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High-Performance Liquid Chromatographic Separation of Heparin-Derived Oligosaccharides

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Heparin has been enzymatically depolymerized with heparinase (heparin lyase (EC 4.2.2.7)) and then separated into di-, tetra-, hexa-, octa-, and decasaccharide mixtures by low-pressure gel-permeation chromatography (GPC). These sized mixtures were resolved by strong anion-exchange (SAX) HPLC into multiple components. The fractions from the SAX-HPLC were collected and characterized for size by GPC-HPLC and sulfate content by ion chromatography. This study provides detailed methodology for the separation of larger and more highly sulfated oligosaccharides than previously reported. It describes the first use of ion chromatography for the accurate determination of the sulfate content of heparin oligosaccharides, a method which can also be applied to heparin and other glycosaminoglycans. © 1985 Academic Press, Inc.

KEY WORDS: heparin; heparinase; ion-exchange chromatography; HPLC; sulfate determination.

High-performance liquid chromatography (HPLC) has greatly aided in the structural determination of complex polysaccharides such as glycosaminoglycans (GAGs)[2] by facilitating the separation of oligosaccharides formed by depolymerization. The approach has been depolymerization of the polysaccharide, followed by fractionation of the resulting oligosaccharides into size-uniform mixtures, then separation of these mixtures to obtain pure samples for analysis. Low-pressure gel-permeation chromatography (GPC) has been effectively used to separate out the various size-uniform mixtures resulting from depolymerization (1). Reverse-phase HPLC has been employed as a method to achieve further separation on a previously sized mixture, as in the case of the disaccharide components obtained from chondroitin sulfate depolymerization (2–4). Strong anion-exchange (SAX) HPLC has also been used to separate the sized mixtures of di-, tetra-, hexa-, octa-, and decasaccharides obtained from hyaluronidase or chondroitinase depolymerization of chondroitin sulfate (5,6). More recently, this technique has been applied to tetrasaccharides resulting from nitrous acid depolymerization of heparin (7). The application of such separation techniques to larger, more highly sulfated oligosaccharides is critical for the isolation and characterization of biologically active molecules. This report examines the use of SAX-HPLC in optimizing separations of the sized mixtures of tetra-, hexa-, octa-, and decasaccharides obtained from the depolymerization of heparin with heparinase (EC 4.2.2.7). We further demonstrate that oligosaccharides within each size class elute from the SAX column in order of increasing degree of sulfation.

EXPERIMENTAL

Materials

Porcine mucosal heparin, sodium salt, was obtained from Hepar Industries, Ohio. Fractogels TSK HW40(F) and HW50(S) were from MCB Manufacturing Chemists, New Jersey.

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[2] Abbreviations used: GAGs, glycosaminoglycans; GPC, gel-permeation chromatography; SAX, strong anion exchange.

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Sephadex G-10, SP-Sephadex C-50, and blue dextran were purchased from Sigma Chemical Company, St. Louis, Missouri. Pyrolysis tubes were Pyrex No. 9860 from Corning Glass, New York. Desalting was done using Spectra/Por dialysis tubing (1000 M_r cutoff) purchased from Spectrum Medical, Indiana. HPLC was performed using two LDC Constametric III pumps with gradient control by D/A interface with an Apple IIe computer equipped with Chromatochart software from Interactive Microware Inc., Pennsylvania, and a LDC low-volume mixing chamber connected to a Rheodyne 7125 injector, Techsphere 5 cm × 4.6-mm guard and 25 cm × 5-mm SAX analytical column from Phenomenex, California. An ISCO Model 1840 variable-wavelength uv detector connected through an A/D converter to the Apple IIe computer was used for continuous monitoring and integration report. High-pressure sizing was done on Toyo Soda TSK-Gel G3000SW 7.5 mm × 50 cm and G2000SW 7.5 mm × 50 cm (in series) with 7.5 mm × 10 cm guard from Phenomenex. Sulfate analysis was performed using Ion Chromatography QIC equipped with an anion HPIC-AS3 column with guard and AFS-1 suppressor from Dionex, California. Sulfate was detected conductametrically, and peak areas were quantitated by Spectra Physics integrator recorder Model 4270. Freeze-drying was performed with Virtis 10-100 lyophilizer, and spectrometric measurements were performed on a Pye Unicam SP8-100. Conductametric measurements of salt gradients were done on conductivity bridge Model RC 16B2 by Industrial Instruments Inc., New Jersey. All chemicals were reagent grade or better.

