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Production and molecular characterization of tailored citrus pectin-derived compounds

Jessika N. Humerez-Flores^{a,*}, Clare Kyomugasho^a, Ana A. Gutiérrez-Ortiz^a, Margot De Bie^a, Agnese Panozzo^a, Ann M. Van Loey^a, Paula Moldenaers^b, Marc E. Hendrickx^a

^a KU Leuven, Department of Microbial and Molecular Systems (M2S), Laboratory of Food Technology and Leuven Food Science and Nutrition Research Centre (LForCe), Kasteelpark Arenberg 22, PB 2457, B-3001 Leuven, Belgium.

^b KU Leuven, Department of Chemical Engineering, Soft Matter, Rheology and Technology, Celestijnenlaan 200F, PB 2424, B-3001 Leuven, Belgium.

Author's emails addresses:

Jessika N. Humerez Flores: jessikanatalia.humerezflores@kuleuven.be

Clare Kyomugasho: clare.kyomugasho@kuleuven.be

Agnese Panozzo: agnesepanozzo@gmail.com

Ana A. Gutierrez Ortiz: ana.gutierrezortiz@gmail.com

Margot de Bie: margot.de.bie@outlook.com

Paula Moldenaers: Paula.moldenaers@kuleuven.be

Ann M. Van Loey: ann.vanloey@kuleuven.be

Marc E. Hendrickx : marceg.hendrickx@kuleuven.be

* Corresponding author

Telephone: +3216376575

E-mail: jessika.humerez@kuleuven.be

31 Graphical abstract (IN COLORS)

HPSEC-RI-MALLS: High Pressure Size Exclusion Chromatography/Refractive Index detection and Multi Angle Laser Light Scattering ; FT-IR: Fourier Transformed Infrared Spectroscopy; ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry; HPAEC-PAD: High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection; ESI/TOF-MS: Electrospray ionization-time-of-flight-mass spectrometry.

ABSTRACT

In this study, tailored-made citrus pectin-derived compounds were produced through controlled enzymatic and/or chemical modifications of commercial citrus pectin with different degrees of methylesterification (DM) and similar average molecular weight (M_w). In the first treatment, degradation of the citrus pectin (CP) materials by endo-polygalacturonase (EPG) yielded pectins with average M_w 's (between 2 and 60 kDa). Separation and identification of the oligosaccharide fraction present in these samples, revealed the presence of non-methylesterified galacturonic acid oligomers with degree of polymerization (DP) 1-5. In the second treatment, exploiting the combined effect of EPG and pectin lyase, compounds with M_w between 2 and 21 kDa, containing methylesterified and non-methylesterified polygalacturonans (DP 1-6), were generated. Finally, CP was sequentially modified by chemical saponification and the action of EPG. A sample of DM 11% and M_w 2.7 kDa, containing POS (DP 1-5), was produced. Diverse pectin-derived compounds were successfully generated for further studies exploring their functionality.

Key words: Citrus pectin, pectin oligosaccharides, structural modifications, endo-polygalacturonase, pectin lyase, HILIC-TOF-MS

1. INTRODUCTION

Pectin is a complex heterogeneous polysaccharide present in the cell walls of higher plants. The polymer is structurally composed of different subdomains including homogalacturonan (HG), rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan (Mohnen, 2008). The structural features of this polymer, such as its neutral sugar content, molecular weight, protein content, and methylesterification, have been reported to govern its functional properties, such as its emulsifying, and gelling properties (Alba & Kontogiorgos, 2017; Kyomugasho et al., 2018). Therefore, in the last decade, structural modifications of the pectin molecule have been exploited with the aim of producing tailored molecules which exhibit enhanced functional properties (Alba & Kontogiorgos, 2017; Schmidt, Koch, et al., 2015). Pectin-derived compounds can be obtained through depolymerization of the pectin molecule by applying different techniques, leading to the generation of a mixture of substituted and/or unsubstituted polymer fragments of different degree of polymerization (DP) (Babbar et al., 2015). The most commonly applied techniques include enzymatic (Coenen et al., 2008; Leijdekkers et al., 2013) and chemical (Coenen et al., 2008; Zhang et al., 2018) pectin modifications. In general, enzymatic processes are highly preferred to produce tailored compounds, due to their mode of action. As enzymes act on specific substrates, undesired structural modifications can be largely avoided, thereby allowing the production of specific tailored pectin-derived compounds (Karaki et al., 2016). However, when the action of the pectin-related enzymes is insufficient to modify the pectin molecule to a certain extent, a combination of the above-mentioned processes has been reported to be successful for the structural modification of pectin (Wu & Mort, 2014; Zhu et al., 2019).

With pectin-derived compounds suggested to possess several functionalities, exploring their structure-function relationship would facilitate exploitation the full potential of these molecules. Pectin-derived molecules, including pectin oligosaccharides (POS), have been proposed as prebiotic candidates, as these compounds have been shown to selectively stimulate the growth and activity of beneficial gut bacteria (Babbar et al., 2015). A number of studies have also focused their research on other physiological properties of pectin-derived compounds, such as their antioxidant and anti-inflammatory properties (Koriem et al., 2014), as well as their antiglycation and antibacterial capacity (Zhu et al.,

2019). Due to the different properties exhibited by POS, these compounds have been suggested to be applied as potential functional food ingredients (Barreteau et al., 2006). Furthermore, physicochemical properties of POS such as their rheological properties (Francielle et al., 2018), fat absorption and ion binding capacity (Aline de Moura et al., 2020) have been recently explored. Nevertheless, existing studies lack the evaluation of the structural characteristics and their effect on the functional properties of specific tailored pectin molecules, containing fractions of POS, generated through structural modification processes. For instance, although research has been performed on the effect of DM of pectin on its emulsifying properties, little research has been performed on the effect of DM on tailored pectin molecules of different chain lengths (Schmidt, Koch, et al., 2015). Similarly, few studies have extensively evaluated the effect of M_w of pectin from different sources, on its emulsifying capacity (Schmidt, Schmidt, et al., 2015). Therefore, establishing a direct relation between structure and functionality remains a challenge.

Therefore, this study aimed at generating a variety of specific tailored pectin-derived compounds of interest, in relation to their potential functional properties, which are aimed to be fully explored in further research. More specifically, the chain length of the mother pectin samples was strategically modified to different extents by applying enzymatic processes. To this end, ten different pectin-derived samples were generated by enzymatic and non-enzymatic modification of three mother citrus pectins. The generated oligogalacturonides were then characterized for their functionality-related properties including galacturonic acid content, neutral sugar profile, degree of methylesterification, molecular weight distribution, ion content and protein content. Furthermore, the different oligosaccharide fractions present in each of the generated samples were identified and quantified. Altogether, this work generates and characterizes strategically tailored citrus pectin-derived samples containing POS as a first step towards the establishment of their structure-function relationships.

2. MATERIALS AND METHODS

2.1. Materials

Commercial citrus pectins (CP) with different degrees of methylesterification (DM 77, 57 and 43%) and M_w (162000, 184000 and 158000 kDa, respectively), were supplied by Cargill (Belgium) and

encoded CP77, CP57 and CP43. The pectin samples were stored in a desiccator until further use. Pure galacturonic acid (GalA) standards (mono-, di- and tri-GalA) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Endo-polygalacturonase M2 (EPG) from *Aspergillus aculeatus* was purchased from Megazyme (Netherlands) and Pectin lyase (PL) from *Aspergillus japonicas* was purchased from Sigma-Aldrich (Japan). Polygalacturonic acid, sodium acetate, L-fucose, L-arabinose, D-glucose, D-mannose, D-xylose, ammonium formate (HCOONH_4) and formic acid (HCOOH) were retrieved from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Acetonitrile (CH_3CN), di-Sodium tetraborate (Borax) decahydrate, hydrochloric acid (0.1 M), nitric acid (65%) and L-rhamnose were purchased from Acros Organics (Geel, Belgium). Concentrated volumetric NaOH solution (convo normadose, 0.1 M) was retrieved from VWR Chemicals (Leuven, Belgium). D-galactose was purchased from Merck KGaA (Darmstadt, Germany). Unless mentioned otherwise, all chemicals used were of analytical grade.

2.2. Production of pectin-derived samples of lower molar mass

Generation of the citrus pectin-derived samples in this study was achieved by means of enzymatic and/or non-enzymatic processes. Given the variation (77% - 43%) in the DM of the starting samples, hydrolases with preference for high DM pectin (PL) or low DM pectin (EPG) were exploited in this study for generation of tailored pectin molecules with different structural properties.

2.2.1. Enzyme activity measurements

The activity of EPG was determined following the spectrophotometric method proposed by Verlent et al. (2004) and Gross (1982). Briefly, to 350 μL of a 0.3% (w/v) polygalacturonic acid (PGA) dissolved in Na-acetate buffer (40 mM, pH 4.4), 50 μL of endo-polygacturonase (EPG) was added and the assay incubated for 10 min at 35 °C. The reaction was stopped by addition of borate buffer (pH 9, 0.1 M). After a subsequent reaction of the released reducing end groups with 1% (w/v) 2-cyanoacetamide solution at 40 °C, EPG activity was spectrophotometrically (Ultrospec 2100 pro, Amersham Bioscience, Buckinghamshire, United Kingdom) determined. One unit (U) of EPG activity was defined as the

amount of enzyme catalyzing the release of 1 μ mol of reducing end groups per minute under the assay conditions. The activity of EPG was 1650 U/milliliter (mL).

