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Mapping sugar beet pectin acetylation pattern

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Abstract

Homogalacturonan-derived partly methylated and/or acetylated oligogalacturonates were recovered after enzymatic hydrolysis (*endo*-polygalacturonase + pectin methyl esterase + side-chain degrading enzymes) of sugar beet pectin followed by anion-exchange and size exclusion chromatography. Around 90% of the GalA and 75% of the acetyl groups present in the initial sugar beet pectin were recovered as homogalacturonan-derived oligogalacturonates, the remaining GalA and acetyl belonging to rhamnogalacturonic regions. Around 50% of the acetyl groups present in sugar beet homogalacturonans were recovered as partly methylated and/or acetylated oligogalacturonates of degree of polymerisation $\leqslant 5$ whose structures were determined by electrospray ionization ion trap mass spectrometry (ESI-IT-MS^n). 2-*O*-acetyl- and 3-*O*-acetyl-GalA were detected in roughly similar amounts but 2,3-di-*O*-acetylation was absent. Methyl-esterified GalA residues occurred mainly upstream 2-*O*-acetyl GalA. Oligogalacturonates containing GalA residues that are at once methyl- and acetyl-esterified were recovered in very limited amounts. A tentative mapping of the distribution of acetyl and methyl esters within sugar beet homogalacturonans is proposed. Unsubstituted GalA residues are likely to be present in limited amounts (~10% of total GalA residues), due to the fact that methyl and acetyl groups are assumed to be most often not carried by the same residues.

Keywords: *Beta vulgaris*; Homogalacturonan; Rhamnogalacturonan; Acetylation; Oligogalacturonates; *endo*-Polygalacturonase

1. Introduction

Pectin is a complex carbohydrate polymer that plays an instrumental role in regulating the mechanical properties of the plant cell wall (McCann and Roberts, 1996) and has also several industrial applications related to their gelling properties (May, 1990). Pectins are made of several structural elements among which homogalacturonans (HGs) and type I rhamnogalacturonans (RGs-I) are the most abundant. Type II rhamnogalacturonan (RG-II), a complex polysaccharide composed of galacturonic acid, rhamnose, galactose and some unusual sugars, is also part of the pectic molecule (Ishii and Matsunaga, 2001). RG-II, although present as a quantitatively minor pectic sub-unit, plays a key role in cell wall architecture (O'Neill et al., 2004). HG is composed of (1 → 4)-linked α-D-Gal*p*A residues that can be partly methyl-esterified at *C*-6 and possibly partly acetyl-esterified at *O*-2 and/or *O*-3 (Voragen et al., 1995). The methyl-esterification of HG has been the subject of several investigations since it determines to a large extent the industrial applicability of pectin and their interaction ability *in muro*. Indeed, many of the properties and biological functions of pectins are believed to be determined by ionic interactions between HGs (Ridley

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et al., 2001; Willats et al., 2001). Acetylation inhibits the ability of pectins to bind divalent cations and thereby modifies their associative properties (Ralet et al., 2003). Several studies about the exact location of acetyl groups on pectins have been carried out but the description of acetylation patterns in pectins still remains far from clear. Keenan et al. (1985) presented a ^{13}C NMR study of sugar beet pectin and concluded that both of the available ring positions (*O*-2 and *O*-3) of GalA residues can be acetyl esterified. More recently, Kouwijzer et al. (1996), on the basis of energy calculations, concluded that acetyl groups at both *O*-2 and *O*-3 of GalA residues in the backbone of RG-I and HG are energetically favourable. Comparison of pectic fragments isolated after enzymatic (Driselase®) hydrolysis of pectins of various tissues from several plant species suggests a high diversity in the degree, distribution and location of acetyl groups. In HG-derived oligosaccharides, acetyl esters were found located on *O*-3, or *O*-2 or even *O*-4 of GalA residues, the latter location being attributed to acyl migration during the digestion and purification steps (Needs et al., 1998; Ishii, 1997; Perrone et al., 2002) (Scheme 1). In RG-I fragments, sole 3-*O*-acetylation was detected by some authors (Komalavilas and Mort, 1989; Perrone et al., 2002) while others (Ishii, 1995, 1997) found predominantly 2-*O*-acetylation together with some 2,3-di-*O*-acetylation.

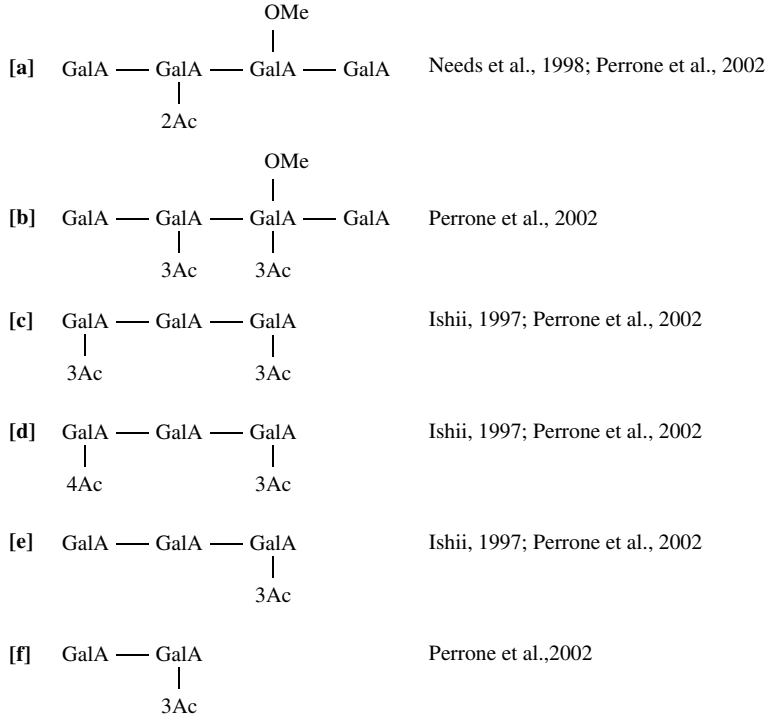
To better understand the relationship between the acetylation of pectins and their associative properties, more information about the position and distribution of acetyl groups on pectin molecules, and more particularly on HGs, is necessary. Enzymatic degradation of pectic polysaccharides is an efficient tool for their structural analysis since enzymes have strict substrate specificities. Moreover, the recovery of oligosaccharides of low degree of polymerisation (d.p.) allows the precise location of *O*-acetyl substituents by mass spectrometry (Quéméner et al., 2003a). *endo*-Polygalacturonases (PGs) from a large number of fungi have been purified and characterised and *Aspergillus niger*, to date one of the best producers of pectin-degrading enzymes, possesses at least seven *endo*-PGs.

