JOURNAL OF MASS SPECTROMETRY, VOL. 33, 334–341 (1998)

Microdialysis Introduction High-performance Anion-exchange Chromatography/Ionspray Mass Spectrometry for Monitoring of On-line Desalted Carbohydrate Hydrolysates

N. Torto,[1]*† A. Hofte,[2] R. van der Hoeven,[2] U. Tjaden,[2] L. Gorton,[1] G. Marko-Varga,[3] C. Bruggink[4] and J. van der Greef[2]

[1] Department of Analytical Chemistry, University of Lund, P.O. Box 124, S-221 00, Lund, Sweden
[2] Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, P.O. Box, 2300 RA Leiden, The Netherlands
[3] Astra Draco AB, Pre-Clinical Research and Development, P. O. Box 34, S-221 00 Lund, Sweden
[4] Dionex BV, P.O. Box 9338, 4801 LH Breda, The Netherlands

A system for the determination of carbohydrates based on on-line microdialysis introduction high-performance anion-exchange chromatography integrated pulsed electrochemical detection/mass spectrometry (MS) using ionspray as the ionization technique, with on-line desalting, is presented. This coupling of techniques utilizes a cation-exchange membrane desalting device (CEMDD) which permitted the exchange of sodium ions with hydronium ions when sodium ion concentrations up to 600 mM were used to separate and elute oligosaccharides. The effective on-line desalting by the CEMDD of the effluent before its introduction into the mass spectrometer was achieved by the electrolysis of water with a 500 mA current. The use of the CEMDD in combination with ionspray eliminated the need for a booster pump and also the regenerating water improved the sensitivity in comparison with the use of sulphuric acid as oligosaccharides could be detected down to 3 μg ml^{-1}. The system was developed and used to monitor cationized oligosaccharides based on their singly $[M + Na]^+$ and doubly sodiated $[(M + 2Na)/2]^{2+}$ molecules during the hydrolysis of wheat starch. The MS/MS properties of maltose, trehalose and sucrose were used in combination with retention times to identify these disaccharides based on their dissociation patterns, which are characteristic of the type of glycosidic linkage. © **1998 by John Wiley & Sons, Ltd.**

J. Mass Spectrom. **33**, 334–341 (1998)

KEYWORDS: mass spectrometry; microdialysis; high-performance anion-exchange chromatography; desalting; carbohydrates; ionspray

INTRODUCTION

The contrast between the structural resemblance of carbohydrates and the differences in their chemical and physical properties because of their different glycosidic linkages (sites), branching characteristics and different properties have put an impetus in the search for an analytical technique that understands their complexity. The unequivocal determination of carbohydrate structures, composition and concentration is very important in the biotechnological, pharmaceutical and medical research areas. More notably, characterization of hydrolytic properties of novel enzymes requires a substrate whose composition is well known since the hydrolysis products have to be identified. Such an endeavour can only be realized if techniques with specific capabilities are combined, as exemplified herein. On-line sampling, sample clean-up and sample introduction by microdialysis are combined with the high separation power of high-performance anion-exchange chromatography (HPAEC). Further, the sensitivity of integrated pulsed electrochemical detection (IPED) allows the direct detection of carbohydrates as a complementary technique to mass-selective mass spectrometry (MS).

Most carbohydrates can be separated in their enolate form at high pH by HPAEC with sodium acetate gradients up to 500 mM. Application of HPAEC is desirable because underivatized carbohydrates can be detected using the sensitive IPED. Simpson *et al.*[1] have shown the feasibility of interfacing HPAEC with MS using an anion micromembrane suppressor (AMMS). Niessen and co-workers[2,3] demonstrated the coupling of HPAEC with thermospray (TSP) MS utilizing two AMMSs for the analysis of a wide variety of neutral and acidic oligosaccharides based on singly and doubly charged cationization of the oligosaccharides. In addition to at least two AMMS modules for interfacing TSP to HPAEC, a booster pump is also required in order to prevent the damage to the membrane owing to the high back-pressure induced by the TSP probe. In addition to off-line sample work-up, the use of large amounts of regenerating 0.1 M sulphuric acid is also undesirable. In

* *Correspondence to*: N. Torto, Department of Analytical Chemistry, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden.
E-mail: nelson.torto@analykem.lu.se
Contract grant sponsor: NUTEK
† On leave from the Department of Chemistry, University of Botswana, P/Bag 0022 Gaborone, Botswana.

CCC 1076–5174/98/040334–08 $17.50

Received 28 October 1997
Accepted 7 January 1998

this paper, we report the coupling of microdialysis introduction HPAEC/ionspray MS with on-line desalting of carbohydrates utilizing a single cation-exchange membrane desalting device (CEMDD) based on the electrolysis of pure water, without using a booster pump.

