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Separation and identification of enzymatic sucrose hydrolysis products by high-performance anion-exchange chromatography with pulsed amperometric detection

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Abstract

An accurate carbohydrate analysis method, namely high-performance anion-exchange chromatography with pulsed amperometric detection was successfully applied to the study of sucrose hydrolysis under enzymatic (baker's yeast invertase) conditions. The hydrolysis was monitored by determining sucrose degradation and the corresponding formation of D-glucose, D-fructose and five intermediate fructans using a CarboPac PA-100 (Dionex) analytical anion-exchange column. Highly reproducible results were obtained. The unknown fructans were collected from a semi-preparative CarboPac PA-100 (Dionex) column, neutralized and then desalted on a column containing mixed bed resin AG 501-X8 (D) before identification of the chemical structure. This procedure permitted us to obtain about 20 μg of pure product which is not enough for NMR analysis. Detailed GC–MS analytical data of the methylated compounds indicated that these oligosaccharides were β-D-Fru-(2→1)-β-D-Fru-(2→1)-α-D-glucopyranoside (1-kestose), β-D-Fru-(2→6)-α-D-glucopyranoside (6-β fructofuranosylglucose), β-D-Fru-(2→1)-β-D-fructofuranoside (inulobiose), β-D-Fru-(2→6)-β-D-Fru-(2→1)-α-D-glucopyranoside (6-kestose) and β-D-Fru-(2→6)-α-D-Glc-(1→2)-β-D-fructofuranoside (neokestose) coeluating with a disaccharide.

Keywords: Carbohydrates; Sucrose; Fructans; Fructose

1. Introduction

Various high-performance liquid chromatography (HPLC) techniques have been developed to facilitate carbohydrate analyses in an effort to better understand their role in biochemical processes. Many neutral oligosaccharides, which differ by only one sugar residue or the composition of a 1→6 branch, can be readily resolved using either reverse-phase (i.e. alkyl-bonded phases) or normal-phase (i.e. amino-bonded phases) liquid chromatography [1–5]. However, resolution among 1→2, 1→3, and 1→4 positional isomers of oligosaccharides is more difficult [4]. In the last decade, high-performance anion-exchange chromatography (HPAEC) in conjunction with pulsed amperometric detection (PAD) has been

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demonstrated to be a powerful method for the separation of sugars [6]. We have already used this technique for the study of sucrose degradation during sugar processing in a local cane sugar refinery (Saint-Louis Sucre in Marseilles) [7–9]. In this work, an example of the HPAEC–PAD, characterization of sugar oligomers obtained by enzymatic degradation of sucrose is described. Attention is mainly focused on the characterization of the reaction products and detailed analytical data are reported.

2. Experimental

2.1. Chemicals

Baker's yeast invertase grade VII, lyophilized salt-free powder freed from α-galactosidase with a specific activity of 1000 U mg^{-1} was a product from Sigma (St-Quentin Fallavier, France) which was used without any further purification. One unit of enzyme activity hydrolysed 1.0 μmol of sucrose into invert sugar per min in a 50 m*M* sodium acetate buffer, pH 4.5 at 55°C. D-Glucose, D-fructose and sucrose were also from Sigma, while the fructooligosaccharide (FOS) standard set containing β-D-Fru-(2→1)-β-D-Fru-(2→1)-α-D-glucopyranoside (1-kestose), β-D-Fru-$(2\rightarrow1)_2$-β-D-Fru-(2→1)-α-D-glucopyranoside (nystose) and β-D-Fru-$(2\rightarrow1)_3$-β-D-Fru-(2→1)-α-D-glucopyranoside (fructofuranosylnystose) was purchased from Wako (Neuss, Germany). The AG 501-X8 (D) resin used for oligosaccharide desalting was provided by Bio-Rad Labs. (Ivry-Sur-Seine, France).

2.2. Apparatus

Carbohydrates (sucrose, D-glucose, D-fructose and fructans) were separated by HPAEC–PAD using a Dionex DX-500 (Sunnyvale, CA, USA) instrument as already reported [10]. The systems consisted of a GP 50 gradient pump and an ED 40 electrochemical detector, working in the PAD mode, equipped with a Dionex eluent degas module that was used to sparge and pressurize the mobile phase with helium. The following pulse potentials and durations were used: $E_1 = +0.05$ V ($t_1 = 0$–400 ms), $E_2 = +0.75$ V ($t_2 =$ 410–600 ms) and $E_3 = -0.15$ V ($t_3 = 610$–1000 ms). This system was connected to a personal computer using the JMBS Developments Borwin chromatography software program.

