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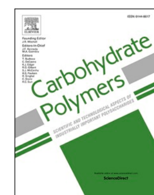

Revealing methyl-esterification patterns of pectins by enzymatic fingerprinting: Beyond the degree of blockiness

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ABSTRACT

Citrus pectins were studied by enzymatic fingerprinting using a simultaneous enzyme treatment with *endo*-polygalacturonase (endo-PG) from *Kluyveromyces fragilis* and pectin lyase (PL) from *Aspergillus niger* to reveal the methyl-ester distribution patterns over the pectin backbone. Using HILIC-MS combined with HPAEC enabled the separation and identification of the diagnostic oligomers released. Structural information on the pectins was provided by using novel descriptive parameters such as degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme}) and degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}). This approach enabled us to clearly differentiate citrus pectins with various methyl-esterification patterns. The simultaneous use of PG and PL showed additional information, which is not revealed in digests using PG or PL alone. This approach can be valuable to differentiate pectins having the same DM and to get specific structural information on pectins and therefore to be able to better predict their physical and biochemical functionalities.

1. Introduction

Polysaccharides are the most abundant elements of the plant cell wall, determining the shape, size and many functional properties of the plant cell (Voragen et al., 2009). Pectin is a complex polysaccharide found in especially plant cell walls from fruits and vegetables (Vincken et al., 2003) and has a key role in controlling the architecture of the primary plant cell wall and steering several plant processes as well as cell functions (Osborne, 2004; Voragen et al., 2009; Willats et al., 2001). Traditionally, pectins are used in food products as a stabilizer, or a gelling and thickening agent. Dietary fibers, such as pectins, also play a significant role in the maintenance of health, both in gut fermentation and in immune modulation (Beukema et al., 2021; Gómez et al., 2016; Tian et al., 2016; Vogt et al., 2016).

Pectins can be built up of four main structural elements, homogalacturonan (HG), rhamnogalacturonan I and II (RG I and RG II) and xylogalacturonan (XGA) (Schols et al., 2009). Alfa-(1,4)-linked D-galacturonic acid (GalA) is the main building block of the HG which is the most prominent section of pectins, commonly present in amounts up to 60% of the total pectin structures (Voragen et al., 2009). The linear HG chain can be methyl-esterified at the carboxyl group at C-6 of GalA and, less commonly, also can be acetylated at the O-2 and/or O-3 position of the GalA residues (Voragen et al., 2001).

Commercial pectin is mainly extracted from apple pomace and citrus peels (May, 1990) and since its structure strongly depends on the pectin source and extraction conditions, pectin structure might be highly diverse (Levigne et al., 2002; Oosterveld et al., 1996). Extracted pectins can be tailored further through targeted chemical- or enzymatic modifications to meet required functionalities (Fraeye et al., 2010). Both the level and the distribution of the methyl-esters in the HG regions are key features within pectin's functionality (Osborne, 2004; Rolin, 2002; Sahasrabudhe et al., 2018; Thibault & Ralet, 2003; Vogt et al., 2016; Voragen et al., 2009). The percentage of methyl-esterified GalA residues within the HG backbone is defined as the degree of methyl-esterification (DM). Two main distribution patterns of methyl-esters have been described as random or blockwise (Guillotin et al., 2005; Levesque-Tremblay et al., 2015; Vincken et al., 2003; Willats et al., 2006).

The methyl-esterification pattern of the pectin backbone was first quantitatively described by Daas et al. (1999) as degree of blockiness (DB) which represents the amount of non-esterified mono-, di- and

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trigalacturonic acids released by enzymatic treatment of pectin using *endo*-polygalacturonase (endo-PG) from *Kluyveromyces fragilis*, relative to the total amount of non-esterified GalA residues present in the pectin (Daas et al., 1999). To enable the action of endo-PG from *Kluyveromyces fragilis* at least four consecutive non-esterified GalA residues are needed (Daas et al., 1999; Pasculli et al., 1991). Until now, DB and the related DB_{abs} (DB related to total amount of GalA residues present in the pectin) has been calculated from the amount of oligomers released as quantified in pectin digests by quite different methods like capillary electrophoresis (CE) and high performance anion exchange chromatography (HPAEC) analyses (Coenen et al., 2008; Daas et al., 2000; Guillotin et al., 2005; Ngouémazong et al., 2011; Ström et al., 2007). Together, DB and DB_{abs} have been commonly used to differentiate methyl-esterification patterns of pectins and are common parameters to characterize non-esterified blocks of GalA residues (Daas et al., 2000; Guillotin et al., 2005; Ralet et al., 2012). Details regarding the non-esterified block length and distribution of methyl-esters of pectins having a similar DM are rather difficult to define (Tanhatan-Nasseri et al., 2011). Pectins with similar DM and DB values can still show different patterns of methyl-esterification by having different sizes of non-esterified blocks (Guillotin et al., 2005). To better understand pectin methyl-esterification patterns Ralet et al. (2012) described the degree of blockiness (DBMe) and absolute degree of blockiness (DB_{abs}Me) for the methyl-esterified regions in the homogalacturonan based on oligomers released upon pectin lyase (PL) digestion to study the highly methyl-esterified residues of pectins. Focusing either on the non-esterified pectin segments via the investigation of *endo*-PG digestion products or on the methyl-esterified sections released by the PL products explores only restricted sections of the entire pectin backbone (Ralet et al., 2012). Next to DB, DB_{abs}, DBMe and DB_{abs}Me, Remoroza, Broxterman, et al. (2014) and Remoroza, Buchholt, et al. (2014) introduced new descriptive parameters, degree of hydrolysis by PG (DH_{PG}) and degree of hydrolysis by PL (DH_{PL}) for the enzymatic fingerprinting methyl-esterified and acetylated GalA sequences in sugar beet pectin. DH_{PG} and DH_{PL} are based on a combined enzymatic digestion by PL and *endo*-PG (Remoroza, Broxterman, et al., 2014). As yet, there has been no detailed investigation of the above-mentioned parameters, DH_{PG} and DH_{PL} for the analysis of non-acetylated pectins.

The main focus of the current research was to characterize and quantify the methyl-ester distribution of citrus pectins in more detail. Digestion using endo-PG acting preferably between unesterified GalA residues and PL requiring two neighboring methyl-esterified GalA residues was performed to describe methyl-ester distribution of 4 selected pectins. HPAEC-PAD/UV was used to identify and quantify GalA-oligomers released, although information on the level and location of methyl-esters are lost during analysis. HILIC-ESI-MS as complementing technique which preserves the methyl-esters present was used to distinguish methyl-esterified fragments, and to identify and quantify the diagnostic oligosaccharides released. The beauty of using this approach is that no pectin part remain high molecular weight and therefore unanalyzed. Novel parameters describing methyl-esterification are introduced and compared and different methyl-esterification patterns of pectins are discussed.

