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Recovery of Low Molecular Weight Carrageenan Fractions by Ultrafiltration Through Semi-Permeable Membranes – A Feasibility Study

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Recovery of Low Molecular Weight Carrageenan Fractions by Ultrafiltration Through Semi-Permeable Membranes – A Feasibility Study

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FOREWORD

This research project was funded by the Food Ingredients Forum (Project Number 00416).

It should not be assumed that any equipment, materials or chemicals specifically mentioned in this report are the only items available, or necessarily the most suitable items on the market, for purpose described.

SUMMARY

The European Commission adapted the purity criteria for carrageenan in April 2004 by imposing a limit of 5% on the low molecular weight fraction (less than 50 kD). These changes have implications for the manufacturers and users of carrageenan who need to ensure that these regulations are met. Currently, various bodies and research institutions are developing suitable methodologies for molecular weight profiling by size exclusion chromatography.

A possible alternative approach is to separate the low molecular weight fraction by transport through a semi-permeable membrane. This report describes the use of Vivaspin disposable membrane devices for separating carrageenan into permeate and retentate fractions. Two types of membrane with cut-off points 100 kD and 50 kD were used to generate permeate fractions and their molecular weights were determined using size exclusion chromatography.

These membrane units produced permeate fractions with lower mean molecular weight than the cut-off level, but these fractions have a broad molecular weight distribution and contained material of much higher molecular weight. These results suggest that the mechanism of transport through the semi-permeable membrane is kinetic in nature, i.e. the longer chains take a much longer time to pass through the membrane than the shorter chains.

These particular Vivaspin membrane units, 100 kD and 50 kD, are not suitable for separating the low molecular weight fraction from carrageenans. Membrane products with lower porosities (30 kD, 10 kD or 5 kD) might have permeation characteristics more suitable for this application, but more work would be required to investigate this possibility.

INTRODUCTION

The European Commission Scientific Committee on Food published an opinion on carrageenan in March 2003, which recognised the desirability of minimising the presence of low molecular weight carrageenan fractions. The Committee concluded that the acceptable intake should remain unchanged, but that the restrictions be tightened by imposing a limit of 5% for the molecular weight fraction less than 50,000 Daltons. Consequently, the European Commission, in its amending Directive of April 2004, adapted the existing regulations for carrageenan by including the tighter purity criteria.

These regulatory changes have implications for the carrageenan manufacturers and users, who now need an accurate method for the quantification of the low molecular weight fraction (< 50 kD) of carrageenans. The trade body representing carrageenan manufacturers, Marinalg, is currently developing a method based on size exclusion chromatography and light scattering photometry (SEC/MALLS). Leatherhead also uses this technique for molecular weight characterisation, and is developing this approach independently of Marinalg.

A possible alternative approach is to separate the low molecular weight fraction using semi-permeable membranes with specified molecular weight cut-off characteristics. This research explores the effectiveness of commercial semi-permeable membranes for separating the low molecular weight fraction of carrageenan. The Vivaspin range of membranes, manufactured by Sartorius, was originally developed to isolate protein fractions and is manufactured with a range of porosities and associated molecular weight cut-off points. As far as we are aware, there is no literature describing the use of these membranes for the separation of polysaccharide fractions, and the manufacturers have no knowledge of their effectiveness for polysaccharide fractionation. In this research, carrageenan samples have been subjected to ultrafiltration through these membranes under centrifugal forces. Both retentate and filtrate have been characterised by size exclusion chromatography to establish their molecular weight distributions. In this way, the potential of this approach for determining the low molecular weight fraction of carrageenans has been assessed.

MATERIALS

This research was carried out using a sample of kappa-carrageenan, obtained from the seaweed *Eucheuma cottonii*, which was partially hydrolysed to provide carrageenans of different broad molecular weights. These were subjected to ultrafiltration through semi-permeable membranes. After extended dialysis, the purified filtrate and retentate were both characterised by size exclusion chromatography and molecular weights estimated using pullulan molecular weight standards. Low molecular weight fraction (filtrate) was quantified by measuring the area under the corresponding elution curve for filtrate.

Table I lists the ingredients that were used in this project.

