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Evaluation of Counterions for Electrospray Ionization Mass Spectral Analysis of A Highly Sulfated Carbohydrate, Sucrose Octasulfate

Nur Sibel Gunay,[§] Keiko Tadano-Aritomi,[‡] Toshihiko Toida,[†] Ineo Ishizuka,[‡] and Robert J. Linhardt*[,§]

[+]*Division of Medicinal and Natural Products Chemistry, Department of Chemistry, Department of Chemical and Biochemical Engineering, The University of Iowa, Iowa City, Iowa, 52242, Department of Biochemistry, Teikyo University School of Medicine, Tokyo, Japan, and Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan*

A systematic approach was used to evaluate the electrospray ionization mass spectral (ESI-MS) analysis of sucrose octasulfate (SOS), an important pharmaceutical agent. SOS represents a model for other sulfated carbohydrates, such as heparin and glycosaminoglycan-derived oligosaccharides that also are highly sulfated and pose difficult analytical problems. A survey of ammonium counterions showed that 1°, 2°, and 3° ammonium salts of SOS gave substantial fragmentation as a result of sulfate loss. In contrast, quaternary ammonium and phosphonium salts gave excellent ESI spectra, particularly in the positive ion mode. This represents the first report of the ESI-MS analysis of sulfated carbohydrates in the positive ion mode.

Polysulfated carbohydrates are an important class of biologically active and pharmaceutically important molecules.[1,2] Glycosaminoglycans, for example, are natural products that regulate many important biological processes, including blood coagulation (heparin) and signal transduction (heparan sulfate), through their interaction with basic amino acid residues in their protein binding partners.[3,4] Synthetic polysulfated carbohydrates, including sucrose octasulfate (SOS), polysulfated phosphomannans, sulfated lactobionic acids, and cyclodextrin sulfates, that can be prepared in large quantities by chemical O-sulfonation of oligosaccharide precursors have found pharmaceutical use as both drug products and excipients.[5−7] The aluminum salt of SOS, in particular, is the drug Sucralfate (Carafate), which is widely used in the treatment of duodenal ulcers. Heightened interest in SOS has resulted from suggestion that its mechanism of action involved the stabilization of fibroblast growth factor, thus, promoting wound healing.[8,9] The crystallization of the sodium salt of SOS within the signal transduction complex[10] and demonstration of its modest oral bioavailability [6] has suggested new and important application for SOS and its analogues in wound healing and in the treatment of cancer. [10]

Mass spectral analysis of sulfated carbohydrates poses a number of unique challenges. The sulfate half-ester has a pK_a <1, ensuring that it carries a formal negative charge under nearly all of the experimental conditions. Moreover, nearly all biologically and pharmacologically relevant sulfated carbohydrates carry six or more (up to 100) sulfo groups,[1] making their mass spectral analysis daunting. Soft ionization methods of mass spectrometry have been applied with some success to this class of polyanions. ^{252}Cf plasma desorption PD-MS,[11] fast atom bombardment FAB-MS,[12] matrix-assisted laser desorption ionization MALDI-MS,[13−15] and electrospray ionization ESI-MS [16−21] have all been applied with some success to the analysis of sulfated carbohydrates. Two major problems plague all of these methods: fragmentation through loss

* To whom correspondence should be addressed. Address: 115 S. Grand Ave, PHAR S328, Iowa City, IA 52242. Fax: 1-319-335-6634. Email: robert-linhardt@uiowa.edu.

[§] The University of Iowa.

[‡] Teikyo University School of Medicine.

[†] Chiba University.

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of sulfo groups and sensitivity problems, particularly when analyzing large oligosaccharides or ones substituted with many sulfo groups. One approach for addressing these problems has been to change the counterions used in these analyses. A study of Na^+ versus NH_4^+ counterions in negative ion ESI-MS analysis of heparin-derived oligosaccharides showed that NH_4^+ salts gave enhanced sensitivity.[18] The sensitivity of negative ion ESI-MS analysis of the Na^+ salt of chondroitin sulfate-derived disaccharides could be enhanced through the addition of acid by eliminating multiple ions resulting from Na^+ adduction.[20] The successful application of negative ion ESI-MS to the analysis of sulfated carbohydrates in complex with basic peptides[5,16] was based on the pioneering work of Biemann and co-workers[13] on the analysis of similar complexes by positive ion MALDI-MS. Other counterions, including cationic surfactants, such as TDMAC,[11] and weakly and strongly basic amines,[22] have been used with some success in the analysis of polyanions.

