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Molecular characterisation of κ- and λ-carrageenan by gel permeation chromatography, light scattering, sedimentation analysis and osmometry

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Abstract

Gel permeation chromatography, in conjunction with a double detection system involving a low angle laser light scattering apparatus (LALLS) and a refractive index monitoring device (RI), has been used to obtain both the molecular weight and the molecular weight distribution of sodium salts of κ-carrageenan and λ-carrageenan in saline solutions. The results, M_w and M_n, are in excellent agreement with independent determinations of molar mass based on static light scattering experiments, sedimentation–diffusion analysis and osmometry. The relevance of the data is discussed with respect to current problems in carrageenan research.

Keywords: Carrageenans; Molecular weight determination; low-angle laser light scattering; Gel permeation; Sedimentation–diffusion analysis; Osmometry

1. Introduction

Carrageenans are water soluble polymers extracted from red algae. They are mainly used in the food industry as thickeners and gelling agents. On the basis of their chemical structure, several groups can be discerned: κ-carrageenan, λ-carrageenan and ι-carrageenan. They differ in chemical composition, which affects the linear charge density of the polysaccharides. κ-Carrageenan has only one negative charge per disaccharide unit, ι-carrageenan two and λ-carrageenan bears on the average 2.7 charges per disaccharide unit. The present work is an extension of previous light scattering studies on κ-carrageenan and λ-carrageenan [1,2]. The case of ι-carrageenan has been deliberately omitted in these works as our own earlier efforts as well as those of others failed to secure reproducible measurements [3]. The current status of our present experience with ι-carrageenan solutions is the subject of a separate research project [4]. The ideal chemical structure of κ-, ι- and λ-carrageenan is shown in figure 1. Their molecular weights depend upon the season and the extraction procedure [5]. The precise mechanism of the salt-induced gelation continues to be a matter of debate and controversy [6,7]. Although there is general agreement on a two-step mechanism of gelation, i.e. a coil-to-helix transi-

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tion followed by a rapid aggregation of the helices, there appears to be neither extensive studies of the polyelectrolyte behaviour of the carrageenans, nor of the so-called coil-to-helix transition apart from indirect information obtained from optical rotation studies.

Meanwhile it has been shown by Smidsrød and Grasdalen [8] as well as by our own work that, in the absence of gelation, the coil–helix transition is essentially an intramolecular process, a finding which contrasts with the domain model involving aggregated double helices as the physical crosslinks of the gels [9]. However, a study by static and dynamic light scattering of the solution behaviour of κ- and λ-carrageenan failed to detect specific differences between the solution behaviour of both species, the experimental findings, however, being difficult to interpret by the complexities of the charge effects arising from the polyelectrolyte nature of the polysaccharides, specific interactions with ions (I^-) and polydispersity effects which obscure a straightforward interpretation of the observed salt-induced changes in the radius of gyration and the hydrodynamic radius [1,2]. Because physical properties of the solution (e.g. the viscosity) and the gel (e.g. the modulus of rigidity [9] and the stress at fracture [10]) are determined by the molecular weight and the molecular weight distribution, knowledge of these quantities is important in many applications. Consequently, there have been a number of efforts in the past to obtain this information from physicochemical data [10,11] as well as from size exclusion chromatography.

Molecular weight characterisation of water soluble polyelectrolytes is mostly performed by such conventional methods as light scattering, sedimentation analysis and osmometry. These methods are rather time consuming on the experimental level and, with respect to a correct evaluation of the data, charge effects have to be accounted for.

Typical experimental problems inherent to the field of carrageenan research have been reported in a previous paper [1]. 'Classical' gel filtration turned out to be very useful for the characterisation of carrageenans. Oligomers of κ-carrageenan were fractionated using modified silica packing material [13]. Intermediate and higher molecular weight carrageenans were separated on sepharose [15]. Porous glass beads as well proved to be practical for chromatography of carrageenans [9]. A serious disadvantage is, that unless a continuous molecular weight detector is combined with the fractionation columns, no real molecular weight distribution can be obtained.