In the present work, *A. niger* PG I was combined with a fungus-pectin methylesterase (f-PME) and side-chain degrading enzymes (*endo*-galactanase and *endo*-arabinanase) in order to allow the release of acetylated oligogalacturonates from sugar beet pectin HGs. The acetylated oligogalacturonates produced were purified for structural characterisation. The repartition of GalA and acetyl groups between HG and RG regions is discussed and a tentative mapping of the distribution of acetyl and methyl esters within sugar beet HGs is proposed.

2. Results

2.1. Enzymatic hydrolysis of sugar beet pectin

An acid-extracted sugar beet pectin (SBP 62-30; Buchholt et al., 2004) was hydrolysed by PG I in combination with a f-PME, an *endo*-arabinanase and an *endo*-galactanase, in fixed conditions of pH, temperature and



Scheme 1.

ratio enzymes/substrate. Hydrolysis was followed by measuring the relative viscosity of the reaction mixture and the reducing sugars (expressed as GalA) produced by the enzymes as a function of time (Fig. 1A). A rapid decrease of relative viscosity (50% decrease in 15 min) associated with a relatively low increase in reducing sugar concentration was observed, showing that PG I, in combination with f-PME and side-chain degrading enzymes, was able to hydrolyse sugar beet pectin efficiently. The reciprocal of the specific viscosity was linearly related to the reaction time in the initial steps of hydrolysis (Fig. 1B), suggesting that the enzymes randomly degraded pectins. 24 h incubation was assumed to be the end time of hydrolysis as no detectable change in the reducing sugar concentration and oligogalacturonate distribution of the enzyme mixture was observed after this time, even after adding fresh enzymes (data not shown).

The 24 h digest was analysed by HPAEC pH 13. Due to the high pH of the eluent, all ester groups were removed and separation of the oligogalacturonates took place according to their dp. Molar response factors determined on pure oligogalacturonates (dp 1–12) were used for quantification. PG I, in combination with a f-PME, an *endo*-arabinanase and an *endo*-galactanase, generated high amounts of oligogalacturonates of dp $\leqslant$ 6 (74, 149, 233, 188, 82 and 66 mg GalA/g of GalA in initial pectin of dp 1, 2, 3, 4, 5 and 6, respectively). Oligogalacturonates of dp 7–12 were also detected (110 mg GalA/g of GalA in initial pectin). Altogether, around 90% of the GalA initially present in sugar beet pectin was recovered as HG-derived oligogalacturonates.

A combination of preparative anion-exchange and size-exclusion chromatography was used to purify the different oligogalacturonates for further analyses.

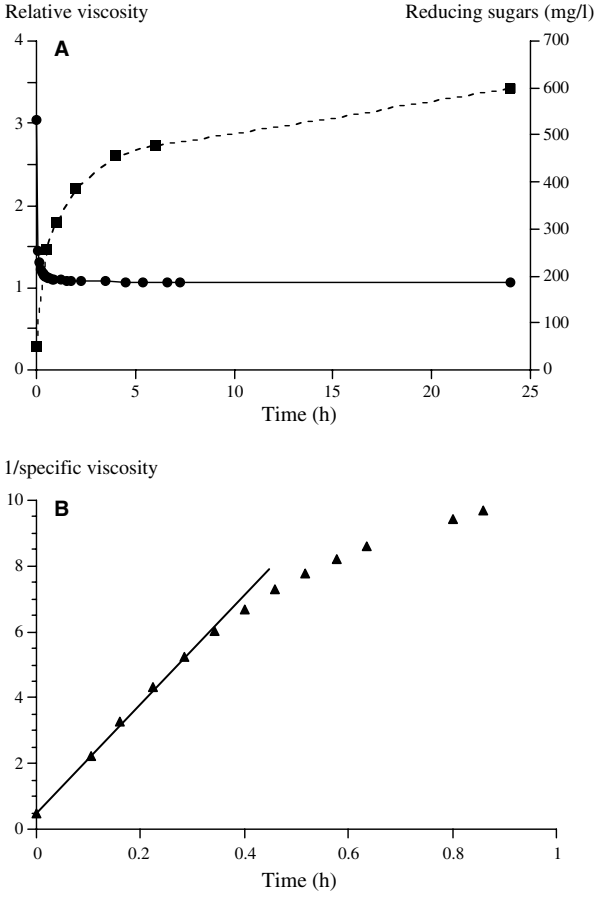


Fig. 1. Time course of hydrolysis: (A) Relative viscosity (-▲-) and reducing sugars (-■-) concentration of the reaction mixture versus time. (B) Reciprocal of specific viscosity of reaction mixtures containing sugar beet pectin incubated with *endo*-A + *endo*-G + f-PME + PG I in admixture.

2.2. Chromatographic fractionations and characterisation of the hydrolysis products

The end-products were fractionated by anion-exchange chromatography and their elution pattern is shown in Fig. 2. Fractions were pooled and desalted, and their yields and compositions were determined (Table 1). Fractions were also analysed for their dp by HPAEC pH 13 (Table 1). Recovery yields for GalA and acetyl groups (compared to GalA and acetyl groups present in the initial sugar beet pectin) were close to 100%. Due to the use of PME, most of the methyl groups were removed and methyl groups recovery was 27%, corresponding to overall mean final DM of 16.

Several fractions (I-1–I-7) contained merely GalA. GalA monomers, dimers and trimers with no methyl-esterification but with minor acetyl-esterification were recovered at 0, 0.08 and 0.15 M NaCl, respectively (fractions I-1, I-3 and I-6, respectively). They represented altogether 34.3% of the initial GalA content. A GalA trimer possibly bearing one methyl and one acetyl group (DM 31, DAc 30) was recovered at 0.06 M NaCl (fraction I-2). A GalA tetramer possibly bearing one methyl

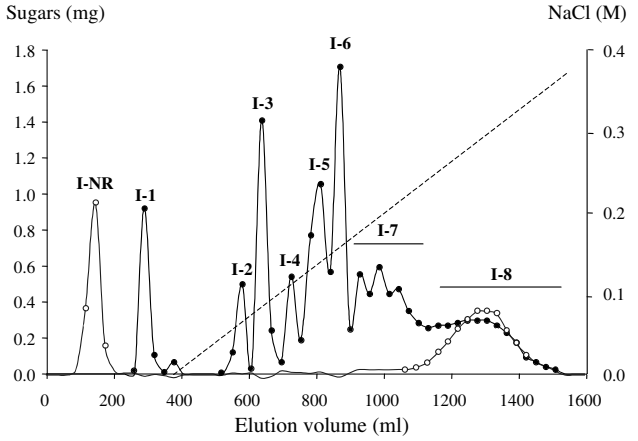


Fig. 2. Anion-exchange (DEAE Sepharose CL-6B) elution pattern of sugar beet pectin hydrolysate (●) GalA; (○) neutral sugars.

