JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. **33**, 713–720 (1998)

Rapid Molecular Mass and Structural Determination of Plant Cell Wall-derived Oligosaccharides Using Off-line High-performance Anion-exchange Chromatography/Mass Spectrometry

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A method has been developed for the rapid molecular mass determination and structural elucidation of mixtures of oligosaccharides derived from plant cell walls. The oligosaccharides were fractionated using gel permeation chromatography and 'analytical' high-performance anion-exchange chromatography (HPAEC), neutralized, dried and the mixtures of eluent salt and oligosaccharides were per-*O*-acetylated directly. The derivatized oligosaccharides were isolated by dissolution in dichloromethane and the salts were removed by aqueous partitioning. The per-*O*-acetylated oligosaccharides were analysed using electrospray (ES) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MS). Exploiting the fact that acid-catalysed per-*O*-acetylation of oligosaccharides can be achieved even under the extremely salty conditions that are found in post-column neutralized HPAEC fractions, and combining this derivatization step with off-line ESMS, allow rapid screening for molecular mass and thus yield information on the composition of the various oligosaccharides in these complex mixtures. Subsequent per-*O*-methylation of the per-*O*-acetylated, salt-free fractions and collision-induced dissociation tandem mass spectrometric analysis was used for additional sequence and branching determination of the oligosaccharides.

KEYWORDS: high-performance anion-exchange chromatography/mass spectrometry; electrospray; oligosaccharides; derivatization

INTRODUCTION

The study of complex plant cell wall polysaccharides requires complex fractionation protocols and enzymatic or chemical degradation of the polysaccharides to obtain workable sized oligosaccharides. The resulting mixtures of oligosaccharides may be fractionated using high-performance anion-exchange chromatography (HPAEC) although the elution behaviour of the various types of oligosaccharides is unpredictable. This results in very complex chromatograms with partially unresolved peaks.[1]

The rapid determination of the molecular masses of the oligosaccharides present in each HPAEC fraction is of importance in screening the products and in identifying those fractions that contain oligosaccharides of interest for detailed structural analysis using nuclear magnetic resonance spectroscopy and fast atom bombardment (FAB) or electrospray (ES) tandem mass spectrometry (MS/MS). Recently, reports describing methods for on-line HPAEC/MS[2–4] have been appearing in the literature, but these approaches are not applicable to routine HPAEC/MS analyses. Since HPAEC fractions contain large amounts of sodium acetate (typically 0.6–0.8 M) owing to the sodium hydroxide- and acetate-containing eluent that is used and the post-column neutralization with acetic acid, oligosaccharides in lyophilized HPAEC fractions are not amenable to direct MS analysis since the salt suppresses the ionization of the oligosaccharides and blocks the capillary of the ESMS system.

Consequently, we have established a protocol for the rapid off-line determination of the molecular masses of

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Contract/grant sponsor: Dutch Technology Foundation (NWO/STW).
Contract/grant sponsor: Gistbrocades.
Contract/grant sponsor: Product Board for Feeding Stuffs (VVR).

CCC 1076–5174/98/080713–08 $17.50

Received 17 March 1998
Accepted 26 April 1998

oligosaccharides in HPAEC profiles using ESMS and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) following acid-catalysed derivatization.

Mixtures of oligosaccharides resulting from the degradation of polysaccharides are first fractionated using gel-permeation chromatography (Bio-Gel P-2) and then each fraction, consisting of oligosaccharides of essentially the same degree of polymerization (d.p.), is subjected to HPAEC. Since acid-catalysed per-*O*-acetylation[5] of oligosaccharides can be achieved even in the presence of large amounts of salt,[6] it can therefore be used as a 'desalting' device since the derivatized oligosaccharides can be extracted into dichloromethane while the salts are removed by partitioning with water.[7] Another benefit of per-*O*-acetylation of oligosaccharides is the improved MS sensitivity and the direction of MS fragmentation[8,9] of oligosaccharides so that unambiguous structural data are obtained. We demonstrate here that acid-catalysed per-*O*-acetylation of oligosaccharides can be achieved even under the extremely salty conditions that are found in post-column neutralized HPAEC fractions, and that it yields derivatized oligosaccharides that can easily be analysed using MS. Per-*O*-methylation of the per-*O*-acetylated oligosaccharides[6] prior to structural analysis using FAB-MS/MS was used to improve the intensity of the precursor ions in these experiments.

EXPERIMENTAL

Cell wall pectic polysaccharides from soybean meal were obtained by extraction with chelating CDTA as described,[10] yielding a fraction which represents a pectic polymer, especially rich in galacturonic acid, galactose and arabinose (ChSS[11]). A solution of this extract (4%) was incubated with endogalactanase, exogalactanase, endoarabinase and arabinofuranosidase B[12] for 48 h and the oligosaccharide-containing supernatant of this digest was fractionated on a Sephacryl S-100 HR column. The arabinogalactan oligomers were pooled, concentrated and fractionated further on a Bio-Gel P-2 column at 60 °C isothermal with an eluent flow-rate of 0.5 ml min^{-1}. The eluate was monitored using a Shodex RI-72 refractive index detector. Fractions of 7.5 ml were collected and appropriate fractions were pooled and freeze-dried.

