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Molecular Weight Distribution of Carrageenans by Size Exclusion Chromatography and Low Angle Laser Light Scattering

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SUMMARY

A procedure to determine the absolute weight average molecular weight and molecular weight distribution of carrageenans by high pressure aqueous size exclusion chromatography coupled with low angle laser light scattering is described. Experimental parameters are successively discussed, particular attention being focused on the absence of shear degradation during elution. The distribution curves were highly reproducible in time and weight average molecular weights integrated along the chromatogram were in good agreement with static light scattering results. A large difference in the molecular weight range between native (food-grade) and acidic degraded carrageenan samples was observed. Weight average molecular weights were found to be in good correlation with viscosity values, for degraded as well as undegraded products. It is also shown that the method described can help people using carrageenans in pharmacological studies by providing information on the real molecular weight distribution of the products they are employing.

INTRODUCTION

Carrageenans are water soluble, cell-wall polysaccharides that can be extracted from certain members of the Rhodophyceae (red seaweeds).

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They are composed of alternating α(1 → 3)- and β(1 → 4)-linked D-galactosyl residues (Anderson *et al.*, 1965). The classification of these polysaccharides is based on the modification of this simple repeating disaccharide unit which can result from the occurrence of ester sulphate, or anhydride formation in the 4-linked residue. If we consider the three usual families, kappa (κ-) and iota (ι-) carrageenans contain 3,6-anhydro-D-galactose, whereas lambda (λ-) carrageenan does not (see Fig. 1). Both κ- and ι-carrageenans can adopt a three-fold right-handed double helical structure in solution, allowing the formation of thermoreversible gels. However, λ- and other carrageenans yield highly viscous solutions

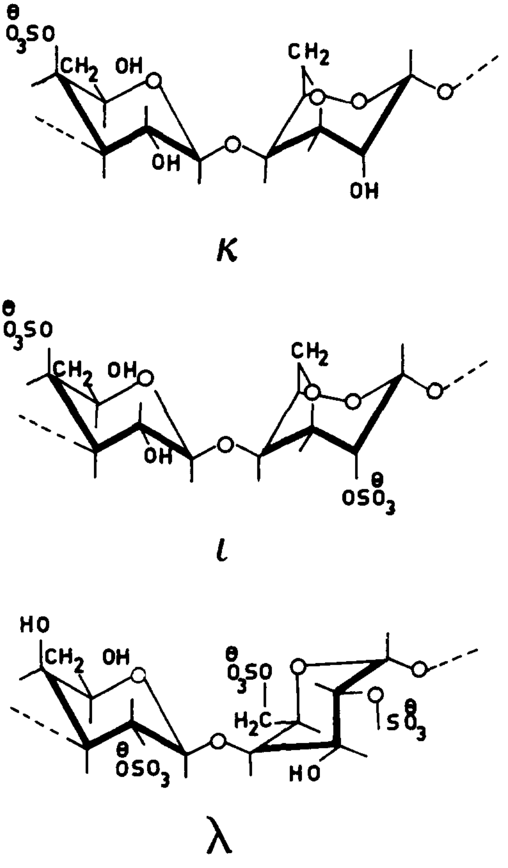

Fig. 1. Primary structure of carrageenans.

that do not gel. With such physical properties, carrageenans form a well-known group of food additives.

Carrageenans can be used not only as food additives but also as drugs for the treatment of gastroduodenal ulcers. For this latter application, carrageenans are voluntarily degraded to reduce their viscosity and thus facilitate oral administration. Much work has been devoted to the toxicological evaluation of carrageenans. For instance, the CNERNA (Centre National de Coordination des Etudes et Recherches sur la Nutrition et l'Alimentation) in France has recently published a review on that question (Causeret *et al.*, 1984) and concluded that the results are still inadequate to give a definitive answer. Problems, if any, will concern essentially low molecular weight products ($M < 20\,000$). Therefore it is natural to enquire whether food-grade products contain low molecular weight components or not and to what extent.

Molecular characterization of carrageenans is currently performed in some scientific laboratories (Rinaudo *et al.*, 1979; Vreeman *et al.*, 1980; Norton *et al.*, 1983*a*,*b*,*c*) or industrial research centres. Viscosity measurements or weight average molecular weights (by classical light scattering) are generally obtained. But it is clear that in both instances the molecular distribution is averaged, and the presence of a limited amount of low molecular weight chains may be obscured by the greater proportion of large molecules. Gel chromatography methods have sometimes been used (Rochas & Heyraud, 1981; Greer *et al.*, 1984). Molecular sieving coupled with low angle laser light scattering detection has been recently reported (Ekstrom *et al.*, 1983). For the first time, absolute distribution curves may be obtained for κ-carrageenans, but the method is very time consuming, resolution is poor and assumptions have to be made about other types of carrageenans.

