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Comparison of dynamic fast atom bombardment/liquid secondary ion mass spectrometry and electrospray mass spectrometry coupled to reversed-phase liquid chromatography for the determination of oligosaccharides in seawater

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Abstract

A method has been developed for determination of non-derivatised oligosaccharides by LC–MS at low concentration levels and in complex matrices of seawater and sediment pore water. Two ionisation techniques, dynamic fast atom bombardment (FAB)/liquid secondary ion (LSI) MS and electrospray ionization (ESI), were investigated and evaluated. The electrospray using Li^+ ions as adducts proved to be superior to the FAB/LSI-MS for the determination of non-derivatised oligosaccharides. The LC separation was accomplished by the use of a C_{18} column and the elution either by a gradient of methanol–acetonitrile–water or pure water, in which latter case the column was held at an elevated temperature. We also report results from measurements of oligosaccharides in pore waters of marine sediment cores. Oligosaccharides of a range from 2 to 6 sugar units were found in a concentration range of 2–100 n*M*, the smaller sugars being more abundant. The depth profiles in the sediment cores indicate a production in the redox boundary layer and from there, diffusion in both directions to the overlaying water and further down into the sediment.

Keywords: Water analysis; Ionization methods; Oligosaccharides

1. Introduction

One of the largest pools of organic matter on the earth is dissolved in the waters of the oceans or buried in the upper sediment layers [1]. Much effort has been directed towards the chemical characterisation of this dissolved organic matter (DOM) and it has been found that a large fraction of it, 15% and up to 35% [2], consists of carbohydrates, mono-, di- and oligo-saccharides, either in their free form or bound to other organic substances such as lipids, proteins and humic substances. The total concentration of organic matter in the open ocean is in the range of 30–100 μ*M*, in deep sea sediment pore water 50–300 μ*M*, measured as carbon. As concentrations are low and the seawater matrix is complex with its content of inorganic salts as well as organic compounds it has not, until now, been possible to analyse the carbohydrate fraction of marine waters further than to determine monosaccharides. However, with a preceding hydrolysation step the total amount of larger saccharides have been estimated.

1.1. Separation of oligosaccharides

Oligosaccharides can be separated by gas chroma-

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tography (GC), supercritical fluid chromatography (SFC), liquid chromatography (LC) and capillary zone electrophoresis (CZE).

The GC and SFC methods demand derivatisation (methylation, ethylation or silylation) prior to separation. For GC, column bleeding and thermal degradation of the analytes, lead to an upper limit of sugar chain length of about 5–10 sugar units, provided the concentrations are reasonably high. Despite these drawbacks GC and GC–MS have been the most frequently used methods.

With the use of CZE it is possible to separate compounds with very similar structures as well as quite large molecules. Serious drawbacks are that the extremely small injection volumes demand high concentrations of the analytes, and derivatisation is needed to improve detection. The lack of sensitivity is the reason, so far, that the CZE–MS method has not been used for seawater samples.

The remaining separation technique mentioned above is LC. During the last decade, two methods of coupling LC to MS have become common, namely dynamic fast atom bombardment (FAB)/liquid secondary ion (LSI) ionization [3] and electrospray ionization (ESI) [4].

Several different separation techniques for saccharides using LC have been published [5]. However, it has not been shown that it is possible to use LC–ESI-MS for low level seawater samples.

We are also, in parallel with our work on analytical methods, developing a technique for desalting and preconcentration of seawater samples.

1.2. Derivatisation

A common technique is pre-column derivatisation of the oligosaccharides before analysis by LC–MS, because saccharides have no chromo- or fluorophoric groups in a suitable wavelength range for spectroscopic detection (the most cost-effective method for routine analysis after mass spectrometric identification of peaks, especially for screening of large amounts of not too complex sample matrices). Neutral sugar molecules have no natural sites for protonation by surrounding water, the existing hydroxyl and carbonyl protons are only slightly acidic (the first $pK_a \sim 13$). Derivatisation by adding a hydrophobic and chargeable group to oligosaccharides was reported to increase the sensitivity in the MS detection (e.g., [6]). It also facilitated on-column preconcentration by the use of reversed-phase chromatography with a gradient elution and large injection volumes of water matrices.

Derivatisation is often, however, a labour intensive method with a time-consuming purification step both before and afterwards. The yields are less than 100% and unequal for different sugars in a mixture, which is a major drawback. Finally, the use of a reducing reagent mixture to split the sugar unit from e.g., a protein or a lipid, decreases the choice of derivatisation method to those where the carbonyl group is not essential. To circumvent these drawbacks, our aim was to develop an analytical method for non-derivatised oligosaccharides.

1.3. Dynamic FAB/LSI-MS

In 1980, FAB was introduced, and has since then been frequently used especially for polar or ionic target compounds of molecular masses up to 10 000. The ion source works under high vacuum ($<10^{-5}$ mbar). In order to maintain low pressure in the ion source, metal ions (Cs^+) instead of xenon gas can be used, but the evaporation from the solvent and the sample matrix, in which the analyte is exposed to the beam of atoms/ions, limits the flow-rate to a few μl/min, all dependent on the pumping capacity of the mass spectrometer. Attempts have been made to increase the MS sensitivity for oligosaccharides by the use of various derivatives. Suzuki et al. [6] have studied detection limits of various derivatives of maltopentaose analysed by positive frit-FAB. The lowest detection limit, 6 pmol/10 μl or 600 n*M*, was accomplished with the derivatisation agent 4-aminobenzoic acid ethyl ester (ABEE), a significant improvement in comparison with earlier methods.

1.4. Electrospray ionization

The basic principles of ESI have often been described as follows: small droplets from the exit of a capillary emerge in a dense electric field, evaporate in a warm counter-flow of nitrogen gas at atmospheric pressure, after which the analyte molecules are ionised in one of the following ways; charged by exchange of protons (dissociation or protonation) or

by adduction of charged groups (NH_4^+, Na^+) added to the liquid phase (e.g. [4,7]).

ESI is a so-called soft ionisation method, no fragmentation of the molecules takes place. Contrary, dimerisation or even larger cluster ions are formed. The pre-acceleration potentials in the ion source determine the intensity ratios between possible ions. This may cause different appearances of spectra from different instruments, but for quantification purposes, it does not raise any problems. When analysing large molecules (e.g., proteins or oligosaccharides), many sites for adduction is an advantage, thereby the m/z value is kept on a reasonably low level. Okamoto et al. [8] have shown spectra achieved by ESI-MS from an injection of trimethyl-(*p*-aminophenyl)ammonium (TMAPA)-derivatised maltopentaose at concentrations down to 20 n*M*. At this concentration level, the peak from MNa^{2+} was about seven times higher than the peaks of other masses.

