ABSTRACT
The use of novel mass spectrometric techniques, off-line coupled to chromatographic techniques is described in order to obtain molecular mass information of compounds present in complex mixtures of oligosaccharides. Such information could be quite useful in studies where pure and well-defined enzymes are used to study the chemical fine structure of polysaccharides. Post source decay fragmentation using a Matrix-assisted laser desorption/ionisation Time of flight Mass Spectrometer may result in more detailed structure information of oligosaccharides without laborious purification protocols. In addition to the valuable contribution of HPAEC in the separation of complex oligosaccharide mixtures, alternatives for the separation of oligosaccharides having alkali-labile substituents are suggested.

INTRODUCTION
Polysaccharides are important constituents of plant cell walls and represent the bulk of plant biomass. They determine quality attributes of fresh fruits and vegetables (ripeness, texture etc.) and their processing behaviour in the manufacture of foods. Polysaccharides are also predominantly present in by-products of the agro-industry (beet pulp, potato fibre, wheat bran, etc.). Much research is focused to reveal the relationship between the chemical structure of the various classes of polysaccharides (e.g. pectin, hemicellulose, and cellulose) and their corresponding functional properties (e.g. stabilising, suspending, thickening, gelling, water-holding capacities). Such knowledge is essential for the food industry to come to improved or novel processes and/or products and to extend the use of (components derived from) agricultural by-products in food or feed application.

The common way to establish the chemical structure of polysaccharides is starting with the determination of their sugar (linkage) composition, the presence of non-glycosidic substituents like methyl esters and acetyl- and feruloyl groups. However, it is recognised that, in addition to these chemical characteristics, the sequence of the composing sugar moieties within a complex polymer or the distribution of the various substituents over the polymer backbone play a major role in determining the physical properties of the polysaccharides. Furthermore, the interaction of the various classes of such complex polysaccharides (with each other and with proteins, etc) within the cell wall is of immense importance for the final behaviour of the plant material. These interactions also determine the enzymic modification/degradation by both endogenous and added enzymes. Therefore, specific strategies are being developed to obtain more information on the precise chemical structure of the native polysaccharides. NMR spectroscopy is gaining interest since information about sequences of building blocks can be recognised within a polymer (e.g. pectins: Neiss et al., 1999) although this is quite difficult for more complex/heterogeneous molecules. For many years, we are using pure and well-characterised enzymes to obtain oligomeric fragments from polysaccharides in a very defined way. These fragments fit better within the analytical range of chromatographic, spectrometric and spectroscopic methods. Following the full characterisation of unknown oligomeric fragments, (hypothetical) structures of the star-
ting polymer can be proposed. The use of enzymes in structural analysis is further stimulated by the continuous discovery and characterisation of novel polysaccharide degrading enzymes.

**ANALYTICAL STRATEGIES**

A major breakthrough for the HPLC-analysis of complex mixtures of oligosaccharides was the introduction of high-performance anion-exchange chromatography using high-pH eluents in combination with pulsed amperometric detection (Rocklin and Pohl, 1983). Ever since, an increasing number of applications for the separation of sugars have been published: both for the separation of ‘normal’ monomeric and oligomeric carbohydrates in food products as well as for the separation of complex mixtures as obtained after enzymic degradation of polysaccharides (Voragen et al., 1993). For a nice overview of mechanisms concerning HPAEC using PAD detection and possible applications, the reviews of Lee (1990, 1996) are recommended. The high resolution found for HPAEC and the fact that the separation is influenced by the type of sugar residue present, the type of linkage and the size of the oligomer explain its wide use in the analysis of complex mixtures. However, these mechanisms make the elution behaviour of different classes of oligosaccharides rather unpredictable and it is quite difficult to identify the nature of an unknown compound on the basis of its position in a chromatogram. As a consequence, to obtain more information about the nature of unknown oligosaccharides, extensive purification steps such as preparative size-exclusion chromatography or HPAEC followed by laborious desalting steps are required prior to characterisation by MS and NMR. Mid nineties, we started to investigate whether we could use mass spectrometric techniques coming available to have a rapid method to examine the nature of compounds present in a complex mixture. At that time, we used HPAEC coupled online with mass spectrometry using a thermospray interface (Niessen et al., 1996). Information about the mass of the oligomers obtained, together with the sugar composition of the polysaccharide under investigation and the specificity of the enzyme used enabled us to predict rather precisely the structure of unknown compounds (Schols et al., 1994).