In the case of pectin lyase, the activity of the enzyme provided by Sigma Aldrich was used for determining the enzyme concentrations used during the sample production process. The enzyme activity was 0.3 U/milligram (mg).

2.2.2. Generation of low molecular weight pectin-derived compounds by means of endo-polygalacturonase (1st treatment)

The three mother pectin materials (CP77, CP57 and CP43) were incubated with EPG in order to facilitate their hydrolysis and therefore the production of pectin-derived samples containing compounds of different molecular weights. EPG is an enzyme that preferentially cleaves the linkage between non-methylesterified GalA residues of the HG backbone of pectin, leading to the generation of GalA residues and shorter chain pectin polymers, including POS, which can be methyl- or non-methylesterified (Pedrolli et al., 2009).

In a preliminary study, determining the optimal concentration of EPG to be added during the process, this enzyme was added at two different concentrations (5 or 25 U per gram of pectin), to pectin previously dissolved in Na-acetate buffer (40 mM, pH 5) at a concentration of 0.8% (w/v). The hydrolysis was performed for a period of 15 min, 30 min, 1 h, 3 h and 22 h at 35 °C with constant stirring at 250 rpm. The enzyme was inactivated through a heat treatment at 95 °C for 5 min. These samples were then characterized for their M_w s to ensure significant differences and facilitate selection of the enzyme concentration and incubation times, to produce the final samples. After enzyme inactivation, the samples were dialyzed against demineralized water to ensure (almost complete) removal of the salt added during the production process. Specifically, the hydrolyzed sample was transferred into Spectra/Por® dialysis tubing (0.1 – 0.5 kDa, molecular weight cut-off (MWCO)) and thoroughly dialyzed against demineralized water for 72 h. Dialyzed samples were then lyophilized (Christ Freeze Dryers, Osterode, Germany) and stored in a desiccator at room temperature until use. Removal of ions, particularly the high amounts of Na^+ added to maintain conditions for EPG action,

was crucial since these ions have been reported to impact different functional properties of pectin, such as cation-binding capacity and gelling potential (Kastner et al., 2020). A summary of the pectin-derived samples generated, and their denotations are presented in **Table 1**.

2.2.3. Generation of low molecular weight pectin-derived compounds by means of endo-polygalacturonase and pectin lyase (2nd treatment)

A second treatment applied for the production of pectin-derived compounds was based on the enzymatic depolymerization of pectin by the combined effect of EPG (hydrolysis) and PL (beta eliminative reaction). PL was aimed at depolymerizing the pectin polymer of high DM, since this enzyme is known to cleave the linkage between methylesterified GalA residues at the HG backbone of pectin (Pedrolli et al., 2009). EPG was added, to each of the pectin solutions, at a constant concentration of 5 U per g of pectin. Therefore, to one gram of pectin (at a concentration of 0.8% (w/v) in Na-acetate buffer (150 mM, pH 5.0)), 5 U of EPG and 0.5 or 2.5 U of PL were added, followed by incubation of the system at 37 °C for 0.5 or 22 h, with constant stirring at 250 rpm. After inactivation of the enzymes at 95 °C for 5 min, purification of the sample was performed through dialysis as described in section 2.2.2. A summary of the pectin-derived samples generated, and their denotations are present in **Table 1**. The codes for each of the samples are based on the initial pectin material from which each of them was originally produced.

2.2.4. Chemical saponification and enzymatic hydrolysis of pectin (3rd treatment)

In the third treatment, pectin-derived compounds were generated through a combination of chemical demethylesterification followed by enzymatic depolymerization of the demethylesterified pectin. First the chemical saponification of a pectin material in order to produce low DM pectin was performed as described by Fraeye et al. (2009). Briefly, CP43 was dissolved in demineralized water at a concentration of 0.8% (w/v) and the pH adjusted to 11 with concentrated volumetric 0.1 M NaOH solution while maintaining the temperature at 4 °C in order to avoid undesired chemical depolymerization of the pectin. Under this alkaline condition, methylester groups underwent saponification by OH-ions, leading to a decrease in pH. The drop in the pH of the pectin solution was titrated with NaOH using an automatic

pH-stat titrator (Titrimo 718, W.Methohm, Herisau, Switzerland) in order to maintain the pH at 11. The amount of 0.1 M NaOH required to achieve a specific DM was theoretically determined. The calculated amount of 0.1M NaOH was added by the pH-stat titrator to the pectin in solution, the reaction was stopped adjusting the pH to 4.4 with 0.1 M HCl. Enzymatic depolymerization of the demethylesterified pectin by EPG then followed in order to produce shorter chain pectin-derived compounds including POS. First, a specific quantity of sodium acetate was added to the system to obtain a 40 mM Na-acetate buffer solution that facilitated maintaining the pH at 4.4 during the subsequent EPG incubation step. After equilibration of the system at 35 °C under constant stirring (250 rpm), EPG was then added at a concentration of 5 U per gram of pectin followed by incubation for 22 h at 35 °C under constant stirring. The enzyme was inactivated by applying a heat treatment at 95 °C for 5 min. Finally, dialysis and freeze drying of the samples was performed as described in section 2.2.2. A summary of the pectin-derived samples generated, and their denotations are present in **Table 1**. Furthermore, a scheme summarizing the three different treatments applied for the degradation of the initial pectin materials CP77, CP57 and CP43, and the correspondent CP-derived compounds generated are presented in **Supplementary Figure 1**.

2.3. Structural characterization of pectin samples

The different pectin samples, mother pectins and samples derived thereof, were subsequently structurally characterized. Specifically, the degree of methylesterification (DM), galacturonic acid content, neutral monosaccharide profile and content, molar weight distribution (MWD), mineral content and protein content were determined as described below.

2.3.1. Molecular weight distribution

The molar mass distribution of the pectin samples was measured based on the method described by Shpigelman et al (2014), using high-performance size-exclusion chromatography (HPSEC) equipped with multi-angle laser light scattering (MALLS) (PN3621, Postnova analytics, Germany) and refractive index (RI) detection (Shodex RI-101, Showa Denko K.K., Kawazaki, Japan). Separation was performed using a series of Waters columns, i.e., Ultrahydrogel 250, 1000, and 2000 with exclusion limits of 8×10^4 ,

4*10⁶, and 1*10⁷ g/mol, respectively (Waters, Milford, MA). Analysis of the samples was done in duplicate and the molar masses were calculated using the Debye fitting method (2nd order) of the operating software (Nova Mals, version 1.0.0.18, Postnova analytics).

2.3.2. Galacturonic acid content

The GalA content was determined after samples hydrolysis (in duplicate) based on the method described by Ahmed & Labavitch (1978) followed by spectrophotometric quantification of GalA (in triplicate per duplicate) as described by Blumenkrantz & Asboe-Hansen (1973).

2.3.3. Monosaccharide composition

The neutral sugar composition of pectin-derived samples was determined using high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) based on the method described by (Houben et al., 2011). For this purpose, a Dionex system (ICS-6000) equipped with a Dionex Single Pump, a CarboPac™ PA20 column (150 X 3 mm) and guard column (30 X 3 mm), and an ED50 electrochemical detector (Dionex, Sunnyvale, CA, USA) was used. Calibration curves of commercial sugar standards (L-fucose, L-rhamnose, L-arabinose, D-galactose, D-glucose, D-xylose and D-mannose), at varying concentrations (1-10 ppm), were used for quantification and identification of the different monosaccharides.

2.3.4. Degree of methylesterification

The DM was determined based on the procedure described by Kyomugasho et al. (2015) using Fourier transform infra-red (FT-IR) spectroscopy (Shimadzu FTIR-8400S, Japan). Determination of the DM was performed by measuring the transmittance at wave numbers between 4000 cm⁻¹ and 400 cm⁻¹. The absorption intensity of the bands situated around 1740 cm⁻¹ (due to carbonyl group stretching) and 1630-1600 cm⁻¹ (due to carboxylate group stretching) were used to generate the ratio $X = [I(1740)/I(1740+1630)]$. The DM of all the pectin samples (Y) was determined using the equation $Y = 136.86X + 3.987$ of the calibration curve described by Kyomugasho et al. (2015).

2.3.5. Determination of ash content

Determination of the ash content of the different pectin samples was calculated based on the method described by Park & Bell (2004) with few modifications. Briefly, incineration of lyophilized pectin material (10 mg) was performed in a muffle furnace at 550 °C for 24 h. Afterwards the samples were cooled in a desiccator and finally weighed. The ash content was expressed in terms of percentage, by calculating the ratio of sample weight after incineration to the weight before incineration. Analysis of the ash content was carried out in duplicate.