Table I
List of Ingredients

Ingredients	Company¹	Specifications
κ-carrageenan	CP Kelco	Genugel X-902-02
Polysaccharide standard kit		
Pullulan 180 D	Polymer Laboratories Ltd	
Pullulan 738 D	Polymer Laboratories Ltd	
Pullulan 5800 D	Polymer Laboratories Ltd	
Pullulan 12200 D	Polymer Laboratories Ltd	
Pullulan 23700 D	Polymer Laboratories Ltd	
Pullulan 48000 D	Polymer Laboratories Ltd	
Pullulan 100000 D	Polymer Laboratories Ltd	
Pullulan 186000 D	Polymer Laboratories Ltd	
Pullulan 380000 D	Polymer Laboratories Ltd	
Pullulan 853000 D	Polymer Laboratories Ltd	
Others		
Ion exchange resin	ICN Biomedicals, Inc.	Amberlite IR-120 Na
Sodium sulphate anhydrous	Vickers Laboratories Ltd	

The Vivaspin range of concentrators is disposable ultrafiltration devices that are designed to separate permeable components under centrifugation. For this study, two Vivaspin concentrators, with molecular weight cut-off points at 50 kD and 100 kD were obtained from VivaScience Sartorius Group.

Dialysis of carrageenan solutions was performed using Visking DVT 03500 tubing with a nominal cut-off molecular weight of 3500 D.

¹ The authors are grateful to the companies for the supply of the ingredients.

METHODS

Preparation of solutions

Pullulans

0.1% (w/w) pullulan solutions were prepared by dispersing the powders in 0.05 M sodium sulphate eluant.

Carrageenans

0.1% carrageenan solutions were prepared by dispersing the powders in either 0.05 M sodium sulphate eluant or distilled water at 85°C with use of magnetic stirring. The solutions were held at 85°C for 10 min, after which ion exchange resin was added to the solutions to remove all gelling cations, free Ca²⁺ and K⁺. The mixtures were stirred for a further 10 min, and then the solutions were decanted.

Partial hydrolysis of carrageenans

The pH of the carrageenan solutions were lowered to pH 1.5 with hydrochloric acid (HCl), and then kept with continuous stirring at 40°C for 3 h and 6 h. The solutions were then neutralised with sodium hydroxide (NaOH).

Centrifugal fractionation of solutions

Aliquots (6 ml) of native and hydrolysed carrageenan solutions (0 h, 3 h & 6 h) were poured into the 50 kD and 100 kD Vivaspin concentrators. These were then centrifuged at 4000 rpm for a period of time sufficient for half the original volume of 6 ml to emerge in the filtrate. The retentate and filtrate fractions were collected and subjected to extended dialysis to remove small molecules that had washed from the membranes. The purified samples were then characterised using High Performance Size Exclusion Chromatography (HPSEC).

Purification of carrageenan solutions by extended dialysis

Fractionated carrageenan solutions were transferred into pre-conditioned Visking DVT dialysis tubing (cut-off molecular weight 3500 D) and dialysed against 0.05 M sodium sulphate eluant (plus sodium azide) for a total of 7 days, the eluant being changed after 1, 5, & 6 days. This extended dialysis routine was required to remove completely all small molecule contaminants from the carrageenan solutions.

High Performance Size Exclusion Chromatography (HPSEC)

All pullulan and carrageenan solutions were filtered through 0.45 µm membrane filters prior to HPSEC analysis.

The size exclusion profiles of the pullulans and purified carrageenan fractions were determined using an autosampler (Waters 2690 Separation Module) coupled with a refractive index detector (Waters 410 Differential Refractometer). Two injections of 100 µl per sample were made, and the samples were eluted in 0.05 M sodium sulphate at 0.5 ml/min through PolymerLab Aqua-Gel-OH columns (PL-Aquagel-OH 30, PL-Aquagel-OH 40 and PL-Aquagel-OH 60; 8 µm; 7.5 mm ID x 300 mm) set at 50°C.

The elution profiles obtained for the pullulan molecular weight standards were used to construct a calibration curve for the column set. This was used to provide estimated values for the molecular weights of the carrageenan fractions after ultrafiltration.

RESULTS

Pullulan molecular weight standards

The elution profiles associated with the pullulan standards, and with glucose (180 D) and raffinose (738 D), are shown in Fig. 1. Pullulans with decreasing molecular weights emerged from the columns at longer elution times. These profiles show that this particular column-set provides for mid-range location of the 50 kD reference point. This means that the column set should be able to separate effectively the molecular weight fractions on either side of this reference point.

