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Comparison of Different Methods for Determination of Molecular Weight and Molecular Weight Distribution of Alginates

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ABSTRACT

The scope of the present work was to assess and compare different methods to determine molecular weight (MW) averages and molecular weight distributions (MWD) of two samples of sodium alginate of different composition.

Weight average ($\bar{M}_w$) and number average ($\bar{M}_n$) molecular weights were determined by totally integrated laser light scattering (LLS), both at wide angles (WA) and at a low angle (LA), and membrane osmometry, respectively.

The two alginates were fractionated by preparative and analytical size exclusion chromatography. Absolute molecular weight distribution

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curves were obtained from SEC, using an on-line LA-LLS detector coupled with an RI concentration detector. Relative MWD curves were determined from 'universal' calibration of the SEC data. $\bar{M}_w$, $\bar{M}_v$, *and* $\bar{M}_n$ *were calculated from the 'universal' calibration curve and* $[\eta]_j$ *values of the fractions. Good correlation between molecular weight averages obtained from the different methods was observed. Mark-Houwink-Sakurada parameters, a and K, for the two different alginate samples have been determined.*

INTRODUCTION

Alginates, which are widely used to prepare viscous solutions and to produce gels with multi-valent cations such as Ca^{2+}, can be regarded as a family of linear, binary copolymers of 1-4 linked β-D-manuronic acid (ManA or M) and its C-5 epimer α-L-guluronic acid (GulA or G). The monomers are arranged in a block-wise pattern along the polymeric chain, in which homopolymeric regions are interspaced with sequences containing both monomers and where the proportion and sequential arrangement of the uronic acids depends upon the source (Haug *et al.*, 1966). While the selectivity for binding of cations and the gel-forming properties of alginate strongly depend on the relative content and distribution of the two monomers, the viscosity is mainly related to the molecular weight (MW). Recently alginate, due to its ability to form gels with cations, has been widely used as immobilization material for living cells (Skjåk-Bræk & Martinsen, 1990). For these applications of alginate, diffusion characteristics, porosity and homogeneity as well as chemical and mechanical stability, are important properties of the gel matrix. It has previously been shown (Martinsen *et al.*, 1989) how these properties strongly depend upon chemical composition and molecular size of the alginates as well as on the gelling condition (Lim & Sun, 1980). When alginate is used for making microcapsules the molecular weight distribution (MWD) may be of importance in controlling the porosity and pore-size distribution of the capsular membrane. It is well established that the population of alginate molecules may differ in chemical composition (Haug, 1959) and that, as in all other polysaccharide preparations, the molecules will be polydisperse with respect to molecular weight. Knowledge of the molecular weight distribution is therefore of great importance for the commercial application of polysaccharides. While the average molecular weights can be obtained from light scattering ($\bar{M}_w$) and osmometry ($\bar{M}_n$), the determination of the molecular weight distribution is not so easily accomplished. Although other methods have been

recently proposed (Wedlock *et al.*, 1986; Ball *et al.*, 1988) analytical gel permeation chromatography (GPC) is the method of choice, but it is normally limited by the lack of suitable calibration substances with defined molecular size, narrow molecular weight distribution, and appropriate hydrodynamic features. Dextrans and pullulans have been used extensively as general calibration substances for polysaccharides, but, due to their highly flexible 1-6 linked glycosidic backbone, they are not representative of more rigid polysaccharides such as alginates. More sophisticated approaches are based on the so-called universal calibration procedure. This calibration relates the elution volume from a size exclusion chromatography (SEC) column of a given polymer fraction, to its hydrodynamic volume, $[\eta]\cdot MW$, where $[\eta]$ is the intrinsic viscosity and MW is the molecular mass, related by the Mark-Houwink-Sakurada (MHS) equation (Harding *et al.*, 1990):

$$\log[\eta] = \log K + a \log M \qquad (1)$$

Accurate and reliable values of a and K need to be available, obtained exactly at the same experimental conditions, both for the calibration sample and the polymer whose MWD curve is to be determined. Such conditions are not always met, and strict requirement of the use of pertinent a and K parameters is often overlooked. For instance, the different compositional and sequential structures in alginate may severely affect the stiffness of the chain, and hence the exponent a and constant K in the MHS equation in different alginate samples (Mackie *et al.*, 1980).

In the present work two types of alginate, prepared from *Macrocystis pyrifera* and *Laminaria hyperborea*, respectively, have been analysed with respect to molecular weigh and molecular weight distribution, using preparative MW fractionation, analytical gel permeation chromatography, and absolute determination of MW averages by light scattering and osmometry. The combined use of different methods will be used to estimate the MHS parameters for the two alginates with different chemical composition.

EXPERIMENTAL

Materials

An alginate with a high content of guluronic acid was prepared from stipes of *L. hyperborea*, obtained from Protan A/S, (Drammen,

Norway). This alginate sample was dissolved in water, sterilized by filtration, extracted with ethanol, dried, and milled. The other alginate sample used in this work was isolated from *M. pyrifera* and was obtained from Kelco Division of Merck (San Diego, CA, USA). The chemical composition, determined by n.m.r. spectroscopy is given in Table 1. All chemicals used were of analytical grade and bi-distilled water was used throughout.

Preparative gel permeation chromatography

The chromatography system consisted of two columns (hardware from Amicon Witten, Germany) attached in series, with an inner-diameter of 9 cm and height of 95 cm. The first column contained Sepharose Cl-6B and the second column Sepharose Cl-4B (Pharmacia Uppsala, Sweden).

Aqueous alginate samples (10 mg/ml) of 50–100 ml were applied to the column and eluted with 0·1 M Na_2SO_4 at a constant flow rate of 340 ml/h using a peristaltic pump. The elution peaks were monitored by UV-absorbance at 206 nm (ISCO model 1840 detector). The void- and total-volume of the column were determined from the elution volume of dextran and glucose respectively. Total content of carbohydrate in each fraction (volume = 114 ml) was determined by the phenol-sulphuric acid analysis (Dubois *et al.*, 1956). These fractions were pooled to give nine final fractions. The nine fractions were then concentrated on a rotary evaporator, dialysed exhaustively against distilled water and freeze-dried.