2.3. Chromatography

Reaction products were quantitatively analyzed using a Dionex CarboPac PA-100 analytical (250×4 mm) anion-exchange column equipped with a CarboPac PA-100 guard column (50×4 mm), which requires lower sodium acetate concentrations for the elution of large sugar oligomers than the old CarboPac PA-1 column. The elution was performed at a constant flow-rate of 1 ml min^{-1} at room temperature using a 20 min linear gradient from 0 to 40% of 80 m*M* NaOH–500 m*M* sodium acetate in 80 m*M* NaOH–5 m*M* sodium acetate. The injection volume was 25 μl. Reaction products were identified and quantified either via standard fructooligosaccharides or by linkage analysis. In the latter case, the unknown products were collected, using a CarboPac PA-100 preparative (250×9 mm) anion-exchange column. The elution was performed at a constant flow-rate of 4 ml min^{-1} at room temperature using a 20 min gradient ranging from 0 to 10% of 80 m*M* NaOH–500 m*M* sodium acetate in 80 m*M* NaOH–5 m*M* sodium acetate. The injection volume was 100 μl. The collected product was neutralized with HCl, then passed through a desalting column (30×1 cm) containing mixed bed resin AG 501-X8 (D). Finally the desalted product was lyophilized.

2.4. Oligosaccharide identification

Permethylation was performed on 10–20 nmol dry samples of each unknown product (1, 2, 3, 4 and 5) using a method similar to that of Lindberg et al. [11] and Ciucanu and Kerek [12]. To verify completion of the methylation the samples were analyzed using an elecrospray ionisation (ESI) mass spectrometer (VG Platform). The mass of each sample was determined.

After hydrolysis with 2 *M* TFA for 2 h at 100°C, the samples were reduced using $NaBD_4$. The now free OH groups were acetylated using pyridine–acetic anhydride. At this point the partially methylated alditol acetates (PMAAs) were dissolved in CH_2Cl_2 and analyzed by GC–MS [13].

2.5. Gas chromatography–mass spectrometry

Combined GC–MS analyses were performed using a Jeol AX505 mass spectrometer fitted with an HP5890 gas chromatograph, a DB-5 GC column (0.25 mm×30 m, J&W Scientific) and using helium as a carrier gas (1 ml min^{-1}). The following temperature program was used: 60°C for 2 min, then ramping at 30°C min^{-1} to 150°C, holding for 1 min, then ramping at 5°C min^{-1} to 260°C. Mass spectra were recorded with an electron impact ionization source in the positive ion mode with an electron energy of 70 eV.

3. Results

3.1. Reproducibility analysis

When an equimolar (4 μ*M*) mixture of sucrose, D-glucose and D-fructose was injected 10 times in the HPAEC–PAD system, the determination of each sugar concentration gave 4.18 μ*M*±0.10, 4.16 μ*M*±0.11 and 4.00 μ*M*±0.18, respectively. The relative standard deviation, (standard deviation/mean value)·100, for carbohydrate analysis averaged between 2.39% and 4.50%. High sucrose as well as low D-glucose containing aqueous solutions were also analyzed for the same purpose. From a 65% (w/w) aqueous sucrose solution, ten samples (3 g/5 ml, 1.14 *M*) were prepared, further diluted (1:40 000) and analyzed by the HPAEC–PAD system. The resulting quantification gave 1.12 *M*±0.08. In the same way, the glucose content of ten samples which were prepared by diluting (1:1000) a (0.5%, w/v; 27.74 m*M*) mother-aqueous D-glucose solution was determined to be 25.96 m*M*±0.41. All these reproducible determinations were in accordance with the data described in the literature [14].

3.2. Characterization of the fructans produced by enzymatic hydrolysis of sucrose in aqueous media

Enzymatic hydrolysis of sucrose (0.2 *M*) by invertase (25 U) was carried out at 55°C in 2 ml of a 50 m*M* sodium acetate buffer (pH 4.5). Fig. 1 shows the HPAEC–PAD chromatograms of an experimental sample obtained by incubating sucrose with pure

