ELSEVIER

Carbohydrate Polymers 44 (2001) 161–165

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Mass determination of oligosaccharides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry following HPLC, assisted by on-line desalting and automated sample handling

M.A. Kabel, H.A. Schols, A.G.J. Voragen*

Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Bomenweg 2, 6703 HD, Wageningen, Netherlands

Accepted 22 March 2000

Abstract

The off-line coupling of analytical high-performance anion-exchange chromatography (HPAEC) to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is described. The system was applied to the analysis of neutral and acidic xylo-oligosaccharides. For MALDI-TOF MS, on-line desalting of the HPAEC eluent was performed using an anion self regenerating suppressor (ASRS) in series with a cation self-regenerating suppressor (CSRS). The ASRS permitted the exchange of acetate ions with hydroxide ions while the CSRS permitted the exchange of sodium ions with hydronium ions. The continuous desalting of the eluent was achieved by the electrolysis of pure water in both suppressors. Following automated fractionation after HPAEC separation using a 96-well plate fraction collector and computer-controlled MALDI-TOF MS sample preparation using a robot are applied as well. The complete process from HPAEC separation at analytical scale to MALDI-TOF MS could be performed most conveniently, giving molecular mass information overcoming the rather unpredictable HPAEC elution behavior of (unknown) oligosaccharides.

Keywords: Xylan; Oligosaccharides; Identification; High-performance anion-exchange chromatography; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; On-line desalting

1. Introduction

A relatively new area of research focuses on the functional properties of oligosaccharides. Some of the qualities attributed to such oligosaccharides are protection against colon cancer bacteria (Crittenden & Playne, 1996; Voragen, 1998), anti-infective properties (Zopf & Roth, 1996) and stimulation of the growth of beneficial gut bacteria (Crittenden & Playne, 1996; Voragen, 1998). Furthermore, the identification of oligosaccharides formed upon enzymatic degradation of polysaccharides has proven to be helpful to elucidate the fine structure of the corresponding polymer (Voragen, Schols & Gruppen, 1993).

HPAEC with pulsed amperometric detection (PAD) is a widely used method for the separation of a variety of oligosaccharides (Lee, 1996). However, since the elution behavior of the various types of oligosaccharides is rather unpredictable and oligomer standards are frequently not available, the identification of complex oligosaccharide mixtures is difficult (Lee, 1996). Consequently, subsequent characterization by using MS or NMR is required in order to reveal the exact structures of the separated oligosaccharides. Nevertheless, for both techniques usually a labor-intensive sample preparation including a desalting step is needed (Brüll et al., 1998; Daas, Arisz, Schols, de Ruiter & Voragen, 1998). Another disadvantage for NMR analysis is that relatively large amounts (0.5–1 mg) of purified material are required (Daas et al., 1998).

From literature, it is known that the on-line coupling of HPAEC to MS, using thermospray or electronspray (ES), is successful in the analysis of oligosaccharides (van der Hoeven et al., 1998; Niessen, van der Hoeven, van der Greef, Schols, Voragen & Bruggink, 1993). Nevertheless, the feasibility of a permanent on-line application is low due to the extended experimental set-up. Also, specific knowledge and experience is required to obtain usable mass spectra. Daas et al. (1998) showed that off-line HPAEC on preparative scale combined with the relatively simply operable MALDI-TOF MS appeared to be quite useful to determine the molecular masses of oligosaccharides in e.g. an enzyme digest. However, due to the high pH of the mobile phase used for HPAEC (pH $\approx$ 12) oligomers will not be

* Corresponding author. Tel.: +31-0-317-482888; fax: +31-0-317-484893.

E-mail address: fons.voragen@chem.fdsci.wau.nl (A.G.J. Voragen).