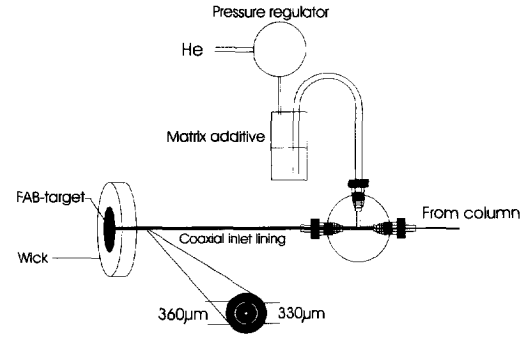


Fig. 1. The set-up for the LSI-MS experiments. The eluent flow from column is 2 µl/min which makes dead volumes critical. The two feedlines from the gradient pumps are directly connected to the injector. Outlet from column is a narrow fused-silica capillary which goes directly to the FAB-target backside. Another fused-silica capillary surrounds the narrow fused-silica capillary and between these two the matrix additive is fed. Mixing ratio of the two is controlled by the dimensions of the capillaries and the pressure applied to the matrix container.

2. Experimental

2.1. Dynamic FAB/LSI-MS

The FAB/LSI-MS work was performed on a high-resolution mass spectrometer (Zab-Spec, VG Analytical, Fisons Instruments, UK) equipped with a dynamic LSI-MS probe bombarded with 30 keV Cs^+ ions. The target was a stainless-steel round net surrounded by a wick (Fig. 1). The liquid sample is drained through the net to the wick by capillary forces, where the solvent evaporates. The FAB probe was gently heated to 50–70°C. In this mode, the acceleration voltage was 8 kV. The detector used for measurements of marine samples and standard solutions was the standard photomultiplication detector system.

The LC separation was performed in a 150 mm× 0.32 mm column (LC Packings, Netherlands) with a 3 µm C_{18} stationary phase. The injection valve was a Valco C14W with an internal loop volume of 0.1 µl. Two computer controlled syringe pumps (Phoenix, Carlo Erba, Italy) were used for the mobile phase gradient. The chromatographic separation is, in this case, easily deteriorated even by small amounts of organic modifiers. Also, the increase in viscosity can decrease the number of theoretical plates in the column [3]. The flow of matrix additive (20–100 nl/min) was therefore mixed after the column directly at the FAB target. For this purpose, a coaxial inlet tube was constructed, with the eluent flow through the inner fused-silica tube and the additional matrix between the inner and outer tubes (Fig. 1). As the matrix is relatively viscous and the gap between the inner and outer tubes is small (~12 µm), an adequate flow-rate was achieved by pressurising the bottle containing the matrix. With this set-up it was possible to control the mixing ratio without interfering with the chromatographic separation.

2.2. Electrospray ionization

The ESI experiments were carried out on the same mass spectrometer as the dynamic FAB/LSI-MS experiments. The electrospray interface (VG Analytical) uses a hexapole placed before the acceleration path of the ions. The hexapole was scanned synchronously with the magnet which increases the transmittance of ions considerably. The LC equipment was the same as that described for FAB, except that the separation column was a 150 mm×1.0 mm TSK column (Japan) with a 5 µm C_{18} stationary phase, and the injection valve was a Rheodyne 7125 with a loop volume of 5 µl. The ESI interface can tolerate a much wider flow range (10–1000 µl/min)

compared to dynamic FAB/LSI-MS. In our investigations 50 μl/min was chosen as a compromise between sample consumption and band broadening. The electric potentials in the ESI interface when run in the positive mode were: spray needle +8000 V, counter electrode +5000 V, sampling cone +4200 V, skimmer electrode +4100 V, hexapole and acceleration voltages +4000 V. The LC column was at ground potential, therefore a 400 mm×0.12 mm I.D. polyether ether ketone (PEEK) capillary was inserted between the LC column and the needle. Current drawn through the capillary sets the limit for maximum conductivity of the sample corresponding to ~10 m*M* of NaCl.

3. Results and discussion

Solutions of oligosaccharide standards—maltose, maltotriose, maltotetraose up to maltoheptaose (Aldrich)—were used for standard curves, while a mixture of oligosaccharides obtained by hydrolysis of starch (oligodextranes, Reppe, Sweden) was used for the evaluation of the chromatographic separation. At room temperature, probably due to mutarotation, each saccharide eluted as two peaks, but when the column was heated (faster equilibration) this effect was suppressed. Another important effect of heating is the decrease of retention times for larger saccharides. When using a C_{18} column with water as the mobile phase at room temperature [9], the monosaccharides were hardly retarded at all, while larger saccharides eluted with retention times increasing in relation to their degree of polymerisation (DP). Here we can demonstrate that a gradient of methanol–water or acetonitrile–water is needed if sugars larger than ~DP 15 are to be eluted with reasonably short retention times.

The re-equilibration of the C_{18} column with pure water after a gradient run was time-consuming. Another difficulty with the use of organic modifiers deals with contamination. Methanol and acetonitrile of highest quality (regardless of brand) gave about a ten-fold increase of the background or matrix ion intensity, which certainly counteracts the low detection limits. Also the quality of the water was very critical and needed to be freshly made every day. The best water used was Milli-Q water distilled in a glass distiller together with 1% sulphuric acid and 1% potassium persulfate ($K_2S_2O_8$).

Small saccharides (DP1–DP6) can, according to our experience, be well separated with water only. In the range DP7–DP15, heating of the column (70°C) will resolve the peaks without use of a gradient. Larger molecules, from DP16, demand a gradient elution with an organic modifier, and acetonitrile was used due to its lower background and its larger effect on retention compared to methanol.