Gel permeation chromatography (GPC) of synthetic polymers in organic solvents has been used since long as a quick means to determine molecular weight distributions. On the contrary, high speed GPC of aqueous solutions is much less straightforward. Problems often referred to are the limited chemical and physical stability of the gels and adsorption of the polymer on the column packing. The first problem is overcome by the development of some high performance columns [16]; adsorption can be suppressed by adding low molar mass solutes to the solvent [17].

For polyelectrolytes, interactions between charges on the polymer and the gel constitute new difficulties. Using water as the solvent, electrostatic repulsion between charges on the gel matrix and the polymer prevent the polyelectrolyte from intruding the pores of the gel [18,19]. This is referred to as ion exclusion. By using a salt solution as the eluent, size exclusion becomes the main origin for fractionation. However, under these conditions, a peak following the chromatogram is observed due to the fact that some pores in the gel are only penetrable for the salt, but not for the polyelectrolyte, which gives rise to a Donnan distribution of the small ions [18,19]. This is referred to as ion inclusion. When the salt concentration in the eluent is relatively high ($c_s > 0.05$ M), a salt peak appears at the total volume (V_t) of the column [20]. Other experimental difficulties when working with newly developed columns for aqueous GPC have been reported elsewhere [21]. Specific problems [17], proper to polysaccharide research, are the formation of aggregates, the high solution viscosity and the elution of a fraction of a high molecular weight polysaccharide near the void volume of the column. Some of these limitations can be recognised when using a continuous molecular weight detector [22]: the molecular weight distribution can be determined on an absolute scale and becomes independent upon artifacts due to polymer–gel interactions and poor column resolution.

In the present paper, the molecular weight distribution of κ- and λ-carrageenan is determined by coupling a GPC to a low angle laser light scattering apparatus (LALLS) and a refractive index monitor (RI). The molecular weight averages obtained are compared with independent data resulting from osmotic pressure, light scattering and sedimentation–diffusion experiments. Some of our results will be compared with another independent GPC-LALLS study of carrageenans [23].

2. Materials and methods

2.1 Sample characterisation

The κ- and λ-carrageenan samples were commercial products obtained from Sigma (lot 92F-0406 and 122F-0520 respectively). The polymer samples were ion-exchanged against Na^+ and treated as described before [1]. Concentrations were determined from C-analysis assuming the ideal structure from Fig. 1.

2.1.1 Sulfatation

The sulfate content was determined from titration of the acid form of the carrageenan samples. A 0.01 *M* NaOH solution, calibrated against oxalic acid, was used for titrations with a Radiometer RTS 822 recording titration system.

2.1.2 Intrinsic viscosity

Intrinsic viscosities were measured at 25 °C using an Ubbelohde viscometer. The shear-rate dependence was accepted to be negligible.

2.2 Molar mass determination

2.2.1 Refractometry

Refractive index increments of the carrageenans in 0.05 *M* and 0.1 *M* NaCl were determined with a Chromatix KMX-16 differential refractometer at a wavelength of 633 nm as outlined by Mandel et al. [24]. Stock solutions of κ- and λ-carrageenan were dialysed against the appropriate salt solutions and succesive dilutions were made using the dialysate.

2.2.2 Light scattering

The excess scattered intensity of the solutions of different polymer concentration was measured with a Chromatix KMX-6 apparatus at a scattering angle of less than 6° using a 4 mW He–Ne laser. The cleaning procedure of the LALLS flow cell was the same as reported before [19]. Polymer solutions for light scattering were filtered through a 0.22 μm MF millipore filter. Alternatively, angular dependent light scattering experiments have been obtained with an AMTEC MM 1000 photometer in the angular range from 25° up to 140°. The details of these experiments, data handling and the results have been published elsewhere [1,19].

2.2.3 Osmometry

Osmotic pressure measurements of carrageenan solutions were carried out on a Knauer Membrane osmometer at 25 °C. Membranes were Sartorius Ultrafilter type SM 11736 and Schleicher & Schuell Standardfilter RC_51 for the Knauer and the Wescan osmometer, respectively.

