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STATUS REPORT ON THE WORK OF MARINALG INTERNATIONAL TO MEASURE THE MOLECULAR WEIGHT DISTRIBUTION OF CARRAGEENAN AND PES IN ORDER TO MEET THE EU SPECIFICATION: LESS THAN 5% BELOW 50,000 DALTONS

FULL REPORT

1.0 INTRODUCTION

The following report describes the results of Marinalg's work concerning the attempted identification of a validated test method to determine the low molecular weight tail (LMT) of carrageenan and PES. The work is described in the chronological order as performed since 2003

The Marinalg Working Group on Molecular Weight Determination (William Blakemore, Celtic Colloids Inc, representing FMC, Chairman; Dr. Harris Bixler, SIAP, Secretary; Arne Graff Anderson, C.P.Kelco; Dr. Markus Klinger, Danisco; Dr. Patrick Boulenguer, Cargill) has been carrying out experiments since April, 2003 to try to measure the molecular weight distribution of commercial carrageenan and PES used in foods. In its March 5, 2003 opinion, the EC-SCF proposed a new specification, "*if feasible*", for these hydrocolloids to augment the 5 cps water viscosity, the surrogate specification for molecular weight.

The purpose of the new specification is for the European Commission to have better control over the molecular weight distribution of carrageenan used in foods. In particular, the EC has decided that there should be no more than 5% of the carrageenan molar mass that is less than 50k Da in molecular weight in carrageenan for food use. Marinalg believes that there is no toxicological evidence to support the establishment of the specific numerical values in the specification, but a brief review of the history will help in the understanding the EC's action.

Starting in the 1960s, evidence was accumulating that very low molecular weight sulphated polygalactose, later named poligeenan, caused ulcerative colitis or its precursor, Kupfer cell proliferation, in certain rodents when they were fed high dosages of poligeenan using non-standard feeding regimens. One such regimen involved feeding the poligeenan in a solution made up with drinking water but with the test animals having no other source of water. Hydrocolloids are well known water absorbers, so the animals were in effect being robbed of water needed for good health. The harmful effect of the poligeenan was compounded by the stress imposed on the animals by the feeding regimen that was used.

The EC imposed the molecular weight distribution specification on carrageenan to assure consumers that carrageenan for food use did not contain appreciable

quantities of poligeenan-like substance in the low molecular weight distribution tail of carrageenan for food use.

As an item of commerce, poligeenan is used in medical imaging; carrageenan is used as a starting material. The carrageenan raw material is deliberately acid hydrolyzed at high temperatures for extended periods of time to produce a finished product with an average molecular weight (Mw) of 10,000 – 20,000 Daltons. Carrageenan extracted from red seaweeds has a normal or Gaussian distribution of molecular weight with average Mw of 300,000 to 1,000,000 (Refs: Annexes IV, VII, and VIII). There is no evidence from the work discussed in this report that carrageenan for food use has a bi-modal or skewed distribution that would result in an abnormally large fraction in the Mw range of poligeenan. Therefore, animals fed carrageenan in chronic and sub-chronic feeding studies ingest this normal distribution of molecular weights. There is neither toxicological evidence from animal studies nor epidemiological evidence from observation of the effects of consumption of carrageenan by a broad segment of the population to indicate that commercial carrageenan for food use is unsafe for human consumption. The effect of the very small amounts of lower molecular weight components in carrageenan is of no significance to human health.

Carrageenan manufacturers have often been questioned about the possibility of adulterating carrageenan with poligeenan and thereby creating a harmful product. There is no commercial incentive for adulterating carrageenan with poligeenan. Poligeenan is much more expensive than carrageenan, and blending it with carrageenan would simply lower the carrageenan's functional properties in foods.

2.0 STARTING THE EXPERIMENTAL PROGRAM

Before the Marinalg Working Group of carrageenan producers had adequate time to determine the feasibility of measuring the new specification, it was formally adopted by the EC as Commission Directive 2004/45/EC on April 16, 2004 for implementation by Member States by April 1, 2005. This specification requires that carrageenan or PES used in food must not contain more than 5% molar mass with molecular weight less than 50,000 Da. The industry refers to this as the "low molecular weight tail" or LMT.

As of December 2011, the Working Group still has not been able to develop a method for molecular weight distribution measurement that is sufficiently accurate and reproducible to yield a validated and defensible method, and not so costly as to act as a barrier to trade for smaller producers.