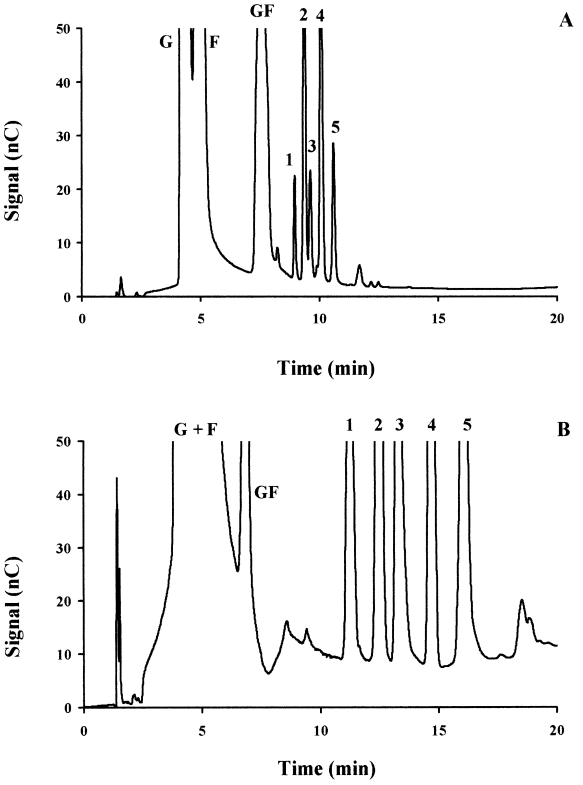

Fig. 1. HPAEC–PAD analysis of the enzymatic degradation products of sucrose. Chromatograph obtained with a CarboPac PA-100 analytical- (A), and with a CarboPac PA-100 preparative anion-exchange columns (B).

invertase for 10 min. Fig. 1A and B show the profile obtained with the CarboPac PA-100 analytical- and the CarboPac PA-100 preparative anion-exchange columns, respectively. D-Glucose, D-fructose, sucrose and 1-kestose (fraction 1) were identified using the corresponding commercial synthetic products. The identity of each intermediate compound corresponding to fractions 1, 2, 3, 4 and 5 were determined by performing linkage analysis after collecting the material under each peak obtained from the preparative column. The ESI mass spectrum of the permethylated fractions reveals an $[M+Na]^+$ ion at m/z 681.5, 477.5, 477.5 and 681.5 for the fraction 1, 2, 3 and 4, respectively, while fraction 5 contains two products with an $[M+Na]^+$ ion at m/z 477.5 and 681.5. Fig. 2 shows the gas chromatogram of fraction 1 giving the five peaks a, a′, b, c and c′ as well as the mass spectra of the corresponding peaks.

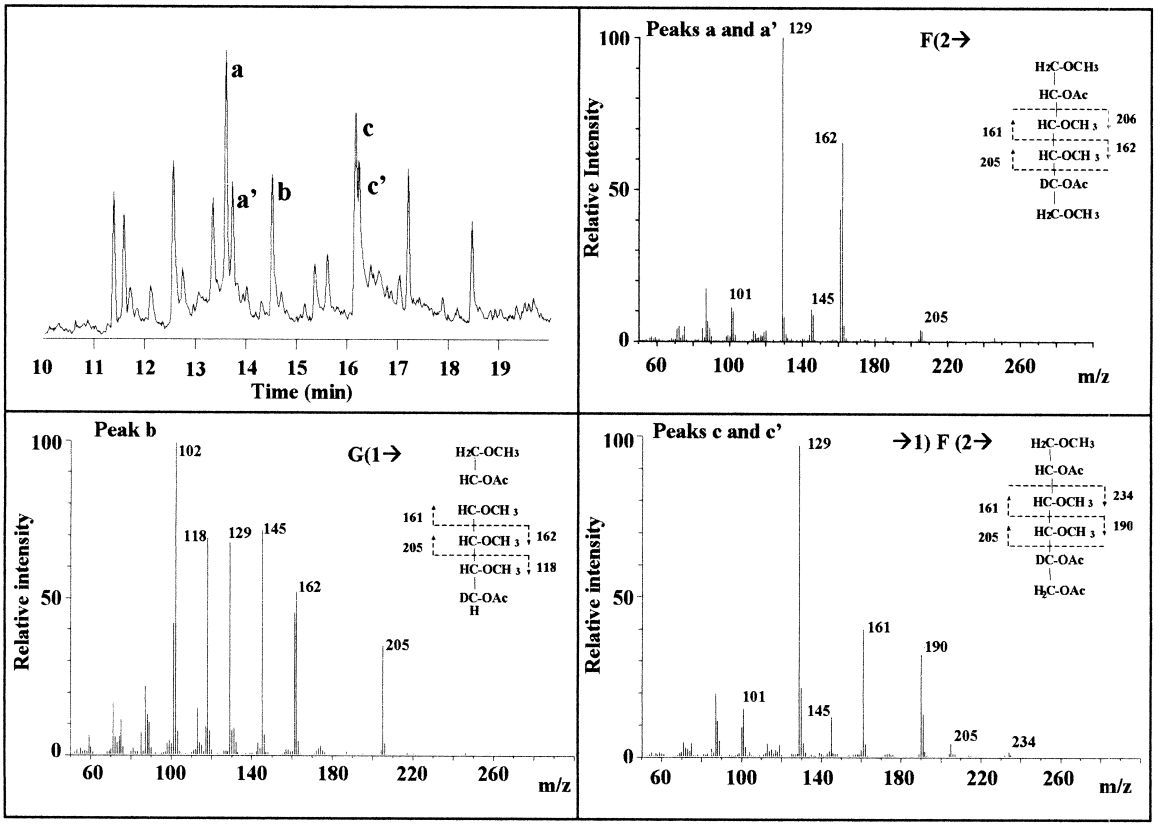

Fig. 2. Gas chromatogram of the partially methylated alditol acetate and mass spectra of fraction 1.

