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Separation and characterization of underivatized oligosaccharides using liquid chromatography and liquid chromatography–electrospray ionization mass spectrometry

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Abstract

Native cyclodextrin-based columns are particularly useful for the analysis of oligosaccharides because the retention of these carbohydrates is based mainly on the hydrogen bonding interactions of oligosaccharide hydroxyl groups with the stationary phase. Thus, the retention time predictably increases with the number of analyte hydroxyl groups, which corresponds to the elongation of the oligosaccharide chain. High-performance liquid chromatography (HPLC) coupled to electrospray ionization (ESI) mass spectrometry (MS) was used for the separation and characterization of underivatized oligosaccharide mixtures. With the limits of detection as low as 50 pg, all individual components of oligosaccharide mixtures (up to 11 glucose units long) were baseline resolved on a Cyclobond I 2000 column and detected using ESI-MS. Low flow rates and narrow I.D. columns increase the ESI-MS sensitivity significantly. The method showed potential usefulness for the sensitive and quick analysis of hydrolysis products of polysaccharides, and for trace levels of individual oligosaccharide or oligosaccharide isomers from biological systems.

Keywords: LC; LC–ESI-MS; Dextran digest; Mannan digest; Cellulose digest; Disaccharide separations; Separation; Cyclodextrin stationary phases

1. Introduction

The separation and characterization of underivatized oligosaccharides have continued to gain attention in recent years due to their ubiquity in nature and their significant roles and uses in industry, consumer products and biology where they are crucial for the development, growth, function or survival of an organism [1,2]. Identification of the carbohydrate substituents on glycoproteins and glycopeptides (which affect or control their function) is an important and often difficult endeavor [3,4]. A variety of chromatographic methods have been used to separate saccharides and oligosaccharides. They include ligand-exchange chromatography [5,6], chromatography based on size exclusion [7–9], and widely used hydrophilic chromatography on silica-based bonded polar stationary phases [10–12]. Often the separations of underivatized carbohydrates require specific types of columns such as alkylated silica-bonded phases [13–15], amino-bonded stationary phases [12,16–18] or various ion-exchange media [19–22] in which specific counter ions are added to affect the separations. However, most of the columns have problems, particularly in terms of column stability, lifetime and separation reproducibility. Stable cyclodextrin-bonded stationary phase columns were first introduced for oligosaccharide analysis by Armstrong and Jin [23] in 1989. It has been shown that these native cyclodextrin-bonded columns are particularly useful for the analysis of oligosaccharides [23–25]. In this paper, a native beta-cyclodextrin stationary phase (Cyclobond I 2000) was used to separate a variety of related oligosaccharides using mass spectrometry (MS)-compatible mobile phases. The retention of oligosaccharides in high acetonitrile-containing mobile phases on cyclodextrin-bonded phases is based on the hydrogen bonding interactions between the oligosaccharide hydroxyl groups and the cyclodextrin hydroxyl groups that constitute part of the stationary phase [23–25]. In this separation approach, the retention time predictably increases with the number of the

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hydroxyl groups possessed by the saccharide or oligosaccharide. Hence, longer/larger saccharides/oligosaccharides tend to be retained more than shorter/smaller ones. In addition, cyclodextrin-based stationary phases are stable, have good efficiency and good reproducibility, and do not react with reducing sugars/carbohydrates as do amine-type bonded stationary phases. Consequently, they are very good for quantitative analysis.

Several liquid chromatography (LC) detection methods have been used for carbohydrate analysis. Oligosaccharides have been labeled with a wide variety of derivatization reagents in order to enhance detectability during chromatographic analysis [26–29]. However, most derivatization techniques require tedious procedures, and often suffer from poor reproducibility. Therefore, detection of underivatized oligosaccharides remains extremely important.