Methods

Heparin depolymerization. A solution was prepared containing heparin (25 mg/ml), 0.025 M sodium acetate, 0.25 mM calcium acetate and adjusted to pH 7.0 with acetic acid. To 1 vol of this solution, 2 vol of heparinase (0.05 unit/ml (1 unit = 1 μmol bonds cleaved/min) in 100 mM sodium phosphate buffer, pH 7.0) was added and the reaction was run at 30°C for approximately 8 h, monitoring at 232 nm until a constant absorbance was achieved (8). The product solution was diluted with an equal volume of distilled water and adjusted to pH 4, and the enzyme was removed by SP-Sephadex C-50 ion-exchange chromatography. After the pH was readjusted to 7, the product solution was immediately frozen and freeze-dried.

Low-pressure GPC of heparin-derived oligosaccharides. Size fractionation (1) was performed by dissolving the freeze-dried digestion mixture into water (0.1 g/2 ml) and applying it to a Fractogel TSK low-pressure column (HW50S 17 × 2.5 cm and HW40F 80 × 2.5 cm run in series) eluted at 0.3 ml/min with 1 M ammonium bicarbonate. The tetrasaccharide fraction was desalted by a low-pressure Sephadex G-10 column (42.5 × 2.4 cm) eluted at 1 ml/min with water. Hexa-, octa-, and decasaccharides were desalted by dialyzing (1000 M_r cutoff membrane) against 100 vol of water. After freeze-drying, the samples were dissolved in a small volume of water and rechromatographed on the same column and a center cut of each component was taken, desalted, and freeze-dried.

SAX-HPLC of sized oligosaccharides. Charge separation of the sized oligosaccharides fractions was carried out by analytical SAX-HPLC using linear gradients of sodium chloride at a pH of 3.5, prepared by a programmed two-pump system at a flow rate of 1.5 ml/min with a y intercept at 0.2 M and a slope of 1.2 $\times 10^{-2}$ M/min. After each run the column was washed with 1.5 M sodium chloride, followed by water, and then reequilibrated with 0.2 M sodium chloride at a pH of 3.5. The detector was set at a fixed wavelength of 232 nm and 0.2 AUFS in all runs. The sized oligosaccharide fractions were dissolved in 20 μl of water and applied to the column in the following amounts (as measured by absorbance $\epsilon_{232} = 5500\ M^{-1}\ cm^{-1}$ (8)): tetra ($M_{r_{av}} = 1157$) 300 μg, hexa ($M_{r_{av}} = 1556$) 560 μg, octa ($M_{r_{av}} = 2023$) 720 μg, deca ($M_{r_{av}} = 2804$) 1.1 mg.

GPC-HPLC size determination. The oligosaccharide components obtained by SAX-HPLC and the sized oligosaccharide mixtures were applied (5 μg in 100 μl water) to GPC-HPLC G3000SW and G2000SW columns, set with a G2000SW guard connected in series. The columns were eluted with carefully degassed 0.5 M sodium chloride solution at a flow rate of 1 ml/min. The detector was set at 232 nm with 0.02 AUFS and retention times measured to ±0.1 s. The void and total volumes were measured by blue dextran and sodium azide.

Sulfate determination by ion chromatography. Samples were prepared for sulfate analysis in triplicate. The precise concentration of each sample was determined by dissolving approximately 10 μg in 1 ml of 0.03 N hydrochloric acid and measuring its absorbance (ϵ_{232} = 5500 M^{-1} cm^{-1}). The samples were placed in Pyrex pyrolysis tubes with 3 μg of disodium phosphate and dried at 100°C. Sodium hydroxide (20 μl at 0.02 N) was added and allowed to evaporate. The samples were then pyrolyzed for 7 s using a Fisher burner (9) and reconstituted to 1 ml with double-distilled water.

Sulfate analysis was performed using anion chromatography (10) by eluting with sodium bicarbonate (3 mM), sodium carbonate (2.4 mM) buffer at a flow rate of 3 ml/min and detected by conductance at 3 μS full scale. The sulfate eluted with a retention time of 8 min resolved from all other contaminating anions.