2.1 SIZE EXCLUSION CHROMATOGRAPHY & LIGHT SCATTERING

The methods studied in detail from 2003 to 2008 were all based on size exclusion chromatography (SEC). SEC is used to spread out the carrageenan molecular size distribution in the flow stream exiting the columns so that narrow molecular weight fractions can be collected for analysis. Note that this separation is by molecular size and not molecular weight, so physical models must be used to convert molecular size data to molecular weights. The stream exiting the SEC columns flows through a series of detectors: refractive index for carrageenan concentration determination, and

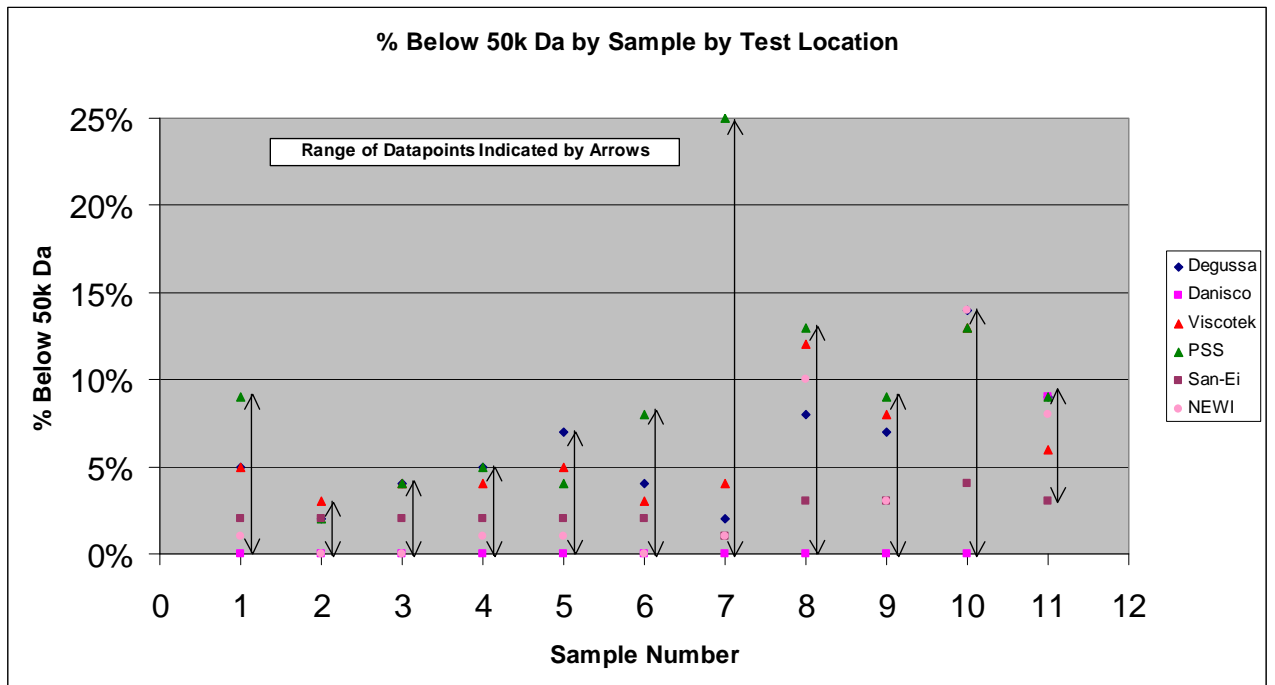
light scattering and/or intrinsic viscosity for molecular weight determination. Some instruments include chemical detectors to be sure only carrageenan is being measured in the flow stream. The Japanese Ministry of Health method mentioned in the Summary is one such method.

These are highly developed commercial research instruments of great technical sophistication. Nevertheless, none met the most important objective of the Working Group, i.e. method validation. Six laboratories participated in this study, Degussa (now Cargill); Danisco; Viscotek, Ltd. (now Malvern Ltd.); Polymer Standards Services, GmbH; San-Ei Gen FFI, Ltd; North East Wales Institute/NEWI, all with state-of-the art equipment and with qualified scientists to run the experiments. Procedure details (sample preparation and concentration, eluent type and concentration, etc.) were recorded for each lab and approved by the Working Group. Eleven different commercial carrageenan and PES samples, representing different sulphated polygalactose types (nominally kappa, lambda and iota) made by five different producers, were tested by all laboratories under "Round Robin" conditions.