As part of our laboratory's ongoing research for improved methods for the analysis of bioactive sulfated carbohydrates, we have undertaken to systematically explore the application of ammonium counterions in ESI-MS analysis. SOS was selected in this study because of its pharmaceutical importance, its availability in gram quantities and at high levels of purity, and its exceedingly high degree of sulfation (a disaccharide substituted with eight sulfo groups).

MATERIALS AND METHODS

Materials. Sucrose octasulfate (SOS), sodium salt of pharmaceutical purity, was a gift from Bukh Meditec (Farum, Denmark). The purity of SOS was confirmed by ^{1}H NMR spectroscopy.[23] Butylamine, 99.5%; diethylamine, 99.5+%; *N*,*N*-dimethylethylamine, 99%; tetramethylammonium hydroxide, 25 wt % solution in water; tetraethylammonium hydroxide, 20 wt % solution in water; tetrapropylammonium hydroxide, 1.0 M solution in water; tetramethylphosphonium chloride, 98%; spermidine, 97%; CGYGPKKKRKVGG (peptide); Dowex 50WX8-100 strongly acidic cation-exchange resin; Dowex MSA-1 strongly basic anion-exchange resin; and deuterium oxide, 99.9 atom % D, were from Sigma-Aldrich (St. Louis, MO).

Preparation of SOS Salts. Sodium SOS was dissolved in water (6 mg/mL) and passed through a Dowex 50WX8-100 strongly acidic cation-exchange resin column (1 × 10 cm, containing 5.1 meq of resin) at a flow rate of 0.3 mL/min. The eluent, having a pH < 2 was collected and immediately neutralized (to pH 7.0) with either alkylamine or the hydroxide form of the ammonium counterion. In the case of the tetramethylphosphonium SOS, tetramethylphosphonium chloride was converted to the hydroxide form using anion-exchange resin for its subsequent reaction with acidic SOS. The resulting neutral salt solutions were freeze-dried and stored desiccated at −60 °C until used.

Characterization of SOS Salts. Each SOS salt was dissolved in D_2O (99.96% of atom) filtered through a 0.45-μm syringe filter and freeze-dried to remove exchangeable protons. After exchanging the sample two times, the sample was dissolved in D_2O (99.96% of atom). One-dimensional (1D) ^{1}H NMR experiments were performed on a Bruker Avance DRX-400 with a BBO probe with XWINNMR version 3.0 software and AMX-600 with a BBI xyz gradient probe with XWINNMR version 2.1 software at 298 K on 700-μL samples at 0.9−2.7 μM.

Each salt was also analyzed by flame atomic absorption spectrometry (Hitachi polarized Zeeman atomic absorption spectrophotometer Z-8000) to determine the amount of residual Na^+ present. Each SOS complex was diluted to 500 μg/mL and was analyzed at 589.0 nm for Na^+.

ESI-MS Analysis of SOS Salts with Ion Trap Analyzer. Positive and negative ion ESI-MS analyses were performed using an LCQ DECA ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) fitted with an ESI probe. The samples (∼50 μg/mL) were introduced either for 0.5−1 min by direct infusion in a solution of methanol/water (1:1) or in a solution of methanol containing 0−70% (v/v) water at a flow rate of 3 μL/min. The heated capillary was set at 250 °C, and the spray voltage was set at 5.0 kV. The sheath gas flow rate was set to 50 in arbitrary units.

RESULTS AND DISCUSSION

Even though the mass range of the instrument was 4000, analysis was performed in a mass range of <2000 to obtain the highest resolution, precision, and sensitivity. Counterions having a mass of <186 amu were selected for this study to keep the analyte mass <2000. Initial experiments focused on the examining 1°, 2°, 3° and quaternary ammonium counterions that contained the same number of carbon atoms (i.e., butylammonium, diethylammonium, *N*,*N*-dimethylethylammonium, tetramethylammonium). Subsequent experiments examined quaternary ammonium counterions with increasing number of carbon atoms having increased hydrophobicity atoms (i.e., tetramethylammonium, tetraethylammonium, tetrapropylammonium). A phosphonium counterion (tetramethylphosphonium), polyamine counterion (spermidine), and a basic peptide counterion (CGYGPKKKRKVGG) were also examined.