RESULTS AND DISCUSSION

Heparin was depolymerized with heparinase (EC 4.2.2.7) prepared fermentatively from *Flavobacterium heparinum* (11) and purified to ensure no contaminating activities were present (11–13). This eliminase is specific for its site of action (Fig. 1) and produces a Δ4,5 site of unsaturation at the nonreducing end of the resulting oligosaccharides (14,15). This chromophore absorbs at 232 nm, allowing easy monitoring as well as quantitation based on the molar absorptivity reported for the disaccharide (16). Chondroitin sulfate and hyaluronate oligosaccharides have also been produced using eliminases including chondroitinases (6,17) and certain hyaluronidases (17) and can similarly be monitored by absorbance. Chondroitin sulfate oligosaccharides prepared using hydrolases such as hyaluronidase (EC 3.2.1.35) do not possess the unsaturated nonreducing end and are therefore often monitored by radiolabeling their reducing end (18). Nitrous acid depolymerization of GAGs results in oligosaccharides, which have been radiolabeled to monitor their separation (19). Detection by refractometry requires higher concentrations of oligosaccharide and can be used only for isocratic elutions (20).

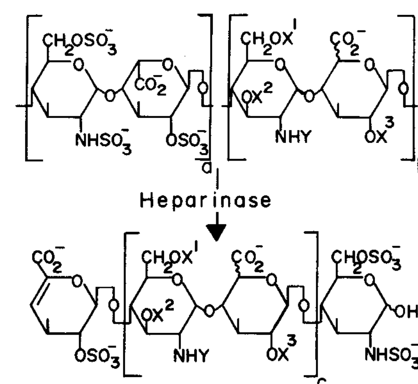

FIG. 1. Heparinase depolymerization of porcine heparin a = cleavable site, b = uncleavable site, a/b = 1.1, c = 0, 1, 2, · · ·). When c = 1, the following five major tetrasaccharides result: tetra 1, $X^1 = X^2 = X^3$ = H, Y = $COCH_3$, iduronic acid; tetra 2, $X^1 = X^2 = X^3$ = H, Y = $COCH_3$, glucuronic acid; tetra 3, $X^1 = X^2 = X^3$ = H, Y = SO_3^-, glucuronic acid; tetra 4, X^1 = SO_3^-, $X^2 = X^3$ = H, Y = SO_3^-, glucuronic acid; tetra 5, $X^1 = X^3 = SO_3^-$, X^2 = H, Y = SO_3^-, iduronic acid (21).

Size fractionation by low-pressure GPC followed by desalting, freeze-drying, and reapplication provided a fraction containing size-pure material. The disaccharide eluted at 262 ml, tetra 231 ml, hexa 212 ml, octa 197 ml, deca 185 ml, and higher oligo < 170 ml with void of 144 ml and total volume of 447 ml as measured by blue dextran and sodium azide. The average molecular weight of sized mixtures was calculated by addition of the measured degree of sulfation for each sized mixture

to the molecular weight of the parent polysaccharide frame. The molecular weight of the disaccharide was determined by mass spectrometry (21). A plot of K_{av} vs log $M_{r_{av}}$ for this column gave an equation of $y = -0.418\ (X) + 1.57$ with a correlation of 0.9997. The average molecular weight of the mixed oligosaccharide fractions was further established by GPC-HPLC (Fig. 2) (21,22) giving a plot of K_{av} vs log M_r ($y = -0.222(X) + 1.47$) with a correlation of 0.996 (Fig. 3). The size-purified oligosaccharide fractions were each applied to an analytical SAX-HPLC column to obtain the complex profile of tetra-, hexa-, octa-, and decasaccharides (Figs. 4a–d). The programmed gradients were checked for accuracy by collecting the mixed salt solution entering the column and measuring its conductance against a standard curve. A sodium chloride gradient at pH 3.5 having a slope of 1.2×10^{-2} M/min and y intercept of 0.2 M produced a good separation. After the column had aged by 50 injections, a twofold reduction in slope was required to reproduce the optimized resolution. Higher slopes produced bunched peaks which were poorly resolved from each other, and lower slopes broadened out peaks causing overlaps. Attempts to sharpen peaks by step gradients reduced resolution, while slightly concave gradients resulted in resolution similiar to that obtained using the optimized linear gradients. Addition of either 5 or 10% methanol only resulted in reduced retention times and broadening of the peaks. The use of sodium chloride as the eluant required extra washing of the pumps to avoid corrosion. The salt solutions were adjusted to pH 3.5 to suppress the ionization of carboxyl groups in the uronic acid residues, and even in the absence of buffer the pH did not exceed 4 in the samples collected. The use of Techsphere 5-μm particle packing material gave improved resolution over a Partisil 10-μm packing on an analytical SAX column of the same dimensions. The higher back pressure associated with the smaller particle size (>3500 psi at 2 ml/min) required a reduction in flow rate by 25%, thus slowing run time.