The coupling of on-line sampling, sample introduction and desalting techniques eliminates the two bottlenecks of carbohydrate analysis using MS. Microdialysis has been demonstrated to be well suited to efficient sampling of polar analytes in a continuously changing biomatrix while precluding larger interfering proteinaceous species in pharmacokinetic and neurochemical studies.[4] Recently, Torto and co-workers[5,6] have shown its applicability to sampling in complex, small-volume enzymatic bioprocesses at temperatures as high as 90 °C. In this paper it is shown that a substantial reduction in analytical steps which are undesirable for low analyte concentrations is achieved by coupling microdialysis introduction HPAEC/ionspray MS to the CEMDD. The CEMDD utilizes a countercurrent flow to exchange on-line, efficiently, effluent sodium ions (Na^+) for hydronium ions (H_3O^+) via a pair of cation-exchange membranes, thus generating acetic acid. The continuous desalting of effluent Na^+ is realized by the electrolysis of pneumatically supplied pure water at 500 mA instead of 0.1 M sulphuric acid which has been utilized in other desalting/suppression systems.[1,2,7,8]

In the work reported here, an efficient methodology for the desalting of oligosaccharides for microdialysis introduction HPAEC/ionspray MS has been developed. This approach utilizes the CEMDD which allowed on-line desalting of oligosaccharide samples during the on-line monitoring of the hydrolysis of wheat starch for 2 h. The chromatographically clean analytes obtained by microdialysis were separated by HPAEC and monitored as cationized molecules. Such a set-up affords higher reproducibility as it eliminates laborious manual sample handling steps. The hydrolysis products and contaminants were confirmed by their glycosidic linkage using MS/MS and chromatographic retention of standards. The advantage of this coupling is the ability to show the different types of saccharides produced during the hydrolysis and also possible contaminants which could affect the characterization of enzyme hydrolytic properties.

EXPERIMENTAL

Reagents

Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, sucrose and trehalose were obtained from Boehringer Mannheim (Mannheim, Germany). Propan-2-ol, sodium hydroxide (50%, w/w) and sodium acetate were obtained from Baker (Deventer, The Netherlands). Termamyl, was a gift from Novo Industries (Bagsvaerd, Denmark) and the wheat starch was a gift from the Swedish Starch Industry (Kristiandstad, Sweden).

Instrumental set-up

The experimental set-up of the microdialysis introduction HPAEC/ionspray MS set-up is shown schematically in Fig. 1. The system consists of a custom-made *in situ* tunable microdialysis probe[9] supplied by Dr Thomas Laurell (Department of Electrical Measurements, Lund University, Sweden). The probe, which is fitted with an SPS 6005 polysulphone membrane (Fresenius, St Wendel, Germany), with a 10 mm effective dialysis length and a cut-off of 30 kDa, is perfused at 1 μl min^{-1} with pure water delivered by a syringe pump (CMA 160; CMA/Microdialysis, Solna, Sweden). The chromatographically clean dialysate is pumped to the metal-free chromatographic system (Dionex 300;

