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Molecular weight distribution of carrageenans: Characterisation of commercial stabilisers and effect of cation depletion on depolymerisation

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ABSTRACT

High performance size exclusion chromatography with multi-angle laser-light scattering detection (HPSEC-MALLS) was used for characterising complete molecular weight distributions for a range of commercial *kappa*-carrageenans. Weight average molecular weights were found to be between 400-560 kDa for refined and 615 kDa for semi-refined carrageenan with polydispersities of 1.2 - 1.5, and only small (< 5 %) amounts of material below 100 kDa. In addition solution hydrodynamics have been probed via radii of gyration measurements.

HPSEC-MALLS was used to follow the depolymerisation effect caused during laboratory purification of carrageenans by dialysis and freeze-drying. Further experiments suggested that depolymerisation was caused by acidification of sulphate groups following exhaustive dialysis against deionised water and subsequent autohydrolysis during freeze-drying. It was shown that carrageenans in the gelled state retained their associated cations, preventing autohydrolysis. Although, it is considered likely that cation-depletion would in general lead to depolymerisation upon drying of sulphated polysaccharides, the levels of cations present in most systems exceed the levels needed for cation-depletion and consequently prevent autohydrolysis upon drying.

INTRODUCTION

Carrageenans are a family of sulphated polysaccharides present in many species of red algae (1,2). They find applications in structuring and stabilising of aqueous phases in numerous food products, *e.g.* chilled, frozen and for fat replacement. *Kappa-* and *iota*-carrageenan are highly valued due to their ability to form thermoreversible gels in the presence of specific cations (3-6). The gelation involves the formation of double helices, from the random coil state, which can aggregate to form a three dimensional network (6,7). Although the general properties of carrageenans are due to their chemical composition, molecular weight (Mw) is known to affect the gel rheology, *e.g.* yield stress and modulus (9,10), and is likely to influence application performance. Like all naturally occurring polysaccharides, carrageenans are heterogeneous with respect to molecular weight (10,11). We now report the use of high performance size exclusion chromatography with multi-angle laser-light scattering detection (HPSEC-MALLS) for characterising complete molecular weight distributions for a range of commercial *kappa*-carrageenans. Some attention will be paid to the reproducibility of the system and the specific problems caused by the presence of minor quantities of low Mw material (< 100 kDa) when using MALLS detection.

Additionally we report that following laboratory purification of *kappa*-carrageenan by dialysis against deionised water and subsequent freeze-drying, a decline and in some instances a complete

loss of gel-strength is found. We report evidence that this is due to an autohydrolysis (12) mechanism.

METHODS

Materials

Commercial (*kappa*-)carrageenan stabilisers used were: Genulacta L100 & K100 (ex Hercules, UK); GP 418 (ex FMC, USA); AubyGel MR 50 (ex Sanofi, France); Sherex 610 and semi-refined carrageenan Sherex IC 109 (ex Quest International, Ireland). All water used was HPLC quality obtained from a Millipore Milli-Q_{plus} unit (resistance >18.2 MΩcm) finally filtered through a 0.22 μm filter. Dialysis tubing (24/32 inch i.d.) with a cut-off of 10-14 kDa was purchased from Fisons (UK). The freeze-drier equipment (Virtis Freezemobile 25 EL (USA) and Leybold trivac B D25B high vacuum pump (Germany)) was running at a condenser temp. of -80°C and a pressure of 13 millitorr.

Absolute molecular weight distribution determination

Carrageenan solutions (0.3% carrageenan w/w) were prepared by dispersing the carrageenan stabiliser in 0.1 M LiCl, heated after swelling at room temperature until boiling using a microwave oven, and filtered hot through a 0.45 μm filter (PVDF, Whatman). The reheated sample was injected hot (200 μL) into the chromatographic system, separated on the basis of hydrodynamic volume by high-pressure size-exclusion chromatography (HPSEC) and molecular characteristics were measured on-line using multi-angle laser-light scattering detection (MALLS). The HPSEC-MALLS system consisted of a Gilson HPLC pump (model 305) connected to an on-line pulse dampener, Rheodyne injection valve (model 7125) and on-line high pressure filter (2 μm, Anachem). Separation of the carrageenans was achieved using Anagel-TSK PW_{XL}G4000, G5000 and G6000 columns in series (7.8 x 300 mm, Anachem), in combination with a TSK PW_{XL} guard column (6.0 x 40 mm). The columns were eluted with filtered (0.02 μm membrane (Anodisc 47, Whatman)) and degassed 0.1M LiCl at a flow rate of 0.5 mL/min. For on-line light scattering detection a DAWN-F MALLS photometer (Wyatt Technology, Santa Barbara, California) was used equipped with a F2 flow cell (n=1.61655), a high temperature read-head, and a He-Ne laser-light source (λ = 633 nm, 5mW). The MALLS detector, on-line filter, tubing and columns were thermostated at 60°C. For on-line sample quantification a Waters 410 differential refractometer was used, thermostated at 40°C. The data were accumulated and processed using Astra for Windows® 4.0 BETA release software.

Prior to the measurements, the DAWN-F MALLS photometer was calibrated and normalised using filtered (0.02 μm membrane (Anodisc 47, Whatman)), HPLC quality toluene and Shodex P20 pullulan standard (23.7 kDa, Machery-Nagle, Germany), respectively. The performance of the SEC-MALLS system was checked using 'mono'-disperse Shodex pullulan standards covering a wide Mw-range (from 5.8 kDa up to 1,660 kDa).

