

Review

Recent progress in carbohydrate separation by high-performance liquid chromatography based on size exclusion

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Abstract

Since the introduction of stationary phases based on microparticulate porous silica and polymeric sorbents, rigid and semi-rigid, size-exclusion chromatography (SEC) has become established as a form of high-performance liquid chromatography. In recent years, there have been revolutionary developments in detection systems for high-performance SEC, which have placed the use of the method for the determination of molecular-size and molecular-weight distributions of polymers on a sound theoretical basis and increased the range of information on molecular characteristics that can be retrieved from SEC data. This review surveys these changes in SEC systems and their application to the separation and molecular-weight distribution analysis of carbohydrates.

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1. Introduction

Size-exclusion chromatography, in which solutes are eluted in order of decreasing molecular size from a stationary phase that comprises a porous, three-dimensional network, which the solute molecules can penetrate to an extent that depends upon the ratio of their molecular dimensions to the average diameter of the pores, has

long been an important technique in the analysis and quality control of polymers. It has the unique advantage over other forms of chromatography that it affords not only a method of fractionation but also, molecular size being the main factor controlling the degree of retention of a solute on the stationary phase, a means of obtaining information on the distribution of molecular sizes in a polymer fraction. If the

relationship between molecular size and molecular weight is known, these data can be used to characterize the polymer in terms of molecular-weight distribution and molecular-weight averages, which are important criteria in many industrial applications of polymers. Until recently, this use of SEC was based largely upon an empirical correlation, established by calibration of the system with a series of standards, and this was a weakness, as standards similar in molecular properties to the polymer being characterized were not always available. However, the advent of detectors based on techniques such as low-angle laser light-scattering (LALLS) and multiple-angle laser light-scattering (MALLS), which permit on-line measurement of molecular weight and other molecular characteristics of the polymer fractions emerging from the column, has now placed the SEC technique on a sound theoretical basis.

In the carbohydrate field, SEC has been widely used in the fractionation and molecular-weight distribution analysis of polysaccharides of industrial or biochemical importance, and also, with stationary phases of lower porosity, in the separation and analysis of mixtures of oligosaccharides, such as those obtained on hydrolysis of starch (maltodextrins) or the oligogalacturonic acids produced by enzymatic digestion of pectins. Recent advances in all of these applications are discussed in this review, with special emphasis on the improvements resulting from the introduction of novel detection systems, some of which are specific for certain carbohydrate analytes.

2. Chromatographic systems for high-performance SEC

2.1. Stationary phases

The term "high-performance liquid chromatography" is conventionally applied to columns having plate numbers above 2000, the typical range being 2000–20 000 [1]. The microparticulate sorbents (average particle diameter between 5 and 13 μm) that are available for high-per-

formance SEC (HPSEC), usually in prepacked columns of length 25 or 30 cm, 4–8 mm I.D., are highly efficient and such columns have plate numbers of 10 000–50 000 per metre [2]; optimal length (50–100 cm) is achieved by coupling two or more columns in series. The range of pore sizes necessary for fractionation of samples having wide distributions of molecular weight may also be provided by coupling columns, containing stationary phases of the same type but differing in porosity. The stationary phases used in high-performance SEC are of two types: silica-based, in which porous silica carries covalently bonded, inert phases (usually glycerylpropyl) which deactivate the adsorption sites on the silica surface, and polymeric sorbents, such as the methacrylate and vinyl types. Some of the gels used in conventional SEC (or gel-permeation chromatography) have been modified to increase their rigidity, so that they are able to withstand the pressures within a high-performance liquid chromatography system. An example of such a gel that has been used in high-performance SEC of polysaccharides is the highly cross-linked form of agarose [3] which, as beads of average diameter 10–13 μm , is commercially available in prepacked columns as Superose 6 and 12 HR (Pharmacia, Uppsala, Sweden).

The pore volume in the stationary phase determines the maximum volume available for chromatographic separation. The relative pore volume of the modern sorbents for SEC varies between 52% and 97% of the column volume, the lower range being typical for silica-based materials, the larger values for polymeric sorbents [2]. Because of their lower pore volume, silica-based stationary phases are more restricted than polymeric sorbents in the number of peaks that can be resolved by a column, so that the resolution achieved is, in general, inferior. For this reason, and because polymeric sorbents are more stable at elevated temperatures and at high pH (conditions that are sometimes necessary in SEC of polysaccharides of low solubility in water at room temperature), these stationary phases are being increasingly preferred over the silica-based types in high-performance SEC.

Some examples of stationary phases that have

been applied in high-performance SEC of carbohydrates are given in Table 1. The fractionation ranges quoted are those for dextrans or polyethylene glycols unless only those for proteins are stated by the manufacturers. In this case it must be stressed that the upper limits for polysaccharides having expanded molecular struc-

tures will be much lower than those for globular proteins.

2.2. Detection systems

The differential refractometer, which has been much used as a universal detector in high-per-

Table 1
Some typical stationary phases^a for high-performance SEC

Sorbent	Matrix ^b	Particle diameter (μm)	Fractionation range (mol.wt.)	Supplier
LiChrospher DIOL 100 Å 500 Å 1000 Å	Silica (glycerylpropyl)	10	$1 \cdot 10^3$ – $1 \cdot 10^5$ $3 \cdot 10^3$ – $2 \cdot 10^6$ $1 \cdot 10^4$ –($> 10^7$)	Merck
SynChropak ^c 100 Å 500 Å 1000 Å	Silica (glycerylpropyl)	10	$1 \cdot 10^3$ – $1 \cdot 10^5$ $3 \cdot 10^3$ – $2 \cdot 10^6$ $1 \cdot 10^4$ –($> 10^7$)	SynChrom
TSK G SW G2000 SW G3000 SW G4000 SW	Silica (not stated)	10 10 13	$1 \cdot 10^3$ – $3 \cdot 10^4$ $2 \cdot 10^3$ – $7 \cdot 10^4$ $4 \cdot 10^3$ – $5 \cdot 10^5$	Toyo Soda
TSK G PW G1000 PW G2000 PW G3000 PW G4000 PW G5000 PW G6000 PW	Methacrylate	10 10 13 13 17 17	$< 1 \cdot 10^3$ $< 5 \cdot 10^3$ $1 \cdot 10^3$ – $5 \cdot 10^4$ $2 \cdot 10^3$ – $3 \cdot 10^5$ $1 \cdot 10^4$ – $1 \cdot 10^6$ $4 \cdot 10^4$ – $8 \cdot 10^6$	Toyo Soda
Ultrahydrogel (range of pore sizes)	Methacrylate	6–13	$1 \cdot 10^2$ – $7 \cdot 10^6$	Waters
Toyopearl (range of pore sizes)	Methacrylate copolymer	25–40	$1 \cdot 10^2$ –($> 10^7$)	Toyo Soda
Superose 12 HR 6 HR	Agarose	10 13	$1 \cdot 10^3$ – $2 \cdot 10^6$ $5 \cdot 10^3$ – $4 \cdot 10^7$ (proteins)	Pharmacia

^a Inclusion of a sorbent in this table does not imply endorsement by the author or the publishers; these serve as examples but other products having similar properties could be substituted in an application.