Sodium SOS was converted to its acid form and neutralized with amines or the hydroxide salts of a variety of counterions to prepare SOS complexes for ESI-MS analysis. ^{1}H NMR analysis confirmed that each SOS ammonium and phosphonium salt contained eight of the desired counterions. The spermidine salt displayed a polyamine to SOS stoichiometry of 2:1. Basic peptide was obtained in a 1:1 complex with SOS. In the case of polyamine and peptide complexes, residual acidity was neutralized with sodium hydroxide. The SOS ammonium and phosphonium salts showed no residual Na^+ contamination by flame atomic absorption spectrometry (detection limit (S/N = 3), 0.05 μg/mL).

ESI-MS analysis was performed on each sample in the negative and positive ionization modes. It was our expectation that the negative mode of analysis would give the best spectral data. To our surprise, the positive ion mode of analysis gave better sensitivity and less fragmentation in every salt on which we obtained spectral data. Sodium SOS was first analyzed under positive and negative ionization modes (Figure 1). The positive ion spectrum of Na^+SOS showed a prominent $[M + Na]^+$ (where M corresponds to Na_8SOS) at m/z 1181 (Scheme 1). In contrast, the negative ion spectrum showed a weak ion at m/z 1135 corresponding to $[M - Na]^-$ and extensive fragmentation associated with loss of $NaSO_3$ with hydrogen transfer[24,25] (102 amu) ((b)−(c) in Scheme 2) at m/z 1033, 931, 829, and 727. We have

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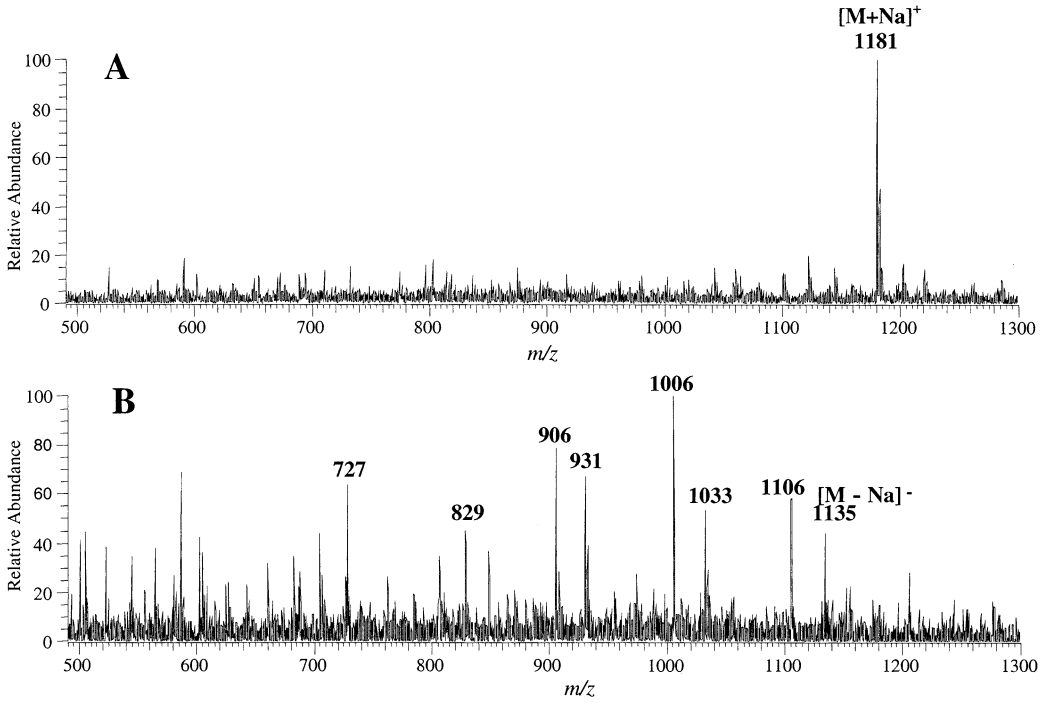
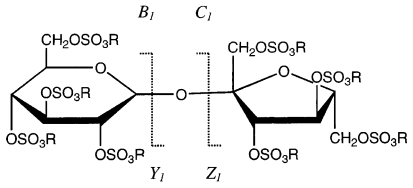


Figure 1. ESI mass spectra of SOS-sodium salt in positive ion (A) and negative ion (B) modes. Expansion of the molecular ion region in all figures gives the expected isotope peak intensities.