These results indicate that fraction 1 is composed of 2-linked, 1,2-linked fructose, and 1-linked glucose, β-D-Fru-(2→1)-β-D-Fru-(2→1)-α-D-glucopyranoside, namely the 1-kestose. Fig. 3 shows the gas chromatogram of fraction 2 giving the three peaks a, a′ and d. The mass spectra indicate that fraction 2 is composed of 1-linked, 2-linked fructose, and 6-linked glucose, β-D-Fru-(2→6)-α-D-glucopyranoside, namely the 6-β-fructofuranosylglucose. Fraction 3 gave the four peaks a, a′, c and c′ (Fig. 4). The mass spectra indicate the presence of a 1-linked, 1,2-linked fructose, β-D-Fru-(2→1)-β-D-fructofuranoside, namely inulobiose. Fraction 4 gave the five peaks a, a′, b, e, e′ (Fig. 5) and the mass spectra indicate the presence of a 2-linked, 2,6-linked fructose, and 1-linked glucose, β-D-Fru-(2→6)-β-D-Fru-(2→1)-α-D-glucopyranoside, namely 6-kestose. Finally fraction 5 gave the three peaks a, a′ and d (Fig. 6) and the mass spectra indicate the presence of a 2-linked, 2-linked fructose, and 6,1-linked glucose, β-D-Fru-(2→6)-α-D-Glc-(1→2)-β-D-fructofuranoside, namely neokestose which was eluted together with a disaccharide. The contaminant disaccharide may be 6-β-fructofuranosylfructose. However, it is present in too small a quantity for the detection of a 6-linked fructose by GC–MS. Reduction of the partially methylated fructose residues resulted in alditols, the configurations of which corresponded to that of gluco- and manno- (a and a′, c and c′, e and e′). Both were found to have identical mass spectra but differed in their retention time. The major mass spectral fragments of the peaks a–e, are shown in Table 1.

The chemical structures of these fructans are shown in Fig. 7. Some of these products, 6-kestose, 6-β-fructofuranosylglucose, inulobiose and 6-β-fructofuranosylfructose have already been detected so far in independent works on baker's yeast invertase