3.1. Fast atom bombardment

For the oligosaccharides, the best signal was achieved when the FAB was running in the negative mode providing only singly charged deprotonated molecular ions and very little fragmentation. With LC–MS, it was possible to separate and detect oligosaccharides with 1 to 17 hexose units in standard mixtures. However, severe drawbacks were the intricate tuning of the instrument for maximal performance and the low flow-rates, which reduce the injection volumes (~100 nl) and make the detection limits quite high (~10 μ*M*). Another noticeable problem, due to the low flow-rate, was broadening of the peaks because the eluent was drying out on the target instead of being washed away to the surrounding wick, where it was meant to evaporate. This forced us to use longer columns in order to achieve baseline separation, which in turn increased the detection limits considerably. In general, stronger but more short-lived signals are achieved when more volatile matrixes are used. On the other hand, the target is better flushed and the background signals are kept lower using less volatile matrixes. Glycerol, nitrobenzylalcohol, thioglycerol, tri- and tetraethyleneglycol were investigated as matrix additives and a mixture of the two latter compounds was found to give the best signal-to-noise ratio. The instrument was scanning over a *m/z* range of 3000 to 150. Singly charged molecular $(M-H)^-$ ions are seen (*m/z* 503, 827, 1151 etc.) in the mass spectra (Fig. 2). The abundance of these ions are declining at higher masses. The low mass region is dominated by matrix ions.

3.2. Electrospray ionization

In positive ESI mode, adduct ions of sodium were

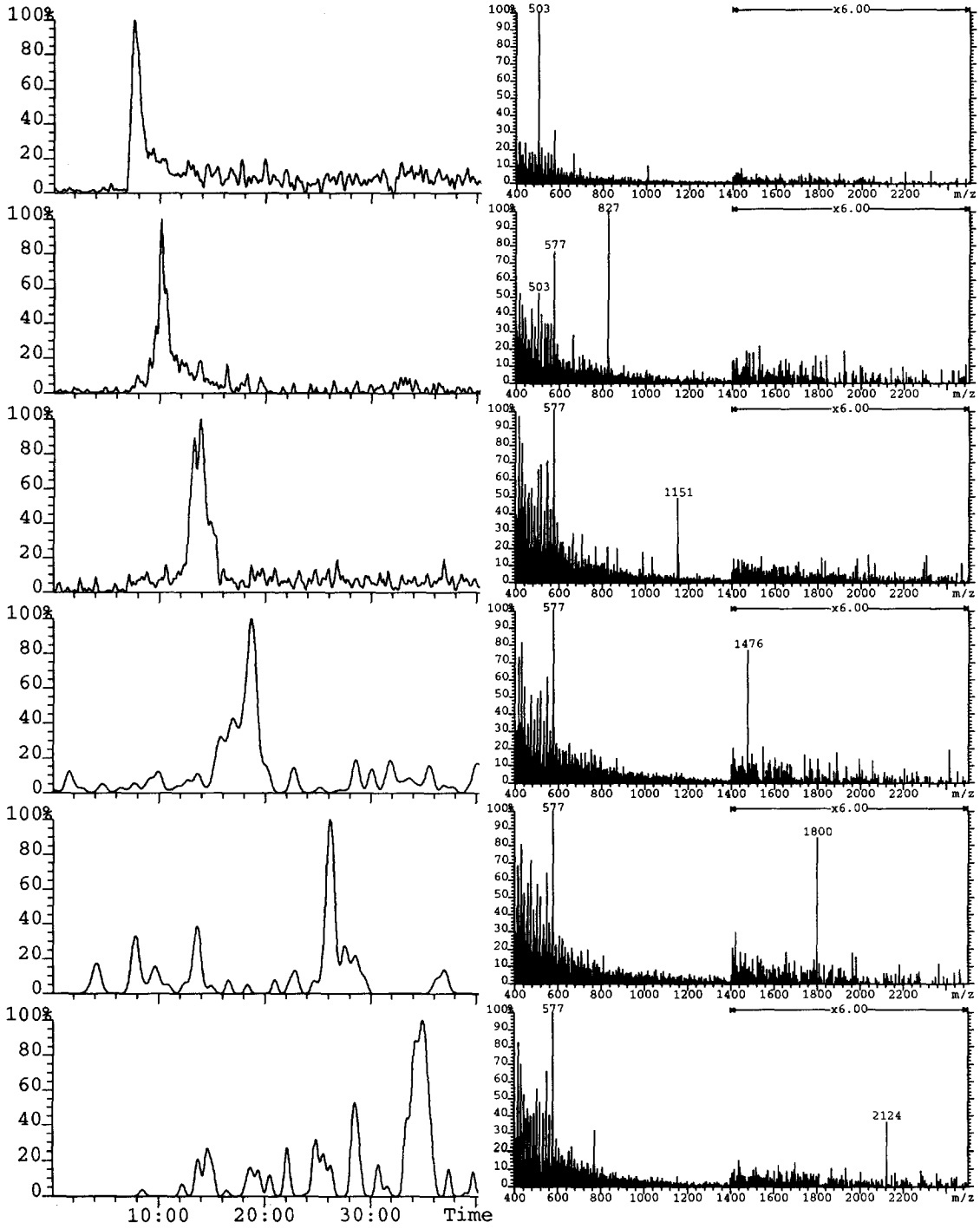


Fig. 2. Oligodextranes run in dynamic FAB mode. A typical mass chromatogram (from top to bottom: *m/z* 503, 827, 1151, 1476, 1800, 2124) and spectra of individual peaks are shown. Concentrations of the oligodextranes are 200–500 μ*M* and injection volume is 100 nl. Mobile phase: 2 μl/min, gradient: 0–2% acetonitrile in water in 15 min, matrix: 40 nl/min of tetra-etylenglycole. Column: 150 mm×0.32 mm I.D. packed with 5 μm C_{18}, column temperature: ambient, back pressure: 70 bar. MS scan from 300–3000 *m/z* in 4 s at a resolution of 1000. The sugar solution contains all DP from 1 to >27, but for clarity only every third peak (DP) is shown.

the most abundant, even if the cleanest water was used. The concentration of adduct cations was shown to be very important for the sensitivity. Additional cations, possibly of other elements, of varying concentrations delivered together with the sample affect the reproducibility of the signal. When the NaCl concentration reaches about 10 m*M*, the background increases and among others $Na(NaCl)_n^+$ ions are seen. Seawater, our start matrix, contains considerable amounts of ions, and had to be desalted before freeze-drying and subsequent analysis. The desalted samples were virtually free of ions, which made it possible to use adduct cations with better properties than sodium. Therefore, other ions were investigated such as Li^+, Ag^+, Ca^{2+} and Pb^{2+}, among which Li^+ gave the highest signal-to-noise ratio. A Li^+ concentration of 1 m*M* in the mobile phase was enough to depress any formation of Na^+ adducts. With Li^+ concentrations higher than 10 m*M*, extra peaks in the mass spectra with a distance of 6 mass units occur, starting at each single charged sugar. This suggests that lithium replaces hydrogen at some of the hydroxyl groups. Another problem with high ion strength was electric discharges through the inlet capillary, which could clog the PEEK feed-line or make the system unstable due to varying potentials in the interface. This sets an upper limit for the lithium concentration to 1 m*M* Li^+. The Ag^+ and Ca^{2+} ions gave weaker signals, while Pb^{2+} ions caused strong signals but with very complicated spectra including M_2Pb^{2+}, $M_3Pb_2^{4+}$ ion peaks as well as all the isotope peaks of lead.