Two important parameters needed to obtain accurate molecular weight information are the refractive index increment (dn/dc) and second virial coefficient (A_2). The dn/dc is necessary for determining the absolute quantities of material eluting at each volume increment. This information is needed for determining the weight fractions and for extrapolation of the light-scattering data to zero concentration (Zimm-plot). A_2 describes the interaction/forces between the solvent and the polymer chain. If the polymer-solvent interactions are large the polymer coil will expand and the solvent is considered good (A_2 is positive). If the solvent is poor polymer-polymer forces dominate causing the polymer chain to collapse either inter- and/or intramolecularly. This parameter is especially important when using poly-electrolytes where repulsive forces play an important role. A_2 is important when calculating absolute molecular

weights by defining the slope of the extrapolation in the Zimm-plot from known concentration of polymer (obtained from RI measurement and dn/dc) to zero concentration (13,14). The dn/dc used for *kappa*-carrageenan in 0.1M LiCl was confirmed to be 0.115 in agreement with published data (11,15). The A_2 of *kappa*-carrageenan in 0.1M LiCl (at 60°C) was taken from literature as 2.62×10^{-3} mLmol/g^2 (11). As shown in Table I, omitting A_2 ($A_2 = 0$) when calculating Mw translates the calculated values towards lower numbers. The deviation seems to be most related to the amount and somewhat to the size of material present.

Within a solvent system the polymer-solvent interaction will vary with the polymer used. This is especially true for the carrageenan family consisting of *kappa-*, *iota,* and *lambda-*carrageenans which vary in charge density. Each family member will interact differently with the solvent used, giving rise to different A_2 values. For carrageenan samples containing mixtures of the different carrageenans the measured Mw will consequently deviate from the 'true' Mw of some of the individual molecules.

Table I. Effect of concentration of polymer and second virial coefficient (mLmol/g^2) on the measured Mw average (kDa) by MALLS (percentage deviation in parenthesis). Data recorded from individual slices through the HPSEC elution profile of Sherex 610 in 0.1 M LiCl at 60°C (see Figure 1).

Elution Volume (mL)	Concentration (g/L)	$A_2 = 0$	$A_2 = 2.62 \times 10^{-3}$
17.0	0.36	985.8	1,118.0 (11.8%)
19.0	1.78	600.1	793.4 (24.4%)
21.0	2.04	400.3	497.7 (19.6%)
23.0	1.32	239.7	257.0 (6.7%)
25.0	0.57	164.1	167.2 (1.8%)

Autohydrolysis experiment

Carrageenan (1.6g or 16g of Sherex 610) was dissolved in 800 mL boiling Milli-Q water, divided into 100 mL aliquots and dialysed against Milli-Q water at 4°C. After 0, 24, 48, 72, 96 and 168 hours a sample of each concentration was removed, the pH measured, and divided into two, approximately equal portions. One portion was freeze-dried, the other adjusted to pH 8, heated to dissolve the carrageenan and precipitated in 2-propanol (IPA) at a final concentration of 70% IPA. The 0.2% carrageenan samples had to be concentrated by rotary evaporation prior to IPA precipitation to obtain a recoverable precipitate. Mw's of the carrageenans recovered were measured by HPSEC-MALLS as described previously. Additionally sodium, potassium, calcium, magnesium, sulphur and carbon levels were determined.

RESULTS AND DISCUSSIONS

HPSEC-MALLS of *kappa*-carrageenan

Using HPSEC, polysaccharides are separated according to their hydrodynamic volume. The hydrodynamic volume is related to the extension of the polysaccharide chain and hydration shell. The amount of chain extension of a linear polysaccharide largely depends on the polymer-solvent interaction. Thus by changing the solvent conditions HPSEC profiles can be manipulated. The eluant conditions used, 0.1 M LiCl at 60°C, were chosen to prevent aggregation and helix