2.3.6. Mineral content

Determination of the mineral content of the incinerated pectin samples (section 2.3.5) was performed based on the method described by Moens et al. (2020), by inductively coupled plasma optical emission spectrometry (ICP-OES) (iCAP 7400 ICP-OES Duo spectrometer, Thermo Scientific, USA). Briefly, the ashed samples were dissolved in ultrapure water (9.9 mL) followed by addition of 100 µL of 65% HNO₃. After 24 h, filtration of the samples was done using a 0.45 µm filter (Chromafil® A-45/25, Macherey-Nagel, Duren, Germany). The mineral content of the samples was expressed as mg per g of pectin sample.

2.3.7. Protein content

Determination of the total nitrogen content of the lyophilized samples was performed based on the method described by Kyomugasho et al. (2017). This analysis was performed by means of the Dumas method using an elemental analyzer (Carlo-Erba EA1108, Thermo Scientific, Waltham, MA, USA). The total protein content was calculated based on the total nitrogen content obtained and applying a correction factor of 6.25. Analysis of each sample was carried out in duplicate.

2.4. Fractionation, separation and identification of citrus pectin-derived samples

2.4.1. Fractionation of hydrolysates

The lyophilized pectin-derived samples were dissolved in 20 mL of ultrapure water (organic free, 18.2 MΩ cm resistance) at a concentration of 1% (w/v). Each sample was then transferred into Spectra/Por® dialysis tubing (MWCO) (3.5 kDa) and dialyzed against ultrapure water for 48 h. The water in which

the membranes were dialyzed, containing the pectin-derived fraction with $M_w < 3.5$ kDa was thereafter freeze dried and stored in a desiccator until further use.

2.4.2 Separation and identification of low M_w compounds

Identification of the generated/fractionated oligosaccharide compounds present in the low molecular weight mixtures ($M_w < 3.5$) was performed based on the work of Prandi et al. (2018) with few modifications. Separation was performed on a hydrophilic interaction liquid chromatography (HILIC) column (Acquity UPLC BEH Amide 1.7 μ m, 30 X 150 mm; Waters, Ireland) coupled to a guard column (Acquity UPLC BEH Amide 1.7 μ m, 2.1 X 5 mm; Waters, Ireland) using a HPLC/ESI-MS system (1200 Series; Agilent Technologies, Diegem, Belgium) and detected using time-of-flight mass spectrometry (TOF-MS). Furthermore, a negative ionization ion mode, capillary voltage of 2.6 kV and a cone voltage of 60 V were applied. Gradient elution with Eluent A ($H_2O:CH_3CN$ 20:80 v/v, 10 mmol/L $HCOONH_4$ + 0.2% v/v $HCOOH$) and B ($H_2O:CH_3CN$ 80 : 20 v/v, 10 mmol/L $HCOONH_4$ + 0.2% v/v $HCOOH$) of 0–60 min linear from 100% to 60% A, 60–65 min isocratic at 60% A, 65–66 min linear from 60% A to 100% A, 66–75 min isocratic at 100% A were used. The optimal run time for separation of various oligosaccharides was 75 min.

2.4.3 Quantification of the low M_w oligosaccharides

Quantification of generated oligosaccharide compounds (mono-, di- and tri-GalA residues) present in the POS mixtures ($M_w < 3.5$) was performed with the same gradient elution described in section 2.4.2, using the aforementioned HPLC system coupled to a charged aerosol detector (CAD) (Corona Veo, Thermo FisherScientific, Geel, Belgium). For quantification, CAD detection was applied as it has been shown that the response generated by this detector is independent of the physicochemical properties of the compound under analysis, this enables the generation of a universal standard calibration for the quantification of the different compounds present in a specific analyte (Vehovec & Obreza, 2010). Therefore, commercial mono-, di- and tri-GalA standards (Sigma-Aldrich, Schnellendorf, Germany) were used in order to obtain a calibration curve which would be further used for the quantification of the different compounds detected. A curve fitting was performed using a power function $f(x) = ax^b$, where

$f(x)$ represents the peak area, 'x' is the concentration of the sample, 'a' is the CAD response factor and 'b' represents the slope (Remoroza et al., 2012).

2.5 Statistical analysis

The statistical software JMP (JMP Pro15, SAS Institute Inc., Cary, NC, USA) was used for analysis of significance. Evaluation of the significant differences was done using one-way ANOVA and Turkey's Studentized Range Post-hoc test. The level of significance was 95% ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Structural properties of mother citrus pectins and samples derived thereof

3.1.1. Molecular weight of pectin-derived compounds generated by the action of endo-polygalacturonase (1st treatment)

Effect of EPG on molecular weight of pectins of different DMs

Molecular weight distributions of the different pectin samples were obtained using high-performance size exclusion chromatography, in which molecules of larger hydrodynamic volume elute earlier than molecules with smaller hydrodynamic volume. In this way, the molecular weight (M_w) of the eluted components decreases with increasing elution time. In order to produce lower molecular weight compounds from the citrus mother pectins (CP77, CP57 and CP43) by action of EPG, a concentration of 5 U/g pectin was selected based on preliminary studies (**Supplementary Figure 2**). Since the aim of this study was to produce pectin-derived compounds with differences in M_w and DM, incubation periods of 1 and 22 h were selected (for all three pectin sources CP77, CP57 and CP43 treated by EPG).

The M_w distribution (full lines) and concentration profiles (dotted lines) for the samples produced through hydrolysis of the three different initial citrus pectin materials (CP77, CP57 and CP43) by EPG are shown in **Figure 1** (desalted samples). Regarding the M_w distribution profiles corresponding to the different initial pectin and pectin-derived samples generated in this study, it can be observed that at a given elution time, there are differences among the different M_w obtained (which indicates different molecular structures with similar hydrodynamic volume). Data beyond 64 minutes (retention time of galacturonic acid) refer to sample impurities (primarily residual salts). The average M_w of peaks prior

to 65 min are summarized in **Table 2**. Nevertheless, to obtain a more accurate picture of the oligosaccharide fraction of low MW, the samples were fractionated and the smallest fraction present was analyzed by Time-Of-Flight Mass Spectrometry as described in section 3.2.

From the concentration profiles of the three initial pectin materials, one population of large pectin molecules (evidenced by a monomodal elution peak with a maximum at 45 min) was observed for each of these samples. The pectin material CP77, CP57 and CP43 exhibited significantly different molecular weights ($1.44 \times 10^5 \pm 4243$, $1.51 \times 10^5 \pm 2828$ and $1.16 \times 10^5 \pm 4213$ Da, respectively). Furthermore, regarding the concentration profiles of the different pectin-derived samples one main polymer population is observed, with peak shoulders on the left or right indicating fractions of respectively higher or lower molar mass. In the case of the compounds generated from CP57 and CP43 after undergoing degradation by EPG (5U) for 22 h (CP57 EPG 5U 22h and CP43 EPG 5U 22h), an additional peak eluting at 50 min could be observed, which is representative for the fraction of larger molecules within these samples. However, these fractions were present in relatively very low amounts (< 5% of the total amount of sample analyzed). **Figure 1** shows that the concentration curves (of larger molecules) of the citrus pectin-derived samples resulting from the hydrolysis of CP77 for 1 h (CP77 EPG 5U 1h) and 22 h (CP77 EPG 5U 22h) elute earlier (peak maxima at 49 and 51 min, respectively) compared to the CP57-based samples, CP57 EPG 5U 1h (54 min) and CP57 EPG 5U 22h (59 min). In a similar way, the second concentration curve (59 min) present in CP57 EPG 5U 22h elutes earlier than that of CP43 EPG 5U 22h (61 min). This pattern indicates that EPG degrades CP77 to a less extent (hence higher average molecular weight derived pectin molecules are generated) compared to CP57 and CP43 for a given incubation period. These results are further supported by the average M_w of the samples produced as shown in **Table 2**. These results can be attributed to the fact that EPG preferentially cleaves the linkage/bond between two non-methylesterified GalA acid residues, with its activity decreasing with increasing DM of the pectin substrate (Duvetter et al., 2009).

3.1.2. Pectin-derived compounds generated by the combined action of endo-polygalacturonase and pectin lyase (2nd treatment)

Molecular weight distribution of pectin-derived compounds

Based on a preliminary experimental stage, in which the effect of DM on the activity of PL was evaluated (**Supplementary Figure 3**), it was observed that the concentration profiles and M_w distribution of the three pectin-derived materials CP77, CP57 and CP43 were similar after undergoing degradation by the combined effect of EPG (5U/g pectin) and PL (2.5U/g pectin) for a period of 22h. Therefore, only the sample CP77, was used for the following experiments involving the degradation of pectin by the combined effect of EPG and PL.