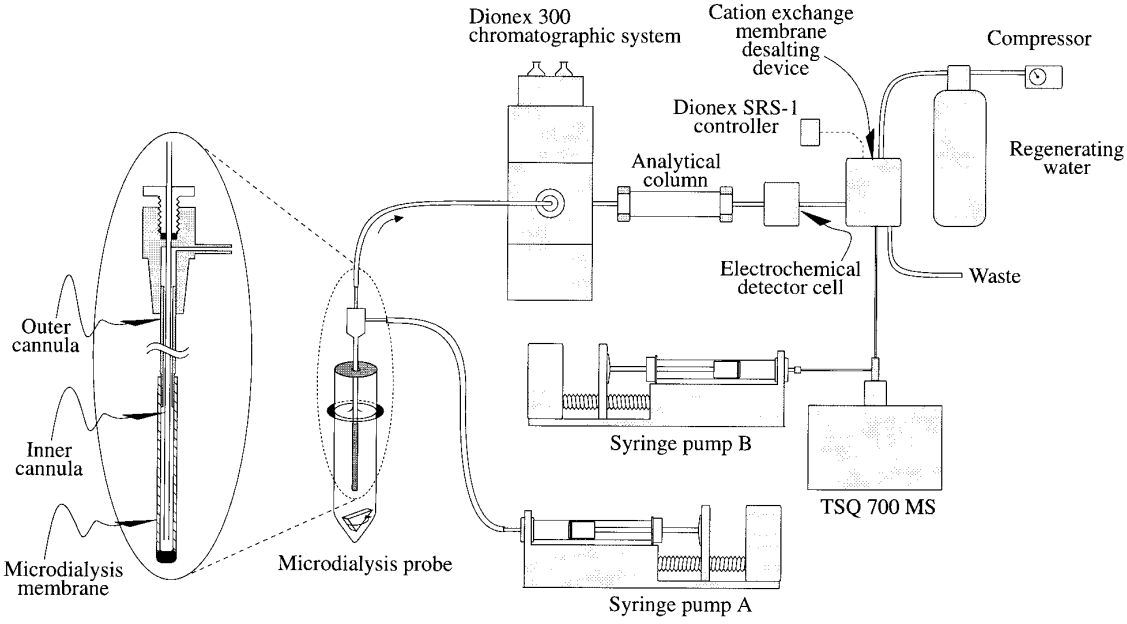

Figure 1. Schematic representation of the experimental set-up based on microdialysis introduction high-performance anion-exchange chromatography/integrated pulsed electrochemical detection/ionspray MS. The on-line desalting of carbohydrate hydrolysates is achieved using a cation-exchange membrane desalting device (CEMDD).

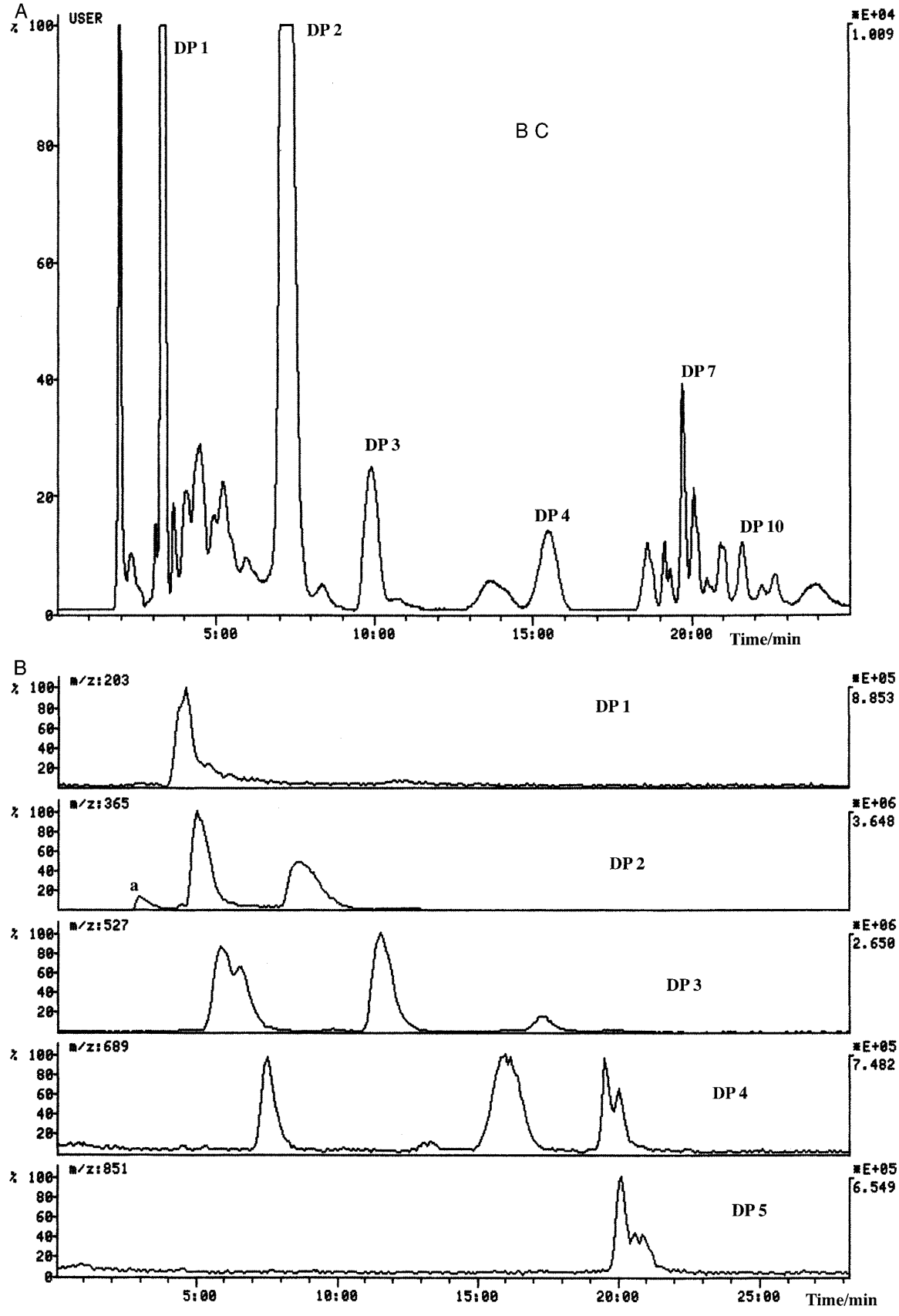

Figure 2. Chromatogram acquired using integrated pulsed electrochemical detection before the hydrolysis of wheat starch (A) and ion chromatograms acquired from wheat starch using ionspray MS before (B) and after hydrolysis (C) for 45 min. The two detection techniques are compared to show the amount of information available. The peak labelled a in the ion chromatograms shows an impurity (identified as trehalose) that elutes with disaccharides and is not a characteristic component of wheat starch.

