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ABSTRACT

Size-exclusion chromatography (SEC) and asymmetric flow field-flow fractionation (AF4) are elution-based techniques for separation and characterization of (bio)macromolecules including plant polysaccharides. These two techniques separate the macromolecules according to their hydrodynamic volume in solution. The primary reason for the separation of polysaccharides with a dispersed nature is the need for accurate information on the molar mass distribution (MMD), which cannot be obtained without the separation of macromolecular species into narrowly distributed fractions. Depending on the detectors coupled to the separation, other information such as size, branching, and conformation can also be obtained across the separated fractions. This review summarizes the SEC and AF4 methodology used for separation and characterization of plant polysaccharides with industrial and/or nutritional importance. The key differences between the two techniques are highlighted and recommendations are given for method selection.

1. Introduction

Plant polysaccharides are renewable biomacromolecules consisting of monosaccharide units which are linked together with glycosidic bonds. Cellulose is the most abundant plant polysaccharide followed by different hemicelluloses (xylans, mannans, glucans, and galactans) [1]. Cellulose and hemicelluloses are structural components in plants [2]. While cellulose has already many industrial applications, hemicelluloses are still less utilized [3]. Starch, a storage polysaccharide produced by most of the green plants, is the most important polysaccharide in nutrition [4]. Pectins are heterogeneous plant polysaccharides which are rich in fruit and tuber peels [5]. Due to the immediate need for fast green transition, polysaccharides will be used even more as an alternative for fossil fuel-based macromolecules. The native structures of polysaccharides are also tailored for improved material properties [6,7].

While some of the plant polysaccharides are homopolymers consisting of only one type of monosaccharide unit (such as cellulose, starch and β-D-glucan), most of the plant polysaccharides are heterogeneous in their structure. Determination of chemical structure, i.e., monosaccharide composition, branching pattern, and linkage type/pattern, is important since the primary structure of a polysaccharide defines most of its properties such as solubility and ability to form macrostructures (self-assembly or interactions with other type of molecules). In addition to primary chemical structure, molar mass distribution (MMD) affects

the end-use properties of a polysaccharide. To obtain information on the MMD, not just the average molar mass (*M*) of all the polymeric chains in the sample, a separation technique such as size-exclusion chromatography (SEC) or field-flow fractionation (FFF) is needed [8,9]. Both techniques are commonly coupled online to multiple detection techniques. Depending on the detectors included in the detector train, other parameters than molar mass can also be obtained [8]. These parameters include branching, size, and conformation of a polysaccharide.

Although SEC is the most used separation technique for all the polymeric substances, including plant polysaccharides, FFF and especially asymmetric flow field-flow fractionation (AF4) have also been widely used for characterization of polysaccharides [10]. AF4 is "a multi-flow" technique and requires more method optimization compared to SEC. In some cases, AF4 is, however, more feasible separation technique over SEC. This review summarizes the SEC and AF4 methodology that has been used for characterization of plant polysaccharides with industrial and/or nutritional importance over the few past decades. The potential of both methods for separation and characterization of plant polysaccharides and the method selection is also critically evaluated.

2. Multi-detector SEC and AF4

SEC is a chromatographic technique for separation and character-

ization of macromolecules. The separation process in SEC differs significantly from the separation in standard interaction chromatography. In interaction chromatography, as suggested by the name, the separation is based on the interactions between the analyte and the stationary phase and there is a significant change in enthalpy (ΔH) [11]:

$$K \sim e^{-\Delta H^\circ / RT} \tag{1}$$

In Eq. 1, K is the analyte distribution coefficient, R, the gas constant, and T, the absolute temperature. In SEC, the separation is due to change in entropy (ΔS) and the standard enthalpy difference can be assumed to be negligible ($\Delta H = 0$):

$$K_{SEC} \sim e^{\Delta S^\circ / R} \tag{2}$$

As seen in Eq. 2, temperature is not affecting the retention in SEC. Even though no enthalpic interactions between the analytes and stationary phase should occur during the SEC analysis, this might not be the case especially when analyzing complex biomacromolecules and charged polysaccharides.

SEC columns contain porous packing material, and the pore size of a column defines the size range for which the column(s) is/are optimal. Larger molecules enter a smaller pore volume or fewer number of pores than the smaller molecules, and thus elute before smaller molecules [8]. In SEC, it is a common practice to increase the resolution by adding several columns, either single-pore or mixed-bed columns, in series. SEC columns are available for both aqueous and organic mobile phases [11]. As discussed more thoroughly in the next paragraphs, the AF4 analyses are largely focused on the water-soluble/water-dispersible samples due to the availability of the membrane materials for AF4 channels [12] and thus, SEC is a more versatile technique than AF4 what becomes to the samples that can only be dissolved in organic solvents.

In AF4, the separation takes place in an open channel instead of a column. An AF4 channel consists of a solid top plate with flow inputs/outputs and a porous bottom plate with a frit [13]. Ultrafiltration membrane, commonly made of regenerated cellulose (RC) or polyether sulfone (PES), covers the frit. The analytes are injected into the channel and focused close to the membrane. The focus flow is opposite to the channel flow and the focus flow rate and focusing time depend on the size of the analytes and the injected amount (longer focusing time is commonly needed for large analytes than for smaller ones). The molecules start to diffuse away from the membrane and the separation is enhanced by the counterforce called cross flow [14]. The smaller molecules, which have higher translational diffusion coefficients (D_T) elute from the channel before the larger ones with lower D_T values. Thickness of the channel ranges commonly from 200 μm to 500 μm and the channel flow has a parabolic flow profile. Since the flow streams are faster towards the center of the channel, the molecules with high D_T reach these faster flow streams before the analytes which diffuse slower [14]. The elution order in AF4 is opposite to the one in SEC.