2. Materials and methods

2.1. Materials

Commercially extracted orange pectins O64 (DM 64%), O59 (DM 59%) and O32 (DM 32%) were provided by Andre Pectin (Andre Pectin Co. Ltd., Yantai, China). Commercially extracted lemon pectin L34 (DM 34%) was provided by CP Kelco (Copenhagen, Denmark). Endo-polygalacturonase (Endo-PG, EC 3.2.1.15; ID: 1027) from *Kluyveromyces fragilis* as described by Daas et al. (1999). A new batch of this enzyme was obtained from DSM (Delft, the Netherlands) and purified according to Pasculli et al. (1991). In addition pectin lyase (PL, EC 4.2.2.10; ID: 1043) of *Aspergillus niger* (Harmsen et al., 1990; Schols et al., 1990) was used to degrade the citrus pectins. Other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), VWR International (Radnor, PA, USA), or Merck (Darmstadt, Germany), unless stated otherwise.

2.2. Characterization of citrus pectins

Neutral sugar composition was analyzed after pretreatment with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by further acid hydrolysis with 1 M H_2SO_4 (3 h, 100 °C). Neutral sugars released were derivatized and analyzed as their alditol acetates using gas chromatography (Englyst & Cummings, 1984), inositol was used as internal standard. Galacturonic acid content of the hydrolysate was determined by the automated colorimetric *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979). For the determination of the degree of methyl-esterification pectin samples were saponified using 0.1 M NaOH for 24 h (1 h at 4 °C, followed by 23 h at room temperature). The methanol released was measured by a head-space gas chromatography (GC) method as previously described and consequently the DM was calculated (Huisman et al., 2004).

2.3. Enzymatic hydrolysis

All citrus pectins were dissolved in 50 mM sodium acetate buffer pH 5.2 (5 mg/ml). The hydrolysis was performed at 40 °C by incubation of the pectin solution with PL for 6 h followed by the addition of *endo*-PG and incubation for another 18 h (Remoroza, Buchholt, et al., 2014). Enzyme doses were sufficient to degrade the entire pectin backbone into monomers within 6 h. Inactivation of enzymes was performed at 100 °C for 10 min and the digests were centrifuged (20,000 ×*g*, 15 min, 20 °C). The supernatants obtained were analyzed by HPSEC, HPAEC-PAD/UV and UHPLC-HILIC-MS.

2.4. High performance size exclusion chromatography (HPSEC)

Pectin before and after enzymatic digestion were analyzed by HPSEC on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA). A set of four TSK-Gel super AW columns was used in series: guard column (6 mm ID × 40 mm) and columns 4000, 3000 and 2500 SuperAW (6 mm × 150 mm) (Tosoh Bioscience, Tokyo, Japan) at 55 °C. Samples (10 μl, 2.5 mg/ml) were eluted with filtered 0.2 M $NaNO_3$ at a flow rate of 0.6 ml/min. The elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan). Pectin standards from 10 to 100 kDa were used to estimate the molecular weight distribution of the pectins (Deckers et al., 1986).

2.5. High performance anion exchange chromatography (HPAEC)

The pectin digests were analyzed and subsequently quantified using an ICS5000 HPAEC-PAD (ICS5000 ED) (Dionex) equipped with a CarboPac PA-1 column (250 mm × 2 mm i.d.) and a CarboPac PA guard column (25 mm × 2 mm i.d.) and UV detection at 235 (Dionex). The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the column temperature was 20 °C (Broxterman & Schols, 2018). GalA DP 1–3 (Sigma–Aldrich, Steinheim, Germany) were used as standards for quantification. Oligomers above DP3 and unsaturated oligomers were quantified using the response from $GalA_3$ standard. Before the analysis pectin digests were diluted using ultra-pure water to 0.5 mg/ml. Samples (10 μl) were injected and eluted at a flow rate of 0.3 ml/min. The gradient profile was as follows: 0–55 min, 20–65% B; 55.1–60 min column washing with 100% B; finally, 60.1 to 75 min column re-equilibration with 20% B.

2.6. Ultra-high pressure liquid chromatography HILIC-ESI-IT-MS

Pectin digests were analyzed using UHPLC in combination with electrospray ionization tandem mass spectrometry (ESI-IT-MS) on a Hydrophilic Interaction Liquid Chromatography (HILIC) BEH amide column (1.7 μm, 2.1 × 150 mm). Pectin digests were centrifuged (15,000 ×*g*, 10 min, RT) and diluted (with 50% (v/v) aqueous acetonitrile containing 0.1% formic acid to a final concentration of 1 mg/ml). The eluents used were (A) 99:1% (v/v) water/acetonitrile (water/ACN); (B) 100% ACN, both containing 0.1% formic acid with a flow rate of 400 μl/min. The following elution profile was used: 0–1 min, isocratic 80% B; 1–46 min, linear from 80% to 50% B; followed by column washing: 46–51 min, linear from 50% to 40% B and column re-equilibration; 52–60 min isocratic 80% B. The oven temperature was set at 40 °C. The injection volume was 1 μl. Mass spectra were acquired over the scan range *m/z* 300–2000 in the negative mode. A heated ESI-IT ionized the separated oligomers in an LTQ Velos Pro Mass Spectrometer (UHPLC-ESI-IT-MS) coupled to the UHPLC.

2.7. Descriptive pectin parameters

2.7.1. Determination of degree of blockiness and absolute degree of blockiness

The degree of blockiness (DB) is calculated as the number of moles of GalA residues present as non-esterified mono-, di- and triGalA released by *endo*-polygalacturonase related to the total amount of non-esterified GalA residues present and expressed as a percentage (Eq. (1)) (Daas et al., 1999; Daas et al., 2000; Guillotin et al., 2005). The absolute degree of blockiness (DB_{abs}) is calculated as the amount of non-esterified mono-, di- and triGalA residues released by endo-PG expressed as the percentage of the total GalA residues present in the pectin (Eq. (2)) (Daas et al., 2000; Guillotin et al., 2005). The amount of GalA monomer, dimer, trimer released from the digested pectins was determined by HPAEC-PAD and corrected for partially methyl-esterified triGalA levels using HILIC-ESI-IT-MS data. GalA and $GalA_2$ and $GalA_3$ (Sigma-Aldrich, Steinheim, Germany) were used for quantification. DB and DB_{abs} were calculated using the following formulas:

$$DB = \frac{\sum_{n=1-3}[\text{saturated GalA}_n\text{released}]_{\text{nonesterified}} \times n}{[\text{total nonesterified GalA in the polymer}]} \times 100 \quad (1)$$

$$DB_{abs} = \frac{\sum_{n=1-3}[\text{saturated GalA}_n\text{released}]_{\text{nonesterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (2)$$