Although underivatized oligosaccharides do not have strong chromophores, they are still detectable by UV at low wavelengths, e.g., 195 nm, with detection limits in the microgram range [23]. Refractive index (RI) detection was frequently used with slightly higher sensitivity compared to UV detection. The limits of detection by RI have been reported to be below 25 ng [30]. However, this detector has a major disadvantage, namely, that the signal is related to the solvent composition, which prevents the use of gradient elution. Also even greater sensitivity is desirable. Evaporative light scattering detection (ELSD) is another preferred universal detector for carbohydrate analysis [31,32] because its response is independent of the spectral properties of the analyte and solvent. Therefore, ELSD is compatible with the solvent variations and it produces stable baselines. However, the non-linear response of ELSD limits the detector's use for high-accuracy quantitative analysis [33].

Recently, mass spectrometry has gained increasing importance as a detection method for oligosaccharide analysis and characterization. So far, several mass spectrometric techniques have been used for the determination of oligosaccharide structure. They are matrix-assisted laser desorption/ionization (MALDI) [34–37], fast-atom bombardment (FAB) [38,39], and electrospray ionization (ESI) mass spectrometry [40–43]. All of aforementioned MS techniques have an advantage over chemical or chromatographic methods in that they yield molecular mass information with relatively high sensitivity. Coupled HPLC–mass spectrometry became the most widely used technique in proteomics and is now beginning to make an impact on the analysis of complex carbohydrates in many fields of biotechnology [44–47]. However, the ionization and sensitive detection of carbohydrates via this approach is not as facile as it has been for protein and peptides. So far, ESI-MS is known as one of the most suitable interfacing techniques for online LC–MS.

Online LC–ESI-MS analyses of underivatized oligosaccharides have so far been performed using graphitized carbon chromatography (GCC) [48], and a nanoscale amide-80 column [43]. We applied a native beta-cyclodextrin stationary phase to the online LC–ESI-MS analysis of a variety of underivatized oligosaccharides. This system is characterized by high chromatographic resolution, high column stability, and low mass spectrometric detection limits at picogram (femtomole) sensitivity. In addition, this method showed potential usefulness for the sensitive and quick analysis of hydrolysis products of polysaccharides, and individual oligosaccharides from biological systems.

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile and water was purchased from Fisher (Pittsburgh, PA, USA), formic acid from J.T. Baker (Philipsburg, NJ, USA). Cellooligosaccharides, maltooligosaccharides, dextran, and all other underivatized oligosaccharides were all purchased from Sigma. Most of the oligosaccharides were dissolved in water at 1 mg/ml, and then further diluted before injection.

2.2. Chromatography

Three Cyclobond I 2000 columns with different I.D. (250 mm × 4.6 mm I.D.; 250 mm × 2.0 mm I.D.; 250 mm × 1.0 mm I.D.) were from Advanced Separation Technology (Astec, Whippany, NJ, USA). Most separations were performed at room temperature on the 250 mm × 4.6 mm I.D. Cyclobond I 2000 column. Mobile phase compositions were acetonitrile–0.1% formic acid in water at varying concentrations. Flow rates varied from 100 to 800 μl/min and 195 nm was used for UV detection.

2.3. Mass spectrometry

2.3.1. ESI conditions for Shimadzu LCMS-2010

Most LC–MS experiments were performed on a Shimadzu LCMS-2010 coupled with an electrospray (ESI) ion source. The single stage quadruple mass spectrometer provides an upper mass unit limit of 2000 Da and unit mass resolution.

Nebulizer gas flow was 4.50 l/min and MS parameters were optimized to the following: probe bias = 4.5 kV, CDL = 25.0 V, aperture = 0 V, CDL temperature = 250 °C, and block temperature was 200 °C.

2.3.2. ESI conditions for Thermo Finnigan LCQ Advantage MS

The effect of column I.D. on sensitivity experiments were performed on a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with ESI ion source.

Sheath and auxiliary gases were at 50 and 40 arbitrary units, respectively. MS parameters were optimized to the following: source voltage = 4.5 kV, capillary voltage = 25.0 V, tube lens offset = 0 V, and capillary temperature = 250 °C.