To evaluate the effect of PL concentration on the M_w of pectin, an experiment was performed on CP77, in the presence of EPG at a constant concentration (5 U/g pectin), and at different concentrations of PL (0.5-2.5 U/g pectin) and incubation times. The concentration profiles and the M_w distributions of the pectin-derived compounds produced through hydrolysis of CP77 under different conditions are presented in **Supplementary Figure 4** (de-salted samples). The concentration profiles of each of the three different samples produced, present two concentration peaks which represent the presence of different population of compounds exhibiting differences in M_w . The third peak (elution time above 64 min), represents sample impurities (primarily residual salts). In this way, the first peaks in the concentration profiles of CP77 EPG 5U PL 0.5U 30 min (45 min), CP77 EPG 5U PL 2.5U 30 min (50 min) and CP77 EPG 5U PL 2.5U 22h (52 min) represent the fraction of larger molecules present in these samples. However, for the depolymerized samples, these fractions were present in relatively very low amounts (< 5% of the total amount of sample analyzed). Furthermore, by comparing the 2nd concentration peak of each of the samples, it can be observed that after hydrolysis of CP77 for 22 h by addition of 2.5 U of PL/g of pectin (CP77 EPG 5U PL 2.5U 22h) the maximum of the concentration profile (61 min) is shifted towards the right in comparison to the samples incubated with PL at concentrations of 0.5 and 2.5 U/g pectin for 30 min (59 and 55 min, respectively), thus indicating a greater extent of depolymerization when applying a higher concentration of PL and/or a longer incubation time. From the M_w distribution profiles corresponding to the different pectin-derived samples generated (**Supplementary Figure 4**), it can be observed that there are differences among the different M_w profiles at a given elution time, which could indicate differences in structure (with similar hydrodynamic volume) among the different pectin-derived samples generated as previously suggested.

Pectin-derived compounds generated by means of a combined chemical-enzymatic treatment (3rd treatment)

Molecular weight distribution of pectin-derived compounds

In the third treatment the citrus pectin of lower DM (CP43) was chemically saponified followed by the enzymatic depolymerization (by EPG), leading to the generation of a pectin material with DM of 11% (CP11 ALK EPG 5U 22h). The latter pectin sample contained three fractions (with peak maxima at 51, 61 and 67 min) as shown in **Supplementary Figure 5** (desalted samples). The fraction containing the largest molecules (96000 ± 2121 Da) in this sample, represented by the elution peak at 51 min, was present in low amounts (< 5% of the total amount of sample analyzed). The peak eluting at 61 min was shifted towards the right in comparison to the concentration peak of the initial pectin sample CP43, thus indicating depolymerization of the mother pectin CP43 after it had been chemically demethylesterified and further incubated with EPG for 22 h. Consequently, the generated sample CP11 ALK EPG 5U 22h contained a substantial fractions of lower average M_w (2710 ± 187) as compared to the pectin CP43 from which it was produced (116000 ± 4243 Da). As indicated above, the peak beyond 64 min represents sample impurities (primarily residual salts).

3.1.3. Galacturonic acid content

The GalA and neutral sugar content for each of the final samples produced are shown in **Table 3**. As expected, GalA was the most abundant monomer in all the pectin-derived samples, as the initial material for the production of these samples was citrus pectin, which is known to be rich in HG (which consists of a linear backbone of consecutive 1,4-linked- α -d-GalA units). In general, the generated pectin-derived samples exhibited similar GalA content in comparison to the mother pectin samples from which they were produced, some small differences were observed on some of the generated samples. For instance, in the case of the GalA content of the samples generated through the combined effect of EPG and PL on CP77 differences in GalA content were found when comparing them to the initial pectin material CP77. These samples exhibited the lowest GalA content among all the samples generated in this work. This outcome was expected since a prolonged depolymerization of pectin with EPG and PL has been

reported to promote the release of mono-, di- and tri-GalA residues (Remoroza et al., 2012). Finally, the sample (CP11 ALK EPG 5U 22h), generated applying the 3rd treatment (chemical saponification followed by an enzymatic degradation in the presence of EPG), exhibited a relatively lower GalA content in comparison to its mother pectin material (CP43). A similar relation between DM and GalA content was observed on demethylesterified sugar beet pectin hydrolyzed by the action of EPG (Buchholt et al., 2004). Since EPG is known to prefer substrates of lower DM, an initial saponification of the pectin material promoted the generation of an increased number of active sites available, along the HG backbone, for optimal cleavage by EPG. Therefore, larger amounts of mono-, di- and tri-galacturonate residues could be produced during the degradation process and further removed during the dialysis step.

3.1.4. Neutral sugar content

The amounts of the different monosaccharides present in the initial pectin samples, as well as in the samples derived from these pectins are presented in **Table 3**. In general, all samples exhibited relatively low amounts of the sugars fucose (Fuc) and xylose (Xyl) (pectin-related) as well as glucose and mannose. On the contrary, appreciable amounts of galactose (Gal), rhamnose (Rha) and arabinose (Ara) were present in all the initial pectins and the derived samples. Three ‘sugar ratios’ of linearity (GalA/(Fuc+Rha+Ara+Gal+Xyl), RG-I contribution (Rha/GalA) and Degree of branching [(Ara+Gal)/Rha], formulated specifically for pectin, were calculated based on the molar amounts of the different sugars present in the pectin and pectin-derived samples.

In this regard, the initial pectin materials CP77, CP57 and CP43 exhibited a linearity of 7.26 ± 0.60 , 11.94 ± 1.08 and 13.71 ± 0.58 , respectively. Relatively similar values are found for the pectin-derived samples generated from these materials after undergoing different degradation procedures. Furthermore, the contribution of RG-I for the sample CP11 ALK EPG 5U 22h (0.03 ± 0.01) is the highest among all the pectin-derived compounds generated. Finally, the degree of branching of the derived samples were relatively similar to those of their respective parent pectin materials.

3.1.5. Degree of methylesterification

The DM was determined to ensure that this property was not modified during the enzymatic production of the samples. In the 3rd treatment which involved chemical saponification followed enzymatic depolymerization of CP43, DM determination was aimed at ensuring a decrease in DM (success of the chemical saponification). The average DM values of the initial pectin materials and the samples generated thereof are presented in **Table 3**. It can be observed that the DM of the initial pectin materials, CP77, CP57 and CP43, after undergoing dialysis for purification purposes, were 74, 53 and 38%, respectively. In general, in presence of EPG, no changes in DM were observed. However, when the initial pectin CP77 was depolymerized by the combined action of EPG and PL, a decrease in DM was observed in the derived samples. This decrease in DM was more pronounced with increasing incubation time or concentration of PL and could be attributed to an increase in the amount of the generated methylesterified residues/molecules (as this enzyme preferentially cleaves the bond between methylesterified GalA residues) and their subsequent elution during the dialysis step, thus leading to a drop in the DM of the samples (Remoroza et al., 2012). Finally, the sample generated by means of chemical saponification exhibited a low DM of $10.80 \pm 0.83\%$, which was aimed in order to produce a suitable substrate on which EPG could act optimally.

3.1.6. Protein content

From the results of protein content (**Table 3**), the initial pectins exhibited very low protein contents, with no significant differences. These results are similar to the results obtained by Kyomugasho et al. (2017), who reported a protein content below 1.9% for commercial citrus pectin samples of different DMs. Analyzing the citrus pectin-derived samples (**Table 3**), no significant difference in the protein content was observed. The protein content of pectin was determined given that certain functional properties of pectin including its emulsifying capacity have been reported to be influenced by the presence of protein (~3% or above) in the pectin polymer (Alba & Kontogiorgos, 2017).

3.1.7. Intrinsic mineral content

The mineral content of the initial pectin materials (CP77, CP57 and CP43) and their derived compounds are presented in **Supplementary Table 1**. Specifically, the contents of Ca^{2+} , Mg^{2+} , Fe^{2+} and Zn^{2+} were

determined, given that these divalent ions are reported to influence the functional properties of pectin (Kyomugasho et al., 2018). Additionally, the content of Na^+ was determined in order to ensure optimal purification of the samples during the dialysis process (this ensure removal of Na^+ added as sodium acetate and NaOH during sample production). Furthermore, Ca^{2+} was present in higher amounts (between 0.129 – 0.832 mg Ca^{2+} /g pectin sample) in comparison to Mg^{2+} , Fe^{2+} and Zn^{2+} , which were present at concentrations of 0.009 - 0.126, 0.008 – 0.117 and 0.002 – 0.037 mg/g pectin or samples derived thereof, respectively. For each of these minerals, the values were not statistically significantly different among the pectin samples. Although, the divalent cations are present in small amounts, attention must be paid for those initial and derived pectin samples of low DM, given that low DM pectins possess a high affinity and thus ion binding capacity (Kyomugasho et al., 2017). Therefore, the presence of divalent cations could interfere with the functionality of low DM pectins, including Ca^{2+} -induced pectin gelation which is based on the ability of pectin to bind divalent cations (particularly Ca^{2+}) and the formation of a three-dimensional network of cross-linked pectin molecules (Thakur et al., 1997).