The osmotic pressure π of a polyelectrolyte of concentration c in a salt solution is related to the number average molar mass M_n of the solute through eq. (1). Here R is the universal gas constant, T the thermodynamic temperature and A_2 and A_3 are respectively the second and third virial coefficient. M_n and A_2 are obtained from a conventional linear plot of π/c vs. c. Such plots deviate at high concentration from linearity due to the increasing influence of the third virial coefficient A_3.

$$\pi/c = RT\left(1/M_n + A_2 c + A_3 c^2 + \dots\right) \qquad (1)$$

However, by using a square root plot, i.e. $(\pi/c)^{1/2}$ is plotted versus c, the linearity is extended over a broader range of concentrations [25]. This is very useful as the osmotic pressure of high molecular weight polymers at low concentration becomes very small, a fact which may lead to a substantial experimental error. By including measurements at a somewhat higher concentration, precision increases without recurring to a quadratic plot. The procedure involves the neglect of virial coefficients of order higher than three and the approximation of A_3 by 0.25 A_2^2 M [26].

2.2.4 Hydrodynamical measurements

Experimental conditions and data treatment of dynamic light scattering, ultracentrifugation and density measurements have been reported elsewhere [2].

2.2.5 Gel permeation chromatography

GPC measurements were carried out on a Waters model 150 C HPLC. Three columns from Toya Soda of different fractionation range i.e. G 6000 PW, G 5000 PW and G 4000 PW were used together with a precolumn GPWPH. These column gels mainly consist of $[CH_2\text{–}CHOH\text{—}CH_2\text{–}O]_n$ groups [16], although some remaining carboxylate groups [27] are present, necessitating the use of a 0.1 *M* $NaNO_3$ solution to suppress electrostatic effects. The columns have a length of 60 cm and an inner diameter of 0.75 cm. The temperature of the columns and the detectors was kept at 25 °C. The injected volume was 0.5 ml, while the concentration of the polymer amounted 0.8 and 1.5 g/l for κ- and λ-carrageenan, respectively. The flow rate was 1 ml/min. As detectors, a refractive index detector (RI) and a low angle laser light scattering (LALLS) detector, which was the same as for static measurements, were used. A 0.22 μm MF filter (Millipore) was placed between the columns and the LALLS cell.

2.3 Gel permeation chromatography combined with low angle laser light scattering

In an ideal gel permeation chromatograph with infinite resolution, the polymer molecules are separated according to their size. By monitoring continuously at the end of the column the concentration c_i by a refractometer and the excess scattered light ΔR_i by a low angle laser light scattering apparatus, the molecular weight M_i at each elution volume is obtained through the use of eq. (2) as given in ref. [19]. Different average molecular weight averages are subsequently calculated in the usual manner:

$$M_n = \left(\sum c_i\right) / \left(\sum c_i/M_i\right) \tag{4}$$

$$M_w = \left(\sum c_i M_i\right) / \sum c_i \tag{5}$$

$$M_z = \left(\sum c_i M_i^2\right) / \left(\sum c_i M_i\right) \tag{6}$$

3. Results

Differences in the charge density of κ- and λ-carrageenan are apparent from a more complex titration behaviour of λ-carrageenan as compared to κ-carrageenan, indicating differences in the p*K* values of the various sulfates on the λ-carrageenan backbone. In Table 1 the total sulfate content of the κ- and λ-carrageenan samples is compared with the values expected for the ideal structures indicated in Fig. 1. The accuracy of all experimental data is indicated for 95% confidence intervals [28]. For κ-carrageenan, the experimental content is then too high, as compared to the ideal struc-

Table 1

Characteristics of κ- and λ-carrageenan

	Sulfate content (eq. H^+/mol dissach.)		[η] in 0.1 *M* NaCl (dl/g)	Refractive index	
	experim.	ideal		0.1 *M* NaCl	0.05 *M* NaCl
κ-Carrageenan	1.11 ± 0.02	1.00	6.7 ± 0.2	0.149 ± 0.005	0.145 ± 0.005
λ-Carrageenan	2.43 ± 0.04	2.70	9.5 ± 0.2	0.108 ± 0.013	0.116 ± 0.002