3. Results and discussion

3.1. LC–ESI-MS of underivatized oligosaccharide mixtures on the Cyclobond I 2000 column

It is well known that mass spectrometry is one of the most sensitive LC detection methods for many ionizable organic compounds, especially those with a poor UV absorptivity. In this paper, cyclodextrin-based LC–ESI-MS was examined as a way to rapidly separate and characterize underivatized oligosaccharides using a MS-compatible mobile phase.

Fig. 1 shows the mass spectra of sodium adduct peaks observed in the ESI positive ion mode for standards of different oligosaccharides (initial test analytes). Cellooligosaccha-

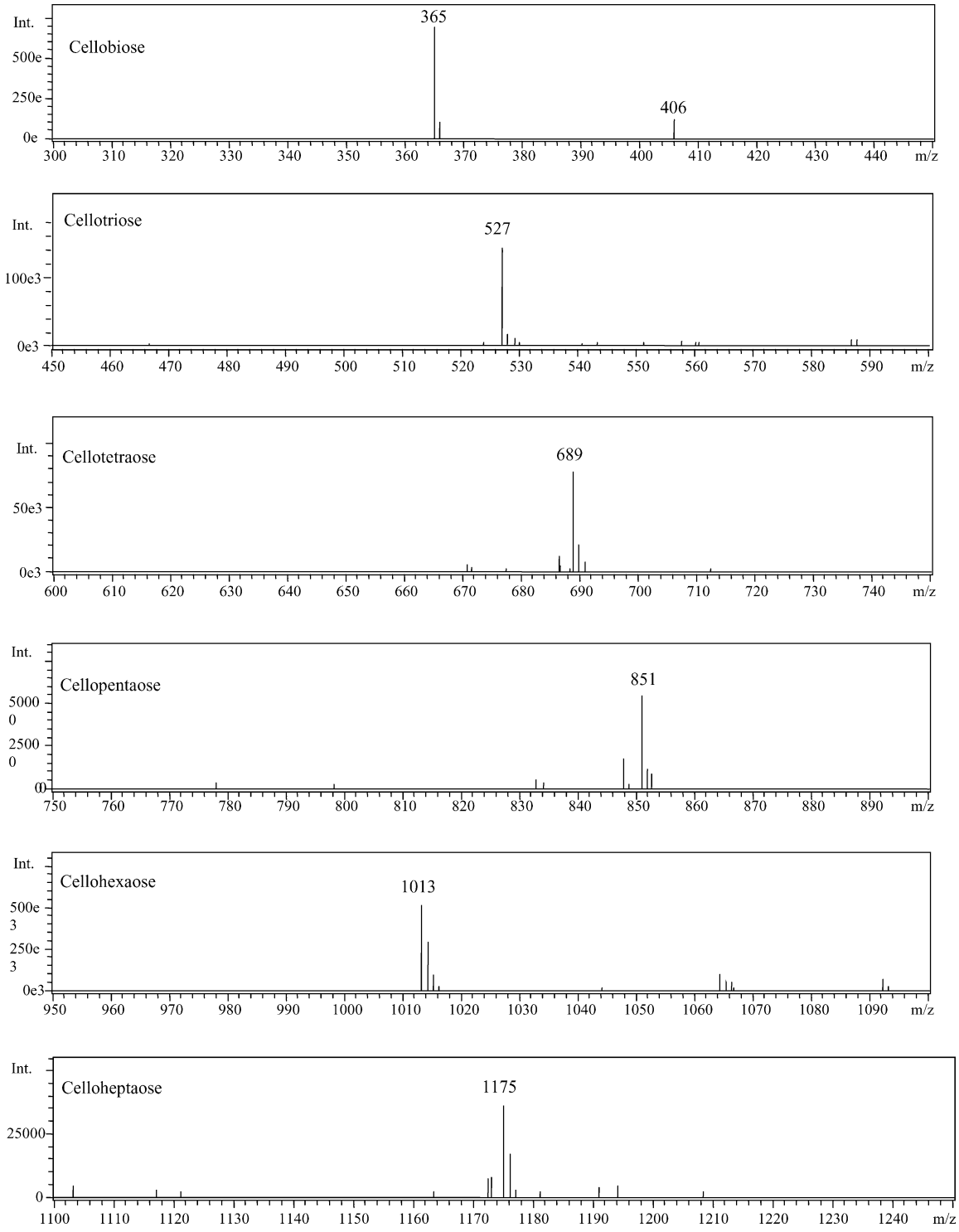

Fig. 1. Mass spectra of sodium adduct peaks observed in ESI positive ion mode for each underivatized oligosaccharide.