Approximately 16 mg of oligosaccharides of essentially d.p. 4 were separated, following the above enzymatic treatment, using a Dionex HPAEC system with a PA-100 column (250 × 22 mm i.d.) and pulsed amperometric detection, applying a 20 min gradient of 0–200 mM sodium acetate in 100 mM sodium hydroxide with a flow-rate of 25 ml min^{-1}. The fractions were neutralized using automated post-column addition of acetic acid.[13] Approximately 5% of the total lyophilized HPAEC fraction (salt and oligosaccharide) was used for further analysis using MS.

Per-*O*-acetylation of the lyophilized oligosaccharides was performed under conditions of acid catalysis in 350 μl of a 2:1 (v/v) mixture of trifluoroacetic anhydride and glacial acetic acid for 20 min at room temperature,[7] then the samples were dried under vacuum. The derivatized oligosaccharides were dissolved in dichloromethane and the salts were partitioned into water, the extraction being repeated four times.

The derivatized oligosaccharides were redissolved in 100 μl of methanol–water (50:50, v/v, containing 1% HCOOH). Positive mode electrospray mass spectra were obtained on a VG Platform II single-quadrupole mass spectrometer. Aliquots of 10 μl of the sample solutions were infused into a mobile phase of methanol–water (50:50, v/v, containing 1% HCOOH) and introduced into the electrospray source at a flow-rate of 5 μl min^{-1}. Spectra were scanned at a speed of 8 s for m/z 200–2000 with a cone voltage of 95 V and recorded and processed using MassLynx software, version 2.0. Mass calibration was performed by multiple-ion monitoring of per-*O*-acetylated maltooligosaccharides derived from corn syrup.

MALDI/MS experiments were performed using a Micromass TofSpec-2E mass spectrometer in the linear mode. A nitrogen laser with an emission wavelength of 337 nm and a 4 ns pulse duration was used. Spectra were recorded in the positive ion mode. Ions were accelerate to an energy of 30 keV before entering the flight tube. The samples for MALDI/TOF analysis were prepared by mixing directly on the target 1 μl of the oligosaccharide solution (10-fold more concentrated than that used for ESMS) and 1 μl of a 10% aqueous 2,5-dihydroxybenzoic acid (DHB) matrix solution. The samples were allowed to dry at room temperature for 15 min.

Per-*O*-methylation[14] of the per-*O*-acetylated oligosaccharides was performed by adding freshly ground sodium hydroxide to the per-*O*-acetylated fractions dissolved in 200 μl of dimethyl sulfoxide; aliquots of 250 μl of methyl iodide were added after 0, 10 and 30 min. The reaction was stopped 20 min after the final addition of methyl iodide by adding 1 ml of sodium thiosulfate solution (100 mg ml^{-1}) and 1 ml of chloroform. The chloroform layer was washed six times with water, after which the organic layer was evaporated to dryness under nitrogen.

The monosaccharide sequence and the branching pattern of the oligosaccharides were determined using positive mode collision-induced dissociation (CID) FAB-MS/MS of the per-*O*-methylated oligosaccharides. FAB-MS/MS was carried out using a JEOL (Tokyo, Japan) JMS-SX/SX102A tandem mass spectrometer (BEBE geometry) at a 10 kV accelerating voltage. A xenon beam of about 4 kV translational energy (gun current 10 mA) was used for FAB ionization. CID tandem mass spectra were obtained by using the collision cell in the third field-free region (FFR) of the mass spectrometer with air as collision gas at a pressure sufficient to reduce the intensity of the selected ion beam by 50%. As the collision cell was held at ground potential, the collision energy in the MS/MS experiments was 10 keV. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged using a Hewlett-Packard HP9000 data system running JEOL COMPLEMENT software. Samples were dissolved in 10 μl of methanol and about 1 μl of the solution was mixed with 0.8 μl of the thioglycerol matrix on the probe tip.