Table 1
Yields, chemical compositions and degree of polymerisation of the fractions recovered after anion-exchange and size-exclusion chromatography

Fractions	NaCl (M)	dp*	DM	DAc	GalA yield (%)**	Acetyl yield (%)***
I-1	0	1	0	5.1	6.6	1.2
I-2	0.06	3	31.0	30.0	4.2	4.7
I-3	0.08	2	0	9.8	11.3	4.1
I-4	0.10	5	33.0	31.1	5.1	5.9
I-5	0.13	4	25.0	25.0	16.6	15.4
I-6	0.15	3	0	11.7	16.4	7.2
I-7	0.17–0.25	4–8	29.0	31.6	23.5	27.5
a	0.17	$6 \gg 3$	nd	37.2	3.4	4.8
b	0.18	5	nd	29.6	3.3	3.7
c	0.19	$8 \gg 4$	nd	26.6	3.0	3.1
d	0.20	7	nd	27.7	3.6	3.7
e	0.21	6	nd	32.9	6.0	7.5
f	0.21–0.24	$8 \gg 7 > 5 > 6$	nd	30.6	4.1	4.7
I-8a	0.25–0.34	–	0	63.5	10.0	23.5
I-8b	0.25–0.34	Up to 18 (centred on 10)	32.9	40.3	6.4	9.6

* Degree of polymerisation of the major oligogalacturonates in each fraction.
** Recovered GalA/GalA present in initial sugar beet pectin.
*** Recovered acetyl/acetyl present in initial sugar beet pectin.

and one acetyl group (DM 25, DAc 25) was recovered at 0.13 M NaCl (fraction I-5). A partly methylated and acetylated pentamer was recovered at 0.1 M NaCl (fraction I-4). This fraction exhibited a DM of 33 and a DAc of 31, suggesting that several pentamers exhibiting different levels of methyl- and/or acetyl-esterification could be present. Fraction I-7 was more heterogeneous with respect to dp and was highly acetylated. This fraction was further purified by anion-exchange chromatography (not shown) and six sub-fractions were recovered (I-7a–I-7f). Most of the sub-fractions (except I-7f) appeared homogeneous with respect to dp on HPAEC pH 13 (not shown). GalA pentamers were recovered in sub-fraction I-7b, GalA hexamers in sub-fractions I-7a and I-7e, GalA heptamers in sub-fraction I-7d, and GalA octamers in sub-fraction I-7c (Table 1).

Fraction I-8, containing GalA together with neutral sugars and eluting between 0.25 and 0.34 M NaCl, was further purified by size-exclusion chromatography. The elution profile (Fig. 3) revealed the presence of two main populations: the first one (I-8a), containing neutral sugars and GalA, eluted between K_{av} 0.2 and 0.4 and the second one (I-8b), containing only GalA, eluted at $K_{av} \sim 0.85$. Fraction I-8a was composed of Rha, Ara, Gal and GalA (molar ratio: 1.0:1.4:1.1:2.7). Moreover, its elution at low K_{av} values indicates a large hydrodynamic volume. This fraction probably encloses the resistant "hairy" fragments of sugar beet pectin. Fraction I-8b represented only 6.4% of the GalA initially present in sugar beet pectin, was highly acetylated, and appeared highly heterogeneous with respect to dp (Table 1). It most probably corresponds to the most resistant HG part towards hydrolysis by *endo*-PG I.

Oligogalacturonates arising from HGs were readily separated from the remaining unaltered RGs-I (fraction I-8a) by anion-exchange and size-exclusion chromatography. 90% of the total GalA present in the initial sugar beet pectin was recovered as HG-derived oligogalacturonates, in consistency with hpaec data. Moreover, ~75% of the acetyl groups present in the initial sugar beet pectin were attached to those HG-derived oligogalacturonates.

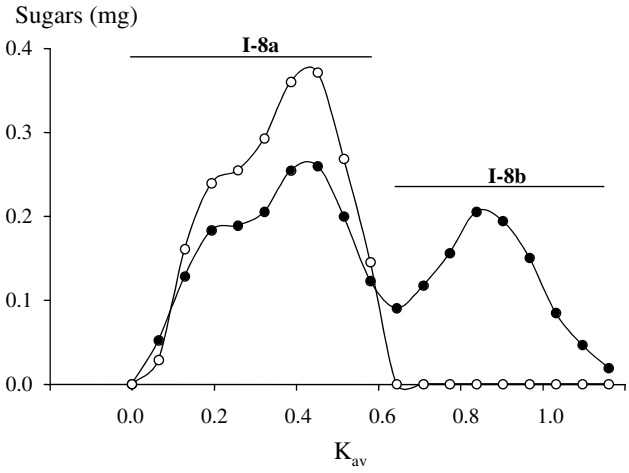


Fig. 3. Size-exclusion (Sephacryl S-200) elution pattern of fraction I-8 from anion-exchange chromatography (●) GalA; (○) neutral sugars.

2.3. Structural assignment by ESI-IT-MS

Purified fractions (I-1–I-7f) were analysed by ESI-IT-MS. Singly $[M - H]^-$ and doubly $[M - 2H]^{2-}$ deprotonated pseudomolecular ions, cluster $[2M - H]^-$ ions, and/or their sodium-form were detected as illustrated in Fig. 4 for fractions I-2 and I-5. The oligogalacturonates detected in each fraction are summarized in Table 2 together with the GalA and acetyl recoveries referred to HG. Besides unsubstituted GalA monomers (fraction

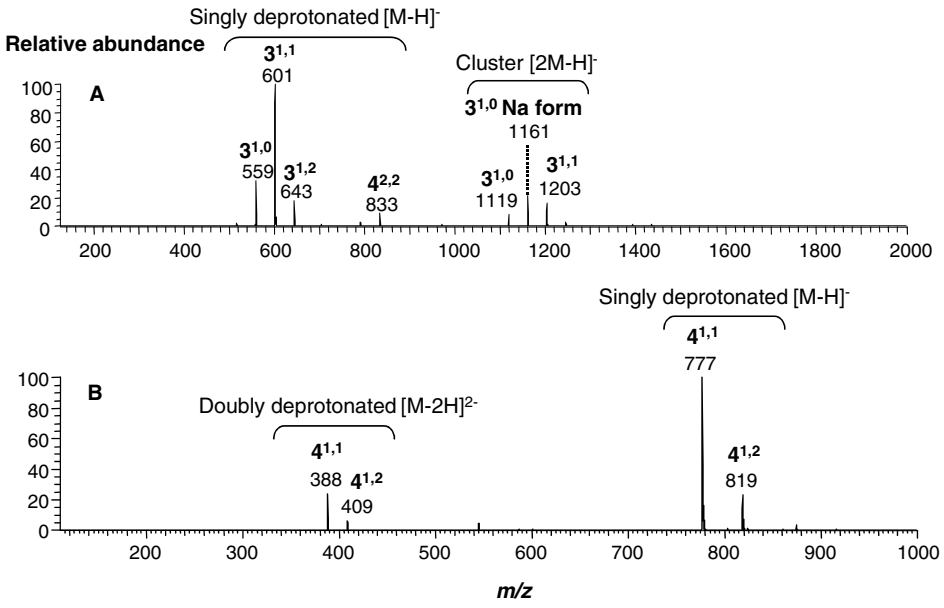


Fig. 4. Negative full MS spectra of: (A) fraction I-2 and (B) fraction I-5.