2.2 RESULTS FOR SEC/LS

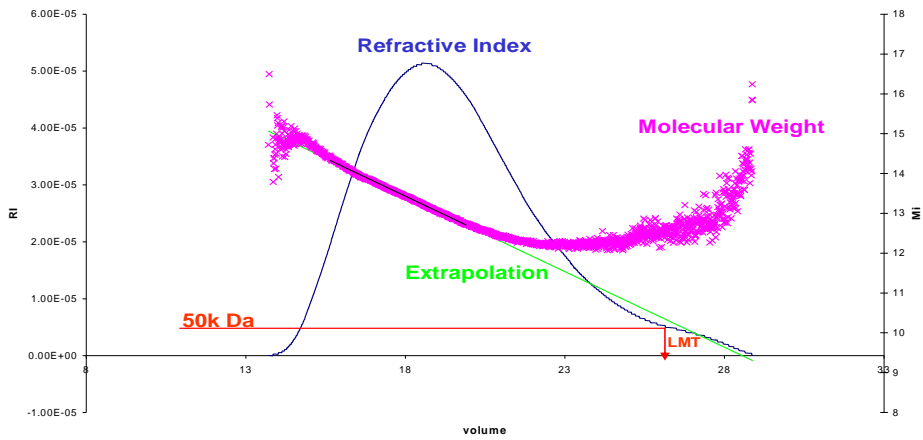
Annex I (Summary of Round Robin #1 and #2) and Annex II (Sample Information) contain test results and physical characteristics of the Round Robin samples, respectively. Despite all the effort to impose technical discipline, inter-lab reproducibility of the LMT was poor as shown in Fig. 1. Detectors downstream of the SEC columns must be able to measure polymer concentration and molecular weight accurately in the range represented by the LMT.

Figure 1



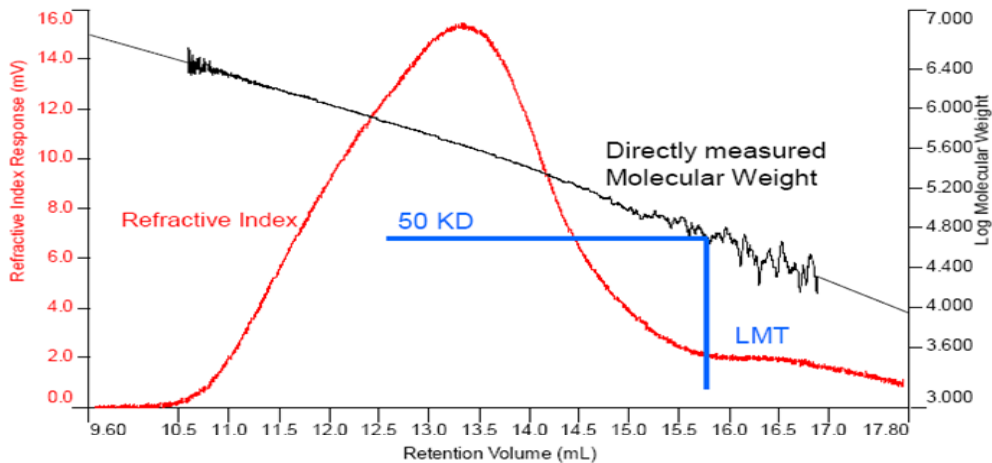
It appears that even under optimum SEC conditions, light scattering detector signal to noise ratio (S/N) in the LMT region is extremely low, and it is this signal upon which molecular weight determination is based (Fig. 2). The LS signal goes non-linear (called the “swish” by some) well before the LMT has exited the column. As if this were not bad enough several test locations experienced drifting baselines. This led to variable recoveries (input of carrageenan to the SEC column did not equal the carrageenan in the effluent stream). These added problems make the LMT estimates even more unreliable.

Figure 2
 Typical output from SEC/RI/MALLS - Degussa Data



Initially the Working Group thought that the Viscotek triple detector method (refractive index, low angle light scattering and intrinsic viscosity) was giving promising results, and as a consequence published the method on the Marinalg website. However, further testing indicated that these same issues applied, but to a much lesser degree (Fig. 3).

Figure 3
 Typical output from SEC/RI/LALS/RALS - Viscotek Data



2.3 DISCUSSION SEC/LS

Various fitting and extrapolation routines (Zimm, Debye, Berry) are being used in the MALS detection systems software to determine Mw where there is enough S/N. This