^b Type of bonded phase shown in brackets.

^c The range given is that applicable to the fractionation of most polysaccharides; a sorbent of pore size 4000 Å is also available, for use in cases where aggregates of very high molecular mass are formed.

formance SEC, as in other HPLC systems, is now frequently replaced by a detection system that is more sensitive or is specific for the analyte, or one that yields information on molecular properties of the analyte. The last-mentioned type is clearly of great value in SEC, which is governed by these factors.

UV detection is applicable if the analyte molecules contain functional groups that absorb in this region, as in the case of glycosaminoglycans, glycoproteins and the oligosaccharides produced by chemical or enzymatic degradation of these biopolymers. Detection at 206 nm, in series with refractometric detection, has also proved useful in HPSEC of polysaccharides containing carboxyl groups or ester carbonyls, such as pectins [4]. The use of refractive index and UV detectors in series in a HPSEC system permits calculation of the mass ratio of carbohydrate to protein in glycoproteins, after calibration of the detector responses with the carbohydrate and protein moieties, or standards similar in properties [5]. Multiple-wavelength detection can be advantageous in yielding information on the purity of the analyte, as well as its molecular-weight distribution. For example, on-line photodiode-array detection in SEC analysis of heparin samples has been used to detect the presence of impurities in these clinically important glycosaminoglycans, by use of techniques such as spectral overlay or the computation of absorbance ratios at two wavelengths in the range 206–280 nm [6].

As in other forms of HPLC, precolumn derivatization can be used to introduce chromophoric or fluorescent groups and thus permit detection by UV photometry or fluorimetry in the SEC system. A recent example of this strategy is the application by Suortti [7] of the specific interaction of β -glucan, the (1 \rightarrow 3),(1 \rightarrow 4)- β -D-glucan that is the major soluble component of the cell walls of barley and oats, with the dye Calcofluor as a detection method in SEC analysis of this polysaccharide. Owing to the high viscosity of solutions of β -glucan, it is necessary to reduce the sample concentration to levels of the order of 0.02% in SEC, to avoid deformation of peaks. At such low concentrations a detector more sensitive than the differential refractometer is required.

The Calcofluor method has been found to be effective in the concentration range 20–200 mg/l (0.002–0.02%) with either fluorimetric detection (excitation wavelength 415 nm, emission 445 nm) or a diode-array detector monitoring at 415 nm. This detection system is compatible with the alkaline eluent (50 mM aqueous sodium hydroxide) that is required in SEC of β -glucan (see Section 3.2); the reagent dye (30 mg/l) is dissolved in the eluent.

Postcolumn derivatization has been effectively applied in a system described by Suortti and Pessa [8] for SEC analysis of starches, also under alkaline conditions. The well-known blue complex formed with iodine has been utilized in the detection of starch fractions eluted with 50 mM aqueous sodium hydroxide in SEC analysis on a polymethacrylate column. The reagent (1.2 mM iodine solution, containing 2 mM potassium iodide, in aqueous 0.5% orthophosphoric acid) was stable in the presence of the alkaline eluent. Monitoring at two wavelengths, 546 and 658 nm, was recommended to check the homogeneity of amylose and amylopectin fractions eluted from the SEC column.

Enzymatic methods have also been used in detection systems for SEC analysis of amyloses. Karkalas and Tester [9] have recently described a method for the continuous monitoring of α -(1 \rightarrow 4)-D-glucan in the alkaline eluents from SEC of components of entire or debranched starches. This detection method involves continuous neutralization of the alkali with dilute acid, followed by hydrolysis of the α -D-glucans with amyloglucosidase (EC 3.2.1.3) and in-flow spectrophotometric determination of the resulting glucose with a modified glucose oxidase-peroxidase chromogenic reagent.

Radiochemical detection is often employed in SEC of glycoproteins, glycopeptides and the oligosaccharides produced in degradative studies of these glycoconjugates, which are usually available only in very small amounts (microgram quantities or less). This involves precolumn labelling and postcolumn scintillation counting of eluate fractions collected directly into scintillation vials, which detects components at subnanomolar levels. The technique of metabolic labelling is being used to an increasing extent:

for example, for SEC analysis of membrane glycopeptides from normal and tumour cells [10] the parent glycoproteins are labelled during the logarithmic growth phase of cell culture by exchange of the medium for fresh medium containing [³H]mannose and [¹⁴C]glucosamine hydrochloride, followed by culture of the cells for 24 h in the presence of the isotopes.

Detection systems giving information on molecular properties of the analyte include the use of a conductivity detector in series with a differential refractometer to determine differences in the degree of esterification of pectins fractionated by SEC [11]. The ratio between the areas of peaks given by the two detectors may be correlated with degree of esterification, that of the refractive index signal being proportional to the total mass of pectin in the sample injected, whereas the area of the signal given by the conductivity detector depends on the content of unesterified galacturonic acid groups. This technique has recently been applied in the determination of the charge density of carboxymethyl-celluloses [12].

The correlation of intrinsic viscosity with the hydrodynamic volume of a polymer (see Section 3.3) is now being applied to an increasing extent in SEC by the coupling of a viscosity detector to the differential refractometer in the on-line detection system. This detector, which is based on the measurement of pressure drop as the eluate from the SEC column flows through a length of capillary tubing, makes possible continuous determination of the intrinsic viscosity of polymer fractions as they are eluted from the column. The technique has been extensively applied by Fishman et al. in characterization of pectins [13–15], and recently its value in quality control of many of the polysaccharides used in the food industry has been recognised [16]. These include carboxymethylcelluloses: the use of three detectors on-line in SEC analysis of such cellulose derivatives by Rinaudo et al. [12] is of special interest, as a new approach to their characterization is afforded by the simultaneous determination of molecular-weight distribution, hydrodynamic volume and charge density from the outputs of refractive index, viscosity and conductivity detectors.