Nuclear magnetic resonance (n.m.r.)

The whole sample and some of the fractions of both alginates were analysed by ^{1}H-n.m.r. spectroscopy, using a Bruker AM-500 spectrometer (500 MHz) or, for a few cases, a Jeol FX 100 (100 MHz). The samples were prepared as described previously (Grasdalen *et al.*, 1979). From these n.m.r. spectra the monomer, diad and 'G-centred' triad frequencies and the average number of consecutive G-units were calculated as described previously (Grasdalen, 1983).

Viscosity measurements

Aqueous solutions of sodium alginate containing sodium chloride (0·1 M) were analysed at 25°C in a Micro-Ubbelohde viscometer, Schott-Geräte (Type No. 53610). Intrinsic viscosity values were determined from the

concentration dependence of the reduced specific and the logarithm of relative reduced viscosity following the Huggins and Kraemer equations.

Membrane osmometry

The osmotic pressure from solutions of sodium alginates in 0·1 M NaCl at 25°C was determined in a Knauer Membrane Osmometer (Type 01.00; membrane diameter 40 mm) fitted with a Sartorius SM 11736 cellulose acetate membrane. Number average molecular weights ($\bar{M}_n$) were determined by extrapolation to infinite dilution.

Wide angle laser light scattering (WA-LLS)

Weight average molecular weight of both alginates were obtained from WA-LLS on a self built instrument (Strand *et al.*, 1982). A Spectra-Physics argon ion laser (model 165-03) was used as the light source, and the green line ($\lambda_0 = 514{\cdot}5$ nm) was chosen as the wavelength (Strand *et al.*, 1982). The alginate solution was made twice the desired starting concentration, then diluted with an equal amount of 0·2 M NaCl. The solutions were centrifuged for 2 h at 230·000 *g* and passed through a membrane filter (0·2 μm) before measurements of the scattered light. Scattered light was measured at 11 different angles in the range 30–130° for three different concentrations.

Low angle laser light scattering (LALLS)

The system consisted of an injection part (Rheodyne 7125), a pump (Jasco BIP/1), a low angle laser light scattering detector (LDC-Chromatix CMX-100 equipped with a He-Ne laser, $\lambda_0 = 632{\cdot}8$ nm) and a concentration sensitive detector (refractive index, RI, Waters Mod. 410). The system could operate in two modes:

(i) Flow injection analysis (FIA) for $\bar{M}_w$ and A_2 determination (Mahn, 1984). A 1000 μl loop was used to reach a steady flow of polymer solution in the scattering cell; this could be repeated for different polymer concentrations to give a plot of KC/R_θ versus C. The flow rate was 0·2 ml/min.

(ii) In the GPC mode a column was used between the injector and the detectors. The RI and the LA-LLS signals for each point of the chromatogram were analysed by an IBM-XT-based software (Chromatix PC-LALLS™) (McConell, 1978) corrected for delay (100 μl, i.e. 6 s *c.*) and instrumental parameters and eventually

provided both the differential and the cumulative MW distribution curves for the given sample. The RI signal was also sent to a Digital P350 *via* an acquisition system Waters SIM, to be processed by means of the Waters 840 software for relative GPC determination. In this way comparison between absolute (i.e. LA-LLS) and relative MWD determination methods was achieved on the very same chromatographic run.

The lay-out of the experimental set-up is given in Fig. 1. The column used in the different experiments were: TSK G 5000 PW and TSK G 6000 PW (stationary phase: cross-linked hydrophillic polymer gel containing the group $-CH_2CHOHCH_2O-$, as the main constituent component; LKB Bromma, Sweden) with column guard TSK PWH; and μ-Bondagel E-125, E-500, E-1000, E-high Å (stationary phase: porous silica which has been bonded with an organic ether; Millipore-Waters, Milford, USA). A post column filter (0·45 μm, Millipore) was present in all GPC LA-LLS experiments.

Solutions, prior to all LA-LLS experiments, were filtered through a Millipore 0·22 μm filter. The loop volume in the GPC-mode was 100 μl. The polymer concentration ranged from 0.5 to 2 g/litre.

RESULTS

In all the following experiments the conditions were: temperature, 25°C; solvent, aqueous 0·1 M NaCl. The only exception will be explicitly mentioned.

Batch average properties

Chemical characteristics

The samples were analysed by ^{1}H-n.m.r. to determine the monomer composition and sequence. The results are reported in Table 1; they indicate that *L. hyperborea* alginate is not only richer in GulA, but that also the number of consecutive G-units is higher than that of the *M. pyrifera* sample.

The n.m.r. results also show that the MW fractionation procedure does not give rise to any separation based on monomer composition.

Water (moisture) content was determined by K. Fischer titrimetry: the percent (w/w) values are reported in Table 1. The values for the two alginates are very close and correspond to about two water molecules bound *per* uronic acid residue.