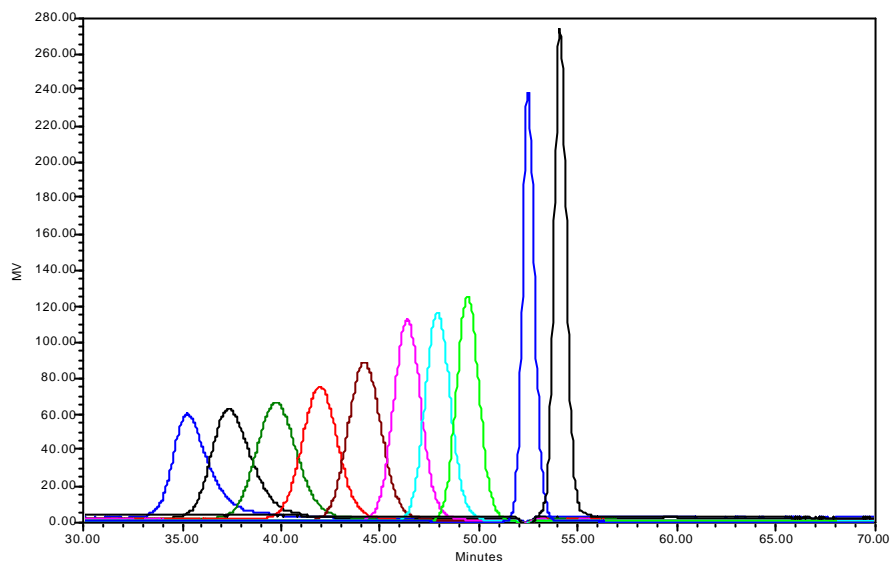


Fig. 1. Elution profiles of standard pullulans with specific molecular weights (180, 738, 5800, 12200, 23700, 48000, 100000, 186000, 380000 and 853000 D)

Fractionation of carrageenans

The elution profiles for the native carrageenan after ultrafiltration through 50 kD and 100 kD membranes are shown in Fig. 2. The filtrate (LL) and retentate (UL) fractions are shown on the same figure. It is clear that native carrageenan did not contain significant quantities of polysaccharide small enough to pass through these membranes, and the elution profiles for these filtrate fractions were devoid of peaks. The second small peak seen at 46 min elution time is attributable to eluant salt (sodium sulphate)

The elution profiles for the partially hydrolysed carrageenan after ultrafiltration through the same membranes are shown in Figs. 3 & 4. The 3 h hydrolysed carrageenan (Fig. 3) contained a small amount of material in the filtrate fraction after passing through both types of membrane. The filtrate fractions were broad and shifted towards the lower molecular weight region compared with the retentate fraction. The filtrate fraction passing through the 50 kD membrane was smaller and of lower molecular weight than the fraction that passed through the 100 kD membrane. Again, there was associated with these carrageenan fractions a small peak attributable to the eluant salt.

