Chemistry of Natural Compounds, Vol. 35, No. 1, 1999

LIQUID CHROMATOGRAPHY OF POLYSACCHARIDES

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UDC 543.544.08

This review gives an analysis of methods of high-performance liquid chromatography for the separation of various polysaccharides.

The development of the chemistry and biochemistry of the polysaccharides is connected mainly with the use of chromatographic methods. At the present time, no single method exists that could be recommended for the chromatographic separation and purification of polysaccharides and the determination of their analytical characteristics. In the main, two types of liquid chromatography are used: ion-exchange chromatography — for the separation of polar and neutral polysaccharides — and steric exclusion chromatography — for the separation of polysaccharides according to their molecular masses. In investigations of polysaccharides, the development of silica gel sorbents with grafted-on nonpolar and polar phases has led to the appearance and use of methods of high-performance liquid chromatography (HPLC): reversed-phase and ligand-exchange variants. Classical methods of chromatography, based on the use of soft gels and low pressures are gradually being displaced by HPLC methods [1].

Two approaches exist to the use of chromatography in investigations of polysaccharides: 1) the chromatographic separation of polysaccharides, and 2) the employment of chromatography for analyzing polysaccharide degradation products. The second approach is widely used in structural investigations of polysaccharides. The task of the present review includes an analysis of the literature on the chromatographic separation of some oligo- and polysaccharides.

Two reviews may be useful in connection with chromatographic methods of analysis of the degradation products of polysaccharides [2 and 3]. They give a critical look at the methodology of the analysis of materials containing sugars, beginning with the stage of sample preparation. Known systems of HPLC are evaluated from the point of view of their effectiveness and their stability. The main mechanisms of chromatographic retention are described, and questions of the influence of the natures of the stationary and the mobile phases on the HPLC of sugars are also considered.

In the present review we generalize information on the HPLC of oligo- and polysaccharides using sorbents containing on their surface various functional groups on which the chromatographic process takes place in the corresponding variants: reversed-phase, ion-exchange, ligand-exchange, and steric exclusion chromatographies. The necessity for generalizing literature information is due to the requirements for the high-performance liquid chromatography of the polysaccharides investigated by the authors of the present review [4—7].

Sorbents Used in the Chromatography of Oligo- and Polysaccharides

The HPLC of oligosaccharides makes use of silica gel with a mean particle diameter of 5—10 μm with a chemically bound nonpolar phase such as octadecylsilyl (Vydac, Bondapak C_{18}, etc.) or polar phase, such as aminopropylsilyl (Lichrosorb NH_2, Bondapak NH_2, etc). Literature references on the use of these sorbents are given in the corresponding sections of the review.

Use is still being made of cross-linked dextran gels (Sephadexes of the G series, Pharmacia) and agarose gels

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0009-3130/99/3501-0001$22.00

(Sepharoses, Pharmacia) for the fractionation of polysaccharides by steric exclusion chromatography (SEC) [8, 9]. A serious deficiency of these sorbents is a degradation of the carbohydrate matrix, leading to the appearance of peaks not corresponding to the substances being separated [1]. Of noncarbohydrate sorbents, the greatest popularity is enjoyed by polyacrylamide gels (Bio-Gels of the P series, Bio-Rad) the rigidity of which permits their use at moderate pressures. Among the "semirigid" polymeric sorbents that have found use are a copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate (Spheron), on which dextrans with MMs of up to 500,000 are separated [10, 11], macroporous copolymers of styrene and divinylbenzene (Shodex) [12], and TSK Gel SW [13—15]. The latters are distinguished by fairly high chemical stability and heat resistance (up to 150°C).

The most rigid sorbents are porous glass and silica gels. which are resistant not only to thermal and chemical actions but also to bacterial degradation [16]. At the present time, silica gels are being produced (Lichrosorb from Merck, Porasil from Waters Associates) with porosities permitting the fractionation of polysaccharides with MMs of up to 2×10^6.

Instruments and Methods of Detection in the HPLC of Oligo- and Polysaccharides

The analysis of oligosaccharides and polysaccharides is conducted on liquid chromatographs from various manufacturing firms (Beckman, Du Pont, Hewlett-Packard, Optilab, Pharmacia, Shimadzu, Varian, Altex, etc.), which produce, besides the instruments themselves, parts complementing them: pumps, detectors, prepared columns, devices for forming gradients, injectors, etc.

In recent years, for anion-exchange chromatography wide use has been made of the Dionex Bio-LC system (Dionex Co. USA) fitted with a pulsed amperometric detector, and for steric exclusion chromatography the systems — Shimadzu LC-6A (Japan) and HLC-803D (Tosoh Co., Japan). Starting from the problems faced, each researcher selects an instrument and equips it with the appropriate columns and detectors.