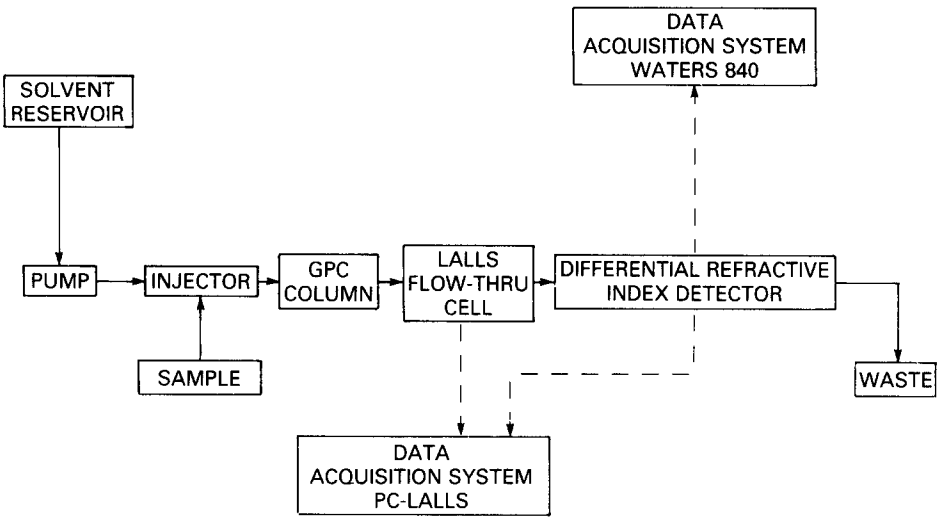

Fig. 1. Diagram of the arrangement of the analytical GPC.

Throughout the following experiments the polymer concentration values have been corrected for the moisture content.

Intrinsic viscosity

Both the Huggins plot ($[\eta]_{sp}/C = [\eta] + k' \cdot [\eta]^2 \cdot C$), and the Kraemer plot ($\ln \eta_{rel})/C = [\eta] + k'' \cdot [\eta]^2 \cdot C$) of the capillary viscosity data gave very good linear behaviour in the range of polymer concentration, C, from about 0·02 to 0·2 g/dl, for both *M. pyrifera* and *L. hyperborea* whole samples. The calculated values of intrinsic viscosity, Huggins' constant (k') and the sum of Huggins and Kraemer's constant ($k' + k''$) are reported in Table 2.

Membrane osmometry

Osmotic pressure experiments were performed using the membrane osmometer. As a routine the build up of osmotic pressure was followed over time to a constant equilibrium value to give an automatic check of possible leakage through the membrane. The reduced osmotic pressure data were recorded as a function of concentration in the range from 0·1 to 0·48 g/dl for *L. hyperborea* and 0·1 to 0·57 g/dl for *M. pyrifera*, and the derived plots were linear in the whole range. The calculated values of $\bar{M}_n$ and the second virial coefficient of *M. pyrifera* and *L. hyperborea* samples are reported in Table 3.

TABLE 1
Chemical Characteristics of Alginate Samples

Sample	F_M	F_G	F_{MM}	F_{GG}	$F_{MG,GM}$	$\bar{N}_{G>1}$	*Moisture (%, w/w ± s.d.)*
L. hyperborea							
Whole sample	0·28	0·72	0·14	0·58	0·14	~20	17·4 ± 1·4
Fraction 2	0·33	0·67	0·24	0·58	0·09		
4	0·35	0·65	0·28	0·58	0·07		
7	0·30	0·70	0·17	0·57	0·13		
M. pyrifera							
Whole sample	0·58	0·42	0·38	0·22	0·20	5·4	18·9 ± 1·2
Fraction 1 + 2	0·61	0·39	0·45	0·23	0·16		
4	0·60	0·40	0·43	0·23	0·17		
7	0·60	0·40	0·45	0·25	0·15		

F_M/F_G = Molar fraction of mannuronic/guluronic acid.
F_{MM}/F_{GG} = Frequency of ManA-ManA/GulA-GulA diads.
F_{MG} = Frequency of alternating diads.
$\bar{N}_{G>1}$ = Average number of consecutive GulA residues.

TABLE 2
Viscosity Parameters of Sodium Alginate Samples in 0·1 M Aqueous NaCl at 25°C

	L. hyperborea	*M. pyrifera*
$[\eta]_{\text{whole sample}}$/dl/g	7·11 ± 0·009	5·48 ± 0·030
k'	0·386 ± 0·007	0·421 ± 0·001
$k' + k''$	0·518 ± 0·008	0·544 ± 0·002
$[\eta]_{\text{fractions}}$/dl/g	7·74 ± n.d.	5·61 ± 0·230

Wide angle laser light scattering
Scattered light intensities at different angles and different concentrations have been collected at 25°C for *M. pyrifera* and *L. hyperborea* samples. As an example, the Zimm plot for *L. hyperborea* is given in Fig. 2(A). No anomaly was observed either in the concentration or in the angular dependence of the scattered radiation intensity for both samples. The derived values of $\bar{M}_w$, of the second virial coefficient A_2 and of the r.m.s. value of the radius of gyration are given in Table 3.

Low angle laser light scattering
Flow-injection analysis (FIA) of dilute solutions was performed in a concentration range from 0·037 to 0·18 g/litre for *M. pyrifera* and from 0·18 to 1·8 g/litre for *L. hyperborea*, measuring the scattered laser

TABLE 3
Light Scattering and Osmometry Parameters of Sodium Alginate Samples in 0·1 M Aqueous NaCl at 25°C

Sample	*Property*	*WA-LLS*	*LA-LLS (FIA)*	*Property*	*Osmometry*
Laminaria hyperborea	$\bar{M}_w$/kilo Dalton	217 ± 10	214 ± 10	$\bar{M}_n$/kilo Dalton	$108{\cdot}5 \pm 10$
	A_2/ml mole g^{-2}	$7{\cdot}0 \times 10^{-3}$	$4{\cdot}7 \times 10^{-3}$	B/ml mole g^{-2}	$7{\cdot}1 \times 10^{-3}$
	$\langle R_G^2 \rangle^{1/2}$/nm	59	—		
	dn/dc	0·150	—		
Macrocystis pyrifera	$\bar{M}_w$/kilo Dalton	210 ± 10	224 ± 3	$\bar{M}_n$/kilo Dalton	84 ± 10
	A_2/ml mole g^{-2}	$5{\cdot}2 \times 10^{-3}$	$1{\cdot}36 \times 10^{-3}$	B/ml mole g^{-2}	$8{\cdot}6 \times 10^{-3}$
	$\langle R_G^2 \rangle^{1/2}$/nm	56	—		
	dn/dc	0·150	0·152		

WA-LLS = Wide angle laser light scattering.
LA-LLS = Low angle laser light scattering.

