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Macromolecular characterisation of three barley β-glucan standards by size-exclusion chromatography combined with light scattering and viscometry: an inter-laboratory study

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Abstract

Six (1 → 3)(1 → 4)-β-D-glucan standards (A–F) isolated from barley were analysed by size-exclusion chromatography (SEC) in five different laboratories with varying columns, solvent conditions and detector systems (low- and multi-angle light scattering and viscometry). Static (batch) measurements by capillary viscometry and laser light scattering were included. Fairly consistent results were obtained for the weight average molecular weights (M_w), radii of gyration (R_G) and intrinsic viscosities [η], demonstrating that the β-glucans may serve as useful standards or reference materials in the study of cereal β-glucans. Average values for M_w were: A,E: 114,000 (±11%); B,C: 374,000 (±9%), D,F: 228,000 (±13%). Some inconsistencies regarding the polydispersity (M_w/M_n) could be ascribed to the influence of peak broadening in certain column/solvent systems. The study further demonstrated that individual researchers tended to use different processing parameters, especially refractive index increments (d*n*/d*c*), due to ambiguities in the literature or to differing experimental values. The need for consistent parameters and processing methods is clearly demonstrated.

Keywords: Barley β-glucan; Size-exclusion chromatography; Multi-angle light scattering; Low-angle light scattering; Viscometry

1. Introduction

The endosperm cell walls of both barley and oats are composed mainly of a polysaccharide known as mixed-linkage β-glucan or simply β-glucan. It is a linear glucan with about 70% β(1 → 4) and 30% β(1 → 3) linkages. The structure is essentially (85–90%) a random assembly of β(1 → 3)-linked cellotriosyl and cellotetraosyl units but the oat β-glucan contains more of the latter structure. About 10–15% of the molecule is more cellulose like, with from 4 to15 or more consecutive (1 → 4)-β-D-linked glucopyranosyl units. The cellulose-like features seem to occur almost identically in oat and barley β-glucan (Wood, Weisz & Blackwell, 1994a).

The β-glucan of barley has been studied extensively because its viscosity adversely affects brewing and the performance of barley as feed for chicks. More recently, oat β-glucan has received considerable attention as a component of oats which lowers serum cholesterol levels (Braaten et al., 1994), a risk factor for heart disease. The mechanism(s) by which oat β-glucan, and other so-called "soluble fibres", lower serum cholesterol levels is not well understood, but again, viscosity is believed to play a role (Jensen, Spiller, Gates, Miller & Whittam, 1993). Additionally, consumption of oat β-glucan attenuates blood glucose and insulin levels in a viscosity related fashion (Wood, Braaten, Scott, Riedel, Wolynetz & Collins, 1994b).

Thus viscosity of β-glucan is identified as of key importance commercially and nutritionally. The solution viscosity depends on the concentration, molecular weight, shear regime, temperature and solvent. Above a critical concentration (the critical overlap concentration, c^*), the viscosity increases very rapidly with concentration and is highly shear dependent. The underlying property of interest is the

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Table 1
Sample preparation

Laboratory	Dissolution procedure	Clarification	Sample conc. before SEC
1	0.1 M $NaNO_3$ added to dried samples; conc. 2 mg/ml; heated 3 h at 70°C	Centrifuged 30,000*g*, 30 min; filtered 0.45 μm	Assumed 2.0 mg/ml
2	Method I: 4 mg dissolved in 0.2 ml 0.1 M NaOH (40°C, 10 min); diluted with 3.8 ml 0.02% NaN_3	Filtered 0.45 μm	1.0 mg/ml
	Method II: wetted with EtOH; 0.02% NaN_3 added; heated at 70°C for 3 h	Filtered 0.45 μm	1.18–1.39 mg/ml[a]
3	Samples (vacuum dried) dissolved in water (6 mg/ml); heated 10–15 min at 100°C; diluted to 3 mg/ml with 0.1 M $NaNO_3$	Centrifuged 12,000 rpm; filtered 0.4 μm	3.0 mg/ml
4	Wetted with EtOH; water added to 1.5–2 mg/ml; heated 30 min at 100°C; diluted to 0.75–1.0 mg/ml with 0.1 M Na_2SO_4 containing 0.02 M EDTA, pH 6	Filtered 0.8 μm (Millex AA)	0.5–1.8 mg/ml; measured by phenol–sulphuric acid method using glucose as standard
5	0.05 M $NaNO_2$ (nitrite) added to dried samples; conc. 2 mg/ml; heated ~2 h at 70°C	Filtered 0.45 μm	3 mg/ml

[a] Corrected for 5% moisture and 0.7% protein.

hydrodynamic volume occupancy of the molecules, which depends primarily on molecular weight (MW) distribution and the shape (conformation) of the molecules.

Notable amongst earlier studies (prior to those using the now readily available commercial on-line MW sensitive detectors) Woodward, Phillips and Fincher (1983) determined the MW of barley β-glucan using sedimentation and osmometry. Vårum and Smidsrød (1988) and Vårum, Smidsrød and Brant (1992) used both osmometry and light scattering to evaluate oat β-glucan. Recently, Gómez, Navarro, Manzanares, Horta and Carbonell (1997a,b) used intrinsic viscosity and size-exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) to characterise barley β-glucan (in pure water).

High performance size-exclusion chromatography (HPSEC) is a powerful technique for analysing molecular weight distributions. It is especially powerful for β-glucan since the use of the specific dye-binding of calcofluor allows determination in mixtures with other polymers such as starch, pentosan and protein (Wood, Weisz & Mahn, 1991). Without appropriate standards, molecular weight sensitive detectors are required to determine MW. Methods available are low-angle laser light scattering (LALLS), multi-angle laser light scattering (MALLS), or right-angle laser light scattering (RALLS) in combination with on-line viscometry. For large polymers ($R_G > \lambda/20$), the zero angle scattering value is required to calculate MW. In the LALLS system a low angle, essentially equivalent to zero is used. In the MALLS system, a series of angles are used and the zero angle value obtained by extrapolation. In the RALLS system, the molecular weight is first calculated by putting the angular scattering function at 1. From this estimate, and from the intrinsic viscosity value obtained by the viscometer, a radius of gyration is calculated, assuming in this case a linear flexible chain. From this, a truer estimate of the angular scattering function can be obtained. The process is repeated until the estimated values of MW converge (usually about three iterations).