In the last few years, considerable progress has been made in the size exclusion chromatography of water soluble polymers. The use of an on-line light scattering detector as a tool for the absolute characterization of fractionated polymers (Ouano & Kaye, 1974) has proved valuable in permitting a deeper insight into the absolute molecular weight distribution of polysaccharides. Examples include guar (Vijayendran & Bone, 1984), starch and other uncharged polysaccharides (Salemis & Rinaudo, 1984) and xanthan (Lambert *et al.*, 1982).

We describe here the examination of a variety of carrageenan samples using high performance aqueous size exclusion chromatography (SEC) coupled to a low angle laser light scattering detector (LALLS).

EXPERIMENTAL

Materials

Attention was focused on two ι-carrageenan samples from *Eucheuma spinosum* (CECA, France), herein after referred to as samples A and B. Sample A was a natural, food-grade product, whereas B was an acid-degraded, low molecular weight sample.

Various undegraded κ-carrageenan samples from *Eucheuma cottonii* or *Chondrus crispus* were analysed. Samples C, D and E were pure carrageenans supplied by CECA. Reference samples F to J were five formulated products obtained from competitors (F and G from Litex, Denmark, H and I from CPF, Denmark, and J from Marine Colloids Inc., USA).

Finally, a comparison was made between various degraded samples from CECA, either commercial (K, L) or pilot plant (M, N, O) products.

Chromatographic apparatus

The high pressure liquid chromatograph consisted of the following components: a Waters 6000 A solvent delivery system, a Waters U6K injector, and a Jobin Yvon Iota differential refractometer as a concentration detector. The whole system was thermostated to a selected temperature with a Lauda electronically controlled water bath. Two columns (Toyosoda, TSK PW 5000 and PW 6000 (each 60 cm in length)) were employed with an added home-constructed water jacket.

A Chromatix CMX 100 LALLS detector was installed on-line between the columns and the refractometer. It was equipped with the high temperature accessory. Detector outputs were recorded by a Sefram dual pen recorder. The water used was taken from a Milli-ro + Milli-q water purification system supplied by Millipore and was carefully degassed and filtered through Millipore HA (0·45 μm) before use. Samples were filtered through membranes of similar type.

Refractive index increment

A Chromatix KMX-16 differential refractometer operating with He/Ne 632·8 nm laser light (the same as in the CMX-100) was used to determine d*n*/d*c* under the conditions selected for the GPC analysis (see ‘Development of Method’).

Viscosity measurements

Dilute solution viscosities were measured using a conventional Ubbelohde-type viscometer. The intrinsic viscosity was deduced from the classical extrapolation to zero concentration. The shear rate was about 1000 s^{-1}. Brookfield viscosities at 30 r.p.m. (36 s^{-1}) were measured for degraded carrageenans on 1·5% and 5% solutions at 75°C in water. A UL adapter was used with the LVT model. Fann viscosities at 3 r.p.m. (5·1 s^{-1}) and 300 r.p.m. (510 s^{-1}) were measured for food-grade carrageenans on 1·5% solutions at 75°C in water. The Brookfield and Fann viscosities are expressed in centipoise units.

DEVELOPMENT OF METHOD

Choice of size exclusion chromatography (SEC) conditions

After some 20 years of gel filtration using cross-linked dextran gels, polyacrylamide gels or agarose, high resolution packings for use in aqueous SEC have become commercially available. The TSK-GEL, PW-type columns, have been chosen for the present study. They are packed with particles, about 15 μm in diameter, of a hydrophilic polymer gel containing the group $-CH_2-CHOH-CH_2O-$ as the main constituent (Hashimoto *et al.*, 1978) and have a very high number of theoretical plates (more than 13 000 plates m^{-1}). A large number of applications have been published in the past few years, for synthetic water-soluble polymers as well as for polysaccharides.

The set G 6000 PW + G 5000 PW was selected because of its high molecular weight fractionation range. A 100% recovery level has been obtained for carrageenans. After 2 years of extensive operation, no loss of resolution has been observed.

Troubles encountered in the SEC of polyelectrolytes have given rise to a considerable number of reports but, nowadays, experimental obstacles have been substantially overcome. Unless otherwise specified, the injected quantity must be as little as possible and the solvent ionic strength not lower than 5×10^{-2} M, typically 10^{-1} M (Rinaudo *et al.*, 1981). As far as carrageenan is concerned, selection of the simple electrolyte used as eluent is not made at random, especially so since evidence from various physical techniques has shown that cations affect the conformation of κ and ι-carrageenan.

Information on the conditions under which helix–coil transitions occur, and gels form, have come mainly from optical rotation, differential scanning calorimetry, intrinsic viscosity and light scattering measurements (Rinaudo *et al.*, 1979; Rochas & Rinaudo, 1980, 1984; ter Meer & Burchard, 1983; Norton *et al.*, 1983*a*,*b*,*c*; Rochas *et al.*, 1983; Rizzo *et al.*, 1984). The role of the co-anion has recently been reviewed too (Norton *et al.*, 1984). It is generally recognized that precise and absolute characterization by SEC requires the total absence of multichain aggregation. We have therefore chosen to operate at 60°C in 0·1 M LiCl. Even though κ-carrageenan is a coil at 25°C (Drifford *et al.*, 1984), elevated temperature conditions will be maintained for all carrageenans, with the aim of standardizing the analytical procedure.

