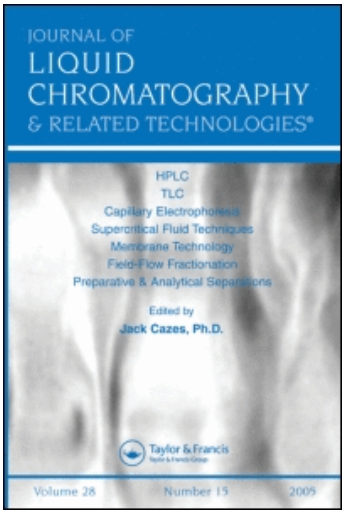

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CHARACTERIZATION OF XYLANSULFATE BY SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

A sulfated, low molecular weight beech xylan was fractionated preparatively on Sephadex G-50 into ten fractions with molecular weights from 17 000 to 1 800. The degree of sulfation, refractive index increment, molecular weight, and specific optical rotation of each fraction was determined. The fractions were then used to calibrate a Sephacryl S-200 gel column for the molecular weight distribution analysis of xylansulfate.

The molecular weights, $\bar{M}_w$, obtained by SEC for samples of xylansulfate were in good agreement (within 4 ± 1%) to the values derived by low angle light scattering.

INTRODUCTION

Sulfated beech xylan is one of the sulfated polysaccharides used therapeutically today. This report deals with characterization of a low molecular weight xylansulfate-Na (XS) marketed under the label SP54 (BeneChemie, Munich, FRG). The high charge density and the low molecular weight range of the product poses

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special problems in the characterization of fractions required for the analysis of the molecular weight distribution (MWD) by size exclusion chromatography (SEC).

In this communication, we describe the preparative fractionation of the xylansulfate, a technique for adapting an ordinary RI-detector for the determination of the refractive index increment, dn/dc, and the calibration of the SEC column. No structural studies on the xylan are included.

MATERIALS AND METHODS

The xylansulfate-Na was obtained from Bene Chemie. The laser light scattering (LALLS) instrument, KMX-6, was purchased from Chromatix, Mountain View, Cal. The chromatographic gels and equipment were products from Pharmacia AB, if not otherwise stated.

Preparative Fractionation

Xylansulfate (ca 15 g) was fractionated on Sephadex G-50, particle size 25 - 56 μm, packed in a jacketed glass column 2.6 x 100 cm. The injector consisted of two adjoining 4-way valves provided with a loop of ca 15 ml volume. With this simple arrangement, the sample loading, rinsing and elution could be performed without interrupting the flow. The flow rate was adjusted to 24 ml/h, with a peristaltic pump (P-3). The column effluent was monitored by the interferometer Multiref 901 (Tecator, Höganäs, Sweden) with 0.2 mm flow cell. Both the column and the RI-monitor were thermoregulated to 25 $\pm$ 0.1°C. The signal from the detector was recorded on a flat bed recorder (REC-1). A programmable fraction collector (FRAC-300) was set to collect 12 ml fractions (30 min). The eluant (0.05M NH_4HCO_3) was chosen in order that the final fractions would be saltfree. One run was completed in 20 hours and 23 consecutive cycles were chromatogra-

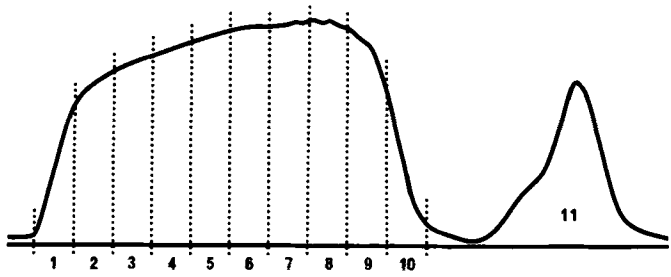

Figure 1. Refractometer profile from the preparative fractionation of xylan sulfate-Na on Sephadex G-50. Column dimensions 2.6 x 100 cm. 23 consecutive cycles were run and fractions pooled as denoted on the chromatogram. The large sample volume (15 ml) results in a considerable broadening of the chromatogram.

phed under similar conditions. Corresponding fractions from the 23 runs were pooled as shown in Fig. 1. Each of the eleven fractions was concentrated by Rotavapor (Büchi, Switzerland), lyophilized and finally dried under vacuum at 70°C until constant weight was attained and the residue entirely free from the smell of ammonia. (The first 3 fractions required repeated treatment).