Parameters obtained from HPSEC with light scattering do not only depend on the macromolecular properties alone. The instrument configuration, the experimental conditions (columns, buffer/salt, temperature, flow rate, in-line filters), as well as the sample preparation, may influence the final results. Correct results on an absolute basis further require that processing parameters (dn/dc, A_2 etc.) and detector responses (calibration factors) are correctly determined. Although light scattering detectors in principle provide absolute MWs without the use of calibration substances, the access to relevant standards remains crucial in order to check the performance of the system.

In this study we report the use of all three HPSEC techniques in addition to static (batch) measurements to determine the MW of three barley β-glucan standards. Five different laboratories took part in the study. The methodologies used were not tightly delineated, as in a collaborative analytical study, since part of the purpose was to determine whether despite using somewhat different methodologies similar values would be obtained. However, one laboratory used both MALLS and RALLS/viscometry, allowing an internal comparison, and one used classical batch

Table 2
Chromatographic conditions and sample recoveries

Lab	Columns	Solvent	T (°C)	In-line filter before column	In-line filter after column	Injected amount (μg)	Recovery (%)
1	PL-aquagel-OH 60/50/40 (3 in series)	0.1 M $NaNO_3$	40	0.45 μm	0.45 μm	300	> 90
2	PL-aquagel-OH 60/60/50/40 (4 in series)	0.02% (3 mM) NaN_3	35	None	None	131–155	87–100
3	Shodex OH-pak SB-806/805/803 (3 in series)	0.05 M $NaNO_3$	45	0.1 μm	None	300	97–100
4	TSK GEL G6000/5000/4000 PWXL, (3 in series)	0.05 M Na_2SO_4, 0.1 M Na_2EDTA	Room temp.	None	0.45 μm	100–360	100
5	Ultrahydrogel, exclusion limit $2\times10^6/5\times10^5/1.2\times10^5$ (3 in series)	0.05 M $NaNO_2$ (nitrite)	30	None	None	200	57

measurements with LALLS as well as on-line use with both LALLS and MALLS, again allowing an internal comparison.

Ultimately, the purpose was for interested workers to have access to well-characterised β-glucan standards for MW determinations. This is of particular importance for the evaluation of products making health claims.

2. Experimental

Three barley β-glucan standards were obtained from Megazyme International, Bray, Ireland. The supplier reports a purity of >95%. Each sample was divided in two portions. The six samples were assigned letters A–F (randomised) and shipped to each participant.

The purity of the β-glucans was investigated by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) using glucose as standard (Lab 4). The β-glucan content was 91% (±2.7%) for samples that had been dried in vacuo over P_2O_5. By using the method of McCleary and Glennie-Holmes (1985) β-glucan contents ranging between 95.1 and 97.1% (dwb) were obtained.

One laboratory (Lab 2) included a correction for 5% moisture and 0.7% protein. Otherwise, the other laboratories made no corrections.

The samples were dissolved and prepared for SEC injection as summarised in Table 1. Table 2 gives a survey of the chromatographic conditions, whereas Table 3 shows the detector systems and combinations used in the study. Note that $NaNO_2$ rather than $NaNO_3$ was used as solvent in Lab 5 (Müller, Pretus, McNamee, Jones, Browder & Williams, 1995).

Injection volumes were typically 50–250 μl, and flow rates were 0.75–1.0 ml/min.

In one case the β-glucans were reacted with Calcofluor by means of a mixing-T (100 mg/l in 0.1 M NaOH at 0.8 ml/min and mobile phase, 0.02% NaN_3 at 0.75 ml/min). A fluorescence detector was the used to monitor the β-glucan elution.

The data were processed by commercial software, depending on the detector type. Data from Dawn DSP or

Table 3
Detection systems and initial processing parameters (dn/dc-values and A_2)

Lab	Concentration detectors	LS detectors	λ (nm) (LS detectors)	Viscosity detectors	A_2	dn/dc
1	Viscotek Mod. 250[a] (RI)	Viscotek Mod. 600 RALLS (90° angle)[a]	670	Viscotek Mod. 250[a]	0	0.140
2	(a) Waters Mod. 401 (RI) (b) Perkin–Elmer LS-5[b]	DAWN DSP-F multi-angle	633	–	0	0.145
3	(a) Shodex SE-61 (RI) (b)Viscotek Mod. 405 (RI)	DAWN F multi-angle Viscotek Mod. 600 RALLS (90° angle)[a]	633 670	– Viscotek Mod. 100	0	0.135 0.135
4	Shodex SE-61 (RI)	KMX-6 low angle (4.88°)	633	–	1.0×10^{-4}	0.150
5	Optilab 903 (RI)	DAWN DSP	488	Viscotek Mod. 200	0	0.166–0.175

[a] The Viscotek system is an integrated unit containing an RI detector, a LS detector and a viscosity detector.
[b] Fluorescence detector used for Calcofluor detection.

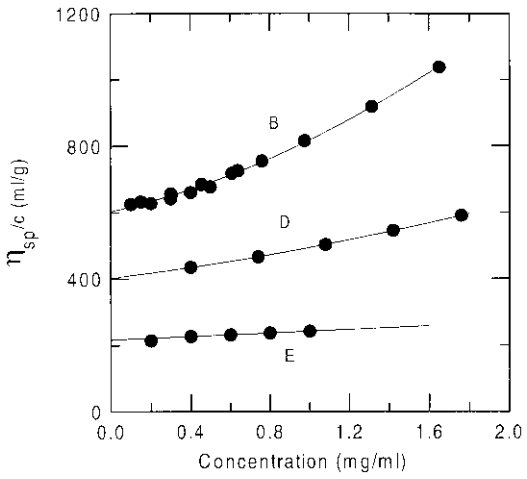

Fig. 1. Concentration dependence of the reduced viscosity (η_{sp}/c) for β-glucans B, D and E obtained in a capillary viscometer at an average shear rate of 110 s^{-1} (Lab 4).