The necessity for a sensitive detection method in SEC of polymers giving viscous solutions, which demands the use of samples of low concentration to avoid errors due to peak deformation, led to the introduction of light-scattering photometers as on-line detectors in SEC of such polymers. Because the output of these detectors is proportional to both the average molecular weight (\overline{M}_w) and the concentration of the sample, lower concentrations may be applied to the SEC column as \overline{M}_w increases. The intensity of the scattered light is a function of the cosine of the scattering angle and is thus greatest with scattering angles close to 0°. This is the principle of the low-angle laser light-scattering (LALLS) photometer that is now much used in SEC.

Coupling of a LALLS detector to a differential refractometer in the detection system not only increases the sensitivity of detection of polymers but also permits direct determination of their molecular-weight distribution and \overline{M}_w by an absolute method, not dependent upon calibration of the SEC column with standards (see Section 3.3). The application of this technique in the determination of the molecular weights of biomolecules, including polysaccharides and proteins, has recently been reviewed [17]. The method is now widely applied in molecular-weight distribution analysis of commercially important polysaccharides such as starch [18] and its hydrolysates [19], carrageenans [20], agarose [21] and pectins [22]. The method is also valuable in determination of the molecular weights of biopolymers such as heparin [23], for which calibration standards for SEC alone are difficult to obtain.

Heparin fractions of low molecular weight (1000–4000) are of clinical importance due to their anticoagulant activity. It has recently been reported [24] that for these samples the LALLS intensity was greater at a slightly wider scattering angle (2°) than that normally employed (1°) and the signal-to-noise ratio was improved. The use of the wider collection angle was therefore recommended for LALLS detection in SEC of smaller biopolymers.

A significant advance in SEC of polymers has been the coupling of the multiple-angle laser light-scattering (MALLS) photometer [25] to the

chromatographic system. This detector is more versatile than LALLS, as it is capable of yielding information on polymer conformation as well as size. The technique of SEC–MALLS has recently been used in the characterization of samples of the cationic biopolymer chitosan [26], for which calibration of the SEC column with dextrans is invalid. The method, which gives values of the radius of gyration (R_g) of polymer molecules, in addition to molar mass, is proving invaluable in the detection of aggregates [27,28]

3. Applications of HPSEC to carbohydrates

3.1. Separation of oligosaccharides

Conventional SEC, on gels of small pore size, has been much used in separation and analysis of the members of homologous series of oligosaccharides, such as the maltodextrins. For this purpose SEC using these gels (e.g. Bio-Gel P-2, P-4 or P-6, Bio-Rad, Richmond, CA, USA) gives much better resolution than does HPSEC, as has been emphasized in two recent reviews [29,30]. However, the long analysis times (several hours) necessary when low-pressure, conventional SEC is used are a serious disadvantage and therefore the newer technique of ion chromatography (high-performance anion-exchange chromatography on pellicular sorbents with strongly alkaline mobile phases; reviewed elsewhere in this issue) is now generally considered the method of choice for analytical chromatography of oligosaccharides, to dp 40 and above.

Some of the newer gels (described as “semi-rigid”), which are capable of withstanding higher pressures than the older dextran and polyacrylamide gels, have been used in medium-pressure LC systems for preparative chromatography of oligosaccharides. This application is exemplified by the use of Trisacryl GF05 (LKB, Bromma, Sweden), a cross-linked polymer of N-acryloyl-2-amino-2-hydroxymethyl-1,3-propanediol [2], in the chromatographic separation of four structurally significant products (having molar masses ranging from 250 to 1250) after Smith degradation of a complex glucuronoman-

noglycan from a plant gum [31]. Another semi-rigid gel that has proved useful in the isolation of oligosaccharides by medium-pressure SEC is Toyopearl HW-40S (Toyo Soda, Tokyo, Japan), which has been effectively applied, for example, in the preparative separation of oligosaccharides obtained by alkaline borotritiide degradation of mucins [32]. In both examples, a volatile buffer, 0.1 M pyridinium acetate, pH 5.0, was used as the mobile phase, so that the oligosaccharides were easily removed from the eluate fractions by evaporation of the solvent.

HPSEC on macroporous diol-modified silica has recently been successfully applied to the analysis of oligogalacturonic acids, dp 2–19 [33]. With a column (500 × 8 mm I.D.) packed with YMC Diol, 120 Å, 5 μm (YMC, Kyoto, Japan), resolution of the oligogalacturonic acids was found to be optimal when the column temperature was 40–45°C. The effects of the ionic strength and pH of the mobile phase were investigated: an ionic strength of at least 0.05 M was necessary and retention times increased with the pH of the mobile phase over the range 3.4 to 5.4. Separation was optimal with a 0.1 M acetate buffer, pH 3.4, as the eluent, at a flow-rate of 2 ml/min. This simple and rapid method should prove invaluable in studies of enzymatic degradation of pectins.

3.2. Fractionation of polysaccharides and glycoconjugates

Recent developments in HPSEC of carbohydrates have been largely directed at the application of the technique to the fractionation of polysaccharides of commercial or clinical importance. Progress in this field has involved the use of novel detection methods in some cases, as has been mentioned (see Section 2.2), and also the introduction of stationary phases compatible with the strong solvents and elevated temperatures that are often required to dissolve the polysaccharides.

Starch and its components serve as prime examples of polysaccharides having restricted solubility in water, so that more rigorous con-

ditions are necessary in SEC analysis. Initially dimethyl sulphoxide (DMSO) was used as the mobile phase, with silica-based stationary phases and a column temperature of 40°C [34], although amylose fractions of relatively low average molecular weight (\overline{M}_w up to 950 000), after dissolution in DMSO and precipitation with methanol, were amorphous and sufficiently soluble in water at ambient temperature to permit their analysis under these mild conditions, on a Superose 6 HR column [35]. The introduction of polymeric sorbents that are stable at high pH has simplified SEC of starch, amyloses and amylopectins, as all are soluble under strongly alkaline conditions, which may be used with these stationary phases [8,36]. The use of polymethacrylate stationary phases with aqueous sodium hydroxide eluents affords a rapid technique for the elucidation of starch composition, the amylopectin components being analysed in the same run as the amylose fractions, which are eluted later, well separated from the high-molecular-weight amylopectins. The alkaline eluent is compatible with the LALLS detector [18], which gives some information on the degree of branching in each polysaccharide fraction, from its hydrodynamic volume (see Section 3.3), and also with the specific spectrophotometric method based on the formation of the blue starch-iodine complex [8] (see Section 2.2). As has been mentioned (Section 2.2) enzymatic detection can also be applied in a system of this type provided that the alkaline eluate is neutralised prior to contact with the amyloglucosidase [9].