2.7.2. Determination of degree of blockiness of methyl-esterified oligomers by PG (DB_{PGme})

To get a clear picture of the partially methyl-esterified HG region of citrus pectins, a new parameter DB_{PGme} was used. Using the amounts of individual saturated and methyl-esterified oligosaccharides present after digestion by *endo*-PG, the formula of degree of hydrolysis by PG was modified (Remoroza, Buchholt, et al., 2014) in order to distinguish between completely non-esterified blocks and partially methyl-esterified regions released by PG. As Eq. (3) shows, DB_{PGme} is calculated as the number of moles of galacturonic acid residues present in the digest as saturated, methyl-esterified GalA oligomers DP 3–8 per 100 mol of the total GalA residues in the pectin polymer (saturated DP 2 is never methyl esterified).

$$DB_{PGme} = \frac{\sum_{n=3-8}[\text{saturated GalA}_n\text{released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (3)$$

2.7.3. Determination of degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme})

Beside the saturated partially esterified residues as degraded by PG, the number of unsaturated oligomers by the simultaneous PL action is determined as well. The DB_{PLme} quantifies the amount of unsaturated and methyl-esterified GalA oligomers (DP 2–8) released by PL. DB_{PLme} is based on the previous concept $DB_{abs}Me$ for highly methyl-esterified stretches (Ralet et al., 2012). $DB_{abs}Me$ is defined as mole of GalA residues present as unsaturated methyl-esterified GalA oligomers per 100 mol of total GalA units in the polymer as released after PL digestion (Ralet et al., 2012). In our study a similar approach of Ralet et al. was used, but in this case PG and PL were used simultaneously instead of PL alone (Ralet et al., 2012) resulting in slightly different PL-derived oligomers. As shown by Eq. (4), all GalA residues present as unsaturated partly methyl-esterified oligomers (DP 2–8), released by PL action were quantified and expressed as degree of blockiness of methyl-esterified oligomers by PL (DB_{PLme}).

$$DB_{PLme} = \frac{\sum_{n=2-8}[\text{unsaturated GalA}_n\text{released}]_{\text{esterified}} \times n}{[\text{total GalA in the polymer}]} \times 100 \quad (4)$$

3. Results and discussion

3.1. Characteristics and parameters of pectin samples used in this study

Pectins used in this study were characterized for GalA content, neutral sugar composition, molecular weight distribution and degree of methyl-esterification. The characteristics of the pectins are given in Table 1.

Two pairs of pectins were selected because each pair have similar DM and similar features. The chemical characteristics of pectins are typical for homogalacturonan type pectins from citrus origin (Kravtchenko et al., 1992; Voragen et al., 2009) and only small variations in the neutral sugar content, GalA content and the DM of HM and LM pectins are present as can be seen in Table 1. The molecular weight distribution of all four pectins is rather similar with a Mw around 90 kDa (see also Fig. 1), which is in accordance with previous studies (Bagherian et al., 2011; Guillotin et al., 2005).

3.2. Enzymatic fingerprinting of citrus pectins

Enzymatic fingerprinting of pectins using one single enzyme activity is a well-known approach for structural characterization since enzymes have established substrate specificities. In this study however, in order to study the methyl-ester distribution in commercial citrus pectins, pectins O64, O59, O32 and L34 were degraded using a combination of two pure and well defined pectin enzymes: *endo*-PG and PL. Pectin degradation was followed by HPSEC with RI detection. The enzyme-treated citrus pectins showed a shift to low molecular weight oligomers (<2.5 kDa) containing information on methyl-esterification as will

Table 1
Characteristics of citrus pectin samples used in this study.

Pectin	Rha	Ara	Gal	Glc	UA[b]	Total (w/w %)[c]	Mw (kDa)[d]	DM (%)[e]
	mol %							
O64[a]	0 ± 0.30	7 ± 0.41	7 ± 0.29	1 ± 0.01	82 ± 1.2	86 ± 2.8	92	64 ± 2.6
O59	1 ± 0.04	3 ± 0.22	9 ± 0.03	3 ± 0.04	84 ± 0.62	83 ± 4.3	87	59 ± 2.1
O32	1 ± 0.04	3 ± 0.05	6 ± 0.09	1 ± 0.02	89 ± 0.02	87 ± 2.9	77	32 ± 1.9
L34	1 ± 0.16	3 ± 0.09	6 ± 0.25	1 ± 0.21	89 ± 0.73	65 ± 2.9	107	34 ± 3.1

[a] O: orange origin, L: lemon origin, Number: DM. O64 = Orange pectin with a DM of 64.
[b] Rha = rhamnose, Ara = arabinose, Gal = Galactose, Glc = Glucose, UA = Uronic Acid.
[c] Total neutral sugar content in w/w%.
[d] Molecular weight (Mw) as measured by HPSEC.
[e] Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample.

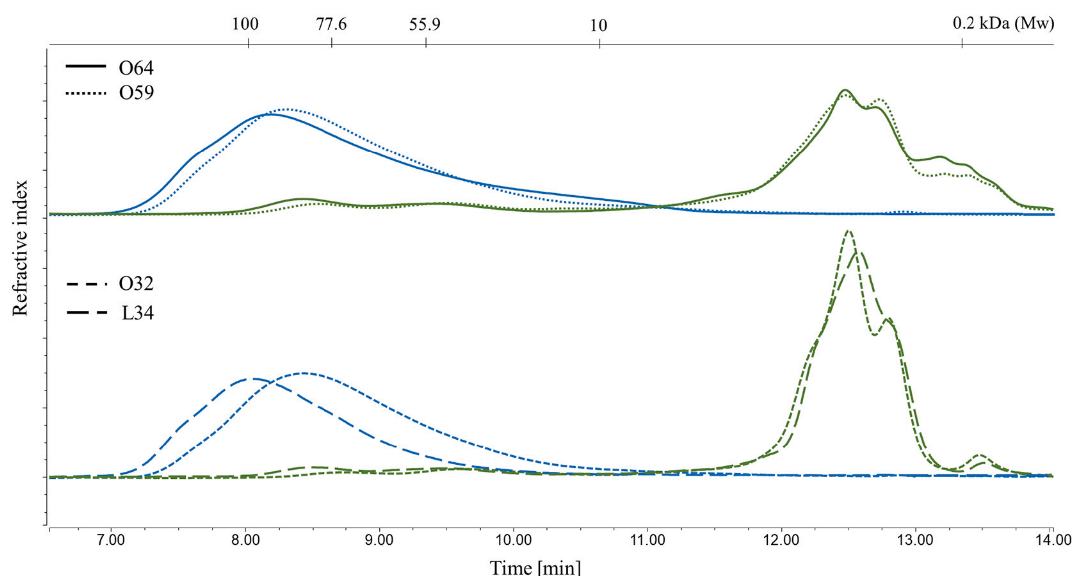

Fig. 1. HPSEC elution patterns of O64, O59, O32 and L34 pectins before (● blue line) and after (● green line) digestion by homogalacturonan degrading enzymes: PL and *endo*-PG. Molecular weights of pectin standards (in kDa) are indicated.

be discussed in Section 3.3.