Routinely, the flow rate was 1 ml min^{-1}. NaN_3 (200 ppm) was added to the solvent as a bactericide. Injected quantities were about 0·3 mg (food grade) or 3 mg (degraded samples), according to the sensitivity of the LALLS equipment.

The first chromatograms which were obtained for samples A and B (see Figs 2 and 3, respectively) seemed promising. A large difference in

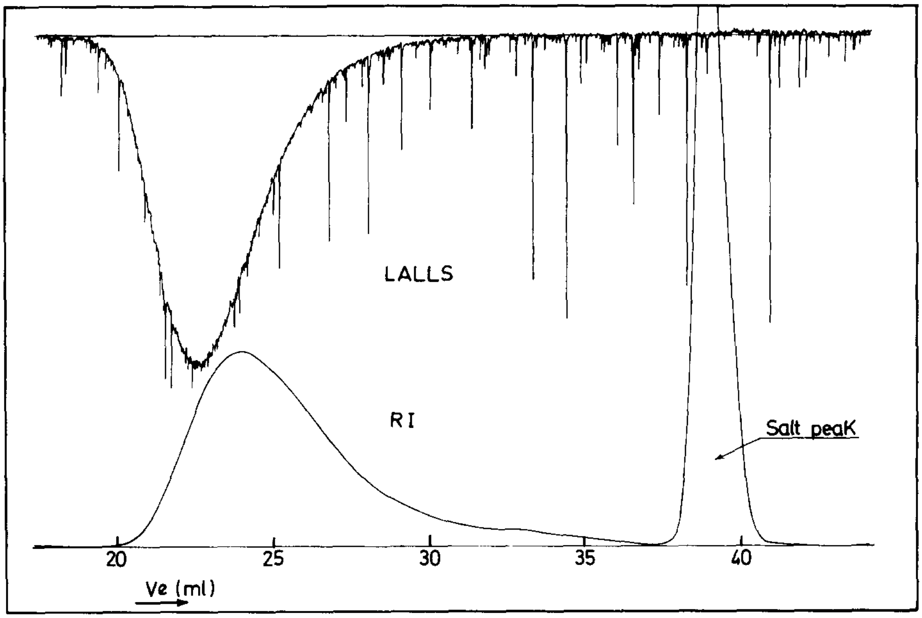

Fig. 2. Dual detector response for sample A (injected quantity 0·4 mg).

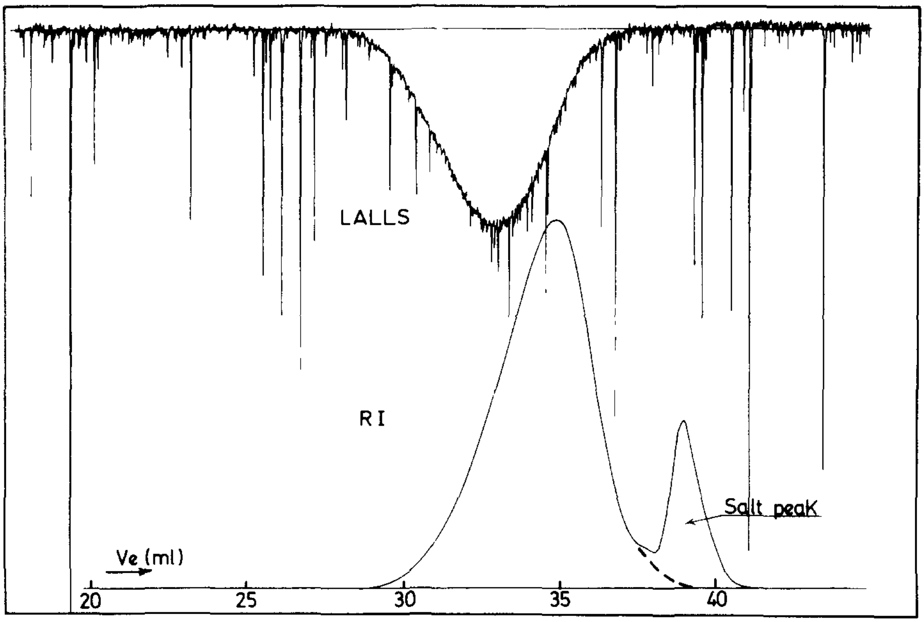

Fig. 3. Dual detector response for sample B (injected quantity 4 mg).

the elution volume area of each product was observed in relation to the molecular weight range gap. The distribution curves could be totally separate, except for the low molecular weight tail of sample A. It should be noted, however, that the presence of low molecular weight species in food-grade carrageenans has been reported by other scientists (Ekstrom *et al.*, 1983; Greer *et al.*, 1984), making our result not surprising. To the best of our judgment, low molecular weight products represent a few per cent of the whole (see next section), but before an insight into absolute characterization we have to confirm whether it is a true image of the sample, or an analytical shortcoming.