Recently the applicability of the MALLS detector (see Section 2.2) to HPSEC of starch with an alkaline eluent (0.1 M aqueous potassium hydroxide) has been demonstrated [27]. The use of this technique afforded evidence of the presence of large aggregates in amylose solutions. Detection by on-line viscometry in series with the differential refractometer, as described in Section 2.2, also yields data on the hydrodynamic volumes of starch fractions separated by SEC, so that contamination of purified amyloses with amylopectin or with subfractions of lower molecular weight is readily discernible by a single analysis [37]. The method has recently

been applied to the characterization of starches dissolved in water by microwave heating in a high-pressure vessel; in this case a neutral salt solution (50 mM aqueous sodium nitrate) could be used as the mobile phase in HPSEC, with a three-column system containing silica-based stationary phases of various pore sizes [38].

The use of an alkaline eluent is necessary in SEC of the (1→3),(1→4)-linked β -D-glucan that is a major structural component of the cell walls in barley and oats. Oat β -glucan is a good source of soluble dietary fibre, whereas barley β -glucan poses problems in the brewing industry, owing to its low solubility. For these reasons, sensitive and rapid analytical methods for β -glucans are required. Suortti [7] recently reported the successful use of a method involving HPSEC on a polymethacrylate column system, eluted with 50 mM aqueous sodium hydroxide at 65°C. The difficulties caused by the high viscosity of solutions of β -glucan were overcome (as discussed in Section 2.2) by the incorporation of a novel detection system based on precolumn derivatization of these polysaccharides with the dye Calcofluor, which permitted sensitive fluorimetric or photometric detection at sample concentrations as low as 20 mg/l.

Cellulose is amenable to SEC analysis only if the stationary phase remains stable in the presence of the special solvent mixtures that are required. A solvent consisting of N,N-dimethylacetamide containing lithium chloride has been used in molecular-weight distribution analysis of cellulose samples by complementary HPSEC and viscometric techniques [39]. It was reported that a column system having a wide fractionation range was necessary for accurate determination of molecular-weight parameters of underivatized cellulose by this method. The use of 5% (w/w) lithium chloride-N,N-dimethylacetamide as solvent and eluent, with a styrene-divinylbenzene copolymer as the stationary phase for HPSEC, has been recently recommended [40] for molecular-weight distribution analysis of not only cellulose, but also chitins from crab shells, which have much higher \overline{M}_w . In this case the solvent used in (off-line) viscometry of the cellulose and chitin fractions was cupriethylenediamine.

Water-soluble derivatives of cellulose, such as hydroxyethylcellulose (HEC) and carboxymethylcellulose (CMC), are more easily fractionated by HPSEC provided that a sensitive detector is available, so that samples of concentrations low enough to obviate peak distortion due to viscosity effects can be used. Another important consideration is that the ionic strength of the mobile phase must be high enough to repress intramolecular electrostatic repulsion between ionic groups in polyelectrolytes such as CMC, which affects the hydrodynamic volume and hence both the viscosity of the polymer and its elution volume in SEC. The research of Barth and Regnier on this problem has been summarised in a recent review [41]: these authors recommend a mobile phase consisting of a 0.12 *M* acetate buffer, pH 3.7, to which sodium sulphate is added to adjust the ionic strength to 0.72. This has been used successfully in HPSEC fractionation of the cellulose derivatives and of pectins on column systems containing silica-based sorbents of various pore sizes. Sample concentrations not exceeding 0.02% for polymers of high intrinsic viscosity, 0.05–0.10% for those of medium to low viscosity, are necessary in HPSEC [41], and therefore the use of sensitive detectors such as the light-scattering photometer is required.

Analysis of sodium carboxymethylcelluloses by HPSEC with three detectors on-line [12] (see Section 2.2) has yielded useful information on the effects of degree of substitution as well as molecular weight on SEC distribution coefficients. It was found that neither these distribution coefficients nor the Mark–Houwink parameters (from viscometry) were strongly affected by degree of substitution in the range tested. Consistency of the ratio between the signals from the conductometric and the refractometric detectors when the two outputs were superimposed showed that charge density did not change with molecular weight for the samples examined.

Water-insoluble polysaccharides that can be analysed by HPSEC on polymethacrylate stationary phases with alkaline eluents include the hemicelluloses extracted from wood with 18% sodium hydroxide solution. Fractionation

and molecular-weight distribution analysis of these polysaccharides, which are mainly xylans, by HPSEC on a polymethacrylate column, with 0.5 *M* aqueous sodium hydroxide as solvent and eluent, has recently been described [42].

The group of polysaccharides that are important in the food industry because of their gel-forming properties, namely carrageenans, agarose, alginates and pectins, require special conditions for HPSEC analysis, as the aggregation of the polysaccharide chains that results in gelation must be prevented during chromatography. In general the presence of electrolyte in the mobile phase is necessary to adjust the ionic strength of the medium, which greatly affects the conformations of the polyelectrolytes, a major factor in gel formation. For the algal polysaccharides column temperatures of 45–60°C are required to prevent gelation and therefore polymeric sorbents are preferable to those based on silica, which are less stable at elevated temperatures. Characterization of carrageenans by HPSEC on LiChrospher DIOL (1000 Å) at 50°C has been reported [43], but in the procedure developed by Lecacheux et al. [20] coupled columns containing polymethacrylate stationary phases, differing in pore size, were used, with the column temperature at 60°C; systems of the latter type have been generally recommended for HPSEC under the conditions required for analysis of gelling polysaccharides. Lecacheux et al. [20] emphasized that the choice of electrolyte to be included in the mobile phase for HPSEC of carrageenans was important, since there is abundant evidence from studies of the helix–coil transitions that govern gel formation by kappa- and iota-carrageenans that these conformations are strongly affected by inorganic cations. Lithium [20] or sodium [43–45] salts, in 0.1 *M* aqueous solution, are suitable eluents; potassium ions, on the other hand, promote gelation [45]. The use of LALLS detection coupled to the HPSEC system is advantageous, not only because it permits simultaneous determination of \bar{M}_w of the fractions by an absolute method (see Sections 2.2 and 3.3), but also because sample concentrations can be lowered, another factor that is important in preventing gel formation.