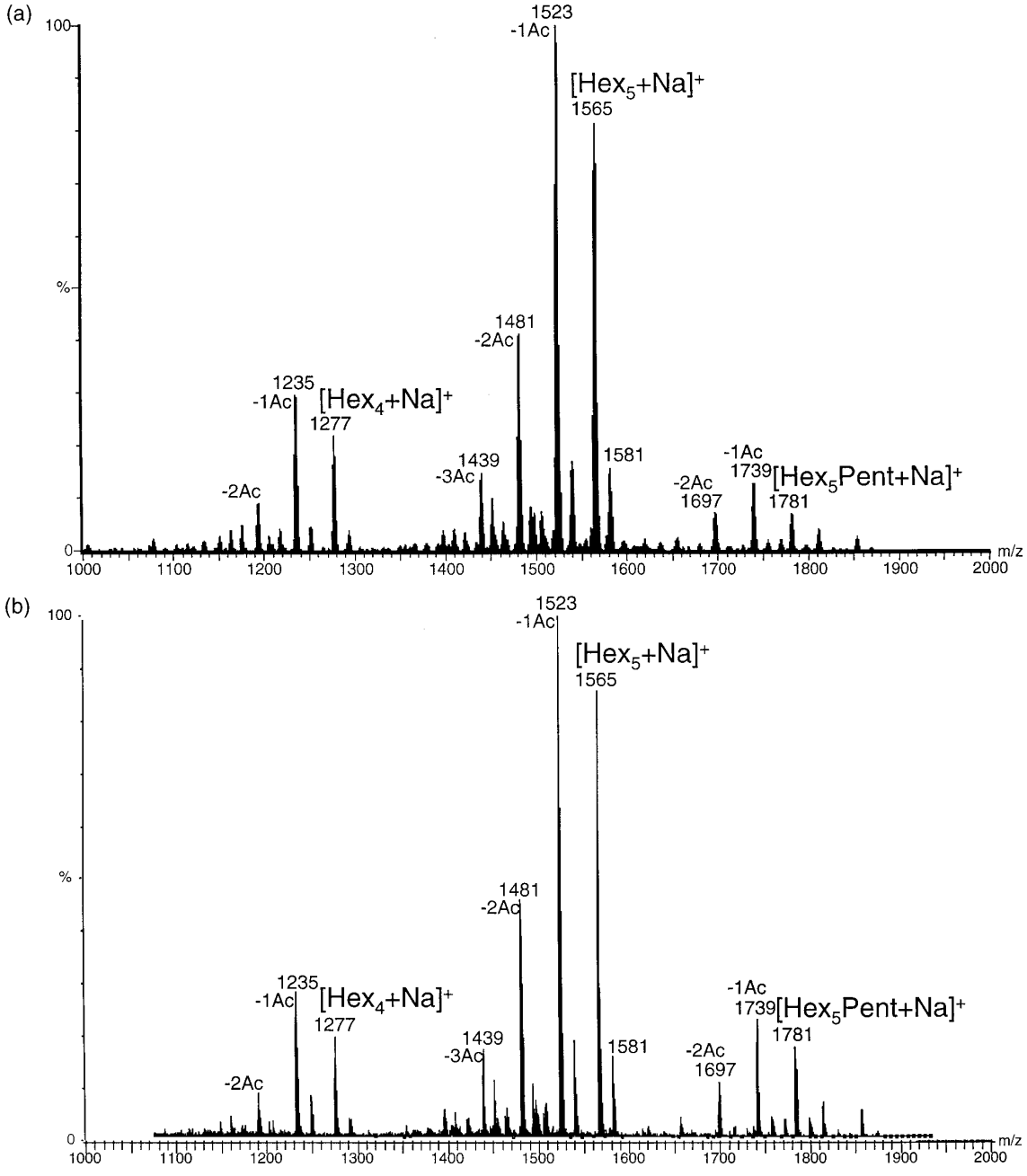

Figure 1. (a) Positive ES mass spectrum of per-*O*-acetylated oligosaccharides in an HPAEC peak obtained on fractionation of arabinogalactan-derived oligosaccharides of essentially d.p. 5. (b) Positive MALDI/TOF mass spectrum of per-*O*-acetylated oligosaccharides in an HPAEC fraction obtained on fractionation of arabinogalactan-derived oligosaccharides of essentially d.p. 5.

Linkage analysis was performed following hydrolysis, reduction and *O*-acetylation of the per-*O*-methylated oligosaccharides.[15] GC/MS analyses were performed using a Fisons MD800 mass spectrometer fitted with a Carlo Erba GC8060 gas chromatograph and an on-column injector and using helium as the carrier gas. Monosaccharide derivatives were separated on a DB-5MS column (30 m × 0.32 mm i.d.) (J&W Scientific). Partially methylated alditol acetates were injected in solution in dichloromethane (1 µl injected) and separated using the following temperature programme: 50 °C for 2 min, then ramped at 40 °C min^{-1} to 130 °C, held for 2 min, then ramped at 4 °C min^{-1} to 230 °C, held for 15 min. Mass spectra were recorded under electron impact conditions in the positive ion mode with an electron energy of 70 eV and were recorded using linear scanning from *m/z* 55 to 400 over 0.9 s.