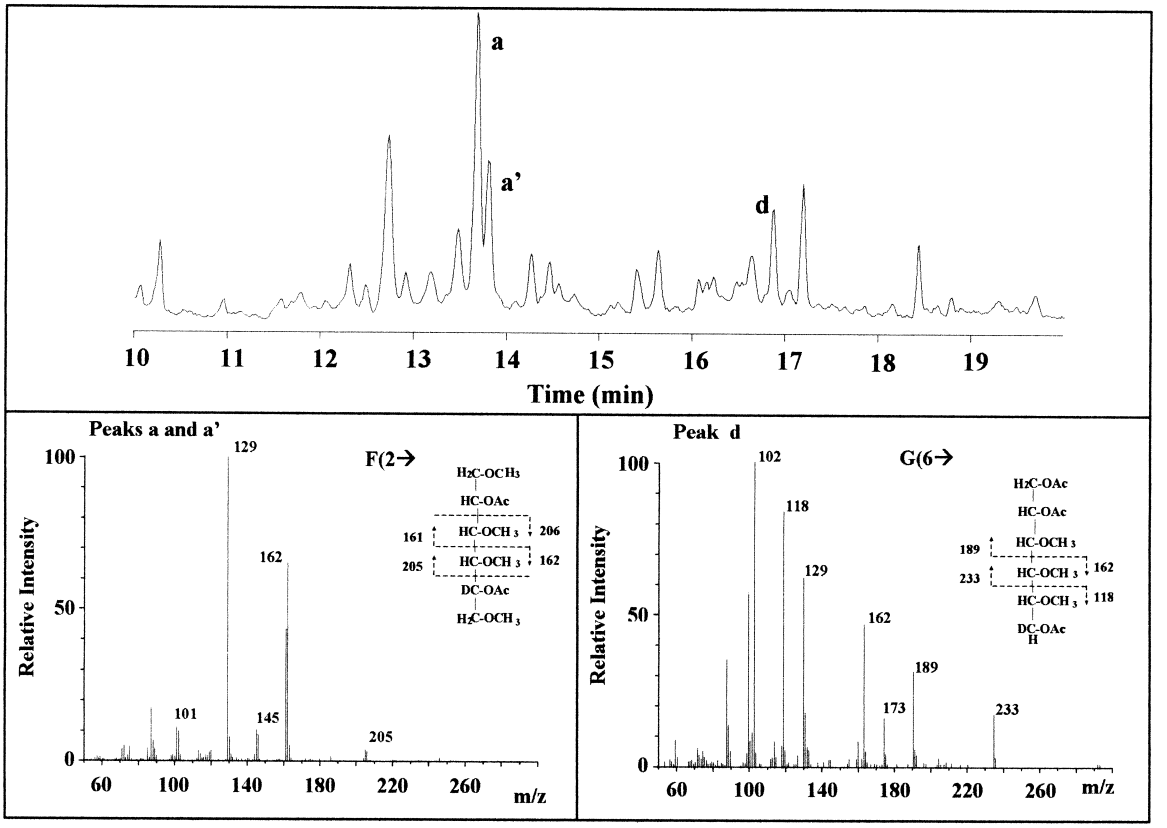

Fig. 3. Gas chromatogram of the partially methylated alditol acetate and mass spectra of fraction 2.

[15–17]. There are, however, no reports of the separation and analysis of all these compounds in a single run as we show in this study.

4. Discussion and conclusions

Early quantitative methods for trisaccharide (kestoses) analysis have been slow, cumbersome, and subject to numerous errors. Current methods for the separation of 1-kestose, 6-kestose and neokestose require thin-layer chromatographic separation and the use of radioactive materials for good quantification. Conventional HPLC does not provide good separation of the three different kestoses [18]. Furthermore, currently employed methods of separation are particularly difficult to adapt for enzyme activity studies [19]. Indeed, the kinetics of the enzymatic hydrolysis of sucrose by invertase have frequently been estimated indirectly by polarimetric measurements of glucose [20]. These measurements, however, neglect the intermediate oligosaccharides formed by the transferase action of invertase [15] and assume equal amounts of glucose and fructose. These oligosaccharides interfere by producing an erroneously low reaction rate [21]. The kinetics have also been estimated indirectly with a spectrophotometric assay using glucose-oxydase [22]. This procedure quantifies changes in glucose concentrations rather than measuring the amount of the different oligosaccharides produced. Since other reactions may cause changes in the concentrations of glucose, measurements from those spectrophotometric assays provide only empirical estimates of the rates of kestose synthesis. Furthermore information is difficult to obtain regarding the nature of the products synthesized in such reactions. A study about the action of invertase at high sucrose concentrations (up