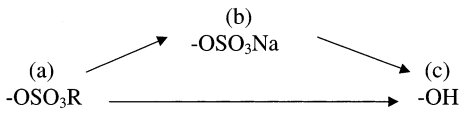
Scheme 1. Molecular-Related Ions and Major Fragment Ions (*m*/*z*) for Various Salts of SOS



R	R	MW	M+Na	M+R	M-R	$B_1(Z_1)$+R	$C_1(Y_1)$+R
H	1.01 1	982.80 982	1005.79 1005	983.81 983	981.79 981	484.41 484	500.41 500
Na	22.99 23	1158.66 1158	1181.65 1181	1181.65 1181	1135.67 1135	594.32 594	610.32 610
$(CH_3)_4N$	74.15 74	1567.91 1566	1590.90 1589	1642.02 1640	1493.76 1492	850.10 849	866.10 865
$(CH_3CH_2)_4N$	130.25 130	2016.77 2014	2039.76 2037	2147.03 2144	1886.52 1884	1130.64 1129	1146.64 1145
$(CH_3CH_2CH_2)_4N$	186.36 186	2465.64 2462	2488.63 2485	2652.00 2648	2279.27 2276	1411.18 1409	1427.18 1425
$(CH_3)_4P$	91.11 91	1703.65 1702	1726.64 1725	1794.76 1793	1612.53 1611	934.94 934	950.94 950

Upper: average mass
Lower: nominal mass

Scheme 2. Major Fragmentation Pathway and Mass Difference for Each Step



R	R	(a)-(b)	(b)-(c)	(a)-(c)
$(CH_3)_4N$	74.15 74	51.16 51	102.04 102	153.20 153
$(CH_3CH_2)_4N$	130.25 130	107.26 107	102 04 102	209.31 209
$(CH_3CH_2CH_2)_4N$	186.36 186	163.37 163	102.04 102	265.42 265
$(CH_3)_4P$	91.11 91	68.12 68	102.04 102	170.17 170

Upper: average mass
Lower: nominal mass

been unable to assign a second series of ions observed at *m*/*z* 1106, 1006, and 906.

The positive ion spectrum of Na_8SOS gave a sensitivity of ~8 ng at S/N = 3. Our initial survey of ammonium salts having four carbon atoms demonstrated that 1°, 2°, and 3° ammonium salts of SOS resulted in extensive loss of SO_3 in both the negative and positive ion modes (data not shown). In contrast, the quaternary ammonium salt, tetramethylammonium SOS (Figure 2) showed a strong positive ion spectrum (1 ng sensitivity at S/N = 3) with a molecular ion at *m*/*z* 1640, corresponding to $[M + R]^+$ (where M corresponds to $[(CH_3)_4N^+]_8SOS$ and R corresponds to $(CH_3)_4N^+$ and a base peak at *m*/*z* 1589 corresponding to $[M + Na]^+$. Surprisingly, a series of molecular ions separated by 51 amu are observed at *m*/*z* 1538, 1487, and 1436, corresponding to the replacement of $(CH_3)_4N^+$ with Na^+. Analysis of the sample by atomic absorption spectrometry showed no residual Na^+, suggesting that these sodiated species resulted from Na^+ contaminating the solvents, glassware, or mass spectrometer. Generally, ESI-MS studies show that molecular-related ions are typically detected as their Na adducts when neutral or acidic compounds are analyzed by positive ion ESI-MS. The negative ion spectra of the same sample showed an intense peak of *m*/*z* 1492, corresponding to $[M - R]^-$, with the unusual loss of 51 and 102 amu, corresponding to the replacement of $(CH_3)_4N^+$ (R) with Na^+ followed by the exchange of SO_3Na with a hydrogen, i.e., $[-SO_3(R - Na)\ (51) - ((SO_3Na) + H)\ (102)]$ or the exchange of SO_3R