I-1), dimers (present in fraction I-3) and trimers (present in fraction I-6), several partly methylated and/or acetylated oligogalacturonates were detected. Most, but not all, acetylated oligogalacturonates isolated were also methylated which suggests, as previously pointed out (Perrone et al., 2002; Bonnin et al., 2003), that the presence of an acetyl group most probably protects the vicinal methyl ester groups from hydrolysis by PME. Partly acetylated tri-, tetra-, penta- and hexa-oligogalacturonates present in fractions I-6, I-5, I-4 and I-7e, respec-

Table 2
Oligogalacturonates detected by ESI-IT-MS in the different fractions

Fractions	GalA* (%)	Acetyl* (%)	Oligogalacturonates detected by ESI-IT-MS $dp^{Me,\ Ac}$**	Molar mass (g/mol)
I-1	7.3	1.6	$1^{0,0}$	194
I-2	4.7	6.2		
			$3^{1,0}$	560
			$3^{1,1}$	602
			$3^{1,2}$	644
I-3	12.6	5.4		
			$2^{0,0}$	370
			$2^{0,1}$	412
I-4	5.7	7.8		
			$5^{2,1}$	968
			$5^{2,2}$	1010
I-5	18.4	20.4		
			$4^{1,1}$	778
			$4^{1,2}$	820
I-6	18.2	9.5		
			$3^{0,0}$	546
			$3^{0,1}$	588
I-7	26.1	36.4		
a	3.8	6.3	**$6^{2,2}$**; $6^{2,3}$	1186, 1228
b	3.7	4.9	**$5^{1,1}$**; **$5^{1,2}$**; $5^{1,3}$	954, 996, 1038
c	3.3	4.1	**$8^{3,3}$**; **$8^{3,4}$**; $4^{0,1}$	1594, 1636, 764
d	4.0	4.9	**$7^{2,2}$**; **$7^{2,3}$**; $7^{2,4}$; $7^{3,2}$; $7^{3,3}$	1362, 1404, 1446, 1376, 1418
e	6.7	10.0	$6^{1,1}$; **$6^{1,2}$**; $6^{1,3}$	1130, 1172, 1214
f	4.6	6.2	$8^{2,2}$; **$8^{2,3}$**; $8^{2,4}$; $10^{3,4}$; $10^{3,5}$	1538, 1580, 1622, 1988, 2030
I-8b	7.1	12.6	–	–

* Recovered GalA or acetyl/GalA or acetyl present in HGs.
** $dp^{Me,Ac}$, degree of polymerisation $^{\text{number of methyl groups,number of acetyl groups}}$ Major oligogalacturonates appear in bold.

tively, appeared as major hydrolysis products, representing altogether close to 50% of the acetyl groups present in HG domains. It is noteworthy that the elution on DEAE-Sepharose CL-6B was governed by the number of overall negative charges and the dp of the oligomers. Indeed, oligogalacturonates were eluted for increasing ionic strength for increasing number of overall negative charges and, for an identical number of charges, oligogalacturonates were eluted for increasing ionic strength for decreasing dp. The DAc does not affect the elution as shown by the recovery in a unique fraction of oligogalacturonates of a given dp and DM and of various DAc (example: $5^{1,1}$, $5^{1,2}$ and $5^{1,3}$ in fraction I-7b).

The main acetylated and/or methylated compounds were analysed by ESI-IT-MS^n in order to perform full structural assignment. The electrospray ionization followed by formation of fragment ions by collision-induced dissociation (CID) allows sensitive mapping and sequencing of oligosaccharides. Carbohydrates undergo two types of fragmentation: those of glycosidic cleavages and those of cross-ring fragmentation. For sequencing, glycosidic cleavages along the chain are major tools, especially after ^{18}O labelling of the reducing end that allows the differentiation of isobaric ions (Quéméner et al., 2003b). According to the cross-ring fragmentation, linkage and branching patterns can be established (Vakhrushev et al., 2004). In particular, we previously demonstrated that ^{0,2}A-type cross-ring cleavage ions are highly diagnostic ions allowing the precise location of acetyl groups on *O*-2 or *O*-3 of GalA residues (Quéméner et al., 2003a). MS^n experiments performed on one partly acetylated and methylated oligogalacturonate ($5^{2,1}$ arising from fraction I-4) after ^{18}O-labelling are described for clear illustration. The full structural characterisation of this compound required a purification step, that was achieved by HPLC on a C18 column.

After isolation and CID of the ^{18}O-labelled $[M^* - H]^-$ parent ion at *m/z* 969, C-ions at *m/z* 791 [C_4 + 2Me + 1Ac], 601 [C_3 + 1Me + 1Ac] and 383 [C_2 + 1Me] and their complementary ^{18}O-labelled Z*-ions at *m/z* 775 [Z^*_4 + 2Me + 1Ac], 585 [Z^*_3 + 1Me + 1Ac] and 367 [Z^*_2 + 1Me] were detected (Fig. 5A). Ions at *m/z* 907 (loss of labeled $C_2H_4O^{18}O$) and ions at *m/z* 407 [Z'_2 + 1Me + 1Ac], arising from subsequent fragmentation of the [C_3 + 1Me + 1Ac] ions at *m/z* 601, were also detected. This fragmentation pattern agrees with the presence of the following structure:

GalA-GalA(OMe)-AcGalA-GalA(OMe)-GalA.