After degradation, the diagnostic oligomers formed show similar low Mw (RT 11–14 min) for both pairs of similar DM pectins, however it can be already seen from the peak shape that the degradation products might differ. What stands out in the chromatogram is that endo-PG combined with PL degraded the citrus pectins almost completely into oligomers of Mw < 2.5 kDa. This complete enzymatic degradation of the pectin by a combination of enzymes to oligosaccharides is a considerable improvement compared to the use of single enzymes like *endo*-PG, *exo*-PG or PL, all having their own DM-dependency, to convert pectins only partly into diagnostic oligomers (Daas et al., 1999; Guillotin et al., 2005; Limberg et al., 2000; Ralet et al., 2012).

3.3. Characterization and quantification of the diagnostic oligomers

The differences between the methyl-ester distribution patterns of pectins have till now mainly been described by the parameters DM, DB, and DBMe and in addition DH_{PG}, DH_{PL} are used to describe acetylated pectins (Daas et al., 1999; Guillotin et al., 2005; Ralet et al., 2012; Remoroza, Buchholt, et al., 2014). HPAEC-PAD/UV of the endo-PG and PL degradation products of citrus pectins allowed the separation, identification and quantification of monoGalA and both saturated and unsaturated GalA oligomers ranging from degree of polymerization (DP) 2–7 (Fig. 2). However, as a consequence of the high pH (pH 12) used during the HPAEC separation, information on the methyl-esterification of the different oligomers is lost.

In the HPAEC saturated oligomers eluted earlier, while unsaturated oligomers eluted later, and in most cases they were nicely separated, however uDP1 (unsaturated GalA DP1) is not present and DP5 and uDP3 are coeluting. Fortunately they can be distinguished with the help of the UV signal. Fig. 2 reveals that the same type of oligomers were released after PG and PL treatment of the citrus pectins, but in quite different quantities for the various pectins. Especially, the similar-DM pectins O32 and L34 show rather different patterns, while patterns are rather similar for O64 and O59. Quantification of the oligosaccharides showed that the amount of saturated DP1–3 produced after degradation was higher in the O64 and L34 than in the similar DM, O59 and O32 pectins which means that O64 and L34 have more non-esterified GalA blocks present being accessible to PG.

What can be clearly seen in Fig. 2 is the difference between the low DM pectins regarding the unsaturated oligomers released. In the O32 digest, there were higher amounts of unsaturated products present such

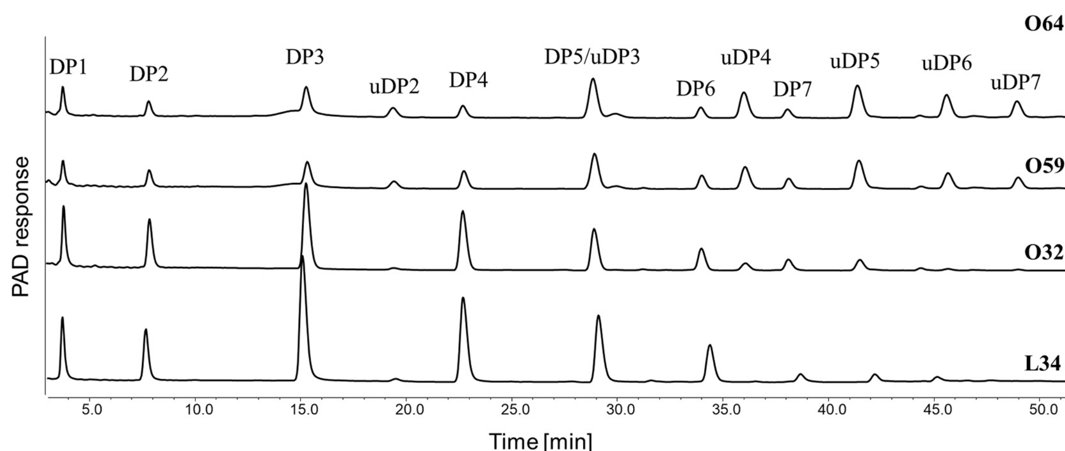

Fig. 2. HPAEC-PAD elution patterns of *endo*-PG and PL digests of O64, O59, O32 and L34 pectins after 24 h incubation detected by PAD. Peak annotation: DP4, saturated DP4 GalA oligosaccharide; uDP4, unsaturated DP4 GalA oligosaccharide.

as uDP2, uDP4 and uDP5 compared to L34. Despite the presence of dominantly non-esterified GalA residues in PG-degradable sequences, the methyl-esters still are positioned differently over the backbone of these two LM pectins causing the PL to act and to act differently. Despite rather similar oligosaccharide structures released for O59 and O64, still small differences can be observed in the amounts released. These results already confirm the presence of different methyl-esterification patterns over the pectin backbone in pectins having similar DM. Which means that, pectins with similar DM can have different patterns of methyl-esterification.

3.4. Structure elucidation of the generated oligosaccharides after enzymatic digestion

To tackle the limitations of HPAEC due to the removal of methyl-esters at high pH (pH 12) (Kravtchenko et al., 1993), HILIC-MS was employed to separate and identify methyl-esterified oligomers (Remoroza et al., 2012). Peak annotation has been done based on the *m/z* of the GalA oligosaccharides, and relative abundance of selected DPs has been obtained after integration of peak areas in the ion chromatograms (Fig. A.1. showing DP3 as an example). Since saturated dimer is only present as non-esterified oligomer, and the saturated DP4 only as methyl-esterified oligomer, saturated DP3 had to be checked for methyl-esters. Following the quantification of DP 1–7 and uDP 2–7 using the HPAEC-PAD, the relative abundance of oligomers obtained from HILIC-MS was used to differentiate between differently methyl-esterified and non-esterified oligomers within one DP.

Fig. 3 illustrates the HILIC elution patterns of the enzyme digests of the four citrus pectins. It is shown that the main degradation products are present in all digests but at different ratios, demonstrating different methyl-ester distribution in the same DM pectins. Besides the unsubstituted dimer (2^0) and trimer (3^0), partially methyl-esterified saturated and unsaturated GalA oligomers of different DPs are present as main degradation products as illustrated by mono-esterified trimer, mono- and di-esterified tetramer, mono-, di- and tri-esterified pentamer and so on. All these oligomers with different levels of methyl-esterification can be easily separated and not only the saturated, but also the unsaturated galacturonic acid oligomers. The sequence of elution of GalA oligomers is based on clustering oligomers of the same charge although larger oligomers eluted slightly later than smaller oligomers having the same net charge, due to small differences in charge density (Leijdekkers et al., 2011). For example: retention times of DP $4^1 < 5^2 < 6^3 < 7^4$ increase with the number of GalA residues present in the oligomer while they all have the same net charge.