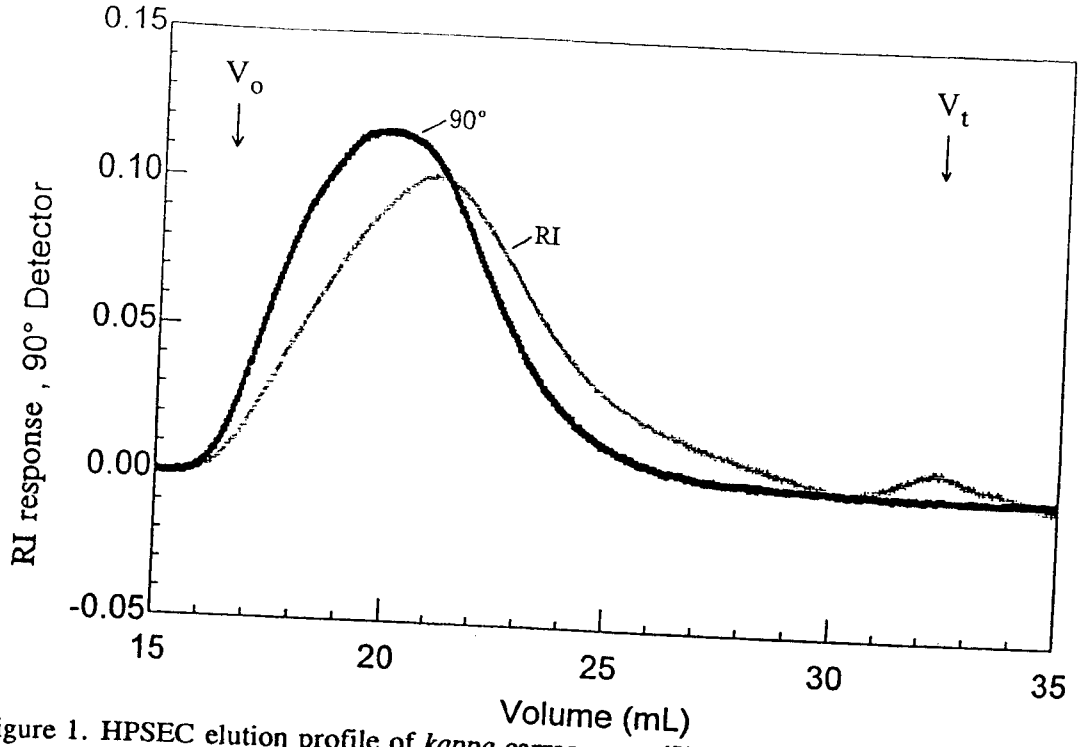

Figure 1. HPSEC elution profile of *kappa*-carrageenan (Sherex 610) in 0.1 M LiCl at 60°C detected by RI and MALLS at the 90° angle. V_0 and V_t mark the void volume and total volume of the HPSEC column used, respectively.

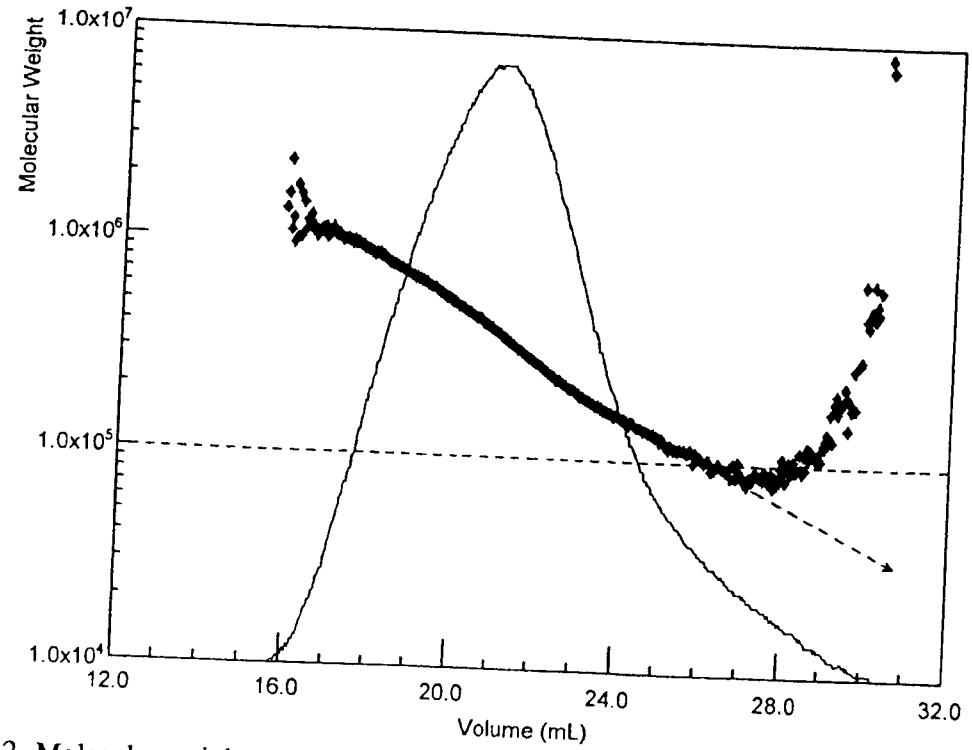

Figure 2. Molecular weight vs elution volume plot overlaid with the HPSEC-RI elusion profile of Figure 1. The linear extrapolation of the Mw vs elution volume plot is given by the arrow.