Testing of method

It is increasingly believed that shear degradation can occur during SEC experiments. Although precise results with water-soluble polymers are not so numerous, it is important to demonstrate that degradation does not take place through the SEC technique itself (Quivoron, 1984). In this connection, sample A was injected at three flow rates, namely 1,

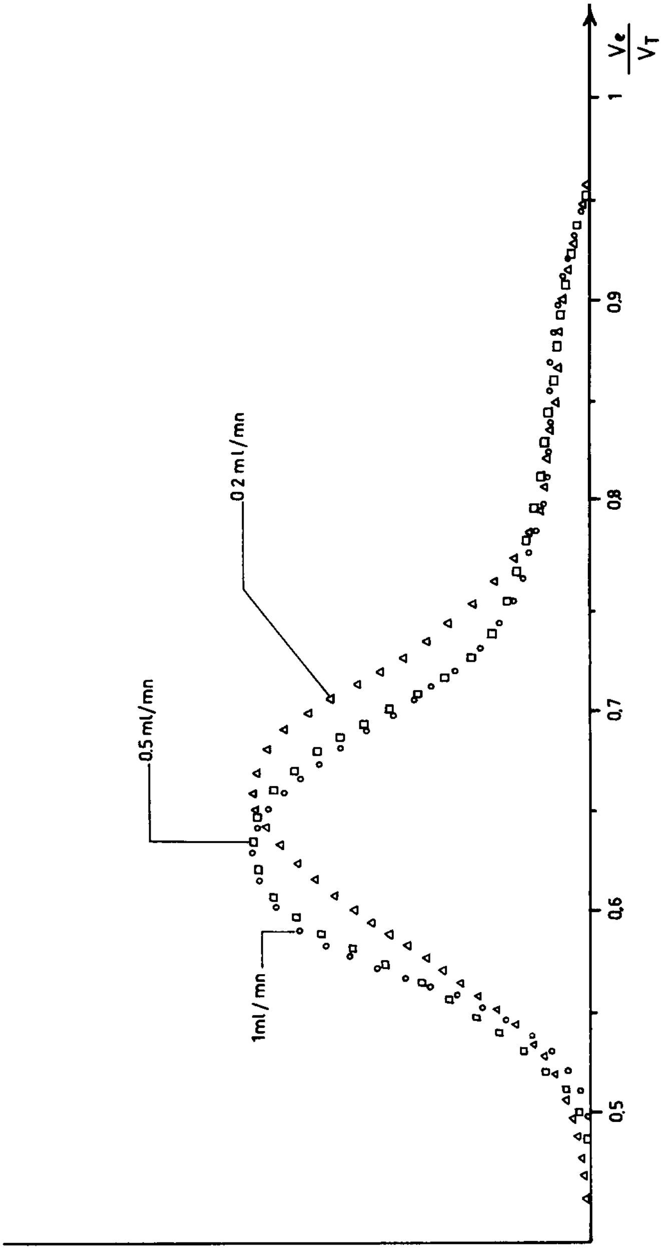

Fig. 4. Study of the flow rate effect on the sample A refractometric trace.

0·5 and 0·2 ml min^{-1}. As can be seen from Fig. 4, the low molecular weight region was absolutely constant. Moreover, integration of the LALLS signal, which gives a value proportional to the weight average molecular weight, led to a constant value. If the area obtained at 1 ml min^{-1} is taken as a reference, 0·999 was found for the intermediate flow rate and 1·008 for the lowest one, which proves the absence of measurable degradation.

Another experiment was performed to ascertain the presence of traces of low molecular weight products in sample A. After checking that the injector was working satisfactorily, sample A was injected about 10 times in a series. Eluate was collected between 20 and 30 ml. This fractionated material was reinjected after concentration. Despite a very prominent salt peak centred on the total permeation volume, a significant decrease of the tail was noticed, close to the base line (see Fig. 5).

Figure 4 shows that the shape of the chromatogram depends on the flow-rate. Conflicting observations on the extent to which the retention volume is flow-rate dependent have been reported (Mori, 1984), but, in the present case, non-equilibrium effects due to slow polymer diffusion

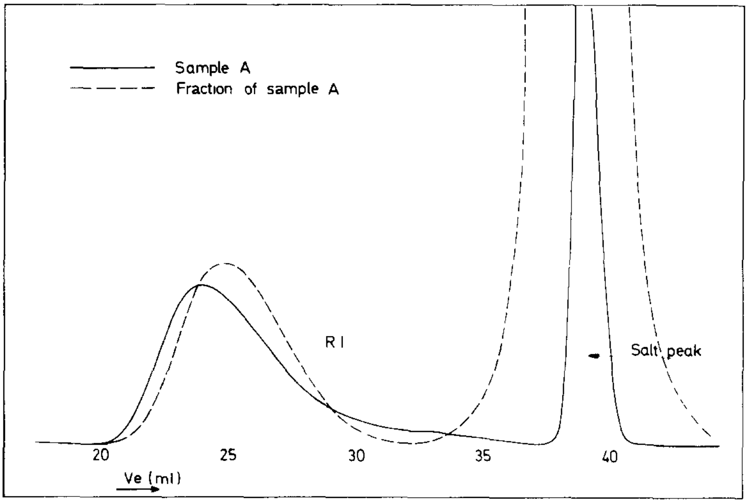

Fig. 5. Comparison of the whole sample A with its 20–30 ml fraction.