The HILIC chromatogram of O64 digest is different from O59 digest since showing different relative intensities for the various oligosaccharides. The amounts of unsaturated highly methyl-esterified oligomers such as uDP5^4, uDP6^4 and uDP7^5 in the O64 digest were higher than in the O59 digest, suggesting more densely methyl-esterified regions in O64 compared to O59. The small saturated non-esterified oligosaccharides DP 2^0 and 3^0 are slightly higher in O64 compared to O59, pointing to a more blockwise distribution non-methyl esterified GalA residues within O64. In addition, the levels of unsaturated low methyl-esterified oligomers such as uDP3^1, uDP4^2 and saturated DP4^1, DP5^2 were higher in O59 pectin pointing to the presence of more randomly distributed methyl-esterified GalA residues in O59 compared to O64. The ratios of different oligomers differ highly in the digests of the two similar-DM pectins. For example, the ratio of uDP4^2: uDP5^3: uDP6^4: uDP7^5 in O64 and O59 are rather different 17:24:38:22 for O64 and 34:51:50:42 for O59. While DM64 has higher amounts of uDP6^4 and uDP7^5, in DM59 uDP4^2 and uDP5^3 are higher.

For the low DM pectins, in the L34 digest hardly any unsaturated products like uDP4^2 and uDP5^3 were detected which is expected as PL has low activity on low DM pectins, however in the O32 digest those unsaturated products were found. This result may be explained by the fact that the number and distribution of methyl-esters affects the activity of PL. The enzyme can cleave partially methyl-esterified GalA residues, but its activity towards pectins having DM < 50 is rather limited (Mutenda et al., 2002; van Alebeek et al., 2002). Surprisingly, O32 must have some PL degradable residues where methyl-esters are more

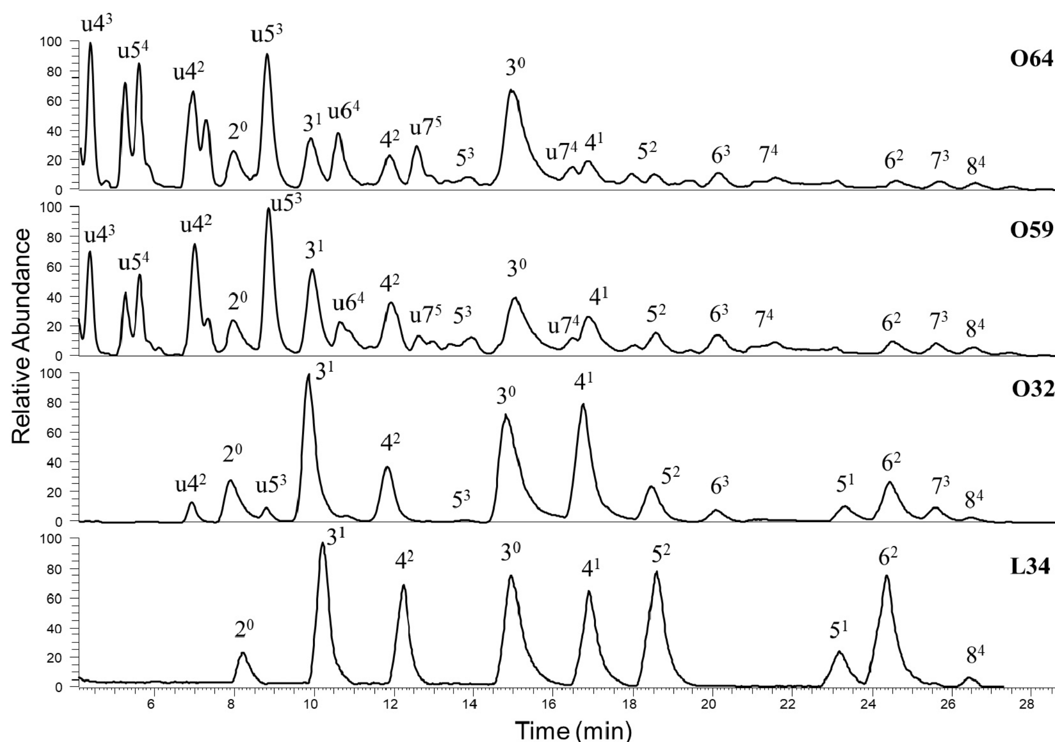

Fig. 3. HILIC-MS base peak elution pattern of O64, O59, O32 and L34 digested by homogalacturonan degrading enzymes *endo*-PG and PL. Peak annotation: 3^1, saturated DP3 GalA oligosaccharide having one methyl-ester; u5^3, unsaturated DP5 GalA oligosaccharide having three methyl-esters.

clustered on the backbone. In L34 pectin digest the quantity of saturated methyl-esterified oligomers released and also the ratio of e.g., saturated DP 4^2: 5^2: 6^2 is higher than in O32. This suggests a specific pattern of methyl-esterification, having stretches of non-esterified GalA residues interrupted with a few methyl-esterified GalA residues. Altogether, absolute as well as relative amounts of the various oligomers clearly differ for different pectins, even when having the same DM and may add to a detailed characterization of pectin's methyl-esterification patterns. Taking the relative abundance of individual oligosaccharides which are identified obtained from HILIC and applying those ratios on the easily separated and quantified oligomers from HPAEC can be beneficial to explain the differences in pectin structure and help to explain pectin functionality.

3.5. Investigation of pectin methyl-esterification patterns

Previously the methyl-esterified segments present in pectins were described by DBMe and DB_{abs}Me based on PL digestion alone and for the non-esterified segments DB and DB_{abs} were used based on oligomers released by PG alone (Daas et al., 1999; Guillotin et al., 2005; Ralet et al., 2012). However, it seems that the precise methyl-ester distribution patterns are not yet clearly revealed, described and understood by these parameters. By the simultaneous PL and PG digestion and by the combination of HPAEC and HILIC high throughput analysis is possible as all the pectin oligomers can be examined in a very short time. By calculating the DB_{PGme} and DB_{PLme} based on simultaneous degradation by PG and PL, additional information can be revealed on the methyl-esterification patterns of the citrus pectins and pectins can more readily be compared based on these parameters. DB_{abs} quantifies unsubstituted mono-, di- and tri GalA oligomers as released by PG related to all GalA present in the pectin, D_{BPGme} does quantify PG released saturated and partly methyl-esterified random segments of the pectin and DB_{PLme} quantifies PL released unsaturated and highly methyl-esterified oligomers released from the pectin, therefore by these three parameters the entire pectin backbone can be described.