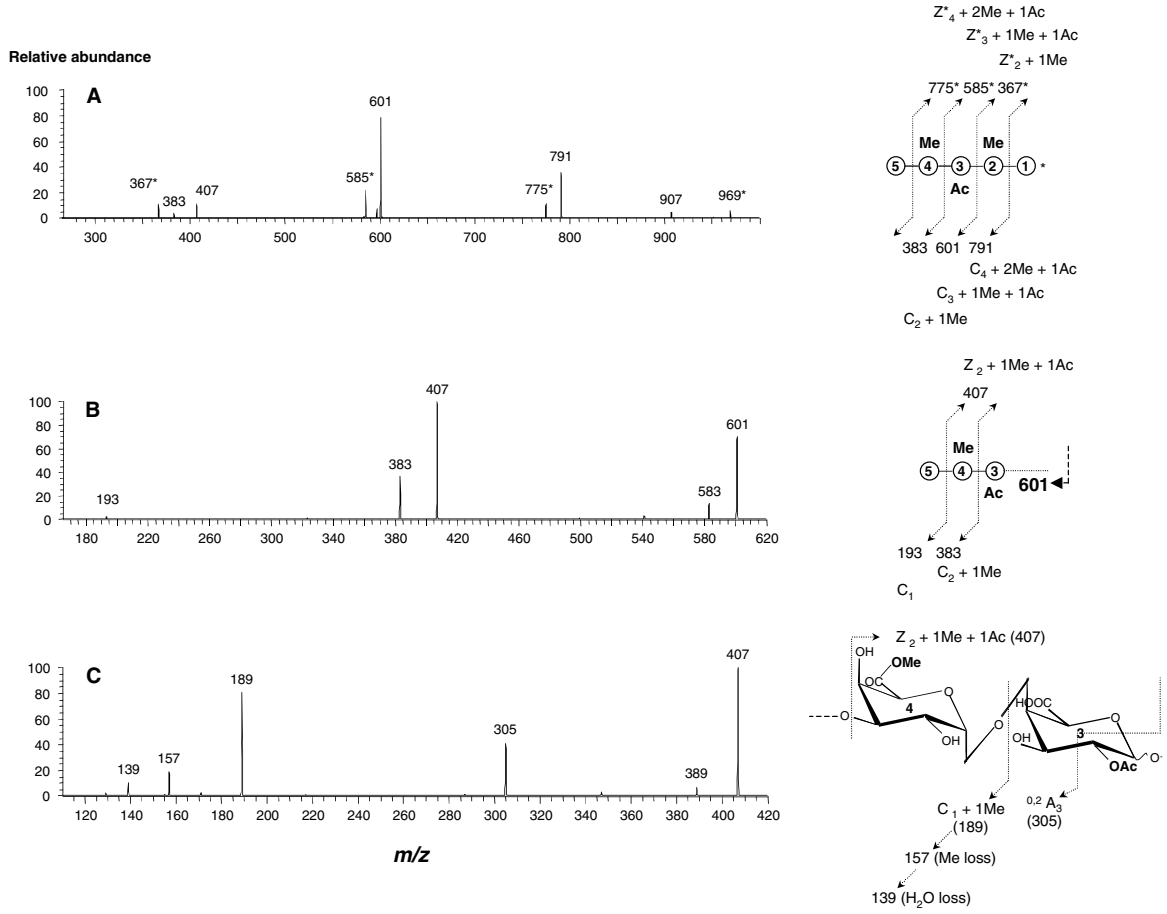


Fig. 5. Negative MS^n experiment spectra of ^{18}O-labelled $5^{2,1}$ (fraction I-4) and observed cleavages at each MS step. (A) MS^2 experiment (*m/z* 969* > products). (B) MS^3 experiment (*m/z* 969* > 601 > products). (C) MS^4 experiment (*m/z* 969* > 601 > 407 > products).

The MS^3 spectrum of the [C_3 + 1Me + 1Ac] glycosidic cleavage ion at *m/z* 601 (*m/z* 969* > 601 > products) (Fig. 5B) was dominated by the [Z'_2 + 1Me + 1Ac] ion at *m/z* 407 and the [C_2 + 1Me] ion at *m/z* 383, which is consistent with the proposed structure. The precise location of the acetyl group onto the third GalA residue was assessed by the fragmentation of the [Z'_2 + 1Me + 1Ac] glycosidic cleavage ion at *m/z* 407 in an MS^4 experiment (*m/z* 969* > 601 > 407 > products) (Fig. 5C). An $^{0,2}A_3$ cross-ring cleavage ion at *m/z* 305 (102 Da loss) was produced. It was demonstrated that the acetyl-ester bond was not broken during the CID process and that the *O*-2-linked acetyl group was eliminated with the $C_2H_4O_2$ neutral fragment leading to a loss of 102 Da (60 + 42) (Quéméner et al., 2003a). When the acetyl group was linked on *O*-3, the sole $C_2H_4O_2$ neutral fragment was eliminated leading to a loss of 60 Da (Quéméner et al., 2003a). The specific loss of 102 Da observed after CID of the [Z'_2 + 1Me + 1Ac] ion at *m/z* 407 is thereby consistent with the presence of an acetyl group located on *O*-2 of the GalA residue. A [C'_1 + 1Me] ion at *m/z* 189 was produced together with fragment ions at *m/z* 157 and 139 that may be attributed to successive methanol and water losses from that [C'_1 + 1Me] ion. The structure of $5^{2,1}$ was assigned to GalA-GalA(OMe)-2AcGalA-GalA(OMe)-GalA (Table 3, compound **4**).

The structure of the eight acetylated oligogalacturonates was established using the same experimental approach (Table 3). In some cases, several isomers were identified: $4^{2,2}$, for example, was present as three different isomers quoted **7a**, **7b** and **7c** (Table 3). The presence of multiple isomeric forms impeded full structural assignment for compounds present in fractions I-7a–I-7f. In these fractions, major compounds were submitted to MS^2 analysis that always revealed the presence of an unsubstituted reducing end and of a methyl-esterification of the second GalA residue (Table 3).

3. Discussion

Homogalacturonans, one of the major pectic domains, is an unbranched chain of (1 → 4)-linked α-D-

Table 3
Structural assignment of the major oligogalacturonates present in the different fractions