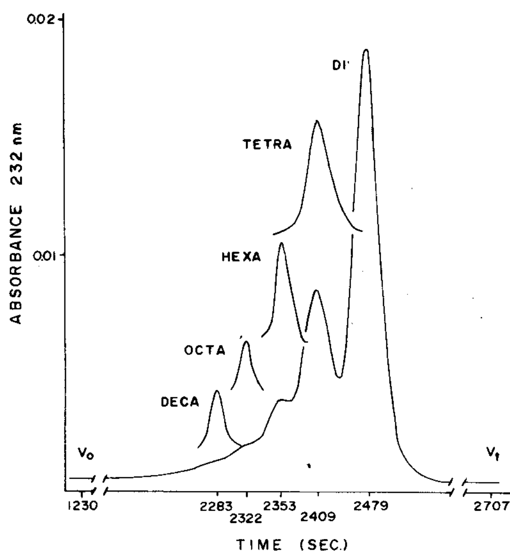

FIG. 2. GPC-HPLC size determination of heparin depolymerized with heparinase, and sized tetra-, hexa-, octa-, and decasaccharide mixtures obtained using low-pressure GPC. Elution was performed at a flow rate of 1 ml/min, peaks were detected at 232 nm (0.02 AUFS), and retention times were calculated by the interfaced Apple IIe microcomputer.

Peak profiles were reproduced on the analytical column many times and always found to contain the same number of components. Scaling the sample loading up to 3 mg on the analytical column only slightly reduced the resolution of peaks. Fractions corresponding to each peak were collected from the 3-mg injections and desalted by dialysis in 1000 M_r bags. Individual fractions from each SAX-HPLC separation were subjected to GPC-HPLC and most eluted with retention times consistent with their expected chain length (Fig. 3). The tetrasaccharide and hexasaccharide SAX-HPLC purified fractions all proved to be size pure by GPC-HPLC analysis. The octasaccharide mixture was contaminated with 12 mol% of decasaccharide, while the decasaccharide mixture contained 5.5 mol% of higher oligosaccharide as determined by GPC-HPLC.

Scaling up to a Partisil M9 10-μm 50-cm semipreparative SAX column resulted in decreased resolution of the sized oligosaccharide mixtures. By repeating preparative chromatography on incompletely resolved compo-

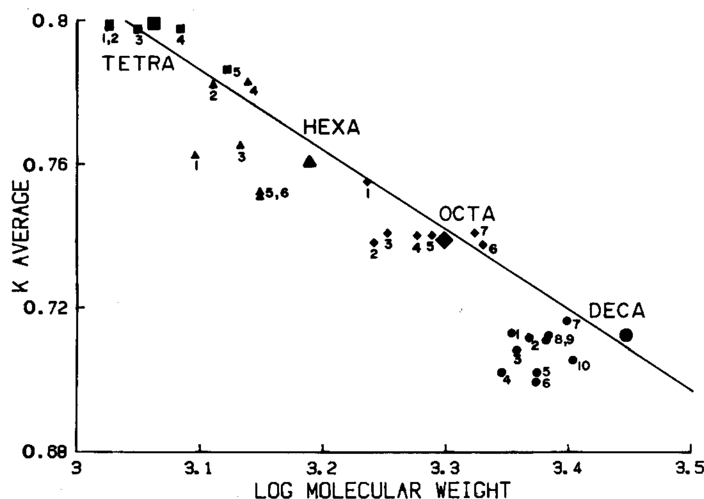

FIG. 3. A plot of K_{av} vs log M_r of sized tetrasaccharide (■), hexasaccharide (▲), octasaccharide (♦), and decasaccharide (●) mixtures. K_{av} was determined by GPC-HPLC, and molecular weight averages were calculated using the observed degree of sulfation for the mixtures. Fractions collected from the SAX-HPLC column ($■_{1-5}$, $▲_{1-6}$, $♦_{1-7}$, $●_{1-10}$) are also plotted based on their observed K_{av} and molecular weight determined from sulfate data.