The instrument used most widely for detection in the HPLC of unsubstituted carbohydrates is the differential refractometer [1].

Its operation is based on the continuous measurement of the difference in the refractive indices of the pure mobile phase and the mobile phase containing the substance. In spite of its lower sensitivity than colorimetric detection, this method is very suitable for the analysis of sugars: it has the advantage of reproducibility and is convenient in use. However, a serious disadvantage of refractometric detectors is the difficulty of their use in gradient elution.

A UV detector permits the detection of sugars and sugar acids at 192 nm. It is more sensitive than a refractometric detector but its intensity depends greatly on the type of eluent and, above all, on it purity, Pre-column derivatization considerably improves detection. Derivatives are detected either at 230 nm or at 254 nm with higher sensitivity [17, 18].

In the high-performance ion-exchange chromatography of polar oligosaccharides the greatest attention has been attracted by the pulsed amperometric detector (PAD) [19, 20].

HPLC on Phases Grafted onto Silica Gel

The HPLC of oligosaccharides on columns with alkyl-grafted phases has not yet come into wide use, but interest in this method is shown by a number of publications [17, 18, 21—37]. Examples of typical separations are given in Table 1. In the main, octyl (C_8) and octadecyl (C_{18}, ODC) alkyl phases were used. Alkylsilanized silica gel containing 12—24 carbon atoms in the alkyl group and with methylated residual silanol groups have been proposed for the separation of oligosaccharides [36].

Water [23, 24, 27, 35, 36] and mixtures of acetonitrile and water in various ratios [22, 25, 26, 30, 34] have been used as the mobile phases. The efficiency of the HPLC of higher oligosaccharides is improved by chromatography in gradient systems [17, 18, 28], but in this case the possibilities of the differential refractometer that is usually employed in the detection of unsubstituted sugars are limited.

Oligosaccharide derivatives, such as fully acetylated oligosaccharides, have been separated successfully under these conditions with the use of a moving wire detector [8]. Perbenzoylated maltooligosaccharides separated on a column of Ultrasphere C-8 using gradient elution in an acetonitrile—water mobile phase were detected at 230 nm [17].

TABLE 1. HPLC of Oligosaccharides on Alkylsilylated Silica Gels

Oligosaccharide	Stationary phase, column, temperature	Mobile phase	Detector	Literature
Maltodextrins	Altex Ultrasphere octyl, 250×4.6 mm	CH_3CN-H_2O, gradient elution 86%→100% CH_3CN	UV, 230 nm	17
Cyclodextrins	Separon 6 C18, 150×3.2 mm	H_2O	Refractometer	23
Oligosaccharides	Spherisorb ODS-2 150×4 mm	H_2O	Refractometer	24
Permethylated oligosaccharides	Nucleosil 100-C18, 250×4 mm, 55°C	CH_3CN-H_2O (7:3)	Differential refractometer	25
Cyclomaltododecaose	Senshu Pak ODS-5251-SS, 250×2.0 mm	CH_3CN-H_2O (6:100)	Differential refractometer	26
Neutral oligosaccharide alditols	Capcell Pak C18, 150×4.6 mm	CH_3CN-H_2O, gradient elution 40%→100% CH_3CN	UV, 230 nm	18
Maltooligosaccharides	Lichrosorb 10RP-18 (Merck), 250×4.6 mm	H_2O	Refractometer	27
Acetylated oligosaccharides	Vydac C18, 100×0.32 cm; Bondapak C18, 65°C	H_2O-CH_3CN, gradient elution (9:1)→(3:7)	Moving wire detector	28
Glucooligosaccharides	Ultrasep C18, 300×5 mm	H_2O-CH_3OH (99:1)	Refractometer	29
Acidic oligosaccharides	μ-Bondapak C18	CH_3CN-H_2O (50:50)	Differential refractometer	30
4-Nitrophenyl-α-*D*-maltooligosaccharides	Hypersil-ODS	Linear gradient H_2O-CH_3CN (100:80) 60 min and H_2O-CH_3OH (96:90) 180 min	UV, 300 nm	31
Pyridylaminated galactooligosaccharides	μ-Bondasphere-ODS, 250×3.9 mm	Sodium citrate buffer solution (0.2 M)pH 4.5-6.0	UV, 310 nm	32
Pyridylaminated xyloglycan oligosaccharides	Zorbax-ODS, 250×4.6 mm	CH_3CN-H_2O (40:60)+0.01% CF_3COOH, linear gradient from 0 to 40% CH_3CN in water	UV, double-beam with variable wavelength	33
Per-N-acetylated chitodextrins	Bondapak/Carbohydrate, 300×4 mm	CH_3CN-H_2O (70:30)	Refractometer	34
Per-N-acetylated chitodextrins	Whatman Partisil P×55/25 ODS, 250×4.6 mm	H_2O	UV, 202 nm	35

Nitrophenyl derivatives [31] and pyridylaminated [32, 33] derivatives of oligosaccharides have been detected at 300—310 nm.

