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## **A simple procedure to obtain a medium-size oligogalacturonic acids fraction from orange peel and apple pomace wastes**

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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 **1. Introduction**

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

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

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

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

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

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

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

## 44 **2. Materials and methods**

## 45 2.1. Chemicals

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

## 51 2.2. Hydrolysis

### 52 2.2.1. Pectin hydrolysis with H<sub>2</sub>O<sub>2</sub>

53 The H<sub>2</sub>O<sub>2</sub> treatment was performed as previously reported (Zhang, Hu, Wang, Liu, & Pan, 2018).  
54 Briefly, a solution of pectin from citrus peel (1 g) in water (100 mL) at pH 9-10 was heated at 90 °C  
55 for 10 min, then H<sub>2</sub>O<sub>2</sub> (30%, 10 mL) was added and the solution was stirred at 90 °C for 6 h. After  
56 workup, a white solid was obtained.

### 57 2.2.2. Pectin hydrolysis with HCl

58 A mixture of pectin from citrus peel (2 g) in water (200 mL) and HCl (37%, 2 mL) was heated at 100  
59 °C for 18 h. After cooling at room temperature, the pH was adjusted to 8-9 with 4 mol/L NaOH,  
60 and 96% EtOH (600 mL) was added. After 4 h, the mixture was centrifuged for 20 min at 9500 rpm  
61 to obtain a brownish pellet that was dissolved in water and lyophilized to afford the OGAs (**Solid 1**,  
62 1.76 g).

### 63 2.2.3. Hydrolysis of **Solid 1** with TFA and separation of OGAs families

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

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

#### 72 *2.2.4. Hydrolysis of orange and apple wastes*

73 Orange peel (FRUSA, Albal, Valencia, Spain) and apple pomace (COVILLASA, La Almuña de Doña  
74 Godina, Zaragoza, Spain) were collected in the fruit juice factories as a fresh residue that was  
75 immediately frozen. Afterwards, they were subjected to coarse milling till a particle diameter of 2-  
76 3 mm and dried in a convection oven for 72 h at 50 °C. Samples of 5 g of the dried residues were  
77 grinded separately in water (500 mL) with a disperser (T 18 digital ULTRA-TURRAX®, IKA) for 15  
78 min. Mixtures were treated with 37% HCl (5 mL) as in section 2.2.2. After adjusting the pH with  
79 NaOH 4 mol/L, the residues were filtered. 96% EtOH was added to the mother liquor and then the  
80 suspensions were treated as mentioned above. OGAs from orange peel (**O-Solid 1**, 793 mg) and  
81 apple pomace waste (**A-Solid 1**, 614 mg) were obtained. The hydrolysis with TFA was performed  
82 using the same protocol detailed in sections 2.2.3 on 500 mg of **O-** and **A-Solid 1**.

#### 83 *2.3. High performance anion exchange chromatography (HPAEC) analysis*

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

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

91 *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  
92 v/v) at a flow rate of 1 mL/min.

93 *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  
94 v/v) at a flow rate of 1 mL/min.

95 *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  
96 v/v), 100-110 min (0:100 v/v) at a flow rate of 1 mL/min.

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

98 *2.3.1. Preparative HPAEC: Preparation of pure OGAs of DP3, 4 and 5.*

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

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

106 *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  
107 (0:100 v/v) at a flow rate of 30 mL/min.



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

## 110 *2.4. Mass Spectrometry*

### 111 *2.4.1. Matrix assisted laser desorption ionization - time of flight - mass spectrometry (MALDI-TOF-* 112 *MS)*

113 MALDI-TOF HRMS experiments were performed using a SYNAPT G2-Si hybrid quadrupole time-of-  
114 flight instrument (Q-TOF) equipped with an intermediate pressure (IP) MALDI ionization source  
115 (Waters, Manchester, UK). The source was operated with a 2.5 KHz solid state UV laser system ( $\lambda =$   
116 355 nm). HRMS data were recorded in the negative ion mode. Mass calibration from 50 to 4000  
117 Da was carried out using 2-[(2E)-3-(4-tert-butylphenyl)-2-methylprop-2-enylidene] malononitrile  
118 (DCTB) matrix mixed with CsI<sub>3</sub> in a 1:2 molar ratio in THF. The scan range was m/z 50-3000 at 1  
119 s/scan. The TOF was operated in the sensitivity mode, providing an average resolving power of  
120 20,000 (FWHM). The source parameters were as follow: sample plate, 30 V; extraction, 30 V;  
121 hexapole, 20 V and aperture, 5V. The HMRS spectra were recorded in the continuum mode. Data  
122 acquisition was performed with MassLynx software (V4.1, Waters).