0144-8617/01/$ - see front matter
PII: S0144-8617(00)00203-4

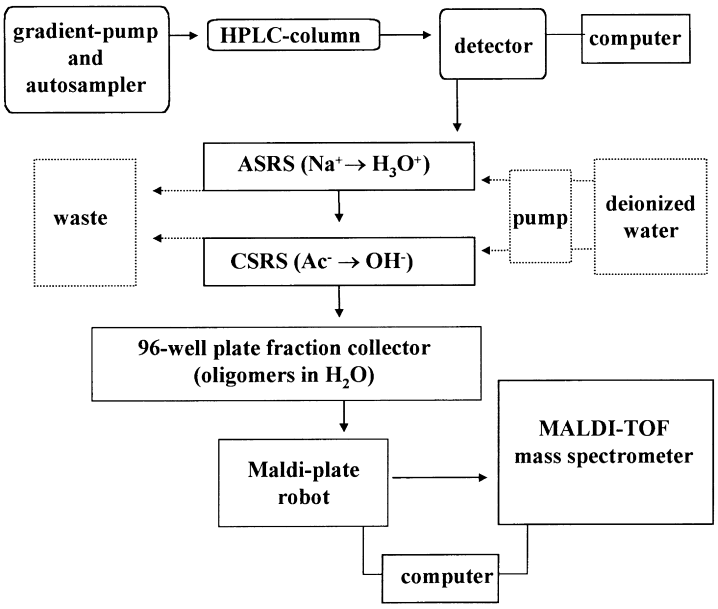

Fig. 1. Schematic overview of the experimental setup for automated coupling of HPAEC to MALDI-TOF MS.

stable for a long time and because of that, the pH of the eluent should be neutralized directly after separation. Additionally, large amounts of sodium acetate, which are present in the mobile phase of HPAEC, decrease the signal on the MALDI-TOF mass spectrometer enormously, making desalting of the mobile phase necessary.

On-line membrane suppressors, which can remove sodium from the mobile phase before analysis with thermospray MS (van der Hoeven, Niessen, Schols, Bruggink, Voragen & van der Greef, 1992; Niessen et al., 1993; Schols et al., 1994a), or before analysis with MALDI-TOF MS (Thayer, Rohrer, Avdalovic & Gearing, 1998) have already been described to be successful. But, in all these studies large amounts of regenerating acids (e.g. H_2SO_4) were used, which may cause artefacts (Niessen et al., 1993; Schols et al., 1994a). Furthermore, in these applications only sodium is removed from the mobile phase of HPAEC, leaving the oligosaccharides in acetic acid. This acetic acid disturbs the crystallization of the samples for MALDI-TOF analysis. In another study, an on-line microdialysis membrane system was used in combination with ESMS. However, this system was only capable of desalting the eluent within a pH range between 4 and 11 at a flow of 10–20 μl/min. Also, only oligomers with a mass above 1000 Da remained for further analysis (Liu & Verma, 1999). Off-line desalting of preparative HPAEC separated oligosaccharides to perform both ESMS and MALDI-TOF MS was achieved by acid-catalyzed per-*O*-acetylation of the oligosaccharides (Brüll et al., 1998). For sequential analysis, a less time-consuming and less laborious sample preparation for MALDI-TOF analysis will be convenient.

In this paper, we describe a rapid method for the on-line desalting of HPAEC eluent at an analytical scale with the use of a cation membrane suppressor in series connected to an anion membrane suppressor. Subsequent analysis of the separated and on-line desalted oligosaccharides by using MALDI-TOF MS after automated MS sample preparation is described as well.

2. Experimental

Xylo-oligosaccharide-mixtures were prepared by hydrothermal treatment of *Eucalyptus* wood (17 min; 175°C) at the Department of Chemical Engineering of the University of Vigo (Spain). The xylo-oligosaccharides (20 mg) were dissolved in 1.2 ml of 0.05 M NaOH and saponified overnight at 4°C. The mixture was neutralized with 0.6 ml of 0.1 M acetic acid and the final buffer concentration was set to 50 mM NaOAc pH 5 with 0.2 ml of 50 mM NaOAc pH 5. The sugar composition of the mixture was determined as described by Verbruggen, Beldman, Voragen and Hollemans (1993).

HPAEC was performed on a Dionex system equiped with a CarboPac PA-1 column (250 × 4 mm) in combination with a CarboPac PA guard column (25 × 3 mm) and PAD-detection (Lee, 1996). Elution (1 ml/min) was performed with a combination of linear gradients of 50–90 mM sodium acetate in 100 mM NaOH during 0–5 min, 90–130 mM sodium acetate in 100 mM NaOH during 10 min, followed by 15-min linear gradient to 520 mM sodium acetate in 100 mM NaOH (Schols, Voragen & Colquhoun, 1994b). One hundred microliters of the saponified sample (15 mg/ml) was injected and separated on the column.