Since the separation in AF4 takes place in an open channel, molecules are exposed to lower shear during the analysis compared to separation with SEC columns. In addition, due to the porous nature of the SEC column packing material, the surface area of the stationary phase in SEC is large and might cause unwanted interactions between the analytes and the packing material [11]. The chemical resistance of the available membrane materials restricts the use of organic solvents in AF4 separations; thus, aqueous applications are the ones commonly found in the literature. Since the bottom plate and the membranes are permeable (molecular weight cut-offs available from 1 kDa to the cut-off of 10 kDa being the most used), the AF4 is not a closed system. This can be either a positive or negative feature; the sample can be purified from some low-molar-mass impurities, but also there is a risk of losing the important analytes from the sample. One additional benefit of AF4 over SEC is that information on size as a form of a hydrodynamic radius (R_H) can, in theory, be obtained directly from the retention time. This approach is, however, prone to errors caused by the anomalies in

separation [15] and nowadays it is more common to obtain size information by online coupling of AF4 to light scattering detector(s).

As mentioned, the cross flow is used in AF4 to enhance the separation. Cross flow can be constant over the time of the experiment, or it can decrease as a function of time; either in a linearly or exponentially decaying fashion. In general, higher cross flow is needed for separation of smaller analytes and vice versa. Cross flow programming is convenient, especially for samples with both small and large analytes; linear and decaying cross flows can be combined, for example to shorten the analysis time. Exponentially decaying cross flow has proven to be useful for separation of various polysaccharides [16–18].

As mentioned in the Introduction, information on the MMD is commonly extracted from the SEC and AF4 data. Both, SEC and AF4 are size-based separation techniques and thus MMD information is obtained either by calibration procedures (conventional calibration or universal calibration) or using light-scattering techniques. Conventional calibration can give reliable results only if the molar mass standards have a similar chemical structure (and consequently a similar mass-to-size relationship in solution) to the analytes. Since this is not commonly a case, as there are only limited number of structurally varying molar mass standards available, the use of MALS together with concentration sensitive detector such as RI (UV detector can also be used as concentration sensitive detector, but due to the lack of chromophores in most polysaccharide structures, RI detector is more applicable for polysaccharides that UV detector) has become a golden standard procedure for MMD determination (Fig. 1, Table 1). MALS/RI method relies on the fact that the MALS detector signal is proportional to M, concentration, and a square of the refractive index increment ($\partial n / \partial c$) [8]. When the concentration for each separated fraction is obtained by the RI detector, M can be determined for each of these fractions assuming that the $\partial n / \partial c$ is known. The $\partial n / \partial c$ values depend on the chemical structure of the analyte, solvent that the analyte is dissolved in, and the wavelength of light and temperature used for the measurements [19].

Other detector that is commonly coupled online to SEC and AF4 is a DLS detector (Fig. 1). While MALS (static light scattering detector) yields radius of gyration (R_G) for large analytes ("anisotropic scatterers"), DLS can give D_T and further R_H for small analytes with just a couple of nanometers in size. Viscosity detector (VISC) is mainly used with SEC since the VISC signal is sensitive to variations in pressure during the analysis. VISC detector yields specific viscosity (η_{sp}) and intrinsic viscosity ([η]) when the data from RI detector is combined with VISC detector data [11].

3. SEC and AF4 separation and characterization of plant-based polysaccharides

This section summarizes the SEC and AF4 methodology used for the most important plant polysaccharides. The used mobile phases, methods for M and MMD determination and used/measured $\partial n / \partial c$ values (in cases where light-scattering detection has been used for M determination) are listed with related references in Table 1. As can be seen from Table 1, MALS/RI method for M determination is prevailing for plant polysaccharides. Also, the $\partial n / \partial c$ values used are quite consistent being in the range of around from 0.13 to 0.15 ml/g for all polysaccharides in aqueous solution.

3.1. Cellulose and cellulose derivatives

Cellulose, the most abundant biopolymer, consists of β-D-glucose units which are linked together with (1→4)-linkages. Due to the zigzag orientation of the glycosidic bonds, cellulose chains pack closely with each other and a strong intermolecular hydrogen bonding network glues the cellulose chains tightly together. Thus, cellulose is insoluble in water. The standard protocol for dissolving the different types of celluloses for SEC analysis is to use saturated lithium chloride (LiCl) in N,N-dimethyl acetamide (DMAc) after the solvent exchange procedure
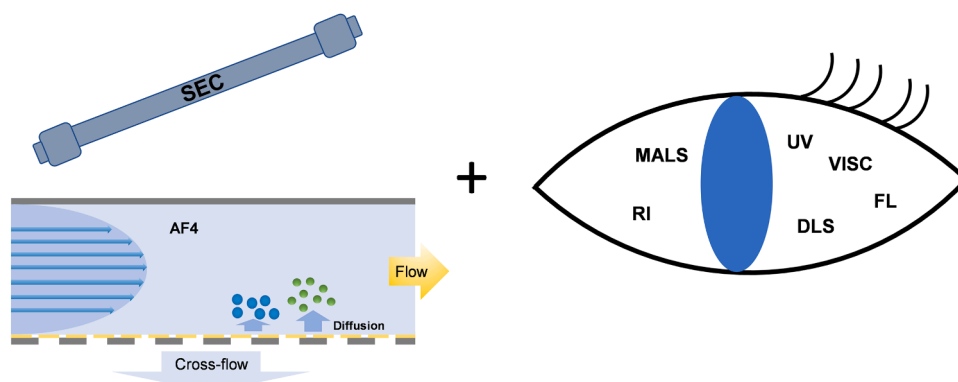
Fig. 1. Size-exclusion chromatography and asymmetric flow field-flow fractionation is commonly coupled to multi-angle light scattering (MALS), refractive index (RI), ultra violet (UV), dynamic light scattering (DLS), viscosity (VISC), and fluorescence (FL) detectors. MALS and RI detectors are needed for absolute molar mass determination. Viscosity detector is commonly used only with SEC.