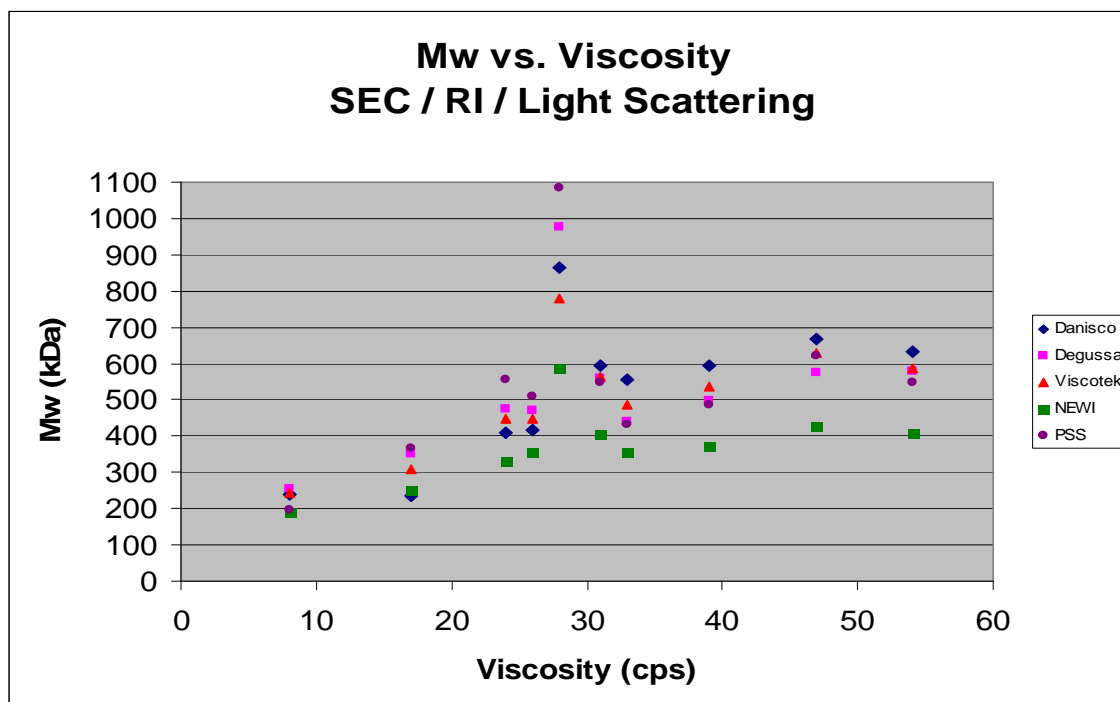
data is then further extrapolated by some routine (usually linear) into the region of poor S/N. For all of the carrageenan samples studied the S/N within the LMT was low and resulted in extrapolations having to be made from well outside the LMT range (Fig. 2, green line). This type of extrapolation is subject to enough error so as not to give defensible results for regulatory purposes. This can be seen in Fig. 2 where the extremely small LMT region is shown graphically. Clearly any shift in baseline or green line extrapolation will have a profound effect on the very small LMT region calculated for commercial carrageenan being used in foods. It is estimated that it is virtually impossible to determine the molecular weight of SEC-spread samples below about 10,000 Da by any of the light scattering techniques, and this is the molecular weight region most likely to raise toxicological concerns.

Whilst no method was found to measure the entirety of the LMT, the Viscotek SEC/LALS method delivered the most consistent and repeatable results when comparing final outputs and data from duplicate runs of the same sample, either from the same or different solution preparations. This is likely due to the LALS approach using direct measurement of the molecular weight without any fitting of the data to molecular size / molecular weight models, because measuring at a low angle avoids the angular dependence of the scattered light. Also, the Viscotek application of RALS in combination with LALS greatly improves the signal to noise ratio, allowing direct measurement of molecular weight to much lower levels than by MALS (compare Figures 2 & 3), thus reducing, but not eliminating the impact of extrapolation techniques. Also, the inclusion of a viscometer by Viscotek to measure eluate intrinsic viscosity helps to maximize sample solution concentration before overloading of the SEC columns. Nevertheless, the Viscotek methodology still did not provide sufficient reproducibility to qualify for a validated method for regulatory use.

The Working Group's experience with SEC/light scattering in no way detracts from its use as a valuable research tool. The technique is widely used for estimating polymer molecular structure in food and industrial applications. A higher level of accuracy, however, is required when it is to be used for specification and regulatory measurements.