It was possible to separate a standard mixture of oligohexoses (DP1 to DP27) in 20 min. Short chain sugars were detected as MLi^+ (e.g., *m/z* 511, 673, 835 etc.) the larger (DP8–DP17) as $(MLi_2)^{2+}$ (e.g., *m/z* 745, 826, 907 etc.) and even longer chains as $(MLi_3)^{3+}$ (e.g., *m/z* 1201, 1255, 1309) as can be seen in Fig. 3. We can therefore detect sugars with molecular masses up to at least 4500 scanning from *m/z* 1700 to 300 only.

3.3. Detection limits

Experiments with a dilution series of raffinose (M_r 504) gave a detection limit of 10–20 n*M* using a 5 μl injection loop which corresponds to only 50–100 fmol. Detection limits for oligopentaose were determined for various Li^+ concentrations by scanning over a single mass unit (Fig. 4). The detection limit was defined as three times the standard deviation of the measured concentration on a series of blanks and was determined to be 12 n*M* in the 1 m*M* Li case (no LC column was used).

It is not easy to compare our detection limits with those previously reported by Suzuki et al. [6] (FAB) and Okamoto et al. [8] (ESI). They define the detection limits by comparing the intensity of other masses in the spectra with the one of interest, a more qualitative or recognition based method but not a quantitative determination of the detection limit. In comparison, their lowest limit for ESI work (20 n*M*, derivatisation of pentaoses) is similar to the detection limit we obtained in this ESI study. On the other hand, the FAB methods with derivatised pentaoses can hardly compete with the methods (600 n*M*, [6]).

3.4. Oligosaccharides in sediment pore water

The first determinations of oligosaccharides in seawater and in marine sediment pore waters have been made in a study of sediment cores from five locations in the Skagerrak. Virtually undisturbed sediment cores, 12 cm deep and with a diameter of 98 mm, were collected using a so called multiple corer, after which the sediment was carefully sliced and centrifuged, all onboard the research vessel immediately after retrieval of the multiple corer. The pore water samples were filtered through pre-washed 0.45 μm celluloseacetate filters and stored frozen until the return to the home laboratory. The pore waters were desalted using a laboratory-built computerised flow-through electrodialysis system. After that, in order to obtain a liquid phase suitable for freeze-drying, 0.1% ethyleneglycol was added in order to prevent losses of carbohydrates during the following freeze-drying step [10]. The freeze-dried samples were dissolved in water to give a total concentration factor of 10–25, after which they were analysed by LC–ESI-MS as described in Section 2.2. Mass spectra and corresponding mass chromatograms of the most abundant peaks are shown in Fig. 5. Blanks (persulfate-distilled water) were analysed the same way as the samples. As the sample composition and the nature of the sample matrix was not precisely known (no similar measurements had been