123 The ionic liquid matrix (ILM) of HABA/TMG<sub>2</sub> used in this study was prepared as previously reported  
124 (Armstrong, Zhang, He, & Gross, 2001; Przybylski et al., 2009). HABA (30 mg) was mixed with TMG  
125 (31.2  $\mu$ L) in methanol (300  $\mu$ L), and the resulting solution was sonicated for 15 min at 40 °C and  
126 dried under vacuum overnight. ILM was then prepared at a concentration of 70-90 mg/mL in  
127 methanol for use as a matrix, without further purification.

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

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

#### 133 *2.4.2. Electrospray ionization – mass spectrometry (ESI-MS)*

##### 134 *2.4.2.1. Ultra performance size-exclusion chromatography (UP-SEC)*

135 Samples were prepared in water at a concentration of 1 mg/mL. Chromatographic separations  
136 were performed on an ACQUITY UPLC H-Class system (Waters, Milford, MA, USA) using an  
137 ACQUITY UPLC Protein BEH SEC column (125 $\text{\AA}$ , 1.7  $\mu\text{m}$ , 4.6 mm x 300 mm). Elution was conducted  
138 in 50 mmol/L ammonium formate, formic acid 0.1% at a flow rate of 400  $\mu\text{L}/\text{min}$  and a column  
139 oven temperature of 40  $^{\circ}\text{C}$ . The injection volume was set to 2  $\mu\text{L}$ .

##### 140 *2.4.2.2. Electrospray ionization high-resolution mass spectrometry (ESI-HRMS)*

141 MS-detection was performed with the SYNAPT G2-Si instrument hyphenated with the ACQUITY  
142 UPLC H-Class system. The ESI source was operating in the negative ionization mode using a  
143 capillary voltage of -2.5 kV and the following conditions: cone voltage, 120 V; source offset, 20 V;  
144 source temperature, 120  $^{\circ}\text{C}$ ; desolvation gas temperature, 450  $^{\circ}\text{C}$ ; desolvation gas flow, 800 L/h,  
145 and cone gas flow, 50 L/h. Nitrogen (> 99.5%) was employed as the desolvation gas. Mass  
146 calibration was carried out using a sodium formate solution (10 mmol/L NaOH in  
147 isopropanol/water/formic acid 49.9:49.9:0.2, v/v/v) and a lock mass correction was applied for  
148 accurate mass measurements using the  $[\text{M}-\text{H}]^{-}$  ion ( $m/z$  554.2615) obtained from a Leu-enkephalin  
149 solution (1 ng/ $\mu\text{L}$  in  $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{formic acid}$  50:49.9:0.1, v/v/v). The scan range was  $m/z$  50-2500  
150 at 0.25 s/scan. The TOF was operated in the sensitivity mode, providing an average resolving

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

### 153 **3. Results and discussion**

#### 154 *3.1. Hydrolysis*

##### 155 *3.1.1. Pectin hydrolysis with H<sub>2</sub>O<sub>2</sub>*

156 It has been reported that H<sub>2</sub>O<sub>2</sub> degradation of pectin produced oligosaccharide mixtures of  
157 different main molecular weight varying H<sub>2</sub>O<sub>2</sub> concentration (Zhang et al., 2018). However, in our  
158 hands this alkaline treatment was hardly repeatable from one run to another as revealed by  
159 HPAEC analysis (data not shown).