The HPSEC–LALLS technique has been effectively applied in the fractionation and characterization of carrageenans [20] and agarose [21]. Aqueous sodium nitrate (0.1 M) is the recommended mobile phase for HPSEC of agarose.

The SEC system introduced by Lecacheux et al. [20] for analytical fractionation of carrageenans has been scaled up to allow preparative fractionation, Toyopearl packings differing in pore size being used in coupled columns of 100 mm I.D. [44]. The eluent was 0.1 M sodium nitrate solution and the column temperature 60°C, as in the analytical SEC system. The eluate was monitored by refractometry and fractions were collected accordingly. These fractions (0.1–1 l) were then concentrated and the polysaccharide components were recovered by precipitation with 2-propanol. The molecular-weight distribution in each fraction was determined by analytical HPSEC–LALLS. With a 2-column set (Toyopearl HW-55S and -75S, both columns 600 × 100 mm I.D.) about 1.25 g could be fractionated on each injection (100–200 ml, depending on the molecular-weight range of the sample). Alginates have been fractionated with the same SEC system under the same conditions [44]. The isolation of fractions of narrow molecular-weight distribution by preparative SEC permits investigation of the variation with molecular weight of structural features that affect the functional properties of the polysaccharides: for example, the ratio of mannuronic to guluronic acid (M/G) in alginates.

The gelling mechanism of pectins is less affected by temperature than is that of the algal polysaccharides. In this case the degree of esterification of the pectin, the pH and ionic strength of the medium and the presence of sucrose or divalent cations such as Ca^{2+} are the main factors governing gelation. It is, therefore, unnecessary to perform SEC of pectins at elevated temperatures, and columns are usually run at ambient temperature or at 30–35°C, so that either silica-based or polymeric sorbents can serve as stationary phases. The application to pectins of the HPSEC system developed by Barth and Regnier [41] for fractionation of water-soluble derivatives of cellulose has been

mentioned; they used coupled columns packed with silica-based sorbents differing in pore size, with the mobile phase of ionic strength (0.72) and pH (3.7) found to be optimal for SEC of the polyelectrolytes. Both high- and low-methoxyl pectins as well as amidated derivatives have been fractionated by this SEC method. Fishman et al. [13–15] have also reported fractionation and characterization of pectins on a system consisting of coupled columns containing silica-based sorbents of different pore sizes; in this case the eluent was only 0.05 M (aqueous sodium chloride or nitrate) but the column temperature was raised to 35°C. Coupled columns packed with polymethacrylate sorbents of different pore size, maintained at 30°C, with a mobile phase in which the ionic strength of an acetate buffer, pH 3.7, was adjusted to 0.34 M by addition of sodium sulphate, were used by Deckers et al. [46] in molecular-weight distribution analysis of pectins. However, with such a column system, as with that containing silica-based sorbents, a simple eluent (0.1 M aqueous sodium nitrate) proved equally effective [22]. The SEC system consisting of coupled columns containing Toyopearl packings, with the same eluent, that proved effective in preparative fractionation of carrageenans and alginates [44] has also been applied to pectins, the columns being operated at room temperature in this case. Variations in the molar proportions of the constituent sugars with \overline{M}_w have been detected by analysis of the pectin fractions thus isolated.

In recent studies of pectins by HPSEC the use of the newer detectors in addition to the differential refractometer has yielded useful information on molecular parameters. As mentioned in Section 2.2, the coupling of a LALLS detector to the SEC system has permitted more sensitive detection and the direct determination of \overline{M}_w of the fractions emerging from the column, and aggregates are readily detected by the light-scattering technique [22]. MALLS, which has proved even more effective in the detection of aggregates, has recently been applied in HPSEC analysis of de-esterified pectins (polygalacturonates) [28]. Any variation in degree of esterification with molecular weight is shown by com-

parison of the outputs of conductometric and refractometric detectors [11].

The introduction of an on-line viscosity detector in series with the refractometer has enabled Fishman et al. [13–15] to characterize pectin fractions in terms of molecular size and shape, by calculation of intrinsic viscosities and radii of gyration of the fractions separated by HPSEC. This technique clearly demonstrates aggregation of pectins in solvents of low ionic strength [13]. Computer-aided curve fitting of the outputs from the detectors on HPSEC with a 0.05 M electrolyte solution as mobile phase has shown that pectins from a variety of fruit and vegetable sources are polydisperse, each containing five polysaccharide components, ranging in \overline{M}_w from 10^3 to about $4 \cdot 10^5$, in various proportions [14,47]. This suggests a molecular structure composed of sub-units held together by covalent and non-covalent interaction. Even in the presence of electrolyte there is some indication of aggregation [14] and of highly asymmetric molecular shape [15].

The technique of using a viscosity detector coupled to HPSEC is now being extended to studies of other commercially important polysaccharides, such as gum arabic, tragacanthin, locust bean gum, sodium alginates and carboxymethylcellulose [16]. In addition to the practical advantage of affording a rapid method of determining rheological properties crucial to the use of these polysaccharides in the food industry, it is evident that information on polydispersity may be obtained from global and component intrinsic viscosities and radii of gyration.

Various microbial polysaccharides, such as xanthan, gellan and scleroglucan, have been characterized by Lecacheux et al. [48] under the conditions developed for HPSEC–LALLS in analytical fractionation of carrageenans [20]. Elution with 0.1 M aqueous lithium chloride at 60°C proved effective in avoiding aggregation of these polysaccharides, which has led to uncertainty in \overline{M}_w values determined by other methods, especially for scleroglucan. All of these polysaccharides form highly viscous solutions, and therefore the injection of samples of low concentration (0.05%) is necessary and the use

of LALLS detection is advantageous. In contrast to the results discussed above for pectin samples, \overline{M}_w values of microbial polysaccharides were found by Lecacheux et al. [48] to vary little with source; these polysaccharides have low polydispersity, in contrast to plant polysaccharides. The synergistic interaction between xanthan and locust bean gum, which is important in the food industry for the manipulation of rheological and textural properties, has been studied by HPSEC of partly depolymerized samples of xanthan and galactomannan [49]. The gelation that occurs when the intact polymers are mixed did not take place, but the appearance of a new peak, corresponding to a component of higher molecular weight, in the chromatogram obtained on SEC of the mixture on a polymethacrylate sorbent, with water as the mobile phase, afforded evidence for interaction between the depolymerized species that could serve as a model for the binding between the native polysaccharides.