Dawn F multi-angle laser light scattering photometers were processed together with the signal from the concentration detector by Astra software. Data originating from the KMX-6 low-angle laser light scattering photometer (and the RI detector) was processed by the PCLALLS software. Viscotek data were processed by Trisec software or Unical software.

The results obtained by means of chromatography software depend strongly on the calculation routines and data fitting as well as system parameters such as the refractive index increment and the second virial coefficient (A_2). The refractive index increment (dn/dc) was determined independently by two groups (Labs 1 and 5) using an Optilab 903 interferometric refractometer (488 nm) operating at 25°C, with 50 mM $NaNO_3$ as solvent. In one case (Lab 3) dn/dc of sample D was determined indirectly by comparing the RI detector response (Viscotek Mod. 405, 670 nm) to that of dextran T110, assigned a value of dn/dc of 0.142 which was obtained from the literature (Yu & Rollins, 1987) The method of determining dn/dc was verified by comparing dextran against pullulan which had a literature value of 0.148 at 488 nm (Kato, Okamoto, Tokuya & Takahashi, 1982). A value of 0.140 for dextran was found by the comparison with pullulan. Results are included in Table 3 together with the other dn/dc-values used.

The sample concentration also enters the calculations of the molecular weight as well as dn/dc. In general, the concentration of the stock solutions (before injection) was assessed on the basis of the amount of dried material which was dissolved in the solvents, assuming that negligible amounts of β-glucan were removed by clarification procedures or by adsorption to the columns. Chromatographic software such as Astra back-calculates the actual concentration in each chromatographic slice by means of dn/dc combined with a detector constant for the concentration detector. In other cases 100% sample recovery is assumed, which, if not compensated for, may give rise to errors.

2.1. Total intensity low-angle laser light scattering measurements (static LALLS)

These measurements were performed using the KMX-6 in the static mode, where the HPLC-cell was replaced by the standard flow cell. Samples were pumped (100 μl/min) through a Millex HA (0.22 μm) filter directly into the KMX-6. The barley β-glucans adsorbed to the filters, and it was necessary to saturate the filters before a constant scattering level (Rayleigh factor) could be obtained. This was most conveniently done by injecting the highest concentrations first.

2.2. Intrinsic viscosity (batch measurements)

Measurements were performed at 20.0°C in a Ubbelohde capillary viscometer (Schott-Geräte capillary no. 0a) equipped with an AVS 310 control unit and PC-operated titrator for automatic and sequential dilution with solvent, as well as for automatic data acquisition and calculations. The solvent flow-through time was 212 s, corresponding to an average shear rate of 110 s^{-1}.

3. Results and discussion

3.1. Intrinsic viscosity (batch measurements in capillary viscometer)

The intrinsic viscosities of samples A–F were determined at 20°C by conventional capillary viscometry to provide a basis for subsequent investigations. The solvent was 0.05 M Na_2SO_4 containing 0.01 M EDTA. Huggins' plots (η_{sp}/c vs. c) for samples B, D and E are shown in Fig. 1, whereas calculated intrinsic viscosities and Huggins' constants (k') are given in Table 4. Due to pronounced curvature, especially at the highest concentrations and viscosities, the data were fitted to a second-order polynomial. The data indicate the pairwise identities A = E ([η] of 219 and 214 ml/g), D = F ([η] of 403 and 393 ml/g) and B = C ([η] of 620 and 619 ml/g), respectively. The calculated Huggins' constants were 0.4–0.5 in all samples.

By using Kraemer's plots ((ln η_r)/c vs. c) straight lines were obtained (not shown), in contrast to the curved Huggins' plots. Nevertheless, the calculated intercepts (intrinsic viscosities) were the same.

3.2. Static (total intensity) low-angle laser light scattering

The weight average molecular weights (M_w) of samples B, D and E were determined by total intensity LALLS using the KMX-6 LALLS photometer in the 'static' mode (flow injection). The solvent was 0.05 M Na_2SO_4, 0.01 M EDTA, and the system was operated at ambient temperature. It turned out that the β-glucans tended to adsorb to the in-line filter (cellulose-based) used between the peristaltic pump and the photometer. For low concentrations no stable

Table 4
Data summary Lab 4

Method	SEC-LALLS				Static LALLS		SEC-MALLS			Static MALLS		Viscometry	
Sample	M_w (unfitted)	M_w (linear fit)	M_n (linear fit)	M_w/M_n	M_w	A_2 (ml mol/g^2)	M_w (unfitted)	R_w (nm)	R_z (nm)	M_w	R_z (nm)	$[\eta]$ (ml/g)	k'
B	410,000	409,000	266,000	1.5	409,000	0.2×10^{-3}	420,000	48	57	402,000	54	620	0.44
C	409,000	404,000	248,000	1.6			415,000	48	57	–	–	619	0.42
D	235,000	238,000	146,000	1.6	260,000	1.1×10^{-3}	239,000	38	46	–	–	403	0.43
F	241,000	240,000	148,000	1.6			239,000	35	44	–	–	393	0.47
A	120,000	118,000	59,000	2.0			112,000	22	32	–	–	218	0.48
E	127,000	120,000	61,000	2.0	132,000	0.9×10^{-3}	116,000	–	–	–	–	214	0.42

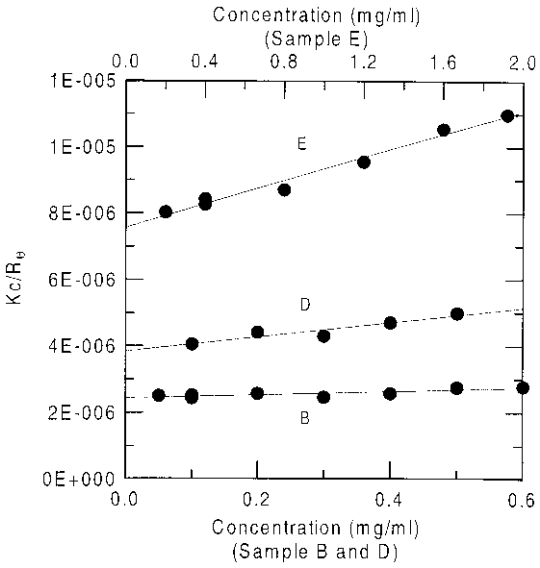

Fig. 2. Plots of Kc/R_θ vs. concentration for β-glucans B, D and E obtained by static LALLS (flow injection mode) (Lab 4).