A satisfactory separation of oligosaccharides by the HPLC method has been achieved on amine-grafted phases [38—46] (Table 2). Most of these publications report the use of mixtures of acetonitrile and water in various ratios as mobile phases, with a refractometer used as detector [39, 42—45]. The use of gradient elution required UV detection at 220 nm [40]. Unsaturated oligomers of galacturonic acid in 0.11 M acetate buffer solution were detected at 235 nm [41]. The possibility has been shown of isolating gram amounts of oligogalacturonates with a purity of 95—99% by preparative HPLC on aminopropylated silica gel (Dynamax-60A NH_2) with isocratic elution by 0.9 M acetate buffer (pH 5) [47].

TABLE 2. HPLC on Amino-Functional Silica Gels

Oligosaccharide	Stationary phase, column, temperature	Mobile phase	Detector	Literature
Sophorodextrins (Glc_2-Glc_{24})	Japan Spectroscopy Finepack SIL NH_2, 250×4.6 mm	CH_3CN-H_2O, (55:45)	Refractometer	39
Cyclodextrins	Lichrosorb NH_2, (Merck), 250×4.6 mm	CH_3CN-H_2O, gradient elution 80%→100% CH_3CN	UV, 220 nm	40
Unsaturated oligomers of galacturonic acid	Lichrosorb NH_2, (Merck), 250×4.6 mm	0.11 M acetate buffer solution, pH 7.5	UV, 235 nm	41
Maltooligosaccharides (degree of polymerization 1—9, 2—12)	μ-Bondapack NH_2, 300×3.9 mm, 35 °C	CH_3CN-H_2O, (65:35)	Refractometer	42
Xyloglucan oligosaccharides	Amino-substituted silica gel, 200×7mm	CH_3CN-H_2O, (70:30)	Refractometer	43
Mannooligosaccharides	Lichrosorb NH_2, preparative column	CH_3CN-H_2O, (75:25)	Refractometer	44
Cyclodextrins	μ-Bondapak NH_2, 150×19 mm	CH_3CN-H_2O, (70:30)	Refractometer	45

The possibility of analyzing oligosaccharides by the HPLC method on phases grafted on to silica gel plays a fundamental role in the methodology of analyzing complex polysaccharides.

Ion-exchange and Ligand-exchange Chromatography

In recent years, in the high-performance ion-exchange chromatography (IEC) of oligo- and polysaccharides preference has been given to an anion-exchange resin with quaternary ammonium functional groups — CarboPac PA-1 — as the column filling. Gradient elution is mainly used for effective separation, in view of which either UV detection [48] or pulsed amperometric detection (PAD) [49] is employed — the latter more frequently, as is shown by the publications in Table 3.

In the separation of oligosaccharides on columns filled with CarboPac PA-1 with detection by PAD, great differences are observed in the detector signals for related oligosaccharides [66].

For the reliable identification of oligosaccharides in mixtures of them and the determination of their relative proportions it is proposed to convert sialylated and phosphorylated oligosaccharides into the corresponding neutral oligosaccharides under the action of alkaline phosphatase or neuraminidase and to perform direct analysis of the hydrolysates with detection limit amounting to 10 pmole.

The possibility of determining sugars containing up to four monosaccharide units on CarboPac PA-1 using simply a 0.1 M aqueous solution of NaOH as the mobile phase and detection with a flow-through refractometer and PAD has been shown by Paskach et al. [67]. By a study of the features of the retention of the sorbates investigated as a function of the structures of their molecules and of the numbers of carbon atoms and of OH groups, correlations were found for homologous series. IEC on CarboPac and detection by PAD have been used for detecting branched compounds in starch amylopectins during investigations of starch connected with genetic changes in cereals [68].