appear a reasonable explanation. Whatever the reason, it is well known that such a phenomenon is of no consequence on the validity of SEC–LALLS results. Similarly, a slight concentration effect has been observed on food-grade products (as sample A) but again the accuracy of the SEC–LALLS coupling results is not at issue. Nevertheless, the use of the proposed method without the LALLS detector should be accompanied with care when using a classical calibration curve.

One experimental feature should be mentioned for potential users. A few hours are required to achieve system equilibrium. If any injection is performed prematurely, distribution curves may be more or less truncated on the high molecular weight side, thus giving rise to such problems as erroneous results and the possibility of filter plugging.

Absolute characterization

Various parameters have been measured on samples A and B under the conditions described above, i.e. 0·1 M LiCl in water as solvent at 60°C. Intrinsic viscosities were determined to compare values for our samples with those reported in the literature (Karimian, 1978; Rochas *et al.*, 1980; Vreeman *et al.*, 1980; Norton *et al.*, 1983*a*,*b*,*c*). The following values were obtained:

Sample A $[\eta] = 870\ \mathrm{ml\ g^{-1}}$

Sample B $[\eta] = 33\ \mathrm{ml\ g^{-1}}$

These results clearly demonstrate the gap in the molecular weight range existing between sample A, representative of food-grade carrageenans, and sample B, representative of acidic degraded products. The difference in the viscosifying capacity of these two types is well known to the producers. Such measurements are made daily, but the same method cannot be used for both product families.

Refractive index increments were measured under the same conditions. Samples A and B, which are mainly sodium salts, but contain other cations, were kept in their commercial form because the authors observed that chromatograms (RI and LALLS) remained unchanged after removal of potassium, calcium and other ions. The following dn/dc were measured:

Sample A $dn/dc = 0{\cdot}113\ \mathrm{ml\ g^{-1}}$

Sample B $dn/dc = 0{\cdot}118\ \mathrm{ml\ g^{-1}}$

Given the relative accuracy (water content, etc.), we decided to consider 0·115 to be a constant for all types of carrageenans, basing this on the few data available in the literature (Karimian, 1978; Vreeman *et al.*, 1980).

The second virial coefficient A_2 was determined on sample A using the CMX-100 LALLS detector as a static light scattering apparatus. Columns were removed and solutions with increasing concentrations were successively pumped at a low flow rate (0·2 ml min^{-1}). Results are shown in Fig. 6. The least squares regression analysis leads to

$$A_2 = 2{\cdot}62 \times 10^{-3} \text{ mole ml g}^{-2}$$

$$M_w = 434\,000 \text{ g mole}^{-1}$$

with a good correlation factor. Such an A_2 value is in accordance with the single published series of results (Vreeman *et al.*, 1980).

The same experiment was tried on sample B, but did not succeed, due to the lack of sensitivity of the CMX-100 in the static mode.

Returning to the SEC–LALLS coupling, it is evident that absolute results can now be reached. The most common method of data treatment was used. The axial dispersion correction as recently proposed

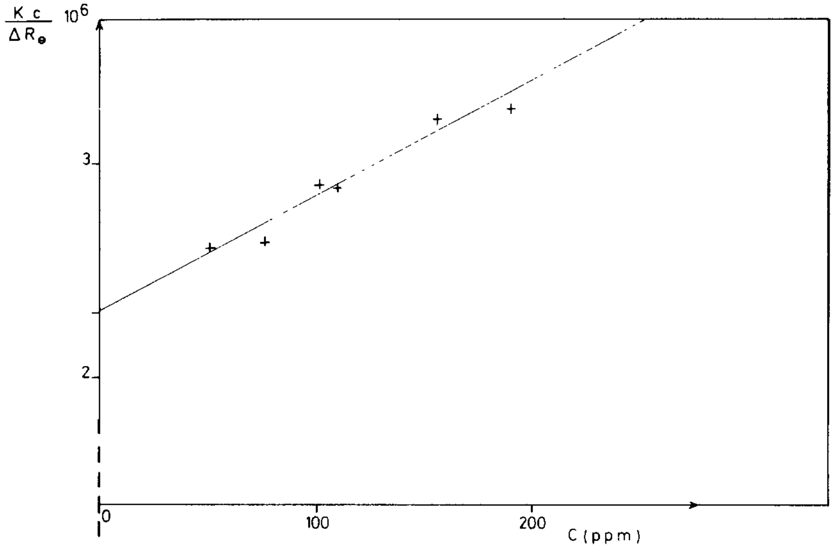

Fig. 6. Static light scattering experiment on sample A.