In the current study, the potential effect of the amounts of cations present was concluded to be negligible, as Celus et al. (2018) who obtained comparable low concentrations (5.13 ± 0.37 mg Ca^{2+} /g pectin and below 0.09 ± 0.04 mg/g pectin of Zn^{2+} , respectively) in citrus pectin samples of different DMs observed that such low cation concentrations did not have a significant contribution to the cation binding capacity of the pectins. Furthermore, although it has been reported that the formation of calcium cross-links between polygalacturonic acid chains could make the substrate less accessible to polygalacturonase (Cabanne & Donche, 2002) and pectin lyase (Sinitsyna et al., 2007), the pectin materials investigated in this work are not expected to cross-link due to the significantly low mineral content they exhibit, as previously explained. Consequently, the activity of the enzymes used during the production process of the samples is not expected to be negatively influenced.

Regarding the Na^+ content, it accounts for the largest percentage among the different cations present in the samples (up to 2.6%). However, considering that high amounts of this cation were present in the sodium acetate buffer (40 mM and 150 mM) and NaOH (0.1 M) used during the production of the

samples (to maintain an optimal pH during the enzymatic hydrolysis), it can be observed that a substantial amount of this cation was removed from the samples during dialysis. Assessing the concentration of Na⁺ ions present in the samples was crucial, since the presence of monovalent ions, specifically Na⁺, have been reported to influence some functional properties of pectin such as its gelling and cation-binding capacity (Kastner et al., 2020). For instance, Kastner et al. (2020) reported that the presence of Na⁺-ions in a low methylesterified pectin material, at a concentration of 1.2%, had a substantial impact on the formation of a gel network.

3.2. Fractionation, separation and identification of POS fractions present in pectin degraded samples

In order to isolate the low molecular weight fractions (POS) present in the different generated pectin samples, having a M_w below 3.5 kDa, a cut-off membrane of 3.5 kDa was used. In this way, separation and quantification of POS fractions containing molecules between 0.1-3.5 kDa was performed. It is important to highlight that during the dialysis process of the generated samples, performed in order to purify them through the removal of added salts during the process, a MWCO of 0.1-0.5 kDa was used, thus the smallest pectin molecules (mono-, di- and maybe some tri-GalA residues) generated were lost (results not shown). Separation and identification of the different pectin oligosaccharides present in the different pectin-derived samples was performed on a HILIC column coupled to TOF-MS. The ions present in each of the generated samples were identified based on their retention time and m/z. Furthermore, the m/z of the ions identified were compared to that of compounds which were already identified in previous studies (Leijdekkers et al., 2011; Prandi et al., 2018).

From the TOF-MS chromatograms (**Figure 2**), it could be observed that unsubstituted mono-, di-, tri- and tetra-GalA, as well as methylesterified oligogalacturonides with DP 3-5 were the main degradation products present in the <3.5 KDa fractions of the purified samples generated by EPG activity. Furthermore, from the TOF-MS chromatogram, it could be seen that mainly unsubstituted methylesterified and non-methylesterified oligogalacturonides were present in the fractionated samples generated by the action of EPG. However, by degrading a high DM pectin (CP77) by the combined effect of EPG and PL, not only methylesterified and non-methylesterified compounds were detected,

but also unsaturated compounds were identified (**Figure 2 B**). Additionally, an oligomer constituted by 3 GalA and 2 Rha units (GalA₃Rha₂) was also identified in the sample CP77 EPG 5U PL 2.5U 22h. This oligomer has been previously identified in other studies when treating sugar beet and apple pectin with a multienzyme complex containing EPG among other enzymes (Leijdekkers et al., 2013). Wu & Mort (2014) suggested that the detected compound GalA₃Rha₂ was likely to represent the junction zones between the HG and RG-I domains of the pectin molecule. Unsaturated products were also identified within each of the samples degraded by EPG and PL. These compounds could be attributed to the mode of action of PL, which leads to loss of a hydroxyl group at the C-4 position during the cleavage of a pectin chain through a beta eliminative reaction (Van Alebeek et al., 2002). Similar unsaturated compounds were also detected in previous studies when digesting sugar beet pectin with a mixture of enzymes including EPG and PL (Leijdekkers et al., 2011; Remoroza et al., 2012). Lastly, in the case of the chemically demethylesterified sample which was subsequently treated by EPG for 22 h (CP11 ALK EPG 5U 22h), some of the same unsaturated and substituted oligogalacturonides identified in the samples degraded by the action of EPG were also identified within this sample (**Figure 2 C**). A summary of the different oligosaccharide fractions identified, m/z values as well as retention times of each of the pectin-derived samples are shown in **Table 4**.

Regarding the retention time of the different compounds present in the POS fractions, in general it could be observed from the MS chromatograms that elution time increases with increasing DP. Furthermore, the presence of methyl ester groups leads to shorter retention times of GalA oligosaccharides. This phenomenon was suggested to occur due to the shielding of the carboxyl group attached to the methyl ester (Leijdekkers et al., 2011). A similar effect was reported when acetyl groups were present, which was attributed to the blocking of hydroxyl groups (Leijdekkers et al., 2011). In addition, unsaturated compounds were found to elute at earlier retention times than saturated ones. Retention times have been suggested to correlate to with the number of hydroxyl groups present in a oligosaccharide (Leijdekkers et al., 2011). In general, the data collected confirmed that a variety of structurally different POS fractions were produced by degrading pectin through different techniques.

3.3. Quantification of identified POS

Quantification of the POS (DP1-5) present in the fractionated samples (section 3.2.) was possible using a calibration curve, which was obtained by correlating the peak area of different GalA standards against their correspondent concentrations, as described by Remoroza et al. (2012). In this way, the CAD response of pure mono, di and tri-GalA standards with DP 1-3 was established. From the results obtained in this work, it was observed that the calibration curves of mono-, di- and tri-GalA residues had similar slopes (0.9681, 0.9526 and 0.8865 respectively). Therefore, a single calibration curve was obtained by plotting all the data points corresponding to the three pure GalA standards. The equation corresponding to this calibration curve is shown in equation (1), it was used to determine the CAD response of the different oligogalacturonides detected in the different pectin degraded samples. The curve fitting was performed using a power function $f(x) = ax^b$, where $f(x)$ represents the peak area, x is the concentration of the sample, a is the CAD response factor and b represents the slope (Remoroza et al., 2012). In this way, the following equation was derived.

$$f(x) = 6.6636 \cdot x^{0.9752} \quad (1)$$

Reorganization of equation (1) was performed as shown below (equation 2), in order to directly calculate the concentration (ppm) of each compound.

$$\text{Concentration (ppm)} = \exp \left[\frac{\ln (\text{CAD peak area}/6.6636)}{0.9752} \right] \quad (2)$$

In this way, the different GalA oligosaccharides present in the fractionated samples ($M_w < 3.5$ kDa) were quantified, the results are presented in **Table 4**. Notice that some of the fractionated samples contained oligosaccharides eluting at similar times (**Supplementary Figure 6**), therefore, the peak areas of such compounds could not be attributed to the oligosaccharides involved. Consequently, quantification of the sum of all compounds of that peak in the CAD chromatogram was carried out and the compounds involved are listed (see Table 7, column compounds identified). From the results obtained, it was observed that a wider range of oligogalacturonides were generated when pectin samples of low DM were incubated with EPG for a long incubation time (22h). This is suggested to be due to the fact that EPG acts in a more efficient way on low DM substrates, thus a variety of smaller compounds are

generated. Additionally, it is possible that a higher amount of oligogalacturonides than the quantities reported were generated after degradation of the different pectin samples, however, certain amount of these small molecules could have been lost during the purification process (dialysis) of the samples.

In the case of CP77 undergoing depolymerization by the combined effect of EPG and PL, it was seen that when a higher concentration of PL at longer incubation time was used, a higher total concentration of oligogalacturonides (<3.5 kDa) was produced. These results show the extensive cleavage of a high DM substrate by the action of PL when PL is added in sufficient amounts and for a longer period of time. At lower concentrations of PL and/or shorter incubation times, it seems that degradation of the pectin molecule is limited, therefore, leading to the production a lower amount of oligosaccharides of small M_w .

4. Conclusions

Characterization of the different pectin-derived samples containing a diversity of compounds demonstrated that structurally different fractions, of different degrees of polymerization and with differences in molecular composition can be produced by applying specific depolymerization processes under different production conditions. HILIC/TOF-MS was successfully applied to elucidate the composition of the acidic POS present in the samples exhibiting differences in DP but carrying the same charge. Based on the revealed composition of the pectin-derived mixtures generated, it was observed that a wider range of compounds were produced when applying an extended enzymatic process on a low DM pectin substrate, by the action of EPG, and when depolymerizing a high DM pectin by means of the combined effect of PL and EPG. As expected, unsaturated compounds were detected in the samples which were treated with a mixture of EPG and PL on a high DM substrate, which were attributed to the β -eliminative action of PL. Based on the structural characteristics of the samples generated, these pectin-derived compounds are expected to exhibit promising functional properties (e.g. prebiotic and emulsifying potential). In this context, further research is required in order to evaluate the potential functionalities of the generated pectin-derived samples, and hence establish different structure-function relationships which enable researches to further understand the impact of specific pectin structural modifications on their functional properties.

Declaration of competing interest

The authors of the present work declare no conflict of interests.

