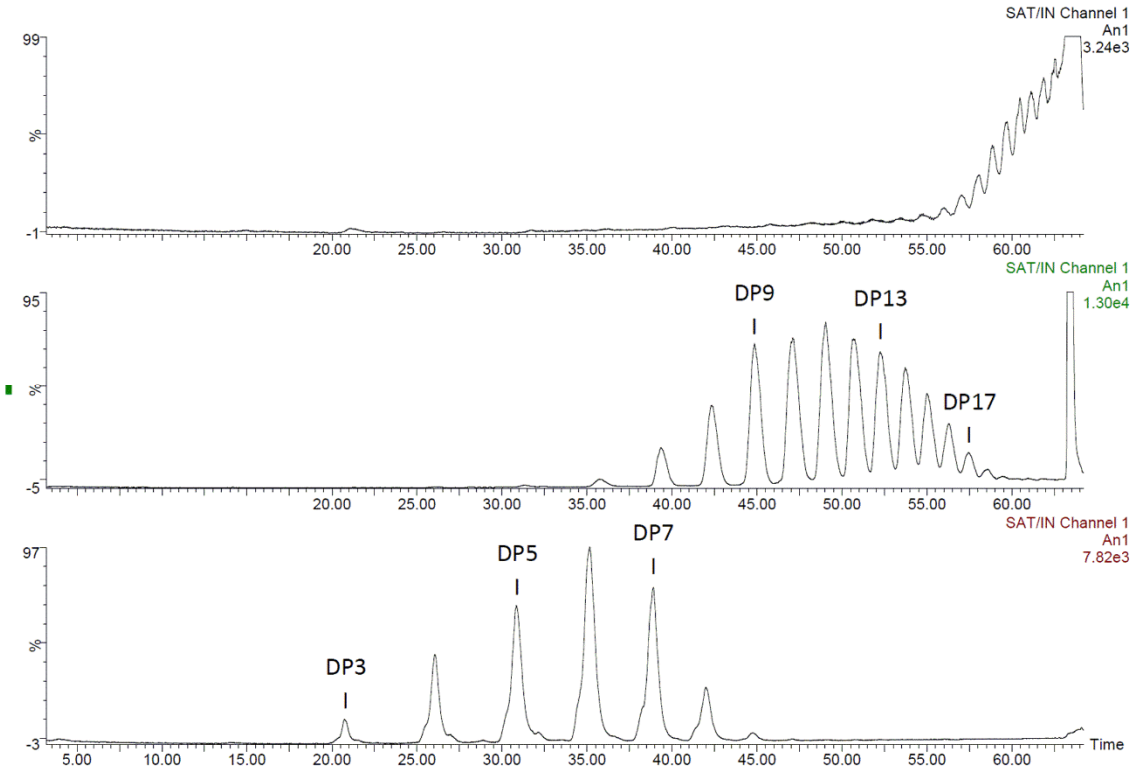

Fig. 3: HPAEC chromatogram of **Solid 2** (top), **Solid 3** (middle) and **Solid 4** (bottom) in *conditions 2*.

OGAs of DP 3, 4 and 5 were used as control samples to estimate the DPs of the oligosaccharides. They were obtained by preparative HPAEC of **Solid 4** and their purity was verified by ^{1}H NMR (Fig. S1). In the fraction containing the larger oligosaccharides (**Solid 2**), OGAs with DP higher than 16 were found. The medium size family (**Solid 3**) was composed mainly by OGAs with estimated DP between 6 and 18, whereas the chromatogram of the fraction with small OGAs (**Solid 4**) showed DP between 3 and 9.

3.3. Hydrolysis of orange and apple waste

The same protocol used for pectin was performed to dried residues of apple pomace and orange peel. With HCl treatment, pectin is extracted and *in situ* hydrolyzed. After the hydrolysis a similar HPAEC profile for **Solid 1** was obtained for both residues (Fig. 4) in 16% yield for orange (**O-Solid 1**) and 12% yield for apple (**A-Solid 1**). These yields are in accordance with the pectin content of each residue, *i.e.* 25-35 % in orange peel and 10-15% in apple pomace (dry basis) (Sundarraj & Ranganathan, 2017).