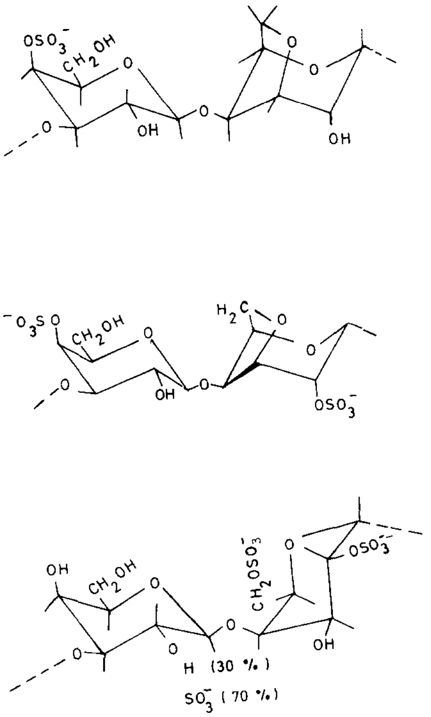

Fig. 1. Ideal chemical structure of κ-carrageenan (a), ι-carrageenan (b) and λ-carrageenan (c).

ture. However, it has been reported that 9% of the 3,6 anhydro-α,D-galactose units in κ-carrageenan might be sulfated on the second carbon [29]. For λ-carrageenan, the lower experimental degree of sulfatation might be due to a smaller amount of sulfate substitution on the second carbon of the 1,3 linked galactose 2-sulfate than the indicated 70% (see Fig. 1). Also included in Table 1 are the intrinsic viscosities of the two samples in 0.1 *M* NaCl.

In Fig. 2, the refractive index above water is plotted as a function of the carrageenan concentration in 0.1 and 0.05 *M* NaCl and the values of the d*n*/d*c* obtained from the slope of these plots are collected in Table 1. According to reference [1], the refractive index increment of κ-carrageenan was taken to be 0.150 ml/g. For λ-carrageenan, we accept the d*n*/d*c* value with the lowest standard deviation namely 0.116 ml/g.

In Fig. 3, the low angle laser light scattering intensities are plotted for κ- and λ-carrageenan in 0.1 *M* NaCl. The weight average molecular weight ($M_{w,LALLS}$) and the second virial coefficient ($A_{2,LALLS}$) obtained from the intercept and the slope respectively are shown in the first and the fifth column of Table 2. Also included in the second and the sixth column of Table 2 are the values $M_{w,LS}$ and $A_{2,LS}$ from angular dependent light scattering as reported elsewhere [1].

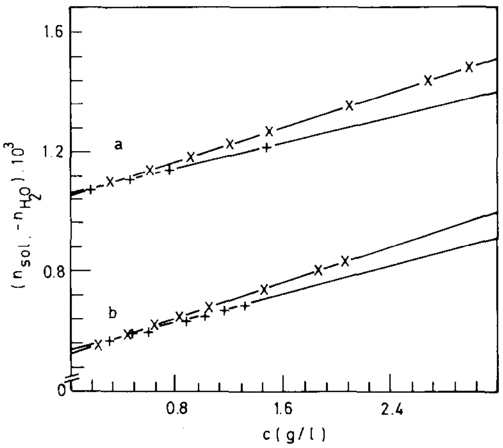

Fig. 2. Refractive index relative to water of κ-carrageenan (×) and λ-carrageenan (+) at different carrageenanconcentration (a: solvent is 0.1 *M* NaCl; b: solvent is 0.05 *M* NaCl).