scattering level was obtained, and R_θ was generally underestimated. This resulted in upward curvatures (with poor reproducibility) in plots of Kc/R_θ vs. c, which might—erroneously—be interpreted in terms of reversible (concentration-dependent) aggregation as seen in other β-glucans (Vårum et al., 1992) and chitosans (Anthonsen, Vårum, Hermansson, Smidsrød & Brant, 1994; Ottøy, Vårum, Christensen, Anthonsen & Smidsrød, 1996). The problem was overcome by measuring the highest concentrations first, whereby the filter became rapidly saturated and reasonable stable measurements were obtained at all concentrations. The plots of Kc/R_θ vs. c are shown in Fig. 2. The calculated M_ws are included in Table 4.

Static LALLS also provided the second virial coefficient (A_2), which was small, but positive for all samples (Table 4), as expected for a neutral polymer. A_2 was the highest for the two lowest molecular weights, which is also to be expected from theory. The results also show that the solvent system (Na_2SO_4/EDTA) used in this study, which originally was developed for alginates, is well suited for the study of barley β-glucans.

3.3. Static (total intensity) multi-angle laser light scattering

An attempt was made to determine M_w, R_G and A_2 for sample B by static MALLS using the DAWN DSP photometer in the static mode. A concentration series was measured (at room temperature) using simple glass vials. The solvent was Na_2SO_4/EDTA as described above. As also noted by Gómez et al. (1997a), difficulties were encountered in the measurements. A low or slightly negative A_2 (-0.8×10^{-4}) indicates that concentration-dependent aggregation takes place under the static conditions. However, the obtained M_w (402,000) is in reasonable agreement with that obtained by static LALLS (flow injection). The calculated z-average radius of gyration was 54 ± 2 nm.

3.4. Size-exclusion chromatography (SEC)—general

Selected elution profiles are shown in Fig. 3a–c. Despite different combinations of columns and solvents the profiles appear quite similar and elution orders are identical in all cases. The sample identities A = E, B = C and D = F were easily demonstrated by total peak overlap. The sample peaks are well resolved between the exclusion limit and the salt peak. Relative peak width, peak asymmetry and tailing (due to polydispersity) are quite similar, showing that all chromatographic systems behave normally and are well suited for the characterisation of mixed (1 → 3)(1 → 4)-β-D-glucans of cereal origin.

3.5. Size-exclusion chromatography (SEC) with LALLS detection

Elution curves (refractive index and LALLS detectors) of samples A–F are given in Fig. 3a. The area under the peaks was integrated, and the sample recovery was calculated by means of a standard curve obtained with pullulan standards. Essentially 100% recovery was obtained for all samples, indicating that no material adsorbed to the columns and that no low molecular weight components eluting in the salt peak were present. The peak area is also proportional to dn/dc, and 100% recovery also indicates that dn/dc of the barley β-glucans is the same as for pullulans. Nordmeier (1993) reports pullulan (and dextran) values of 0.148 at 633 nm. A value of 0.150 was therefore used in this part of the study. It may be noted that Lab 3 also obtained 97–100% recovery with a dn/dc of 0.135.

The elution curves derived from the LALLS detector (Rayleigh factor, R_θ) are given in the same figure. The pairwise sample identity is also seen here, except for samples A and E, where the 'shoulder' occurring at 20–21 ml is somewhat larger for sample E. This shoulder is attributed to very small amounts of aggregates which could not be observed in the other samples.

The calculated plots of log M_w (calculated from the relation $Kc/R_\theta = 1/M_w + 2A_2c$) versus elution volume (hereafter termed 'calibration curve') are included in Fig. 3a. For the elution interval where both detectors yield a good signal we obtain basically the same calibration curve for all samples. This demonstrates that the columns cover the entire molecular weight range, and that peak broadening does not influence the results. It further shows that all samples belong to a family of polymer chains with the same basic conformation, only differing in the distribution of chain lengths. The curve is essentially linear in the elution range 22–30 ml.

At lower elution volumes (19–21 ml) a slight upward curvature is observed in the calibration curves, particularly for sample A, whereas samples B and C just show a very slight curvature. This effect is attributed to some aggregation,