[20–29] but in addition, 1,3-dimethyl-2-imidazolidinone (DMI) with LiCl has been used as a cellulose solvent [21]. It has been postulated that the hydroxyl protons of anhydroglucose units associate with the chloride anion by hydrogen bonding. The hydrogen bond network of cellulose is disrupted when lithium forms a macrocation with DMAc and this macrocation then interacts with the chloride anion [23]. Different solvent exchange procedures prior dissolution in DMAc/LiCl are also proposed. Commonly, the first step is activation in water followed by the activation step in acetone. After acetone, the samples are soaked in pure DMAc [26]. Some alternative solvent exchange procedures have also been proposed for improved dissolution of regenerated cellulose fibers and softwood pulps [30,31] In these protocols, dimethyl sulfoxide (DMSO), ethylene diamine (EDA) and methanol, water and ethanol, and a combination of water, tert-butyl alcohol, and ethanol were tested for improved solubility.

As only cello-oligomers with low M are commercially available, the use of MALS and RI detectors for absolute M determination of celluloses is commonly a method of choice. Since many celluloses have quite high average M, the MALS detector gives good signal-to-noise levels in general. The determination of $\partial n/\partial c$ for cellulose in DMAc/LiCl is somewhat challenging due to the uneven distribution of LiCl in the cellulose solutions [24,26,32] and $\partial n/\partial c$ is heavily affected on the LiCl concentration. In most cases, the final LiCl concentration in SEC mobile phase range from 0.5 % to 0.9 % and the $\partial n/\partial c$ values of celluloses for this concentration range have been determined to range from 0.104 to 0.136 so that the $\partial n/\partial c$ value is higher for solutions with higher LiCl concentration [26,27].

Due to the water-insolubility of cellulose, AF4 has only been used to characterize cellulose nanocrystals (CNCs) in aqueous suspensions and cellulose derivatives. Rod-shaped CNCs can be produced from various cellulose sources, such as from cotton and wood, by hydrolysis, and they have a wide range of applications, e.g. in composite materials. Guan et al. [33] optimized the AF4 separation conditions for CNCs, which had the rod lengths up to around 300 nm. The rod lengths obtained by MALS were confirmed by transmission electron microscopy (TEM) of the fractions collected from the AF4 separations. In a later study of Mukherjee and Hackley [34] the molar mass and recovery of CNC samples were measured in addition to the rod length/size. Determination of both molar mass and recovery requires an adequate signal-to-noise level for a refractive index detector signal. That might be a challenge for high-molar-mass / large-size analytes due to the saturation of light scattering detector signals if higher concentration is used to improve the signal-to-noise level of RI detector.

In addition to CNCs, chemically modified celluloses have also been characterized with AF4 besides the more commonly used technique, SEC. Most common cellulose derivatives are cellulose ethers, such as carboxymethyl cellulose (CMC), methyl cellulose (MC), hydroxyethyl

cellulose (HEC), hydroxypropylmethyl cellulose (HPMC), and hydroxypropyl cellulose (HPC) and cellulose esters including cellulose acetate (CA), cellulose nitrate, and cellulose sulfate [35]. Most cellulose derivatives are soluble in water, and they have high or ultra-high molar mass. Thus, AF4 is an ideal separation technique for cellulose derivatives. Leeman et al. [16] used pullulan mixture for optimization of AF4 flow conditions for highly disperse polysaccharide samples. Both constant cross flows and programmed cross flows (linear and exponential decays with and without initial constant cross flow step) were tested and M selectivity (log retention time vrs log M) was used as a measure to judge which condition gave the best separation. Exponentially decaying cross flow gave the most uniform molar mass selectivity across the separated range and higher selectivity was observed especially for high molar masses (ranging from 3.8×10^5 g/mol to 1.6×10^6 g/mol) compared to the constant or linearly decaying cross flow conditions. In addition to pullulans, the exponentially decaying cross flow program separated well the HPC samples which were shown to contain a low number of aggregated chains.

Filtration is not always necessary for samples that will be analyzed by AF4. Andersson et al. [36] analyzed ethylhydroxyethyl cellulose samples with AF4 with and without filtration using programmed cross flow including several linearly decaying steps. The samples contained ultra-high-M chains (with M up to 10^9 g/mol) and part of this material was lost when the sample was filtered before the analysis. The coupling of AF4 to MALS detector enabled the determination of R_G and further conformation plots (double logarithmic plot of R_G vs. M). Conformation plots revealed the dense structure of the ultra-high-molar mass chains.