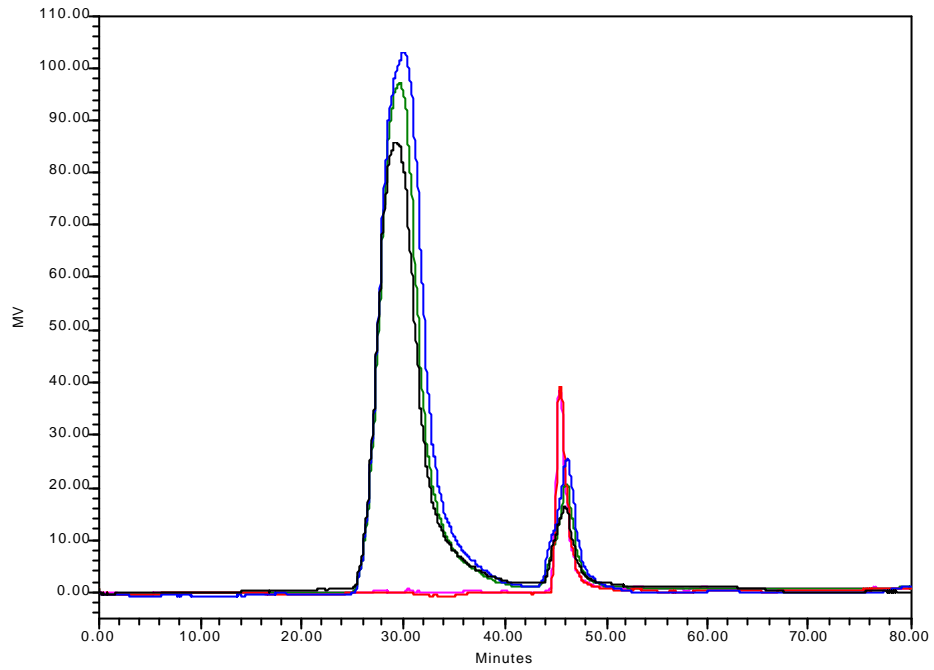


Fig. 2. Elution profiles for native carrageenan (0 h) after ultrafiltration retentate (UL), filtrate (LL)

0h 0h UL 50kD 0h LL 50kD 0h UL 100kD 0h LL 100kD

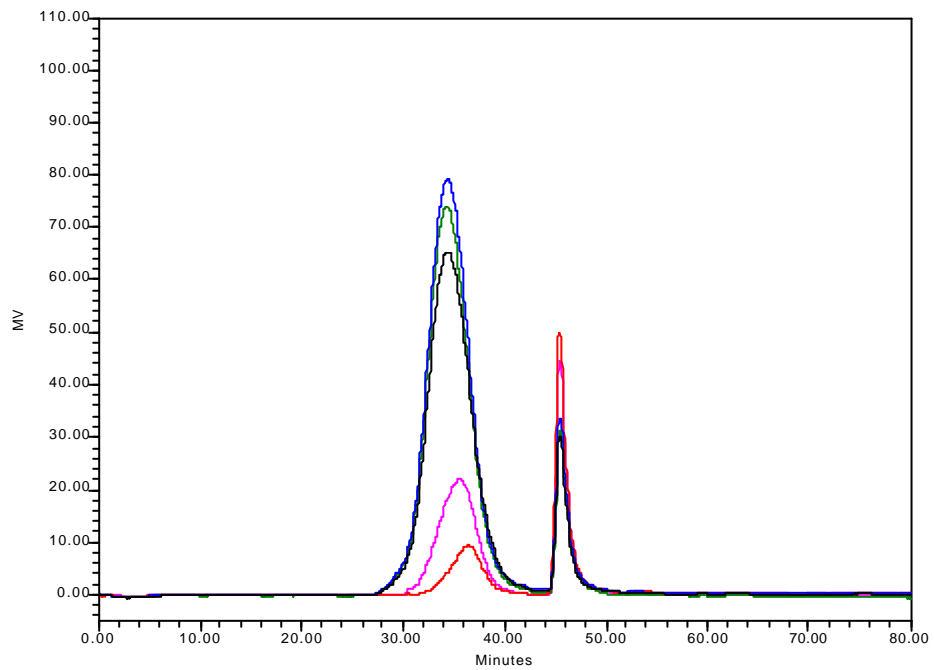


Fig. 3. Elution profiles for 3h-hydrolysed carrageenan after ultrafiltration retentate (UL) & filtrate (LL)