##### 160 *3.1.2. Pectin hydrolysis with TFA*

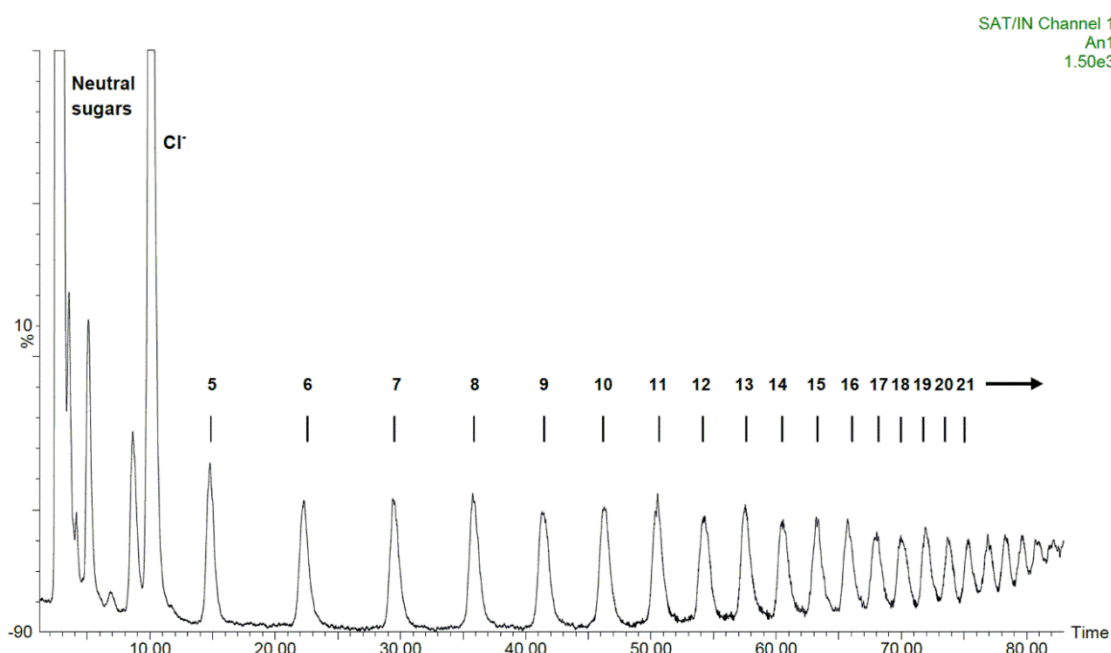
161 Direct TFA treatment of pectin (in similar conditions to those described in 2.2.3) produced partial  
162 degradation and gave mixtures containing medium to large size oligosaccharides, from which it  
163 was not possible to obtain clear cut fractions. Probably, under these conditions the neutral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

##### 166 *3.1.3. Pectin hydrolysis with HCl*

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

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

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



179

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

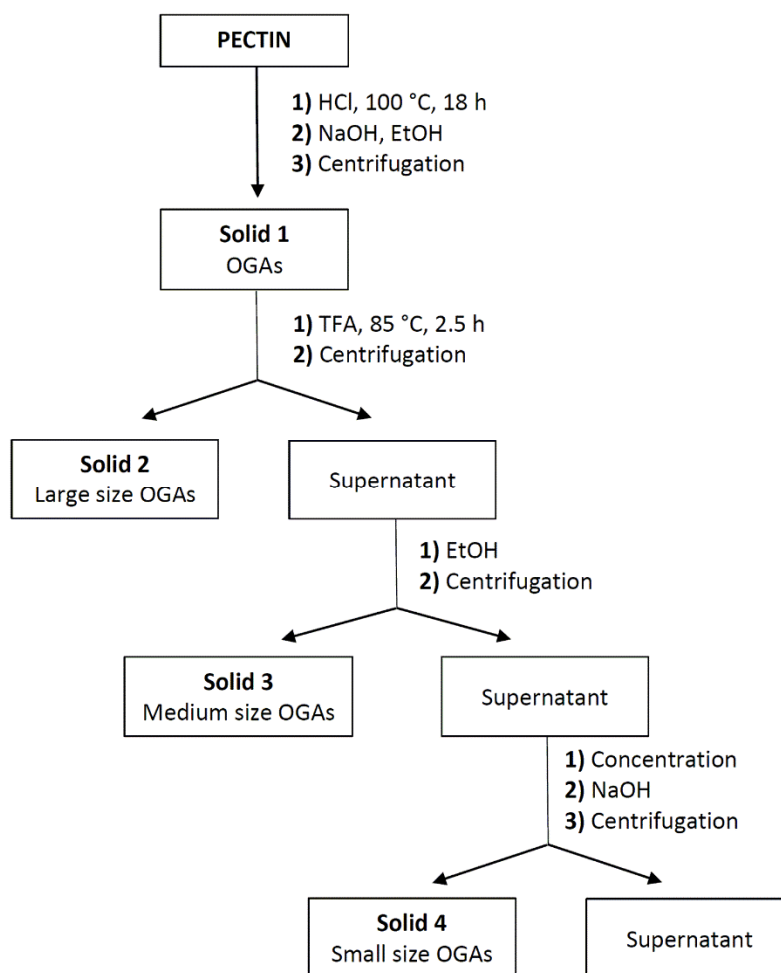
180

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

184 *3.2. Pectin depolymerization followed by TFA hydrolysis: production of different size families of*