Fractions	Oligogalacturonates submitted to MS^n analyses $dp^{Me,Ac}$ [b]	Structure by MS^n***	Compound
I-1	$1^{0,0}$	–	–
I-2			
	$3^{1,0}$	GalA-GalA(OMe)-GalA	–
	$3^{1,1}$	3AcGalA-GalA(OMe)-GalA	1
	$3^{1,2}$	3AcGalA-3AcGalA(OMe)-GalA	2a
		3AcGalA-GalA(OMe)-3AcGalA	2b
I-3			
	$2^{0,0}$	–	–
	$2^{0,1}$	GalA-3AcGalA	3
I-4			
	$5^{2,1}$	GalA-GalA(OMe)-2AcGalA-GalA(OMe)-GalA	4
	$5^{2,2}$	GalA-2AcGalA-2AcGalA(OMe)-GalA(OMe)-GalA	5
I-5			
	$4^{1,1}$	GalA-2AcGalA-GalA(OMe)-GalA	6
	$4^{1,2}$	GalA-2AcGalA-3AcGalA(OMe)-GalA	7a
		3AcGalA-2AcGalA-GalA(OMe)-GalA	7b
		GalA-2AcGalA-GalA(OMe)-3AcGalA	7c
I-6			
	$3^{0,0}$	–	–
	$3^{0,1}$	GalA-3AcGalA-GalA	8a
		3AcGalA-GalA-GalA	8b
I-7			
a	$6^{2,2}$	?-GalA(OMe)-GalA	–
b	$5^{1,1}$; $5^{1,2}$	?-GalA(OMe)-GalA	–
c	$8^{3,3}$; $8^{3,4}$	?-GalA(OMe)-GalA	–
d	$7^{2,2}$; $7^{2,3}$	?-GalA(OMe)-GalA	–
e	$6^{1,2}$	?-GalA(OMe)-GalA	–
f	$8^{2,3}$	?-GalA(OMe)-GalA	–
I-8b	–	–	–

Several acetylated oligogalacturonates (quoted compound **1**–**8b**) were fully identified.
* Recovered GalA or acetyl/GalA or acetyl present in HGs.
** $dp^{Me,Ac}$, degree of polymerisation $^{\text{number of methyl groups, number of acetyl groups.}}$
*** Structures are written with the reducing terminus to the right; OMe = methyl ester; 2Ac = *O*-acetyl on the 2-position; 3Ac = *O*-acetyl on the 3-position.

Gal*p*A residues that are partially substituted with methyl and *O*-acetyl ester groups. Oligogalacturonates arising from HGs were readily separated from the remaining unaltered RGs-I by anion-exchange and size-exclusion chromatography. Around 90% of the total GalA present in the initial sugar beet pectin was recovered as HG-derived oligogalacturonates, in agreement with previous findings (Thibault et al., 1993; Bonnin et al., 2002). Moreover, ~75% of the acetyl groups present in the initial sugar beet pectin were attached to those HG-derived oligogalacturonates. Although RGs-I isolated in the present work carry only ~25% of the acetyl groups present in the initial sugar beet pectin, the DAc of this pectic region is particularly high (~60). It is noteworthy that no methyl-esterification was detected on these RGs-I. Indeed, Komalavilas and Mort (1989) and Perrone et al. (2002) reported that there is no evidence that the GalA residues in RGs-I are methyl-esterified. Another explanation might be that the f-PME used was able to remove methyl groups from RGs-I. Such a hypothesis was previously raised by Bonnin et al. (2002).

The quantitative recovery, with respect to both GalA and acetyl contents, of several HG-derived fractions, the sequencing of most of the compounds present in these fractions by ESI-IT-MS^n, and the known chain length of sugar beet pectin HG domains (dp ~ 80 GalA residues) (Thibault et al., 1993; Bonnin et al., 2002), allowed us to draw a quantitative representation of the HG-derived oligogalacturonates recovered after enzymatic (PG I + f-PME + side-chain degrading enzymes) hydrolysis of sugar beet pectin (Fig. 6A).

Among the multiple compounds identified, a tetragalacturonide containing one 2-*O*-acetylated GalA residue adjacent to which was a methyl-esterified GalA residue (compound **6**) appeared as a major product from sugar beet pectin. This oligosaccharide was also a major product from carrot and spinach pectin (Needs et al., 1998; Perrone et al., 2002). Similarly, a digalacturonide 3-*O*-acetylated on the reducing end, that was previously observed on spinach pectin (Perrone et al., 2002), was identified (compound **3**). Several representative new compounds were also revealed. Overall, it appears that:

(i) *O*-2- and *O*-3-acetylation are present in roughly similar amounts.
(ii) 2,3-di-*O*-acetylation is absent.
(iii) GalA residues that are at once *O*-acetyl- and methyl-esterified are rare; in that case, 2-*O*-acetylation (compound **5**) or 3-*O*-acetylation (compounds **2a** and **7a**) can be present.
(iv) Methyl-esterified residues downstream acetylated GalA residues are rare (compounds **2b**, **4**, **7c**).
(v) Methyl-esterified residues upstream 3-*O*-acetylated GalA residues are rare (compounds **1**, **2a**, **2b**).

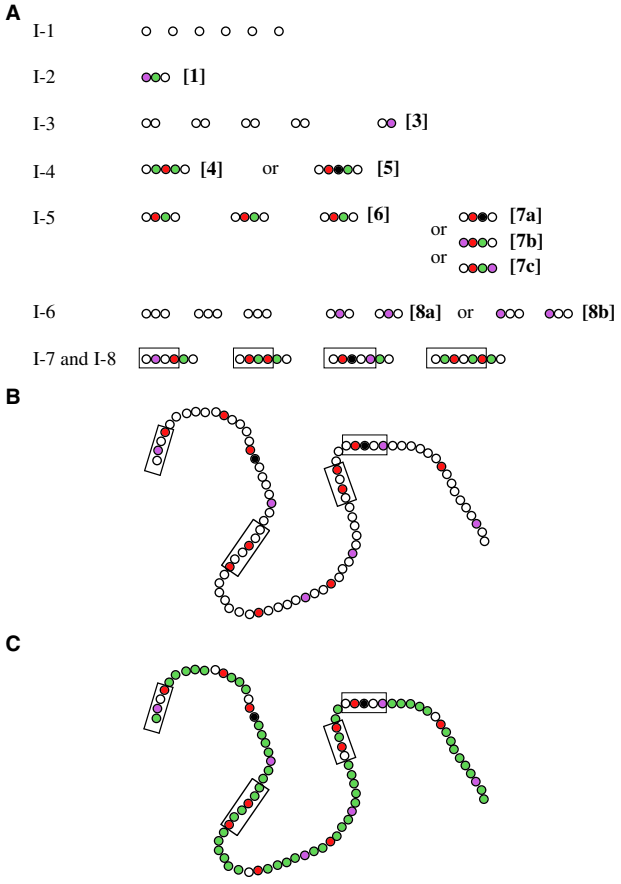


Fig. 6. Mapping sugar beet pectin acetylation and methylation patterns (A) Quantitative representation of the HG-derived oligogalacturonates recovered. (B) Reconstructed HG domain with proposed acetylation pattern. (C) Reconstructed HG domain with proposed acetylation and methylation patterns (○) unsubstituted GalA residue; (●) methylated GalA residue; (●) 2-*O*-acetylated GalA residue; (●) 3-*O*-acetylated GalA residue; (●) methylated and acetylated GalA residue.