Table 2 shows these descriptive parameters for the four pectins used in this study. It can be seen that, even though the DM of both low DM and high DM pectins are rather similar, especially the DB_{PGme} and DB_{PLme} parameters differ from each other. The DB_{PGme} is 40% lower while the DB_{PLme} is 23% higher in O64 than in pectin O59. O64 thus has, next to non-esterified blocks, also blocks with methyl-esterified residues. In contrast to first thoughts that an equal DB_{abs} of two pectins would indicate similar pectin methyl-ester distributions, the different DB_{PLme} and DB_{PGme} of O64 and O59 suggest much more refined structural differences. In O59 there are more PG degradable methyl-esterified GalA residues, which indicates more randomly methyl-esterification next to having highly methyl-esterified residues degradable also by PL. DB_{PLme} and DB_{PGme} complement the previous research describing pectins using DB (Daas et al., 2000; Guillotin et al., 2005) while also adding an extra dimension by revealing differences in the methyl-esterified regions of pectins using both PL and PG simultaneously. Fig. 4 visualizes the differences in methyl ester patterns from the two high DM pectins. The oligomers released by PG and PL in the digests are highlighted as also included in the formulas and the hypothetical representation of the parental molecule is visualized.

The relative abundance of the different oligomers as released by the combination of endo-PG and PL differs to a large extent in the pectins studied. As expected O64 served as good substrate for PL. Interestingly, also oligomers such as $DP7^5$ and $uDP7^5$ were present in the digests, which in theory could have been degraded further by PL, but this may be explained by the pattern of methyl-ester distribution within the oligomer, not matching with the specificity of the enzyme (Kravtchenko et al., 1993; van Alebeek et al., 2002). Larger differences were found between pectin digests having highly methyl-esterified oligomers. Good examples for the densely methyl-esterified segments are the unsaturated $uDP4^3$, $uDP5^4$ and $uDP6^5$ oligosaccharides released by PL from O64 pectin in 30–50% higher amounts than from O59 pectin. O59 pectin has less methyl-esterified GalA stretches, degradable by PL, in addition to non-esterified stretches, degradable by PG, releasing both more non-esterified GalA DP1–3 and more methyl-esterified GalA sequences which could not be released/degraded by PL (Fig. 4). The presence and length of the methyl-esterified oligomers released by PG represent the pattern of methyl-esterification outside any block and are not covered by DM nor DB, but are now covered by DB_{PGme} and $DB_{PLme.}$

For low DM pectins it was found, as hypothesized, that they are favorable substrates for *endo*-PG and mainly saturated oligomers were released. However, more unexpected, differences can still be found in methyl-ester distribution patterns. Interestingly in case of O32, the level of methyl-esterified products released by PL, the DB_{PLme}, is more than doubled compared to L34 having a similar DM, at the expense of partly methyl-esterified GalA oligomers being released by PG. Together, this suggests a less random pattern of methyl-esterification for L34. Furthermore, in O32 mainly less methyl-esterified oligomers are present like $DP4^1$, $DP5^1$ or $DP6^3$ which relates to a more random pattern of methyl-esters in lower DB pectin.

Table 2
Descriptive parameters of citrus pectins used in this study.

Pectin (DM)	DB (%)[b]	DB_{abs} (%)[c]	DB_{PGme} (%)[d]	DB_{PLme} (%)[e]
O64[a]	37	13	18	65
O59	28	11	30	53
O32	41	27	67	11
L34	50	33	95	5

[a] O: orange pectin, L: lemon pectin. Number: DM. O64 = Orange pectin with a DM of 64.
[b] Degree of blockiness (DB): the amount of non-esterified mono-, di- and tri-GalA per 100 mol of the non-esterified GalA in the sample.
[c] Absolute degree of blockiness (DB): the amount of non-esterified mono-, di- and triGalA per 100 mol of total GalA in the sample.
[d] Degree of blockiness by endo-PG (DB_{PGme}): the amount of saturated methyl-esterified galacturonic residues per 100 mol of total galacturonic acid in the sample.
[e] Degree of blockiness by PL (DB_{PLme}): the amount of methyl-esterified unsaturated galacturonic oligomers per 100 mol of total galacturonic acid in the sample.

4. Conclusion

The main goal of the current study was to elucidate pectin methyl-esterification patterns by using combined *endo*-PG and PL digestion on two pairs of commercial citrus pectins representing either high or low DM pectins. When using HPAEC alone, the saturated and unsaturated GalA oligomers can be easily separated and quantified. In addition, with HILIC the different methyl-esterified oligomers in pectin digests having the same DP can be easily differentiated. Information on both the saturated (non)methyl-esterified oligo galacturonides released by PG and the methyl-esterified unsaturated oligo galacturonides released by PL, can now be used to simply characterize pectins with various structural parameters faster and in more detail. It was demonstrated that pectin methyl-esterification patterns differ highly, even in pectins having similar DM and DB. The efficient separation and identification of oligomers using HILIC demonstrate the value of the analysis of citrus pectin digests and can provide understanding between pectin fine structure and functionality. Combing endo-PG and PL digestion of pectin and consequently quantifying the entire homogalacturonan region, provided more details on the methyl-esterification patterns in citrus pectins, beyond the degree of blockiness. It is possible now to characterize methyl-esterified pectins on a higher level by recognizing patterns between fully non-esterified and fully esterified segments. This approach can be useful to differentiate between pectins having the same levels of methyl-esterification but different physical and biochemical functionalities and to explain these differences in applications.

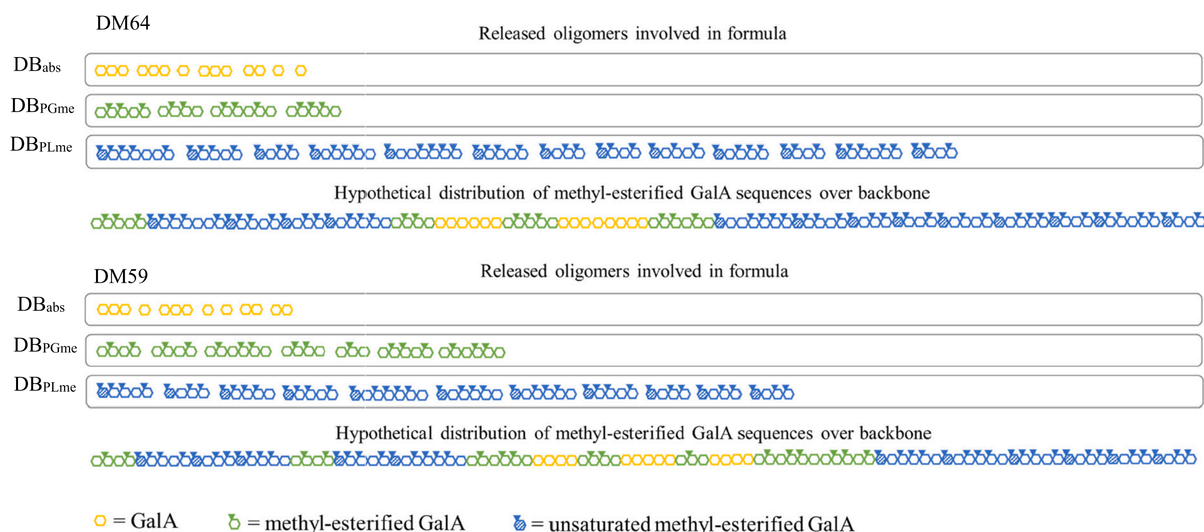

Fig. 4. Schematic representation of enzymatic digestion with *endo*-PG of *Kluyveromyces fragilis* and PL on two ~60% DM pectins, O64 and O59 having different methyl-ester distributions. The released diagnostic oligomers can be analyzed and quantified on HPAEC and HILIC and consequently the descriptive parameters, such as DB, DBabs, DBPGme and DBPLme can be calculated. The precise location of the released oligomers could not be determined.