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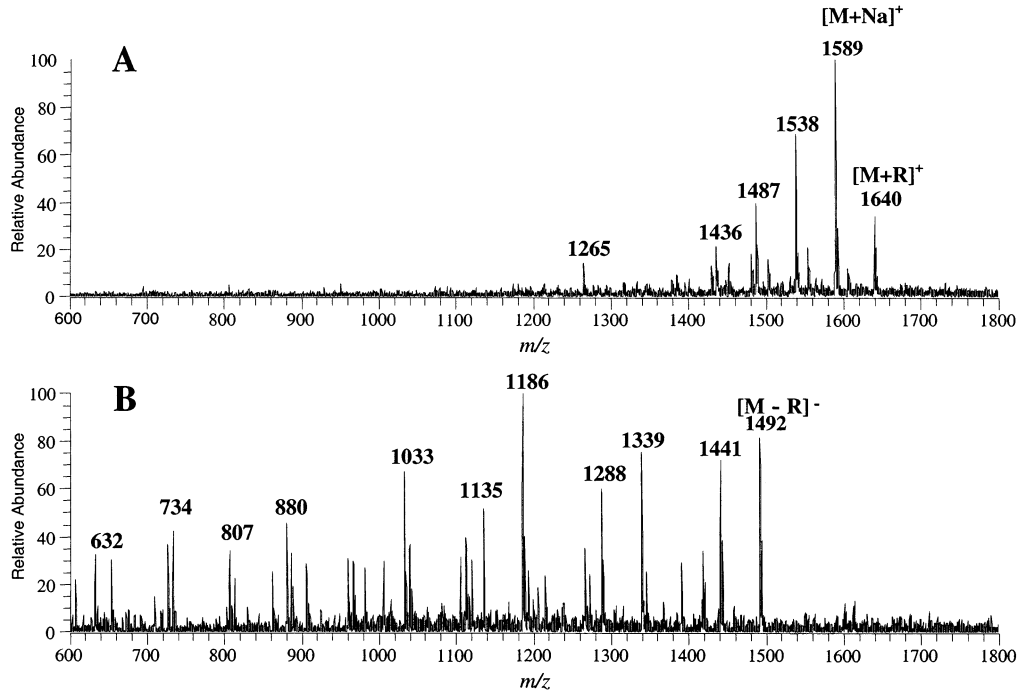

Figure 2. ESI mass spectra of SOS-tetramethylammonium salt in positive ion (A) and negative ion (B) modes.

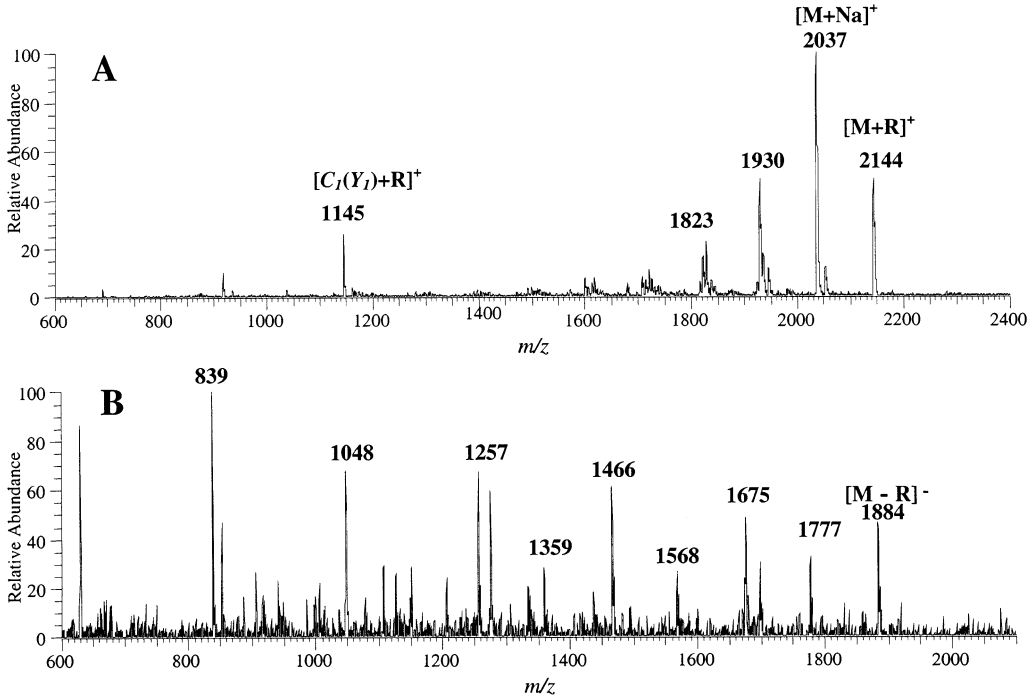

Figure 3. ESI mass spectra of SOS-tetraethylammonium salt in positive ion (A) and negative ion (B) modes.