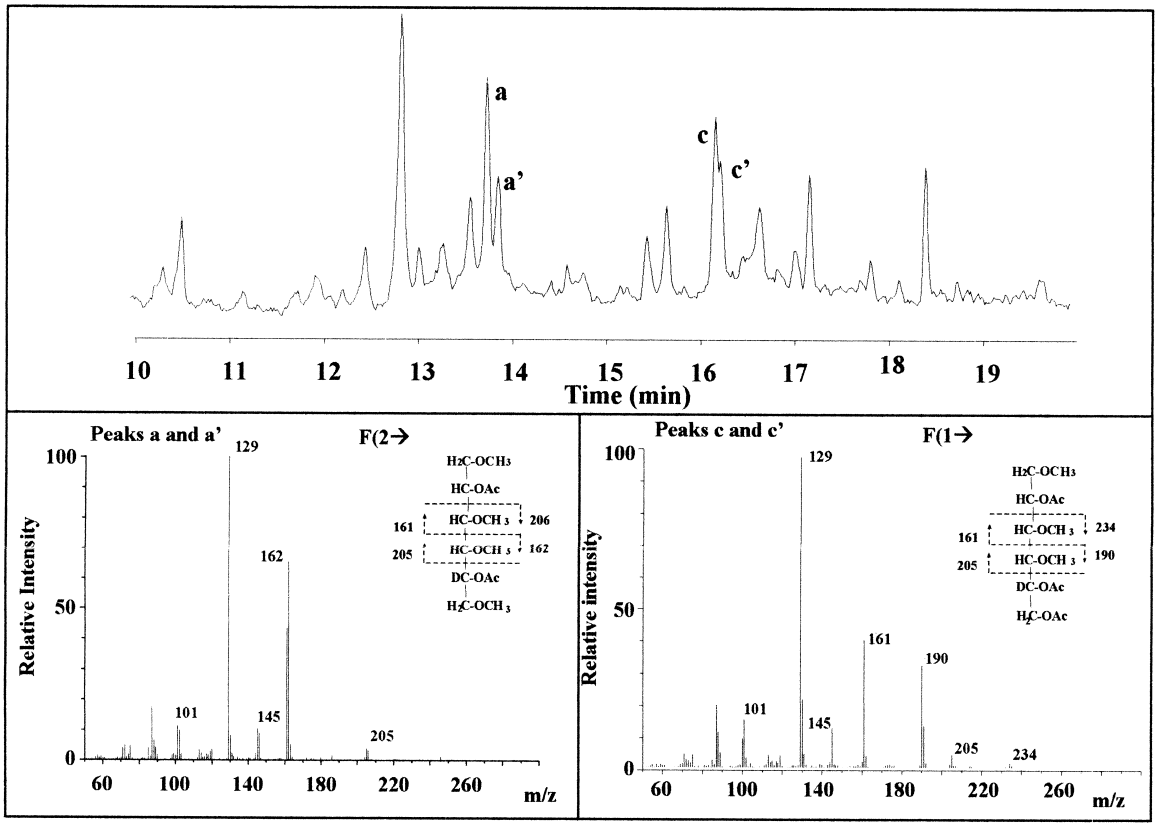

Fig. 4. Gas chromatogram of the partially methylated alditol acetate and mass spectra of fraction 3.

to 2.34 *M*) followed by HPLC analysis allowed the quantitative determination of mixtures of D-glucose, D-fructose, 6-kestose, inulobiose and sucrose [23]. In this case, only two intermediate fructans were detected, namely 6-kestose and inulobiose, as compared to the four intermediates 1-kestose, 6-β-fructofuranosylglucose, inulobiose, and 6-kestose which were identified in this study, in addition to neokestose which was eluted with a disaccharide. The use of HPAEC–PAD resolves these difficulties.

Nevertheless, one of the remaining problems of HPAEC–PAD in this area of interest, is the difficulty of predicting the elution order of heterogeneous sugar oligomers since, in most instances, no standards are available. Further, a full characterization of sugar oligomers requires the determination of the sugar composition and the sugar linkage positions. The abilities of mass spectrometry, especially in an on-line combination with HPAEC, are expected to be promising. However, the solvent conditions generally used in the HPAEC of sugar oligomers are not compatible with on-line MS detection. The problems with the high salt concentrations of the mobile phase, e.g., 0.1 mol l^{-1} sodium hydroxide and a gradient of 0–0.5 mol l^{-1} sodium acetate, as used in the separation of sugars oligomers, have already been solved for conductivity detectors in ion chromatography by the introduction of a micromembrane suppressor [24] which efficiently replaces either cations or anions by protons or hydroxyl ions. An anion micromembrane supressor (AMMS) can be used in combination with HPAEC in sugar analysis to remove the excess sodium ions. On-line desalting with an AMMS prior to fraction collection for off-line fast atom bombardment MS or ^{1}H NMR analysis has already been described [24]. With an AMMS the sodium concentration can be decreased from 0.1 *M* to a level compatible with on-line thermospray mass spec-