AF4 can be an advantageous separation technique over SEC for cationic polysaccharides. Common SEC stationary phases carry negative charges, which might contribute to the unwanted interactions between cationic analytes and column stationary phase. Cationic hydroxyethyl cellulose derivatives (quaternary ammonium salt of hydroxyethyl celluloses) were separated with AF4 using acidic eluent (0.135 M NaCl in 0.012 M HNO$_3$) [37]. The commonly used membrane type in AF4, RC, has the isoelectric point of 3.4. Thus, in the acidic conditions with pH lower than 3.4, the RC membrane is weakly positively charged [38,39] and repulsion between the positively charged analytes and the membrane prevented the unwanted interactions. Eluent with pH closer to neutral, namely 0.8 M NaNO$_3$ (aq.), was also tested in the study of Pitkänen and Tenkanen [37]. The recovery values were, however, lower with 0.8 M NaNO$_3$ indicating the interactions with the RC membrane. Similar cationic polysaccharide samples have been analyzed with SEC, and it was found that abnormal elution behavior occurred depending on the elution conditions and the M of the samples. Adding organic solvent to the mobile phase together with high buffer content reduced the interactions between the cationic analytes and column stationary phase [40].

Table 1

Separation of plant polysaccharides by SEC and AF4.

Plant polysaccharide	SEC				AF4			
	Eluent	Method for M determination	$\partial n/\partial c$ (ml/g)	Refs	Eluent	Method for M determination	$\partial n/\partial c$ (ml/g)	Refs
Cellulose	DMAc + 0.5 wt% LiCl, DMAc + 0.9 wt% LiCl, DMI + 1 wt% LiCl	MALS/RI Column calibration with pullulans	0.104-0.136[1] 0.062 (in DMI/LiCl)	[20–28]				
Cellulose nanocrystals					H₂O, 0.01 N NaCl	MALS (for rod length determination) MALS/RI	0.148	[33,34]
Carboxymethyl cellulose	0.1 M NaCl, 0.1 M NH₄OAc	MALS/RI	0.140–0.178[2]	[42]				
Hydroxypropyl cellulose					0.01 M NaNO₃	MALS/RI	0.138 0.139 (depending on the sample)	[16]
Hydroxypropyl-methyl cellulose					0.01 M NaCl[3]	MALS/RI	0.133 0.134 0.135 (depending on the sample)	[43]
Ethylhydroxyethyl cellulose					0.01 M NaCl	MALS/RI	0.138	[36]
Quaternary ammonium salt of hydroxyethyl celluloses	0.1 M TEA/1 % HOAc, 0.5 M NaOAc/ACN (80:20)[3]	Triple detection (LS/ VISC/RI)[4]	0.144	[40]	0.135 M NaCl in 0.012 M HNO₃[3]	MALS/RI	0.132 0.138 0.141 (depending on the sample)	[37]
Dialdehyde cellulose	NaNO₃ + MeOH, NaNO₃ + Na₂EDTA, NaOAc/ HOAc	MALS/RI	0.145	[41]	0.1 M NaNO₃	MALS/RI	0.145	[41]
Wheat arabinoxylan (from flour and bran)	0.05 M NaNO₃, 0.1 M NaNO₃, 0.4 M NaOAc buffer pH 4, DMSO + 0.01 M LiBr	MALS/RI Triple detection (LS/ VISC/RI)	0.146 (in NaNO₃) 0.150 (in NaOAc) 0.064 (in DMSO)	[44–46]	0.1 M NaNO₃	MALS/RI	0.146	[17]
Rye arabinoxylan (from flour)	0.05 M NaNO₃, 0.1 M NaNO₃, DMSO + 0.01 M LiBr	MALS/RI Triple detection (LS/ VISC/RI)	0.146 (in NaNO₃) 0.064 (in DMSO)	[46,47]				
Barley arabinoxylan (from flour and husks)	0.05 M NaNO₃, DMSO + 0.01 M LiBr	MALS/RI Triple detection (LS/ VISC/RI)	0.146 (in NaNO₃) 0.064 (in DMSO)	[47,48]				
Oat spelt arabinoxylan	DMSO/H₂O 90/10 + 0.05 M LiBr[3]	Universal calibration with pullulans		[49]				
Glucuronoxylan (from birch and aspen)	DMSO + 0.5 % LiBr, DMSO + 0.01 M LiBr, 0.1 M NaNO₃	Column calibration with pullulans		[50,51]				
Galactomannan	0.02 wt% NaN₃, 0.1 M NaNO₃	MALS/RI Triple detection (LS/ VISC/RI)	0.150 0.146	[18,52, 53]	0.1 M NaNO₃	MALS/RI	0.150	[18]
Galactoglucomannan (from spruce)	0.2 M NaCl, DMSO + 0.01 M LiBr, 0.1 M NaNO₃	Column calibration with dextrans Column calibration with pullulans		[50,54]	25 mM sodium citrate buffer, pH 4.5	MALS/RI	0.145 0.148 (depending on the sample)	[55]
B-D-glucan	0.1 M NaNO₃, DMSO + 0.01 M LiBr	Triple detection (LS/ VISC/RI)	0.146 (in NaNO₃) 0.151 (in NaNO₃) 0.062 (in DMSO)	[56–59]	0.1 M NaNO₃, 0.01 M NaNO₃, 0.05 M NaOH, 0.5 M NaOH	MALS/RI	0.146	[59–62]
Gum Arabic	0.1 M LiNO₃, 0.5 M NaCl	MALS/RI Column calibration with dextrans	0.141	[63,64]	0.1 M LiNO₃	MALS/RI	0.141	[63]
Pectin	0.1 M NaCl, 0.01 M NH₄OAc, 0.1 M NaNO₃, 0.2 M NaNO₃	Column calibration with pullulans Triple detection (LS/ VISC/RI) MALS/RI	0.146 0.147	[65–67]				
Starch	DMSO + 0.5 wt% LiBr, 0.02 wt% NaN₃, 0.05 M NH₄OAc (pH 5.2),	Column calibration with pullulans (also R_H calibration)	0.145 (in aq. solution)	[68–71]	0.02 wt% NaN₃,	MALS/RI	0.145 0.146 0.147	[72–75]