TABLE 3. High-Performance Ion-Exchange Chromatography of Polysaccharides

Oligosaccharide	Stationary phase, column, temperature	Mobile phase	Detector	Literature
Mixture of polysaccharides	CarboPac PA-1, 25 cm ×4.6 mm	Gradient elution (75 mM AcONa in 0.15M NaOH)→(0.25M AcONa in 0.15M NaOH)	UV	48
Xyloglucan oligosaccharides	CarboPac PA-1, 25 cm ×4.6 mm	AcONa in 100mM NaOH, linear gradient 25→50mM, 60 min	PAD	43
N-Linked oligosaccharides	CarboPac PA-1, 25 cm ×4.6 mm	Linear gradient 10% 1M NaOH/2% 1M AcONa/88% H_2O→10% 1M NaOH/14% 1M AcONa/76%/H_2O, 57 min	PAD	50
Galactomannan oligosaccharides	CarboPac PA-1, 25 cm ×4.6 mm	Linear gradient AcONa in 35mM NaOH, 0→25mM, 40 min	PAD	51
Rhamnogalacturonan-II	CarboPac PA-1, 25 cm ×4.6 mm	0.1M NaOH (0-2 min linear gradient (2-30 min) AcONa 0→0.2M in 0.1M NaOH, then linear gradient (30-54 min) AcONa	PAD	52
Galactoglucomannan oligosaccharides	CarboPac PA-1, 25 cm ×4 mm	Linear gradient AcONa (0-250mM) in 150mM NaOH, 100 min	PAD	53
Galactoglucomannan oligosaccharides	CarboPac PA-100, 25 cm ×4 mm	0.05M NaOH with gradient of 0.25M AcONa from 10 to 80%	PAD	54
Arabinooligosaccharides	CarboPac PA-100, 25 cm ×4 mm	Linear gradient AcONa in 150 mM NaOH: 0-3 min, 0 mM; 3-5 min, 0-100 mM; 5-25 min, 100-500 mM	PAD	55
Oligoglycosylaldonic acids	CarboPac PA-100, 25 cm ×4 mm	Gradient AcONa (0-700 mM) in 100 mM NaOH; 100 mM NaOH (0-2 min), 0→500 mM AcONa (2-45 min and 500-700 mM AcONa (46-50 min)	PAD	56
2-Deoxy- and 3-deoxy-*D*-hexonic acids	CarboPac PA-100, 25 cm ×4 mm	0.1M NaOH with gradient of AcONa from 0→0.25M, 25 min	PAD	57
Feruloylated oligosaccharides	CarboPac PA-100, 25 cm ×4 mm	150 mM NaOH (A) and 150 mM NaOH , with gradient: 600 mM AcONa (B); gradient t=0, A=90%, B=10%, t=40 min, A=35%, B=65%	PAD	58
Maltooligosaccharides	CarboPac PA-100, 25 cm ×4 mm	160 mM NaOH with 10→50% gradient from 600 mM NaOH/AcONa, 15 min	PAD	59
Maltooligosaccharides	CarboPac PA-100, 25 cm ×4 mm	0.1M NaOH→100mM AcONa+0.1M NaOH, linear gradient, 100 min	PAD	60
Acidic oligosaccharides	CarboPac PA-100, 25 cm ×4 mm	Gradient elution: deionized water, 100 mM NaOH, 300 mM AcONa→100 mM NaOH, 300 mM AcONa	PAD	61
Chitotetraose	CarboPac PA-1, 25 cm ×4 mm	30 mM NaOH	PAD	62
O-Antigen oligosaccharides	CarboPac PA-1, 25 cm ×4 mm	Gradient, 100mM NaOH (100mM NaOH+500mM AcONa), 85:15→75:25	PAD	63
Oligogalacturonic acids	Cyclobond I, 25 cm ×4.6mm	Acetonitrile—sodium acetate buffer solution (100 mM, pH 5.0)	PAD	64
Chitodextrins	Beckman PA-35	Gradient of 0.2 M citrate buffer containing 1.0 M NaCl, pH 6.4→pH 7.2	Ninhydrin, 570 nm	65

TABLE 4. Ligand-Exchange Chromatography of Polysaccharides*

Oligosaccharide	Stationary phase, column, temperature	Mobile phase	Literature
Maltodextrins	Aminex HPX-42C, 6% cross-linkage, Ag^+- form, 10—15 μm, 30 cm × 7.8 mm, 95°C	Water	77
Oligosaccharides	Aminex HPX-42C, 6% cross-linkage, Ag^+- form, 10—15 μm, 30 cm × 7.8 mm, 76-98°C	0.005M H_2SO_4	79
Cyclodextrins (α, β, γ)	Aminex 50w-x4, Ca^{2+}- form, 20—30 μm, 25 cm × 6.2 mm, 2 in series, 90°C	Water	81
Cyclodextrins	Aminex 50w-x4, 4% cross-linkage, Ca^{2+}- form, 30 cm ×7.8 mm	Water	82
Oligosaccharides in starch fermentation products	Aminex HPX-87H, Ca^{2+}- form	Water	83

*Detector — refractometer

Depending on the conditions used, ligand exchange may take place on ion-exchange resins during chromatographic separation in addition to ion exchange [69, 70]. This is observed on the use of the following combinations of stationary and mobile phases:

1. Silica gel with grafted-on amino groups (weak anion-exchanger) and water—acetonitrile [46, 71—73]. The volume content of acetonitrile ranges from 50 to 85% The main retention mechanism apparently consists in the formation of hydrogen bonds [71]. An increase in the volume of water in the eluent decreases retention. The retention of oligosaccharides increases with a rise in their molecular mass.