3h 3h UL 50kD 3h LL 50kD 3h UL 100kD 3h LL 100kD

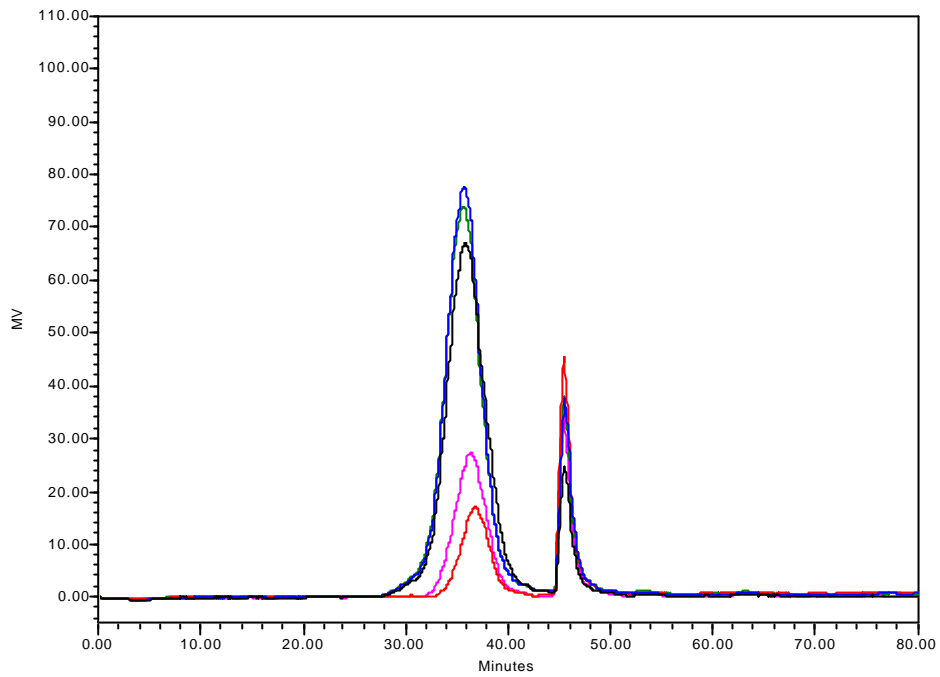


Fig. 4. Elution profiles for 6h-hydrolysed carrageenan after ultrafiltration retentate (UL) & filtrate (LL)
 6h 6h UL 50kD 6h LL 50kD 6h UL 100kD 6h LL 100kD

The filtrate fractions of the 3 h and 6 h hydrolysed carrageenans were characterised by peak elution time and peak area, expressed as a percentage of the total area for both filtrate and retentate. The peak elution times were converted into peak molecular weights using the calibration curve (Fig. 5) that was generated using the peak elution times for the pullulan molecular weight standards.

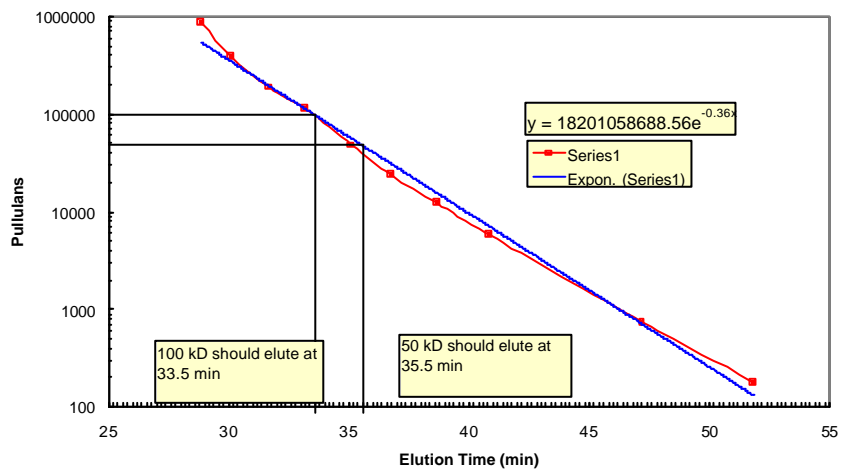


Fig. 5. HPSEC calibration plot of pullulan molecular weight against elution time

The primary elution data obtained for retentate and filtrate fractions of both 3 h and 6 h hydrolysed carrageenans are recorded in Table II, which shows the areas and elution times associated with the carrageenan fractions (duplicate injections). Average values for peak position and peak areas were used to calculate peak molecular weights and percentages for the filtrate and retentate fractions.

Table II. Elution data for hydrolysed carrageenans after ultrafiltration: retentate (UL) & filtrate (LL)