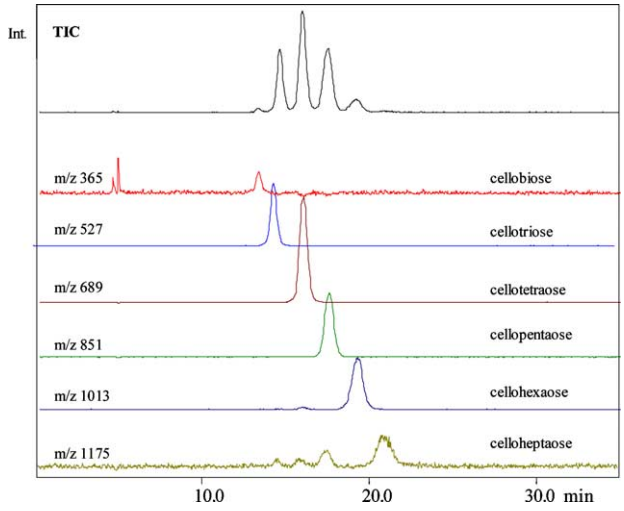

Fig. 2. LC–ESI-MS of underivatized cellooligosaccharide standard mixture (dp 2–7). Selected ion chromatography at $[M + Na]^+$. Column: Cyclobond I 2000 column (250 mm × 4.6 mm I.D.); flow rate at 400 μl/min. Mobile phase: 65:35 acetonitrile–water (0.1% formic acid).

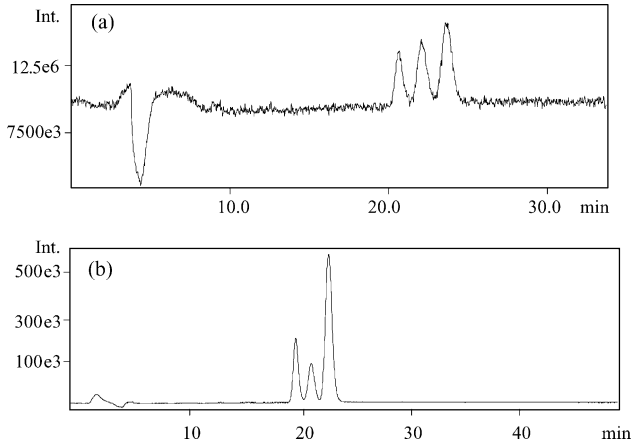

Fig. 3. LC–ESI-MS of disaccharide separation. (a) Total ion chromatography. Scanned from *m/z* 200 to 500. (b) Selected ion chromatography at $[M + Na]^+$ (*m/z* 365). Under both conditions, peaks from left to right correspond sucrose, mixture of cellobiose and maltose, and lactose, respectively. Column: Cyclobond I 2000 column (250 mm × 4.6 mm I.D.); flow rate at 400 μl/min. Mobile phase: 80:20 acetonitrile–water (0.1% formic acid).

ride and maltooligosaccharide with the same degree of polymerization (dp), or the same number of glucose units, have similar mass spectra (results not shown). Fig. 2 shows the LC–ESI-MS separation of an underivatized cellooligosaccharide standard mixture (dp 2–7) using selected ion chromatography. The retention of cellooligosaccharides increases with the elongation of the chain, because the retention of the oligosaccharides on cyclodextrin-bonded phases is based on the hydrogen bonding interactions of oligosaccharide hydroxyl groups with those of the stationary phase. In general, the more hydroxyl groups the oligosaccharide has, the greater the number of hydrogen bonding interactions with the stationary phase and the longer is the retention. Within a homologous series, this allows the prediction of elution positions in terms of the number of the analyte's constituent monosaccharide units.