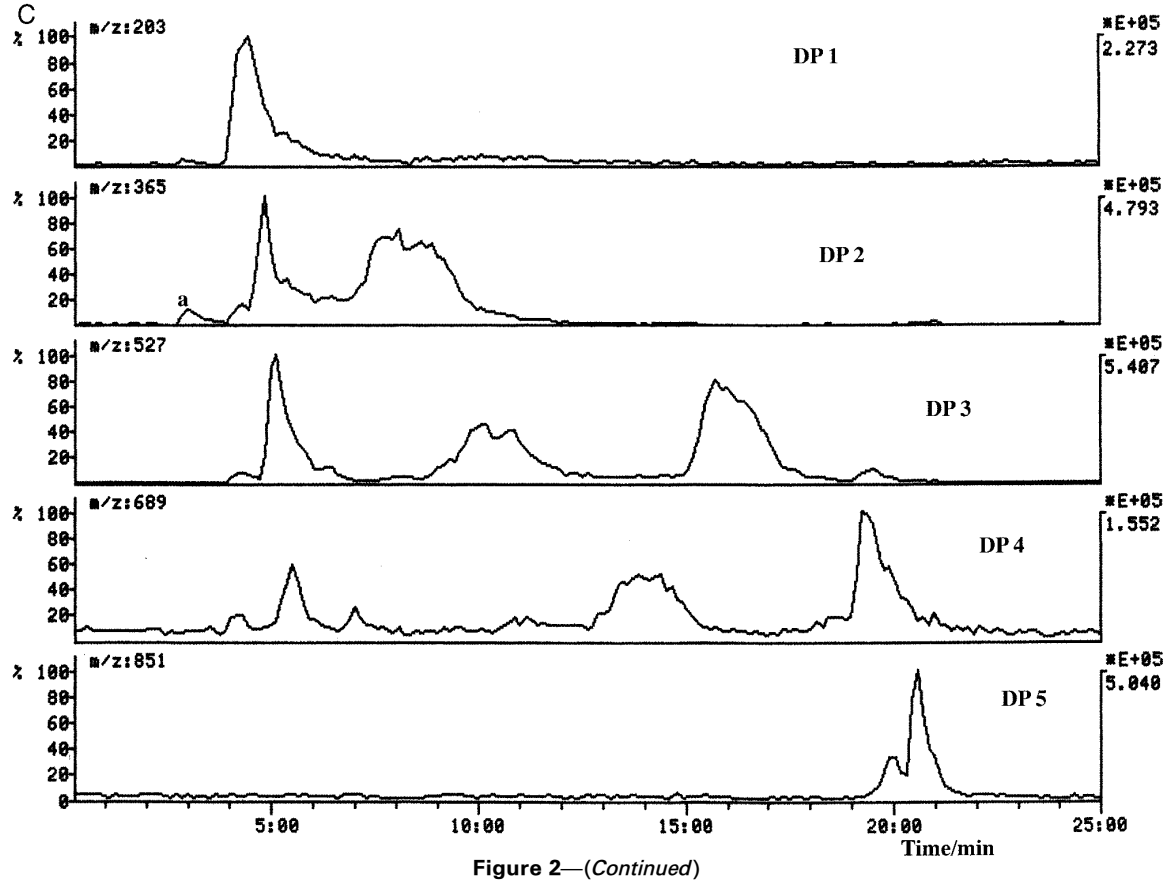

Figure 2—(*Continued*)

Dionex, Sunnyvale, CA, USA). The Dionex 300 system consists of an EDM-2 solvent-degassing unit, an AGP pump module, an LCM-3 chromatography module with a Rheodyne (Cotati, CA, USA) Model 9126 PEEK injector equipped with a 6.3 µl injection loop and a pulsed electrochemical detector. The carbohydrate hydrolysates were separated on a narrow-bore (250 × 2 mm i.d.) CarboPac PA1 column (Dionex) using a sodium hydroxide–sodium acetate gradient at a flow rate of 0.2 ml min^{-1}. The gradient composition for the separation of hydrolysates consisted of 75% water and 5% 625 mM sodium acetate for 10 min. The composition was then changed to 40% water and 40% sodium acetate and maintained thus until the end of the run. The concentration of sodium hydroxide was kept constant by a 20% contribution of 500 mM sodium hydroxide. A step gradient was then used to separate the hydrolysates after 24 h. The step gradient was used to increase the sensitivity since a mass-selective detector was used for these investigations. The proportion of the 500 mM sodium hydroxide was kept at 20%, that of water was 70% and that of 625 mM sodium acetate was 5% for 10 min. The proportion of the sodium acetate was then increased to 80% between 10 and 15 min and maintained at 80% until the end of the run.