After the detector, two desalting units (Dionex) were connected in series (Fig. 1). The ultra-self-regenerating anion suppressor 4 mm-unit (ASRS) was connected first to exchange the sodium ions for hydronium ions (H_3O^+). Next,

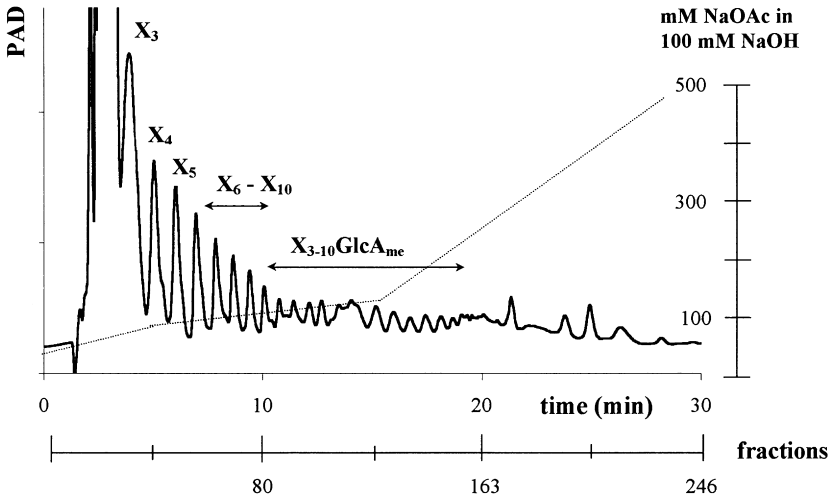

Fig. 2. HPAEC elution profile of a xylo-oligosaccharide mixture obtained after hydrothermal treatment of *Eucalyptus* wood, including the used HPAEC gradient and fractionation (X = xylose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid).

the ultra-self-regenerating cation suppressor 4 mm-unit (CSRS) was installed to exchange the acetate ions for hydroxide ions (OH^-). The continuous desalting of the eluent was achieved by the electrolysis of deionized water (8 ml/min) in both suppressors. Fractions (120 μl) were collected in a 96-well plate, using a Gilson FC-203B fraction collector.

For MALDI-TOF MS analysis, 1 μl of each fraction was automatically transferred from the 96-well-plate to a MALDI-sample-plate and mixed with 1 μl of matrix by using a Symbiot-I robot (PerSeptive Biosystems) and was allowed to dry at room temperature. The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg 1-hydroxyisoquinoline in a 1-ml mixture of acetonitrile:water (300 μl:700 μl). MALDI-TOF mass spectra were obtained from all fractions using a Voyager-DE RP workstation (PerSeptive Biosystems), operated as described by Daas, Meyer-Hansen, Schols and Voragen (1999). The mass spectrometer was calibrated with a mixture of maltodextrins.

3. Results and discussion

The elution profile of the HPAEC separation of an *Eucalyptus* wood hydrolysate, prepared by hydrothermal treatment, is shown in Fig. 2. The sugar composition of the mixture showed that the oligomers in the mixture mainly consist of xylose and uronic acid (xylose 71 mol%; uronic acid 12 mol%), while hardly any arabinose is present (3 mol%). Van der Hoeven et al. (1992) described that β-1,4-xylo-oligomers up to DP 10 elute within 10 min from the column at the gradient used. Therefore, the first 10 peaks were expected to be xylo-oligomers up to DP 10. Nevertheless, the chromatogram (Fig. 2) presents a complex pattern and further characterization was needed to define the separated oligosaccharides. Furthermore, since xylan from *Eucalyptus* wood is reported to be branched with 4-*O*-methylglucuronic acid, xylo-oligosaccharides containing a glucuronic acid are expected to be present in the hydrolysate after hydrothermal treatment (Timell, 1964). While these acidic oligomers all elute after the neutral decamer of β-1,4-xylose residues (Puls, Tenkanen, Korte & Poutanen, 1991), identification of the various components on basis of their elution behavior was impossible (Fig. 2). Subsequent MALDI-TOF MS is performed to confirm the presence and elution time of the expected oligomers.