nents obtained from the tetrasaccharide mixture, the five major tetrasaccharides have been obtained at greater than 95% purity and completely characterized (Fig. 1) (21). The analysis of these five major tetrasaccharides shows that SAX-HPLC retention at pH 3.5 is related to degree of sulfation. Tetrasaccharides 1 and 2 are trisulfated, tetrasaccharide 3 is tetrasulfated, tetrasaccharide 4 is pentasulfated, and tetrasaccharide 5 is hexasulfated (21). The ability of SAX-HPLC to resolve the trisulfated tetrasaccharides, 1 and 2, may be due to conformational factors resulting in differences in charge accessibility affecting the retention time. A direct relationship between SAX-HPLC elution time and degree of sulfation for various components can be seen for all the sized oligosaccharide mixtures (Figs. 5a–d).

The single disaccharide, which represents heparin's major repeating unit, has been characterized using this method with reference to chondroitin sulfate standards, and its exact structure determined (21,23,24). SAX-HPLC has been used by Delaney *et al.* (5) to fractionate oligosaccharides resulting from the depolymerization of chondroitin sulfate with hyaluronidase hydrolase. The oligosaccharides, radiolabeled at their reducing end, were eluted with linear gradients of potassium phosphate and monitored by scintillation counting.

Bienkowski and Conrad (7) reported a stepwise elution, using phosphate buffer, from SAX-HPLC to effect a separation of radiolabeled tetrasaccharides resulting from the nitrous acid cleavage of heparin. Linker and Hovingh (18) used low-pressure anion-exchange chromatography to achieve separations of *N*-desulfated heparin-derived tetrasaccharides to isolate and characterize three of the major tetrasaccharide components.

We have made improvements on these previously reported anion-exchange separations of sulfated oligosaccharides. By eliminating phosphate buffers we have avoided their interference with oligosaccharide characterization (25) and have achieved improved resolution permitting the isolation and characterization of five major tetrasaccharides (21). We have also presented a rapid analytical technique for the estimation of the molecular weight of heparin-derived oligosaccharides by obtaining GPC-HPLC data and correlating these with the degree of sulfation. Ion chromatography is a sensitive analytical method for sulfate determination of heparin oligosaccharides and is generally applicable in the analysis of GAGs.