(Hamielec *et al.*, 1981) was not applied but the A_2 contribution (Kim *et al.*, 1982; Martin, 1982) was certainly not negligible. To enhance the accuracy of treatment along the tail of sample A, a calibration curve was derived from A and B over the entire range of elution volume (Fig. 7). The following results were obtained:

Sample A	$M_n \simeq 130\,000$	$M_w = 432\,000$
Sample B	$M_n = 7\,900$	$M_w = 18\,100$

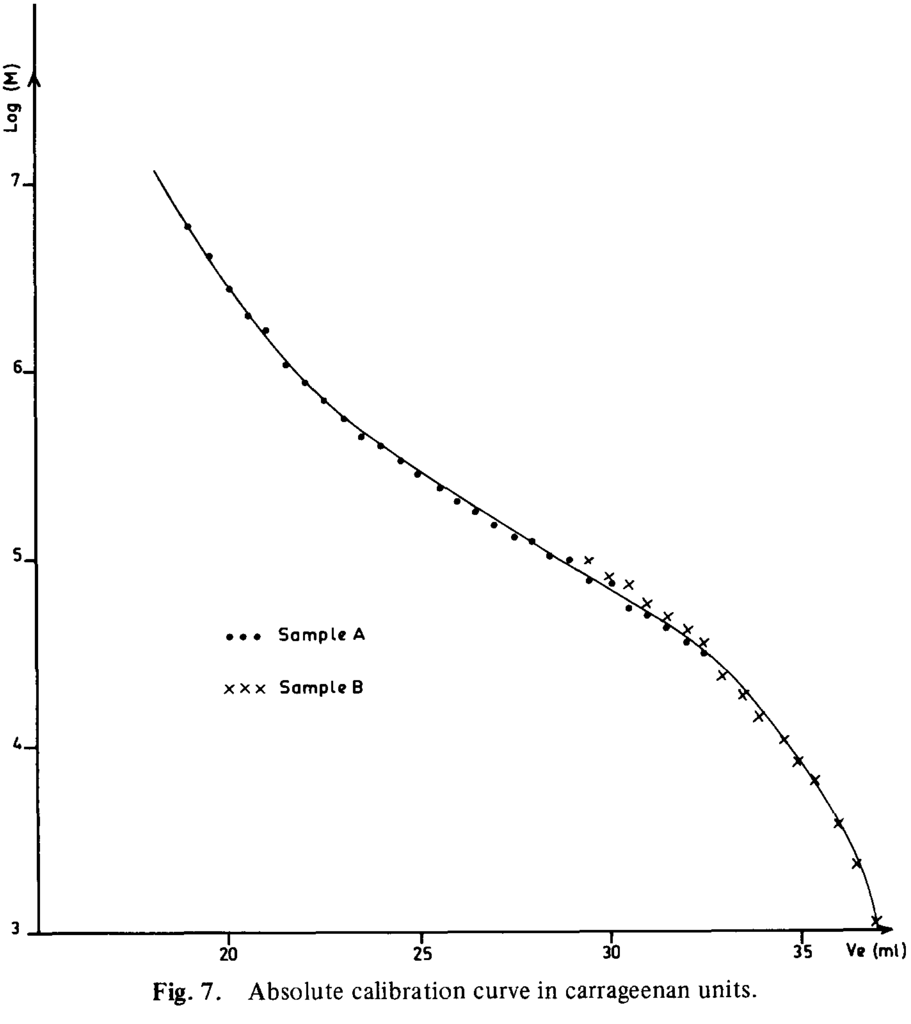

Fig. 7. Absolute calibration curve in carrageenan units.