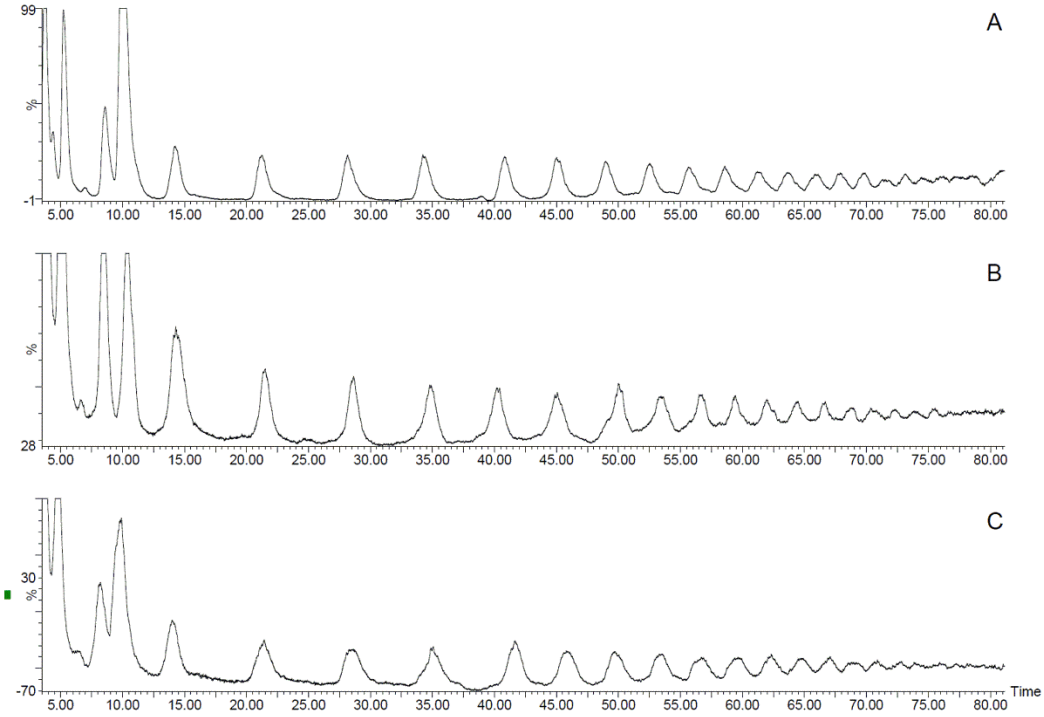

Fig. 4: Chromatograms obtained by HCl hydrolysis of **A**: Commercial pectin (citrus peel); **B**: Orange peel waste; **C**: Apple pomace waste in *conditions 1.*

When these solids were treated with TFA, both orange and apple gave the same HPAEC chromatograms. The three DP families of OGAs were obtained, as it was observed for commercial pectin. Large size OGAs in **O-** and **A-Solid 2**, were obtained in 35% yield, whereas the medium size families (**O-** and **A-Solid 3**) were obtained in 20% yield. The chromatograms of these solids are

identical with OGAs with DP between 6 and 18 (Fig. S2). Finally, for the small size fractions, oligosaccharides with DP between 2 and 8 were found for both fruit wastes.

3.4. Characterization of the fractions

3.4.1. HPAEC

Several reports consider the medium size OGAs as the most promising fraction to act as elicitor in plants (Aziz, Heyraud, & Lambert, 2004; Bellincampi, Dipierro, Salvi, Cervone, & De Lorenzo, 2000; Davis, Darvill, Albersheim, & Dell, 1986). We therefore decided to further characterize this fraction, corresponding to **Solid 3**. The chromatogram of this oligosaccharide mixture was first expanded using a slower gradient (*Condition 3* section 2.3.). The resulting elution profile is shown in Fig. 5. The fraction is composed mainly by OGAs of DP 6-18, whereas the presence of very small amounts of DP 4, 5, 19 or even 20 cannot be completely excluded. The relative ratios of the different DPs have been determined by integration of the chromatogram (Table S1).