2. A strongly basic anion-exchange resin, usually of low capacity, with superficial functional groups and an aqueous solution of caustic soda, frequently with a concentration of 0.15 M [67, 74]. In such solution, sugars are deprotonated, forming anions separable on an anion-exchanger [74]. Detection with a high sensitivity is ensured with the aid of a PAD. Oligosaccharides are retained the more strongly the higher their molecular mass, with good resolution; however, decomposition of the substances in the column is possible [75].

3) Cation-exchange resins with counter-ions forming coordination complexes with sugars. Sulfonated copolymers of styrene and divinylbenzene are used in the Ca^{2+}- and Ag^+- forms [76—79]. A linear relationship has been found to exist between the logariphms of the corrected retention volumes and the molecular masses of the sugars (with a number of glucose units in the oligomer, n = 1—6) [80]. This shows that the nature of the separation here is determined mainly by a molecular exclusion mechanism. Because the approach of oligosaccharide molecules to the counter-ion of the resin in this chromatographic system is sterically hindered, chelation does not, to all appearance, play an appreciable role.

The use of silver in a resin with a degree of cross-linking of 4% ensures a more effective separation of oligosaccharides, corresponding to a larger number of theoretical plates, than the use of calcium in the same resin [77]. The use of a resin charged with calcium enables individual peaks of oligosaccharides with a degree of polymerization of 8 to be obtained, while the use of a resin charged with silver gives clear peaks up to a degree of polymerization of 12. Experiments have shown that the use of a resin charged with 70% of silver ions and 30% of calcium ions (calculated as equivalents) increases separation efficiency [77].

In such columns, separations are usually conducted at 76—98°C with the use of a refractometric detector. Water or a dilute (0.005 M) solution of sulfuric acid is used as the mobile phase (Table 4).

The mechanism describing this type of chromatography includes ligand exchange: a molecule of a polyhydroxy compound (oligosaccharide) is exchanged for a molecule of water and is retained in the hydrate shell of the counter-ion of the resin through interactions the force of which depends on the capacity of the hydroxy groups for forming coordination bonds [84—86].

TABLE 5. Steric Exclusion Chromatography of Polysaccharides

Polysaccharides	Stationary phase, column, temperature	Mobile phase	Detector	Literature
Neutral				
Laminarodextrins	Sephadex G-15, 100 cm × 5 mm	Water	Refractometer	90
Galactomannan, galactan	Bio-Gel P-100, 48 cm × 1.0 mm	50 mM (pH 5.2) AcONa	UV 490 nm	93
Galactomannan	2 columns of TSK PW (6000 and 5000)	0.1M $LiNO_3$	Refractometer	94
Galactooligosaccharides	Shodex KS-802, 30 cm × 8 mm, 80°C; Bio-Gel P-2, 90 cm × 4.4 mm, 60°C	Water	Refractometer	91
Galactomannan oligosaccharides	Bio-Gel P-2, 100 cm × 2.6 mm, 60°C	Water	Refractometer	3
Oligosaccharides	Bio-Gel P-4, 25 cm × 4.6 mm	0.1 mM pyridinium acetate buffer solution, pH 5	Differential refractometer	95
Dextran, soluble starch, methylcellulose, hydroxymethylcellulose	TSK GM PW, 60 cm × 7.5 mm	0.1 M $NaNO_3$	Differential refractometer	92
Amylose, amylopectin	Silica gel with a diol-bound phase	Dimethyl sulfoxide + MeOH (15% by volume) + $AcONH_4$	Differential refractometer	96
Acidic				
Pectin	TSK PW 6000, 5000, 4000 and 3000, 30 cm × 7 mm, 30°C	0.44 M AcOH + 0.06 M AcONa + 0.1 M Na_2SO_4 + 1 ml of propionic acid (pH 3.7)	Differential refractometer	96
Rhamnogalacturonic fraction of a pectin hydrolysate	Shodex OH-pak SB-802.5, 30 cm × 8 mm	50 mM AcONa buffer solution (pH 4)	Differential refractometer	12
Rhamnogalacturonic fraction of a pectin hydrolysate	TSK HW 50S or HW 55S, 50 cm × 2.25 cm	50 mM $AcONH_4$ buffer solution (pH 5.2)	Differential refractometer	97
Sodium alginate, sodium hyaluronate, carboxymethylcellulose, sodium chondroitin sulfate	TSK GM PW, 60 cm × 7.5 mm	0.1 M $NaNO_3$	Differential refractometer	92
Basic				
Per-N-acetylated chitodextrins	Bio-Gel P-6, 25 cm × 7 mm.	Water	UV, 220 nm	98
Glycolchitosan	TSK GM PW, 60 cm × 7.5 mm	0.5 M AcOH containing 0.3 M Na_2SO_4 or 0.8 M $NaNO_3$	Differential refractometer	92
Diethylaminoethyl(DEAE) dextran	TSK GM PW, 60 cm × 7.5 mm	0.8 M $NaNO_3$	Differential refractometer	92
Chitosan acetate, quaternized amylopectin (with a degree of substitution of 0.075)	Porous silica gel with grafted-on quaternary ammonium groups	0.05 $AcONH_4$	Differential refractometer	99
Water-insoluble polysaccharides (cellulose, amylose, amylopectin, chitin)	3 Mixed B columns (Burdick & Jackson/Polymer Laboratories	N,N-Dimethylacetamide + 0.5% LiCl	Viscometric and refractometric	100