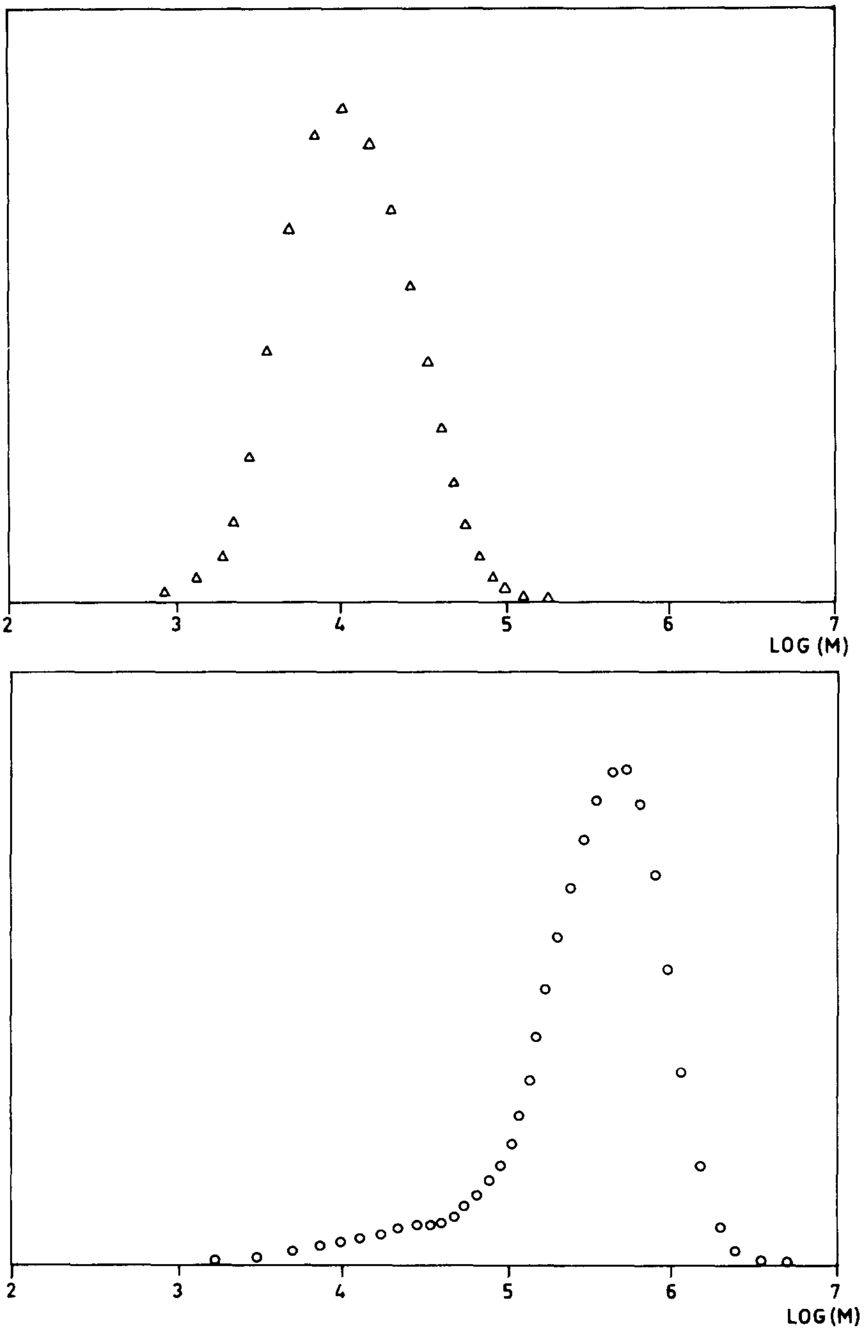

Fig. 8. Molecular weight distributions of samples A (○) and B (△).

The number average molecular weight of sample A is not very precise but the weight average molecular weight is in perfect agreement with the static result and a very satisfactory reproducibility has been reached, i.e. fluctuations within less than 3% over a long period of time.

A ratio of about 25 is again obtained for the two samples. Their respective distribution curves are grouped in Fig. 8. They seem to be each on one side of the molecular weight scale, with the exception of the tail, whose order of magnitude can now be precisely stated:

weight fraction with $M <$ 10 000:	0·5%
weight fraction with $M <$ 20 000:	1·8%
weight fraction with $M <$ 50 000:	4·8%
weight fraction with $M <$ 100 000:	10%

The values are much lower than the results reported elsewhere on another ι-carrageenan sample (Ekstrom *et al.*, 1983).

RESULTS

Food-grade carrageenans

Samples C, D and E from CECA (κ-type) were analysed in the same way. As in the case of sample A, the shape of the distribution curves was the same for the three carrageenans, with the bulk of the product in the high molecular weight region and a low molecular weight tail at

TABLE 1
Results on Food-grade Carrageenans Supplied by CECA

Sample	$\overline{M}_n$	$\overline{M}_w$	*Weight fraction with $M < 20\,000$*	*Fann viscosity at 3 r.p.m.*	*Fann viscosity at 300 r.p.m.*
A	130 000	432 000	1·8%	250	65
C	110 000	575 000	2·5%	400	95
D	90 000	340 000	2·5%	100	37
E	200 000	530 000	1%	300	86