The separated carbohydrate hydrolysates were either detected directly on-line with the IPED or they were desalted using the CEMDD and introduced into the MS system. The CEMDD was connected to the waste outlet of the electrochemical detector cell, while a helium pressure of 50 kPa was maintained using a compressor to achieve a water flow rate of 2 ml min^{-1}. Desalting of the effluent was carried out by the electrolysis of pure water at 500 mA by means of the Dionex SRS-1 controller. The H_3O^+ ions produced at the anode were exchanged for Na^+ ions via two cation-exchange membranes. The H_3O^+ ions combined with the OH^- and OAc^- ions from the effluent to form water and acetic acid, respectively. The Na^+ ions combined with OH^- ions from the cathode to form sodium hydroxide, which was collected as waste. The desalted effluent was directed to the electrospray ionization interface, without splitting, via a fused-silica capillary of 100 µm i.d. with a total length of 0.4 m. Mass chromatograms were acquired using a Finnigan MAT (San Jose, CA, USA) TSQ-700 mass spectrometer equipped with a custom-made electrospray interface as described elsewhere.[10] Owing to the absence of back-pressure in the electrospray interface, the outlet of the CEMDD was coupled directly to the MS system. The sheath liquid was propan-2-ol–water (80:20) containing 10^{-4} M sodium acetate, at a flow rate of 10 µl min^{-1}, together with nitrogen as sheath gas at a flow rate of 6 l min^{-1}. The MS conditions were optimized with respect to the sample capillary temperature, repeller voltage, helium pressure and collision energy and the spectra were acquired in the single ion monitoring (SIM) mode.

RESULTS AND DISCUSSION

During the characterization of the hydrolytic properties of an enzyme or substrate, the knowledge of the structure or composition of the starting material is very important. For a new enzyme, its hydrolytic properties

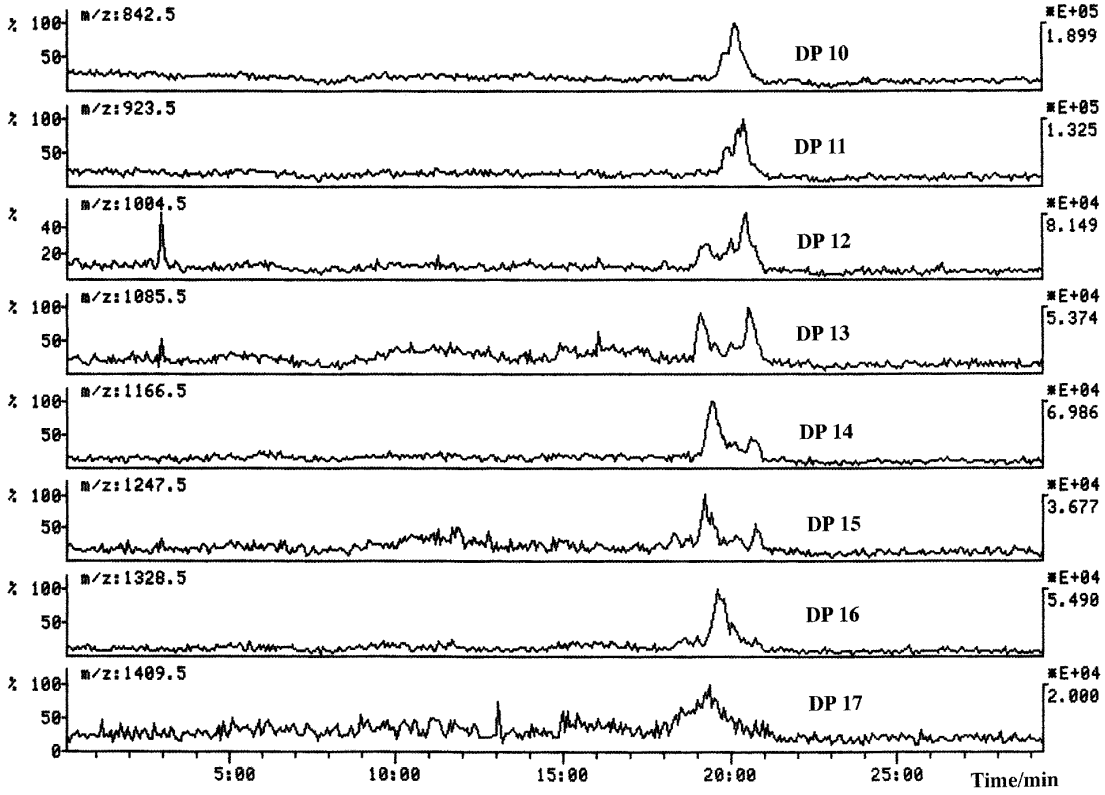

Figure 3. Ion chromatograms acquired after hydrolysis of wheat starch for 24 h and detected using ionspray MS. A single ion monitoring program for the DP 10–DP 17 oligosaccharide window was used to demonstrate the presence of α-limit dextrins which are not hydrolysed by the enzyme. The chromatogram obtained using electrochemical detection is not included as it was not informative [see Fig. 2(A)].