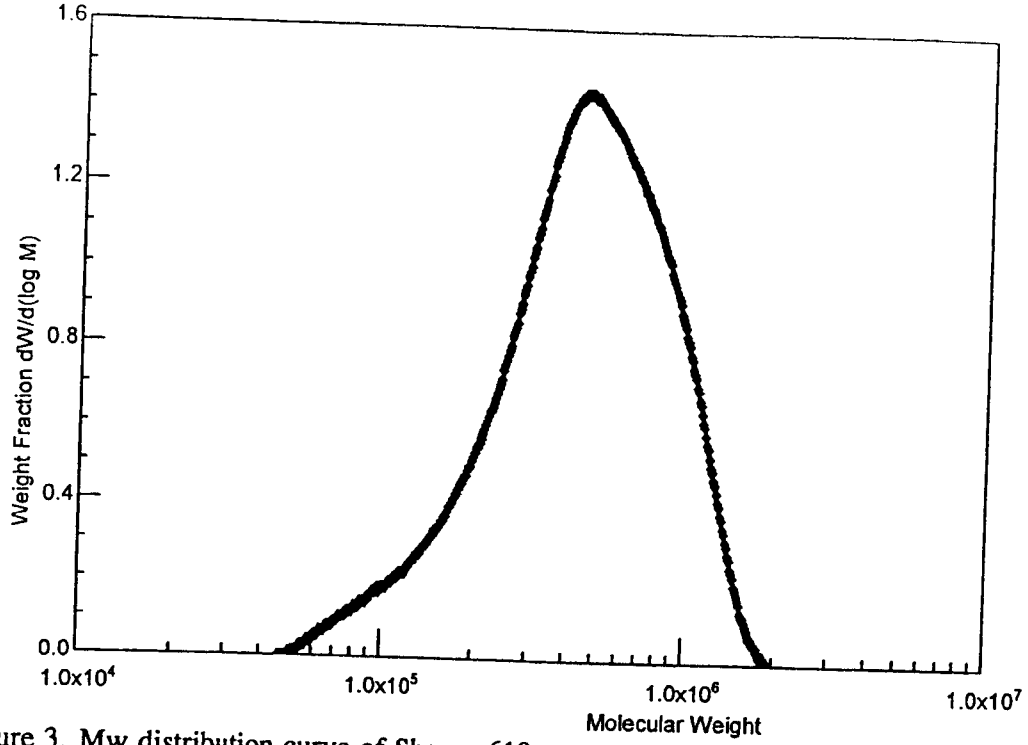

Figure 3. Mw distribution curve of Sherex 610 constructed from the data given in Figure 2.

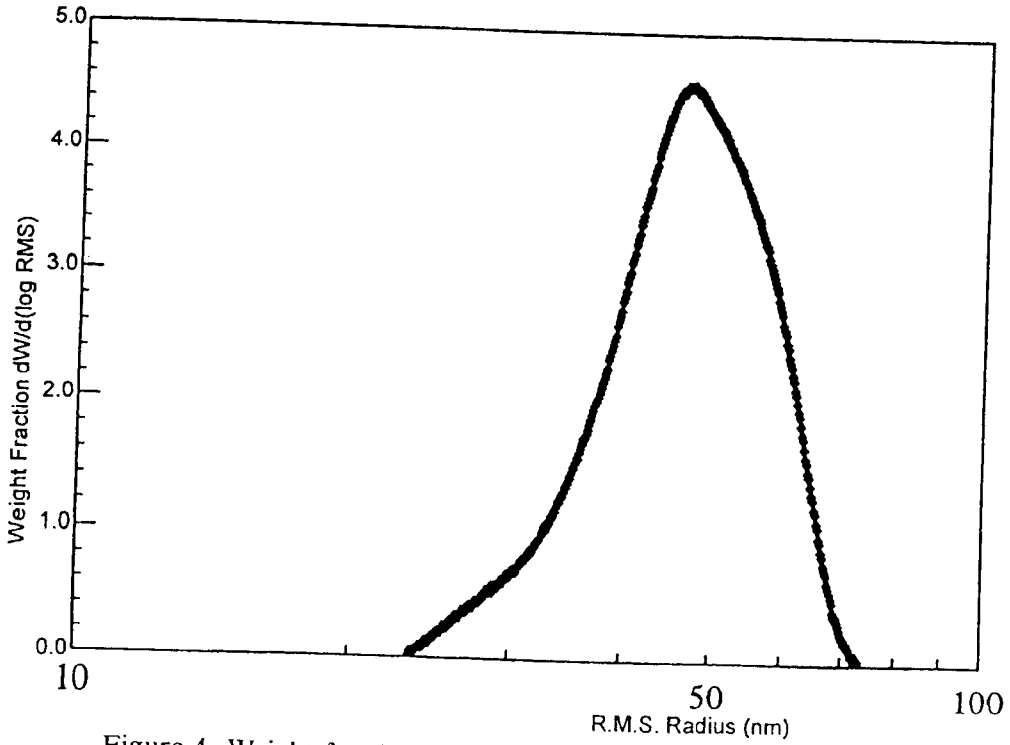

Figure 4. Weight fraction vs radius of gyration plot of Sherex 610.

formation of the *kappa*-carrageenans during HPSEC (10), ensuring that Mw's of individual chains are measured only. Using the given HPSEC set-up essentially all carrageenans elute in the included volume, as shown in Figure 1. The refractive index (RI) trace given in Figure 1 clearly shows that most of the carrageenan elutes early after the void volume (V_0) with a tail towards higher retention times. This tail reaches baseline level (at approx. 30.5 min elution time) before the total volume (V_t). For carrageenan samples which do not contain added sugars a RI-peak, sometimes negative, can still be observed at V_t. No carbohydrate was detected in this fraction by colorimetric assays and it is assumed that this RI-peak is caused by dissolved air or salts co-injected with the carrageenans. Due to the absence of any carrageenan in this fraction the V_t signal will be omitted in future calculations of carrageenan parameters.