Even in the present study useful information (from the Round Robin samples) was obtained on molecular structure. For instance, fairly good consistency was seen for inter-lab results obtained for the weight average molecular weight (M_w) (Fig. 4), except for one sample in Fig. 4 and one laboratory not included in Fig. 4. The aberrant sample was a lambda type that is known to be a more rigid rod in solution than the kappa and iota types, and thus making the calculated M_w more sensitive to the model used to convert molecular size to molecular weight. Furthermore there was fairly good correlation for M_w versus water viscosity (Fig. 4), except for the one aberrant sample already noted. The laboratory whose results seemed out of line was San-Ei. On average the San-Ei calculated M_w were about 2x those from other labs except for the lambda aberrant sample where San-Ei reported M_w values generally less than those from the other labs. San-Ei was the one lab using column calibration, and that calibration was done with pullulan samples rather than simply extrapolating LS results into the LMT region. The higher reported M_w by San-Ei is consistent with literature references on determining M_w of other hydrocolloids with pullulan column calibration. (Ref: Benoit, Annex IX)

Figure 4



The causes of these inconsistencies and poor correlations when using SEC/LS need to be identified before further progress on method development can be attempted. In addition to the mathematical errors from extrapolation, possible interferences would also include counting non-carrageenan components as part of the LMT. No steps were taken to remove the salts and other carbohydrates generally present as standardizing agents in commercial carrageenans for food use. Other inaccuracies crept in by inconsistent measurement of cut-off points and incomplete dissolution of the carrageenan (e.g. gelation and aggregation) leading to poor SEC separation. SEC column selection and previous uses of columns also contribute to variable results.

3.0 COLUMN CALIBRATION TO AUGMENT LIGHT SCATTERING

While the work to date with light scattering has led to frustrating results, it has pointed in a direction of potentially more promise which will be explored by the Working Group. Light scattering became dominant in the measurement of polymer molecular weight distributions because molecular sizes exiting a SEC column over most of a samples' range (except the LMT) could be determined directly. Prior to this advancement, column calibration with molecular weight standards had to be used.

This technique involves preparing a calibration curve of exit time from the SEC column versus molecular weight for a set of standards of very narrow molecular weight distribution ($M_w/M_n < 1.2$) (M_w/M_n is also referred to as polydispersity index or PDI). The molecular weight of the polymer standards is now usually determined by light scattering. No SEC is required when the molecular weights of the standards are being determined because of their low polydispersity, and sample concentrations can be adjusted to optimize the S/N ratio. The polymer standards must encompass the molecular weight range of interest, i.e. the range represented by those used in the current SEC study (Annex I), and most particularly must include one or more samples in the LMT range

For water soluble hydrocolloids, the most widely used standards are eight pullulans ranging in M_w from 5,300 to 760,000 Daltons that are commercially available from Shodex. This method has been tested on commercial carrageenans, and the results have been reported in the scientific literature by Japanese scientists (Uno, *et al*, Annex VIII). No correction was applied in this work for the differences between pullulan and carrageenan molecular sizes versus molecular weights, so validation of the LMTs reported by Uno remains in question.

Of course, having a set of carrageenan standards would be preferable and the Working Group is exploring the preparation of such a set. It should be noted, however, that producing carrageenan standards with $PDI < 1.2$ will be very difficult, and from past experience PDI values would be expected to be 1.6 at best and more likely closer to 2.0, probably outside the range of PDI needed for LMT accuracy.

The difficulties in obtaining carrageenan standards has lead the Working Group to explore the application of a technology referred to as "universal calibration", a physical model for converting a pullulan calibration curve to a carrageenan calibration curve (Grubisic, *et al*, Annex XI). The model takes into consideration size and shape differences for the two polymers when their molecular weights are the same. Initial work applying this technique to the Uno data shows some promise, but it is too early to draw any conclusions.

A related technology referred to as "polydisperse or broad standard calibration" is also under investigation (Malawer, *et al*, Annex X). For this purpose, a very broad molecular weight distribution carrageenan is prepared as a standard that has relatively high concentrations of carrageenan in the low and high molecular weight tails and spans the range of M_w of interest. Again, physical modeling and computer analysis is employed to convert SEC exit time to a carrageenan molecular weight.

There is no assurance until additional experiments can be run to know whether the poor accuracy of LMT calculation from light scattering can be improved upon by use of either universal or polydisperse calibration. The potential to apply carrageenan standards to such calculations is reviewed in the next section.

3.1 PREPARATION AND ANALYSIS OF CARRAGEENAN STANDARDS

In 2009/2010 carrageenan standards were prepared by the deliberate degradation of kappa carrageenan extract from *K. alvarezii* using a range of concentrations of ammonium persulphate. The analyses of these standard samples are detailed in Table 1, focusing on standard water viscosity (Brookfield Viscometer 1.5%, 75°C), weight average molecular weight (Mw), number average molecular weight (Mn), and polydispersity index (PDI, Mw/Mn), and shown graphically in Fig. 5. The trendlines are logarithmic with excellent correlations.