(continued on next page)

Table 1 (*continued*)

Plant polysaccharide	SEC				AF4			
	Eluent	Method for *M* determination	∂n/∂c (ml/g)	Refs	Eluent	Method for *M* determination	∂n/∂c (ml/g)	Refs
	DMSO:DMAc (10:90) + 0.05 wt% LiBr	MALS/RI Column calibration with polystyrene and poly(methyl methacrylate)			0.05 M NaNO$_3$			
Carboxymethyl starch					0.05 M NaNO$_3$	MALS/RI	0.146	[76]

[1] Depending on the LiCl concentration and wavelength of light.
[2] Variation in the ∂n/∂c values regarding degree of substitution and used mobile phase.
[3] Best eluent according to the authors; other eluents were tested as well.
[4] Triple detection: Combination of right-angle light scattering, viscometry, and refractometry, for further detail please see e.g, Striegel et al. [11].

Periodate oxidation of cellulose yields dialdehyde cellulose (DAC) which can be further modified for various derivatives. Highly oxidized DACs do not dissolve in DMAc/LiCl but can be dissolved in water upon heating. According to the results from the work of Sulaeva et al. [41], the eluent composition strongly affects the results in SEC. Hemiacetal cross-links were found to be stable in near-neutral and low ionic strength eluent while they opened in acidic eluents or eluents with stronger ionic strength. AF4 offered better separation efficiency compared to SEC, but SEC provided representative results besides AF4.

3.2. Hemicelluloses

3.2.1. Xylans

Xylans are a group of plant cell wall polysaccharides with large structural variation. As an example, glucuronoxylans, containing xylose chains with glucuronic acid side chains, are abundant in hardwoods, and arabinoxylans, consisting of xylose backbone with arabinose substituents, are common in grasses and cereals. In xylans, the β-D-xyloses are attached to each other with (1→4)-linkages similarly as glucose units in cellulose. Many xylans are, however, water-soluble (at least partly) due to side groups attached to the free hydroxyl groups of the xylose units. Also, the *M* of xylans is lower compared to cellulose. Wood xylans have a molar mass less than 10^5 g/mol or even lower, being below 10^4 g/mol [50], but cereal arabinoxylans can have a molar mass up to a few hundred thousand g/mol [45] depending on the extraction method.

Molar mass analyses of xylans are commonly done with SEC using aqueous mobile phase, dimethyl sulfoxide (DMSO), or mixture of DMSO and water [44–47,77]. It should be noted here that preferential solvation might occur when using mixed solvents such as a mixture of DMSO and water. Preferential solvation complicates the accurate *M* determination with MALS/RI [78] method and thus MALS/RI method should be avoided when working with mixed solvents. Depending on the structure, some xylans have been found to form aggregates in aqueous solutions. This is true especially for xylans with a relatively low degree of substitution [46]. The aggregation tendency is less pronounced in DMSO and DMSO/water, but time-dependent aggregation of xylans has been shown to occur even in DMSO [79]. AF4 has not been widely used for characterization of xylans. Wheat arabinoxylans were separated both using SEC and AF4 in similar aqueous conditions (0.1 M NaNO$_3$), and the results from two separation techniques were compared with each other. Low amounts of large-sized aggregates were separated from the individual polysaccharide chains with good resolution using AF4. The resolution of these aggregates in SEC was much worse and thus the molar mass data from SEC were found to be overestimated [17].

When analysing polysaccharides with SEC using aqueous mobile phases, non-size-exclusion effects might hamper the separation process. These effects include ion exchange, ion inclusion, ion exclusion, intramolecular electrostatic interactions, and adsorption [11]. Many of these non-size-exclusion effects can be eliminated by adding electrolyte to the mobile phase. When characterizing xylans with SEC, the commonly used

electrolyte is NaNO$_3$. It has been used as an eluent modifier in SEC of e. g., wheat flour arabinoxylan [45,46], barley flour arabinoxylan [47], and rye flour arabinoxylan [47]. Eluent modifiers have also been used with DMSO, the most common salt added being lithium bromide (LiBr) [48,51].

Hardwood glucuronoxylans have lower *M* than cereal arabinoxylans. Similar to the characterization of arabinoxylans, *M* analysis of glucuronoxylans has commonly been done with SEC using DMSO or water (with some eluent modifiers) as eluent. Due to low molar mass (which results in relatively low signal-to-noise for MALS detector signals), column calibration with pullulan standards is commonly used for low-*M* glucuronoxylans [50,51].

3.2.2. Mannans

The cell walls of seeds are rich in mannans and galactomannans. Galactomannans consist of a β-(1→4)-linked D-mannose chain with D-galactose units α-(1→6)-linked to the mannose backbone. The ratio between mannose and galactose varies depending on the origin. Guar galactomannan, which is widely used as a thickener and stabilizer in the food industry, has a galactose-to-mannose ratio of around 0.6 [80]. The main softwood hemicellulose is galactoglucomannan. Softwood galactoglucomannans have β-(1→4)-linked backbone with both D-mannose and D-glucose residues in the main chain and α-(1→6)-linked D-galactose units attached as side chains. The ratio between galactose, glucose, and mannose in spruce galactoglucomannan is around 0.5:1:4. Some of the hydroxyl groups of mannose units are acetylated [81].