(vi) Methyl-esterified residues upstream 2-*O*-acetylated GalA residues are frequent (compounds **4**, **5**, **6**, **7a**, **7b**, **7c**).
(vii) GalA residues present as reducing and non-reducing ends of the oligogalacturonates can be acetylated on *O*-3 (compounds **1**, **2a**, **2b**, **3**, **7b** and **7c**) but not on *O*-2, showing that the PG I used in this work is able to accommodate acetyl groups in its active site cleft on position (+1) or (−1) only when this acetyl group is located on *O*-3 of GalA residues.

Those findings, together with calculated average dp, DM and DAc, were taken into account for constructing putative structures for the oligogalacturonates present in fractions I-7 and I-8 (Fig. 6A).

When sugar beet pectins are submitted to blockwise demethylation with a plant-PME, the sequences of demethylated GalA residues generated are not long enough to induce abnormal polyelectrolyte behaviour or

to promote calcium-induced dimerisation of those pectins (Ralet et al., 2003). A random repartition of the acetyl groups is thus most probable. A hypothetical HG was created using the recovered oligogalacturonates as randomly distributed building blocks. Methyl-esterification was then discarded to give a clear overview of the acetylation pattern (Fig. 6B). *O*-2 acetylation was statistically slightly more frequent than *O*-3 acetylation and, as pointed out above, GalA residues that are at once methyl- and acetyl-esterified are rare. The use of f-PME has however to be kept in mind although f-PME is likely to be hindered by the presence of an acetyl group on a methyl-esterified GalA residue. It is noteworthy that 2,3-di-*O*-acetylation was not detected in any of the isolated HG-derived oligogalacturonates. Although the presence of 2,3-di-*O*-acetylation cannot be totally precluded in compounds arising from fractions I-7 and I-8, they are most probably very scarce if not absent in HG domains. Non-acetylesterified stretches ranging from 1 to 7 GalA residues were obtained.

Turning to the distribution of methyl esters within HG domains, a DM of 69% was calculated for the HG domains taking into account (i) the DM of the whole pectin sample (62%); (ii) the lack of methyl groups in the RG-I domain (Komalavilas and Mort, 1989; Perrone et al., 2002; this work), and (iii) the GalA repartition between HGs and RGs-I (90/10; Thibault et al., 1993; Bonnin et al., 2002; this work). Methyl groups were tentatively positioned considering the premise that an acetyl group would protect the neighbouring upstream and downstream methyl ester groups from hydrolysis by f-PME (Perrone et al., 2002). A maximum theoretical DM of 57% could be obtained, revealing that the above premise is most probably at least partly incorrect. We have shown that methyl-esterified residues downstream acetylated GalA residues and upstream 3-*O*-acetylated GalA residues were scarce, contrary to methyl-esterified residues upstream 2-*O*-acetylated GalA residues. This could mean that solely acetyl groups on *O*-2 could be able to protect a neighbouring upstream methyl group from hydrolysis by f-PME. The distribution of methyl esters was mapped taking this new premise into consideration and a DM of 71%, close to the expected DM of 69% was obtained (Fig. 6C). One can see that unsubstituted GalA residues are present in limited amounts (~10% of total GalA residues), due to the fact that methyl and acetyl groups are most often not carried by the same residues. This is in good agreement with the well-established statement that native sugar beet pectins are very poor substrates for *endo*-PGs.

In summary, this is, according to our knowledge, the first attempt to map concomitantly the distribution of methyl and acetyl esters within pectins. It has however to be kept in mind that a diverse range of esterification patterns can occur, with possible taxonomic or ontological variation (Perrone et al., 2002).

4. Experimental

4.1. Pectins

Sugar beet pectin was kindly supplied by Danisco (Brabrand, DK). It consisted of 508 mg/g of GalA. Arabinose was the main neutral sugar (121 mg/g), beside galactose (98 mg/g) and rhamnose (54 mg/g). Sugar beet pectin exhibited high DM (59.7) and DAc (33.5).

4.2. Oligogalacturonates standards

Polygalacturonic acid from orange (PGA) (purity 89%, dry matter basis) was purchased from Sigma (St. Louis, MO, USA). PGA (5.45 g) was suspended in ultrapure water (500 ml) and autohydrolysis was carried out under magnetic stirring for 48 h at 100 °C. After centrifugation, the supernatant was freeze-dried. Separation of oligogalacturonates (dp 1–12) was achieved by chromatography on combined Bio-Gel P-6 and P-4 columns mounted in series as previously described (Renard et al., 1995). Each oligogalacturonate was collected, brought to pH 5 and kept at −18 °C.

4.3. Enzymes

Recombinant fungal pectin methyl esterase (f-PME, EC 3.1.1.11, Swiss-Prot Q12535), *endo*-PG I (EC 3.2.1.15), *endo*-arabinanase (EC.3.2.1.99) and *endo*-galactanase (EC 3.2.1.89) were provided by Novozyme (Copenhagen, Denmark). The f-PME was cloned from *A. aculeatus* and expressed in *A. oryzae*. PG I (Swiss-Prot P26213) was purified in our laboratory from a crude preparation of a cloned *A. niger* (Sakamoto et al., 2003). *endo*-Arabinanase (EC.3.2.1.99) and *endo*-galactanase (EC 3.2.1.89) were purified from *A. niger* as described previously (Bonnin et al., 2002).

4.4. Enzymatic hydrolysis of sugar beet pectin

Sugar beet pectin was dissolved in 0.05 M succinate buffer pH 4.5 by overnight shaking at room temperature. This pectin solution was incubated with an enzymatic mixture containing f-PME, *endo*-galactanase, *endo*-arabinanase and PG I (25.7, 2.1, 2.1 and 2.1 nkat/ml of final reaction mixture, respectively). The reaction mixture (12 ml, 4.3 mg sugar beet pectin/ml) was incubated at 30 °C for 24 h.

4.5. Viscosity measurements

The viscosity of an aliquot (2 ml) of the reaction mixture was measured using an automated viscosimeter (AVS 310, Schott-Geräte, Germany) fitted with a Micro-Ubbelhode (Schott-Geräte, 538-10) capillary tube (diameter 0.4 mm). Time courses of relative ($\eta_{rel} = t/t_0$)

and specific ($\eta_{sp} = t - t_0/t_0$) viscosities were determined (t: flow time of the reaction mixture; t_0: flow time of buffer).