Another development of the last years includes the introduction of Matrix-assisted laser desorption/ionisation Time of flight Mass Spectrometry (Bahr et al., 1994; Kaufmann, 1995) giving good mass accuracy, being easy to operate and requiring hardly any expertise in mass spectrometry. MALDI-TOF MS has also shown to be very suitable to study the presence of ‘unstable’ substituents in oligosaccharides providing information which might be lost using other methods of analysis (Daas et al., 1998).

Below, some examples will be given where the above techniques have been combined in such a way that important information on the structure of unknown oligosaccharides become available more easily. Attention is also paid to alternative chromatographic separations allowing us to obtain information on the presence of alkali-labile substituents.

**MATERIAL AND METHODS**

HPAEC conditions used for the separation of (partially methyl esterified) oligogalacturonic acids are described by Daas et al. (1998) and Van Alebeek et al. (2000 a + b) while they also described in detail in which way the MALDI-TOF Mass Spectrometry was performed using a Voyager-DE RP apparatus (Perseptive Biosystems). Off-line HPAEC MALDI-TOF MS facilitated by on-line desalting using two commercially available ultra self regenerating suppressors (ASRS and CSRS; Dionex) and automated handling of fractions collected in a 96-well-plate using a Symbiot-I robot (Perseptive Biosystems) is described in more detail by Kabel et al. (2000). Reversed Phase chromatography of

![Figure 1: Schematic overview of the experimental set-up for automated coupling of HPAEC to MALDI-TOF MS.](image-url)
acetylated xylo-oligosaccharides was performed on an ODS-2 column (Chrompack) using a methanol-water gradient at a flow rate of 1 ml/min. The same samples were also analysed using a TSK-gel Amide-80 column (Toso Soda) using a water-acetonitril gradient at 1 ml/min. Effluents were monitored using an evaporative light scattering detector (ELS: Sedex model 55, Severe).

**COMPLEX ARABINOXYLANS**

Xylans consist of a backbone of (1,4)-linked β-D-xylopyranosyl residues. Depending on their origin, e.g. Gramineae (grasses and cereals), Gymnosperms (softwoods) or Angiosperms (hardwoods), the backbone is substituted with α-L-arabinofuranose, α-D-glucopyranosyl uronic acid or its 4-methyl derivative, and acetyl groups (Kormelink, 1992). The amount and type of substituents present and their distribution over the xylose backbone determine properties of xylan like solubility and degradability by enzymes and consequently the processing characteristics of xylan-rich raw materials. For this reason, our Laboratory of Food Chemistry has studied various types of (glucurono)arabinoxylans already (ex wheat, barley, sorghum) or is working on it at the moment.

**Off-line HPAEC MALDI TOF MS coupling**

In order to obtain molecular mass information of unknown oligosaccharides, an automated system to measure these masses directly from fractions as collected from an analytical HPAEC run was developed. Typical HPAEC conditions include gradients using 100 mM sodium hydroxide in combination with sodium acetate gradients up to 0.5 or 1 M, while such high salt concentrations are known to hinder co-crystallisation of your analyte molecules with the matrix molecules (e.g. 2,5-dihydroxybenzoic acid) for MALDI-TOF MS analysis. To overcome this problem, the HPAEC eluent was desalted on-line using an anion self regenerating suppressor (ASRS) in series with a cation self regenerating suppressor (CSRS). In this way, acetate ions were exchanged by hydroxide ions and sodium ions by hydronium ions as obtained by electrolysis of water. The resulting eluent (separated oligosaccharides in water) was fractionated and the collected fractions were analysed by MALDI-TOF MS using a robot-system to mix 1 µl of each fraction with 1 µl of matrix solution. A schematic overview of the experimental set-up is presented in figure 1. Using conductivity measurements, it was demonstrated that the combination of suppressors used were able to remove ions almost completely when the concentration of NaAc in 100 mM NaOH does not exceed 300 mM at a flow rate of 1 ml/min. When higher concentrations of salts have to be removed, a lower flow rate is recommended, while also more suppressors in series could be used.