To perform MALDI-TOF MS, without being hindered by huge amounts of salts, the HPAEC eluent was desalted online by using two suppressors in series. The ASRS permitted the exchange of sodium ions for H_3O^+ and the CSRS the exchange of acetate ions for OH^-. The conductivity of the eluent was measured after collecting 5 ml-fractions. Before and after desalting, the conductivity was 20 and 0.07 mS (0–5 min); 180 and 0.06 mS (5–10 min); 190 and 0.08 mS (10–15 min); 200 and 0.05 mS (15–20 min); 230 and 0.52 mS (20–25 min); 260 and 6 mS (25–30 min), respectively. These numbers illustrate that the combined use of an ASRS and a CSRS resulted in an almost complete desalting of the eluent, leaving the separated oligosaccharides in almost pure water. However, the concentration of sodium-ions in the desalted eluent was still high enough to form sodium adducts of the oligosaccharides after crystallization, which is necessary for detection in MALDI-TOF analysis. To allow good performance of MALDI-TOF MS, the desalting was sufficient up to fraction 170 (~21 min), which corresponds with an eluent concentration of 300 mM NaOAc in 100 mM NaOH. Similar results have been reported by van der Hoeven et al. (1992). They describe that by using two anion membrane suppressors in series, the sodium ions could be removed sufficiently to allow thermospray MS detection as long as the sodium concentration in the HPAEC eluent did not exceed 0.4 mol/l (van der Hoeven et al., 1992). The pH of all fractions up to fraction

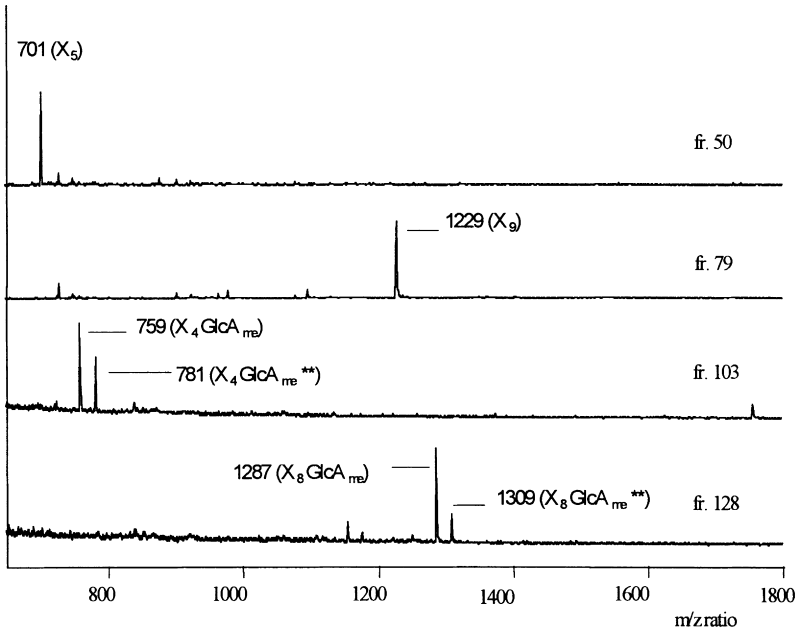

Fig. 3. MALDI-TOF mass spectra of four fractions separated by HPAEC of a xylo-oligosaccharide mixture obtained after hydrothermal treatment of *Eucalyptus* wood (see Fig. 2). ** indicates the acidic oligomers including two sodium ions.

170 (~21 min) had a value between 6 and 7. Only when the ASRS power supply was set at 500 mA and the CSRS power supply at 300 mA, the pH of the remaining eluent was kept neutral. The continuous desalting of the eluent was achieved by the electrolysis of deionized water in both suppressors. Large amounts of regenerating acids, used in most desalting units described in literature, were avoided in this way (Niessen et al., 1993; Schols et al., 1994a).