These molecular parameters can be determined on-line by measuring the angular dependence and intensity of scattered light at preset intervals by MALLS. Due to band-broadening, caused by longitudinal and Eddy diffusions, polysaccharides eluting from a HPSEC column at each volume increment are generally polydisperse. As a result only average molecular parameters are measured. Determining the Mw at each volume increment a curve can be constructed showing Mw vs elution volume. Figure 2 shows the HPSEC-RI trace of Sherex 610 stabiliser with overlaid the Mw measured in each volume increment. The linear relationship between Log Mw vs elution volume is clearly shown which corroborates the separation power of the HPSEC system used. At the extremes a scatter of the Mw data can be seen. The scatter at the high Mw end of the distribution is caused by the very low amounts of material present, making the system too dilute to be measured accurately. Because only a minute quantity of the total carrageenan present is involved no action is taken to correct for the errors made. At the low molecular-weight end the problem is more acute. Due to the nature of light-scattering the measured Rayleigh scatter increases proportionally with particle size, thus the MALLS detector is much less sensitive to small sized molecules. The effect of this can be seen in Figure 1, where the low-molecular-weight tail is observed in the RI trace but not by the MALLS detector. The combination of low sensitivity and minor amounts are the cause of the deviation in the linearity of the Mw's measured in the low molecular weight range (approx. <100 kDa; Figure 2). It was observed that the Mw's measured were generally overestimated. This was presumably caused by minute quantities of 'impurities', *e.g.* dust, convection striation, etc, resulting in an underestimation of low molecular weight material. Using 'mono'-disperse pullulan standards the linearity of the HPSEC system was established down to 5,000 Da (results not shown). This linearity justifies linear extrapolation from the Mw curve. As given in Table II, linear extrapolation increases the amount of low-molecular weight material accounted for, from 1.8% to 6.2%, and increases the reproducibility in a dramatic way, from a deviation of 130% to 2.5%.

Combining the RI and MALLS data a weight fraction vs average Mw plot (Figure 3), *i.e.* molecular weight distribution plot, can be constructed and the Mw average can be calculated. The reproducibility of the HPSEC-MALLS system used was tested by multiple injection of the same sample on various days. The accumulated results are given in Table II. The values for the number average molecular weight (Mn), weight average molecular weight (Mw) and Z-average molecular weight (Mz) only vary between 2 - 4% between the different runs. The reproducibility is also reflected by the small deviation found for the polydispersity index (Mw/Mn).

When particles have a principal radial axis, *i.e.* radius of gyration (not to be confused with the hydrodynamic volume) larger than *c.* 1/20 of the wavelength (λ) of the laser-light source used for the light-scattering measurements, the intensity of scatter has an angular dependence (14,16,17). Making use of the multi-angle detection of the MALLS system this angular dependence can be measured and directly related to the size, *i.e.* radius of gyration, of the molecule. In Figure 4 the radius of gyration is plotted against the weight fraction, showing that

Table II. Reproducibility data of HPSEC-MALLS system. Data collected from six individual separations

	$\bar{x}$	σ	% Error
Mn	280,180	9,940	3.6
Mw	415,200	8,690	2.1
Mz	562,530	14,690	2.6
Peak Mw	384,130	15,240	4.0
% <100 kDa	1.83	2.38	130
[Extrapolated]	[6.19]	[0.16]	[2.5]
Mw / Mn	1.48	0.046	3.1

the radius of gyration of carrageenans in the solvent system used ranges from ca. 70 nm to below 30 nm. Although particles with a radius of gyration smaller than approx. 30 nm could not be measured (λ = 633 nm) the close fit of Figure 4 with Figure 3 indicate that only a minute portion of the carrageenan population is not accounted for. Constructing a double logarithmic plot of radius of gyration vs. average Mw a shape factor can be determined. Under the conditions used this value is approx. 0.45, which indicates that the carrageenans are in a random-coil conformation (16), as would be expected for the solution conditions.

HPSEC-MALLS of commercial (*kappa*)-carrageenan stabilisers

The molecular weight distributions of several commercial, refined carrageenan stabilisers currently used in the food industry were investigated using the HPSEC-MALLS system outlined above. Relevant molecular parameters were collected and are given in Table III, and Mw distribution plots (Figure 5) were constructed. It is surprising how similar the Mw distributions shown in Figure 5 are, especially when taking into account that different weed sources will have been used for the production of the various stabilisers. The similarity is also reflected with respect to the Mw average, with the lowest for Genulacta K100 (410.6 kDa) and the highest for AubyGel MR 50 (557.5 kDa). These Mw averages for carrageenans correlate well with published data (10,11). As shown (*kappa*)-carrageenan polymers give a narrow molecular-weight distribution with minute amounts of very high molecular-weight material (>2MDa) and a minor low molecular-weight tail. The polydispersity (Mw/Mn) of the carrageenans was found to vary between 1.23 (GP 418) and 1.46 (Sherex 610). A higher Mw/Mn value does however not correlate with the presence of larger amounts of low-molecular weight material, as seen in Table III. As given in Table III the low-molecular weight tail found for all carrageenans accounted for less than 4.5% of the total carrageenan moiety, and no material with a Mw <40 kDa was detected (see Figure 5). Mw distributions and Mw averages of commercial carrageenans may vary between batches due to variations in the natural algal source, induced by seasonal, environmental and/or post-harvesting effects or by variations in process conditions.