RESULTS AND DISCUSSION

Lyophilized HPAEC fractions contain relatively large amounts of sodium acetate owing to the sodium hydroxide-containing eluent that is used and its post-column neutralization with acetic acid. Therefore, salt tolerant per-*O*-acetylation[6] was used as a means of removing the salts from the samples prior to MS analysis. In spite of the fact that this method of per-*O*-

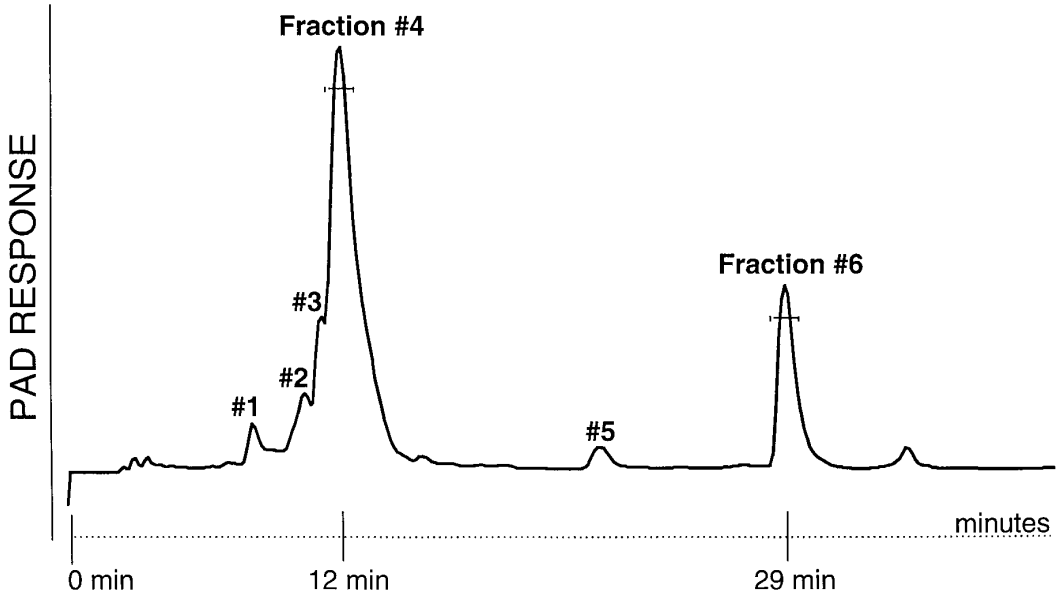

Figure 2. HPAEC profile of a mixture of arabinogalactooligosaccharides with a d.p. of essentially 4. Bars on the peaks indicate pooling.

Table 1. Composition of per-*O*-acetylated oligosaccharides determined using positive ESMS

Per-*O*-acetylated HPAEC fraction No.	*m/z* $[M + Na]^+$	Composition assigned
1	989	Hex_3
2	1205	Hex_3Pent_1
3	1277	Hex_4
4	1277	Hex_4
5	773	$Pent_3$
6	773	$Pent_3$
	989	$Pent_4$

acetylation is described as being salt tolerant, when applied to the extremely salty HPAEC-derived fractions, the oligosaccharides were not fully per-*O*-acetylated. This could be deduced from mass spectra obtained after the first derivatisation (data not shown), where a prominent series of ions corresponding to multiply underacetylated oligosaccharides were observed. Therefore, the per-*O*-acetylation may be readily and rapidly performed a second time following the first partitioning with water, to reduce the extent of the underacetylation of the oligosaccharides.

MALDI/TOF-MS and ESMS were used to determine the molecular masses of a series of oligosac-