In general, the *M* analysis of galactomannans is conducted with SEC using aqueous mobile phase [18,52,53]. The M_w for unmodified guar galactomannan has been reported to range from around 950,000 g/mol to 1,900,000 g/mol depending on the study and the analytical method used. The average *M* closer to 2×10^6 g/mol have been reported to be biased due to the aggregates present in the galactomannan solutions. The aggregation was revealed by the combination of *M* and [η] data. The galactomannan solution was heated, and the results obtained in different temperatures indicated the decrease in *M*, but only minor change in [η]. In case of the chain degradation, the [η] would decrease in line with *M*. Thus, this behaviour was expected to represent the disaggregation phenomenon [53]. Similar observation was done when characterizing the series of enzymatically modified guar galactomannans. The weight-average *M* of native guar galactomannan was around 1.85×10^6 g/mol, and the weight-average *M* of the partially debranched sample was 0.9×10^6 g/mol. The [η] values of the samples were, however, close to each other. Since the partial debranching of the sample was not expected to decrease the *M* drastically, and because the [η] values of the two samples were almost identical, it was concluded that the native galactomannan solution contained aggregates [18]. Investigation of the presence of aggregates in galactomannan solutions is complicated by the fact that galactomannans are only soluble in aqueous solutions and not e.g, in DMAc or DMSO, which are commonly used for characterization of other (1→4)-linked polysaccharides. Characterization of the same

sample in two different solvents is a convenient way of confirming the presence of aggregates. Even though galactomannans have relatively high *M,* the use of AF4 instead of SEC did not show additional benefits over SEC; the results from both techniques were well in accordance [18].

Galactoglucomannans are soluble in DMSO in addition to water. Ho et al. [50] analyzed spruce galactoglucomannans using both DMSO containing 0.01 M LiBr and 0.1 M NaNO3 (aq.) as eluents. The *M* results for samples with high acetyl content were higher in DMSO than when the aqueous eluent was used. The situation was opposite for samples with low acetyl content. Due to the use of conventional calibration in these studies, it was difficult to speculate the reason for observed differences. The presence of aggregates, however, most likely affected the results. Since galactoglucomannans are associated with lignins in native wood, a low amount of lignin is commonly coextracted with polysaccharide fraction [82]. The multi-detector AF4 study of the spruce galactoglucomannan revealed the colloidal features of this polysaccharide, and the colloidal structures were more pronounced when the lignin content of the sample was high [55]. The colloidal material had very large size with $R_G > 100$ nm and these structures may play an important role, e.g. in the pickering emulsions. Individual spruce galactoglucomannan chains have molar mass less than 10^4 g/mol [50].

3.2.3. *β-Glucans*

The water-soluble cereal dietary fiber component $(1\rightarrow3)(1\rightarrow4)$-β-D-glucan, or in brief β-glucan, is abundant especially in oats and barley and has gained a lot of interest due to its bioactive properties. The food authorities have given several health claims to β-glucan related to its ability to lower the blood cholesterol level and to attenuate the serum glucose and insulin response after meals [83]. These bioactive properties stem from the fact that β-glucan increases the solution viscosity. Many food processing technologies might cause degradation of β-glucan and further the decline of its functionality. Thus, the accurate determination of MMD for β-glucan is vital. β-glucans from oats and barley, however, are known to form fringed micelle-type aggregates in dilute aqueous solution, which grow side-to-side via hydrogen bonding of the cellotriose sequences [57,84]. This aggregate formation complicates the accurate MMD determination of β-glucans.

The average *M* values reported for β-glucans range from 2000 g/mol to 40×10^6 g/mol depending on the sample and analysis method used (Grimm et al., 1995). Aqueous SEC is the most used technique for *M* determination of β-glucans [56–58], but DMSO containing 0.01 M of LiBr has also been used as eluent for β-glucans [59]. Other SEC approaches include the use of "cellulose solvent" DMAc containing LiCl [85] and a post-column addition of calcofluor to create fluorescent derivatives (which are then detected with fluorescence detector) [86]. While inter-laboratory study on evaluation of different *M* determination methods for β-glucans showed that results from organic and aqueous SEC are comparable with each other, the calcofluor SEC seemed to underestimate the *M* of high-*M* samples [85,87]. Based on the inter-laboratory study it was also concluded that the *M* values represented the masses of individual polysaccharide chains, and the results were not biased by aggregates.

Due to the relatively high *M* of β-glucans, AF4 seems a suitable alternative for SEC. All the AF4 studies on cereal β-glucans have indicated the presence of aggregates in β-glucan solutions [59–62]. Mäkelä et al. [59] studied the effect of oxidation to β-glucans in aqueous solution by AF4, and noticed that oxidation decreased the *M* of barley β-glucans but also increased the aggregation tendency. Unoxidized β-glucan solutions seemed to be free of aggregates since the average *M* values were similar in SEC and AF4 and comparable with the values from the literature. Higher *M* values for native barley β-glucan were obtained from AF4 by Ulmius et al. [61]. This difference might have been due to the difference in the dissolution protocols. It seems that a temperature of 85 °C for 2 h is needed to obtain aggregate-free β-glucan solutions. Too high temperature (120 °C), however, might cause thermal degradation of β-glucan chains [85]. Sodium hydroxide (0.05 M and 0.5

M) was also used as eluent in AF4 for β-glucans, but it was found to be insufficient to eliminate the aggregated structures from the solution [60, 61].