Table 1 – Analysis of Kappa Carrageenan Standards

Reference	Viscosity (cps) (1.5%, 75°C)	Mw (Da)	Mn (Da)	PDI
G-2409-145	60	599,700	379,200	1.58
G-2409-144	39	561,000	417,800	1.34
G-2409-146	8.8	246,500	165,000	1.49
G-2409-143	5.2	187,000	108,500	1.72
G-2409-147	4.1	103,000	82,000	1.26
G-2409-148	3.3	86,000	68,000	1.26
G-2409-149	3.1	73,000	45,000	1.62
G-2743-5	2.9	56,000	14,000	4.00
G-2743-6	2.6	24,000	4,800	5.00
G-2743-7	2.1	12,000	3,400	3.53

Figure 5

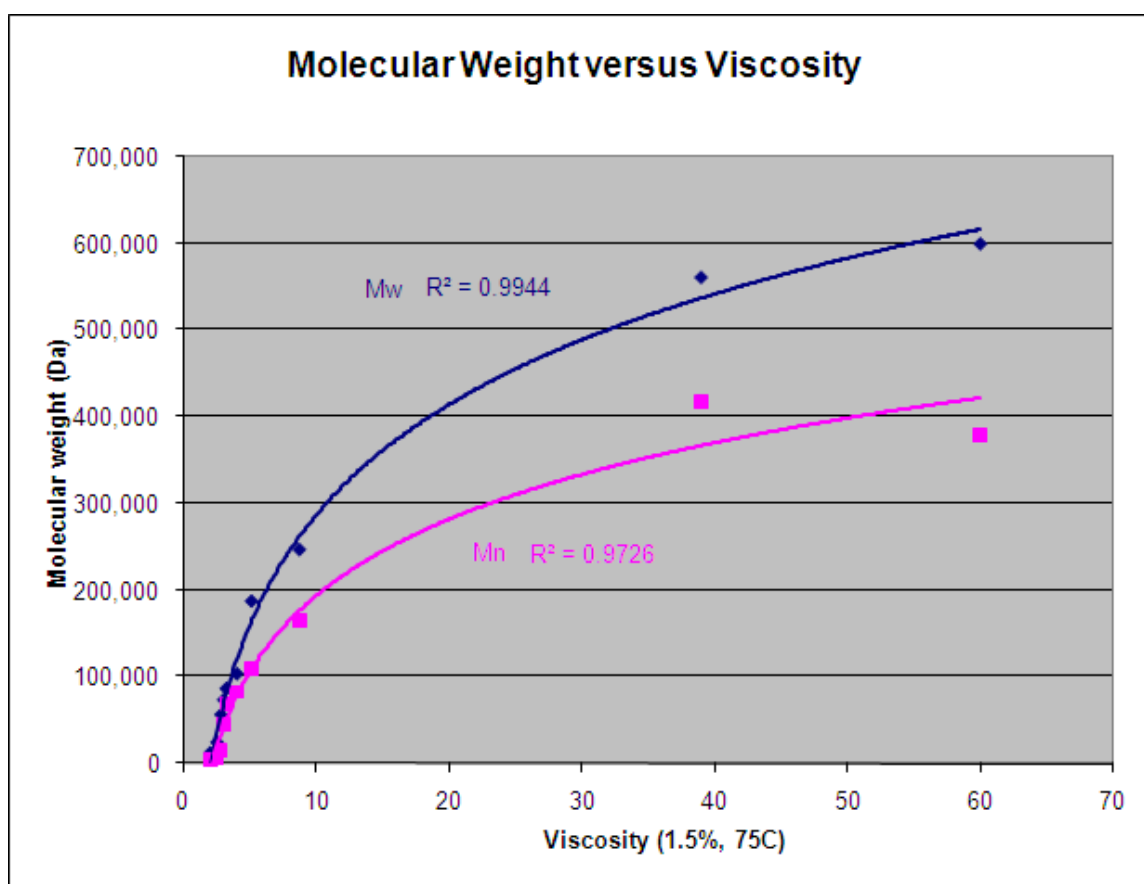


Table 1 shows that the PDI values of the lower molecular weight carrageenan standards are in the range 3.5 – 5.0, much higher than the pullulan equivalents, and too polydisperse for the accurate application of “universal or polydisperse column calibration”. It seems doubtful at this time lower PDI values can be obtained for low Mw carrageenans with the current technology being used to make the carrageenan standards.