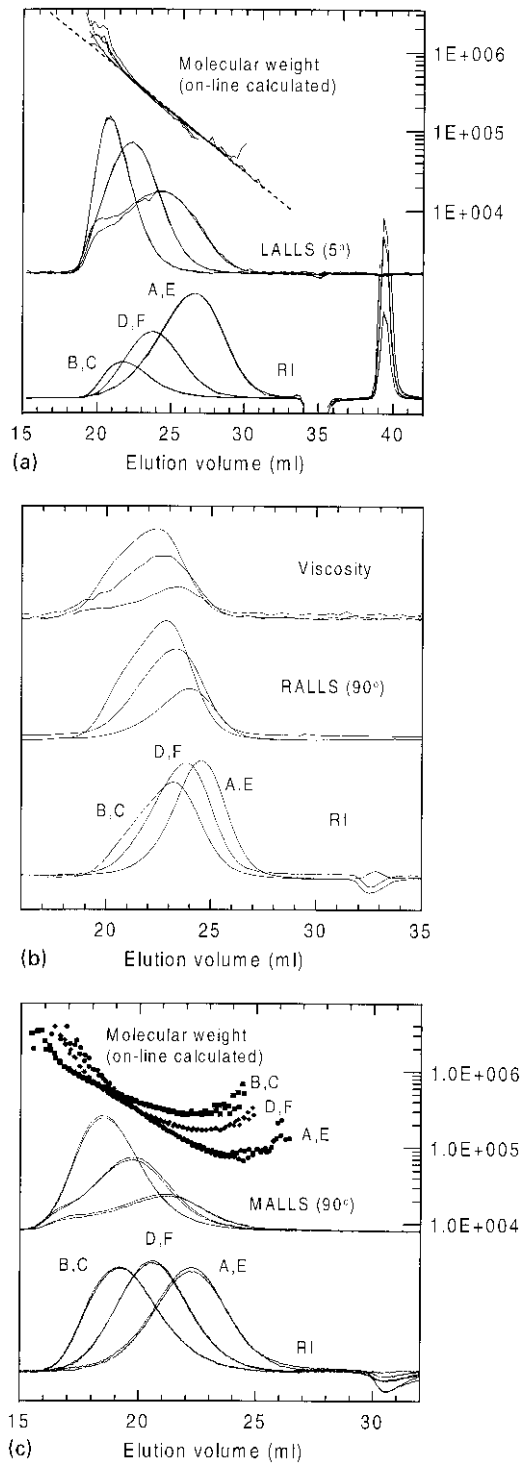

Fig. 3. SEC chromatograms of barley β-glucans A–E obtained by (a) SEC-LALLS (Lab 4); (b) SEC-RALLS (Lab 3); (c) SEC-MALLS (Lab 5). RI, refractive index detector; LALLS, low-angle laser light scattering detector (ca. 5°); RALLS, Right angle laser light scattering detector (90°); viscosity, viscosity detector.

especially in samples A and E, whereas for the samples containing the largest chains (B and C) the curvature may well be due to reduced column resolution near the void volume.

The M_w for the entire distributions was calculated from the formula $M_w = \sum M_i c_i / \sum c_i$, where M_i is the molecular weight (weight average) calculated for each 'slice' of the chromatogram (which is assumed to be monodisperse), whereas c_i is the corresponding concentration. The results are given in Table 4 as M_w (unfitted).

In order to obtain accurate estimates of M_n and the polydispersity index (M_w/M_n) it is necessary that a calibration curve be correctly assigned across the entire distribution. This is usually not the case at the low molecular weight tail of the distribution because of a weak light scattering signal. In the present case, where we investigate a homologous series of unbranched chains, it is possible to construct a single calibration curve based on samples of different MW distributions. It may be noted in Fig. 3a that the linear range of samples A and E are exact extensions of that obtained for D and F, as well as those of sample B and C for the elution interval 21–23 ml. The dotted line in Fig. 3a represents a linear fit based on the overlapping linear regions of all six samples, and the data were reprocessed on the basis of this calibration curve to give M_w and M_n. Data are included in Table 4. The values for M_w are basically the same as for the unfitted data, meaning that high molecular weight aggregates, despite their presence, contribute very little to the average M_w. The polydispersity indices (M_w/M_n) are 2.0 for samples A and E, whereas samples B and C as well as D and F have indices of 1.5–1.6.

3.6. Size-exclusion chromatography (SEC) with MALLS detection

Laboratories 2–5 all included a combination of SEC with a DAWN DSP or DAWN F MALLS detectors (operating at 488 or 633 nm), although the selection of columns and operating temperatures differed somewhat (Tables 2 and 3). In all cases the elution orders and profiles were qualitatively identical to those obtained with SEC-LALLS (Fig. 3).

The SEC-MALLS results obtained by Lab 4 are included in Table 4. These data may be directly compared to the SEC-LALLS results from the same laboratory, since sample preparation, sample injections, SEC configuration and processing parameters are identical, the difference being that a MALLS detector replaces LALLS, and an Optilab DSP RI detector (independently calibrated with NaCl) replaces the Shodex RI detector (calibrated with pullulans). Excellent agreement between the two sets of data is obtained, as both methods yielded very similar M_ws. In this case the Zimm procedure (first-order fit) was chosen to extrapolate to zero angle, since plots of Kc/R_θ vs. $\sin^2(\theta/2)$ were generally linear. The alternative Debye procedure (first-order fit) was rejected because plots of R_θ/Kc vs. $\sin^2(\theta/2)$ became non-linear at the highest molecular weights, with an apparent underestimation of the molecular weight. However, a second-order Debye fit gave results that were essentially identical to the first-order

Table 5
Processing routines and results (recoveries, M_w and polydispersity indices) obtained by SEC-MALLS

	Lab 2	Lab 3	Lab 4	Lab 5		Comments
dn/dc	0.145	0.135	0.150	0.166–0.175		Initial parameters and calculations as reported by each investigator
Angular fit	Zimm[a], first-order	Debye[b], first-order	Zimm[a], first-order	Debye[b], first-order		
Recovery (%)	87–100	97–100	100–102	54–58		
A	117,000	134,000	114,000	194,000 (200)[c]	154,000 (400)	
E	119,000	143,000		191,000 (200)	163,000 (400)	
B	370,000	492,000	419,000	535,000 (200)	467,000 (600)	
C	363,000	486,000		534,000 (200)	456,000 (600)	
D	223,000	277,000	239,000	344,000 (200)	336,000 (400)	
F	222,000	280,000		340,000 (200)	416,000 (600)	
A	109,000	109,000	114,000	146,000	129,000	
E	111,000	116,000		151,000	110,000	Recalculated to dn/dc = 0.150 and 100% recovery (Lab 5); Zimm procedure Lab 5
B	346,000	399,000	419,000	369,000	305,000	
C	339,000	394,000		433,000	349,000	
D	208,000	224,000	239,000	264,000	263,000	
F	207,000	227,000		287,000	434,000	

[a] Zimm: Extrapolation of $Kc_i/R_{\theta,i}$ (plotted as function of $\sin^2(\theta/2)$) to zero angle.
[b] Debye: Extrapolation of $(Kc_i/R_{\theta,i})^{-1}$ (plotted as function of $\sin^2(\theta/2)$) to zero angle.
[c] Refers to the amount of sample injected (μg).

Zimm procedure. It may be noted that sample recoveries were essentially 100% (Table 5), as with SEC-LALLS.