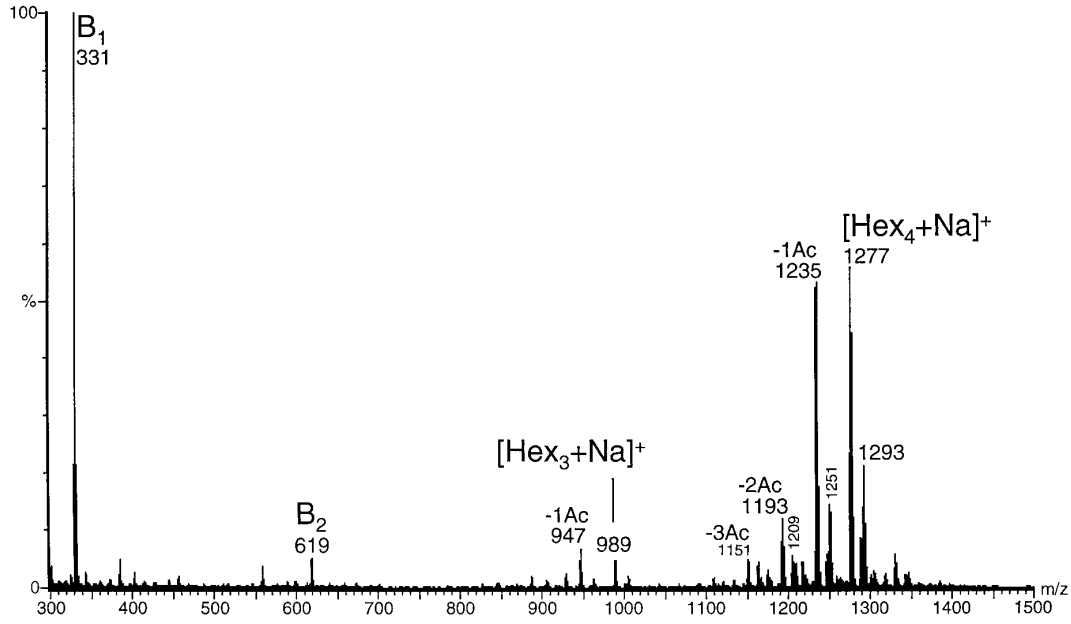

Figure 3. Positive ES mass spectrum of per-*O*-acetylated oligosaccharides in HPAEC fraction 4 originating from a mixture of arabinogalactan-derived oligosaccharides of essentially d.p. 4.

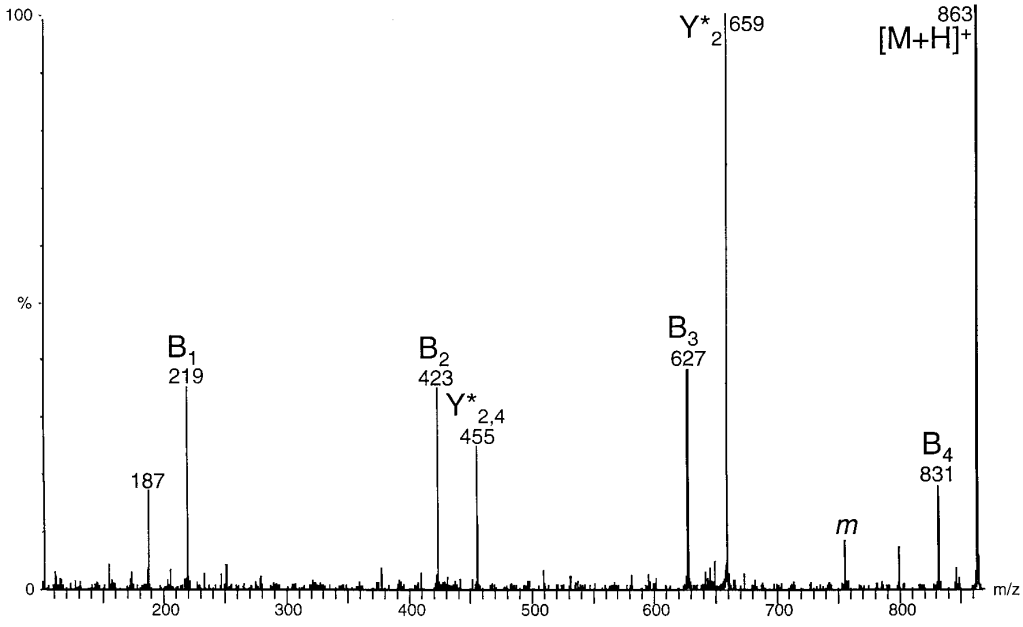

Figure 4. Positive FAB CID tandem mass spectrum of per-*O*-methylated Hex_4 $[M+H]^+$ at *m/z* 863, from HPAEC fraction 4 (*m* is matrix ion).

charides that were derived from plant cell walls following enzymatic degradation, Sephacryl S-100 and Bio-Gel P-2 fractionation, HPAEC separation and per-*O*-acetylation. MALDI/TOF-MS and ESMS analyses of the per-*O*-acetylated oligosaccharides obtained following HPAEC fractionation of fragments of essentially d.p. 5 gave very similar spectra [Fig. 1(a) and (b)]. Sample handling for ESMS only requires the derivatized oligosaccharides to be redissolved, whereas the samples for MALDI/TOF-MS require co-crystallization with the matrix, followed by drying. No clear MS sensitivity advantage in using MALDI/TOF-MS over ESMS for these samples is evident and therefore we chose to analyse per-*O*-acetylated oligosaccharides originating from HPAEC fractionation routinely using ESMS.

Fractions 1–6, obtained as HPAEC fractions from a mixture of oligosaccharides essentially with a d.p. of 4 (Fig. 2), were analysed following per-*O*-acetylation and gave good quality positive ES mass spectra. This molecular mass screening gave clear results, from which the compositions in terms of monosaccharide types of the oligosaccharides can be assigned (Table 1).