Recently, a new, less processed *kappa*-carrageenan stabiliser has been introduced to the market. This stabiliser is made using a solid-state process, in contrast with a solution process generally applied. During this process the algal cell-wall integrity is maintained and the carrageenan is locked into the helical/aggregated state. Restraining the chains in the ordered form might be expected to stabilise the carrageenan molecules and make them more resistant to degradation, thus minimizing depolymerisation. From this semi-refined carrageenan, also called

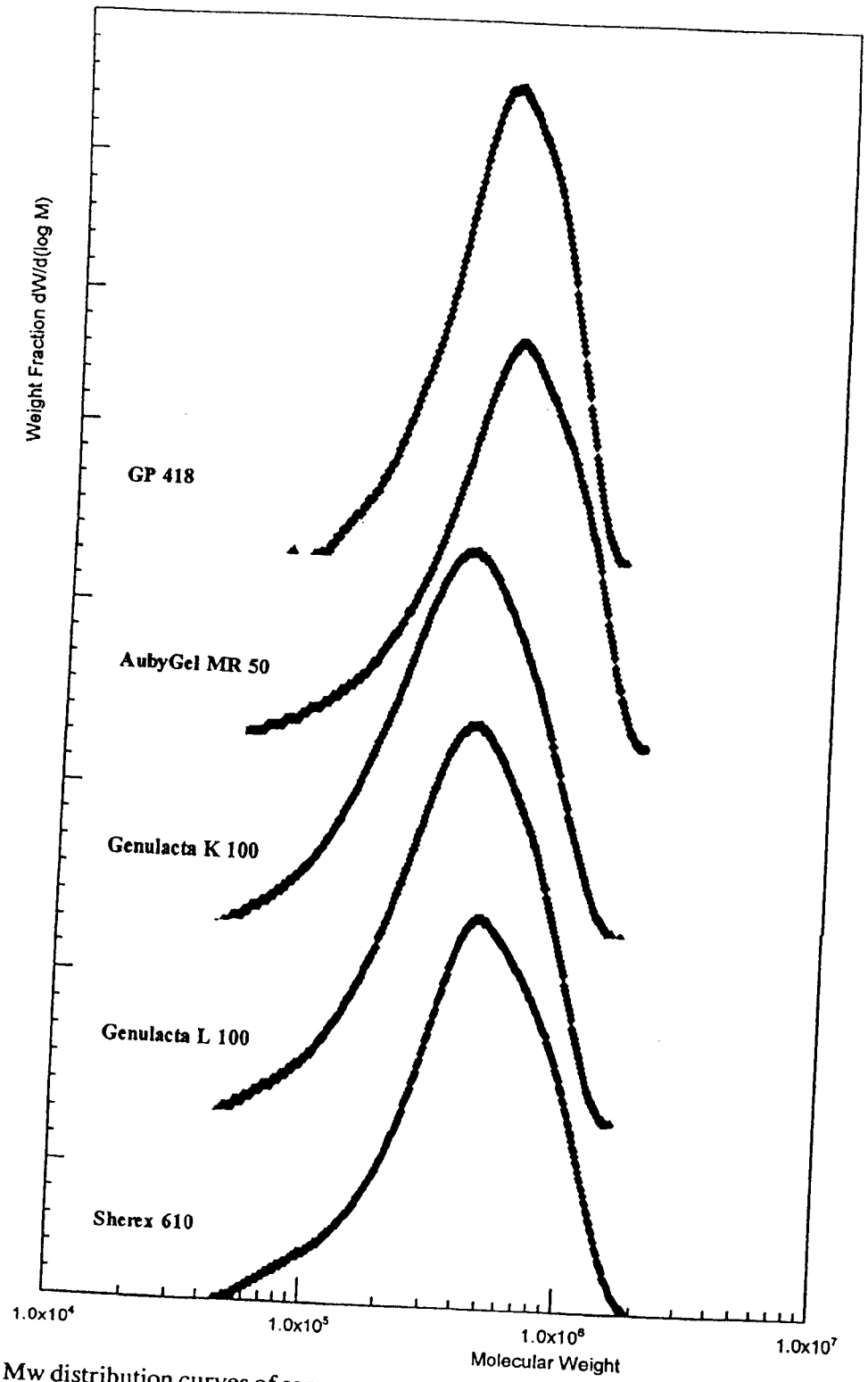

Figure 5. Mw distribution curves of some commercial (*kappa*)-carrageenan stabilisers measured using HPSEC-MALLS and eluted in 0.1 M LiCl at 60°C.