Sample	UL & LL	Cut-off	Inject	Area	Peak	Av Area	Av Peak
0h	-	-	1	24458063	29.318		
0h	-	-	2	24342898	29.351	24400481	29.3
0h	UL	50000	1	32390062	30.079		
0h	UL	50000	2	32381494	30.076	32385778	30.1
0h	LL	50000	1				
0h	LL	50000	2				
0h	UL	100000	1	27303235	29.698		
0h	UL	100000	2	27305455	29.574	27304345	29.6
0h	LL	100000	1				
0h	LL	100000	2				
3h	-	-	1	20396883	34.412		
3h	-	-	2	20452955	34.409	20424919	34.4
3h	UL	50000	1	23714978	34.395		
3h	UL	50000	2	23673717	34.395	23694348	34.4
3h	LL	50000	1	2219915	36.416		
3h	LL	50000	2	2128334	36.403	2174125	36.4
3h	UL	100000	1	22318488	34.307		
3h	UL	100000	2	22341097	34.318	22329793	34.3
3h	LL	100000	1	6047048	35.574		
3h	LL	100000	2	6023791	35.574	6035420	35.6
6h	-	-	1	18641139	35.88		
6h	-	-	2	18570998	35.882	18606069	35.9
6h	UL	50000	1	19928354	35.733		
6h	UL	50000	2	19992778	35.733	19960566	35.7
6h	LL	50000	1	3422873	36.795		
6h	LL	50000	2	3412244	36.792	3417559	36.8
6h	UL	100000	1	19375647	35.652		
6h	UL	100000	2	19322111	35.662	19348879	35.7
6h	LL	100000	1	6210573	36.385		
6h	LL	100000	2	6255052	36.375	6232813	36.4

The calculated values for peak molecular weights and percentages for both retentate and filtrate fractions are shown in Table III. The percentages associated with the lower molecular weight filtrate fractions were calculated using twice the area associated with the filtrate peak (LL) and the total area associated with both filtrate and retentate peaks (UL + LL). The filtrate peak only represents one half of the lower molecular weight fraction as only half the sample volumes were allowed to pass through the membranes on ultrafiltration, which means that half the lower molecular weight fractions remained in the upper layer.

Table III. Estimated percentages and peak molecular weights of low molecular weight fractions (LL) obtained from hydrolysed carrageenans by ultrafiltration

Sample	Area av.	% area LL	Peak av.	Mol.wt.kD
0h	24400481		29.3345	471.8
0h UL 50kD	32385778		30.0775	361.1
0h LL 50kD				
0h UL 100kD	27304345		29.636	423.3
0h LL 100kD				
3h	20424919		34.4105	75.9
3h UL 50kD	23694348		34.395	76.3
3h LL 50kD	2174125	16.8	36.4095	37.0
3h UL 100kD	22329793		34.3125	78.6
3h LL 100kD	6035420	42.6	35.574	49.9
6h	18606069		35.881	44.7
6h UL 50kD	19960566		35.733	47.2
6h LL 50kD	3417559	29.2	36.7935	32.2
6h UL 100kD	19348879		35.657	48.4
6h LL 100kD	6232813	49.2	36.38	37.3

The 3 h hydrolysed carrageenan had a peak molecular weight around 76 kD. The 50 kD membrane separated this carrageenan into high and low molecular weight fractions peaking at 76 kD and 37 kD respectively. The lower molecular weight fraction represented roughly 17% of the total carrageenan. The 100 kD membrane produced a low molecular weight fraction of 50 kD and a quantity of 43%.

As would be expected, the 6 h hydrolysed carrageenan had a lower molecular weight (45 kD) and contained more of the lower molecular weight fraction, roughly 29% of the total according to the 50 kD membrane and 49% of the total according to the 100 kD membrane. The low molecular weight fraction that passed through the 50 kD membrane had a peak molecular weight of 32 kD. The fraction that passed through the 100 kD membrane had a molecular weight of 37 kD.

The breadth of the elution profiles for the filtrate fractions suggests that these membranes are permeable to a broad range of molecular weights, exceeding their nominal cut-off points. It is likely that membranes with a lower cut-off point than 50 kD would be required to retain all carrageenan polymers with molecular weight greater than 50 kD.

DISCUSSION

The membranes used in this study are normally used for fractionating globular proteins and have been calibrated using molecular weight standards such as bovine serum albumin, immunoglobulins and latex beads. Globular proteins are compact, spherical and uniform in size. They can be separated efficiently from bodies of different size using semi-permeable membranes. Carrageenans, in contrast, are highly extended, flexible chain polysaccharides having a broad range of chain lengths and correspondingly broad molecular weight distributions. Being extracted from the native state by a process of random hydrolytic scission, carrageenans have a normal (gaussian) molecular weight distribution, and the peak molecular weight can be described as the mean of the distribution. The breadth of the distribution typically ranges across decades of molecular weight ($5 \times 10^5 - 5 \times 10^3$ Daltons)

The transport of flexible chain molecules through semi-permeable membranes is determined largely by the effective occupied volume of the chains and by their ability to move through the porous network structure of the membrane. This is to some extent a kinetic process as large macromolecules are subject to viscous-drag, and to shear-dependent deformation.