Light Scattering ($\bar{M}_w$)

The molecular weights of the fractions 1 to 10 as well as for the parent material were measured by the low angle laser light scattering (LALLS) technique (1, 2). Fraction No 11 did not contain carbohydrate and was not examined further (9). The optical design and calibration of LALLS-instrument KMX-6 is described by Kaye and Havlik (2) and summarized by the manufacturer (3).

Preparation of Solutions and Measurement of the Scattered Intensity ($\bar{R}_\theta$)

Stock solutions of the substance and fractions, respectively, were prepared in 0.5M NaCl which was used as solvent used throughout this work. For equilibration, the stock solutions (c_1) were

dialyzed against 0.5M NaCl in acetylated Viscing tubing for 40 hours. The acetylation was carried out according to Vink (5) in order to obtain an extremely tight membrane able to retain even the shortest chains present in the XS. No carbohydrate was detected in the dialysate of any of the samples. Four to five concentrations of each sample were measured by LALLS. All solutions and the solvent were filtered through Millipore filter (0.1 μm) before injecting into the flow-through measuring cell (15 mm, 150 μl). The scattered light was collected within 6 - 7^{o} from the incident beam. The excess Rayleigh Factor $\bar{R}_{\theta}$ (= R_{θ}solution - R_{θ} solvent) was then obtained as the ratio of the scattered to the attenuated illuminating beam, respectively. (The absolute value of R_{θ} includes, besides the measured signals, the optical and geometrical constants for the instrument given in the manufacturers manual). To deduce $\bar{M}_{w}$ and the second virial coefficient A_2, the relation $Kc/\bar{R}_{\theta}$ for the measured solutions (C_1 to C_5) is calculated and plotted against their concentration. Intercept with Y-axis gives $1/\bar{M}_{w}$ and the slope A_2. Fig. 2.

Determination of the Concentration of XS

The colorimetric analysis using orcinol/H_2SO_4 reaction has proved a sensitive and reliable procedure for determining low concentrations of pentoses. The flow scheme modified for Auto-Analyzer by John et al (4) was applied here. A series of solutions of dialyzed XS, dried in vacuum at 70^{o}C were used as concentration standards. The concentrations of LALLS-solutions were measured after passing through the scattering cell. Similarly the dialyzed solutions used for determination of dn/dc were measured after the interferometer flow-cell.

Determination of the Specific Refractive Index Increment (dn/dc)

The refractive index increment of the solute/solvent system should be determined with high accuracy since it appears in the