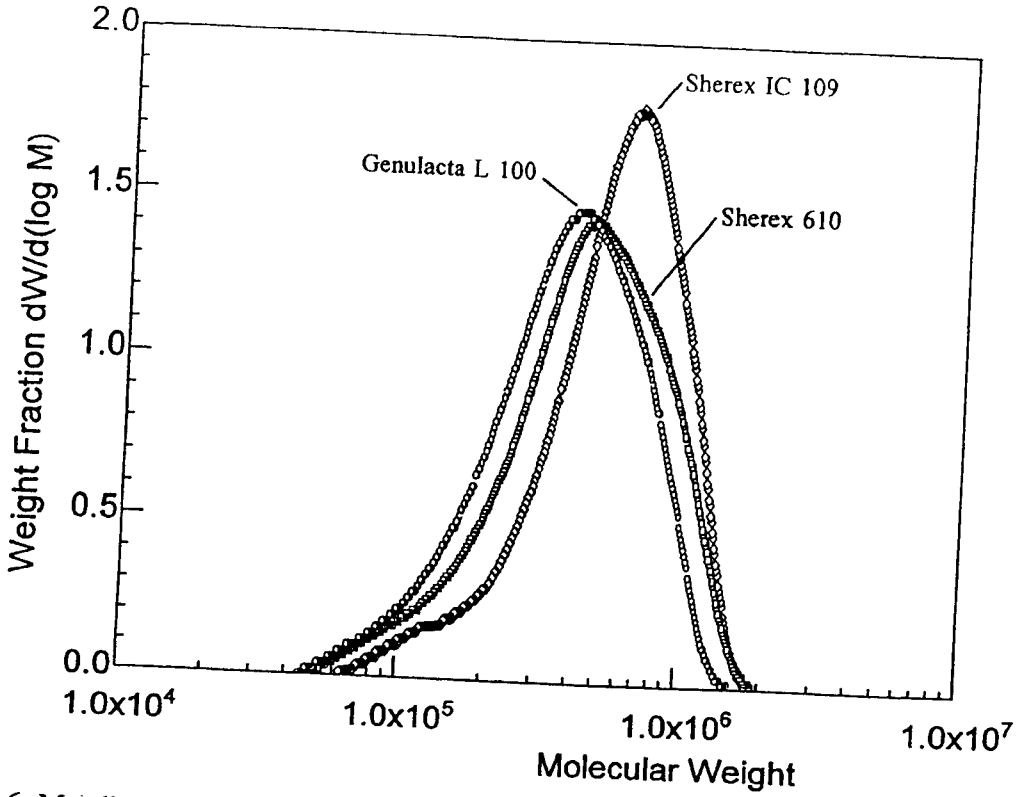

Figure 6. Mw distribution curves of two commercial, refined (Sherex 610 and Genulacta L 100) and semi-refined (Sherex IC 109) (*kappa*)-carrageenan stabilisers measured using HPSEC-MALLS and eluted in 0.1 M LiCl at 60°C.

Table III. Number-, weight- and Z-average molecular weights, peak Mw, polydispersity index, and weight percentage material < 100 kDa of some commercial (*kappa*)-carrageenan stabilisers.

	refined					semi-refined
	GP 418	AubyGel MR 50	Genulacta K 100	Genulacta L 100	Sherex 610	Sherex IC 109
Mn[a]	408.9	411.5	303.4	325.5	357.2	481.0
Mw[a]	502.3	557.5	410.6	444.7	521.6	614.2
Mz[a]	610.1	707.7	542.6	582.0	680.2	735.8
Peak Mw[a]	477.7	530.9	368.3	401.0	467.4	675.4
Mw / Mn[b]	1.23	1.36	1.35	1.37	1.46	1.28
% < 100 kDa[c]	0.1	2.2	3.0	4.6	2.0	1.2

[a]x10^{-3}; [b]polydispersity index; [c]extrapolated.

Philippine natural grade carrageenan, refined material can be produced by hot water extraction and filtration, separating the carrageenan from insoluble algal cellulose (18). In Figure 6 Mw distribution plots of Sherex IC 109 (semi-refined), Sherex 610 (refined material from Sherex IC 109) and Genulacta L 100 are given. A shift to higher Mw of the semi-refined product compared to the refined material can be seen. This shift is reflected by the higher Mw shown in Table III. The slightly narrower Mw distribution of the semi-refined carrageenan seen in Figure 6 is also captured by the low Mw/Mn value of 1.28 (Table III). As the Mw distribution shifts to higher Mw the low-molecular weight tail is more visible. Although the calculated amounts of material < 100 kDa is lower than detected for most other commercial stabilisers used in this study this tail is still detected. We speculate that some of the low molecular-weight fraction is an inherent part of carrageenans in the native algal weed. The Mw averages determined for the commercial carrageenan stabilisers was shown to be very similar to the average Mw found for other natural polysaccharides, *e.g.* Locust bean gum (19).

Kappa-carrageenan autohydrolysis

Autohydrolysis of carrageenans has been used previously for controlled fragmentation, with the aim to cleave specifically the 3,6-anhydrogalactose linkage (13). This was achieved by conversion of the carrageenan into the acid form and heating the solution. Generally autohydrolysis is an unwanted phenomenon responsible for the loss of gel-strength (yield stress). We found that standard laboratory practices, *e.g.* dialysis and freeze-drying, under certain conditions gave rise to a loss in gel-strength of carrageenans. To identify the cause of the observed effect two concentrations of *kappa*-carrageenan solutions (Sherex 610), one at gelling concentration (2% w/v) and one below (0.2% w/v), were dialysed extensively in ultra pure water over a period of 7 days, at 4°C, and aliquots were recovered at set intervals by freeze-drying or IPA precipitation. Both pH and ion levels were monitored and the Mw averages of the recovered carrageenans were measured using HPSEC-MALLS. As shown in Figure 7, in the 0.2% carrageenan solution a steady pH drop was detected over the measured time period.This drop was mirrored by the decline of the sodium and potassium concentration, from 0.98% to 0.82% w/w (of carrageenan) and from 6.43% to 4.54% w/w, respectively, at constant sulphate levels (19.2% w/w). Calculating the molar percentage of total cation (Na^+, K^+ and Ca^{2+}) to sulphate, it was found that prolonged dialysis of the 0.2% carrageenan solution against deionised water depleted the carrageenan from an excess of cations (*c.* 12%) to an excess of sulphate (*c.* 5%) within 72 hours reaching *c.* 13% after 168 hours dialysis. Consistent with the observed pH drop (Figure 7) we presume that the excess sulphate groups are in the acid form. Freeze-drying of the solutions caused a significant loss in the average Mw of the carrageenans present once the pH of the solutions had dropped below neutral. When after 168 hours dialysis the alkaline pH of the solution was restored, pH≥8, before recovering the carrageenan by IPA precipitation only a minor Mw loss was detected (Figure 7).