These results indicate that carrageenans can be separated into fractions of different molecular size by passage through semi-permeable membranes. The partially-hydrolysed carrageenans illustrate this fact as they contain higher levels of low molecular weight material. The elution profiles suggest that the membranes allow through a low molecular weight fraction and retain a high molecular weight fraction. Both fractions have a broad distribution of molecular sizes.

The 50 kD membrane is designed to allow passage to macromolecules less than 50 kD in size. The data shows that a carrageenan having a mean molecular weight of 76 kD (3 h hydrolysed) yields a permeate fraction with a mean molecular weight of 37 kD. However, the breadth of the elution profile suggests the presence of very much higher molecular weights within this permeate fraction. The estimated content of this fraction is 17%. The hydrolysed carrageenan with a mean molecular weight of 47 kD (6 h hydrolysed) contains a permeate fraction with a mean molecular weight of 32 kD, at 29% content. Again, the breadth of the distribution indicates that molecules much larger than 50 kD are able to permeate these membranes.

The 100 kD membrane should allow much larger molecules to pass through, but the mean molecular weights associated with the permeate fractions suggest that these membranes discriminate less on the basis of molecular size than do the 50 kD membranes.

The ability of this type of semi-permeable membrane to separate and quantify the low molecular weight fraction of carrageenan, as defined by the EU purity criteria, remains open to question. The absence of a permeate fraction in the native carrageenan is surprising since analysis by size exclusion chromatography suggests that a small percentage (< 5%) of this fraction is present. The reason for this may lie in the kinetics of the transport of short chains through the membrane. The transport of short chains may be restricted by the high molecular weight chains that are retained by the membrane. Further work using extended centrifugation to completely separate the permeate from retentate fractions would be necessary to throw light on this aspect. Also, the breadth of the permeate elution profile suggests that significant amounts of carrageenan with molecular weights far in excess of the cut-off size (say 50 kD) are present in the permeate.

Membranes of reduced pore size might display cut-off characteristics closer to the required level of 50 kD. The Vivaspin disposable ultrafiltration devices have a range of porosities with nominal cut-off points of 100 kD, 50 kD, 30kD, 10 kD and 5 kD. An extension of this work to investigate the effectiveness of the 30 kD, 10 kD and 5 kD membranes for separating the low molecular weight fraction of commercial carrageenans is recommended so that their potential value for the analysis of carrageenans in relation to current regulations can be established.

CONCLUSIONS

Vivaspin membrane ultrafiltration devices are capable of separating native carrageenan into permeate (high molecular weight) and retentate (low molecular weight) fractions. The permeate fraction has a lower mean molecular weight than the retentate fraction, showing that transport through the membrane is determined partly by molecular size. However, the permeate fraction has a broad molecular weight distribution. These membranes are not characterised by a sharp cut-off point in terms of molecular size.

The transport mechanism is probably kinetic in nature, that is, in any given carrageenan sample, the shorter chain molecules move through the membrane faster than the longer chains. This is partly due to the time-dependent distortions in the flexible carrageenan chains and also the viscous drag associated with the longer chains. Further work on separations carried out for different times and higher centrifugation speeds would be required to clarify this point.

The 50 kD Vivaspin membrane may not be suitable for the determination of the low molecular weight (< 50 kD) fraction of commercial carrageenans. Membranes with lower cut-off points might be more suitable but further work is needed to establish their effectiveness.

ACKNOWLEDGEMENTS

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REFERENCES

Haines, J. and Patel, P. D. (1996) *Rapid estimation of degraded carrageenans by simple enzyme-linked protein-binding assays* Leatherhead Food International Report N°. 730.

European Commission Health & Consumer Protection Directorate-General. Scientific Committee on Food. SCF/CS/ADD/EMU/199 Final. 21st February 2003. *Opinion of the Scientific Committee on Food on Carrageenan*.
<http://europa.eu.int/comm/food/fs/sc/scf>.