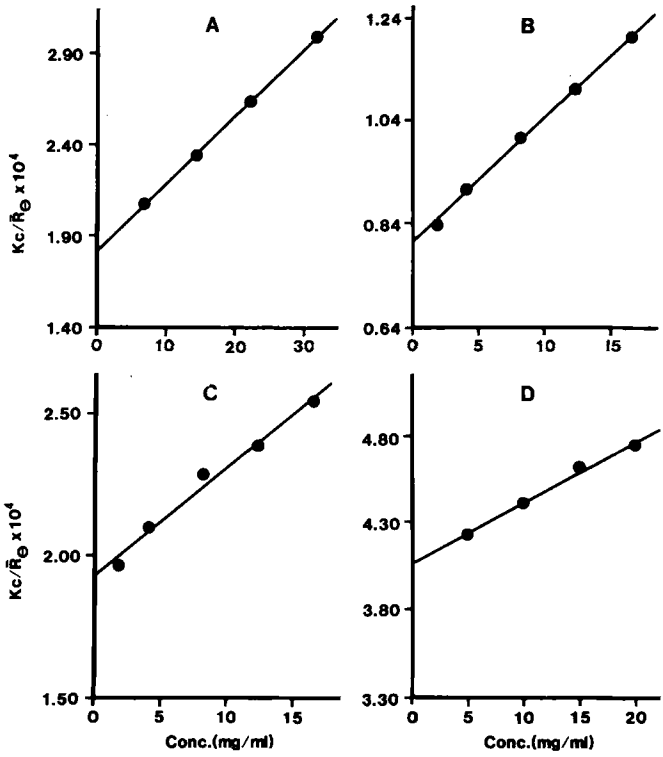

Figure 2. Graph for the calculation of molecular weight ($\bar{M}_w$) from Low Angle Light Scattering measurements. Examples: Unfractionated material (a) and fractions 2, 5 and 9 (b).

optical constant K as its square. Furthermore, the same wavelength of light should be applied in the determination of dn/dc as is used in LS-measurements.

The dn/dc has been determined for each fraction and for the unfractionated material as well. The measuring solutions are considered as three-component systems (1). For this reason each concentration of the sample was dialyzed against the solvent (0.5M NaCl) before the refractive index difference (Δn) was measured for each pair of retentate/dialysate.

We used an assembly of four chambers as dialysis device. The chambers were made of perspex and consisted of two circular compartments separated by a semipermeable membrane and sealed with an O-ring. A sheet of Viscing tubing treated with acetic anhydride-pyridine (5) was used as membrane. After equilibrating with 0.5M NaCl, 15 ml of the solution (~0.6 - 0.2 mg/ml in 0.5M NaCl) and the solvent, respectively, were loaded on each side of the membrane. The chambers were rotated slowly for about 48 hours, with several changes of the solvent. For the measurement of Δn the Multiref 901 RI-monitor was used, modified to operate at 633 nm (the wavelength of the He-Ne laser in KMX-6) by providing a suitable photomultiplier (PM 446) and optical filter (Baltzer 632.8 nm). The recorder signal (mV) for each pair of solution/dialysate was converted, applying the instrument constant determined with NaCl (Suprapur® Merck) and a non-linearity correction, to the corresponding refractive index difference Δn (6).

The concentration of the solutions was determined with orcinol/H_2SO_4 reagents. The slope of the regression line for the plot of Δn vs. c, gives the numerical value of dn/dc. Fig. 3. The values of dn/dc for the fractions and the parent sample of SP54 are compiled in Table 1.

Molecular Weight Distribution by SEC-Analysis

A jacketed glass column (1.6 x 70 cm) was packed with Sephacryl S-200 superfine and equilibrated and eluted with 0.5M NaCl. The column and the cell compartment of the RI-detector were maintained at 25°C ± 0.05 by a water thermostat. The flow rate was adjusted to about 20 ml/h with a peristaltic pump. The sample (3 mg/2 ml) was applied through a 2 ml sample loop. The effluent passed through the flow cell of a RI-detector (Knauer, Berlin, FRD) connected to a recorder. Fractions of the effluent (~1.6 ml) were collected by a time-controlled fraction collector (Stålprodukter, Uppsala, Sweden) provided with a cover to minimize eva-