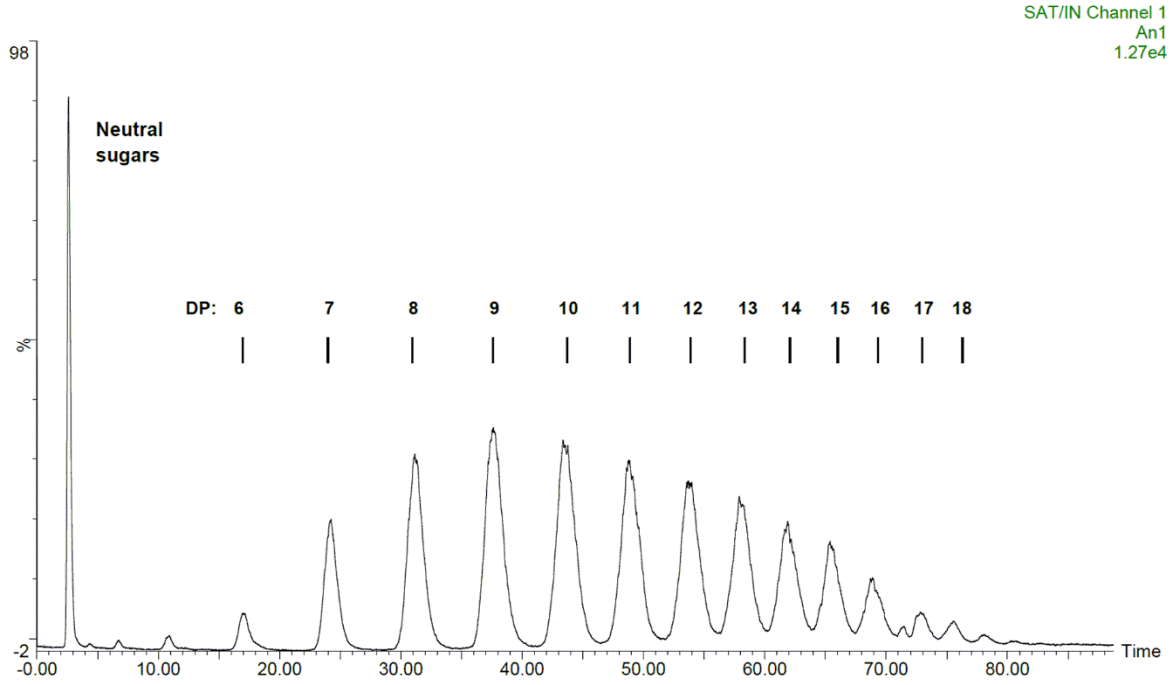

Fig. 5: HPLC-ELSD chromatogram of **Solid 3** in *conditions 3*.

Using these conditions, the different peaks corresponding to the DPs are completely resolved, opening the possibility to isolate the individual oligosaccharides as pure compounds.

3.4.2. MALDI-TOF mass spectrometry

In order to characterize **Solid 3** in detail, and to confirm the presence of all medium size OGAs, MALDI-TOF MS was performed in the negative mode. For these experiments an ionic liquid matrix of HABA/TMG_2 was used. MALDI spectrum (Fig. S3, Table S2) showed peaks of molecular ions $[M-H]^-$ from DP 3 (*m/z* = 545.0995) to DP 14 (*m/z* = 2481.4531). The compounds with higher DP were not detected in the spectrum, due to a probable better desorption ionization process of the lower DP when analyzing disperse OGAs mixtures. In addition, the peaks corresponding to a loss of a water molecule of almost all oligosaccharides were found. They are more abundant for DP 3 and DP 4 (at *m/z* = 527.0895 and *m/z* = 703.1215, respectively), and their intensity decreases for higher DPs.