The separated and desalted fractions were collected on-line in three 96-well plates. Automatic transfer of the fractions from the 96-well plates to MALDI-sample-plates was performed by using a Symbiot-I robot. This process is fully automated and the three plates were prepared for MALDI-TOF analysis within 1 h. MALDI-TOF mass spectra were collected for all fractions. Examples of such spectra are shown in Fig. 3. The first 10 compounds, which were eluted from the column, were separated well giving unambiguous mass spectra. Indeed, the mass spectra confirmed that these compounds were xylose-oligomers with a DP from 3 up to 10. After an elution time of 10 min (fraction 80), the mechanism of separation became less distinct, since more homologous series are eluted with decreasing resolution. This resulted in the detection of two or more masses per fraction. However, a series of xylo-oligomers containing one 4-*O*-methyl-glucuronic acid group (DP 2 up to 9) could be clearly distinguished. Spectra of fraction 103 and 128 (Fig. 3) show the presence of the acidic xylo-oligosaccharides, represented as single sodium adducts. In addition to the strong signal of the single sodium adduct of the acidic oligomers (e.g. mass 759), also a weaker signal of oligomers including two sodium ions were detected (Fig. 3). According to their masses, these ions were not doubly charged ions but single sodium adducts including a second sodium minus a mass of 1. It is anticipated that a hydronium ion from the acid group is exchanged with a sodium and a mass of the sugar plus two sodium ions minus a hydronium occurred.

After separation of the oligomers in the mixture, good signals were detected of masses, which were less abundantly present and were not recognized in the mass spectrum of the initial mixture (results not shown). This is most likely due to both the higher background in the mass spectrum of the initial mixture and the fact that if the number of components per sample decreases, the laser energy per component increases and therefore also the intensity of the detected mass (Harvey, 1996).

At this moment, the quantification of the oligomers present is still difficult, since complex oligomer standards are hardly available and quantification with MALDI-TOF MS is not possible yet (Harvey, 1996). Also, mono- and disaccharides are not detectable, because MALDI-TOF MS only provides masses above 400 Da, as a result of interference with the matrix.

Because fractions are collected in a 96-well plate, the oligosaccharides remain for MALDI-TOF analysis as well as for other MS analysis. Even MS/MS, to characterize the separated oligosaccharides in more detail, could be performed much easier compared to on-line LC/MS analysis because analysis could be performed per fraction. Another possibility to characterize the separated oligomers in more detail is post-source decay fragmentation, observed on a MALDI-TOF apparatus. Molecular masses of fragments, caused by glycosidic cleavages between the monomer residues, can be analyzed to obtain information on the

original chemical structure of the oligosaccharides (Harvey, Naven, Kuster, Bateman, Green & Critchley, 1995).

Instead of HPAEC at high pH, other HPLC-methods and separation of various (non-sugar) components (e.g. peptides) could be used very easily. Separation of charged galacturonic acid oligomers already has been carried out in our department using HPAEC at pH 5 (Daas et al., 1998, 1999). An advantage of HPAEC at pH 5 instead of at pH 12 is that acetyl- or methyl-groups will not be removed by the eluent. Coupling to MALDI-TOF MS according to the method described provide data about the number of ester-groups linked to the separated oligomers. The results obtained were promising, although the level of current supply of the CSRS and ASRS to desalt eluent with different compositions may differ for optimal results. Even when elution is performed using only water and/or organic modifiers (e.g. methanol and acetonitrile), of which no desalting is required, the described, automated method provides a rapid alternative for the characterization of fractionated oligosaccharides.

The HPAEC separation, on-line desalting, automated preparation of the MALDI sample-plates and collecting of the MALDI-TOF mass spectra could all be performed without much effort, allowing a convenient first characterization of all oligomers in a complex mixture. Moreover, only a minimum of sample is required before analysis because the method used is based on separation and identification at an analytical scale.

Acknowledgements

This research is part of the EC/ FAIR-project n^{o} PL973811. The authors would like to thank Dr J.C. Parajó and G. Garrote (University of Vigo, Spain) for kindly providing the *Eucalyptus* wood hydrolysate. We are grateful to Dr A. Oosterveld and Ing. E.J. Bakx for their contribution to this work.

References

Brüll, L., Huisman, M., Schols, H., Voragen, F., Critchley, G., Thomas-Oates, J., & Haverkamp, J. (1998). Rapid molecular mass and structural determination of plant cell wall derived oligosaccharides using off-line high-performance anion-exchange chromatography/mass spectrometry. *Journal of Mass Spectrometry, 33*, 713–720.

Crittenden, R. G., & Playne, M. J. (1996). Production, properties and applications of food-grade oligosaccharides. *Trends in Food Science & Technology, 7*, 353–361.

Daas, P. J. H., Arisz, P. W., Schols, H. A., de Ruiter, G. A., & Voragen, A. G. J. (1998). Analysis of partially methyl-esterified galacturonic acid oligomers by high-performance anion-exchange chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Analytical Biochemistry, 257*, 195–202.