CRediT authorship contribution statement

Éva Jermendi: Methodology, Investigation, Writing – Original Draft.
Martin Beukema: Writing – Review & Editing.
Paul de Vos: Writing – Review & Editing.
Marco van den Berg: Writing – Review & Editing.
Henk Schols: Supervision, Funding acquisition, Conceptualization, Writing – Review & Editing.

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Appendix A

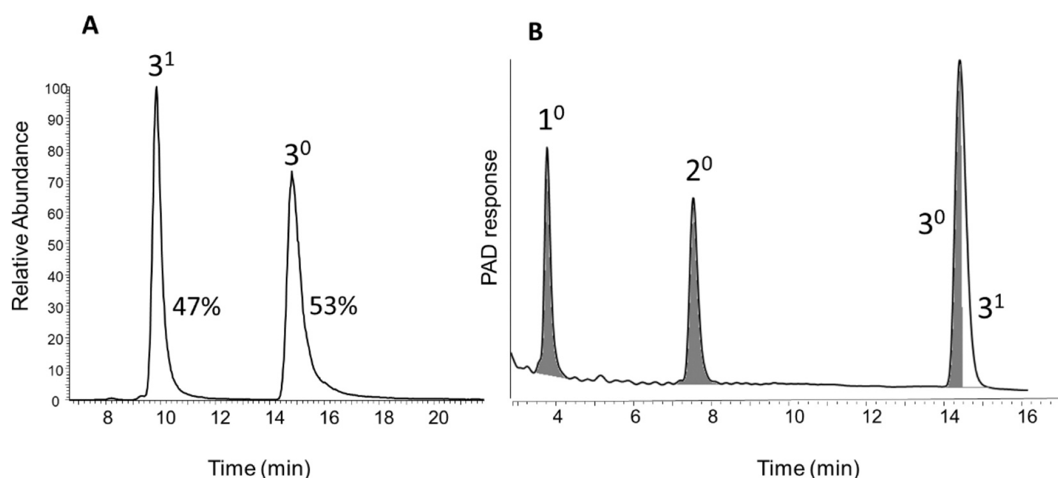

Fig. A.1. A. UPLC-HILIC-MS profile of O32 digested by *endo*-PG and PL enzymes with the selection of saturated $GalA_3$ masses. Peak annotation: 3^1: saturated DP 3 having one methyl-ester. Showing the relative abundance of GalA DP 3^0 and 3^1.
B. HPAEC-PAD elution pattern of DP1–3 from the same O32 pectin after PG and PL digestion indicating that the $GalA_3$ area covers DP 3^1 and 3^0 in different proportions.

References

Bagherian, H., Ashtiani, F. Z., Fouladitajar, A., & Mohtashamy, M. (2011). Comparisons between conventional, microwave-and ultrasound-assisted methods for extraction of pectin from grapefruit. *Chemical Engineering and Processing: Process Intensification, 50*(11–12), 1237–1243.
Beukema, M., Jermendi, É., van den Berg, M., Faas, M., Schols, H., & de Vos, P. (2021). The impact of the level and distribution of methyl-esters of pectins on TLR2-1 dependent anti-inflammatory responses. *Carbohydrate Polymers, 251*, Article 117093.
Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry, 54*(2), 484–489.