185 *OGAs*

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



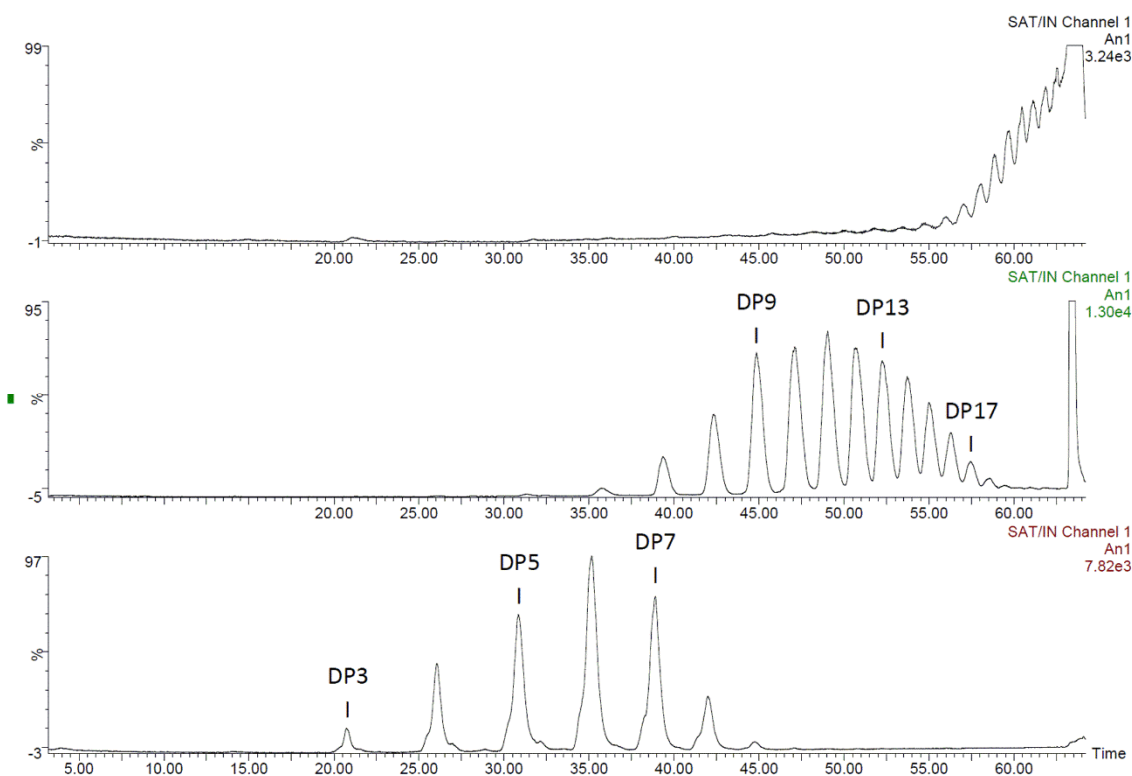
189

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

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

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

198



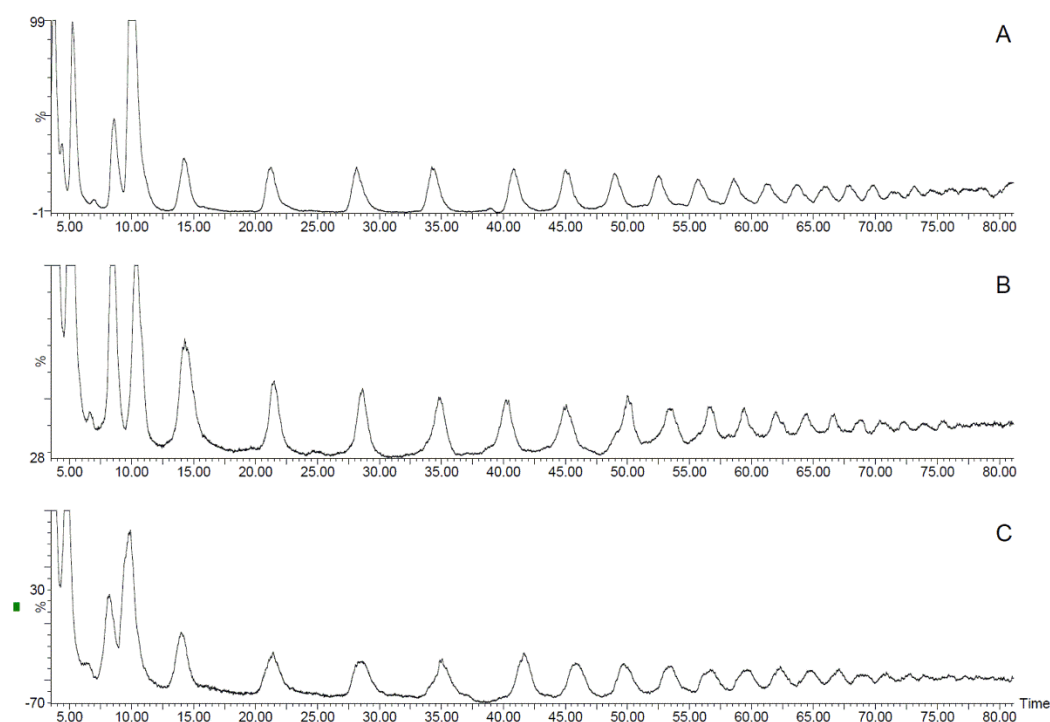
199

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

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

207 *3.3. Hydrolysis of orange and apple waste*

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



214

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

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

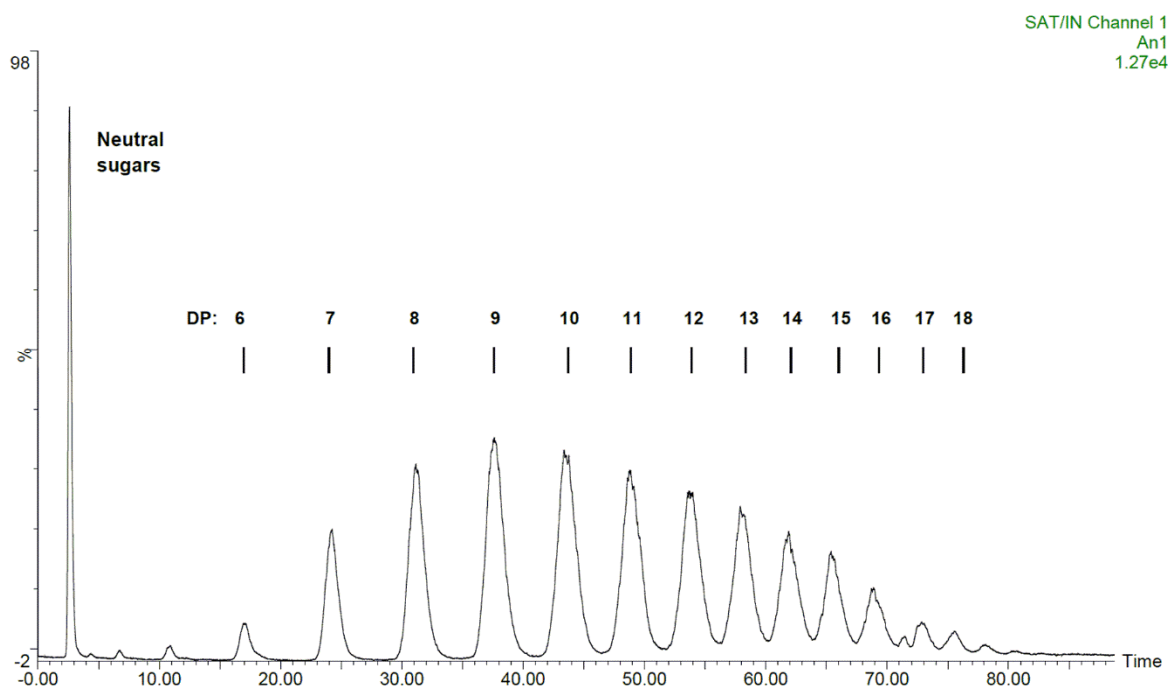
221 identical with OGAs with DP between 6 and 18 (Fig. S2). Finally, for the small size fractions,  
222 oligosaccharides with DP between 2 and 8 were found for both fruit wastes.

### 223 3.4. Characterization of the fractions

#### 224 3.4.1. HPAEC

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

233



234



235

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

236

Using these conditions, the different peaks corresponding to the DPs are completely resolved,

237

opening the possibility to isolate the individual oligosaccharides as pure compounds.

238

#### *3.4.2. MALDI-TOF mass spectrometry*

239

In order to characterize **Solid 3** in detail, and to confirm the presence of all medium size OGAs,

240

MALDI-TOF MS was performed in the negative mode. For these experiments an ionic liquid matrix

241

of HABA/TMG<sub>2</sub> was used. MALDI spectrum (Fig. S3, Table S2) showed peaks of molecular ions [M-

242

H]<sup>-</sup> from DP 3 ( $m/z = 545.0995$ ) to DP 14 ( $m/z = 2481.4531$ ). The compounds with higher DP were

243

not detected in the spectrum, due to a probable better desorption ionization process of the lower

244

DP when analyzing disperse OGAs mixtures. In addition, the peaks corresponding to a loss of a

245

water molecule of almost all oligosaccharides were found. They are more abundant for DP 3 and

246

DP 4 (at  $m/z = 527.0895$  and  $m/z = 703.1215$ , respectively), and their intensity decreases for higher

247

DPs.