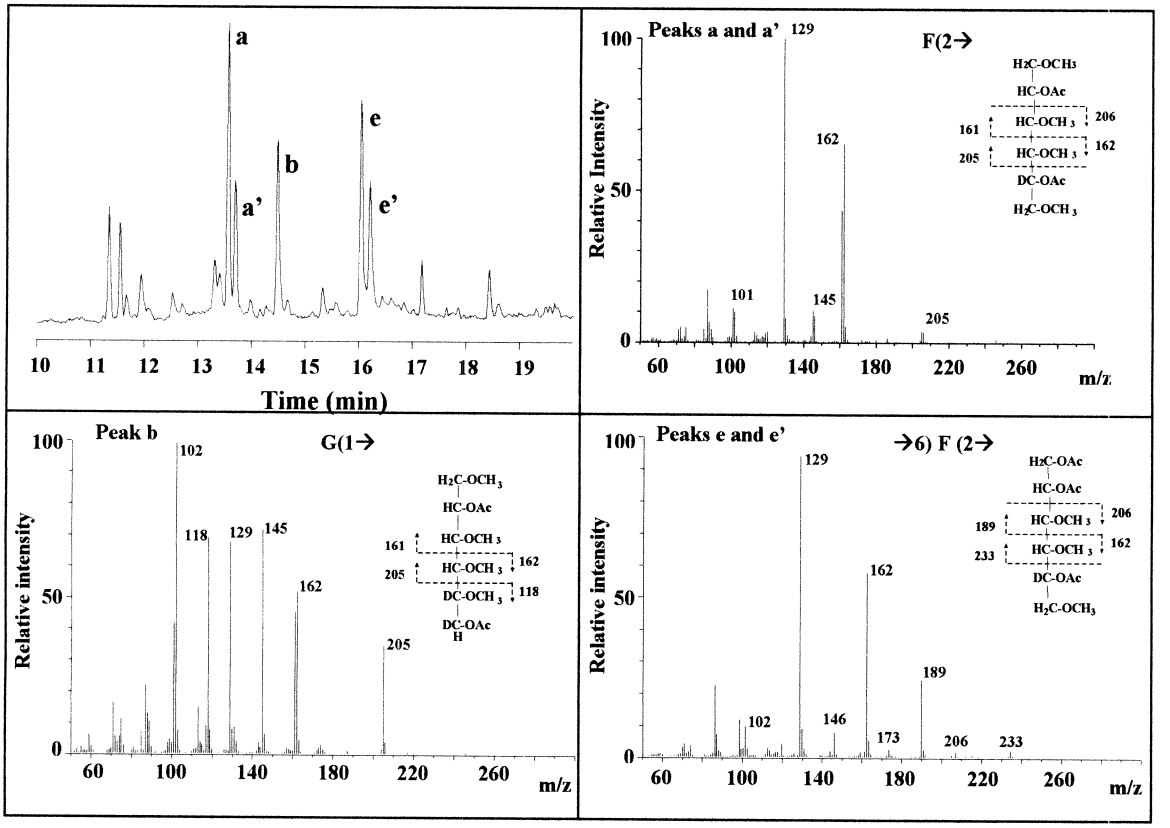

Fig. 5. Gas chromatogram of the partially methylated alditol acetate and mass spectra of fraction 4.

trometry [25]. This system has proved to be a powerful tool in the characterization of sugar oligomers obtained by enzymatic digestion of plant cell wall polysaccharides [26]. The on-line coupling of HPAEC and MS where two AMMS systems are used in series for the detection of β-1,4-xylose oligomers up to a degree of polymerization of 25 has already been tested [27]. In this system the sodium ions could be removed sufficiently to allow thermospray MS detection as long as the sodium concentration in the mobile phase did not exceed 0.4 mol l^{-1}. Otherwise, significant salt deposits will occur in the ion source, which hampers further analysis. As a result, the maximum degree of polymerization that can be determined by HPAEC–MS is limited. Several studies have then reported the application of electrospray interfacing for detection of neutral and acidic sugars [28], showing substancial better performance when using a cation-exchange membrane desalting device (CEMDD) which replaces sodium for hydronium ions [29,30]. The latter method enables the routine use of sodium acetate gradients up to a total sodium concentration of 0.6 *M*. However, this system was never used in the analysis of more complex oligosaccharide samples, containing mixed oligomers having the same molecular mass and differing only by the linkage position as our enzymatic reaction products. Indeed the combination of HPAEC with MS will result in native oligosaccharides in the mass spectrometer. Running single MS will give only the molecular mass. Performing MS–MS of the $[M+H]^+$ or $[M+Na]^+$ ion will give information about the size of the building blocks, branching and unknown intermediates (e.g. disaccharides twice coupled with each other) of oligosaccharides but will not give information about which hexose is present. It can give only some information about linkage in the case of $1\rightarrow4$ coupling. Recent results on the