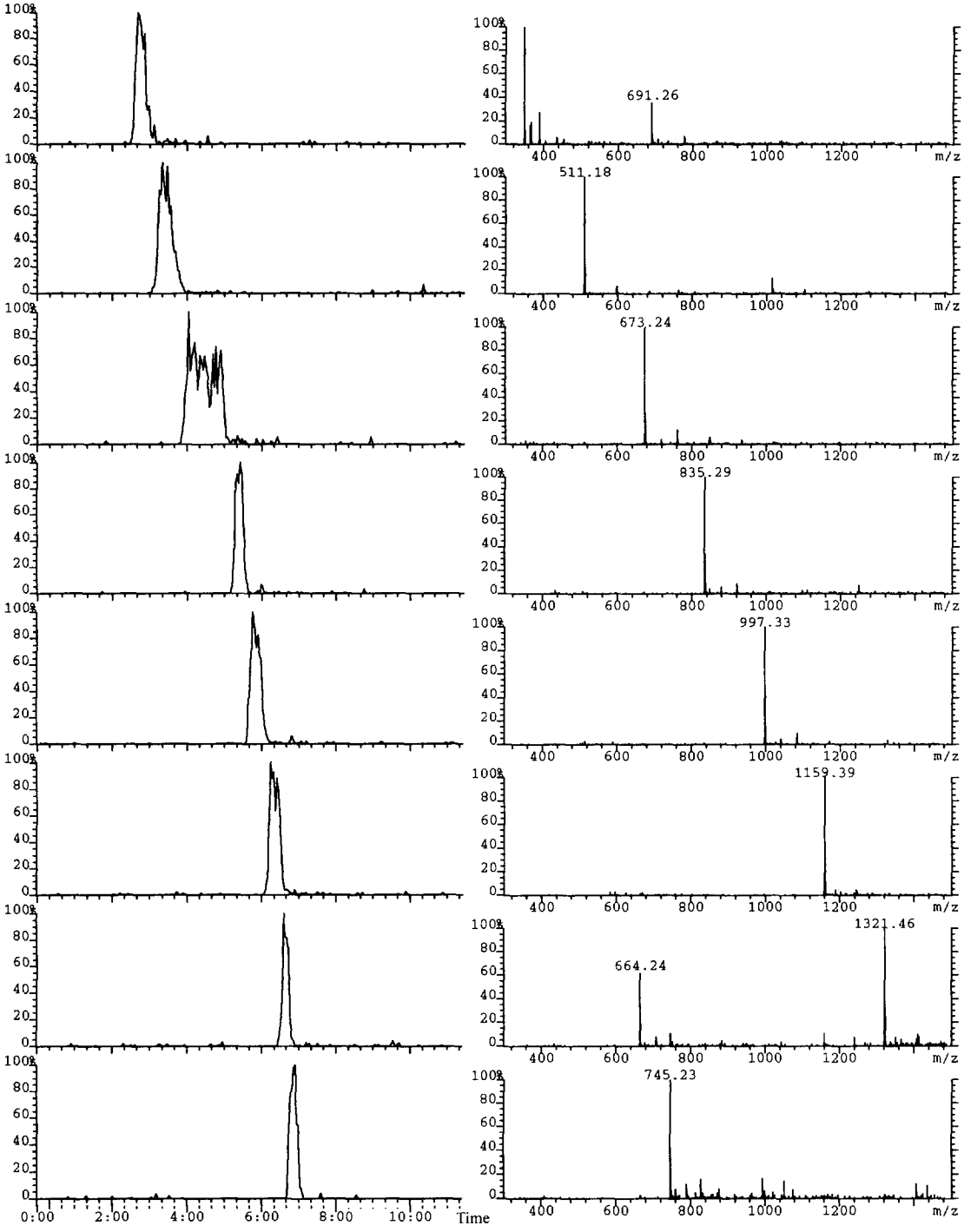
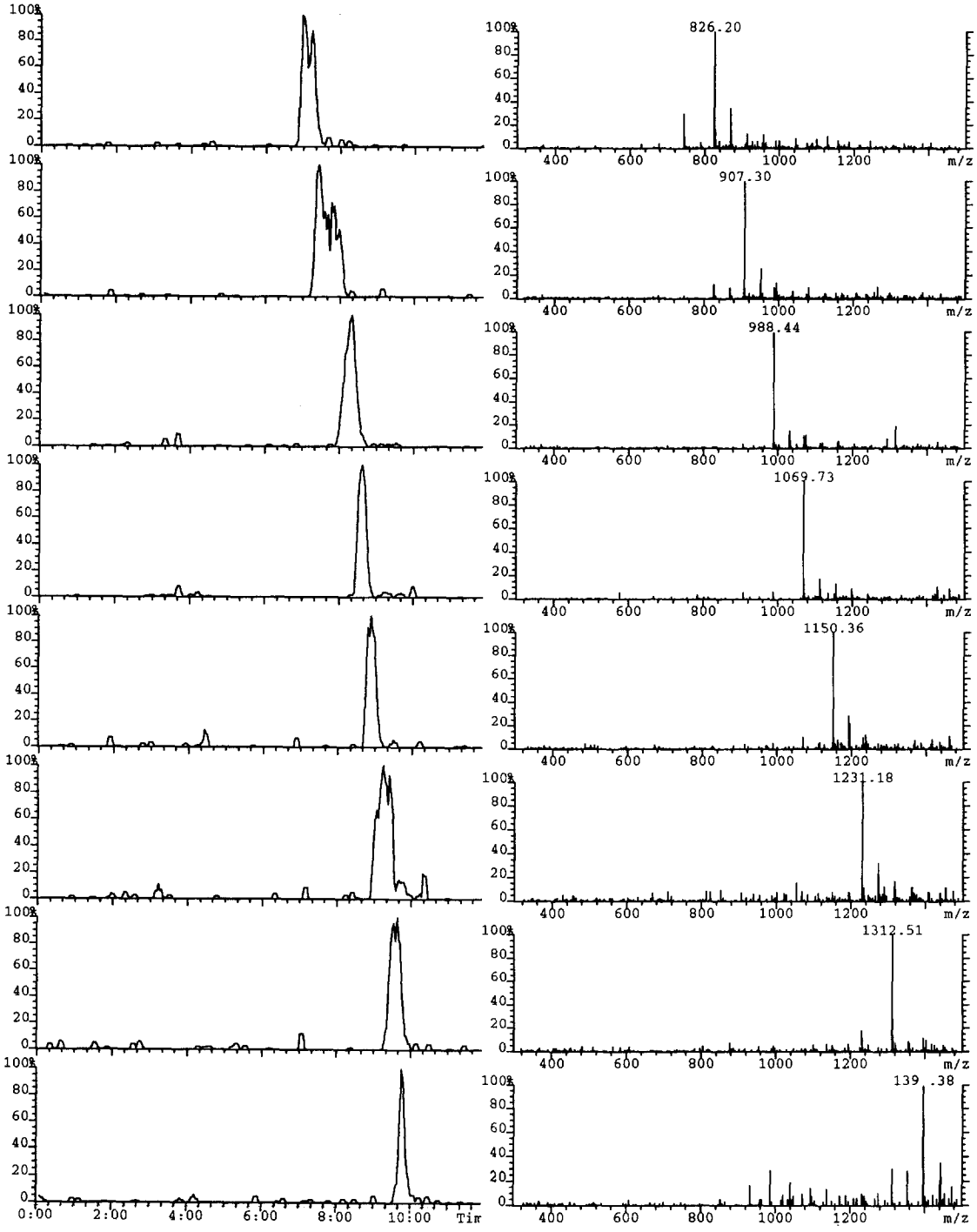


Fig. 3 (*continued on p. 148*)