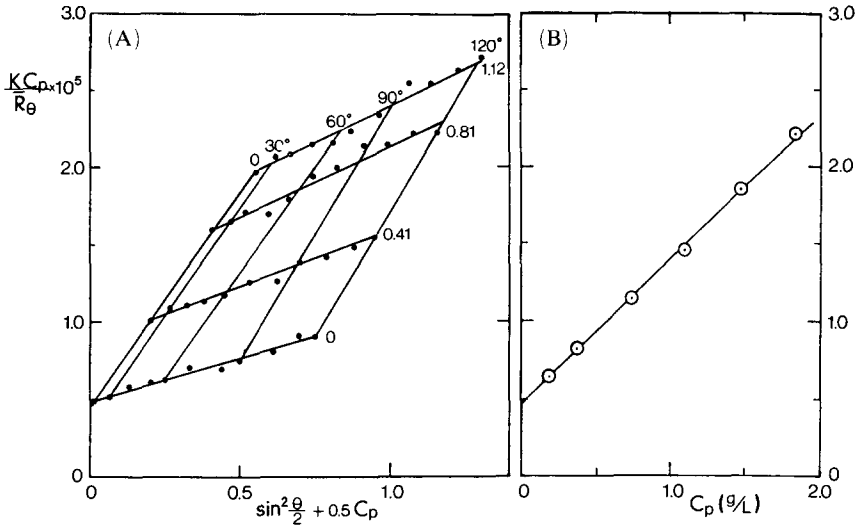

Fig. 2. Laser light scattering results for alginate from *L. hyperborea*. (A) Zimm plot from the WA-LLS experiments; (B) $K \cdot C_p/R_\theta$ versus $K \cdot C_p$ from the FIA/LA-LLS experiments.

radiation intensity at low angle. The experimental results for *L. hyperborea* are reported in Fig. 2(B). The very low angle of observation and the linear concentration dependence of scattering allow the calculation of $\bar{M}_w$ and A_2 from the plots of *L. hyperborea* and *M. pyrifera* which are reported in Table 3.

Differential refractive index increment

Values of *dn/dc* for *M. pyrifera* and *L. hyperborea* were both determined at the wavelength used in the WA-LLS and in the LA-LLS experiments. The values are in all cases close to 0·150 as already reported by other authors (Mackie *et al.*, 1980; Strand *et al.*, 1982) they are listed in Table 3.

GPC: Absolute MWD determination in the analytical mode

Using a high-performance GPC system in connection with a LA-LLS and a RI detector and by manipulation of data with an on-line computer as described in the Experimental, the MWD curve for both the alginate samples was obtained. Triplicate experiments were carried out for each separation condition (i.e. column and/or solvent). For the *L. hyperborea* alginate three different systems were used: (i) a single 60 cm TSK G5000

PW column; (ii) a series of one TSK G5000 PW and one TSK G6000 PW columns; (iii) a series of four modified silica columns, μBondagel type, as described in the Experimental. In all cases 0·1 M NaCl was used as a solvent. The chromatographic runs were performed as described in the Experimental and no anomaly was noticed in any experiment. A typical MWD curve for *L. hyperborea* alginate is reported in Fig. 3(A), whereas the significant distribution parameters (i.e. $\bar{M}_w$ and $\bar{M}_n$) for all the different experiments are reported in Table 4(a). The reproducibility of the results is very good for each set of experimental conditions; moreover, there is no statistically significant difference between the $\bar{M}_w$ and the $\bar{M}_n$ values obtained using either a single PW column or a series of two of them, nor between these and the results obtained using a μBondagel column set. Similar conclusions can be drawn from inspections of the results for *M. pyrifera* alginate (Fig. 3(B) and Table 4(B)), where either a single PW column or a set of silica-based columns were used. In the latter case, a ten-fold lower concentration of $(CH_3)_4N^+Cl^-$ (TMA) (i.e. 0·01 M) was used, but without noticing any

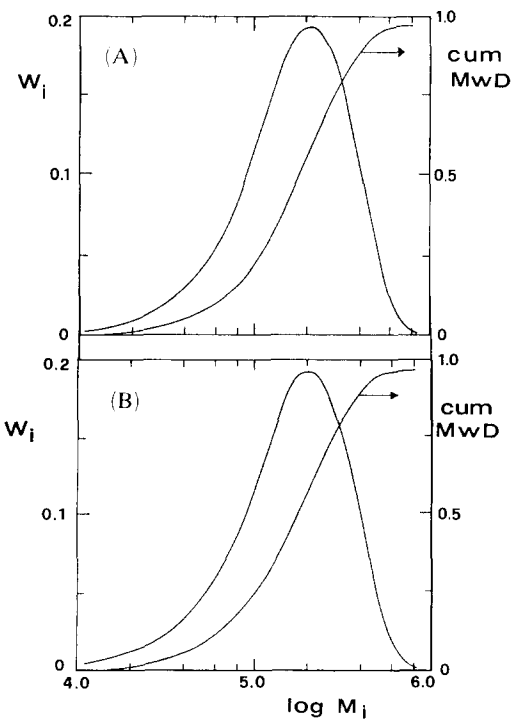

Fig. 3. Differential (w_i) and cumulative (cum MwD) molecular weight distribution curves from GPC/LA-LLS experiments. (A) Alginate from *L. hyperborea*; (B) alginate from *M. pyrifera*.