Fractions 4 and 6 represent the major peaks in the HPAEC trace (Fig. 2). In the positive ES mass spectrum obtained from per-*O*-acetylated fraction 4 (Fig. 3), the ion at *m/z* 1277 represents the sodium-cationized molecule for fully per-*O*-acetylated Hex_4. The derivatization of the oligosaccharides is, however, not complete and a series of underacetylated sodium-cationized molecules are observed at *m/z* 1235, 1193 and 1151. Analogously, a series of ions are present for the potassium-cationized species at *m/z* 1293, 1251 and 1209. In spite of the fact that acetylation is not complete, the molecular mass determination and thus composition assignment for the oligosaccharides in HPAEC fraction 4 are unambiguous. This assignment is further supported by the presence of B_1 and B_2 ions[16] at *m/z* 331 and 619 corresponding to Hex_1^+ and Hex_2^+, respectively. Additional, low-abundance sodium-cationized molecules at *m/z* 989 and 947 are indicative for per-*O*-acetylated and monounderacetylated Hex_3. Since *m/z* 989 in the mass spectrum of fraction 4 does not correspond to a fragment ion but to a fully per-*O*-acetylated trisaccharide, we assume that this ion is not the product of mass spectrometric fragmentation. The origin of the small amount of Hex_3 in this fraction is probably hydrolysis during sample handling as Hex_3 is not expected to co-elute with Hex_4 under the HPAEC conditions used.

In the spectrum of per-*O*-acetylated HPAEC fraction 6 (data not shown), a series of ions corresponding to the sodium-cationized molecules for fully and underacetylated $Pent_4$ is observed (*m/z* 989, 947 and 905). However, $Pent_4$ is not the only component present in fraction 6 since a sodium-cationized molecule for $Pent_3$ is also observed (*m/z* 773). The fact that $Pent_3$ is present in HPAEC fraction 6 is probably due to partial hydrolytic breakdown of the $Pent_4$ component during sample handling after fractionation. Since the polysaccharide from which the oligosaccharides were generated was arabinogalactan derived from soy bean plant cell walls by the action of endogalactanase, exogalactanase, endoarabinase and arabinofuranosidase B, the pentoses are presumably arabinoses in the furanose form, while the hexoses are expected to be galactopyranose.[17] Partial hydrolysis of oligopentofuranoses is observed to a greater extent than the analogous breakdown of oligohexopyranoses since the glycosidic linkage of a furanose is more labile[18] than that of a pyranose. This lability means that breakdown of a tetrasaccharide of arabinofuranose residues after HPAEC separation is not surprising.

In order to determine the monosaccharide sequence and branching patterns of the oligosaccharides, fragmentation information is necessary. Fragmentation was

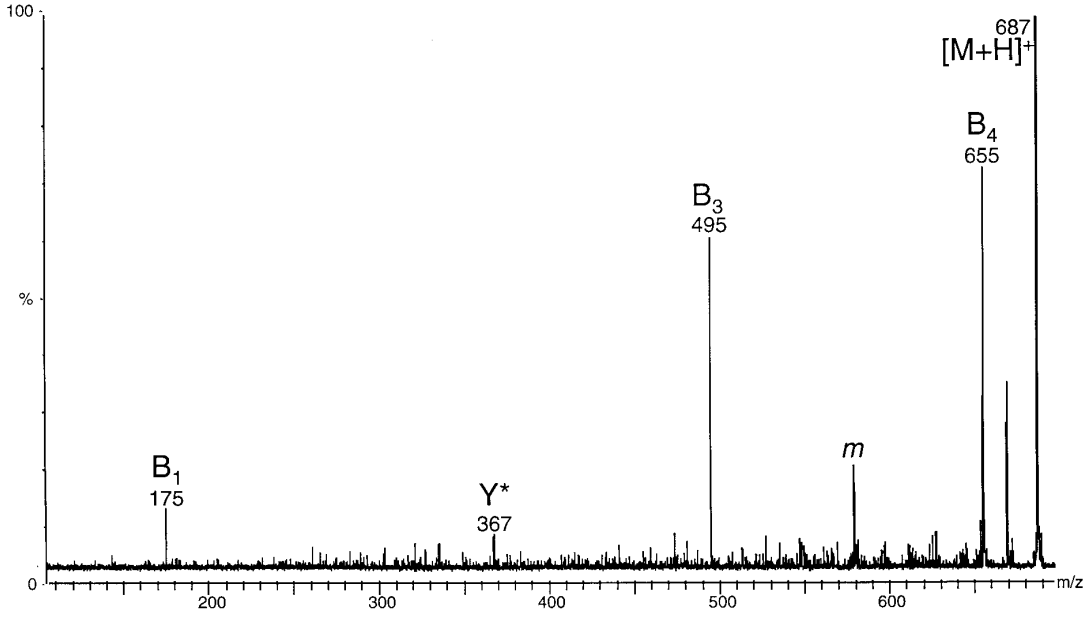

Figure 5. Positive FAB CID tandem mass spectrum of per-*O*-methylated $Pent_4$ $[M + H]^+$ at *m/z* 687, from HPAEC fraction 6 (*m* is matrix ion.)