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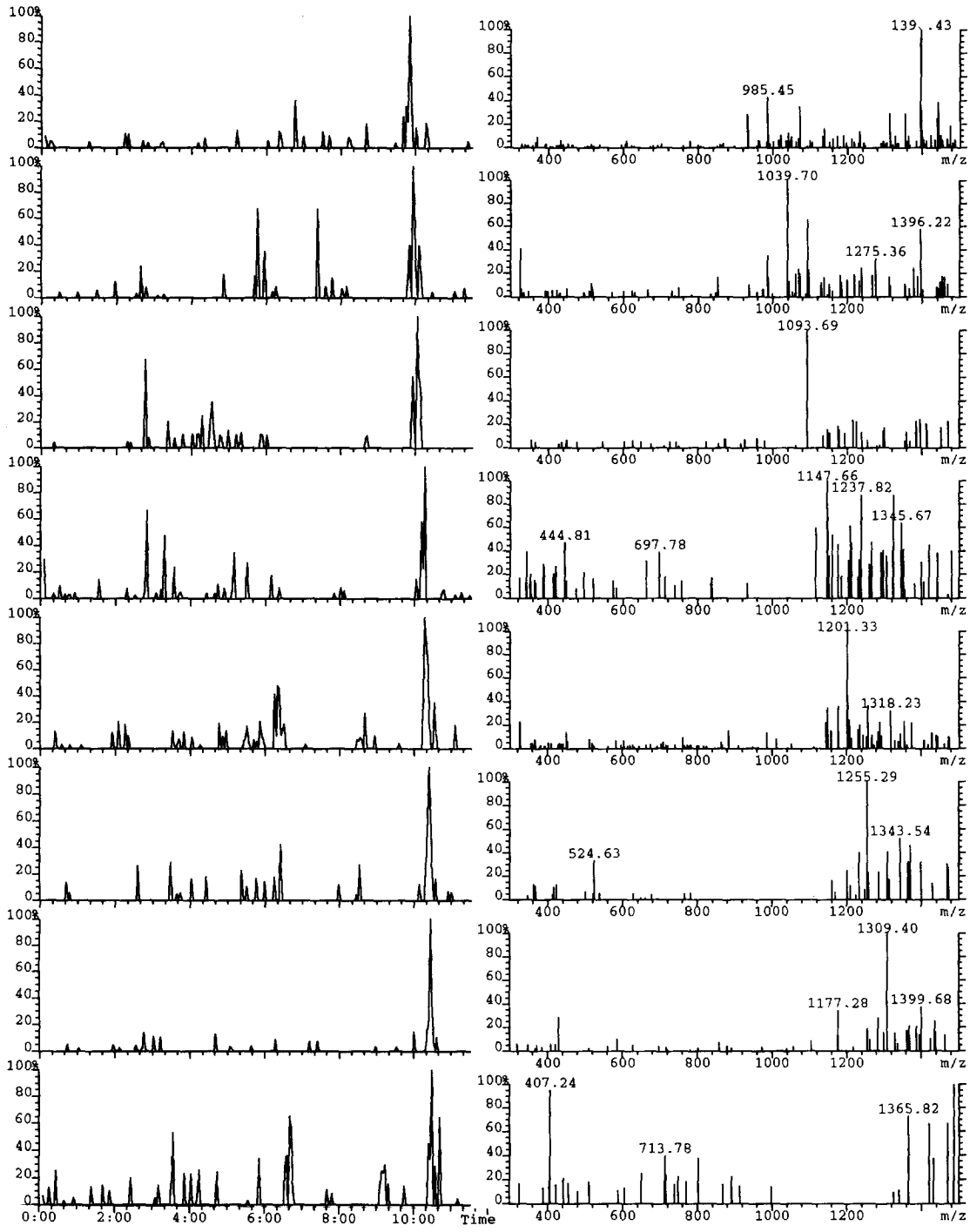

Fig. 3. The same sugar solution as in Fig. 2 run in ESI mode. Mass spectra (to the right) and corresponding mass chromatogram of the most abundant peak in the spectrum (to the left). Injection volume is 5 μl, mobile phase 40 μl/min, gradient of 0–2% acetonitrile in water during 15 min. Column: 140 mm×1 mm I.D. packed with 5 μm C_{18} TSK phase. Column temperature: ambient. MS scan from 300 to 2000 *m/z* in 4 s/decade at a resolution of 1000.

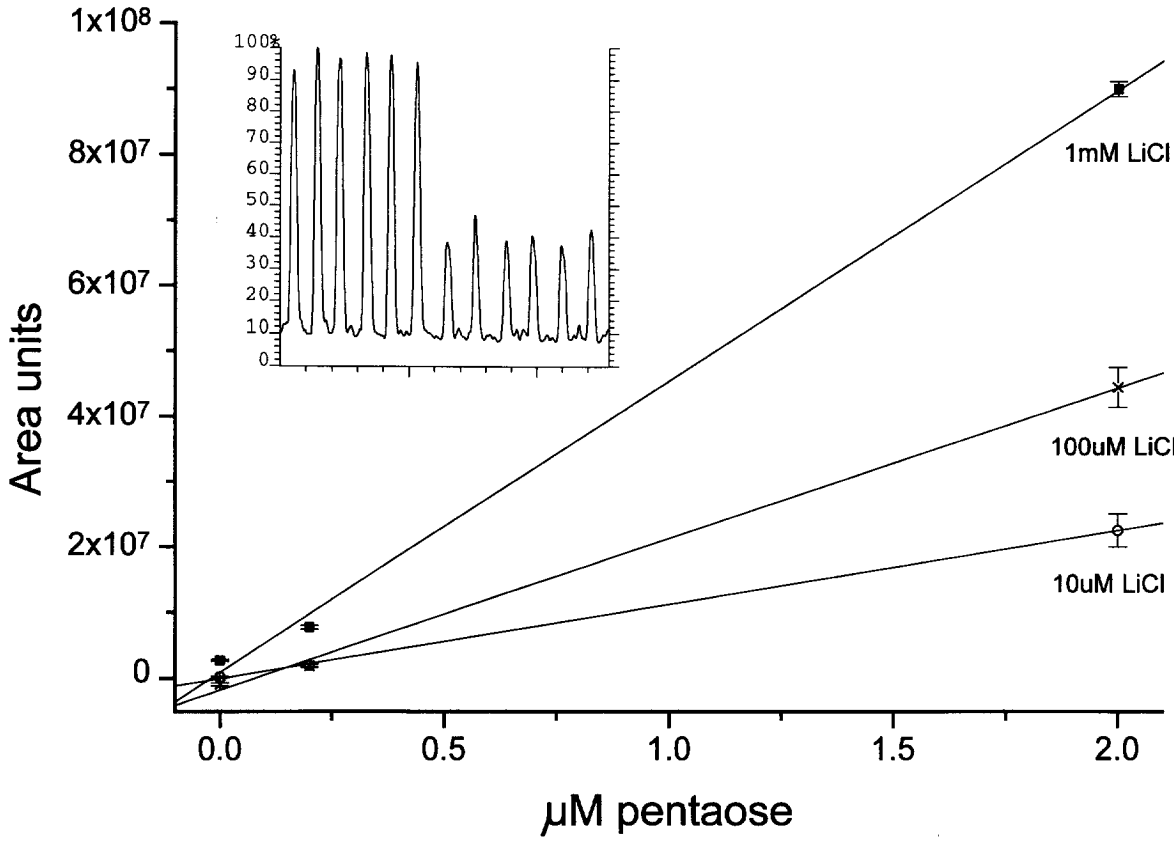

Fig. 4. A measurement of detection limits using the ESI interface is shown. 5 µl of pentaose solutions (200 n*M* and 2 µ*M*) and blanks was injected together with varying (10–1000 µ*M*) concentrations of LiCl in a flow of re-distilled water. An example of a total ion current (TIC) mass chromatogram for 0.2 µ*M* pentaose and 1 m*M* Li is also shown in the picture. Mobile phase flow-rate was 50 µl/min and the MS was scanned over one single mass (997–998) with 0.5 s/scan with a resolution of 1500. All samples and blanks were injected six times and standard deviation is shown (±1 S.D.).

done before), it was regarded premature to select a suitable internal standard for evaluation of the sample preparation step. For example, peaks were found in the mass spectra with m/z values coinciding with one hexose and one pentose unit as well as two hexoses and one pentose. For future work on natural samples, suitable internal standards will be searched, which do not interfere with any of the possible target compounds in our marine samples. A possible choice could be a set of oligoheptoses or oligopentoses.