3.2. LC–ESI-MS separation of disaccharides

The separation of structural isomers in mixtures of oligosaccharides is frequently not easy. Cellobiose, maltose, lactose, and sucrose are all disaccharides with the same molecular weight of 342, and they all gave the same *m/z* 365 peak ($[M + Na]^+$ MS response) in the ESI positive ion mode. Therefore, it was not possible to identify each disaccharide from the mixture simply using mass spectrometry. Fig. 3 shows the LC–ESI-MS separation of mixtures of the four isomeric disaccharides. Three peaks were observed. By spiking pure standards, it was found that cellobiose could be separated from sucrose and lactose, but could not be separated from maltose. Sucrose has the shortest retention time because it has the smallest size due to the combination of six-member ring (glucose) and five-member ring (fructose), while lactose has the longest retention time. Cellobiose and maltose are both composed of two glucose units and differ only in the type of linkage. Cellobiose is connected by a beta-(1,4)-glycosidic linkage, while maltose has an alpha-(1,4)-glycosidic linkage. It seems that the native cyclodextrin column can only differentiate the position of hydroxyl groups, but cannot differentiate the type of linkage. Similar results were also obtained for mixtures of maltotriose and cellotriose, mixtures of maltotetraose and cellotetraose, etc. Separation of compounds with identical molecular weights and numbers of hydroxyl groups can be accomplished by cyclodextrin-based stationary phases provided each compound has different numbers of hydroxyl groups available for simultaneous interactions with the stationary phase. This can occur for geometrical reasons since different saccharides with the same number of hydroxyl groups can have different ring sizes, configurations and stereochemistry.

3.3. Effect of acetonitrile concentration on retention factor

The concentration of organic modifier in the mobile phase controls the retention and selectivity of oligosaccharides on cyclodextrin columns. Unlike traditional reversed-phase LC, the retention of each underivatized oligosaccharide increases when acetonitrile concentration increases from 50% to 90%, as shown as in Fig. 4. This is because the retention of oligosaccharides on cyclodextrin columns is based on the hydrogen bonding interactions between the oligosaccharide hydroxyl groups and the stationary phase. A high percentage of acetonitrile (a poor hydrogen bonding solvent) accentuates hydrogen bonding interactions between the analyte and the stationary phase. With higher percentages of water in the mobile phase, the hydroxyl groups of the oligosaccharides are preferentially solvated by the water in the mobile phase, thus, diminishing hydrogen bonding interactions between the oligosaccharide and the stationary phase, resulting in shorter

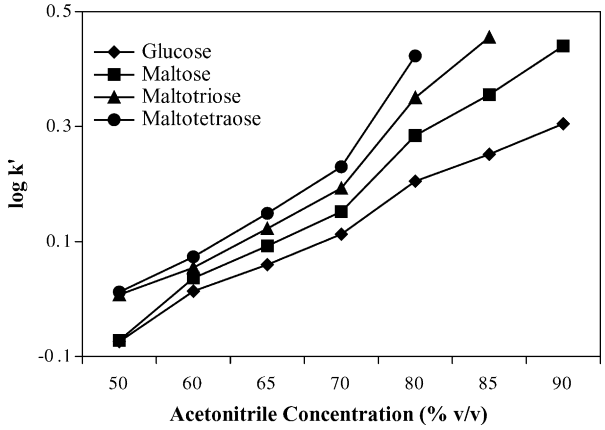

Fig. 4. Effect of acetonitrile concentration on retention factor. Experimental conditions: Cyclobond I 2000 column (250 mm × 4.6 mm I.D.); UV detection at 195 nm; sample concentration for each oligosaccharide is 10 mg/ml; injection volume is 10 μl.

retention times. In addition, better analyte solubility in water (of the mobile phase) also shortens retention times. Finally, the retention/solvent effect is always greater for longer disaccharides compared with their smaller analogues as seen by the steeper slopes in Fig. 4. This retention behavior is analogous to that reported for sugars on alkylamine, polyol and diol columns.