Broxterman, S. E., & Schols, H. A. (2018). Interactions between pectin and cellulose in primary plant cell walls. *Carbohydrate Polymers, 192*, 263–272.
Coenen, G. J., Kabel, M. A., Schols, H. A., & Voragen, A. G. (2008). CE-MSn of complex pectin-derived oligomers. *Electrophoresis, 29*(10), 2101–2111.
Daas, P. J., Meyer-Hansen, K., Schols, H. A., De Ruiter, G. A., & Voragen, A. G. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research, 318*(1–4), 135–145.
Daas, P. J., Voragen, A. G., & Schols, H. A. (2000). Characterization of non-esterified galacturonic acid sequences in pectin with endopolygalacturonase. *Carbohydrate Research, 326*(2), 120–129.
Deckers, H., Olieman, C., Rombouts, F., & Pilnik, W. (1986). Calibration and application of high-performance size exclusion columns for molecular weight distribution of pectins. *Carbohydrate Polymers, 6*(5), 361–378.
Englyst, H. N., & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst, 109*(7), 937–942.
Fraeye, I., Duvetter, T., Doungla, E., Van Loey, A., & Hendrickx, M. (2010). Fine-tuning the properties of pectin–calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology, 21*(5), 219–228.
Gómez, B., Gullón, B., Yáñez, R., Schols, H., & Alonso, J. L. (2016). Prebiotic potential of pectins and pectic oligosaccharides derived from lemon peel wastes and sugar beet pulp: A comparative evaluation. *Journal of Functional Foods, 20*, 108–121.
Guillotin, S., Bakx, E., Boulenguer, P., Mazoyer, J., Schols, H., & Voragen, A. (2005). Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydrate Polymers, 60*(3), 391–398.
Harmsen, J., Kusters-van Someren, M., & Visser, J. (1990). Cloning and expression of a second aspergillus Niger pectin lyase gene (pelA): Indications of a pectin lyase gene family in a. Niger. *Current Genetics, 18*(2), 161–166.
Huisman, M., Oosterveld, A., & Schols, H. (2004). Fast determination of the degree of methyl esterification of pectins by head-space GC. *Food Hydrocolloids, 18*(4), 665–668.
Kravtchenko, T., Penci, M., Voragen, A., & Pilnik, W. (1993). Enzymic and chemical degradation of some industrial pectins. *Carbohydrate Polymers, 20*(3), 195–205.
Kravtchenko, T., Voragen, A., & Pilnik, W. (1992). Analytical comparison of three industrial pectin preparations. *Carbohydrate Polymers, 18*(1), 17–25.
Leijdekkers, A., Sanders, M., Schols, H., & Gruppen, H. (2011). Characterizing plant cell wall derived oligosaccharides using hydrophilic interaction chromatography with mass spectrometry detection. *Journal of Chromatography A, 1218*(51), 9227–9235.
Levesque-Tremblay, G., Pelloux, J., Braybrook, S. A., & Müller, K. (2015). Tuning of pectin methylesterification: Consequences for cell wall biomechanics and development. *Planta, 242*(4), 791–811.
Levigne, S., Ralet, M.-C., & Thibault, J.-F. (2002). Characterisation of pectins extracted from fresh sugar beet under different conditions using an experimental design. *Carbohydrate Polymers, 49*(2), 145–153.
Limberg, G., Körner, R., Buchholt, H. C., Christensen, T. M., Roepstorff, P., & Mikkelsen, J. D. (2000). Quantification of the amount of galacturonic acid residues in blocksequences in pectin homogalacturonan by enzymatic fingerprinting with exo-and endo-polygalacturonase II from aspergillusniger. *Carbohydrate Research, 327*(3), 321–332.
May, C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers, 12*(1), 79–99.
Mutenda, K. E., Körner, R., Christensen, T. M., Mikkelsen, J., & Roepstorff, P. (2002). Application of mass spectrometry to determine the activity and specificity of pectin lyase a. *Carbohydrate Research, 337*(13), 1217–1227.
Ngouémazong, D. E., Tengweh, F. F., Duvetter, T., Fraeye, I., Van Loey, A., Moldenaers, P., & Hendrickx, M. (2011). Quantifying structural characteristics of partially de-esterified pectins. *Food Hydrocolloids, 25*(3), 434–443.
Oosterveld, A., Beldman, G., Schols, H. A., & Voragen, A. G. (1996). Arabinose and ferulic acid rich pectic polysaccharides extracted from sugar beet pulp. In *Pectic substances from sugar beet pulp: structural features, enzymatic modification, and gel* (p. 17).
Osborne, D. (2004). Advances in pectin and pectinase research, 2003. In F. Voragen, H. A. Schols, & R. Visser (Eds.)*, Vol. 94*. *Annals of Botany: Oxford University Press* (pp. 479–480). The Netherlands: Kluwer Academic Publishers.
Pasculli, R., Geraeds, C., Voragen, A., & Pilnik, W. (1991). Characterization of polygalacturonases from yeast and fungi. *Food Science and Technology Lebensmittel-Wissenschaft und Technologie, 24*, 63–70.
Ralet, M.-C., Williams, M. A., Tanhatan-Nasseri, A., Ropartz, D., Quéméner, B., & Bonnin, E. (2012). Innovative enzymatic approach to resolve homogalacturonans based on their methylesterification pattern. *Biomacromolecules, 13*(5), 1615–1624.
Remoroza, C., Broxterman, S., Gruppen, H., & Schols, H. (2014). Two-step enzymatic fingerprinting of sugar beet pectin. *Carbohydrate Polymers, 108*, 338–347.
Remoroza, C., Buchholt, H., Gruppen, H., & Schols, H. (2014). Descriptive parameters for revealing substitution patterns of sugar beet pectins using pectolytic enzymes. *Carbohydrate Polymers, 101*, 1205–1215.
Remoroza, C., Cord-Landwehr, S., Leijdekkers, A., Moerschbacher, B., Schols, H., & Gruppen, H. (2012). Combined HILIC-ELSD/ESI-MSn enables the separation, identification and quantification of sugar beet pectin derived oligomers. *Carbohydrate Polymers, 90*(1), 41–48.
Rolin, C. (2002). Commercial pectin preparations. In *Pectins and their manipulation* (pp. 222–241).
Sahasrabudhe, N. M., Beukema, M., Tian, L., Troost, B., Scholte, J., Bruininx, E., & Schols, H. A. (2018). Dietary fiber pectin directly blocks toll-like receptor 2–1 and prevents doxorubicin-induced ileitis. *Frontiers in Immunology, 9*, 383.
Schols, H. A., Posthumus, M. A., & Voragen, A. G. (1990). Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydrate Research, 206*(1), 117–129.
Schols, H. A., Visser, R. G. F., & Voragen, A. G. (2009). *Pectins and pectinases*. Wageningen Academic Pub.
Ström, A., Ribelles, P., Lundin, L., Norton, I., Morris, E. R., & Williams, M. A. (2007). Influence of pectin fine structure on the mechanical properties of calcium− pectin and acid− pectin gels. *Biomacromolecules, 8*(9), 2668–2674.
Tanhatan-Nasseri, A., Crépeau, M.-J., Thibault, J.-F., & Ralet, M.-C. (2011). Isolation and characterization of model homogalacturonans of tailored methylesterification patterns. *Carbohydrate Polymers, 86*(3), 1236–1243.
Thibault, J.-F. (1979). *Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphényl*.
Thibault, J.-F., & Ralet, M.-C. (2003). Physico-chemical properties of pectins in the cell walls and after extraction. In *Advances in pectin and pectinase research* (pp. 91–105). Springer.
Tian, L., Scholte, J., Borewicz, K., van den Bogert, B., Smidt, H., Scheurink, A. J., & Schols, H. A. (2016). Effects of pectin supplementation on the fermentation patterns of different structural carbohydrates in rats. *Molecular Nutrition & Food Research, 60*(10), 2256–2266.
van Alebeek, G.-J. W., Christensen, T. M., Schols, H. A., Mikkelsen, J. D., & Voragen, A. G. (2002). Mode of action of pectin lyase a of aspergillus nigeron differently C6-substituted oligogalacturonides. *Journal of Biological Chemistry, 277*(29), 25929–25936.
Vincken, J.-P., Schols, H. A., Oomen, R. J., Beldman, G., Visser, R. G., & Voragen, A. G. (2003). Pectin—the hairy thing. In *Advances in pectin and pectinase research* (pp. 47–59). Springer.
Vogt, L. M., Sahasrabudhe, N. M., Ramasamy, U., Meyer, D., Pullens, G., Faas, M. M., & de Vos, P. (2016). The impact of lemon pectin characteristics on TLR activation and T84 intestinal epithelial cell barrier function. *Journal of Functional Foods, 22*, 398–407.
Voragen, A. G., Coenen, G.-J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry, 20*(2), 263.
Voragen, F., Beldman, G., & Schols, H. (2001). In B. V. McCleary, & L. Prosky (Eds.), *Advanced dietary fibre technology*.
Willats, W. G., Knox, J. P., & Mikkelsen, J. D. (2006). Pectin: New insights into an old polymer are starting to gel. *Trends in Food Science & Technology, 17*(3), 97–104.
Willats, W. G., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: Cell biology and prospects for functional analysis. *Plant Molecular Biology, 47*(1–2), 9–27.