In the first step, oligohexoses up to DP6 were quantified with a normal standard curve technique and the assumption that no analytes were lost during the concentration step. This is supported by an experiment, in which the recovery of the desalting and freeze-drying was evaluated for mono- and disaccharides and was found to be above 90% (with only few exceptions due to instrumental failures).

The depth distributions of the measured oligosaccharides (DP2–DP6) are shown in Fig. 6. Concentrations of the dissolved oligosaccharides is in the range of 6 to 114 µg/l (1.2–25 n*M*). Earlier measurements (unpublished results) of the concentration of dissolved organic carbon in pore waters in adjacent areas indicate that 0.5–5% of the DOC consists of saccharides of 2 to 6 hexose units. As can be seen in the figure, the concentrations of the different oligohexoses are similar and follow a pattern with the highest concentrations in the slice 2–4 cm. From this layer the sugars diffuse both

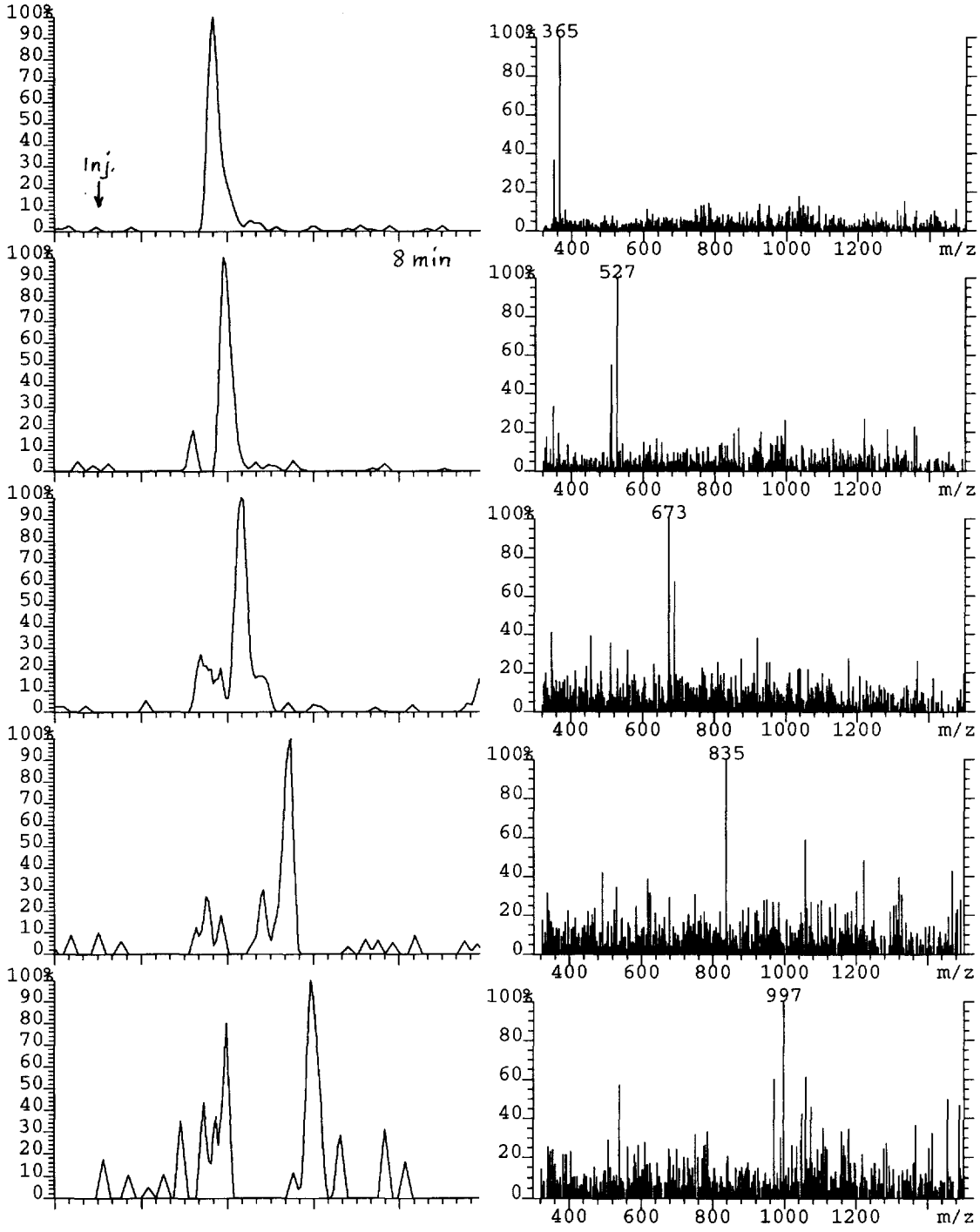

Fig. 5. Typical ESI mass spectra (to the right) and corresponding mass chromatogram of the most abundant peak in the spectrum (to the left) for one of the marine samples. Column temperature 70°C, mobile phase: only water with 0.1 m*M* Li^+. Other conditions as in Fig. 3. This sample was pre-concentrated by a factor of 25. A closer look shows that traces of sodium adducts are found together with the Li adducts (16 u higher). This was not the case when increasing Li concentration to 1 m*M*.