directly with a hydrogen (Scheme 2). The addition of tetramethylammonium hydroxide (5−20 equiv) to the same sample solution in both positive ion and negative ion modes resulted significantly increased $[M + R]^+$ ion at m/z 1640 and $[M - R]^-$ at m/z 1492. A decreased relative intensity of Na adduct ions was observed when 20 equiv of tetramethylammonium hydroxide was added (data not shown). The tetraethylammonium salt (Figure 3) of SOS gave ~5-fold improved sensitivity over the tetramethylammonium salt in both the positive ion and negative ion spectra, showing characteristics similar to those described for the tetramethylammonium salt (Figure 2). Again, the replacement of $(CH_3CH_2)_4N^+$ with Na^+ produced the molecular-related ions at m/z 2037, 1930, and 1823 in the positive ion mode. The same unusual fragmentation pattern was observed in the negative ion spectrum with $[M - R]^-$ losing 107 and 102 amu corresponding to $[-SO_3(R - Na)$ (107) $-$ $((SO_3Na) + H)$ (102)] (Scheme 2). Extension of the quaternary ammonium series to the tetrapropylammonium salt markedly reduced sensitivity but afforded a reasonable positive ion spectrum showing $[M + R]^+$ and $[M + Na]^+$ at m/z 2648 and 2485, respectively. The negative ion spectrum of this sample showed a $[M - R]^-$ ion at m/z 2276 and the loss of 265 amu, corresponding to $[-SO_3(R - Na)$ (163) $-$ $((SO_3Na) + H)$ (102)]

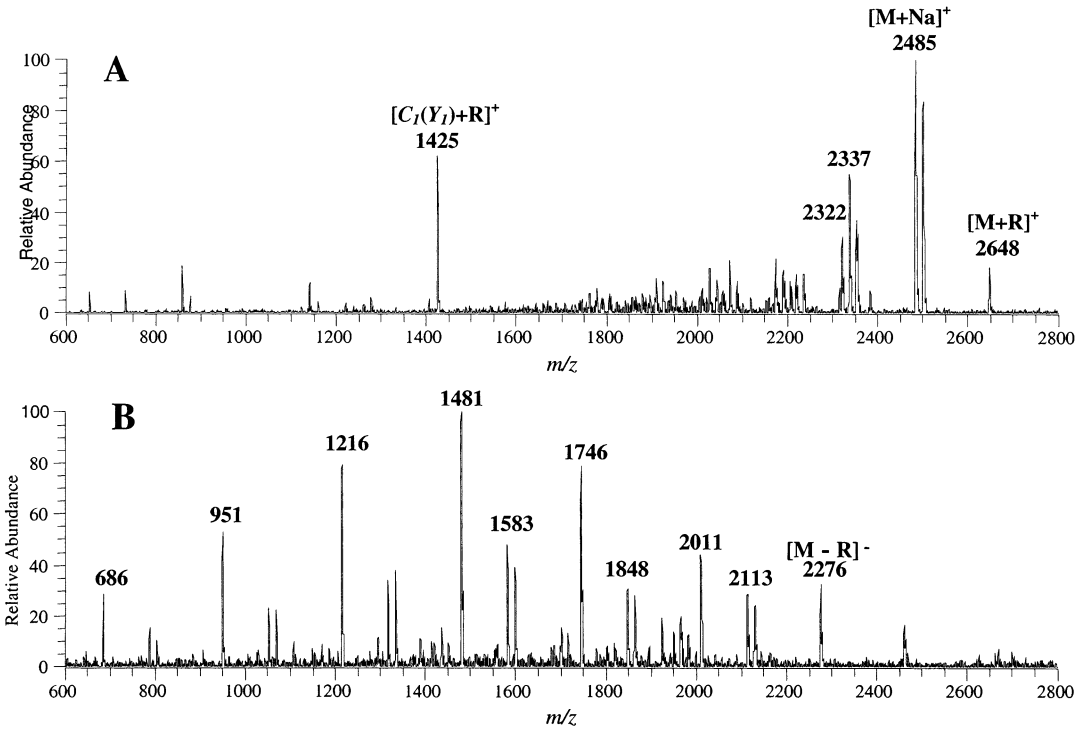

Figure 4. ESI mass spectra of SOS-tetrapropylammonium salt in positive ion (A) and negative ion (B) modes.