TABLE 4
GPC — LA-LLS Parameters of Sodium Alginate at 25°C

Parameter	*(a) Laminaria hyperborea*										
	Run 1	*Run 2*	*Run 3*	*Average 1–3*	*Run 4*	*Run 5*	*Run 6*	*Average 4–6*	*Average 1–6*	*Run 7*	*Average 1–7*
$\bar{M}_w$/kD	206·3	203·0	195·4	201·6 ± 5·6	201·4	200·9	197·3	199·9 ± 2·2	200·7 ± 3·9	201·5	200·8 ± 3·6
$\bar{M}_n$/kD	126·8	100·6	106·1	111·7 ± 13·8	102·1	113·2	112·0	109·1 ± 6·1	110·1 ± 9·6	111·2	110·3 ± 8·8

Run 1–3: 1 TSK G5000 PW column, solvent 0·1 M NaCl (aq).
Run 4–6: 1 TSK G5000 PW column + 1 TSK G6000 PW column, solvent 0·1 M NaCl (aq).
Run 7 : 4 μBondagel columns, solvent 0·1 M NaCl (aq) (see text).

Parameter	*(b) Macrocystis pyrifera*					
	Run 1	*Run 2*	*Run 3*	*Average 1–3*	*Run 4*	*Average 1–4*
$\bar{M}_w$/kD	202·6	199·3	208·3	203·4 ± 4·6	211	205·3 ± 5·3
$\bar{M}_n$/kD	103·5	96·6	100·3	100·1 ± 3·5	75	93·9 ± 12·9

Run 1–3: 1 TSK G5000 PW column, solvent 0·1 M NaCl (aq).
Run 4 : 4 μBondagel columns, solvent 0·01 M TMACl (aq) (see text).

significant difference of the values of the molecular weight parameters from those obtained in the former system.

Relative MWD determination by GPC

Fractionation procedure

One sample of *L. hyperborea* and three samples of *M. pyrifera* were fractionated by the set of columns described in the Experimental. All the four samples were divided into nine fractions, except run 2 in the case of *M. pyrifera*, where fractions 1 and 2 were pooled. The elution pattern/profile of *L. hyperborea* and *M. pyrifera* (run 1) are given in Fig. 4(A) and (B). In this figure the elution volume is also given for glucose and blue dextran, showing satisfactory separation over the entire range of molecular weights. The two samples were divided into nine fractions as illustrated in Fig. 4. Intrinsic viscosity $[\eta]_j$ and polysaccharide content w_j

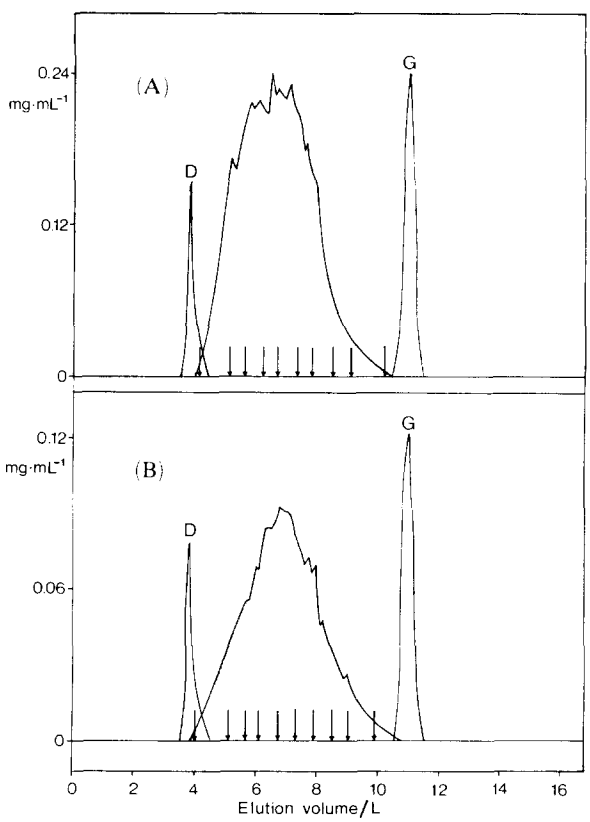

Fig. 4. Elution profile of preparative GPC of alginate from *L. hyperborea* (A) and *M. pyrifera* (B). Arrows indicate the fraction limits; D and G indicate the elution volume of blue dextran and glucose, respectively.

of each fraction, j, were determined, and are reported in Table 5. The intrinsic viscosity of the batch samples, $[\eta]_{\text{fractions}}$, was recalculated from the $[\eta]_i$ values of fractions according to the weight-average:

$$[\eta]_{\text{fractions}} = \frac{\Sigma_i w_i \cdot [\eta]_i}{\Sigma_i w_i} \quad (1)$$

The $[\eta]_{\text{fractions}}$ values for the two samples are reported in the last row of Table 6. The percentage recovery of fractionated material was 71 and 77% for *L. hyperborea* and *M. pyrifera*, respectively.

Determination of the MHS equation parameters

Due to the insufficient amount of fractionated polymer to perform WA-LLS experiments on each fraction, a different procedure for obtaining K and a values in the MHS equation for alginates was resorted to. The new method strongly relies upon the absolute MWD curve obtained in the above section on GPC and it combines the $w_j(M_j)$ data with the $[\eta]_j$ values described in the section on fractionation procedure according to the following scheme;

(i) For each run and for both alginates the $w_j(M_j)$ curve is calculated according to the fractionation procedure.