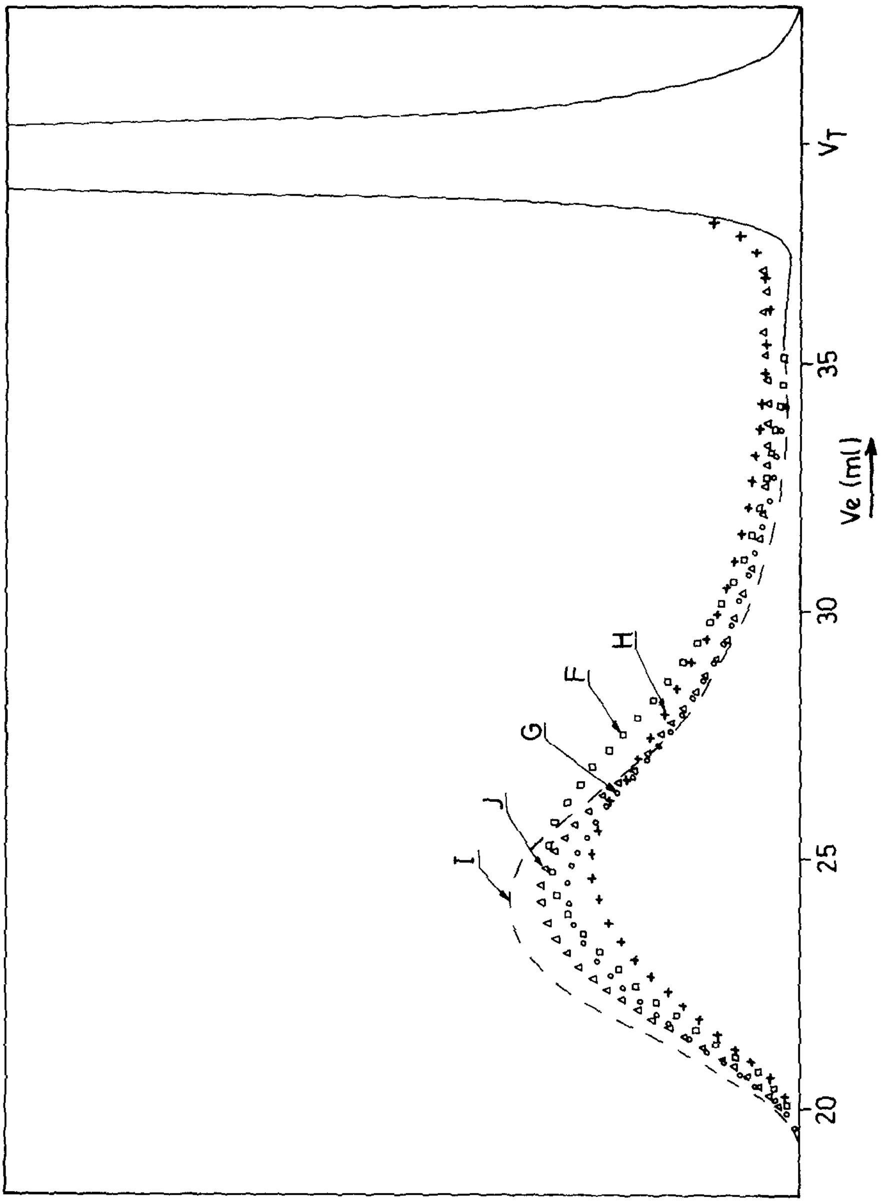

Fig. 9. Refractometric traces of competitors' products.

trace level. The calibration curves resulting from the dual detector response were the same as that for the ι-type (Fig. 7) within the limit of experimental error.

The results obtained from SEC–LALLS are collected in Table 1, together with Fann viscosities. Sample A is included too. Fortunately, classification according to weight average molecular weight is the same as according to viscosity. Consequently, differences between samples, seaweed nature or manufacturing process are perfectly clear.

Formulated products obtained from competitors were then chromatographed in their commercial form (which included the presence of sugar and electrolyte). Again they all exhibited the same typical distribution (see Fig. 9). The weight average molecular weights were in the range of 300 000–500 000 and the fraction with $M < 20\,000$ varied between 1 and 3%. More precise results cannot be given here, due to a lack of precision regarding the nature and concentration of the carrageenan in the mixture.

Nevertheless, results obtained clearly indicate that the distribution curves are all very similar in shape, thus enabling users, unless otherwise stated, to qualitatively compare different products by means of viscosity measurements.

Degraded carrageenans

Degraded carrageenans were finally compared. Though the column set used was not quite suitable (TSK-G 4000 PW would have been the proper one), results are satisfactory. It appears from Table 2 that

TABLE 2
Results on Degraded Carrageenans Supplied by CECA

Sample	$\overline{M_n}$	$\overline{M_w}$	$\overline{M_w}/\overline{M_n}$	*η(1·5%)*	*η(5%)*
M	2 900	4 600	1·6	0·6	0·8
N	4 100	8 000	2	0·70	1·1
O	4 400	9 300	2·1	0·74	1·4
K	4 600	10 000	2·2	0·72	1·5
B	7 900	18 100	2·2	0·76	1·7
L	8 000	23 000	2·8	1·06	3·1

molecular weights and viscosities give the same classification, in spite of the low accuracy of viscosity measurements (note that viscosity of water at 75°C is 0·5 cP). This is a confirmation that one can manage to choose the right product for the right purpose by controlling the extent of degradation.

CONCLUSION

Coupling high performance SEC columns with absolute LALLS detection has afforded a new insight into the molecular weight distribution of carrageenans. The existence of a very large gap between native and degraded products is confirmed. All food-grade samples have the same peculiar shape, with a low molecular weight tail, accounting for about 2% of the bulk. As a result, viscosity measurements are a good means of gaining a qualitative comparison of samples.

We are now working to extend the method to other polysaccharides, namely alginates, pectins and xanthan gum, which will be published in due course.

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