An independent estimate of the weight average molecular weight, obtained from sedimentation data, is indicated in the third column of Table 2. The *z*-average diffusion coefficients used here [2], were obtained from the angular dependence of the

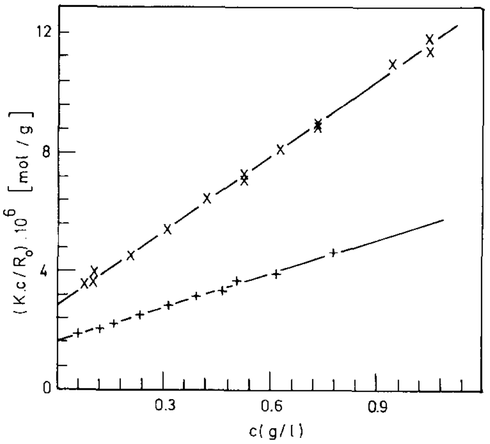

Fig. 3. Low angle laser light scattering of κ-carrageenan (×) and λ-carrageenan (+) in 0.1 *M* NaCl.

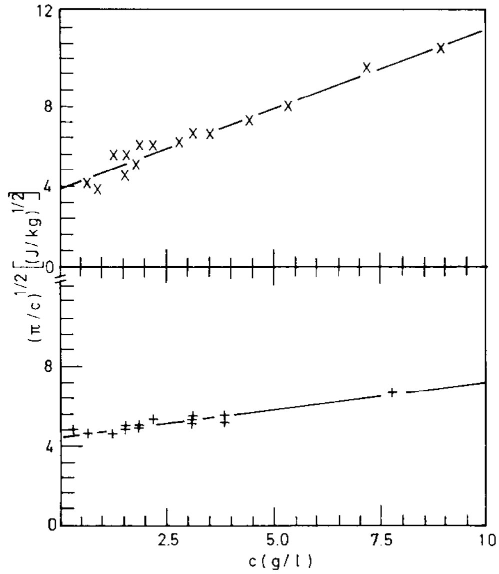

Fig. 4. Reduced osmotic pressure of κ-carrageenan (×) and λ-carrageenan (+) in 0.1 M NaCl.

first cumulant when autocorrelation functions are fitted to a three cumulant expression. The weight average sedimentation coefficients were determined [2] from ultracentrifugation experiments.

Osmotic pressure measurements of κ-carrageenan and λ-carrageenan in 0.1 M NaCl are reported in Fig. 4. The number average molecular weight ($M_{n,\pi}$) and the second virial coefficients ($A_{2,\pi}$) for the two samples obtained from the square root plot are indicated in the fourth and the seventh column of Table 2. These results do not deviate substantially from the results obtained when using only the lowest concentrations in a normal π/c vs. c plot, but the standard deviations are much lower.

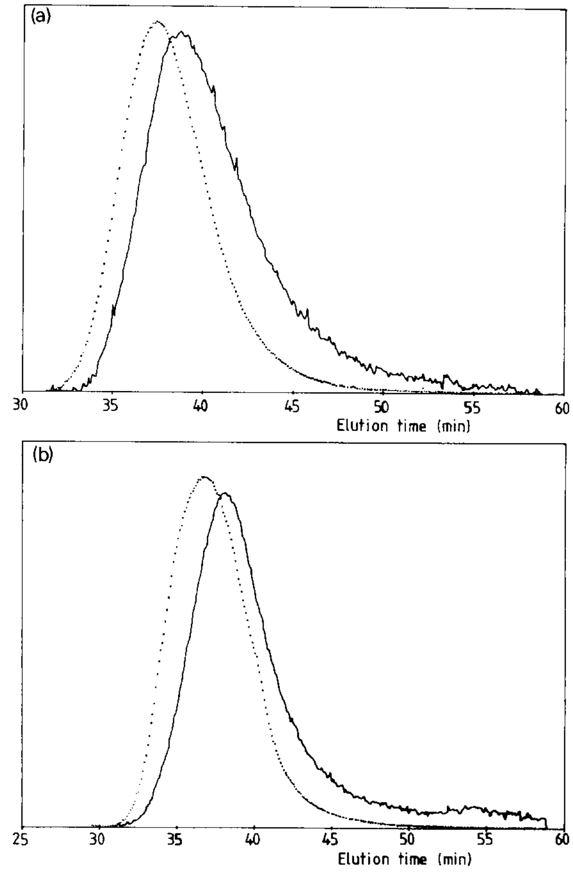

Fig. 5. GPC LALLS signal (···) and RI signal (— — —) of κ-carrageenan (a) and λ-carrageenan (b) in 0.1 M NaNO$_3$.