TABLE 5
Characteristics of Fractions of Alginates from Preparative GPC

Fraction	*L. hyerborea*		*M. pyrifera*					
			Run 1		*Run 2*		*Run 3*	
	w_j	$[\eta]_j$	w_j	$[\eta]_j$	w_j	$[\eta]_j$	w_j	$[\eta]_j$
$j=1$	67·5	10·2	23·0	11·0	—	—	57·0	10·3
2	100·5	12·0	27·3	10·0	99·5[a]	9·6[a]	72·6	8·4
3	129·0	9·4	29·2	8·6	59·6	7·9	32·5	7·8
4	122·7	8·3	48·9	6·8	83·8	7·7	70·0	7·0
5	126·4	6·6	51·4	5·4	101·1	6·0	50·0	4·4
6	102·9	4·7	42·1	3·4	100·2	5·1	57·9	4·1
7	58·4	2·3	28·2	1·6	86·3	3·1	67·3	2·2
8	27·1	0·9	16·9	1·4	46·0	1·3	69·4	1·3
9	17·3	0·8	15·8	1·2	46·0	1·7	20·0	0·8

[a] Collective fraction numbers 1 + 2.
w_j = Weight of fraction, mg.
$[\eta]_j$ = Intrinsic viscosity of fraction, dl g^{-1}.

TABLE 6
Macromolecular Parameters of Sodium Alginate Samples in 0·1 M Aqueous NaCl at 25°C. Values Obtained from Narrow Fractions

			M. pyrifera		*L. hyperborea*
K			$7{\cdot}3\times10^{-5}$		$6{\cdot}9\times10^{-6}$
a			0·92		1·13
$\bar{M}_v$/kD (unfract. sample)			198·1		209·5
	Run 1	*Run 2*	*Run 3*	*Average 1–3*	
$\frac{\Sigma_j w_j[\eta]_j}{\Sigma_j w_j}$ / dl g^{-1}	5·72	5·77	5·34	5·61 ± 0·23	7·74
$\bar{M}_v$/kD	208·8	211	193·7	204·5 ± 9·4	218·9
$\bar{M}_w$/kD	212	213·2	197·1	207·4 ± 9·0	216·2
$\bar{M}_n$/kD	120·2	138·4	102·3	120·3 ± 18·1	150·0

(ii) The average value of the intrinsic viscosity of the alginate sample under study, $[\eta]_{calc.}$, is calculated according to the equation

$$[\eta]_{calc.}=\frac{\Sigma_i w_i \cdot K \cdot M_i^a}{\Sigma_i w_i} \tag{2}$$

for a set of a and K values as described in (iii); i refers to the infinitesimal ith fraction of the continuous MWD curve, and w_i and M_i are its normalized weight fraction and molecular mass, respectively.

(iii) For each case, the exponent a is parametrically given an arbitrary value and then by recursive calculation, the value of K, called $K_{[\eta]}$, is numerically calculated to satisfy the following bound:

$$[\eta]_{calc.}=[\eta]_{whole} \tag{3}$$

where $[\eta]_{whole}$ is the experimentally determined value of the intrinsic viscosity of the unfractionated sample (see Table 2). The range of a values which was considered is from 0·8 to 1·2, following the fact that in 0·1 M NaCl the values of a reported in the literature do not greatly differ from 1 (Mackie *et al.*, 1980; Smidsrød & Haug, 1968).

A curve expressing the functional correlation between $K_{[\eta]}$ and $a(K_{[\eta]}=f(a))$ under the bound given by eqn (3) is then obtained for each case considered: as an example, the insert of Fig. 5 (Fig. 4(A)) shows

such a correlation (conveniently expressed as log $K_{[\eta]}$ versus a) for the *L. hyperborea* sample.

(iv) To find the particular K and a values of the MHS equation for our sample, the $\bar{M}_{w,calc.}$ from the fractionated sample data has been calculated for a number of couples of $K_{[\eta]}$ and a obtained in step (iii) by means of the equation

$$\bar{M}_{w,calc.} = \frac{\Sigma_j w_j \cdot ([\eta]_j / K_{[\eta]}(a))^{1/a}}{\Sigma_j w_j} \tag{4}$$

The results of the calculation for the sample case of *L. hyperborea* alginate are reported in Fig. 5. It can be easily seen that the M_w(calc.)

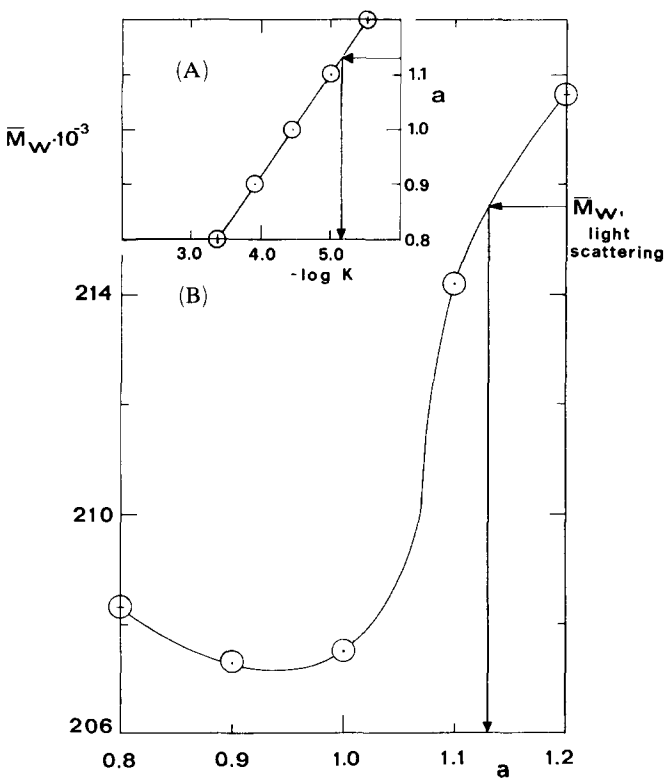

Fig. 5. Alginate from *L. hyperborea*. (A) (incert). Linear dependence of a on log K to satisfy the boundary condition of eqn (3) (see text). Arrows indicate the final a^* and K^* values of the M.H.S. equation. (B) Calculated values of M_w using eqn (4) for a range of a values from 0·8 to 1·2 (see text). Arrows indicate the choice of the a value determined by the experimental M_w.

versus (a) curve is continuous, and that a unique value of a, a^*, corresponding to the M_w value calculated from the WA-LLS experiment can be easily determined by interpolation. Then, by further interpolation on the $\log K_{[\eta]}(a)$ curve of Fig. 5(A), also the K parameters of the MHS equation, K^*, is determined.