3.4. Effect of additives on sensitivity

The effect of acidic additives on MS detection sensitivity was investigated, as shown in Fig. 5. The addition of small amount of acidic additives to the mobile phase greatly increased MS sensitivity in the positive ion mode (to over 10 times that without the additive). The addition of either formic acid or acetic acid produces a similar enhancement in sensitivity. The concentration of the additives does not have a significant effect on sensitivity since the role of the additive

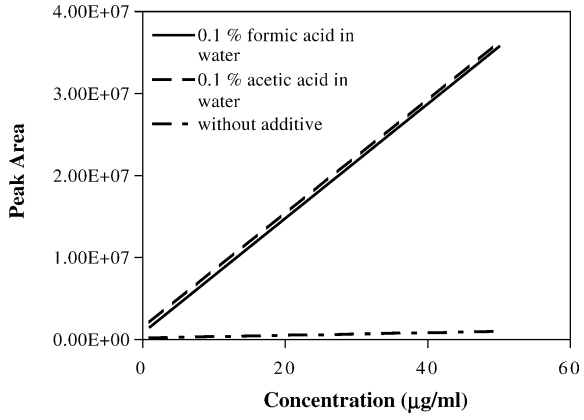

Fig. 5. Effect of additive on sensitivity for maltotriose. Column: Cyclobond I 2000 column (250 mm × 4.6 mm I.D.); flow rate at 400 μl/min. Mobile phase: 60:40 acetonitrile–water (0.1% formic acid). MS: ESI positive SIM at $[M + Na]^+$ (*m*/*z* 527).

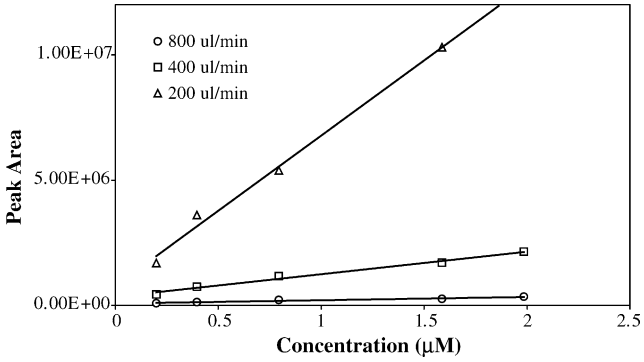

Fig. 6. Effect of flow rate on sensitivity for maltotriose. Mobile phase: 60:40 acetonitrile–water (0.1% formic acid). Column: Cyclobond I 2000 (250 mm × 4.6 mm I.D.). MS: ESI positive SIM at $[M + Na]^+$.

is to aid in ionization in ESI-MS techniques. Therefore, all the experiments were performed with the addition of an acidic additive for enhanced sensitivity.

3.5. Effect of flow rates on sensitivity

The effect of flow rates on the detection sensitivity of maltotriose was investigated using the Cyclobond I 2000 column (25 cm × 4.6 mm I.D.). The flow rate was varied from 200 to 800 μl/min. As seen in Fig. 6, the sensitivity at 200 μl/min is about 5 times higher than that found at 400 μl/min, and about 20 times higher than that found at 800 μl/min. This observed behavior has been discussed previously [49–52]. A lower flow rate is known to reduce the size of the charged droplets initially produced in the electrospraying process. These smaller initial droplets require fewer droplet fission events and less solvent evaporation prior to ion release into the gas phase. Thus, lower flow rates, especially with water–acetonitrile mobile phases, favor higher sensitivity in LC–ESI-MS.