induced following FAB ionization using CID MS/MS. However, since FAB-MS is about 10 times less sensitive than ESMS,[19] and since the ES mass spectra of HPAEC fractions 4 and 6 show that after per-*O*-acetylation the samples are heterogeneous due to the presence of both completely and partially underacetylated species, the heterogeneity in the samples caused by the underacetylation needs to be reduced. Per-*O*-methylation of the *O*-acetylated oligosaccharides[6] resulted in completely methylated oligosaccharides so that all of the ion abundance is to be found in the fully methylated pseudomolecular ion, thereby improving the relative ion abundance of the pseudomolecular ion. Per-*O*-methylation directly following HPAEC separation is not possible, since methylation is not tolerant to sample contaminants such as salts.[20]

To elucidate the branching pattern of the oligosaccharides, the protonated molecule for completely per-*O*-

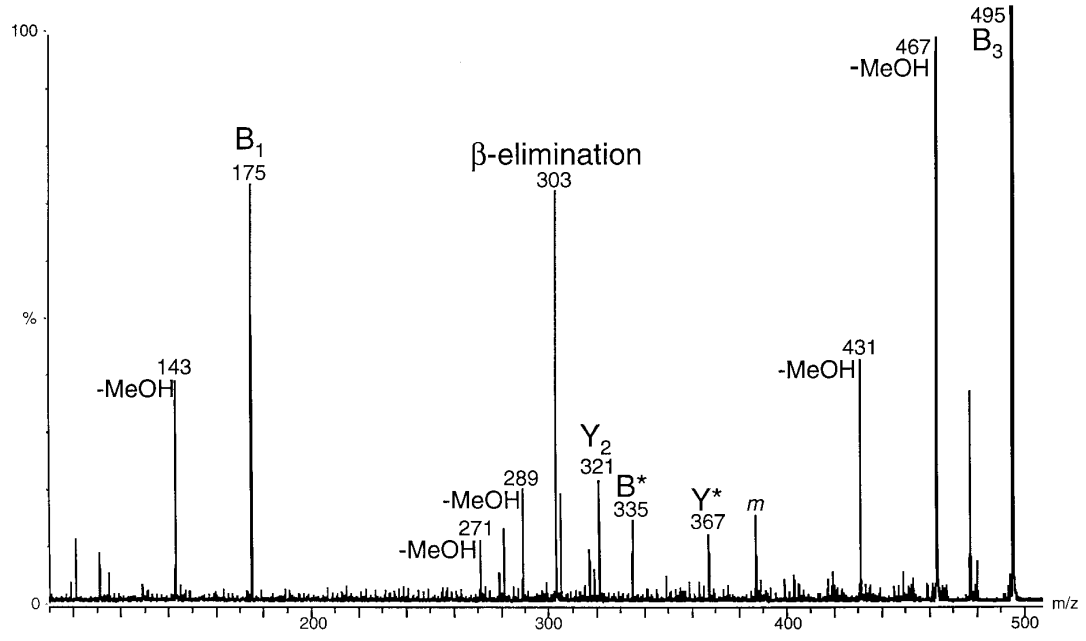

Figure 6. Positive FAB CID tandem mass spectrum of per-*O*-methylated $Pent_4$ B_3 ion *m/z* 495, from HPAEC fraction 6 (*m* is matrix ion.)

methylated Hex_4 (m/z 863) in HPAEC fraction 4 was selected as the precursor. A complete series of B_n-type ions is present (m/z 219, 423, 627 and 831; Fig. 4), consistent with a linear Hex_4 oligosaccharide (see structure **I**, Fig. 7). The ions labelled Y* are the result of 'internal residue loss'[21,22] from $[M + H]^+$.

The FAB tandem mass spectrum (Fig. 5) obtained from the protonated molecule for completely per-*O*-methylated $Pent_4$ in HPAEC fraction 6 (m/z 687) contains B- and Y*-type ions. The relatively high abundances of B_1 (m/z 175), B_3 (m/z 495) and B_4 (m/z 655) ions in the spectrum, together with the absence of a B_2 ion (m/z 335), are indicative of a branched oligosaccharide (see structures **II** and/or **III**, Fig. 7.).