The samples containing 2% w/v carrageenan had gelled in the dialysis tubing and only started to show a pH decline after 72 hours dialysis. Potassium concentrations levelled off after an initial drop during the first 24 hours (down to 5.4% w/w), whilst sodium levels continued to drop to the same level as found for the 0.2% carrageenan solution (0.79% w/w after 168 hours). Calculating the percentage cations to sulphate showed that within the first 24 hours of dialysis the excess of cations diminishes from the initial *c.* 12% to *c.* 5%, reaching equimolar amounts after 72 hours. Prolonged dialysis (168 hours) eventually removed some additional cations, resulting in an excess of sulphate groups (*c.* 5%). Taking into account the potassium selectivity of *kappa*-carrageenan these data indicate that prolonged dialysis is required to liberate carrageenan bound ions. No Mw loss was found for either freeze-dried or IPA precipitated material.

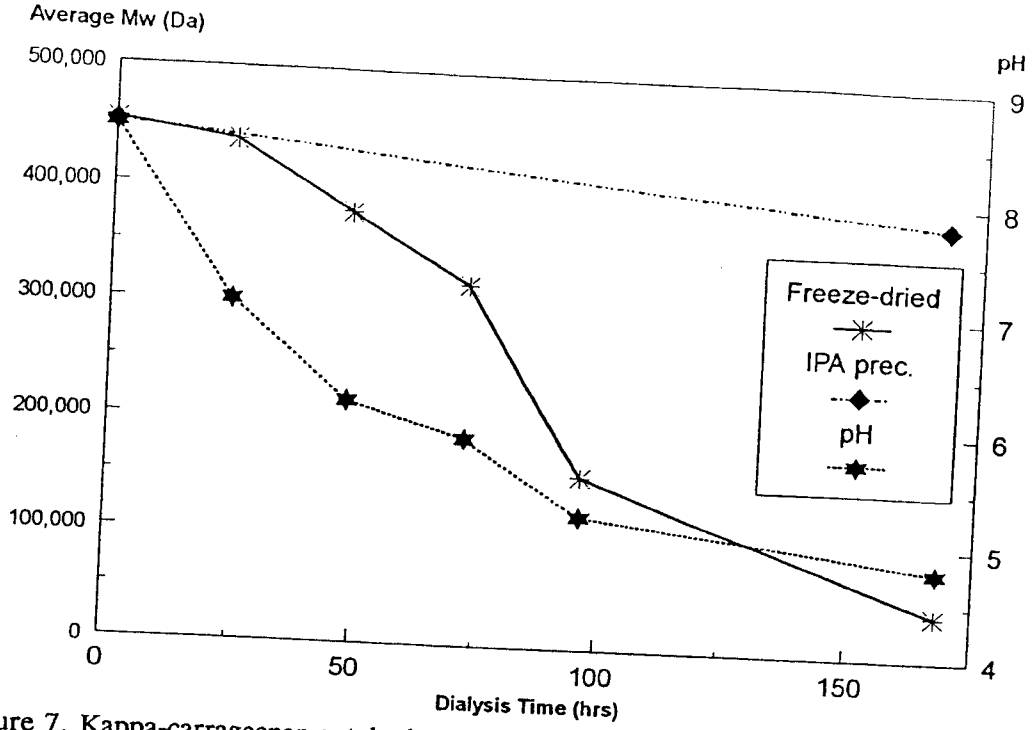

Figure 7. Kappa-carrageenan autohydrolysis at 0.2% w/v carrageenan concentration after extensive dialysis at 4°C and subsequent freeze-drying.

These observations indicate that (*kappa*-)carrageenans in the non-gelled state undergo cation-depletion, *i.e.* acidification, causing the solution pH to drop. At the dialysis temperature (4°C) this pH drop already induces some carrageenan depolymerisation. In *kappa*- (and *iota*-) carrageenan the acid sensitive α(1→3) linkage between the 3,6 anhydrogalactose and the β-galactose 4-SO_4 is in close vicinity of the sulphate at the C-4 position of the galactose unit. Concentrating the sample during freeze-drying will lower the pH at a local level (when sulphate is in excess) causing a further, more dramatic depolymerisation. However, if the carrageenans are kept in their aggregated state they retain most of the sulphate-bound cations (potassium). This prevents acidification of the carrageenan and thus depolymerisation during concentration/drying.

That depolymerisation continued after drying was clearly seen when storing the depolymerised sample (Mw average 41.3 kDa, water content *c.* 7% w/w), at room temperature. The measured Mw average declined even further, down to approx. 3 kDa in 3 weeks.

Although it is considered likely that cation-depletion would in general lead to depolymerisation upon drying of sulphated polysaccharides the presence of sufficient (*i.e.* excess) cations in most systems prevents autohydrolysis. Removing cations from a poly-anion is energetically unfavourable and specific conditions are needed, *e.g.* very low ionic-conditions or ion-exchange resins. The production of commercial carrageenans does not include any such step, consequently commercial carrageenan samples contain an excess of cations. This is illustrated above, showing that Sherex 610 has *a c.* 12% excess of cations. Dialysis of this carrageenan sample and subsequent freeze-drying did not show any Mw loss.

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