248

249

#### *3.4.3. ESI mass spectrometry*

250

A recent developed method using SEC coupled to ESI-MS (Voxeur et al., 2019) allowed us to detect

251

all the OGAs that were shown in the HPAEC chromatogram of **Solid 3** (Fig. 6, Fig. S4), as mono- or

252

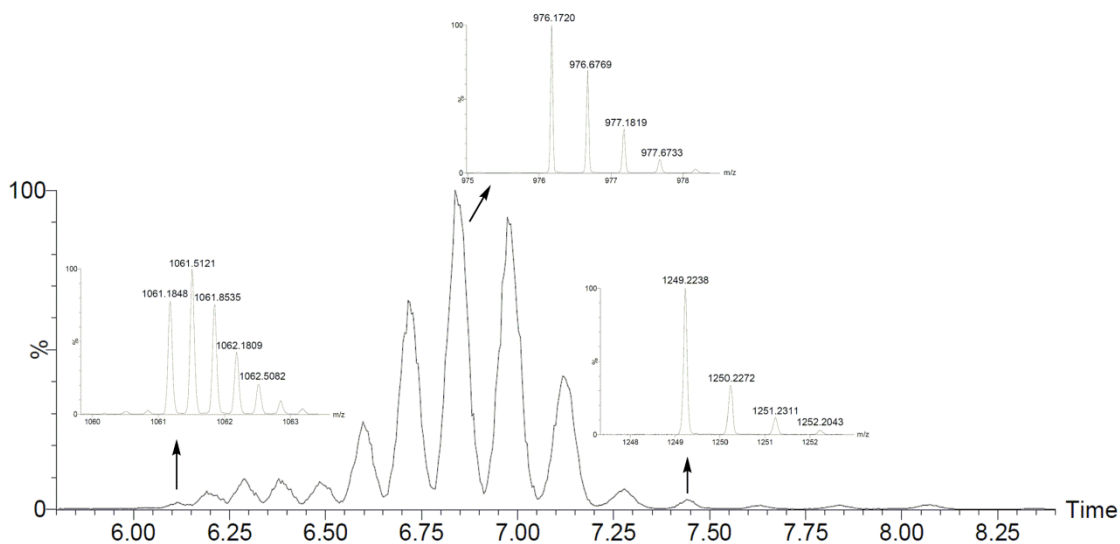
multi-charged ions. For example, DP 7 appeared as the mono-charged ion at  $m/z 1249.2238$  [M-H]<sup>-</sup>

253

, DP 11 as the di-charged ion at  $m/z 976.1720$  [M-2H]<sup>2-</sup> and the DP 18 as the tri-charged ion at  $m/z$

254

$1061.1848$  [M-3H]<sup>3-</sup>.



**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

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

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## 280 **Appendix A. Supplementary data**

281 <sup>1</sup>H NMR spectra of DP 3, DP 4 and DP 5, relative composition of **Solid 3**, MALDI-MS spectra, SEC  
282 chromatograms and ESI-MS spectra.

## 283 **References**

- 284 Armstrong, D. W., Zhang, L. K., He, L., & Gross, M. L. (2001). Ionic liquids as matrixes for matrix-  
285 assisted laser desorption/ionization mass spectrometry. *Analytical Chemistry*, 73(15), 3679–  
286 3686. <https://doi.org/10.1021/ac010259f>
- 287 Aziz, A., Heyraud, A., & Lambert, B. (2004). Oligogalacturonide signal transduction, induction of  
288 defense-related responses and protection of grapevine against *Botrytis cinerea*. *Planta*,  
289 218(5), 767–774. <https://doi.org/10.1007/s00425-003-1153-x>
- 290 Barreira, J. C. M., Arraibi, A. A., & Ferreira, I. C. F. R. (2019). Trends in Food Science & Technology  
291 Bioactive and functional compounds in apple pomace from juice and cider manufacturing :

292 Potential use in dermal formulations. *Trends in Food Science & Technology*, 90(December  
293 2018), 76–87. <https://doi.org/10.1016/j.tifs.2019.05.014>