4.0 SEC/LS WORK OF OTHERS

The only additional relevant publication using SEC/LS to analyze carrageenan is by Spichtig and Austen of Nestle Research Centre in Lausanne, Switzerland (Annex VII). This research work essentially confirms the conclusions of the Marinalg Working Group with respect to measuring the LMT of carrageenan using SEC/LS. Their method developed using HPSEC/RI to measure the LMT is an excellent research tool for the relative comparison of similar carrageenan samples. However, although the generated data confirm consistency of results, method validation, including spiking and recovery, was not attempted. Absolute and accurate measurement of the LMT for specification compliance would require significant additional work with no guarantee of success. In addition, the reported LMT range of 3.4 - 7.9% for carrageenan samples with Mw 535k to 889k Da. respectively is higher than the most probable LMT range determined by the Marinalg Working Group for carrageenans of

comparable Mw. This suggests that their LMT data may include components other than pure carrageenan, but confirmation would require the measured recovery data from the SEC/RI spectra.

The suggestion to consider a specification of “no more than 8% LMT” rather than “no more than 5% LMT” endorses the conclusion that we do not yet have a viable method.

The data indicated that processing at pH values above 4.0 had little or no effect on Mw or LMT values. This endorses the well-established position to avoid processing carrageenan at low pH for long times at high temperatures.

5.0 CONCLUSIONS FOR SEC/LS

The Marinalg Working Group concludes that SEC/LS as currently practiced cannot measure the LMT with the accuracy required by EC-SCF (now EFSA) and corresponding LMT specification. In reaching its conclusions, the Working Group has conferred with several world class scientists (Prof. Wayne Reed, Tulane University, Dr. Phillip Wyatt and his staff at Wyatt Technology, and the group consisting of Drs. Chi-San Wu, E. Malawer and L. Senak at ISP and Dr. Maguarite Rinaudo at CMRV) who have been involved in developing and using SEC/light scattering for a variety of research purposes. While some were confident that the Working Group’s goal could be reached, none had ever done so. Through this process consensus was gradually reached that the current equipment employing light scattering and the attendant software will not measure the EC specification with sufficient accuracy to survive the necessary validation protocols.

Preliminary attempts have been made to try to measure the LMT in a commercial kappa carrageenan deliberately blended with known levels of LMT derived from poligeenan. However, measurements of the total LMT in these synthetic blends by the Degussa, Danisco, San-Ei, and Viscotek facilities were all inconsistent with and far removed from the calculated values. The earlier glimmer of hope with the Viscotek method was dimmed by these results. The failure to accurately detect and quantify known levels of LMT confirms the position that development of a validated method using current SEC/light scattering technology is not feasible.

6.0 ULTRAFILTRATION

Ultrafiltration (UF) is the application of semi-permeable membranes to the separation of solutes of different molecular sizes. Pressure is exerted on a solution of the test material in contact with a membrane of known porosity, and solutes of high molecular weight are retained (retentate), while water and low molecular weight solutes pass through the membrane (permeate or filtrate). Pressure may be caused directly by hydrostatic means or indirectly by centrifugation.

There are many complications in applying UF technology to the LMT specification. First and foremost, membranes do not have a uniform porosity passing only molecules smaller than the manufacturer’s nominal cut-off. Furthermore, applying

pressure to induce flow through the membrane can build up a gel layer of retained molecules that has the effect of reducing the nominal molecular size cut-off of the membrane. This effect, known as polarization, can be alleviated to some extent by applying shear to the upstream membrane surface through stirring or high shear flow across the membrane surface.

There was no expectation that ultrafiltration could yield a permeate containing all of the carrageenan in solution less than 50k Da (the LMT). However, it was hoped that the carrageenan in the permeate was in some way proportional to the total LMT

6.1 LEATHERHEAD FOOD INTERNATIONAL

This work titled “Recovery of low molecular weight carrageenan fractions by ultrafiltration through semi-permeable membranes – a feasibility study” (Annex IV) was published in October, 2005, and pre-dates the Marinalg work on UF. The full report appears in Annex IV. At the time this report was restricted to LFI’s members, so the Marinalg Working Group did not learn about it until they had decided to try UF. Two semi-permeable membrane units (Vivaspin), with cut-offs of 100k Da and 50k Da, were used to generate permeate fractions and their molecular weight profiles determined using size exclusion chromatography (HPSEC).

The native carrageenan used was kappa carrageenan from “*Eucheuma cottonii*” (*Kappaphycus alvarezii*), and several samples of deliberately hydrolyzed “carrageenan” were made from this starting carrageenan.

Pullulan standards were used to generate a calibration curve, which produced the same validation errors as discussed in the previous section on SEC/LS.

The membranes used in the study are normally used for fractionating globular proteins, and calibrated as such using molecular weight standards such as bovine serum albumin, all of these proteins being compact, spherical, and uniform. On the other hand, carrageenans are highly extended, flexible chain polysaccharides with much broader molecular weight distribution. This means that the nominal membrane cut-offs bear no correlation to the actual molecular weight of carrageenan molecules. Membrane separation is based exclusively on molecular size (and shape) rather than molecular weight, not unlike what occurs in size exclusion chromatography (SEC).