3.3. *Gum arabic*

Gum arabic is an exudate polysaccharide from *Acacia senegal* trees and it is widely used as a hydrocolloid in food and beverage industries. The chemical structure of gum arabic is complex. The highly branched main chain consists of $(1\rightarrow3)$- and $(1\rightarrow6)$-linked β-D-galactopyranosyl units along with $(1\rightarrow6)$-linked β-D-glucopyranosyl uronic acid units. Side branches attached to the main chain may contain α-L-rhamnopyranose, β-D-glucuronic acid, β-D-galactopyranose, and α-L-arabinofuranosyl units with $(1\rightarrow3)$, $(1\rightarrow4)$, and $(1\rightarrow6)$ glycosidic linkages. In addition, a small amount of protein (around 2 %) is covalently attached to the polysaccharide [5,63]. Due to the branched structure with acidic monosaccharide units and attached protein moiety, the gum arabic is highly water soluble. Gum arabic has been shown to have a broad, multimodal *M* distribution ranging from around 6000 g/mol to more than 1,000,000 g/mol [64]. Both SEC and AF4 have been used for separation and characterization of gum arabic and it has been postulated that the highly branched material (lower *M*; major part of the sample) elutes separately from the high-*M* complex of arabinogalactan and protein [63]. This was further confirmed by the two-dimensional chromatographic separation in which narrow fractions from SEC were separated with hydrophobic interaction chromatography. The high-*M* fraction corresponded to the high hydrophobicity caused by the protein fraction [64].

3.4. *Pectin*

Pectins are cell wall polysaccharides in higher plants, and they are rich in fruits and vegetables. Commercial pectins are recovered from by-products of the food industry: mainly from apple and sugarbeet pulp and citrus peels. Pectins are heteropolysaccharides with complex branched structure consisting of galacturonans and rhamnogalacturonans. The carboxylic acids in galacturonans may be methyl esterified, and the degree of esterification plays an important role in the solution properties and conformation of pectins, and consequently affect to the gelling behavior. Pectins have relatively low *M* and thus they are used as gelling agents in the food industry rather than thickening agents [5].

Due to the highly branched structure, pectins are soluble in water and various aqueous SEC methods have been employed for pectin characterization [65–67]. Despite the structural variation in pectins (and consequently a small variation in the $\partial n/\partial c$ values), light-scattering detection most likely yields most accurate *M* and MMD data. Conventional column calibration with pullulan standards has also been used to give pullulan equivalent *M* values [65], but structural difference between pectins and pullulans likely causes systematic bias to the results. The characteristic feature of pectins is high *M* dispersity up to around 10 [67].

3.5. *Starch*

Starch, the most important polysaccharide in nutrition, forms a challenge in respect of the size-based separations. Starch is a mixture of a linear amylose (α-$(1\rightarrow4)$-linked D-glucose) and a branched amylopectin (α-$(1\rightarrow4)$-linked D-glucose with α-$(1\rightarrow6)$-linked D-glucose branches). Amyloses in some plant sources might also have a few branching points, but branching points in amylopectin are more abundant, being in the range of around 5 % of the linkages [88]. The degree of polymerization for amylose has found to be between 100 and 10,000, and the *M* of amylopectin ranges commonly from 10^6 to 10^8 g/mol [4]. Due to the overlapping MMDs and structural difference, amylose and amylopectin are difficult to separate from each other with size-based separation techniques.

The SEC separation of starch can be accomplished by using aqueous mobile phases and DMSO/LiBr (similarly to many other polysaccharides), and a mixture of DMSO and DMAc [68–71]. Linear amylose chains can pack closely with each other and form intermolecular hydrogen bonds, which reduces the solubility in water and water solutions containing amylose might become opaque. Due to coelution of the linear and branched components, local M dispersity of the separated fractions is evident and thus, no true MMD for the starch sample can be obtained. Therefore, SEC data is sometimes presented as a function of the hydrodynamic volume (or R_H) and not M [69,73].

The shear forces might cause on-column degradation of high-M analytes during SEC analysis [69,89]. Cave et al. [69] studied the effect of SEC flow rate (from 0.1 ml/min to 1 ml/min) to the R_H distribution of rice starch with amylose content of 27 % using DMSO containing 0.5 % of LiBr as eluent. The results showed the extensive shear scission of the molecules in the high-M amylopectin region when higher flow rates were used. Amylose-rich low-M region was not affected by the shear forces. However, the validity of the SEC results for amylopectin was questioned due to the breakage of the chains during the analysis. Thus, AF4 (together with hydrodynamic chromatography) offers a gentler separation method with lower probability of analyte degradation during the analysis [73].

AF4 has been widely used for separation of unmodified and modified starches [72–75,90]. Rolland-Sabate et al. [73] compared the results from SEC and AF4 separations for cereal and potato starches. The data from two separation techniques were consistent, however, the AF4 enabled better separation for amylopectins than SEC. Dissolving high-M starch components (similar to other high-M polysaccharides such as galactomannans or β-glucans) might be challenging, and high temperatures and/or pressure have been used to assist the dissolution process [72]. While AF4 enables the separation of high-M and large size analytes, the challenges in the light scattering detection might be met when the size of the analytes approaches the wavelength of the light source (and when the Rayleigh–Gans–Debye approximation is not valid anymore). Both R_G and R_H have been shown to reach several hundreds of nm for amylopectins and thus, the R_G values for the largest amylopectin molecules may not be accurate. In case of R_H, collection of online DLS data might become a challenge for molecules which have R_H larger than around 50 to 60 nm [70]. In case of AF4, the R_H values for the separated range can be obtained from the retention theory even when the cross flow programming is used. This approach allows the estimation of R_H for large-sized analytes and brings additional advantage over the separations with SEC [72].