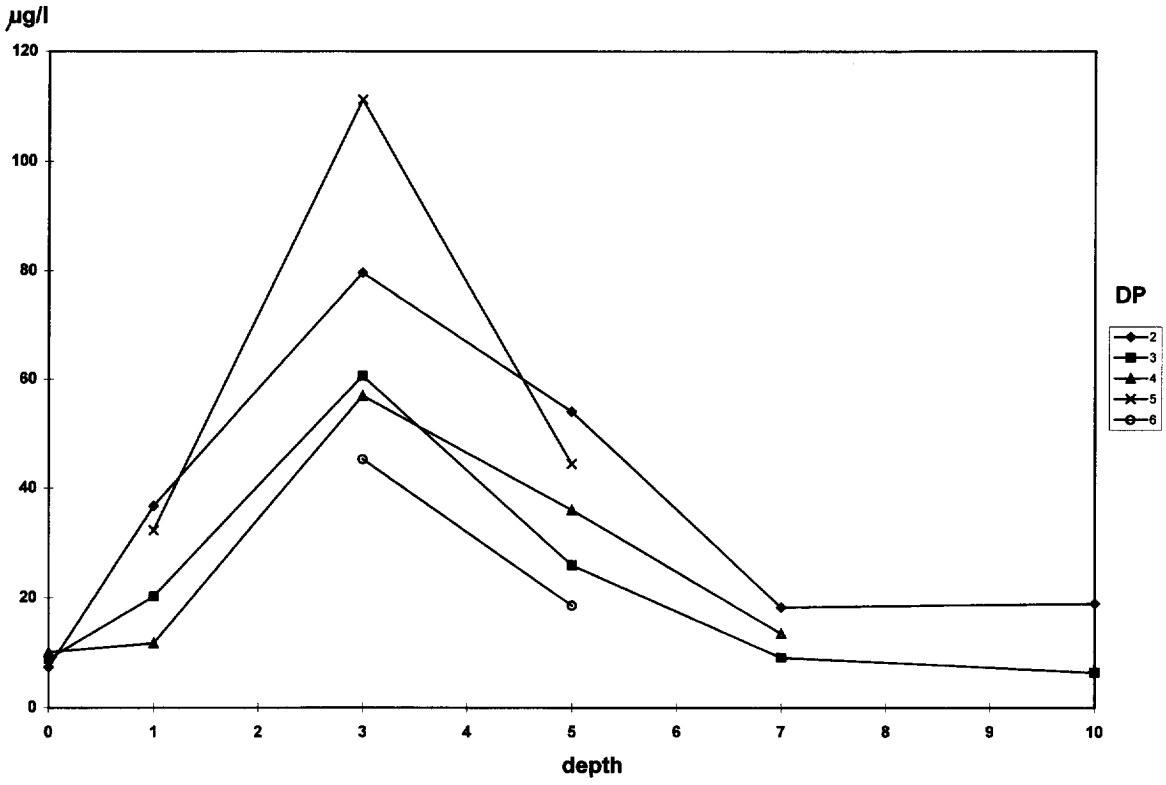

Fig. 6. A depth profile of oligohexoses DP2–DP6 in sediment pore water is shown. Each curve represents the concentration of oligohexoses with 2, 3, 4, 5 and 6 units (DP), respectively. The sediment was sliced in 0–2 cm (here called depth=1), 2–4 cm (3), 4–6 cm (5), 6–8 cm (7) and 8–12 cm (10). The depth 0 was seawater.

upwards out into the bottom water, and down into the core. The most likely source is particulate organic matter undergoing a hydrolysation by exoenzymes released from bacteria covering all surfaces and taking their food from the dissolved phase. The upper molecular mass limit of food that bacteria can transport into their cells is normally 400–600, which means that further hydrolysation steps have to take place before higher fractions of food can be oxidised. A normal DOC profile in sediments does not decline the way the oligosaccharides in our profiles do, but increases further down. This suggests that this oligosaccharide fraction is an easily degradable food resource that is quicker released and probably quicker decomposed (e.g., hydrolysed to smaller compounds) than other substances [11,12]. From this sediment profile, it is not possible to determine hydrolysation rates further than saying that it is declining downwards. For that purpose a higher resolution (thinner slices) would be needed. A further discussion on the results from a biogeochemical point of view will be published elsewhere.

4. Conclusions

Two techniques, dynamic FAB/LSI-MS and ESI-MS have been evaluated for the analyses of oligosaccharides in water samples. The best results were achieved with ESI-MS, whereby a sample containing saccharides up to 27 sugar units could be separated and measured within 20 min. A comparison of detection limits achieved by single ion monitoring of MLi^+ ions with those obtained in previous studies indicates that Li^+-complexed native sugars is a good alternative to derivatisation when applying LC–ESI-MS. This is to a large extent a consequence of the good sensitivity offered by modern ESI techniques.

The method proved to be useful for determination of carbohydrates in the pore waters of marine sediment. The depth profiles in the sediment cores indicate a production in the redox boundary layer and from there, diffusion in both directions, to the overlying water and further down into the sediment.

References

[1] J.I. Hedges, Marine Chem. 39 (1992) 67–93.
[2] J.D. Pakulski, R. Benner, Limnol. Oceanogr. 39 (1994) 930–940.
[3] J.P. Gagné, A. Carrier, L. Varfalvy, M.J. Bertrand, J. Chromatogr. 647 (1993) 13–20.
[4] A.P. Tinke, R.A.M. van der Hoeven, W.M.A. Niessen, J. van der Greef, J.-P. Vincken, H.A. Schols, J. Chromatogr. 647 (1993) 279–287.
[5] A.A. Ben-Bassat, E. Grushka, J. Liq. Chromatogr. 14 (1991) 1051–1111.
[6] S. Suzuki, K. Kakehi, S. Honda, Anal. Chem. 68 (1996) 2073–2083.
[7] K.L. Duffin, J.K. Welply, E. Huang, J.D. Henion, Anal. Chem. 64 (1992) 1440–1448.
[8] M. Okamoto, K.-I. Takahashi, T. Doi, Rapid Commun. Mass Spectrom. 9 (1995) 641–643.
[9] E. Rajakylä, J. Chromatogr. 353 (1986) 1–12.
[10] K. Mopper, Marine Chem. 5 (1977) 585–603.
[11] C. Arnosti, D.J. Repeta, N.V. Blough, Geochim. Cosmochim. Acta 58 (1994) 2639–2652.
[12] R. Benner, J.D. Pakulski, M. McCarty, J.I. Hedges, P.G. Hatcher, Science 255 (1982) 1561–1564.