can only be fully understood if there are no other inhibiting or enhancing compounds in the reaction medium. For this reason, the use of HPAEC alone to study hydrolysates is limited because of the inability to separate the carbohydrates completely. Selectivity in IPED is achieved by choice of the proper detection potential and the integration time; however, owing to the presence of the same moiety, the analytes have to be separated completely to avoid overlap of the oxidized species. MS can be used to separate compounds with identical masses and, in the case of carbohydrates, using MS/MS properties, it has the capacity to differentiate between the types of glycosidic linkages. The power of MS was employed in the study of the hydrolysis of wheat starch as a complementary technique to HPAEC–IPED to allow the unequivocal characterization of oligosaccharides.

On-line hydrolysis of wheat starch

Preliminary investigations (results not shown) showed that use of water as a regenerant for the CEMDD instead of 0.1 M sulphuric acid resulted in increased sensitivity. As expected, the leakage of sulphuric acid through the membrane[8] hampered the formation of cations in the positive ionization mode. The sampling capillary was cleaned thoroughly before conducting the experiments by washing it with a water–methanol mixture. Although the sampling capillary showed some discoloration during the day, this was not associated with any decrease in signal sensitivity. For monitoring the hydrolysis, positive ionization was used with a SIM procedure based on singly $[M + Na]^+$ and doubly sodiated $[(M + 2Na)/2]^{2+}$ molecules up to a degree of polymerization (DP) of 15, based on aldohexoses.

Figure 2(A) is a chromatogram acquired using HPAEC–IPED before the hydrolysis of wheat starch [see Fig 2(B)]. This chromatogram clearly demonstrates the limitations of just using this combination of techniques because of the lack of specific information. The use of the retention times only for identification of the oligosaccharides is not sufficient. The standard additions method is not recommended as some oligosaccharides could co-elute. Figure 2(B) and (C) show the ion chromatograms of DP 1 (m/z 203)–DP 5 (m/z 851) from wheat starch acquired before and after hydrolysis for 45 min, respectively. Owing to the large dead volume at the outlet of the electrochemical detector, which appeared to be more than 100 µl, band broadening was observed. Therefore, the ion chromatograms were then acquired after disconnecting the electrochemical cell from the system. The DP 1–5 window is the most interesting in terms of the hydrolytic properties of the enzymes as it is supposed to give mostly DP 2. From the mass chromatograms, the m/z 203 (glucose) peak is broader after 45 min, showing that this industrial enzyme is not pure, as from the theoretical point of view the amounts of glucose are not supposed to increase.[11] DP 2, however, shows an increase but there is no change in size for the peak labelled a in both Fig. 2(B) and (C). The DP 3 (m/z 527), DP 4 (m/z 689) and DP 5 (m/z 851) peak profiles in Fig. 2(C) show a different profile compared with Fig. 2(B), indicating an increase in hydrolysis products.

From the mass chromatograms in Fig. 2(B) and (C), there is a type of DP 2 which elutes before DP 1, in