294 Bellincampi, D., Dipierro, N., Salvi, G., Cervone, F., & De Lorenzo, G. (2000). Extracellular H<sub>2</sub>O<sub>2</sub>  
295 induced by oligogalacturonides is not involved in the inhibition of the auxin-regulated rolB  
296 gene expression in tobacco leaf explants. *Plant Physiology*, 122(4), 1379–1385.  
297 <https://doi.org/10.1104/pp.122.4.1379>

298 Bhushan, S., Kalia, K., Sharma, M., Singh, B., & Ahuja, P. S. (2008). Processing of apple pomace for  
299 bioactive molecules. *Critical Reviews in Biotechnology*, 28(4), 285–296.  
300 <https://doi.org/10.1080/07388550802368895>

301 Ciriminna, R., Chavarría-Hernández, N., Rodríguez Hernández, A. I., & Pagliaro, M. (2015). Pectin :  
302 A new perspective from the biorefinery standpoint. *Biofuels, Bioproducts and Biorefining*,  
303 9(4), 368–377. <https://doi.org/10.1002/bbb>

304 Clausen, M., & Madsen, R. (2004). Synthesis of oligogalacturonates conjugated to BSA.  
305 *Carbohydrate Research*, 339(13), 2159–2169. <https://doi.org/10.1016/j.carres.2004.06.012>

306 Côté, F., & Hahn, M. G. (1994). Oligosaccharins: structures and signal transduction. *Plant*  
307 *Molecular Biology*, 26(5), 1379–1411. <https://doi.org/10.1007/BF00016481>

308 Davis, K. R., Darvill, A. G., Albersheim, P., & Dell, A. (1986). Host-Pathogen Interactions. XXIX.  
309 Oligogalacturonides released from sodium polypectate by endopolygalacturonic acid lyase  
310 are elicitors of phytoalexins in soybean. *Plant Physiology*, 80, 568–577.

311 Doares, S. H., Syrovets, T., Weiler, E. W., & Ryan, C. A. (1995). Oligogalacturonides and chitosan  
312 activate plant defensive genes through the octadecanoid pathway. *Proceedings of the*  
313 *National Academy of Sciences of the United States of America*, 92(10), 4095–4098.  
314 <https://doi.org/10.1073/pnas.92.10.4095>

- 315 Ferrari, S., Savatin, D. V., Sicilia, F., Gramegna, G., Cervone, F., & De Lorenzo, G. (2013).  
316 Oligogalacturonides: Plant damage-associated molecular patterns and regulators of growth  
317 and development. *Frontiers in Plant Science*, 4(49), 1–9.  
318 <https://doi.org/10.3389/fpls.2013.00049>
- 319 Hodroge, A., Trécherel, E., Cornu, M., Darwiche, W., Mansour, A., Ait-Mohand, K., ... Ausseil, J.  
320 (2017). Oligogalacturonic Acid Inhibits Vascular Calcification by Two Mechanisms: Inhibition  
321 of Vascular Smooth Muscle Cell Osteogenic Conversion and Interaction with Collagen.  
322 *Arteriosclerosis, Thrombosis, and Vascular Biology*, 37(7), 1391–1401.  
323 <https://doi.org/10.1161/ATVBAHA.117.309513>
- 324 Hotchkiss, A. T., Lecrinier, S. L., & Hicks, K. B. (2001). Isolation of oligogalacturonic acids up to DP  
325 20 by preparative high-performance anion-exchange chromatography and pulsed  
326 amperometric detection. *Carbohydrate Research*, 334(2), 135–140.  
327 [https://doi.org/10.1016/S0008-6215\(01\)00170-7](https://doi.org/10.1016/S0008-6215(01)00170-7)
- 328 Lobo, M. G., & Dorta, E. (2019). *Utilization and Management of Horticultural Waste. Postharvest*  
329 *Technology of Perishable Horticultural Commodities*. Elsevier Inc.  
330 <https://doi.org/10.1016/b978-0-12-813276-0.00019-5>
- 331 Luzio, G. A., & Cameron, R. G. (2013). Determination of degree of methylation of food pectins by  
332 chromatography. *Journal of the Science of Food and Agriculture*, 93(10), 2463–2469.  
333 <https://doi.org/10.1002/jsfa.6061>
- 334 Ognyanov, M., Remoroza, C., Schols, H. A., Georgiev, Y., Kratchanova, M., & Kratchanov, C. (2016).  
335 Isolation and structure elucidation of pectic polysaccharide from rose hip fruits (*Rosa canina*  
336 L.). *Carbohydrate Polymers*, 151, 803–811. <https://doi.org/10.1016/j.carbpol.2016.06.031>
- 337 Przybylski, C., Gonnet, F., Bonnaffé, D., Hersant, Y., Lortat-Jacob, H., & Daniel, R. (2009). HABA-  
338 based ionic liquid matrices for UV-MALDI-MS analysis of heparin and heparan sulfate