Steric Exclusion Chromatography of Polysaccharides

The practical importance of steric exclusion chromatography (SEC) is due to the possibility of an effective separation of oligo- and polysaccharides according to their molecular dimensions and also of using it to determine molecular mass distributions. In the main, dextran and pullulan standards are used to calibrate the columns in the analysis of molecular mass distributions [87, 88]. An ideal calibration should be made with well-characterized monodisperse standards of the polymer under investigation, which requires a considerable expenditure of time and effort and is therefore rarely done [89].

In the SEC of water-soluble oligo- and polysaccharides the use of water as mobile phase is possible only when the interactions of the substances to be separated with the surface of the sorbent or with one another are minimal. Water is used mainly for the SEC of neutral oligosaccharides in fractionation on the rigid gels Sephadex G-15 [90] and the polyacrylamide gels of series P [3, 91]. To suppress electrostatic interaction of the dissolved substance with the gel, recourse is had to a rise in the column temperature [3, 91]. However, when water is used for the fractionation of polysaccharides containing acidic or basic functional groups, a rise of the temperature does not give the expected effect. For this reason, solutions of electrolytes are used as eluents, it being necessary to determine their optimum concentration for each concrete case [1].

Various eluents are used in TSK PW columns, depending on the polysaccharides to be separated. According to the results of titration, TSK PW contains approximately 12 μ-equivalents of negative charges per 1 ml, showing the presence of carboxyl groups. On elution with water, normal chromatograms are obtained only for low-molecular-mass dextran. To exclude adsorption phenomena, other nonionic polysaccharides are fractionated with 0.1 M $NaNO_3$. In the separation of anionic polysaccharides into fractions with this eluent there is no interaction with the sorbent.

In the fractionation of cationic polymers, no interactions with the sorbent were observed when 0.5 M acetic acid containing 0.3 M Na_2SO_4 was used as eluent. When the concentration of Na_2SO_4 was lowered to 0.1 M, the area of the peak diminished and the results became unreproducible. The hydrophilic cationic polymers glycolchitosan and DEAE-dextran have been successfully fractionated in 0.8 M $NaNO_3$. However an $NaNO_3$ concentration below 0.4 M is insufficient to prevent adsorption.

Blue Dextran, one of the amphoteric oligosaccharides, is fractionated in a mixture of 0.1 M $NaNO_3$ and CH_3CN (80:20, by volume). In the absence of acetonitrile a peak of small area is obtained and this, moreover, with unreproducible results [92].

Example of the separation of some oligo- and polysaccharides are given in Table 5.

The poor solubility of such complex polymers as cellulose, amylose, amylopectin, chitin, etc., is responsible for limitations to their use. One of the few solvents not degrading chitins [101] that dissolve all the polysaccharides mentioned above is N,N-dimethylacetamide containing 0.5% LiCl [100]. By using this solvent, Striegel and Timpa [100] have developed a procedure for dissolving and determining molecular mass distributions by the SEC method with dual viscometric and refractometric detection.

Problems Connected with the Use of Aqueous Eluents in the SEC of Polysaccharides

In the SEC of polysaccharides, problems may arise such as ionic interactions between the polysaccharides and the sorbent and intramolecular electrostatic effects. Below, we discuss these problems and propose methods for their solution or for reducing their influence.

The ionic interactions between polysaccharides and a sorbent are ion exchange, ion exclusion, and ion inclusion.

Ion Exchange. Ion-exchange effects are observed on Sephadex and polyacrylamide gels [101]. In the case of surface-modified silica gels, the ion-exchange effects due to the silanol group are eliminated, but such effects may arise through ionized groups of the surface coating [102]. In order to weaken the ion-exchange properties of the sorbent, it is recommended to regulate the pH and to raise the ionic strength of the electrolytes of the mobile phase.