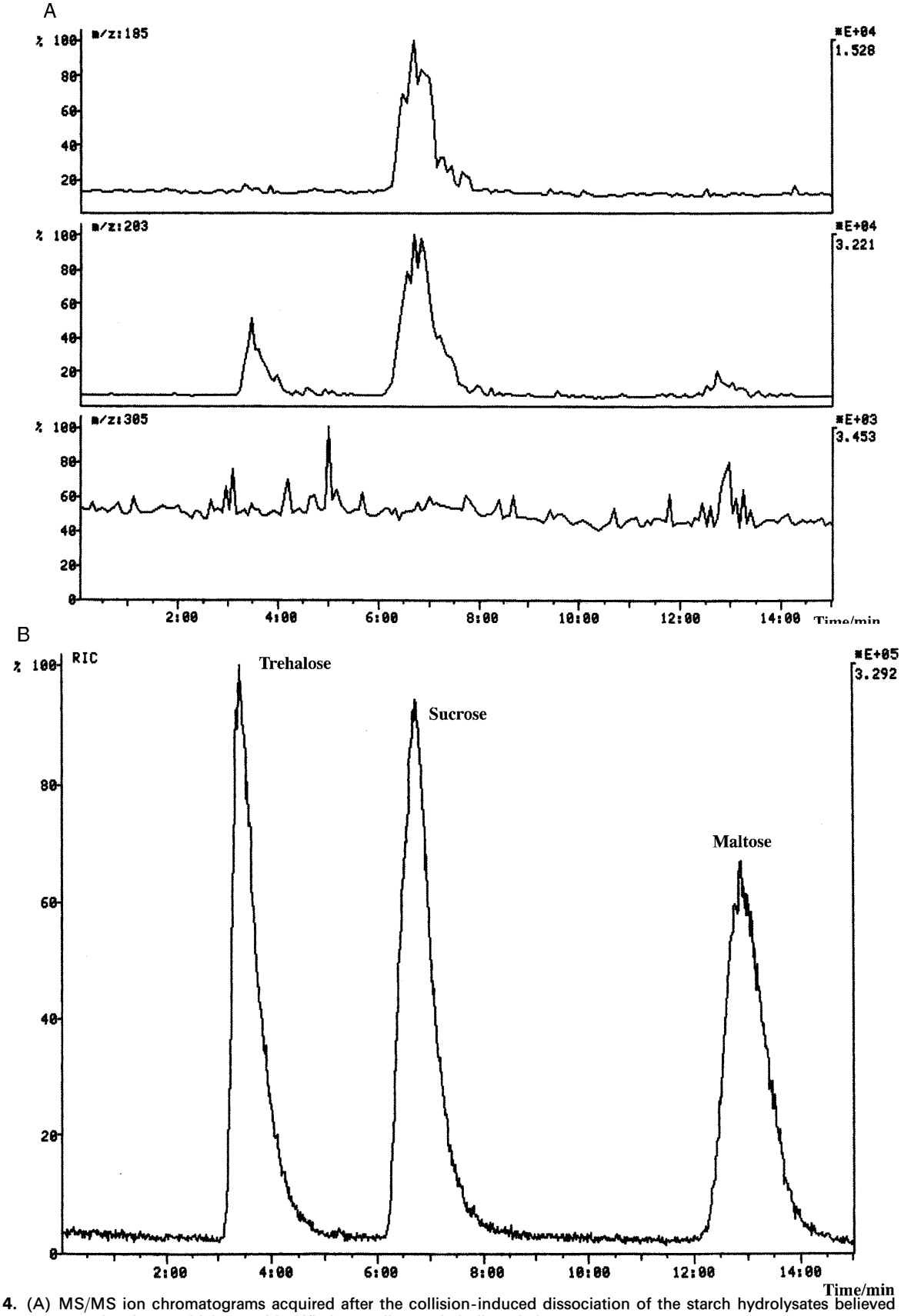

Figure 4. (A) MS/MS ion chromatograms acquired after the collision-induced dissociation of the starch hydrolysates believed to be (B) trehalose, sucrose and maltose, which were identified according to their dissociation patterns.

addition to types of DP 2, 3, 4 and 5, which elute at the same time as DP 1, although they are totally resolved by MS. From DP 2–5, the mass chromatograms show that there is more than one type of these oligasaccharides, which could be differentiated by examining the type of glycosidic linkage. Such information cannot be obtained from HPAEC–IPED alone, which shows the need to use MS as a complementary technique.

The hydrolysis of wheat starch was monitored on-line for 2 h and left to incubate for a further 22 h so that a sample taken at 24 h could be analysed using MS and HPAEC. From the initial chromatograms of unhydrolysed wheat starch, only saccharides up to DP 8 were observed, but as soon as the enzyme was added, up to DP 15 could be detected. When using HPAEC–IPED, the sample has to be diluted to allow the quantitative determination of higher oligosaccharides and owing to their low concentrations it is never clear whether there are oligosaccharides greater than DP 10 after 24 h of hydrolysis, unless post-column switching methodologies are employed. A chromatogram (not shown) acquired after 24 h with HPAEC–IPED using a sodium acetate gradient up to 500 mM shows the same characteristics as that obtained before hydrolysis [see Fig 2(A)]. The chromatogram had multiple component peaks which are difficult to identify since they co-elute. The ion chromatograms acquired after 24 h with MS are much more informative (see Fig. 3). A SIM procedure based on aldohexoses was used to acquire the ion chromatograms of DP 10–17. Although the oligosaccharides co-elute, the power of MS was used to show that although the hydrolysis of wheat starch was complete, α-limit dextrins up to DP 17 were still present in solution after 24 h. This window of ion chromatograms demonstrates that the use of a CEMDD allowed the effective desalting of effluents containing Na^+ ions up to a concentration of 600 mM, without significant contamination of the ion source or compromising the sensitivity as oligosaccharides up to 3 $\mu g\ ml^{-1}$ could be detected.