After an adsorbtion test, κ- and λ-carrageenan samples were both two times injected in the GPC. The RI and LALLS signals of κ- and λ-carrageenan from the GPC unit are shown in Fig. 5. After the RI peak corresponding to the main concentration of the polymer emerged, the RI

Table 2

Molecular weight averages (10^3 g/mol) and second virial coefficients (10^{-3} mol ml/g^2) of κ- and λ-carrageenan samples in 0.1 M NaCl and 0.1 M NaNO$_3$ as obtained from different methods

	LALLS	LS	Sed–diff	Osm.	LALLS	LS	Osm.	GPC-LALLS		
	M_w	M_w	M_w	M_n	A_2	A_2	A_2	M_z	M_w	M_n
κ-car.	353±25	331±26	332±25	165±25	4.17±0.29	4.55±0.18	2.5±0.3	565	323	142
λ-car.	614±17	600±25	500±45	123± 9	1.89±0.06	2.59±0.13	1.0±0.2	1034	664	131
								981	636	216

signal does not return to its original value. The base line is reached at last after elution of the salt peaks (here not shown). The LALLS signal is a single peak, hence excluding the existence of very large aggregates or gel particles eluting in the void volume of the column. The results of the GPC-LALLS coupling are shown in Table 2.

4. Discussion

The refractive index increment values reported here depend upon the type of carrageenan. Indeed, an analysis of earlier data suggests that dn/dc probably increases in the order $\lambda > \iota > \kappa$ [3], an observation which contrasts with literature information [23]. Systematic investigations concerning the dependence of the dn/dc upon the type of polysaccharide and the experimental conditions are unfortunately lacking. The ratio of the values of the dn/dc of κ- and λ-carrageenan reported here (1.29 ± 0.05) from refractometry agrees with the ratio of the RI areas from the RI detector normalised to the same concentration (1.23 ± 0.18). Hence 0.150 and 0.116 are expected to be close to the true values. The accuracy of the light scattering measurements depends upon the statistical error involved in determining the scattered intensity (ΔR_θ) and the error in the determination of the refractive index increment ($\Delta(dn/dc)$). These errors may be estimated from linear least squares fitting of the light scattering and the refractive index data, delivering the variance [30] from which then 95% confidence intervals are constructed [28]. The errors in M_w and A_2 are calculated as normally from the propagation of errors. As for LS and LALLS, the refractive index increment plays the same role in the molecular weight determination, the only source of difference being the uncertainty due to the scattered intensity. The error bars around M_w are then nearly halved, but even then $M_{w,LALLS}$ and $M_{w,LS}$ are indistinguishable.

The values of M_w have also been obtained by the sedimentation-diffusion technique where the absolute error of $M_{w'sed\text{-}diff}$ is estimated as indicated in literature [31]. The values of M_w for κ-carrageenan are in any case confirmed. Moreover, an independent estimate of the molecular weight of κ-carrageenan by high performance size-exclusion chromatography on the same sample yielded the value of 303×10^3 g · mol^{-1} with an uncertainity of 15% [21]. For λ-carrageenan, some deviation is observed which might be due to the fact that hydrodynamic measurements could not be performed at low enough concentrations to reach the dilute regime as discussed in a former paper [2]. Experimental agreement between M_w from light scattering and sedimentation-diffusion was already found for alginates [32]. From the GPC-LALLS coupling, the true weight average molecular weight is obtained, not influenced by axial dispersion effects [19] as for GPC alone. Values of the second virial coefficient ($A_{2,LALLS}$), needed for the evaluation of the GPC-experiment, are obtained from independent LALLS measurements. From Table 2, it is clear that fair agreement is found between M_w from GPC-LALLS and other methods. The limited column resolution (diffusion) affects the values of M_n and M_z and normally, a too small molecular weight distribution is predicted [33,34]. The uncertainty of the M_n value from GPC-LALLS is rather large. This is mainly due to the limited sensitivity of the LALLS signal to low molar mass polymer, further obscured by a low RI signal. When only the elution before 55 ml is taken into account, the M_n values are much higher and the scatter in the GPC-LALLS data (M_n) is consequently reduced. It was, however, shown [23] that the long tail observed in chromatograms of carrageenans is not due to polymer adsorption or degradation, but corresponds to low molar mass polysaccharide present in the sample. When the values of $M_{n,GPC\text{-}LALLS}$ are compared with $M_{n,\pi}$, the same order of magnitude is observed. From the GPC-LALLS measurements, in principle the whole calibration curve of the column can be reconstructed. This is shown in Fig. 6. It can be seen that the λ-carrageenan calibration curve is definitely different from the κ-carrageenan calibration curve. This is ascribed to interactions between the polymer and the gel being different for the two polymers due to differences in charge density and chemical composition. These interactions also show up in the slightly reduced reproducibility of