HPSEC–MALLS has recently been applied [26] in the characterization of samples of the cationic polysaccharide chitosan, obtained by de-N-acetylation of chitin. Chitosan has unique physicochemical properties that are currently being studied for possible exploitation in pharmaceutical and other, novel, applications; these properties are dependent on molecular-weight distribution and therefore a reliable method of characterization is important in quality control. Since none of the polysaccharides generally used as calibration standards in molecular-weight distribution analysis by SEC (see Section 3.3) is sufficiently similar to chitosan to afford a valid calibration, SEC must be coupled with an absolute method of molecular-weight determination. The use by Beri et al. [26] of MALLS on-line with the HPSEC system (3-column system packed with polymeric sorbents, eluted with a 0.33 M acetate buffer, pH 4.2) afforded data on the average molecular weight and radii of gyration of a series of chitosans, differing in both molecular-weight distribution and degree of acetylation. The technique is likely to prove useful not only in quality control but also in establishing structure–function relationships for chitosans.

The use of HPSEC in microbiology is exemplified by a study of the extracellular polysaccharides excreted by moulds of the order *Mucorales*, in which samples of the isolated and purified polysaccharides were fractionated on a 3-column system packed with polymeric sorbents, with a 0.4 M acetate buffer, pH 3.0, as eluent [50]. Glucuronic acid is a major component of these polysaccharides and therefore the ionic strength and pH of the eluent was chosen to minimize variation in hydrodynamic volume due to repulsion between ionized carboxylic acid groups. The effluent was monitored by a differential refractometer and fractions collected accordingly were subsequently screened by enzyme-linked immunosorbent assay (ELISA) to locate the immunochemically active components of the polydisperse polysaccharides.

HPSEC has proved invaluable also in the evaluation of meningococcal polysaccharide vaccines, which consist of one or more of the purified polysaccharides from strains of *Neisseria meningitidis*. These polysaccharides are polymers of (1→6)-linked N-acetylmannosamine phosphate, (2→9)-linked N-acetylneuraminic acid, (2→6)-linked 4-O-glucosyl-N-acetylneuraminic acid or (2→6)-linked 4-O-galactosyl-N-acetylneuraminic acid, according to the strain of origin; all have the α -D configuration and contain some O-acetyl groups. The immunogenicity of the polysaccharides as antigens is directly related to their molecular weight, for which the SEC distribution coefficient serves as a criterion and is a pharmacological requirement in testing vaccines. The standard method of evaluation was, until recently, SEC on the cross-linked agarose gel Sepharose CL-4B (Pharmacia, Uppsala, Sweden), but in 1989 Newland et al. [51] reported that HPSEC on a 2-column set packed with TSK G5000 PW and TSK G2500 PW (Toyo Soda, Tokyo, Japan) in series, with 0.2 M aqueous ammonium acetate as eluent, gave comparable results in a much shorter time and with a smaller sample requirement. Changes in molecular-weight distribution due to depolymerization during storage of the vaccines are readily detectable by the HPSEC technique.

HPSEC is finding increasing application in the

fractionation of glycosaminoglycans, proteoglycans, glycopeptides and glycoproteins. The molecular-weight distribution of glycosaminoglycans is of clinical importance, as it is skewed towards lower molecular weights in certain pathological conditions. Until recently glycosaminoglycan fractions separated by HPSEC were characterized by other methods, such as polyacrylamide gel electrophoresis [52], owing to the difficulty of finding suitable calibration standards for SEC. However, the application of HPSEC–LALLS to the determination of the molecular weights of heparins [23,24] has demonstrated the potential of this technique in the rapid characterization of glycosaminoglycans in general. The use of the photodiode-array detector [6] in assessing the purity of the low molecular-weight heparins used as antithrombotic agents has been mentioned (see Section 2.2); this detection method is a valuable complement to the LALLS detector. Recently Kvam et al. [53] described a rapid method for the characterization of hyaluronan (sodium hyaluronate) in synovial fluid, which involved HPSEC on a set of columns packed with polymethacrylate sorbents differing in pore size, with three detectors coupled in series, namely a differential refractometer, a multi-wavelength photometer operating at 210 and 260 nm, and a LALLS detector. This permitted simultaneous determination of the molecular weight and the purity of the hyaluronan fractions, isolated from the synovial fluid after removal of lipids and proteins. The method should greatly facilitate the investigation of depolymerization of hyaluronate in conditions such as arthritis.

Because of the ionic groups present in glycosaminoglycans the ionic strength and pH of the eluent are important in determining their behaviour on SEC. Many authors have recommended the use of aqueous solutions of sodium chloride (0.02–0.15 M) [6,52,54], but for SEC analysis of hyaluronan Kvam et al. [53] used phosphate-buffered saline (PBS), pH 7.4, and Ovrisky et al. [55] also employed a phosphate buffer (50 mM, pH 7.8).

HPSEC on a preparative scale is now recognised as a major step in the fractionation and

isolation of glycosaminoglycans and proteoglycans, in which one or more glycosaminoglycan chains are attached to a protein core. For example, SEC on Toyopearl columns was used by Fedarko et al. [56] in the isolation and purification of hyaluronan and four proteoglycans synthesised by cultured human bone cells: rapid desalting and buffer exchange were effected by chromatography on Toyopearl HW-40S and fractionation was achieved by ion-exchange chromatography followed by HPSEC on Toyopearl HW-75F. The Toyopearl HW-40S column was also used in monitoring enzymatic digestion of the glycosaminoglycan chains. For the fractionation the mobile phase was 50% formamide, containing potassium chloride (0.1 M), potassium dihydrogen phosphate (0.04 M, pH 6.0) and a trace (0.5%) of the non-ionic detergent Triton X-100, to eliminate hydrophobic interaction between the polymer matrix and the protein moieties of the proteoglycans. Other HPSEC columns that have proved effective in rapid fractionation and size-distribution analysis of proteoglycans include Superose 6 HR, which was recently used by Galis et al. [57] in studies of the sulphated proteoglycans from rabbit aorta. In this case the eluent (0.1 M Tris-HCl, pH 8) contained guanidinium hydrochloride (4 M) in addition to Triton X-100 (0.5%) to eliminate interactions involving the protein components. HPSEC before and after digestion of samples of the proteoglycans with chondroitinase ABC (EC 4.2.2.4), chondroitinase AC (EC 4.2.2.5) or nitrous acid yielded information on the distribution of the sulphated glycosaminoglycan moieties.