For *L. hyperborea* alginate K^* and a^* are $6{\cdot}9 \times 10^{-6}$ and 1·13; by the same procedure, the calculated values for the *M. pyrifera* sample are $7{\cdot}3 \times 10^{-5}$ and 0·92, respectively.

Molecular weight averages by MHS equation

By use of the K^* and a^* values determined in the previous section, the following molecular weight averages have been calculated for both alginate samples:

(i) $\bar{M}_v$ average values from intrinsic viscosity data on the unfractionated sample, $[\eta]_{whole}$.

(ii) $\bar{M}_w$, $\bar{M}_v$ and $\bar{M}_n$ average values from w_j and $[\eta]_j$ data on fractionated samples according to the equations:

$$\bar{M}_w = \frac{\Sigma_j w_j \left(\frac{[\eta]_j}{K}\right)^{1/a^*}}{\Sigma_j w_j} \tag{5}$$

$$\bar{M}_v = \left[\frac{\Sigma_j w_j \left(\frac{[\eta]_j}{K}\right)}{\Sigma_j w_j}\right]^{1/a^*} \tag{6}$$

$$\bar{M}_n = \frac{\Sigma_j w_j}{\left[\Sigma_j \frac{w_j}{([\eta]_j/K)^{1/a}}\right]} \tag{7}$$

The results of steps (i) to (ii) are reported in Table 6.

Molecular weight averages by GPC-universal calibration method

The determination of the MHS equation parameters has then enabled us to build a calibration curve for the determination of the MWD curve by

analysis of the GPC data. The procedure which was followed is given here:

(i) The concentration-sensitive RI detector data collected in a GPC experiment for the *L. hyperborea* sample were processed by the Waters 840 software. The computer program generates a calibration curve, using as the chromatographic 'Broad Standard' the particular array of GPC experimental data, together with the $\bar{M}_w$, $\bar{M}_n$, K^* and a^* values separately determined and provided by the operator. The result is a 'Universal Calibration' curve (called UC1) in which a plot of the logarithm of the product $[\eta]_i \cdot M_i$ is fairly well linear with V_i. $[\eta]_i$, M_i and V_i are the intrinsic viscosity, the molecular weight and the elution volume of the ith fraction, respectively.

(ii) To test the validity of the calibration, three samples of *L. hyperborea* were rerun. From V_i values the product $[\eta]_j \cdot M_j$ is obtained.

TABLE 7
Macromolecular Parameters Calculated by Relative GPC 'Broad Standard — Universal Calibration' Method

Parameters	*Calibration sample*	*'Recalculated'[a] samples*	*'Unknown'[b] samples*
		(a)	
	L. hyperborea	*L. hyperborea*	*M. pyrifera*
K	$6{\cdot}9 \times 10^{-6}$	$6{\cdot}9 \times 10^{-6}$	$7{\cdot}3 \times 10^{-5}$
a	1·13	1·13	0·92
$\bar{M}_w$/kD	217·4 (WA-LLS)	213·2 ± 0·6	187·6 ± 1·2
$\bar{M}_n$/kD	108·5 (osmom.)	107·3 ± 1·3	74·2 ± 1·1
$[\eta]_{whole}$	7·11 (viscom.)	7·50 ± 0·03	5·05 ± 0·03
$\bar{M}_v$/kD	209·5	219·6 ± 0·6	182·3 ± 1·2
		(b)	
	M. pyrifera	*M. pyrifera*	*L. hyperborea*
K	$7{\cdot}3 \times 10^{-5}$	$7{\cdot}3 \times 10^{-5}$	$6{\cdot}9 \times 10^{-6}$
a	0·92	0·92	1·13
$\bar{M}_w$/kD	210 (WA-LLS)	202·8 ± 1·3	228·0 ± 0·7
$\bar{M}_n$/kD	84·2 (osmom.)	84·1 ± 1·3	119·2 ± 1·6
$[\eta]_{whole}$	5·45 (viscom.)	5·43 ± 0·03	8·07 ± 0·02
$\bar{M}_v$/kD	198·1	197·3 ± 1·3	234·5 ± 0·6

[a]Values of parameters of three different samples of the same alginate used for calibration, independently determined using that 'Universal Calibration' curve.
[b]Values of parameters of three different samples of the alginate other than the one used for calibration.

From the known MHS parameters, the product can be substituted by:

$$[\eta]_j \cdot M_j = K \cdot (M_i)^a \cdot M_i = K \cdot (M_i)^{a+1}$$

following the 'Universal Calibration' procedure and their molecular weight averages calculated by use of eqns (5)–(7). The results, reported as averages ± s.d. in Table 7, are in excellent agreement with the separately determined values of $\bar{M}_w$ (from WA-LLS), $[\eta]_{whole}$ (from capillary viscometry), $\bar{M}_v$ (from capillary viscometry and MHS parameters) and $\bar{M}_n$ (from osmometry).

(iii) Three samples of *M. pyrifera* underwent GPC analysis: the raw experimental data and the appropriate values of the MHS equation parameters of the *M. pyrifera* samples were then processed using the 'Universal Calibration' UC1 curve determined in step (i), using the MHS parameters obtained on p. 000. The average results (± s.d.) are reported in Table 7(a) as well; the reproducibility is very good, and also the accuracy for the $\bar{M}_w$, $\bar{M}_v$, $\bar{M}_n$ and $[\eta]$ estimates is well within the currently accepted standard in GPC.