339 oligosaccharides. *Glycobiology*, 20(2), 224–234. <https://doi.org/10.1093/glycob/cwp169>

340 Putnik, P., Bursać Kovačević, D., Režek Jambrak, A., Barba, F. J., Cravotto, G., Binello, A., ...  
341 Shpigelman, A. (2017). Innovative “green” and novel strategies for the extraction of bioactive  
342 added value compounds from citruswastes - A review. *Molecules*, 22(5), 680–704.  
343 <https://doi.org/10.3390/molecules22050680>

344 Ridley, B. L., O’Neill, M. A., & Mohnen, D. (2001). *Pectins: Structure, biosynthesis, and*  
345 *oligogalacturonide-related signaling. Phytochemistry* (Vol. 57).  
346 [https://doi.org/10.1016/S0031-9422\(01\)00113-3](https://doi.org/10.1016/S0031-9422(01)00113-3)

347 Round, A. N., Rigby, N. M., MacDougall, A. J., & Morris, V. J. (2010). A new view of pectin structure  
348 revealed by acid hydrolysis and atomic force microscopy. *Carbohydrate Research*, 345(4),  
349 487–497. <https://doi.org/10.1016/j.carres.2009.12.019>

350 Senit, J. J., Velasco, D., Manrique, A. G., Sanchez-barba, M., Toledo, J. M., Santos, V. E., ... Ladero,  
351 M. (2019). Industrial Crops & Products Orange peel waste upstream integrated processing to  
352 terpenes , phenolics , pectin and monosaccharides : Optimization approaches. *Industrial*  
353 *Crops & Products*, 134, 370–381. <https://doi.org/10.1016/j.indcrop.2019.03.060>

354 Sundarraj, A. A., & Ranganathan, T. V. (2017). A Review - Pectin from Agro and Industrial Waste.  
355 *International Journal of Applied Environmental Sciences*, 12(10), 1777–1801.

356 Thibault, J. F., Renard, C. M. G. C., Axelos, M. A. V., Roger, P., & Crépeau, M. J. (1993). Studies of  
357 the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydrate Research*,  
358 238(C), 271–286. [https://doi.org/10.1016/0008-6215\(93\)87019-O](https://doi.org/10.1016/0008-6215(93)87019-O)

359 Van Cutsem, P., & Messiaen, J. (1994). Biological effects of pectic fragments in plant cells. *Acta*  
360 *Botanica Neerlandica*, 43(3), 231–245. <https://doi.org/10.1111/j.1438-8677.1994.tb00749.x>

361 Virgen-Ortiz, J. J., Morales-Ventura, J. M., Colín Chávez, C., Esquivel-Chávez, F., Vargas-Arispuro, I.,

362 Aispuro-Hernández, E., & Martínez-Téllez, M. A. (2020). Postharvest application of pectic-  
363 oligosaccharides on quality attributes, activities of defense- related enzymes, and  
364 anthocyanin accumulation in strawberry. *Journal of the Science of Food and Agriculture*,  
365 *100*(5), 1949–1961. <https://doi.org/10.1002/jsfa.10207>

366 Voxeur, A., Habrylo, O., Guénin, S., Miart, F., Soulié, M.-C., Rihouey, C., ... Vernhettes, S. (2019).  
367 Oligogalacturonide production upon *Arabidopsis thaliana*–*Botrytis cinerea* interaction.  
368 *Proceedings of the National Academy of Sciences*, *116*(39), 19743–19752.  
369 <https://doi.org/10.1073/PNAS.1900317116>

370 Weiler, E. W. (2003). Sensory principles of higher plants. *Angewandte Chemie - International*  
371 *Edition*, *42*(4), 392–411. <https://doi.org/10.1002/anie.200390125>

372 Willats, W. G. T., Knox, J. P., & Mikkelsen, J. D. (2006). Pectin: New insights into an old polymer are  
373 starting to gel. *Trends in Food Science and Technology*, *17*(3), 97–104.  
374 <https://doi.org/10.1016/j.tifs.2005.10.008>

375 Zhang, S., Hu, H., Wang, L., Liu, F., & Pan, S. (2018). Preparation and prebiotic potential of pectin  
376 oligosaccharides obtained from citrus peel pectin. *Food Chemistry*, *244*(1), 232–237.  
377 <https://doi.org/10.1016/j.foodchem.2017.10.071>

378