3.6. Effect of column internal diameters (I.D.) on sensitivity

The effect of column I.D. on sensitivity of maltotriose was also investigated. The experiments were performed on three different 25 cm long analytical columns with inner diameters of 1.0, 2.0, and 4.6 mm, respectively. As is known, Columns of different sizes need different optimized experimental parameters to produce the optimum MS sensitivity. However, in order to simplify the experiment, all the LC and MS conditions were kept consistent for each individual column in this assay. For better comparison, a flow rate of 100 μl/min was used in order to obtain an acceptable backpressure when using the small I.D. column. As seen in Fig. 7, under the experimental conditions selected, the sensitivity on the 2.0 mm I.D. column is about two times higher than that on the traditional I.D. column (4.6 mm I.D.), while the sensitivity on the 1 mm I.D. column is about five times higher compared to that found with the traditional I.D. column. Although each

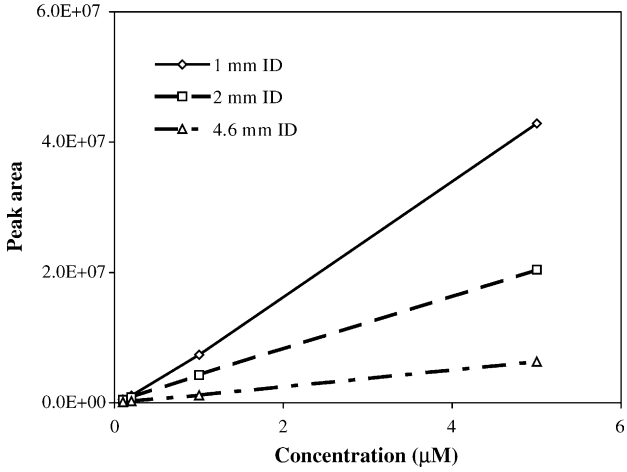

Fig. 7. Effect of column I.D. on sensitivity for maltotriose. Column: Cyclobond I 2000 column (250 mm × 4.6 mm I.D.); flow rate at 100 μl/min. Mobile phase: 60:40 acetonitrile–water (0.1% formic acid). MS: ESI positive SIM at $[M + Na]^+$ (*m*/*z* 527).

separation was not individually optimized, these experiments demonstrate the general trend that a smaller inner diameter column produces an increase in sensitivity over the conventional I.D. columns due to the relatively increased sample concentration at the detector. It should be noted, however, that the use of a 100 μl/min flow rate is not optimal for the conventional 4.6 mm I.D. At this low flow rate, longitudinal diffusion can affect the efficiency of the conventional column while having little effect on that of the microbore column.

Under microbore LC conditions, the flow rate is reduced by a factor of 4, from 400 to 100 μl/min. This reduction brings an increase in MS sensitivity of up to eight-fold (results not shown). Compared to the traditional ID column, the total gain in sensitivity using online LC–ESI-MS can be as high as 40 times. The present data indicate that lower flow rates and narrow I.D. columns favor high ESI-MS sensitivity for these oligosaccharide analyses.

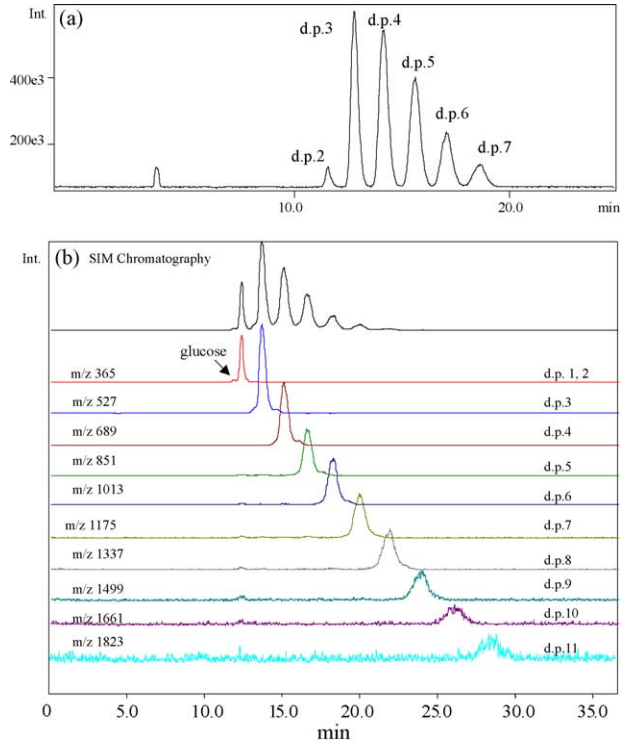

Fig. 8. LC–ESI-MS of dextran ladder on Cyclobond I 2000 column (250 mm × 4.6 mm I.D.). (a) LC–ESI-MS of underivatized maltooligosaccharide standard mixture (dp 2–7). (b) LC–ESI-MS of hydrolysis product of dextran. Experimental conditions: selected ion chromatography at $[M + Na]^+$; 65:35 acetonitrile–water (0.1% formic acid) mobile phase; flow rate at 400 μl/min.