Ion Exclusion. If the surface of the sorbent has a low charge, substances with a similar charge are excluded from the pores thanks to electrostatic repulsion. This phenomenon can be used to separate ionic and nonionic compounds [102]. On the other hand, this phenomenon may interfere with the interpretation of the results of chromatographic separation. Buyenhuis and Van Maeden [103] have observed that part of dextran (a nonionic water-soluble polymer) with a molecular mass of 20×10^3 is excluded from the pores of silica gel when water is used as the mobile phase. They assumed the presence of negatively charged groups on the substance. The ion exclusion of ionized substances may also be shown on the hydrophilic surface of a

modified silica gel when water is used as the mobile phase. This may be due to the influence of negatively charged groups in the stationary phase or to residual silanol groups [103]. This effect is eliminated by increasing the ionic strength of the mobile phase.

Ion Inclusion. This term was introduced by Stenlund [104] for describing phenomena in which the sorbent acts as a semipermeable membrane. The effect of ion inclusion is based on the Donnan membrane equilibrium.

A Donnan equilibrium exists when a polyelectrolyte and an electrolyte are present on one side of a semipermeable membrane that is permeable only for the electrolyte. Since the excluded polyelectrolyte cannot pass through the pores, while its counter-ion is capable of diffusing through the "membrane," the activity of the excluded molecules rises. If the permeating ions are present in the mobile phase, a Donnan equilibrium is established such that the ion of the same charge as the excluded polyelectrolyte is forced into the pores, thus leading to a permeation peak. If the mobile phase contains no electrolytes, the retention volume of the smaller permeating polyelectrolytes present in the added sample will rise [104, 105].

Thus, if a polydisperse polyelectrolyte is added to a mobile phase of low or zero ionic strength the elution volume of a polymer of lower molecular mass will increase, ensuring nonionic exclusion. With a rise in the ionic strength of the mobile phase, a sample of lower molecular mass will become more excluded [104].

In agreement with Donnan's hypothesis, the amount of excluded electrolyte (Q_s) appearing in the form of the total permeation peak can be estimated by using the following equation [106]:

$$Q_S = \Phi z C_p V/4 = (\Phi z/4) Q_p$$

where Φ is the osmotic coefficient,

z is the ionic charge density of the polyelectrolyte (eq/g),

C_p is the concentration of the polymer (g/cm^3), and

V is the volume of the sample.

Q_S can be decreased by lowering the ionic charge density of the polyelectrolyte by neutralizing its charge through regulation the pH. The effect of ionic exclusion is difficult to eliminate. To guarantee complete separation of the permeation peak from the polymer it is recommended to use, in a set of columns, a column with a microporous sorbent [105, 107].

Intramolecular Electrostatic Effects. A specific feature of solutions of polyelectrolytes is the change in the viscosity of the solutions on the addition of small amounts of an electrolyte. As shown in the literature [105], the intrinsic viscosity (η) of a sample of carboxymethylcellulose (CMC) is determined by the ionic strength (μ) of the solvent (Table 6)

As can be seen from Table 6, a more than tenfold decrease in viscosity is observed on increasing the ionic strength from 0 to 0.1, and a seventeen-fold decrease on increasing it from 0 to 0.7. In aqueous solutions the counter-ions (Na^+) form a diffuse and weakly associated layer surrounding the CMC molecule.

The electrostatic repulsion between neighboring carboxyl groups along the polymer chain arising as a result of this causes a stretching of the molecule. In addition, it increases the solvation of the molecule, which leads to a rise in its hydrodynamic volume.

When an electrolyte is added to the majority of solutions of polyelectrolytes, the electrostatic repulsive forces at the protecting ionic groups decrease, the layer of counter-ions diminishes, and the intramolecular osmotic forces weaken. As a result, the hydrodynamic volume of the polymer decreases. Thus, with an increase in the ionic strength of the mobile phase the polyelectrolyte contracts, which leads to a greater penetration into the pores of the sorbent.

TABLE 6. Influence of the Ionic Strength of the Solvent on the Intrinsic Viscosity of CMC in Acetate Buffer Solution (pH 3.7) [105]

μ	η
0	80
0.01	7.5
0.05	5.7
0.10	5.2
0.35	4.9
0.70	4.6

The sensitivity of polyelectrolytes to the presence of electrolytes depends on the degree of substitution and the compositional heterogeneity of the ionic groups, and also on the configuration and flexibility of the polymer chain. In order to select an appropriate ionic strength of the mobile phase it is necessary to determine the intrinsic viscosity of the sample as a function of the ionic strength of the mobile phase. An ionic strength at which the intrinsic viscosity approaches a constant magnitude should be used for the mobile phase. In the case of CMC [105] and pectin [107], to ensure the maximum contraction of the polyelectrolyte molecules the ionic strengths should be >0.3 and >0.05, respectively.