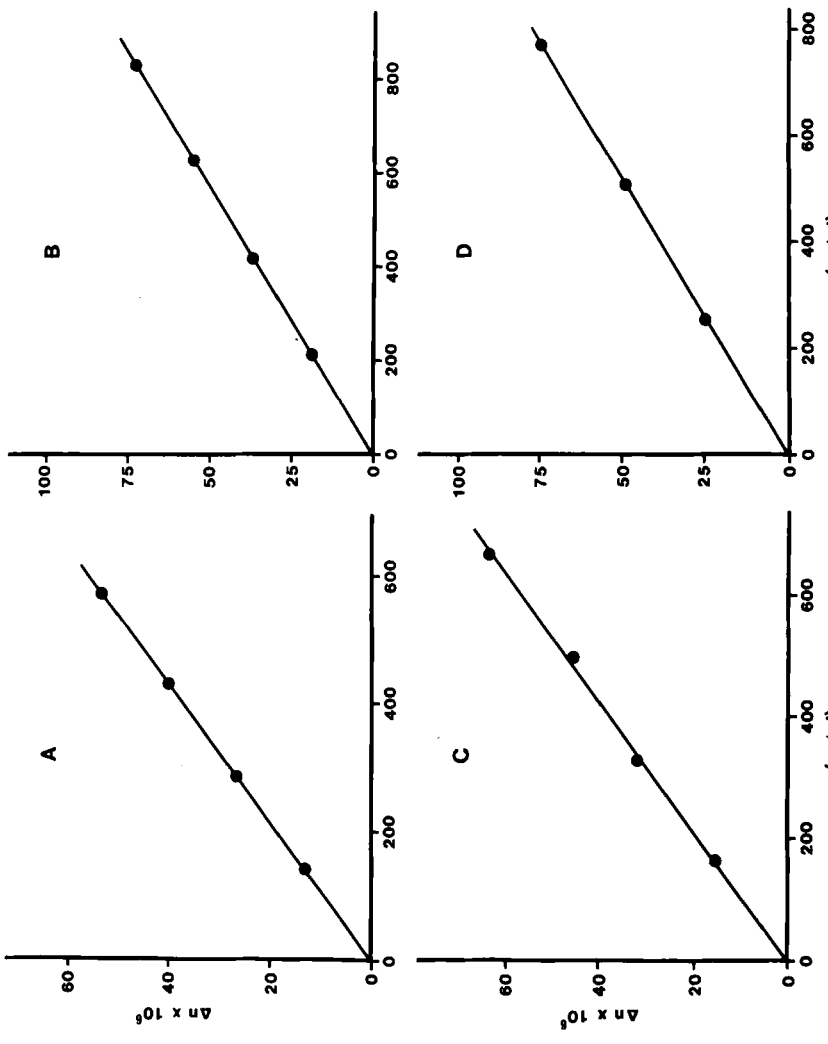

Figure 3. Graph for the calculation of dn/dc, determined with interferometer (Multiref 901). Examples: Unfractionated material (a) and fractions 2, 5 and 9 (b).

poration. The carbohydrate content of these fractions was determined with orcinol.

The column parameters V_o (void volume) and V_t (total solvent volume of the bed) were measured using native dextran (total exclusion from the gel pores, $K_{av} = 0$) and NaCl (penetrating all pores, $K_{av} = 1$), respectively. V_e denotes the elution volume of molecular species i. $K_{av} = V_e-V_o/V_t-V_o$ = distribution coefficient, since V_t-V_o is the total pore volume of the gel matrix and V_e-V_o = volume within the pores available to molecules i eluting at V_e. The elution diagram, where the y-axis = concentration of the eluted species and the coordinate x = distribution coefficient K_{av}, is then converted to the molecular weight distribution by means of a suitable calibration curve. The average molecular weights $\bar{M}_w$ and $\bar{M}_n$ are computed according to their definitions. $\bar{M}_w = \Sigma w_i m_i$ (weight average) and $\bar{M}_n = 1/\Sigma \frac{w_i}{m_i}$ (number average) where w_i = weight fraction of species i.

Calibration of the Gel Bed

The fractions 1 to 10 of the xylansulfate, fractionated and characterized ($\bar{M}_w$) as described above, were used in the calibration of the Sephacryl S-200 column. Each of the ten fractions was chromatographed under conditions described above. The refractometric elution diagrams (solute conc. vs K_{av}) served for construction of a preliminary calibration curve. The Gaussian form of the elution profiles allowed a fair approximation of the position of $\bar{M}_w$ on the curve (log-linear molecular weight distribution) and consequently each fraction gave one point on the calibration curve, M vs K_{av}. The full calibration curve was then constructed by extrapolating to K_{av} 0 and 1, respectively. Minor iterative adjustments were required to obtain a curve which generated molecular weights of optimal agreement with the $\bar{M}_w$-values measured by means of LALLS. The final calibration curve is shown in Fig. 4. The curve, expressed by a polynomial least squares regression (7), was used together with V_o, V_t and other primary data, such as frac-