CRedit authorship contribution statement

J.N. Humerez-Flores: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **C. Kyomugasho:** Supervision, Methodology, Writing - review & editing, Visualization. **P. Agnese:** Methodology. **A.A. Gutiérrez-Ortiz:** Investigation. **M. De Bie:** Investigation. **A.M. Van Loey:** Acquired project funding, Methodology, Review. **P. Moldenaers:** Acquired project funding, Methodology, Review. **M.E. Hendrickx:** Acquired project funding, Project administration, Supervision, Conceptualization, Methodology, Validation, Writing - review & editing.

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FIGURE CAPTIONS

Figure 1. Molecular weight distribution (full lines) and concentration profiles (dashed lines) of CP77 (---), CP57 (---), CP43 (---) and their derived depolymerized compounds (dotted lines) CP77 EPG 5U 1h (...), CP77 EPG 5U 22h (...), CP57 EPG 5U 1h (...), CP57 EPG 5U 22h (...), CP43 EPG 5U 1h (...) and CP43 EPG 5U 22h (...), produced by means of an enzymatic hydrolysis process with EPG for different incubation times of 1 and 22 h. The color of the molecular weight profiles corresponds to the one of each concentration profile, respectively. **(FIGURE CAPTURE TO BE PRINTED IN COLORS)**

Figure 2. Representative TOF-ESI-MS chromatographs of compounds identified in citrus pectin-derived oligosaccharides of $M_w < 3.5\text{kDa}$. Chromatographs of CP43 EPG 5U 22h (A), CP77 EPG 5U PL 2.5U 22h (B) and CP11 ALK EPG 5U 22h (C).

FIGURES

Figure 1 (TO BE PRINTED IN COLORS)

Figure 2

$DP^{Me,Ac}$: degree of polymerization (DP), number of methyl groups (Me), number of acetyl groups (Ac).

TABLES

Table 1

Generated sample	Description of initial and generated pectin-derived samples
CP77 ⁰	Pectin sample DM 77%
CP57 ⁰	Pectin sample DM 57%
CP43 ⁰	Pectin sample DM 43%
CP77 EPG 5U 1h ¹	Pectin sample (CP77) enzymatically hydrolyzed for 1 h with EPG (5 U/g)
CP77 EPG 5U 22h ¹	Pectin sample (CP77) enzymatically hydrolyzed for 22 h with EPG (5 U/g)
CP57 EPG 5U 1h ¹	Pectin sample (CP57) enzymatically hydrolyzed for 1 h with EPG (5 U/g)
CP57 EPG 5U 22h ¹	Pectin sample (CP57) enzymatically hydrolyzed for 22 h with EPG (5 U/g)
CP43 EPG 5U 1h ¹	Pectin sample (CP43) enzymatically hydrolyzed for 1 h with EPG (5 U/g)
CP43 EPG 5U 22h ¹	Pectin sample (CP43) enzymatically hydrolyzed for 22 h with EPG (5 U/g)
CP77 EPG 5U PL 0.5U 30 min ²	Pectin sample (CP77) enzymatically hydrolyzed with a mixture of EPG (5 U/g) and PL (0.5 U/g) for a period of 30 minutes
CP77 EPG 5U PL 2.5U 30 min ²	Pectin sample (CP77) enzymatically hydrolyzed with a mixture of EPG (5 U/g) and PL (2.5 U/g) for a period of 30 minutes
CP77 EPG 5U PL 2.5U 22h ²	Pectin sample (CP77) enzymatically hydrolyzed with a mixture of EPG (5 U/g) and PL (2.5 U/g) for a period of 22 h
CP11 ALK EPG 5U 22h ³	Pectin sample (CP43) chemically demethylesterified to a final DM of 11%, followed by enzymatic hydrolysis with EPG (5 U/g) for a period of 22 h

0: initial pectin materials.

1: pectin-derived samples generated during the 1st treatment.

2: pectin-derived samples generated during the 2nd treatment.

3: pectin-derived samples generated during the 3rd treatment.

Table 1. List of codes of generated pectin-derived samples by means of enzymatic/chemical treatments

Table 2

Treatment	Elution peak #	M _w (Da)
CP77	1	144000 ± 4243 ^a
CP57	1	151000 ± 2828 ^a
CP43	1	116000 ± 4213 ^b
CP77 EPG 5U 1h	1	61600 ± 990 ^c
CP77 EPG 5U 22h	1	40000 ± 1556 ^d
CP57 EPG 5U 1h	1	27000 ± 849 ^c
CP57 EPG 5U 22h	1	83700 ± 113
CP43 EPG 5U 1h	1	18800 ± 1980 ^f
CP43 EPG 5U 22h	1	74100 ± 1626
	2	2520 ± 240 ^g
CP77 EPG 5U PL 0.5U 30 min	1	129000 ± 212
	2	20500 ± 283 ^{e,f}
CP77 EPG 5U PL 2.5U 30 min	1	94900 ± 1650
	2	2850 ± 156 ^g
CP77 EPG 5U PL 2.5U 22h	1	60600 ± 1131
	2	3430 ± 191 ^g
CP11 ALK EPG 5U 22h	1	96000 ± 212
	2	2710 ± 187 ^g

Different superscript letters indicate significant differences ($p < 0.05$) among the average M_w of the most abundant pectin molecule fractions (fractions accounting for > 5% of the total amount of sample analyzed) present in the different pectin and pectin-derived samples.

Table 2. Average molecular weight of initial pectin materials and pectin-derived samples. Results are expressed as average values ± standard deviation.