In the FAB mass spectrum of the per-*O*-methylated $Pent_4$ species (data not shown), and also in its CID mass spectrum (Fig. 5), the B_3 ion (m/z 495) has a relatively high abundance. Selecting the B_3 ion (m/z 495) as the parent ion for a FAB CID MS/MS experiment resulted in the spectrum shown in Fig. 6. In addition to an abundant B_1 ion (m/z 175), a very abundant ion is observed at m/z 303. The ion arises by β-elimination[8,9] of the branching residue (labelled D in Fig. 7) and is consistent with the $Pent_4$ oligosaccharide having a branched structure (either 2- or 3-linked). The minor ion at m/z 335 (Fig. 6) we assign as resulting from 'internal residue loss.' The process resulting in the ion at m/z 335 involves loss of either residue A or residue D together with transfer of a methyl group (i.e. to yield B–D or A–B) resulting in a B-type ion that we therefore assign as B*. The ion at m/z 321 arises by β-cleavage of a pentose from m/z 495 and thus corresponds to a Y-type fragmentation. The ion at m/z 367 (Fig. 6) is another example of an ion arising by 'internal residue loss' and corresponds to a completely per-*O*-methylated protonated $Pent_2$ species. Selecting B_3 as precursor ion, this m/z 367 ion can only be produced by the mass spectrometric loss of residue B to yield a fully per-*O*-methylated $Pent_2$ which is composed of residues A and D, probably forming a $1 \rightarrow 1$ linkage.[23]

Linkage analysis was carried out following hydrolysis, reduction and peracetylation of the per-*O*-methylated fractions. The resulting partially methylated alditol acetates were analysed and identified using GC/MS. In HPAEC fraction 4, derivatives corresponding to terminal Hex and 4-substituted Hex were identified, consistent with the linear Hex_4 assigned. In HPAEC fraction 6, derivatives for terminal Pent, 5-substituted Pent and two branched residues (2,5- and 3,5-substituted) were observed, again consistent with the presence of the two branched tetrasaccharides assigned (Fig. 7).

CONCLUSIONS

A rapid molecular mass determination of oligosaccharides after HPAEC fractionation using ESMS has been described that exploits per-*O*-acetylation as a 'desalting' device. Per-*O*-acetylation of oligosaccharides permits the extraction of the derivatized oligosaccharides into dichloromethane, while the salts can be removed by partitioning with water. With this derivatization, time-consuming, wasteful and strongly acidic desalting steps are avoided, with the intention of keeping degradation of the oligosaccharides to a minimum. A further advantage of this approach is the appropriateness of the derivatized samples for rapid MS analysis using ES. Fractions requiring further structural elucidation may be per-*O*-methylated for monosaccharide sequence and branching pattern using MS/MS. The derivatives produced are also useful for further MS and chemical analyses and so our approach dovetails nicely with existing laboratory strategies for oligosaccharide analysis.

Acknowledgements

This research was supported by the Dutch Technology Foundation (NWO/STW), Gistbrocades and the Product Board for Feeding Stuffs (VVR).

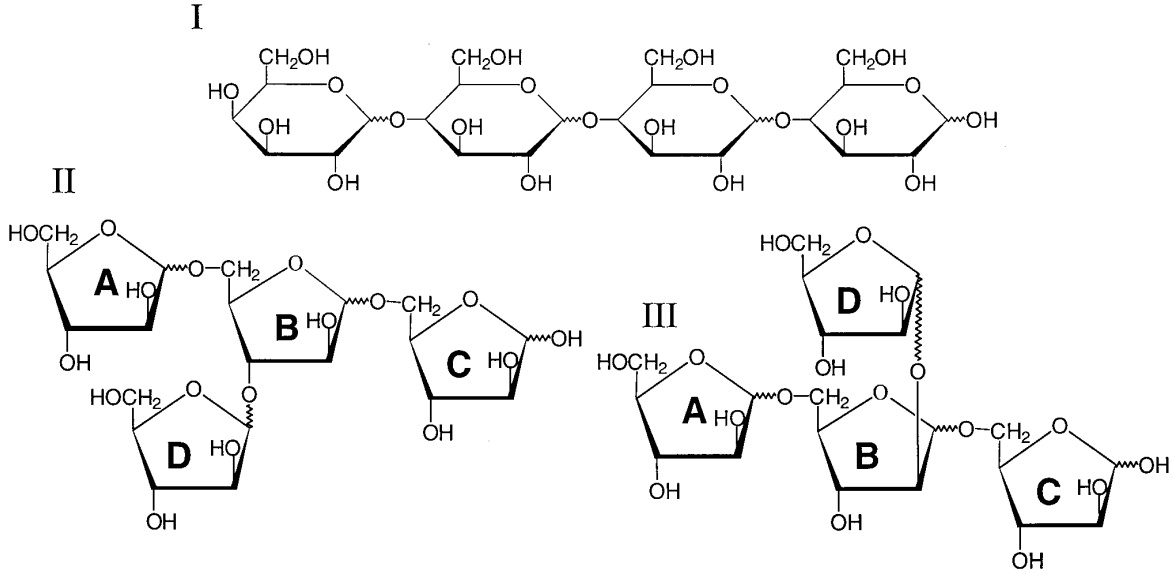

Figure 7. Structures of the arabinogalactan-derived oligosaccharides found in HPAEC fractions 4 and 6.

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