Fig. 4a shows SEC-MALLS chromatograms of β-glucans A and B obtained by Lab 4 with different amounts of injected material. The elution profiles and the corresponding calibration curves and plots of log R_G vs. elution volume are essentially independent of the amount of sample injected, although the signal-to-noise ratios of the light scattering decreases with decreasing sample concentrations. The calculated M_w was also independent of the injected amount, except at very low concentrations, where M_w apparently increased (Fig. 4b). However, this increase is associated with a rapid increase in the calculated standard error due to the low signal-to-noise ratio, and is therefore not considered further. The calculated z-average radii of gyration of sample B were 55–57 nm for the three highest concentrations, in good agreement with the value of 54 nm obtained by static MALLS for the same sample in the same solvent.

Although the raw data obtained by Labs 2, 3 and 5 appeared to be qualitatively similar to those of Lab 4, different molecular weights were initially calculated, as summarised in Table 5. Individual researchers initially selected calculation procedures and parameters on the basis of their own experience. Indeed, different values of dn/dc explains most of the differences between Labs 2, 3 and 4. When the data were recalculated to dn/dc = 0.150, better agreement was obtained (Table 5).

The SEC-MALLS results of Lab 5 differed from the other laboratories (Table 5). Reported recoveries were initially 54–58%, and M_w values were much higher than the other laboratories. In addition, the results seemed to depend more on the amount of β-glucan injected than in the other laboratories. A recalculation of the data using dn/dc = 0.150 and assuming 100% recovery, and using a first-order Zimm procedure gave results that were in some cases reasonably close to the other laboratories.

In the case of Lab 3 it was found that the first-order Debye formalism gave the best precision for M_w and the best agreement with the independent determination of M_w by the RALLS method. Comparison of the recalculated data at the bottom of Table 5 revealed that M_ws from first-order Debye data obtained by Lab 3 fell between M_ws obtained by first-order Zimm data from Labs 2 and 4. Hence, the results obtained by Lab 3 appeared to show no systematic underestimation of M_w due to the use of the first-order Debye formalism. On the other hand, if it is assumed a priori that all procedures performed and values of dn/dc obtained by the various laboratories are equally probable, then all M_w values measured by Lab 3 fall between upper and lower extreme values found by the other laboratories.

Table 6 summarises the polydispersities (M_w/M_n) obtained by SEC-MALLS and SEC-LALLS. Excellent agreement between Labs 2, 3 and 5 is observed for samples A, E and B, C. Lab 4 produced generally higher polydispersities, but SEC-LALLS values are in good agreement with those obtained by SEC-MALLS.

Fig. 3a (or Fig. 4a) and 3c allows a comparison between

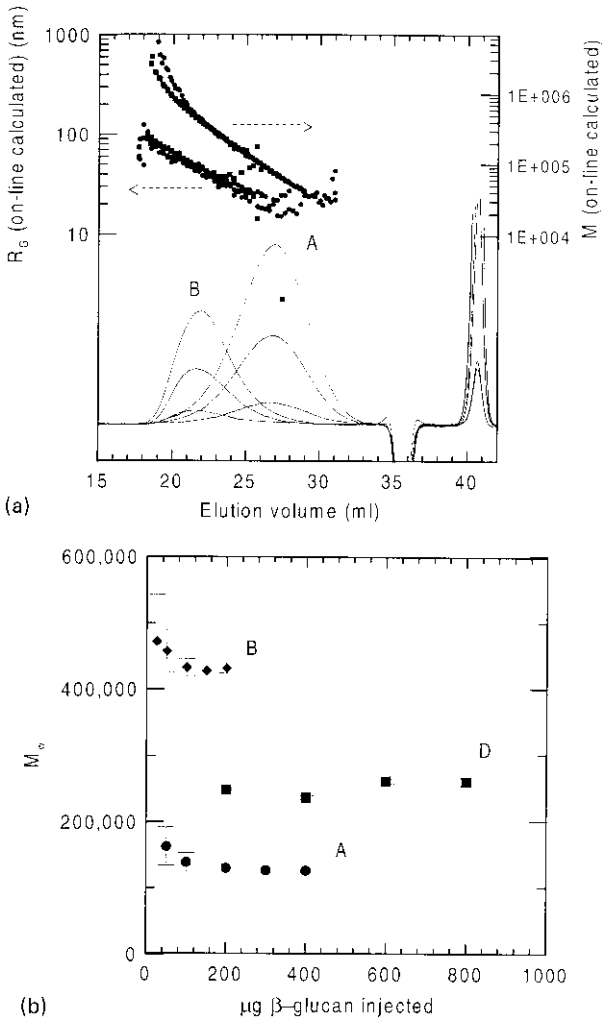

Fig. 4. (a) SEC-MALLS chromatograms including on-line calculations of log M vs. elution volume and log R_G vs. elution volume obtained for different amounts of barley β-glucans A and B (Lab 4). Columns and elution conditions are otherwise identical to those in Fig. 3a. (b) Dependence of M_w on the amount of injected material for β-glucans A, B and D.

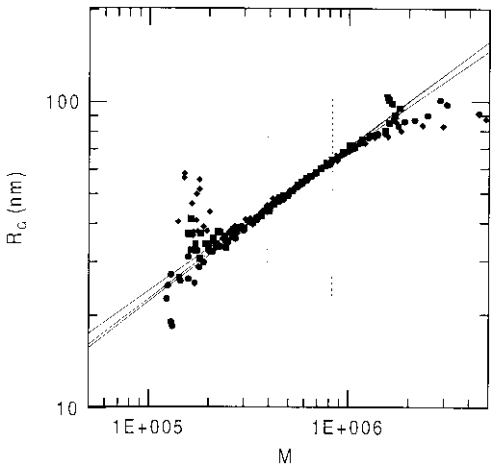

Fig. 5. Dependence of the radius of gyration (R_G) on M_w for β-glucan B as calculated for each elution slice in the chromatogram (SEC-MALLS, Lab 4). Vertical lines refer to the data interval used in the regression analysis. Results for every fourth slice are presented. Symbols refer to different injected amounts (100–200 µg).

elution curves and calibration curves) obtained for Labs 4 and 5, respectively. The calibration curves differ considerably in the two cases. For Lab 4, SEC-MALLS gives the same type of results as in SEC-LALLS, namely a single calibration curve for all samples (Figs. 3a and 4a), independent of the MW distribution (or elution profile). This is expected when column dispersion (band broadening) plays a negligible role as compared to polydispersity. In comparison, monodisperse pullulan standards gave the expected horizontal calibration curves (not shown). The lack of a single calibration curve for the column system used by Lab 5 (Fig. 3c) suggests that significant band broadening occurs. This effect will always contribute to an underestimation of M_w/M_n, as recognised by van Dijk, Varkevisser and Smit (1987).