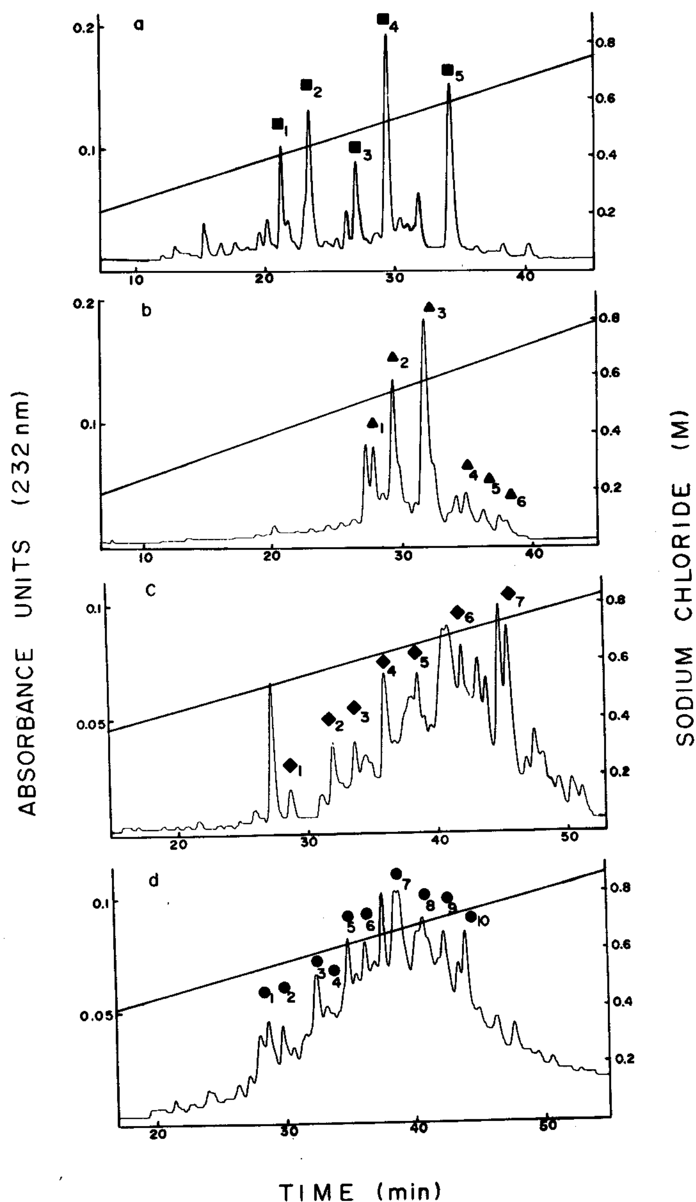

FIG. 4. SAX-HPLC of heparin-derived sized mixtures of tetra- (a), hexa- (b), octa- (c), and decasaccharides (d). The flow rate is 1.5 ml/min, chart speed 15 cm/h, 0.2 AUFS, with a linear sodium chloride gradient at pH 3.5. Numbers above the peaks indicate the fraction of hexa-, octa-, and decasaccharides that were used for sulfate determination and GPC-HPLC. Components eluting from the octa mixture (c) after 47 min and the deca mixture (d) after 48 min were contaminating higher molecular weight oligosaccharides.

The techniques described have important applications in the rapid isolation of small amounts of heparin-derived oligosaccharides (up to decasaccharides) to be screened for biological activities (26–29). Quantitation of the relative amounts of each component present in a mixture of sized oligosaccharides can be performed by computer-assisted integration and is helpful in determining heparin's primary structure (21). Improvements in separation techniques are essential to structural determination, sequencing, and defining the structure activity relationships of complex GAGs such as heparin.

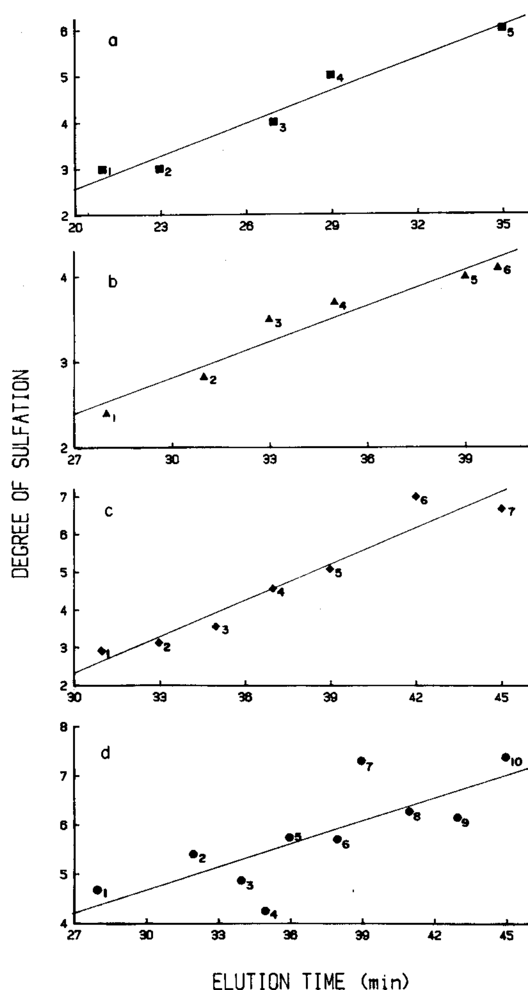

FIG. 5. Plots of degree of sulfation vs elution time from the SAX-HPLC for tetrasaccharides (a), hexasaccharides (b), octasaccharides (c), and decasaccharides (d). Sulfation was determined by placing integrated peak area obtained by ion chromatography (except for the tetrasaccharides (21)) against a standard curve of μg of sulfate vs peak area with a correlation of 0.988. A standard trisulfated disaccharide (21) was used to establish complete pyrolysis in determining the degree of sulfation.

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