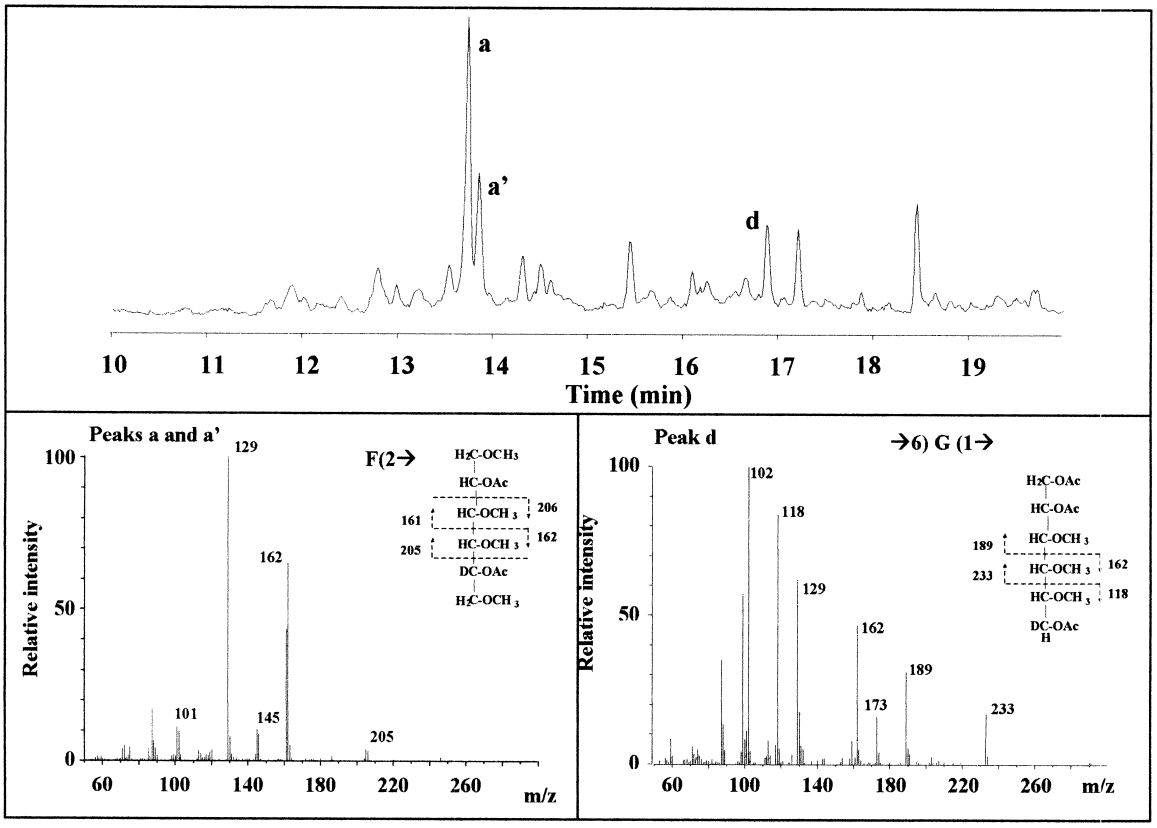

Fig. 6. Gas chromatogram of the partially methylated alditol acetate and mass spectra of fraction 5.

Table 1
The identification of the partially methylated alditol acetates of the unknown products[a]

Peak n°	PMAA	Major mass-spectral fragment (*m*/*z*)	Structural feature
a, a′	2,5-Di-*O*-acetyl-1,3,4,6-Tetra-*O*-methyl	101, 102, 129, 145, 146, 161, 162, 205, 206	→2) F; F (2→
b	1,5-di-*O*-Acetyl-2,3,4,6-Tetra-*O*-methyl	101, 102, 118, 129, 145, 161, 162, 205	→1) G; G (1→
c,c′	1,2,5-Tri-*O*-acetyl-3,4,6-Tri-*O*-methyl	101, 129, 145, 161, 190, 205, 234	→1) F (2→; F (1→
d	1,5,6-Tri-*O*-acetyl-2,3,4-Tri-*O*-methyl	102, 118, 129, 162, 173, 189, 233	→6) G (1→; G (6→
e,e′	2,5,6-Tri-*O*-acetyl-1,3,4-Tri-*O*-methyl	102, 129, 146, 162, 173, 189, 206, 233	→6) F (2→

[a] F: fructose; G: glucose.

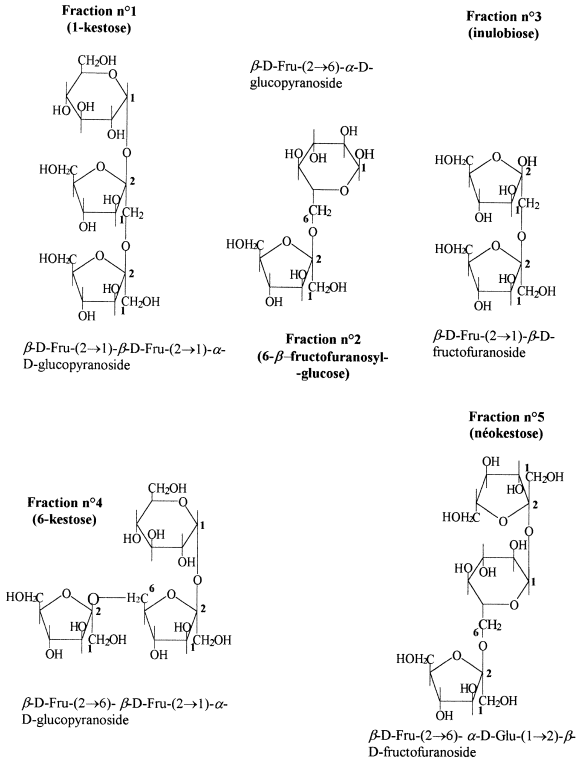

Fig. 7. Chemical structure of the enzymatic degradation products of sucrose.

characterization of linkage positions in small oligosaccharides by means of electrospray ionisation and an ion-trap multistep mass spectrometry ($MS–MS^n$) provide structural insights previously inaccessible by tandem mass spectrometry [31,32], indicating that this approach could potentially be successful in our field.

Finally, optimized on-line desalting is necessary between HPAEC and MS, as other methods can be time consuming and result in poor product yield.

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