Daas, P. J. H., Meyer-Hansen, K., Schols, H. A., de Ruiter, G. A., & Voragen, A. G. J. (1999). Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research, 318*, 135–145.

Harvey, D. J. (1996). Assisted laser desorption/ionization mass spectrometry of oligosaccharides and glycoconjugates. *Journal of Chromatography A, 720*, 429–466.

Harvey, D. J., Naven, T. J. P., Kuster, B., Bateman, R. H., Green, M. R., & Critchley, G. (1995). Comparison of fragmentation modes for the structural determination of complex oligosaccharides ionized by matrix-assisted laser desorption ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 9*, 1557–1561.

van der Hoeven, R. A. M., Niessen, W. M. A., Schols, H. A., Bruggink, C., Voragen, A. G. J., & van der Greef, J. (1992). Characterization of sugar oligomers by on-line high-performance anion-exchange chromatography–thermospray mass spectrometry. *Journal of Chromatography, 627*, 63–73.

van der Hoeven, R. A. M., Tjaden, U. R., van der Greef, J., van Casteren, W. H. M., Schols, H. A., Voragen, A. G. J., & Bruggink, C. (1998). Recent progress in high-performance anion-exchange chromatography/ionspray mass spectrometry for molecular mass determination and characterization of carbohydrates using static and scanning array detection. *Journal of Mass Spectrometry, 33*, 377–386.

Lee, Y. C. (1996). Carbohydrate analysis with high-performance anion-exchange chromatography. *Journal of Chromatography A, 720*, 137–149.

Liu, C., & Verma, S. S. (1999). Direct coupling of ionic high-performance liquid chromatography with electrospray ionization mass spectrometry utilizing a microdialysis junction interface. *Journal of Chromatography A, 835*, 93–104.

Niessen, W. M. A., van der Hoeven, R. A. M., van der Greef, J., Schols, H. A., Voragen, A. G. J., & Bruggink, C. (1993). Recent progress in high-performance anion-exchange chromatography–thermospray mass spectrometry of oligosaccharides. *Journal of Chromatography, 647*, 319–327.

Puls, J., Tenkanen, M., Korte, H. E., & Poutanen, K. (1991). Products of hydrolysis of beechwood acetyl-4-*O*-methylglucuronoxylan by a xylanase and an acetyl xylan esterase. *Enzyme Microbiology Technology, 13*, 483–487.

Schols, H. A., Mutter, M., Voragen, A. G. J., Niessen, W. M. A., van der Hoeven, R. A. M., van der Greef, J., & Bruggink, C. (1994a). The use of combined high-performance anion-exchange chromatography–thermospray mass spectrometry in the structural analysis of pectic oligosaccharides. *Carbohydrate Research, 261*, 335–342.

Schols, H. A., Voragen, A. G. J., & Colquhoun, I. J. (1994b). Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. *Carbohydrate Research, 256*, 97–111.

Thayer, J. R., Rohrer, J. S., Avdalovic, N., & Gearing, R. P. (1998). Improvements to in-line desalting of oligosaccharides separated by high-pH anion exchange chromatography with pulsed amperometric detection. *Analytical Biochemistry, 256*, 207–216.

Timell, T. E. (1964). Wood hemicelluloses: part I. *Advanced carbohydrate chemistry. Biochemistry, 19*, 247–301.

Verbruggen, M. A., Beldman, G., Voragen, A. G. J., & Hollemans, M. (1993). Water-unextractable call wall material from Sorghum: isolation and characterization. *Journal of Cereal Sciences, 17*, 71–82.

Voragen, A. G. J. (1998). Technological aspects of functional food-related carbohydrates. *Trends in Food Science & Technology, 9*, 328–335.

Voragen, A. G. J., Schols, H. A., & Gruppen, H. (1993). Structural studies of plant cell-wall polysaccharides using enzymes. In F. Meuser, D. J. Manners & W. Seibel, *Proceedings of an international symposium on plant polymeric carbohydrates* (pp. 3–15). Cambridge: Royal Society of Chemistry.

Zopf, D., & Roth, S. (1996). Oligosaccharide anti-infective agents. *The Lancet, 347*, 1017–1021.