Table 3

Property	Sample Code												
Pectin Related Sugars (mg/g sample)	CP77	CP57	CP43	CP77 EPG 5U 1h	CP77 EPG 5U 22h	CP57 EPG 5U 1h	CP57 EPG 5U 22h	CP43 EPG 5U 1h	CP43 EPG 5U 22h	CP77 EPG 5U PL 0.5U 30 min	CP77 EPG 5U PL 2.5U 30min	CP77 EPG 5U PL 2.5U 22h	CP11 ALK EPG 5U 22h
GalA	779.52 ± 13.87 ^{c, d}	863.45 ± 26.83 ^a	853.43 ± 12.07 ^{a, b}	789.14 ± 18.88 ^{b, c, d}	791.83 ± 17.82 ^{b, c, d}	781.14 ± 26.04 ^{c, d}	777.92 ± 17.98 ^{c, d}	809.84 ± 24.80 ^{a, b, c}	785.96 ± 15.04 ^{c, d}	730.08 ± 15.62 ^d	780.97 ± 18.40 ^{c, d}	736.25 ± 13.91 ^d	790.91 ± 8.72 ^{b, c, d}
Fucose	0.03 ± 0.02 ^d	0.02 ± 0.01 ^d	0.14 ± 0.06 ^d	0.09 ± 0.03 ^d	0.17 ± 0.01 ^{c, d}	0.02 ± 0.02 ^d	0.04 ± 0.01 ^d	0.01 ± 0.01 ^d	0.08 ± 0.01 ^d	0.31 ± 0.04 ^{b, c}	0.41 ± 0.04 ^b	0.78 ± 0.03 ^a	0.69 ± 0.05 ^a
Rhamnose	11.49 ± 0.93 ^d	10.71 ± 0.17 ^{d, e}	8.70 ± 0.34 ^{c, f}	10.28 ± 0.23 ^{d, e}	11.07 ± 0.67 ^d	8.08 ± 0.12 ^{f, g}	7.89 ± 0.23 ^{f, g}	6.40 ± 0.14 ^g	10.03 ± 0.80 ^{d, e, f}	11.93 ± 1.08 ^{c, d}	13.94 ± 0.16 ^c	18.72 ± 0.45 ^b	21.71 ± 0.30 ^a
Arabinose	15.10 ± 1.01 ^{a, b}	7.79 ± 0.88 ^c	2.83 ± 0.07 ^c	14.19 ± 0.24 ^b	14.20 ± 0.35 ^b	5.49 ± 0.01 ^d	5.10 ± 0.02 ^d	2.04 ± 0.04 ^c	2.34 ± 0.32 ^c	15.37 ± 0.75 ^{a, b}	16.48 ± 0.02 ^a	14.85 ± 0.37 ^{a, b}	4.97 ± 0.26 ^d
Galactose	68.17 ± 5.66 ^{a, b, c}	45.43 ± 5.32 ^d	43.57 ± 1.99 ^d	70.96 ± 2.86 ^{a, b}	72.16 ± 1.98 ^{a, b}	42.64 ± 0.16 ^d	47.03 ± 0.78 ^d	36.14 ± 1.36 ^{d, e}	39.36 ± 1.89 ^d	58.72 ± 3.23 ^c	62.90 ± 2.10 ^{b, c}	27.55 ± 0.79 ^e	76.96 ± 1.53 ^a
Xylose	0.89 ± 0.28 ^{d, e, f}	0.68 ± 0.08 ^f	0.08 ^{d, e, f}	0.14 ^{e, f}	0.05 ^{c, d, e, f}	0.72 ± 1.15 ^c	0.72 ± 0.74 ^c	0.73 ± 0.74 ^c	1.73 ± 1.32 ^c	0.06 ^{c, d, e}	0.04 ^{b, c, d}	2.02 ± 1.47 ^b	2.73 ± 0.09 ^a
Other sugars													
Glucose	5.48 ± 1.22 ^a	3.66 ± 0.31 ^{b, c, d}	3.88 ± 0.10 ^{b, c}	3.53 ± 0.13 ^{b, c, d, e}	3.62 ± 0.09 ^{b, c, d, e}	2.16 ± 0.16 ^{c, f}	2.23 ± 0.37 ^{d, e, f}	2.40 ± 0.01 ^{c, d, e, f}	2.44 ± 0.09 ^{c, d, e, f}	2.29 ± 0.08 ^{d, e, f}	1.85 ± 0.01 ^f	1.69 ± 0.03 ^f	3.94 ± 0.16 ^b
Mannose	1.03 ± 0.39 ^{b, c, d}	0.82 ± 0.04 ^{c, d}	0.93 ± 0.06 ^{b, c, d}	0.95 ± 0.19 ^{b, c, d}	1.19 ± 0.02 ^{b, c}	0.46 ± 0.04 ^d	0.57 ± 0.36 ^{c, d}	0.64 ± 0.06 ^{c, d}	1.04 ± 0.01 ^{b, c, d}	0.82 ± 0.05 ^{c, d}	0.73 ± 0.01 ^{c, d}	1.46 ± 0.05 ^b	2.22 ± 0.02 ^a
Sugar ratios													
Linearity	7.26 ± 0.60 ^c	11.94 ± 1.08 ^{b, c}	13.71 ± 0.58 ^b	7.30 ± 0.19 ^c	7.14 ± 0.22 ^c	12.29 ± 0.02 ^{b, c}	11.51 ± 0.15 ^{c, d}	16.17 ± 0.44 ^a	13.21 ± 0.84 ^{b, c}	7.36 ± 0.43 ^c	7.23 ± 0.09 ^c	9.87 ± 0.09 ^d	6.63 ± 0.14 ^c
RG-I contribution	0.02 ± 0.01 ^{c, d}	0.02 ± 0.01 ^{d, e, f}	0.01 ± 0.01 ^{c, f, g}	0.01 ± 0.01 ^{d, e}	0.02 ± 0.01 ^{c, d}	0.01 ± 0.01 ^{c, f, g}	0.01 ± 0.01 ^{f, g}	0.01 ± 0.01 ^g	0.01 ± 0.01 ^{d, e, f}	0.02 ± 0.01 ^{b, c}	0.02 ± 0.01 ^b	0.03 ± 0.01 ^a	0.03 ± 0.01 ^a
Branching of RG-I	6.84 ± 0.01 ^{a, b}	4.66 ± 0.62 ^{e, f}	4.92 ± 0.03 ^{d, e}	8.13 ± 0.45 ^a	7.35 ± 0.25 ^a	5.56 ± 0.10 ^{c, d, e}	6.14 ± 0.09 ^{b, c}	5.49 ± 0.32 ^{c, d, e}	3.83 ± 0.10 ^{f, g}	5.9 ± 0.22 ^{b, c, d}	5.41 ± 0.31 ^{c, d, e}	2.21 ± 0.11 ^h	3.48 ± 0.03 ^g
DM (%)	74.40 ± 0.83 ^a	52.93 ± 2.19 ^b	37.77 ± 0.64 ^c	72.74 ± 1.09 ^a	73.02 ± 0.47 ^a	52.19 ± 1.51 ^{b, c}	49.36 ± 0.71 ^c	35.99 ± 0.29 ^{c, f}	38.33 ± 0.18 ^c	52.93 ± 1.16 ^b	42.18 ± 0.04 ^d	34.06 ± 0.05 ^f	10.80 ± 0.83 ^g
Protein content (% w/w)	1.39 ± 0.48 ^a	0.89 ± 0.08 ^a	0.91 ± 0.01 ^a	1.16 ± 0.21 ^a	0.82 ± 0.07 ^a	0.63 ± 0.08 ^a	0.77 ± 0.25 ^a	0.73 ± 0.03 ^a	0.74 ± 0.05 ^a	0.80 ± 0.15 ^a	0.74 ± 0.03 ^a	1.21 ± 0.13 ^a	1.60 ± 0.34 ^a
M _w (Da)	144000 ± 4243 ^a	151000 ± 2828 ^a	116000 ± 4213 ^b	61600 ± 990 ^c	40000 ± 1556 ^d	27000 ± 849 ^c	15300 ± 707 ^f	18800 ± 1980 ^f	2520 ± 240 ^g	20500 ± 283 ^{c, f}	2850 ± 156 ^g	3430 ± 191 ^g	2710 ± 187 ^g

Different superscript letters indicate significant differences ($p < 0.05$) among the values of a structural property for the different pectin and pectin-derived samples.

Table 3. Structural properties of generated pectin-derived samples, including galacturonic acid content (GalA), neutral sugar content, sugar ratios, degree of methylesterification (DM), and M_w . Results are expressed as average values ± standard deviation. All samples were de-salted prior to analysis.

Table 4

Degraded pectin samples	(M-H) ⁻ m/z	Elution time (min)	C (ppm)	Compound identified
CP77	193	5.7	38.8	1 ⁰⁰
	369	12.3	20.1	2 ⁰⁰
	545	16.1	13.0	3 ⁰⁰
CP57	193	6.2	7.4	1 ⁰⁰
CP43	193	5.9	11.2	1 ⁰⁰
CP77 EPG 5U 1h	193	6.4	56.3	1 ⁰⁰
CP77 EPG 5U 22h	193	6.4	29.4	1 ⁰⁰
CP57 EPG 5U 1h	193	6.4	5.9	1 ⁰⁰
CP57 EPG 5U 22h	545/735/925	15.3	18.7	3 ⁰⁰ /4 ¹⁰ /5 ²⁰
CP 43 EPG 5U 1h	193	6.1	7.4	1 ⁰⁰
	545	15.9	3.6	3 ⁰⁰
	193	6.5	41.6	1 ⁰⁰
CP 43 EPG 5U 22h	369/559/749	12.4	147.4	2 ⁰⁰ /3 ¹⁰ /4 ²⁰
	545/735/925	15.8	121.7	3 ⁰⁰ /4 ¹⁰ /5 ²⁰
	194	6.4	5.9	1 ⁰⁰
CP77 EPG 5U PL 0.5U 30 min	369/559/749/935/545	15.1	10.1	2 ⁰⁰ /3 ¹⁰ /4 ²⁰ /U5 ⁴⁰ /3 ⁰⁰
	194	6.4	40.9	1 ⁰⁰
CP77 EPG 5U PL 2.5U 30 min	559/749/907/1097	12.7	122.6	3 ¹⁰ /4 ²⁰ /U5 ²⁰ /U6 ³⁰
	735/925	14.2	45.6	4 ¹⁰ /5 ²⁰
	194	6.5	20.5	1 ⁰⁰
CP77 EPG 5U PL 2.5U 22h	351/559/749/907	12.7	177.8	U2 ⁰⁰ /3 ¹⁰ /4 ²⁰ /U4 ¹⁰ /U5 ²⁰
	735/837/925/893	16.7	67.2	4 ¹⁰ /GalA ₃ Rha ₂ /5 ²⁰ /U5 ¹⁰
	194	6.6	24.2	1 ⁰⁰
CP11 ALK EPG 5U 22h	369/559	12.4	44.4	2 ⁰⁰ /3 ¹⁰
	545/735	15.7	126.0	3 ⁰⁰ /4 ¹⁰
	911	21.7	24.5	5 ¹⁰

DP^{Me,Ac}: degree of polymerization (DP), number of methyl groups (Me), number of acetyl groups (Ac).

Table 4. Summary of m/z values, elution time, concentrations (C) and structure of identified compounds

Declaration of competing interest

The authors of the present work declare no conflict of interests.

CRedit authorship contribution statement

J.N. Humerez-Flores: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **C. Kyomugasho:** Supervision, Methodology, Writing - review & editing, Visualization. **P. Agnese:** Methodology. **A.A. Gutiérrez-Ortiz:** Investigation. **M. De Bie:** Investigation. **A.M. Van Loey:** Acquired project funding, Methodology, Review. **P. Moldenaers:** Acquired project funding, Methodology, Review. **M.E. Hendrickx:** Acquired project funding, Project administration, Supervision, Conceptualization, Methodology, Validation, Writing - review & editing.

Appendix - Supplementary material

Supplementary Figure 1 (GRAPH AND FIGURE CAPTURE PRINTED/SHOWN IN COLORS)

Supplementary Figure 1. Scheme of the three different treatments applied for the degradation of the initial pectin materials CP77, CP57 and CP43, and the correspondent CP-derived compounds generated.