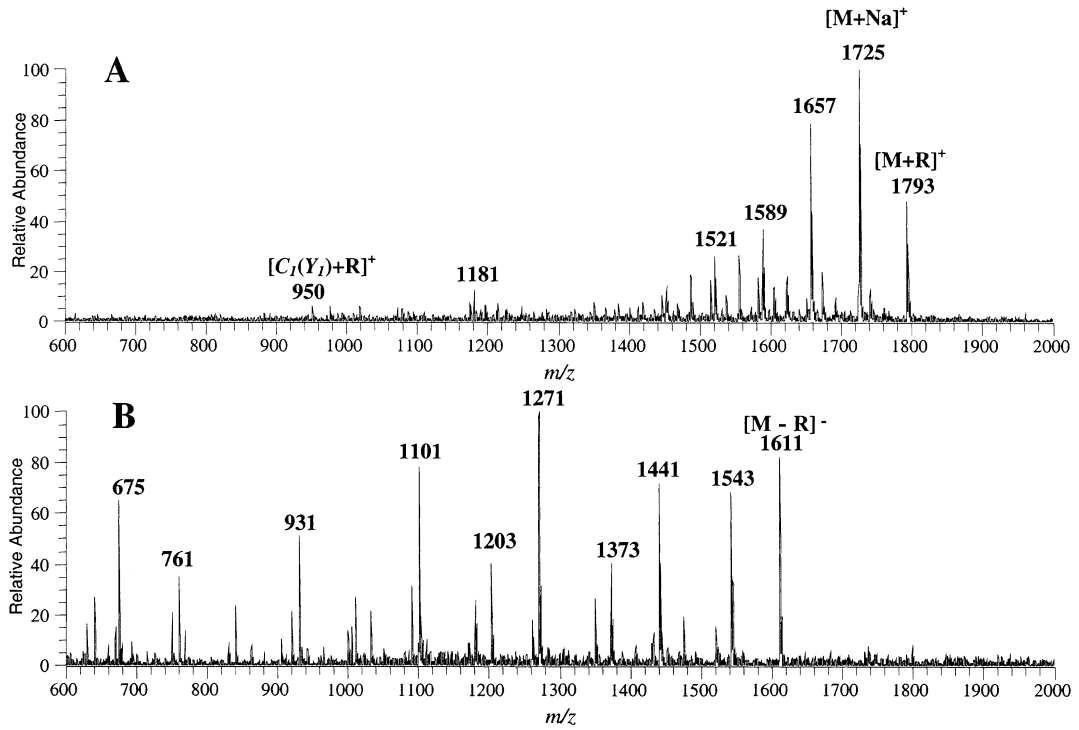

Figure 5. ESI mass spectra of SOS-tetramethylphosphonium salt in positive ion (A) and negative ion (B) modes.

(Figure 4 and Scheme 2). The tetramethylphosphonium salt of SOS (Figure 5) afforded sensitivity comparable to the tetramethylammonium salt. The positive ion spectrum showed $[M + R]^+$ and $[M + Na]^+$ (base peak) at m/z of 1793 and 1725, respectively. A series of ions observed at 68-amu intervals, at m/z 1657, 1589, and 1521, were the result of the replacement of $(CH_3)_4P^+$ with Na^+. The negative ion spectrum of the tetramethylphosphonium SOS showed a $[M - R]^-$ ion at m/z 1611 and a fragmentation pattern of $[-SO_3(R - Na)\ (68) - ((SO_3Na) + H)\ (102)]$ (Scheme 2), similar to that observed in the tetramethyl, tetraethyl, and tetrapropylammonium complexes.

As shown in Scheme 2, major fragmentation pathways include (1) replacement of R by Na ((a)−(b)), (2) elimination of Na and sulfate ((b)−(c)) with hydrogen transfer, and (3) exchange of SO_3R by hydrogen ((a)−(c)). In addition, the positive ion spectra of tetraethylammonium and tetrapropylammonium SOS contained an intense ion corresponding to $[C_1(Y_1) + R]$, produced by glycosidic cleavage (Scheme 1).