The slight difference in polydispersity between SEC-MALLS and SEC-LALLS (obtained by Lab 4) is primarily ascribed to differences in the processing of the calibration curve. For SEC-LALLS a linear fit is used as described above. The ASTRA software used in SEC-MALLS does not allow exactly this type of fitting. In this case an unfitted calibration curve is used as reported in Table 6. However, a linear fit based on the entire calibration curve is possible, which yields polydispersities that are closer to those obtained by SEC-LALLS (data not shown). Thus, it may be concluded that SEC-MALLS and SEC-LALLS in the case of barley β-glucans yield the same results for weight average molecular weights and polydispersities.

3.7. SEC–viscometry

Three laboratories (1, 3 and 5) included in-line viscometers together with light scattering and RI detectors. The calculated intrinsic viscosities (weight average in the case of SEC) are given in Table 7. Labs 1 and 3 report

Table 6
Polydispersities obtained by SEC-LALLS, SEC-MALLS and the Viskotek system. Polydispersities (M_w/M_n) calculated directly from the software without curve fitting

	Lab 1 Viskotek	Lab 2 SEC-MALLS	Lab 3 SEC-MALLS	Lab 4 SEC-MALLS	Lab 4 SEC-LALLS	Lab 5 SEC-MALLS
A,E	1.6	1.6	1.6	1.8	2.0	1.6
B,C	1.4	1.3	1.3	1.5	1.5-1.6	1.3
D,F	1.3	1.4	1.4	1.6	1.6	1.2

Table 7
Intrinsic viscosities (ml/g). Data obtained by SEC–viscometry are reported as weight average intrinsic viscosities, $[\eta]_w$. $[\eta]_{capillary}$, average value Lab 4; $[\eta]_{SEC\text{-}viscometry}$, average value for Labs. 1, 3 and 5

	SEC-viscometry			Capillary viscometry	$[\eta]_{capillary}/[\eta]_{SEC\text{-}viscometry}$
	Lab 1	Lab 3	Lab 5	Lab 4	
A	183	184	198	214	1.12
E	186	192	218	218	
B	471	474	506	620	1.27
C	472	481	516	619	
D	309	321	340	403	1.22
F	310	327	351	393	

almost identical results, whereas Lab 5 report slightly (approx. 10%) higher values. The values obtained by conventional capillary viscometry (Lab 4) are 15–30% higher. One source of systematic differences lies in the concentration determination. In the latter case a 10% correction for water and non-glucan impurities has been incorporated into the data. This may be an overestimate of impurity and results in a corresponding increase in the calculated intrinsic viscosity. A second difference may possibly be ascribed to shear thinning (non-Newtonian behaviour) caused by the different shear rates operating in the viscosimetric detectors in SEC, where values may be as high as 1800 s^{-1} (calculated for radius 0.02 cm, 1 ml/min) (Reed, 1996), and in the capillary viscometer, where the average value is approximately 110 s^{-1}. Shear thinning may on a qualitative basis explain the observed increase in the ratio $[\eta]_{capillary}/[\eta]_{Viscotek}$ with increasing molecular weight (Table 7). However, further experiments are needed to clarify the observed differences.

Table 8 gives the M_w values obtained by Labs 1 and 3 using the combination of a viscometric detector and a 90° light scattering detector. In this case M_w is calculated through an iterative calculation where the angular dependency of the scattered light (extrapolation to zero angle) is calculated from the relation between $[\eta]$ and M_w as described above. For Lab 3 values close to those obtained by SEC-MALLS were obtained, whereas those of Lab 1 are generally lower. Such difference may, for example, be caused by different detector response constants (calibration constants).

Lab 3 obtained molecular weights and radii of gyration by both SEC-MALLS and SEC-RALLS which allows for comparison of the two methods. As indicated by the data in Table 9., M_w and R_w values agree within about 10% or better except for one R_w value. For M_w, the largest discrepancy was for the high M_w samples B and C. For R_w, the largest discrepancy was for the low R_w samples A and E. In this case, the discrepancy may arise from the lower sensitivity in measuring R_w by its angular dependence on light scattering, as employed in the MALLS method, as compared to the higher sensitivity of measuring R_w by viscosity. It should be noted that R_w values determined by the RALLS method agree more closely with MALLS values of R_w using the first-order Debye formalism than values of R_w using the Zimm formalism (cf. data in Tables 4 and 9).

3.8. $M–R_G$ and $M–[\eta]$ relationships

The data obtained in the present study give the possibility to investigate the $M–[\eta]$ and $M–R_G$ relationships in some detail. With an estimated persistence length in the range of 4 nm (Gómez et al., 1997b), the barley β-glucans are expected to behave as simple random coils for chain (contour) lengths exceeding 30 nm ($M >$ ca. 10,000 g/mol). It may thus be expected that $R_G \propto M^{0.5}$ and $[\eta] \propto M^{0.5–0.6}$. Exponents for the $R_G–M$ relationships reported in the literature vary from 0.22 obtained for gel-forming β-glucan isolated from beer (Grimm, Krüger & Burchard,

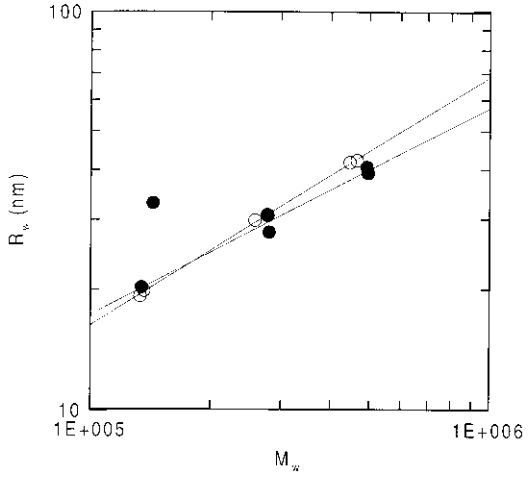

Fig. 6. Relationship between the weight average radius of gyration (R_w) and M_w obtained by SEC-MALLS (●) and SEC-RALLS/viscometry (○) (Lab 3).