The off-line coupling of HPAEC to MALDI-TOF MS was used to analyse a complex mixture of glucuronoxylan oligomers as obtained after a hydrothermal treatment (17 min.; 175ºC) of Eucalyptus wood. It has been shown that such a treatment mainly solubilize xylans leaving the abundantly present cellulose rather unchanged. The HPAEC elution pattern of the hydrolysate is rather complex (not shown). Measuring the fractions collected (120 µl), quite unambiguous results were obtained which show that most fractions only contained one single compound as illustrated for some fractions in figure 2. Mass spectra of a pentamer and a nonamer of pentoses (i.e. xylose since no arabinose is present in the hydrolysate) are easily identified while other spectra/fractions represent xylo-oligomers containing one 4-O-methyl glucuronic acid moiety. In addition to the strong signal of the single sodium adduct of the acidic oligomers (mass +23), also a weaker signal of oligomers
including two sodium ions were detected. An important advantage of this off-line coupling compared to on-line LC-MS is, that the fractions (although representing only quite small amounts of material due to the analytical column used) remain available for further characterisation using e.g. other mass spectrometric techniques (Iontrap MSⁿ or post source decay MALDI-TOF MS: see below).

**Acetylated xylan oligosaccharides**

Although peak identification in a rather complex elution pattern as obtained from a mixture of oligomers originating from a glucuronoxylan is quite successful, a substantial part of the information concerning the precise structure of the glucuronoxylan oligomers is lost by the elution conditions (pH12) used for the HPAEC separation. As can be seen in figure 3 representing the 500-750 Dalton range of the MALDI-TOF mass spectrum of the crude preparation, many acetylated oligomers are present as well. This drawback, together with the fact that PAD-detection is not very quantitative when appropriate response factors are not known (e.g. for unknown oligosaccharides; Lee, 1990), forced us to look for other strategies to separate acetylated xylo-oligosaccharides.

Using a column material modified with amide groups, it was demonstrated that a separation of maltodextrins could be obtained using a gradient of acetonitril and water. However, refractive index detection of the eluent failed when a gradient was necessary to obtain a good separation. PAD-detection was not favoured, since alkali has to be added post-column to have any response. For this reason, an evaporative light scattering (ELS) detector was used. An example of the separation of maltodextrins over the TSK amide column as monitored by an ELS detector shows that oligomers up to a DP (degree of polymerisation) of 20-25 could be separated nicely (Figure 4a). The research concerning this type of detection of oligosaccharides differing in size and composition is on-going. To evaluate whether this TSK amide-column could be used as well for the separation of acetylated oligosaccharides, a neutral (non-bound) fraction from the eucalyptus wood hydrolysate obtained after dowex-treatment was injected. It is shown in figure 4b that a complex elution pattern was obtained. Analysis of fractions collected during this chromatographic run shows that the peaks represent various types of acetylated xylan-oligomers (not shown). Xylans having the same number of acetyl groups but differing in size (e.g. X₃Ac, X₄Ac, and X₅Ac) were separated nicely but co-eluted with oligomers having a higher acetyl substitution (e.g. X₄Ac, X₅Ac, and X₆Ac). Oligomers containing more acetyl groups were retained less than oligomers of the same size without or with less acetyl substitution. More research is ongoing, but it could state already that a useful method is available to separate acetylated oligomers without modification, as was the case under alkaline conditions. The precise location of the acetyl groups within a given xylo-oligomer has to be investigated as well. Rather similar results as described for the TSK amide column has been obtained for a reversed phase ODS-2 column as well (not shown). However, the mechanism of elution is totally different since the presence of acetyl groups resulted in enormous retention on the column and a high concentration of methanol is necessary to elute all acetylated xylo-oligosaccharides.