3.4.3. ESI mass spectrometry

A recent developed method using SEC coupled to ESI-MS (Voxeur et al., 2019) allowed us to detect all the OGAs that were shown in the HPAEC chromatogram of **Solid 3** (Fig. 6, Fig. S4), as mono- or multi-charged ions. For example, DP 7 appeared as the mono-charged ion at *m/z* 1249.2238 $[M-H]^-$, DP 11 as the di-charged ion at *m/z* 976.1720 $[M-2H]^{2-}$ and the DP 18 as the tri-charged ion at *m/z* 1061.1848 $[M-3H]^{3-}$.

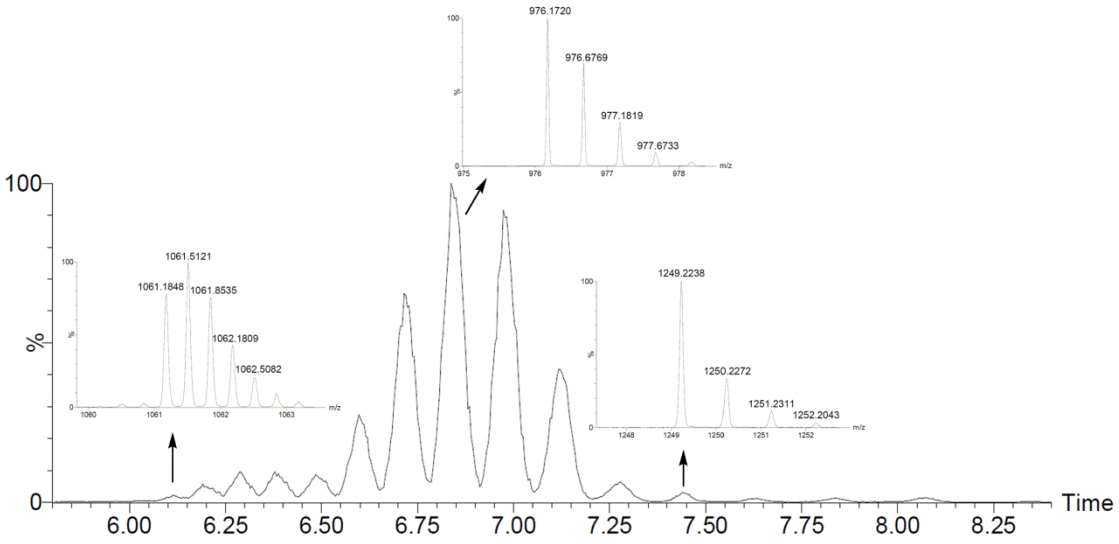

Fig. 6: SEC of **Solid 3**. Insets: ESI-MS spectra of DP 7, DP 11 and DP 18.

This method allowed us to confirm the DP distribution in this fraction observed by HPAEC. ESI-MS is the most sensitive technique to confirm the presence of all the DPs present in the oligosaccharide mixture.

Solid 2 was also analyzed by SEC-ESI-MS (Fig. S5). MS spectra of different peaks (Fig. S6) showed the presence of DPs 14 to 55, in good accordance to the profiles obtained by HPAEC. The small size fraction **Solid 4** was analyzed directly by ESI-MS (Fig. S7), confirming the HPAEC profile.

4. Conclusions

Using a simple method of two subsequent acid treatments with HCl and TFA, followed by EtOH precipitation and centrifugation, three different families of OGAs were obtained from commercial pectin and orange and apple wastes without the need of chromatographic separation. The same families were obtained in good yields regardless of the sources used. The medium size OGAs fraction was analyzed by HPAEC-ELSD and MS (MALDI and ESI). These methods allowed to characterize **Solid 3** as containing OGAs with DP between 6 and 18. This fraction has been considered as the most active to elicit plant defense by different authors. As the different studies

are difficult to compare, this work could be a key contribution to generate size-defined fractions able to elucidate the active OGA species for elicitation. This protocol can be considered as a good start for the valorization of food residues and food industry by-products, in particular from fruit juice industries, reusing valuable components at the farm and at industrial level.

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Appendix A. Supplementary data

^{1}H NMR spectra of DP 3, DP 4 and DP 5, relative composition of **Solid 3**, MALDI-MS spectra, SEC chromatograms and ESI-MS spectra.

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