A rapid method for the separation and analysis of glycopeptides is also based upon HPSEC in the presence of detergent. Fuhrer [10] obtained optimal separation of glycopeptides, derived by exhaustive pronase digestion from glycoproteins of human tumour and normal cell lines, when the mobile phase used in HPSEC on the silica-based stationary phase Zorbax GF-450 (Du Pont, Wilmington, Delaware, USA) was a 20 mM Tris buffer at neutral pH, containing the detergent NP-40 at a concentration of 1%. Glycopeptides isolated by this method were

further analysed by HPSEC on Zorbax GF-250 after desialylation.

Glycoproteins containing high proportions of carbohydrate, such as the mucins of the gastrointestinal tract (60–80% carbohydrate), are amenable to SEC without addition of detergent. The standard method of isolation of mucins has long been chromatography on Sepharose CL-4B, followed by caesium chloride density gradient ultracentrifugation. This is a lengthy procedure, requiring several days, and recent work has shown that HPSEC can effectively replace one or both of these steps. Substitution of a polymeric sorbent (Bio-Gel SEC-40XL; Bio-Rad, Richmond, California, USA) for the agarose column permitted the separation of microgram to milligram quantities of mucin within 15 min on a 300 mm × 7.5 mm I.D. column, eluted with 0.05 M Tris-HCl, pH 8.0, at 1 ml/min [58]. The mucin fraction (M_w $2 \cdot 10^6$ or higher) is eluted at the void volume of the column (retention time about 7 min) and therefore, even if several injections are required, in preparative SEC, the total separation time is only 2–3 h. More recently, it has been claimed [59] that the use of a Superose 6 HR column affords a one-step method for the purification of mucins, since the non-mucin proteins and other low-molecular-weight contaminants are removed so completely by SEC on this column that density gradient ultracentrifugation is rendered unnecessary. This technique therefore makes possible the purification of mucins from lyophilized extracts in only 2 h, which should greatly facilitate comparative studies of changes in mucus glycoproteins in disease states.

Details of some of the chromatographic systems and conditions that have proved successful in recent studies of HPSEC of polysaccharides and glycoconjugates are listed in Table 2.

3.3. Molecular-weight distribution analysis

The calculation of molecular-weight averages and molecular-weight distribution of polymers from SEC data has been greatly facilitated by the availability of computer software for interpolation of calibration plots. However, the validity of

Table 2

Examples of HPSEC systems and conditions used in fractionation of polysaccharides and glycoconjugates

Analyte	Stationary phase	Mobile phase	Flow-rate (ml/min); temperature (°C); detector	Ref.
Starch	Ultrasphydrogel, 2000 and 250; 2 columns (each 300 × 7.8 mm I.D.) coupled in series	50 mM NaOH	0.5; 60; RI and I ₂	[8]
Hydrolysed starch	TSK G5000 PW and G4000 PW; 2 columns (each 600 × 7.5 mm I.D.) coupled in series	50 mM NaCl	0.8; RT; RI and LALLS	[19]
β -D-Glucan, (1 → 3), (1 → 4)- linked	Ultrasphydrogel, 2000 and 250; 2 columns (each 300 × 7.8 mm I.D.) coupled in series	50 mM NaOH	0.5; 65; UV or fluorescence detection of Calcofluor complex	[7]
Water-soluble cellulose derivatives	SynChropak columns (each 250 × 4 mm I.D.) coupled in series; 100 Å and 500, 1000 or 4000 Å, depending on molecular size range of analytes	0.12 M acetate buffer, pH 3.7; ionic strength adjusted to 0.72 with Na ₂ SO ₄	0.5; RT; RI	[41]
Agarose	Shodex OHpak B-804 and/or B-805 (polymethacrylate); single or coupled columns (each 500 × 8 mm I.D.)	0.1 M NaNO ₃	1; 45; RI and LALLS	[21]
Carrageenans	TSK G6000 PW and G5000 PW; 2 columns (each 600 × 7.5 mm I.D.) coupled in series	0.1 M LiCl	1; 60; RI and LALLS	[20]
Carrageenans and alginates, preparative SEC	Toyopearl HW-55S and HW-75S; 2 columns (each 600 × 100 mm I.D.) coupled in series	0.1 M NaNO ₃	8–12; 60; RI	[44]
Pectins, preparative SEC	As for carrageenans and alginates	0.1 M NaNO ₃	8; RT; RI	[44]
Pectins, analytical SEC	SynChropak columns (each 250 × 4 mm I.D.) coupled in series; 100 Å and 500 or 1000 Å, depending on molecular size range	As for cellulose derivatives	0.5; RT; RI	[41]
	TSK G6000 PW and G4000 PW; 2 columns (each 600 × 7.5 mm I.D.) coupled in series	0.1 M NaNO ₃	1; 30; RI and LALLS	[22]
	Ultrasphydrogel, 2000 and 250; 2 columns (each 300 × 7.8 mm I.D.) coupled in series	0.1 M or 10 mM sodium acetate	1; 35; RI and conductivity	[11]
	μ -Bondagel, E-High and E-1000 (silica-based); 2 columns (each 300 × 4 mm I.D.) coupled to SynChropak, 100 Å (column 250 × 4 mm I.D.)	50 mM NaNO ₃	0.5; 35; RI and viscosity detector	[14]
Chitosans	Bio-Gel SEC-60XL (polymeric sorbent) coupled to 2 SEC-50XL columns (each column 300 × 7.5 mm I.D.)	Buffer, 0.33 M acetic acid + 0.1 M sodium acetate, pH 4.2	0.6; RT; RI and MALLS	[26]
Heparin	TSK G2000 SW and/or G3000 SW; single or coupled columns (each column 300 × 7.5 mm I.D.)	0.1 M NaCl	0.5; RT; diode-array detector	[6]
Hyaluronic acid	Shodex OHpak B-806 and B-805; 2 columns (each 500 × 8 mm I.D.) coupled in series	20 mM NaCl	0.5; 40; RI and UV (280 nm)	[54]
Hyaluronan	TSK G5000 PW and G3000 PW; 2 columns (each 600 × 7.5 mm I.D.) coupled in series	Phosphate-buffered saline, pH 7.4	0.8; 40; RI, UV (210, 260 nm) and LALLS	[53]
Proteoglycans	Toyopearl HW-40S (250 × 10 mm I.D.) for desalting, buffer exchange and analysis of enzymatic digests; Toyopearl HW-75F (500 × 10 mm I.D.) for fractionation Superose 6 HR (300 × 10 mm I.D.)	50% aqueous formamide, containing 0.1 M KCl, 40 mM KH ₂ PO ₄ , pH 6.0 and 0.5% Triton X-100 0.1 M Tris-HCl, pH 8.0, containing 4 M guanidinium hydrochloride and 0.5% Triton X-100	0.5–1; RT; scintillation counting of radiolabelled samples 0.2; RT; scintillation counting of radiolabelled samples	[56] [57]