AF4 has also been used to investigate the retrogradation behavior of starch [75]. Starch retrogradation is a process in which the amylose and amylopectin chains realign themselves to form ordered structures. Depending on the application, retrogradation might be an unwanted process (e.g. firming of bread) or desired process (e.g. production of breakfast cereals with desired sensory properties). Due to the ability of AF4 to separate large molecules and aggregates (including aggregated starch), AF4 proved to be a feasible tool to obtain knowledge on this phenomenon which is not well understood. Form factor R_G/R_H across to separated molecules gave additional insight into conformation of the aggregated chains.

Like cellulose, starch has been chemically tailored to alter its functional properties. AF4 was found to be useful in monitoring the M and size changes of carboxymethyl starches during the derivatization. A decrease in M was observed due to the molecular degradation during the derivatization in alkaline conditions [90]. As for native starch, AF4 would be a useful tool for characterization of other modified starches with large molecular size.

4. Critical evaluation of the separation methods – when AF4 is a more beneficial choice than SEC for separation of plant polysaccharides?

As seen from the references cited above, both SEC and AF4 have been widely used for separation and characterization of structurally different plant polysaccharides. First paper on the AF4 was published in 1987 by Wahlund and Giddings [13] and commercial AF4 instrumentation has been widely available for around a quarter of a century; thus, more reports in the field of AF4 separations have been published in this millennium whereas older references report the use of more conventional SEC. As already pointed out, AF4 is a multi-flow technique which requires more optimization and operator skills. In addition, the AF4 instrument is more expensive than the SEC since the instrument must maintain several flows in AF4, but in SEC isocratic elution with a single pump is sufficient. Regarding plant polysaccharide characterization, when is it then beneficial to use AF4 instead of SEC?

Many polysaccharides (depending on the structure) are soluble in water. Most of the AF4 applications are aqueous, mainly due to the membrane material availability. Most common membrane materials include RC and PES. Other polysaccharide solvents include DMAc and DMSO. Since DMAc dissolves cellulose, regenerated cellulose membrane is not suitable with DMAc. The high viscosity of DMSO restricts its flow through the membrane and is thus not applicable for AF4. Therefore, native polysaccharides have been solely characterized by AF4 in aqueous solution. Polysaccharide derivatives which can be dissolved in "AF4 compatible" solvents, such as THF, could be characterized with AF4 in organic solvent [91].

Since the separation in AF4 takes place in an open channel, separation is very gentle and not likely to cause breakage of the glycosidic bonds in polysaccharide chains. On the contrary, breakage of large-sized starch components was observed during SEC analysis [69]. In general, the upper size limit in SEC lies at around 300 nm expressed as R_G [92]; if large-sized polysaccharides need to be separated, AF4 would be a more suitable technique. In addition to starch, many cellulose derivatives and cellulose nanocrystals benefit from the broader separation range of AF4. Cellulose nanocrystals are rod-like particles which cannot be separated with SEC. From polysaccharides derivatives, especially cationic analytes are more feasible to be separated with AF4 than with SEC. Analytes with positive charge may interact with negatively charged SEC stationary phase (non-size-exclusion effects). Adsorption of molecules into the column stationary phase causes lower recovery values, peak tailing, and biased M values to name a few detrimental impacts.

Commonly, plant polysaccharides include non-carbohydrate material attached to the carbohydrate part. This is true, e.g. for spruce galactoglucomannans containing lignin or gum arabic containing protein. As discussed in the previous section, galactoglucomannans form colloidal structures in an aqueous environment and these structures likely play an important role in applications such as in emulsions. AF4 is the method of choice for highly disperse colloidal samples whereas SEC is commonly sufficient for separation of samples dissolved in the level of individual polymeric chains (being in the moderate size range). The same is true for the samples containing polysaccharide aggregates, which can be important e.g. in respect of the material functionality.

5. Conclusions

Size-exclusion chromatography and asymmetric flow field-flow fractionation are size-based separation techniques which are often complementary to each other. Both techniques are widely used for separation and characterization of polysaccharides with structural variety. Due to the disperse nature of polysaccharides, separation based on size is of importance to obtain molar mass, size, and conformation information over the separated range of macromolecules. Batch measurements, such as static and dynamic light scattering experiments, cannot provide detailed information on the distributions or ranges of

molecules. While most of the plant polysaccharides can be separated either using size-exclusion chromatography or asymmetric flow field-flow fractionation, the latter provides broader separation range and is a better method for large-sized polysaccharides, samples with colloidal features, polysaccharide aggregates, and rod-like particles. Since most of the asymmetric flow field-flow fractionation applications are aqueous, more traditional size-exclusion chromatography is still applicable for polysaccharides and polysaccharide derivatives, which dissolve in organic solvents. Due to green transition, detailed characterization of biomacromolecules, such as plant polysaccharides with complex structures, will have even more emphasis in the future.

CRediT authorship contribution statement

Leena Pitkänen: Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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