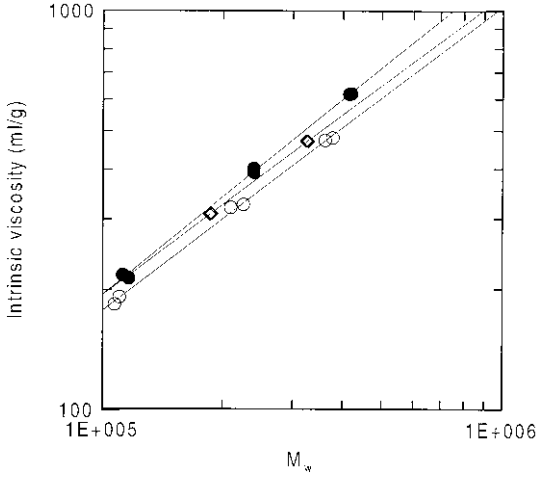

Fig. 7. Relationship between the intrinsic viscosity and M_w obtained by SEC-MALLS and capillary viscometry (●, Lab 4); SEC-RALLS/viscometry (○, Lab 1; ◇, Lab 3).

Table 8
Molecular weights (M_w) obtained by SEC–viscometry combined with RALLS (Viscotek triple detector)

	Lab 1	Lab 3	Comments
dn/dc	0.140	0.135	Initial parameters and calculations as reported by each investigator
A	106,000	133,000	
E	106,000	136,000	
B	376,000	446,000	
C	372,000	465,000	
D	213,000	258,000	
F	215,000	278,000	
A	92,000	107,000	Recalculated to dn/dc = 0.150
E	92,000	110,000	
B	328,000	361,000	
C	324,000	377,000	
D	185,000	209,000	
F	187,000	225,000	

1995) to 0.59 for oat β-glucan (Vårum et al., 1992), whereas Gómez et al. (1997a) report an exponent near 0.4. On the basis of SEC-MALLS/RALLS/viscometry these scaling relationships may be obtained either by analysing log M, log R_G and log η_{sp}/c ($\approx [\eta]$) along the SEC profile (assuming that each elution slice is monodisperse), or by comparing the appropriate averages obtained by summation over all elution slices. A major advantage using SEC is that the scaling relations may be obtained from polydisperse samples, and that interference from aggregates or particles eluting early in the chromatogram may be excluded from the analysis.

Fig. 5 gives SEC-MALLS results (log R_G vs. log M for each elution slice) from Lab 4 for three injections (with varying concentrations) of sample B. Data with $M > 8 \times 10^5$ were excluded from further analysis because of the downward curvature of the plot and because this fraction constituted less than 10% of the total sample. Data with $M < 250,000$ were also excluded because of excessive noise, particularly in the R_G data. Data between these values were fitted to a power function yielding scaling coefficients of 0.47, 0.50 and 0.50, respectively (lowest to highest concentration). These data support the general notion that barley β-glucans largely behave as random coils under the experimental conditions used here.

Fig. 6 contains double-logarithmic plots of R_w vs. M_w obtained by Lab 3 using both SEC-MALLS and SEC-RALLS (data in Table 9). In this case averages over the entire distributions were calculated. Weight average radii of gyration (R_w) were calculated (rather than R_z) to provide the correct type of average for scaling against M_w. Regression analyses provided scaling exponents of 0.52 and 0.62 from SEC-MALLS and SEC-RALLS, respectively, in reasonably good agreement with values obtained from Fig. 5.

Double-logarithmic plots of $[\eta]$ versus M_w obtained by Labs 1, 3 and 4 are given in Fig. 7. Regression analyses provided scaling exponents (a) between 0.75 and 0.81 for the Mark–Houwink relation $[\eta] \propto M_w^a$. The lower values agree well with literature values of 0.75 (Vårum, Martinsen & Smidsrød, 1991), 0.72 (Grimm et al., 1995) and 0.71 (Gómez et al., 1997b).

Table 9
Comparison of SEC-MALLS and SEC-RALLS (Lab 3)

	M_w		R_w (nm)	
	MALLS	RALLS	MALLS	RALLS
A	134,000	133,000	20.3	19.3
E	143,000	136,000	33.0	19.9
B	492,000	446,000	40.7	41.8
C	486,000	465,000	39.3	42.3
D	277,000	258,000	30.9	29.9
F	280,000	278,000	27.9	30.7

3.9. General conclusions

In general we have shown that corrections for concentration, using the same value for dn/dc and; in the case of multi-angle measurements, using the same formalism for extrapolation to zero angle gives reasonably good agreement in M_w among five laboratories in spite of using various light scattering equipment in batch mode and/or connected to an SEC column. Furthermore, samples were analysed by various dissolution procedures, sample concentrations, column sets and combinations of on-line detectors. Based on these results, we conclude that the β-glucans employed herein are satisfactory standards for use in the characterisation of these polysaccharides. Moreover, the scaling behaviour of either $[\eta]$ or R_G against molecular weight revealed that the fractions behaved as if they were random coils or somewhat expanded coils in solution over the molecular weight range measured.

Another aspect of the study was that it highlighted areas in which standardisation is necessary when one attempts to evaluate polymers as standards for molecular size and mass determinations by means of an inter-laboratory study. Key areas appear to be measurement of dn/dc and selection of an appropriate formalism by which to extrapolate multi-angle data. The latter is probably the most daunting problem. This choice often depends on the reliability and necessity of using scattering angles below 45°. Particularly in aqueous solution, accurate measurements of scattering intensities at low angles are difficult due to background scattering. What is lacking here is a quantitative statistical procedure for choosing the appropriate formalism under a given set of circumstances. More research is required to solve this problem.

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