(Continued on p. 164)

Table 2 (continued)

Analyte	Stationary phase	Mobile phase	Flow-rate (ml/min); temperature (°C); detector	Ref.
Glycopeptides	Zorbax GF-250 or GF-450 (350 × 9.4 mm I.D.)	20 mM Tris-HCl, pH 7.0, containing 1.0% NP-40;	RT; scintillation counting of radiolabelled samples	[10]
Glycoproteins	Zorbax GF-250; 2 columns (each 250 × 9.4 mm I.D.) coupled in series	10 mM phosphate buffer, pH 7.0, containing 0.5 M Na ₂ SO ₄	0.5; RT; RI and UV (280 nm)	[5]
Mucus glycoproteins	Bio-Gel SEC-40XL (300 × 7.5 mm I.D.)	50 mM Tris-HCl, pH 8.0	1; RT; UV (230, 280 nm)	[58]

RI = Refractive index; RT = room temperature; UV = ultraviolet photometry.

these plots can be questionable unless appropriate standards are used in calibration. Ideally, these should be narrow fractions of the polymer being characterized, and it is possible to obtain these by preparative HPSEC (see, for example, Ref. [44]) of abundant polysaccharides such as carrageenans and pectins. In particular, fractions of the same polymer are essential in calibration of SEC systems for molecular-weight distribution analysis of glycosaminoglycans, for which no suitable secondary standards exist. Such glycosaminoglycan standards, varying in \bar{M}_w over a wide range, are prepared by SEC fractionation of polydisperse samples [60] or samples that have been partially depolymerized by enzymatic digestion [52]. For hyaluronan degradation can be effected by ultrasonication [54,61]. The behaviour of glycoconjugates on SEC varies widely with their composition and structure [62] and therefore determination of the mass ratio of carbohydrate to protein in glycoproteins [5] is essential in evaluating standards.

The use of secondary standards, consisting of characterized fractions of another polysaccharide, more readily available than the analyte, in SEC calibration has value mainly as a means of estimating molecular-weight averages as a basis for comparison of different samples of a polysaccharide, or following changes on degradation or aggregation. Commercially available dextran standards have been much used as calibrants for this purpose, and pullulans have been recommended as secondary standards for linear polysaccharides, especially amyloses [63]. Hydroxyethyl starches have served as secondary

standards in HPSEC of carboxymethylated derivatives of the clinically important β -(1→3)-linked D-glucans schizophyllan and lentinan [64].

An approach now widely adopted in correlating SEC data with molecular parameters of polymers is to apply the so-called "universal calibration", in which SEC elution volumes or distribution coefficients are plotted against $\log \eta \cdot \bar{M}_w$, the product of intrinsic viscosity and molecular weight being a measure of the hydrodynamic volume of the polymer molecule. In 1984, Kuge et al. [65] reported that when HPSEC data obtained with dextran standards were plotted in this manner, the same correlation held for pullulans, amyloses, amylopectins and polyethylene oxides. These data were obtained with a column system containing polymeric sorbents; the universal calibration was subsequently found by Fishman et al. [66] to hold in HPSEC of dextrans and pullulans on silica-based stationary phases also. Application of the universal calibration in SEC requires the acquisition of viscometric data in addition to the chromatographic data, unless the Mark-Houwink constants for the polymer are known. As has been mentioned (see Section 2.2), incorporation of a viscosity detector in tandem with the differential refractometer in the HPSEC system has greatly facilitated the computation of hydrodynamic volumes of polysaccharides fractionated by SEC [13–16].

The universal calibration has been found to be valid in SEC of many polysaccharides of diverse structural types, including pectins [13–15,46], carrageenans [43], plant-gum exudates such as

gum arabic and gum tragacanth [16], the xylan components of wood hemicelluloses [42], and chitosans [67]. Dextrans are widely applicable as standards for universal calibration in SEC of polysaccharides but for glycosaminoglycans sodium polystyrene sulphonate has been the recommended standard [68]. More recently, however, the validity of universal calibration with this standard in HPSEC of other anionic polysaccharides has been called into question [69]. It has also proved impossible to apply a calibration established with pullulans, which have flexible chains, to rigid, rod-like polysaccharides such as the (1→3)-linked β -D-glucan schizophyllan [70]. Thus, the “universal calibration” is evidently not, in fact, applicable to all polysaccharides, although its use has given greater validity to calibration with secondary standards in determinations of molecular weight or size by SEC.

The coupling of light-scattering detectors (LALLS or, more recently, MALLS) to the HPSEC system is the only method of molecular-weight distribution analysis by SEC that is fundamentally sound and applicable to all polymers, including polysaccharides and glycoconjugates. The values of \bar{M}_w , or of the radius of gyration, R_g , which is strongly influenced by molecular conformation, are determined on-line by an absolute method, without the assumptions implicit in methods using secondary standards for calibration. Used in conjunction with viscometry, now also available as an on-line detection system, SEC and LALLS or MALLS afford a powerful technique for the determination of the size and shape of macromolecules.

4. Conclusion: summary of main trends

Recent progress in high-performance SEC has resulted in the application of the technique in the rapid fractionation of a wide diversity of polysaccharides and glycoconjugates. It is evident from Table 2 that polymeric stationary phases are now used much more extensively than those based on silica, the latter being less stable under the conditions (high pH and/or raised temperature) required for SEC of polysaccharides that are

only slightly soluble, or form gels, under milder conditions. Some chemical detection methods, specific for certain polysaccharides (e.g. starch), that have been incorporated into HPSEC systems also demand the greater stability of the polymeric sorbents. It is in detection systems that the most important developments are taking place: the introduction of LALLS, MALLS and viscosity detectors coupled to HPSEC systems has made possible direct characterization of polysaccharide fractions as they emerge from the columns. The range of molecular parameters that can be calculated from the chromatographic data is consequently increasing, so that HPSEC has become a pivotal step in the characterization and quality control of polysaccharides and glycoconjugates of industrial or clinical importance, especially those having functional properties governed by molecular conformation and size distribution.

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