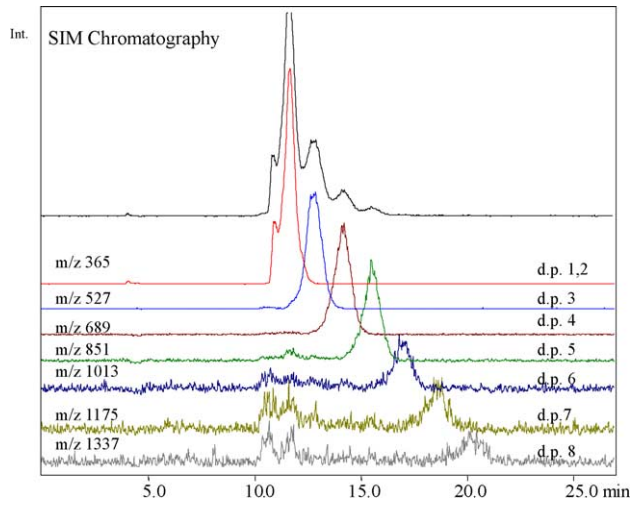

Fig. 9. LC–ESI-MS of mannan digest on Cyclobond I 2000 column (250 mm × 4.6 mm I.D.). Mannan was prepared by incubating mannan in 0.5 M hydrochloride acid at microwave (1.1 kV) for 30 s. Mobile phase composition: 65:35 acetonitrile–water (0.1% formic acid). ESI SIM mode; flow rate at 0.4 ml/min.

3.7. LC–ESI-MS of hydrolysis products of dextrin

Fifty milligrams of dextran was treated in 1 ml of 0.1 M trifluoacetic acid for 16 h at room temperature, and the hydrolysis products were analyzed using online LC–ESI-MS (Fig. 8). All oligosaccharides (up to 11 glucose units in the mixture) were separated and identified. With this simple dextran "ladder", all of the elution positions in terms of the number glucose units are fixed and easily determined. The hydrolysis product of mannan was also analyzed using this method. Mannan digestion products were prepared by incubating mannan in 0.5 M hydrochloride acid in a microwave oven (1.1 kV) for 30 s. Fig. 9 shows the LC–ESI-MS of mannan digest separated on the Cyclobond I 2000 column. Up to eight mannose units of the oligosaccharide mixture was observed. Clearly, this method is very suitable for most low to moderate molecular weight oligosaccharide mixtures produced by digestion of polysaccharides.

4. Conclusions

Native beta-cyclodextrin stationary phases have proven to be particularly useful for the analysis of oligosaccharides because the retention of these carbohydrates is based on the hydrogen bonding interactions of the oligosaccharide hydroxyl groups with those of the stationary phases. Thus, the retention time predictably increases with the elongation of oligosaccharide chain. This system is characterized by its selectivity, which also allows the separation of certain isomers, e.g., of some disaccharides. HPLC coupled with MS was shown to be a viable alternative to traditional detection techniques for the separation and characterization of underivatized oligosaccharides. The limits of detection can go down to as low as picogram levels for ESI-MS, which are orders of magnitude lower than low-wavelength UV detection. The use of low flow rates and narrow bore columns can further enhance the ESI-MS sensitivity. All individual components of oligosaccharide mixtures (up to 11 glucose units long) can be successfully separated using this method in less than 30 min. The method showed potential usefulness for the sensitive and quick analysis of hydrolysis products of polysaccharides, and individual oligosaccharides from biological systems as well.

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