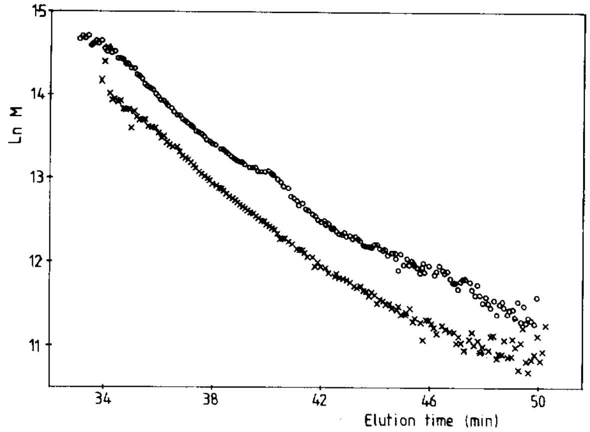

Fig. 6. GPC-calibrationcurve of κ-carrageenan (×) and λ-carrageenan (○) in 0.1 M $NaNO_3$.

the RI traces of duplo measurements. Recently [35], it was found for polystyrene sulphonate, using porous glass beads as gel matrix, that the calibrationcurve was strongly influenced by the salt concentration (even at 0.11 M NaCl). Only for salt concentrations extrapolated to infinite ionic strength, the mechanism for separation was purely hydrodynamic and for molecular weights below 10^5, a single calibrationcurve was obtained. Similar interactions may be important in our carrageenan system, warranting against the use of gel permeation chromatography without LALLS detection. These findings contrast with the identical calibration curve for κ- and ι-carrageenan reported by Lecacheux et al. [23]. However, their measurements were made at 60 °C, where interactions are probably more or less suppressed.

From Table 2, differences in A_2 can be observed dependent upon the type of experiment used. Unless for very high polydispersity, $A_{2,\pi}$ is expected to be equal within experimental error to $A_{2,\mathrm{LALLS}}$ and $A_{2,\mathrm{LS}}$ [25]. However, the values for $A_{2,\pi}$ were obtained at a higher concentration in order to improve the fit and use has been made of a square root plot. The difference may then be ascribed to the fact that in the osmotic pressure measurements, the semidilute concentration regime is reached. By now, one can only stress that interpretation of the concentration dependence of the osmotic pressure in terms of virial coefficients should be done with great care.

5. Conclusions

Good agreement is obtained for the weight average molar mass of κ- and λ-carrageenan as obtained from different methods, all based upon the measurement of light scattering (LS, LALLS, GPC-LALLS). Moreover, these weight average molecular weights are as well in fair agreement with independent sedimentation-diffusion measurements. The determination of number average molecular weights of carrageenans by a GPC unit connected to a low angle laser light scattering detector remains difficult due to the long tail in the carrageenan chromatograms and the finite resolution of the columns. However, the same order of magnitude is found as for osmometry. The use of a GPC unit without a LALLS detector might lead to erroneous results. This is due to interactions between the polysaccharide and the gel, making it impossible to establish a universal calibration curve.

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