Influence of the Concentration of Added Sample. The form of the peak and the elution volume of a polyelectrolyte depend on the concentration of the added sample [107]. The influence of this dependence on the distribution of the polyelectrolyte molecules along the chromatographic column and on their outflow from it are more considerable than the influence of their molecular masses. Thus, on the rechromatography of narrow fractions of a polydisperse sample of a polyelectrolyte obtained after its single passage through a chromatographic column, they issued with one and the same retention volume, which was equal to the smallest V_R of the unfractionated sample [108]. This is explained by the fact that, on moving along the chromatographic column, the polyelectrolyte macromolecules experience the action of two factors. In accordance with the molecular-sieve effect, molecules with a higher molecular mass occupy a position in the leading part of the chromatographic zone. Then an effect connected with the dependence of the size of the polyelectrolyte molecules on their concentration in the solution begins to act.

Molecules of higher molecular mass that have passed into the leading part of the zone, where the concentration of the solution is smaller than in the center, are additionally unfolded, with an increase in their dimensions. This leads to their even faster migration together with the flow of solvent. Molecules of small molecular mass, moving with the rear part of the zone and experiencing there the action of the concentration effect are likewise additionally unfolded and, increasing their dimensions, overtake the molecules migrating in the central part of the chromatographic zone. As a result, the zone acquires a highly extended leading front and a sharply abrupt rear. A chromatogram obtained at the outlet from the chromatographic column has the same form, and from this form it is possible to draw a first qualitative conclusion on the polyelectrolyte nature of the substance being analyzed.

In the interpretation of such a chromatogram, to determine the molecular mass distribution one must take into account not only the calibration curve determined for the given system but also the concentration effect connected with polyelectrolyte swelling that has been described. Such an interpretation is a complex task; it is simpler to suppress the effect of polyelectrolyte swelling and, after having eliminated the concentration dependence, carrying out the interpretation of the chromatograms by the standard method on the basis of a calibration curve. It is possible to eliminate polyelectrolyte swelling in two ways: by adding to the solution ≈0.01 M of a neutral electrolyte screening the ionic groups, and by suppressing their dissociation through a change in the pH of the solution [105, 107].

Adsorption in the SEC of water-soluble polysaccharides may arise as a result of the formation of hydrogen bonds, and also of hydrophobic and ionic interactions. Apart from this, for polysaccharides adsorption may be very severe because of the multiple contacts with the surface of the sorbent. Adsorption caused by Coulomb forces can be decreased by increasing the ionic strength of the mobile phase and by regulating its pH. Hydrogen bonds are weakened by the addition of urea or guanidine hydrochloride [102]. Hydrophobic interactions have been observed in the SEC of pectins on a TSK SW 2000 column. The addition of small amounts of an organic modifier (5—10% of MeOH) enables adsorption effects of this type to be overcome. The formation of aggregates in solutions of polysaccharides, which depends on the pH or the ionic strength of the solution, is another of the problems in the preparation of samples for analysis by the SEC method.

The forces responsible for the association of polysaccharides may be Coulomb, hydrophobic or hydrogen-bond forces. The methods employed for eliminating or diminishing these effects on the appearance of adsorption effects can be used for preventing aggregation [102]. Aggregates may break down even when the solution is heated, as, for example, in the case of a dextran solution [109].

The technique of preparing the solutions plays an importnat role in the formation of nonaggregated solutions of polysaccharides. Because of the rapid hydration of many polysaccharides agglomeration takes place when a polysaccharide is added to water too rapidly. The samle of polysaccharide must be added slowly to the vigorously stirred solution. Insoluble material or aggregates present in the solution must be filtered off. If there is an appreciable amount of insolubles, the risk exists of the appeance of concentration polarization at the membrane, which may lead to the ultrafiltration of the material. As a result, the filtrate may prove not to be the dissolved fraction of the sample. When the phenomenon of ultrafiltration is suspected, the sample solution is first centrifuged, or a filter with large pore dimensions and a large specific surface is used.

Thus, for example, in the case of water-soluble celluloses [105] filtration of the solution through a membrane with 0.65-μm pores is sufficient.

High-performance liquid chromatography has become an important analytical tool for preparative separations and for analyzing the purity of the end-product in polysaccharide studies. The value of chromatographic methods consist in the possibilities of effective fractionation, the study of structural changes; the determination of molecular mass distributions; identification, kinetic investigations of processes in which polysaccharides are involved; the monitoring of processes of enzymatic hydrolysis; the checking of the purity of polysaccharides intended for the food and pharmaceutical industries; etc.

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