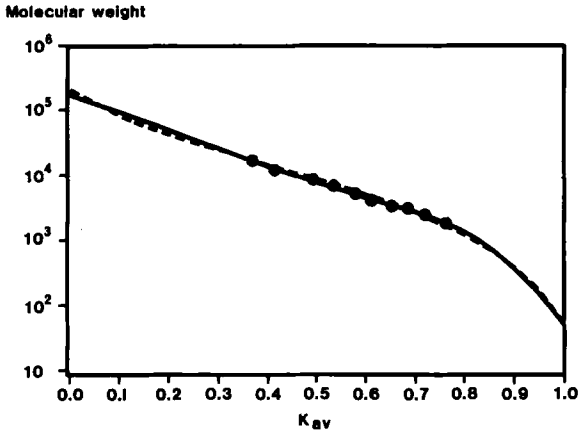

Figure 4. Calibration curve log M vs K_{av} for xylan sulfate-Na on Sephacryl S-200, Superfine, with 0.5M NaCl as eluant.
Solid line (—): The best fit through the calibration points, extended to cover K_{av} from 0 to 1. The curve is described by means of 30 linear cuts.
Dashed line (---): The calibration curve constructed through a polynomial equation (7).

tion size and readings (heights of the orcinol peaks) to compute the molecular weight distribution. Thereafter the MWD of the calibration fractions or of any sample of xylansulfate of comparable degree of substitution and range of molecular sizes could be derived.

The calculated average molecular weights $\bar{M}_w$ and $\bar{M}_n$ of the fractions and the parent material are given in Table 1.

The content of sulfur (%S) was determined with Combustion-GC-Carlo Erba Elemental Analyser.

Specific optical rotations $[\alpha]_{436\ nm}$ were measured with a Perkin-Elmer polarimeter Model 241. The unfractionated dialyzed material gave $[\alpha]_{436\ nm}$ -65^{o}. The specific optical rotation decreased from -83^{o} to -48^{o} in the m.wt. range 17 000 to 1 800 inspite of the uniform degree of sulfation.

TABLE 1

Characterization of fractions of xylansulfate-Na by Size Exclusion Chromatography. Comparison with measurements by light scattering.

Fr. No	Fr. size %	Sulfur %S	LALLS		SEC	
			dn/dc ml/g	$\bar{M}_w$	$\bar{M}_w$	$\bar{M}_n$
1	4.5	n.d.	0.087	16 900	17 500	14 500
2	8.3	n.d.	0.089	12 500	13 200	11 500
3	9.7	n.d.	0.089	8 400	8 160	7 520
4	9.8	16.3	0.092	7 000	6 470	6 100
5	11.3	16.8	0.094	5 200	5 060	4 800
6	11.3	16.6	0.096	4 100	4 330	4 120
7	12.0	16.5	0.096	3 500	3 520	3 330
8	12.0	16.7	0.097	3 100	2 960	2 740
9	11.3	16.7	0.098	2 460	2 370	2 070
10	6.8	16.7	0.106	1 800	1 770	1 360
11	3.0	-	-	-	-	-
parent material, dialyzed		16.6 [1)]	0.093	5 500	5 720	3 830

[1)] Estimated degree of substitution, DS ~1.7.

RESULTS

Xylansulfate sodium salt was preparatively fractionated on Sephadex G-50 gel using ammonium bicarbonate as eluant. The yield was quantitative and the fractions fairly narrow despite of the large number of pooled runs. For each fraction, the refractive index increment (dn/dc) was measured and the limits of error were estimated as $< \pm 0.001$ ml/g (except for Fr. 10). The molecular weight dependence is evident from Fig. 3 and Table 1.