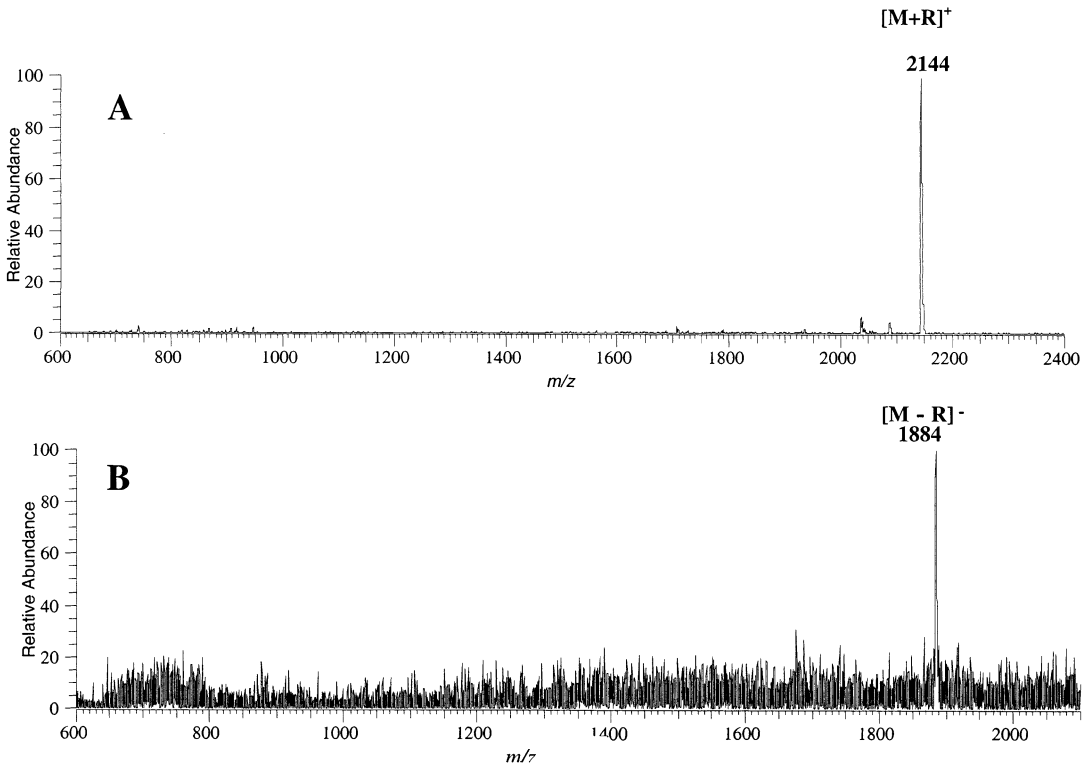

Figure 6. ESI mass spectra of SOS-tetraethylammonium salt in the presence of 10 mM tetraethylammonium hydroxide in positive ion (A) and negative ion (B) modes.

The polyamine complex spermidine$_2$SOS gave no definable mass spectrum in either the positive ion or the negative ion mode. Similarly, no mass spectrum was observed for the peptide$_1$SOS complex, even with analyte amounts of up to 1 μg.

Added methanol 30−100% (v/v) in the presence of 0−100 mM tetramethylammonium hydroxide or tetraethylammonium hydroxide was examined to enhance the sensitivity of this method in the analysis of the tetramethylammonium and tetraethylammonium salts of SOS. The best sensitivity was obtained in 90% aqueous methanol (methanol/water of 9:1) for both salts of SOS. Next, solutions of methanol/water (9:1) containing 5, 10, 20, and 100 mM tetramethylammonium (or tetraethylammonium) hydroxide were used for analysis. The addition of 5−10 mM (150−400 equiv) tetramethylammonium (or tetraethylammonium) hydroxide gave the best results. In the negative ion mode, both tetramethylammonium and tetraethylammonium salts of SOS with 10 mM tetramethylammonium and tetraethylammonium hydroxide afforded exclusively the $[M - R]^-$ ion but did not improve the sensitivity of analysis (Figure 6B). In the positive ion mode, tetraethylammonium SOS with 10 mM added tetraethylammonium hydroxide gave exclusively the $[M + R]^+$ ion (no peak for $[M + Na]^+$ was detected) with a more than 10-fold enhancement in sensitivity (Figure 6A). The tetramethylammonium salt of SOS with 10 mM added tetramethylammonium hydroxide gave a base peak of $[M + R]^+$ at m/z 1640.

In summary, this work presents the first example of positive ion ESI-MS spectra in the analysis of sulfated carbohydrates. Furthermore, the quality of the positive ion data is superior to the negative ion spectra of the same compounds. These studies also confirm the instability of the ammonium salts of sulfate half-esters, but suggest that this problem may be limited to 1°, 2°, and 3° ammonium salts. The ideal counterion in these analyses appears to be tetraethylammonium, with other quaternary ammonium and phosphonium salts also performing well. The sensitivity for SOS-tetraethylammonium salt is more than 20-fold higher than that for SOS-sodium salt. The presence of sodiated ions Na^+, coming from contaminants in the system, can be avoided by addition of tetraalkylammonium hydroxide. The highest sensitivity, more than 200-fold improvement over SOS-sodium salt, was obtained in the presence of 10 mM tetraethylammonium hydroxide. Future studies will examine whether sodium chelators can be used to improve ESI-MS analysis and the application of MS-MS to these analyses. The negative ion spectrum of quaternary ammonium and phosphonium salts also exhibit an unusual fragmentation that requires further investigation. Finally, it appears that the use of polyamine and peptide counterions in the current study is of little value in improving analysis. This is in contrast to the previously reported successful analysis of such complexes by our laboratory[16] and others.[13−15] We suggest that the instrumentation and specific experimental parameters applied in this study may simply not allow the sensitive detection of such complexes.

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