4.6. Low pressure chromatography

Anion exchange chromatography was performed at room temperature on DEAE-Sepharose CL 6B column (30 × 2.6 cm) equilibrated (i) with degassed 0.05 M Na-succinate buffer pH 4.5 or (ii) with degassed 0.075 M NaCl in 0.05 M succinate buffer pH 4.5, at a flow rate of 90 ml/h. Samples ((i) 10 ml of sugar beet pectin hydrolysate at 4.3 mg/ml or (ii) desalted fraction I-7) were loaded onto the column and the gel was washed with 400 ml of 0.05 M Na-succinate buffer containing (ii) or not (i) 0.075 M NaCl. The bound material was eluted with a linear NaCl gradient ((i) 0–0.5 M NaCl in 0.05 M Na-succinate buffer, 1600 ml or (ii) 0.075–0.175 M NaCl in 0.05 M succinate buffer, 1600 ml). Na-succinate buffer containing 0.5 M NaCl (400 ml) was then applied. Nine milli liter fractions were collected and analysed for their content in GalA and neutral sugars (Thibault, 1979; Tollier and Robin, 1979). Appropriate fractions were combined, concentrated by in vacuum rotary evaporation at 40 °C and desalted using a column (1.6 × 100 cm) of Sephadex G-10 at 1 ml/min eluted by deionised water.

Size exclusion chromatography was performed at room temperature on Sephacryl S-200 column (60 × 1.6 cm) equilibrated with degassed 0.05 M Na-succinate buffer pH 4.5. The sample (desalted fraction I-8; 15 ml) was loaded and eluted at a flow rate of 25 ml/h; 1.5 ml fractions were collected and analysed for their content in GalA and neutral sugars. Appropriate fractions were combined, concentrated and desalted as already described.

4.7. High performance anion exchange chromatography

HPAEC was performed using a Waters (Milford, MA, USA) 626 pump equipped with a Waters 600S controller and an autosampler Waters 717 plus. Oligomers were monitored using a pulse amperometric detector (PAD) (EC 2000, Thermo Separation Products). The mobile phases were all degassed with helium in order to prevent absorption of carbon dioxide and transformation to carbonate. Borwin software (JMBS Developments, Grenoble, France) was used for data acquisition and processing.

The purified oligogalacturonates produced from PGA autohydrolysis were individually applied (20 μl) on an analytical Carbopac PA-1 column (4 × 250 mm) equipped with a Carbopac PA-1 guard column (4 × 50 mm) at 1 ml/min (pH 13). The elution was carried out with 500 mM Na-acetate containing 100 mM NaOH (0–20 min), followed by two linear gradient phases of 500–600 mM Na-acetate in 100 mM NaOH (20–40 min) and 600–700 mM Na-acetate in 100 mM NaOH (40–60 min). The column was reconditioned by washing with 1 M Na-acetate containing 100 mM NaOH and then re-equilibrated with the starting buffer. A Carbohydrate Membrane Desalter (Dionex) was placed just after the PAD instrument and before collection (Quéméner et al., 2003b). The GalA content of purified desalted oligogalacturonates was determined colorimetrically (Thibault, 1979) and each oligogalacturonate solution was immediately reinjected onto the HPAEC system to allow peak area measurement. Response factors were thereby calculated for oligogalacturonates from dp 1–12.

Appropriate pools from anion-exchange and size-exclusion chromatography were filtered (0.45 μm) and analysed by HPAEC at pH 13 under the conditions explained above. Previously calculated response factors were used to quantify oligogalacturonates in the different pools.

4.8. Reversed phase HPLC separation of partially methylated and acetylated oligogalacturonates present in fraction I-4

Partially methylated and acetylated oligogalacturonates present in fraction I-4 were separated by HLPC on a C18 column (Superspher 100 RP-18 end capped, (250 × 4 mm) Merck) with a guard column (LiChroCART, 4 × 4 mm) and 100 mM acetic acid (pH 2.5) as eluent at a flow rate of 0.45 ml/min at 7 °C. The effluent was monitored with a refractive index detector.

4.9. Determination of the DM and DAc

For polymeric substrates, methanol and acetic acid were released by alkaline deesterification in presence of $CuSO_4$ and quantified by HLPC on a C18 column as previously published (Levigne et al., 2002). Isopropanol was used as internal standard. DM and DAc were calculated as the molar ratio of methanol and acetic acid to GalA, respectively.

The presence of oligogalacturonates of low dp in some samples impeded the determination of acetic acid by the method described above. In these cases, acetic acid was quantified by the enzymatic method of Boehringer Mannheim/R-Biopharm. When samples of polysaccharides were analysed by both methods, no significant differences were found between DAc values.

4.10. Analytical methods

Reducing ends were quantified by the Nelson's method using GalA as standard (Nelson, 1944). GalA and neutral sugars (expressed arbitrarily as Ara) contents were determined by the automated *m*-hydroxybiphenyl method

(Thibault, 1979) and orcinol methods (Tollier and Robin, 1979), respectively, the latter being corrected for interfering GalA. GalA was quantified after saponification of the pectin samples (0.05 M NaOH, 30 min) and neutralisation (0.05 M HCl). Individual neutral sugars were quantified after hydrolysis by 2 M trifluoroacetic acid at 120 °C for 2 h, derivatisation in their alditol acetates (Blakeney et al., 1983) and analysis by gas chromatography on a DB-225 fused-silica capillary column (J&W Scientific, Courtaboeuf, France; 30 m × 0.32 mm i.d.) mounted in a DI 200 chromatograph (Delsi Nermag Instruments, Argenteuil, France).

4.11. Electrospray mass spectrometry analysis

ESI-IT-MS experiments were achieved on a LCQ Advantage ion trap mass spectrometer (ThermoFinningan, USA) using negative electrospray as the ionization process. The reducing end of the purified and desalted oligogalacturonates was ^{18}O-labelled by adding about 30 µl of $H_2{}^{18}O$ and 0.1 µl of pure formic acid to 20–30 µg of freeze dried oligomer. The sample was incubated with $H_2{}^{18}O$ for at least 72 h at ambient temperature in a dessicator. Sample solutions (30 µl) were diluted 4 times by H_2O and 120 µl of pure methanol were added to favor the spray formation into the electrospray source. Infusion was performed at a flow-rate of 2.5 µl/min. Nitrogen was used as sheath gas (20 arbitrary units). As the exchange kinetic is low, no significant back-exchange ^{16}O was observed during the analysis duration (about 30 min). The MS analysis were carried out under automatic gain control conditions, using a typical needle voltage of 4.2 kV and a heated capillary temperature of 200 °C. For MS^n experiments, the various parameters (collision energy, activation qz, activation time) were adjusted for each sample in order to optimise the signal and get maximal structural information from the ion of interest. A total of 30–50 scans were summed for spectra acquisition.

4.12. Sample handling

A lot of precaution was taken to avoid acyl migration. Fractions were stabilised by adding ethanol (final concentration 20% v/v) and kept at 4 °C. When freeze-drying was necessary (^{18}O-labelling), the MS-fragmentation spectra of freeze-dried samples were compared to "never frozen-samples" spectra. No acyl migration was detected.

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