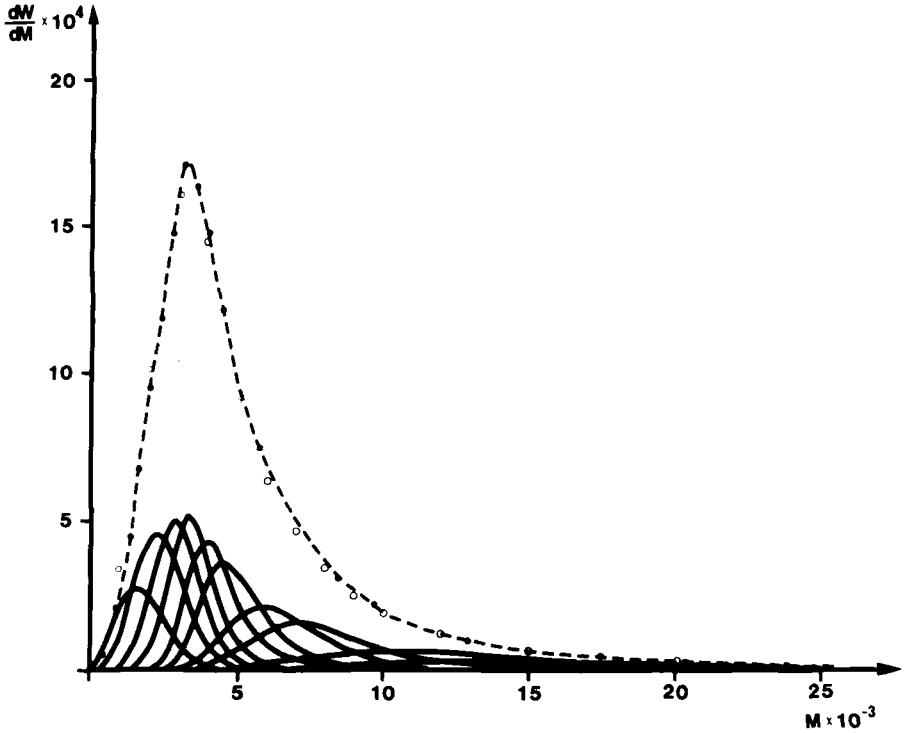

Figure 5. Molecular weight distributions of constituent fractions of xylan sulfate-Na (—). The dashed curve (●--●) represents the MWD of the unfractionated material. The open circles (o) represent the profile of the MWD of the sample calculated by integrating the distributions of the individual fractions 1 to 10.

TABLE 2

Average molecular weights of the unfractionated sample xylan-sulfate-Na.

	LALLS	SEC	
	$\bar{M}_w$	$\bar{M}_w$	$\bar{M}_n$
Calculated from the values of the ten comprising fractions (Table 1, col. 2, 5, 6, 7)	5 713	5 707	3 960
Parent material. Dialyzed, measured	5 500	5 720	3 830

If a mean value of dn/dc is used, the errors in the calculated molecular weights from light scattering are far from negligible. Further the refractometric elution profile of the high m.wt. fractions would be slightly under-dimensioned and the low m.wt. fractions, correspondingly, would be over-dimensioned if a constant dn/dc is used. The calculated $\bar{M}_w$ value would be slightly too low (1.5 - 2.0 % for some of the XS samples, when comparing the orcinol- and RI-diagram). Instead of attempting to apply a correction to the digitizing program, we have used the concentration of the fractions, analysed by orcinol to compute the MWD and the average molecular weights. The orcinol response was found to be practically independent of molecular weight and is not disturbed by non-carbohydrate material. However, the high electrolyte concentration in the effluent causes ghost peaks (gas evolution) on the AutoAnalyzer histogram and the fractions should be diluted 2 to 3 times with distilled water before the assay.

The $\bar{M}_w$-values of the ten fractions calculated from SEC are in good agreement with those determined by LALLS as seen in Table 1. The average values of $\bar{M}_w$ and $\bar{M}_n$ obtained by integrating the ten individual fractions agree well also with results obtained for the parent material, as seen in Table 2 below and illustrated by Fig. 5.

ACKNOWLEDGEMENT

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9. Footnote: The non-carbohydrate peak of the chromatogram in Fig. 1 is considerably larger (~8% of the total area when corrected for an approx. difference in dn/dc) than expected from the amount of non-carbohydrate additives reported by the manufacturer. It is suggested that some of the eluant-electrolyte is excluded from the strong polyelectrolyte XS (8) and appears in the total volume of the column together with the salt components of XS.