Supplementary Figure 2 (GRAPH AND FIGURE CAPTURE PRINTED/SHOWN IN COLORS)

Effect of EPG concentration and incubation time on molecular weight

In **Supplementary Figure 2**, representative results of the mother pectin CP57 and the samples derived thereof by hydrolysis by EPG at different concentrations as well as incubation times are shown. Please note that for this experiment non-desalted samples are shown, meaning that data beyond 62 minutes refer to impurities (salts) in the sample (the retention time of galacturonic acid is 64 min). Analyzing the chromatograms of the citrus pectin derived samples produced by action of EPG at concentrations of 5 U/g pectin (**Supplementary Figure 2A**) and 25 U/g pectin (**Supplementary Figure 2B**), it can be observed that the elution profiles of the different samples produced in presence of 25 U EPG/g pectin eluted at similar times, thus exhibiting average M_w 's within a narrower range in comparison to the samples produced by addition of 5 U/g pectin. These results indicate that addition of EPG at lower concentrations (5 U/g pectin) for different incubation times leads to the generation of compounds having a significantly ($p < 0.05$) different M_w . Therefore, to produce lower molecular weight compounds from the citrus mother pectins (CP77, CP57 and CP43) by action of EPG, a concentration of 5 U/g pectin was selected. Since the aim of this study was to produce pectin-derived compounds with differences in M_w and DM, incubation periods of 1 and 22 h were selected (for all three pectin sources CP77, CP57 and CP43 treated by EPG).

Supplementary Figure 2. Molecular weight distribution (full lines) and concentration profiles of CP57 mother pectin (dashed line) and its derived samples (dotted lines) generated by action of EPG at 35 °C for incubation times of (•••••) 15 min, (••••) 30 min, (•••••) 1 h, (•••••) 3 h and (•••••) 22 h, at two different EPG concentrations: A) 5 U/g pectin and B) 25 U/g pectin.

Supplementary Figure 3 (GRAPH AND FIGURE CAPTURE PRINTED/SHOWN IN COLORS)

Effect of PL concentration and incubation time on molecular weight

The M_w distribution and concentration profiles of the pectin-derived compounds, produced by hydrolyzing the three different citrus pectin materials by the simultaneous action of EPG and pectin lyase (PL), are presented in **Supplementary Figure 3** (non-desalted samples). Analyzing the results of the concentration profiles of this first experimental stage (enzymatic depolymerization of CP77, CP57 and CP43 through the synergistic effect of 5 U EPG /g pectin and 2.5 U PL/g pectin), comparable profiles were observed after incubation of each of the initial pectin materials with a mixture of EPG and PL for a period of 22 h. The concentrations are complemented by similar average M_w , irrespective of the DM of the starting material. As previously mentioned (see 3.1.2), EPG has a predominant preference for substrates of lower DM, while PL preferentially cleaves the linkage between methylesterified GalA residues of the HG backbone of pectin (Pedrolli et al., 2009). Therefore, when EPG and PL synergistically depolymerize the high DM pectin material (CP77), shorter polymer fractions are generated from both the methylesterified and non methylesterified regions. In the case of the pectin material of lower DM (CP57 and CP43), extensive degradation by the action of EPG was observed and the generated methylesterified GalA stretches (intact pectin chains which were not degraded by EPG) became the optimal substrate for PL activity. Therefore, promoting the formation of shorter pectin chains. Since the concentration profiles and M_w distribution of the three pectin-derived materials (CP77 EPG 5U PL 2.5 U 22h, CP57 EPG 5U PL 2.5 U 22h and CP43 EPG 5U PL 2.5 U 22h) were similar, only the sample CP77, was used for the following experiments involving the degradation of pectin by the synergistic effect of EPG and PL.

Supplementary Figure 3. Molecular weight distribution (full lines) and concentration profiles (dashed lines) of CP77 (---), CP57 (---), CP43 (---) and their derived depolymerized compounds (dotted lines) CP77 EPG 5U PL 2.5 U 22h (---), CP57 EPG 5U PL 2.5 U 22h (---) and C43 EPG 5U PL 2.5 U 22h (---), produced by means of an enzymatic hydrolysis process of the initial pectin materials with EPG and PL for 22h. The color of the molecular weight profiles corresponds to the one of each concentration profile, respectively.

Supplementary Figure 4 (GRAPH AND FIGURE CAPTURE PRINTED/SHOWN IN COLORS)

Supplementary Figure 4. Molecular weight distribution (full lines) and concentration profiles of CP77 (---) and depolymerized derivative compounds (dotted lines) CP77 EPG 5U PL 0.5U 30 min (---), CP77 EPG 5U PL 2.5U 30 min (---) and CP77 EPG 5U PL 2.5U 22h (---) produced by means of an enzymatic hydrolysis process with EPG and PL for 22 h. The color of the molecular weight profiles corresponds to the one of each concentration profile, respectively.

Supplementary Figure 5 (GRAPH PRINTED/SHOWN IN COLORS)

Supplementary Figure 5. Molecular weight distribution (full lines) and concentration profiles of CP43 (---), and CP11 ALK EPG 5U 22h (---). The color of the molecular weight profiles corresponds to the one of each concentration profile, respectively.

Supplementary Figure 6

Supplementary Figure 6. Representative CAD chromatographs of compounds identified in citrus pectin-derived oligosaccharides of $Mw < 3.5$ kDa. Chromatographs of CP43 EPG 5U 22h (A), CP77 EPG 5U PL 2.5U 22h (B) and CP11 ALK EPG 5U 22h (C).

Supplementary Table 1. Content of Ca^{2+} -, Mg^{2+} -, Fe^{2+} -, Zn^{2+} -and Na^{+} -ions present in both the initial pectin materials and pectin-derived samples, produced by enzymatic and non-enzymatic methods.

Sample	Mineral content (mg/g sample)				
	Ca ²⁺	Mg ²⁺	Fe ²⁺	Zn ²⁺	Na ⁺
CP77 0h	0.470 ± 0.001 ^a	0.044 ± 0.003 ^a	0.019 ± 0.001 ^a	0.016 ± 0.009 ^a	7.028 ± 0.551 ^{b, c, d}
CP57 0h	0.422 ± 0.062 ^a	0.042 ± 0.004 ^a	0.038 ± 0.004 ^a	0.023 ± 0.003 ^a	9.699 ± 2.807 ^b
CP43 0h	0.129 ± 0.015 ^a	0.010 ± 0.005 ^a	0.008 ± 0.004 ^a	0.002 ± 0.001 ^a	1.398 ± 1.011 ^e
CP77 EPG 5U 1h	0.207 ± 0.079 ^a	0.029 ± 0.028 ^a	0.019 ± 0.011 ^a	0.003 ± 0.001 ^a	1.826 ± 0.571 ^e
CP77 EPG 5U 22h	0.319 ± 0.016 ^a	0.014 ± 0.001 ^a	0.012 ± 0.003 ^a	0.003 ± 0.001 ^a	1.167 ± 0.254 ^{c, d, e}
CP57 EPG 5U 1h	0.832 ± 0.360 ^a	0.126 ± 0.082 ^a	0.024 ± 0.004 ^a	0.011 ± 0.001 ^a	25.843 ± 0.680 ^a
CP57 EPG 5U 22h	0.322 ± 0.216 ^a	0.050 ± 0.033 ^a	0.117 ± 0.012 ^a	0.005 ± 0.003 ^a	4.549 ± 1.608 ^e
CP43 EPG 5U 1h	0.386 ± 0.134 ^a	0.012 ± 0.002 ^a	0.016 ± 0.008 ^a	0.005 ± 0.001 ^a	7.395 ± 0.414 ^{b, c}
CP43 EPG 5U 22h	0.323 ± 0.046 ^a	0.015 ± 0.013 ^a	0.019 ± 0.006 ^a	0.037 ± 0.004 ^a	2.541 ± 1.832 ^{c, d, e}
CP77 EPG 5U PL 0.5U 30m	0.313 ± 0.106 ^a	0.010 ± 0.001 ^a	0.015 ± 0.007 ^a	0.002 ± 0.001 ^a	2.183 ± 1.498 ^{d, e}
CP77 EPG 5U PL 2.5U 30m	0.210 ± 0.099 ^a	0.009 ± 0.001 ^a	0.020 ± 0.006 ^a	0.003 ± 0.002 ^a	2.082 ± 0.596 ^{d, e}
CP77 EPG 5U PL 2.5U 22h	0.503 ± 0.275 ^a	0.118 ± 0.010 ^a	0.044 ± 0.033 ^a	0.005 ± 0.003 ^a	2.876 ± 0.772 ^{c, d, e}
CP11 ALK EPG 5U 22h	0.350 ± 0.119 ^a	0.010 ± 0.010 ^a	0.010 ± 0.003 ^a	0.003 ± 0.001 ^a	2.384 ± 0.974 ^{d, e}

Different superscript letters indicate significant differences ($p < 0.05$) among the values of a specific ion for the different pectin and pectin-derived samples.

- A diversity of citrus-pectin derived samples were successfully generated through enzymatic and chemical processes.
- The generated pectin molecules exhibited differences in structure, including molecular weight and degree of methylesterification.
- The pectin-derived samples generated contained different fractions of oligosaccharides of degree of depolymerization between 1 and 6.