MS/MS of the hydrolysates

Tandem mass spectrometry has already been demonstrated as a useful tool for the structural elucidation of carbohydrates by comparing the fragmention of the protonated molecules in the collision cell.[12] The dissociation pattern is associated with a certain type of glycosidic linkage and can be used qualitatively.[13,14]

Within a short list of possible disaccharide glycosidic linkages according to previously acquired tandem mass spectra, the dissociations of trehalose (1,1-α-linked glucose units), sucrose (1,4-β-linked glucose and a fructose unit) and maltose (1,4-α-linked glucose units) were effected. This investigation utilized a SIM procedure that focused on the following ions counting from the $[M + Na]^+$ at *m/z* 365: *m/z* 185 (−hexose and water), 203 (−hexose), 305 (−60), 347 (−water) and 365 (precursor). Figure 4 shows the MS/MS ion chromatograms of the disaccharides from the wheat starch hydrolysate which compared with the disaccharide standards (not shown) and retention times of the standards. The dissociation profile for trehalose (see Fig. 4) gave the sodiated monosaccharide (*m/z* 203) and sucrose dissociates to a sodiated monosaccharide with (*m/z* 203) or without (*m/z* 185) water. Maltose, however, loses the C-2 and forms the sodiated saccharide. The retention times of the disaccharides were compared with those of the standards and they were found to elute at the same time, indicating that the disaccharides observed during the hydrolysis of wheat starch were possibly trehalose, sucrose and maltose. Trehalose is not expected to be a constituent of wheat starch; however the chromatograms (see Fig. 2) do not show an increase as the hydrolysis continues, indicating that it is a contaminant. The amount of sucrose increased as the enzyme was added. This increased sucrose is associated with the stabilization of the enzyme and is not a product of the hydrolysis of wheat starch, but rather a constituent of this industrial enzyme.[11] This preliminary characterization of the oligosaccharides in the bioreactor shows that although the MS/MS properties are invaluable for unequivocal identification, more standards need to be screened if this technique has to achieve something more than just "staring' into the bioreactor.

CONCLUSION

A system for the determination of carbohydrates based on microdialysis introduction HPAEC/IPED/MS using ionspray as the ionization technique, with on-line desalting, has been presented. The system utilizes an on-line cation-exchange membrane desalting device (CEMDD), which allowed the exchange of sodium ions with hydronium ions when sodium ion concentrations up to 600 mM were used to separate and elute oligosaccharides. On-line monitoring of the hydrolysis of wheat starch for 2 h was performed without any major contamination of the ion source. This opens up the possibility of structure elucidation and unequivocal identification of unkown oligosaccharides/carbohydrate polymers using MS/MS, since there is improved sensitivity that allows the handling of low concentrations of analytes without any tedious sample handling.

Acknowledgements

Dr Thomas Laurell is acknowledged for the supply of the tunable microdialysis probe, Dionex (Breda, The Netherlands) for the loan of the Dionex 300 chromatographic system and NUTEK for financial support.

REFERENCES

1. R. C. Simpson, C. C. Fenselau, M. R. Hardy, R. R. Townsend, Y. C. Lee and R. B. Cotter, *Anal. Chem.* **62**, 248 (1990).
2. W. M. A. Niessen, R. A. M. van der Hoeven, J. van der Greef, H. A. Schols, G. Lucas-Lokhorst, A. G. J. Voragen and C. Bruggink, *Rapid Commun. Mass Spectrom.* **6**, 474 (1992).
3. W. M. A. Niessen, R. A. M. van der Hoeven, J. van der Greef, H. A. Schols and A. G. J. Voragen, *Rapid Commun. Mass Spectrom.* **6**, 197 (1992).
4. C. E. Lunte, D. O. Scott and P. T. Kissinger, *Anal. Chem* **63**, 773 (1991).

5. N. Torto, T. Laurell, G. Marko-Varga and L. Gorton, *J. Membr. Sci.* **130**, 239 (1997).
6. N. Torto, G. Marko-Varga, L. Gorton, H. Stålbrand and F. Tjerneld, *J. Chromatogr. A* **725**, 165 (1996).
7. J. J. Conboy, J. D. Henion, M. W. Martin and J. A. Zweigenbaum, *Anal. Chem.* **62**, 800 (1990).
8. W. M. A. Niessen, R. A. M. van der Hoeven, J. van der Greef, H. A. Schols, A. G. J. Voragen and C. Bruggink, *J. Chromatogr*. **647**, 319 (1993).
9. T. Laurell and T. Buttler, *Anal. Methods Instrum.* **2**, 197 (1995).
10. R. A. M. van der Hoeven, B. A. P. Buscher, U. R. Tjaden and J. van der Greef, *J. Chromatogr*. **647**, 319 (1993).
11. M. Rank, Novo Nordisk, personal communication (1997).
12. R. Orlando, C. Allen and C. Fenselau, *Biomed. Environ. Mass Spectrom.* **19**, 747 (1990).
13. G. E. Hofmeister, Z. Zhou and J. A. Leary, *J. Am. Chem Soc.* **113**, 5964 (1991).
14. D. Garozzo, M. Giuffrida, G. Impallomeni, A. Ballistreri and G. Montaudo, *Anal. Chem.* **62**, 279 (1990).