**METHYL ESTERIFIED PECTIN OLIGOMERS**

Pectin is a complex polysaccharide that is widely distributed in plant material, determining plant cell wall strength and flexibility. Because of its excellent gelling, thickening, and stabilizing properties, pectin is extensively utilized in the food industry after extraction on an industrial scale from e.g. lime and lemon peel (Voragen et al., 1995). One of the most important features of pectins to be used as food ingredient is the number of α-1,4-linked galacturonic acid residues

![Figure 3: Part of the MALDI-TOF mass spectrum of an eucalyptus wood hydrolysate obtained after hydrothermal treatment. GlcAme₃Ac represents a tetramer consisting of three xylose residues and one 4-O-methyl-glucuronic acid moiety carrying one acetyl group.](image-url)
carrying a methyl ester and the distribution of these esterified uronides over the pectin backbone. Such information could be obtained by enzymic degradation of unesterified sequences of the pectin by the well-characterised enzyme polygalacturonase from Kluyveromyces fragilus. Such an approach need procedures to separate and quantify the various (partly methyl esterified) galacturonic acid oligomers. Since HPAEC analysis under alkaline conditions removed the methyl esters during the chromatographic run, Daas et al. (1998) developed a method where HPAEC was performed on a CarboPac PA1 column (Dionex) at pH 5, followed by post column addition of alkali to enable PAD detection. A rather nice separation of the complex mixture of methyl esterified oligogalacturonides was obtained. Peaks were collected (skipping the post column alkali addition) and identified by MALDI-TOF MS. The various compounds were ‘identified’ with respect to the size of the oligomer and the number of methyl esters present although at that time, no further information on the precise location of the esters could be obtained. Nevertheless, this approach proved to be quite successful in characterising pectins from various sources (Daas et al., 1999 + 2000). Recently, we re-examined the esterification reaction of oligogalacturonic acids in a mixture of sulphuric acid / anhydrous methanol by using the HPAEC pH5 method as well as MALDI-TOF MS (Van Alebeek, 2000a). The elution pattern of a reaction mixture after 3 hours of reaction of a pure trimer of galacturonic acid is shown in figure 5. While only four different peaks could be expected based upon the overall degree of methyl-esterification, seven peaks appeared. These peaks were identified by MALDI-TOF MS and the size/number of esters is indicated in the same figure. Subsequently, the collected fractions were further identified using post source decay MALDI-TOF MS where structural information can be obtained from internal fragmentation of the oligosaccharide. To have a more unambiguous interpretation of the data, the reducing end of the oligosaccharide was labelled with H$_2^{18}$O (Körner et al., 1999; Van Alebeek et al., 2000b). A PSD fragmentation pattern of trigalacturonic acid carrying two methyl esters on both ends is shown in figure 6. This clearly shows the enormous developments made in the identification of oligosaccharides by mass spectrometric methods. Although such MS techniques could also applied on more crude mixtures, it is usually desired to have also chromatographic separation methods available to enable more accurate quantification. More efforts are directed towards the identification of isomers of partly methyl-esterified oligomers having a DP up to 10.

Figure 5: HPAEC pH5 elution pattern showing methyl-esterified trigalacturonic acid after 3 h of incubation (4°C) in 0.02 N sulphuric acid in anhydrous methanol. 3 indicates a trimer containing 1 methyl ester.
Also the recent availability of commercial and 'easy-to-use' LC-iontrap-mass spectrometric equipment enabling MS$^3$ till MS$^5$ of carbohydrates looks rather promising.

**CONCLUSIONS**

HPAEC still is a method which is of great importance in the separation of oligosaccharides as present in food products or in enzyme digested polysaccharides. When standards are available, quantification (and identification) is not really a problem. In the case of unknown carbohydrate compounds, especially when alkali labile substituents are present, additional elution strategies including other column media in combination with rather 'inert' eluents could be quite useful. When no inorganic salts are included in such eluents, evaporative light scattering detection looks rather promising in detecting and quantifying (unknown) compounds. Off-line coupling of HPAEC / HPLC to MALDI-TOF MS (different columns and eluents tested), assisted by on-line desalting (when salts are present) and automated handling of the fraction makes peak identification relatively simple. Mass spectrometric fragmentation of collected fractions obtained from an analytical separation may permit an almost univocal identification of oligosaccharides when additional information is available about the sugar composition of the starting polysaccharide, the mode of action of the enzyme used, etc. When less background information is present or in case of quite complex structures, additional efforts (e.g. laborious purification protocols followed by NMR spectroscopy) are necessary to reveal the absolute chemical fine structure.

**REFERENCES**