(iv) The procedure outlined hereabove in step (i) to (iii) was repeated in the opposite way, now using the *M. pyrifera* alginate as the chromatographic 'Broad Standard' and then obtaining a second 'Universal Calibration' curve (UC2). Again, three samples of *M. pyrifera* were rerun and analysed, as well as three samples of *L. hyperborea* treated as 'unknowns'. The average values (± s.d.) of $\bar{M}_w$, $\bar{M}_v$, $\bar{M}_n$ and $[\eta]$ for all these samples are given in Table 7(b); once more the agreement with the separately determined values is rather good.

Figure 6 collectively reports the calibration curve for both the 'universal calibration' curves UC1 and UC2 obtained from analytical GPC and the preparative fractionation columns (points with different symbols). Linear regression of the *L. hyperborea* data points and separate regression analysis for each of the *M. pyrifera* runs, have not yielded any difference from one single regression analysis to all the points which is reported as a solid line in Fig. 6(B).

DISCUSSION

Table 8 summarizes all the results about the different molecular weight averages, for both the *L. hyperborea* and the *M. pyrifera* alginates obtained by the different methods.

It is useful to shortly comment on the physico-chemical features of the two alginate samples. The WA-LLS and LA-LLS results show that they

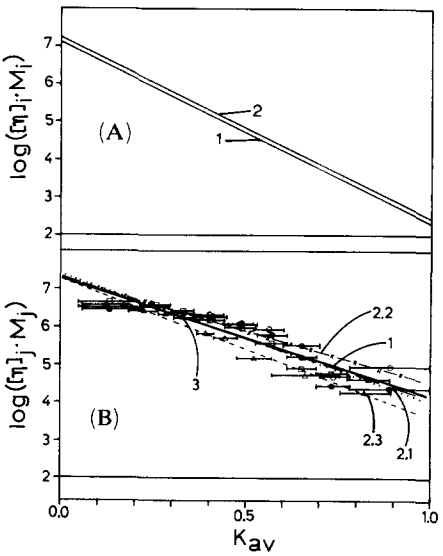

Fig. 6. 'Universal calibration' plot of the GPC results from the analytical (A) and the preparative (B) modes. (A) (1) *L. hyperborea* and (2) *M. pyrifera*. (B) (1) *L. hyperborea*; —— and (□); (2.1) *M. pyrifera*: ---- and (△); (2.2) *M. pyrifera*: —·—· and (○); and (2.3) *M. pyrifera*: ..... and (●).

do not differ significantly in molecular weight thus justifying their choice for the comparative study.

The higher G-block content of *L. hyperborea* seems to render it stiffer, as shown by a slightly larger value of the radius of gyration and a higher a^* exponent in the MHS equation (1·13 versus 0·92). Mackie (1980) had already reported an a value larger than unity ($a = 1{\cdot}11$) for a G-rich alginate, whereas a was as low as 0·91 for an M-rich sample, in all cases at 25°C in 0·1 M NaCl.

Comparing the results from different techniques, one should firstly underline the very good agreement between the $\bar{M}_w$ values obtained for both samples from laser light scattering in the wide angle and in the low angle modes. In particular, for the *L. hyperborea* sample the agreement is within 1·5% of the average value, whereas for the *M. pyrifera* alginate the M_w from LA-LLS is less than 7% higher, indicating that in this case only a very slight presence of higher molecular weight material may not be ruled out. This seems to be confirmed by the significantly lower value of the second virial coefficient (Dingsøyr & Smidsrød, 1977) for *M. pyrifera* alginate from LA-LLS experiments.

TABLE 8
Comparison of Molecular Weight Averages from Different Methods for Samples of Sodium Alginate

Parameter	*Method*							
	WA-LLS	*LA-LLS*	*Osmom.*	*GPC-LALLS*	*M.H.S. (unfractionated sample)*	*M.H.S. (fractions)*	*GPC Broad Universal*[a]	*Standard Calibration*[b]
				(a) *L. hyperborea*				
							UC2	UC1
$\bar{M}_w$/kD	217 ± 10	214 ± 10	—	201 ± 4	—	216	228 ± 1	213 ± 1
$\bar{M}_v$/kD	—	—	—	—	210	219	235 ± 1	220 ± 1
$\bar{M}_n$/kD	—	—	109 ± 10	110 ± 9	—	150	119 ± 2	107 ± 1
				(b) *M. pyrifera*				
							UC1	UC2
$\bar{M}_w$/kD	210 ± 10	224 ± 3	—	205 ± 5	—	207 ± 9	188 ± 1	203 ± 1
$\bar{M}_v$/kD	—	—	—	—	198	205 ± 9	182 ± 1	197 ± 1
$\bar{M}_n$/kD	—	—	84 ± 10	94 ± 13	—	120 ± 18	74 ± 1	84 ± 1

[a]Values determined by 'Universal Calibration' curve obtained by the other alginate sample.
[b]Values determined by 'Universal Calibration' curve obtained by the same alginate sample.

The capillary viscosity data reported in Table 2 are in line with what is said above. Both the analytical columns used in this work, made of two TSK columns or by four μBondagel columns, seems to give satisfactory separation as shown by the calculated averages and the MWD profiles. Similarly, the preparative SEC system seems to give reliable separation efficiency in good agreement with the analytical ones. The higher $\bar{M}_n$ $[\eta]_{fraction}$, values obtained from preparative SEC results with respect to all other estimates can be reasonably attributed to a selective loss of low molecular weight material.

A warning seems necessary to be made on the use of so-called 'universal calibration' methods which have to be based upon reliable MHS a and K values. As shown also in this paper, two alginates of similar M_w but with different chemical composition have different MHS parameters. MWD curves of alginates using relative GPC and 'universal calibration' may give erroneous results if proper choice of a and K values is not made in relation to composition.

This work has reported a very detailed comparison of different methods to determine the molecular weight averages and distributions of carefully selected alginate samples. Far from being limited to a merely analytical scope, it will provide safer tools for further studies on the correlation between solution and gelling properties of alginates and their molecular weight distribution.

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