In addition, the authors concluded that the transport mechanism across the membrane has some kinetic factor, with the shorter chain molecules moving through faster than the longer chains, and that this was impacted by time-dependent distortion (chain flexibility) and viscous drag of the longer chains.

The native carrageenan should have produced a small permeate fraction, but none was detected. The hydrolyzed samples did produce permeates, but the molecular weight profiles were much broader than expected and not in line with the nominal cut-offs. The analytical details are in Table 2.

Table 2 – Analytical Results – Leatherhead Ultrafiltration

Carrageenan	Mw (k Da)	Membrane Cut-off (k Da)	Cgn Filtered (%)	Cgn Filtered (k Da)
Original	472	100	0	-
Degraded 3h	76	100	43	50
Degraded 3h	76	50	17	37
Degraded 6h	45	100	49	37
Degraded 6h	45	50	29	32

The report concluded that the cut-offs of the two membranes tested (100k Da and 50k Da) were too high and that lower cut-off membranes should be investigated. To our knowledge, no additional work has been carried out at Leatherhead.

6.2 ULTRAFILTRATION – MARINALG INTERNATIONAL

In 2010/2011 Marinalg International investigated the potential of ultrafiltration technology using the carrageenan standards generated in the previous section, and along similar lines as carried out at Leatherhead in 2005. This work was carried out by William Blakemore at FMC (wet chemistry) and Markus Klinger / Jesper Wichmann at Danisco (molecular weight profiling).

6.3 PROCEDURES AND RESULTS

Dilute solutions of two of the kappa-carrageenan standards (detailed in previous section) with Mw of 73k Da (G-2407-149) and 12k Da (G-2743-7) were centrifuged through Millipore centrifuge tubes fitted with nominal membrane porosities of 30k Da and 100k Da. The centrifugates were analyzed for carrageenan concentration using the toluidine blue method (Annexes V and VI) and molecular weight profile by SEC/MALS.

The initial concentrations of the carrageenan standards used were selected in order to provide specific filtrate concentrations in the range that could be used directly into the SEC/MALS system. Solutions were prepared in 0.1M NaCl with heating to 85°C for 15 minutes and cooling to 20°C before adjusting to the final target concentration.

Concentration calibration charts were prepared for the two carrageenan standards using the toluidine blue method for absorbance versus carrageenan concentration and these are shown in Figures 6 & 7. The linear ranges were used to determine the carrageenan concentration in the test filtrates.

Figure 6

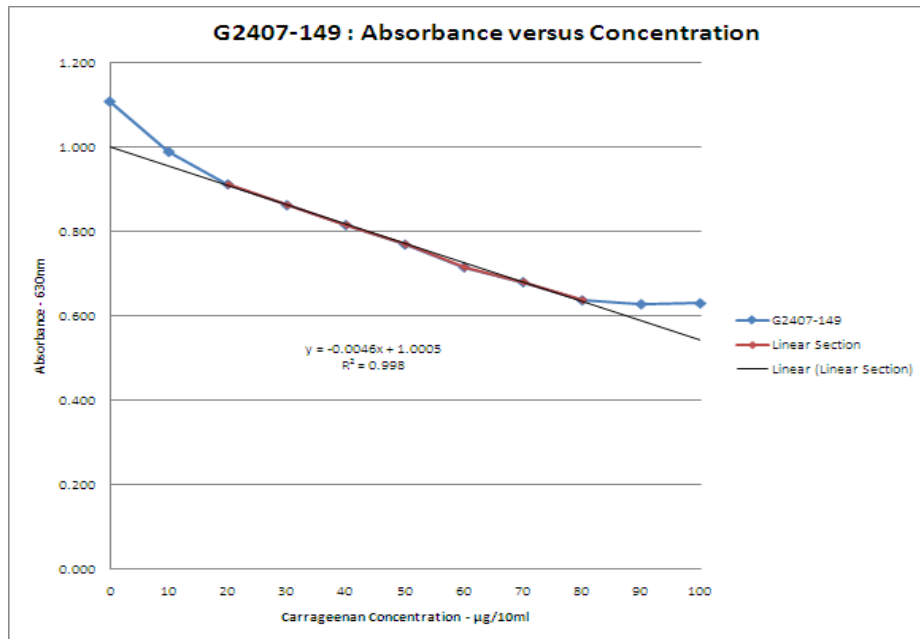
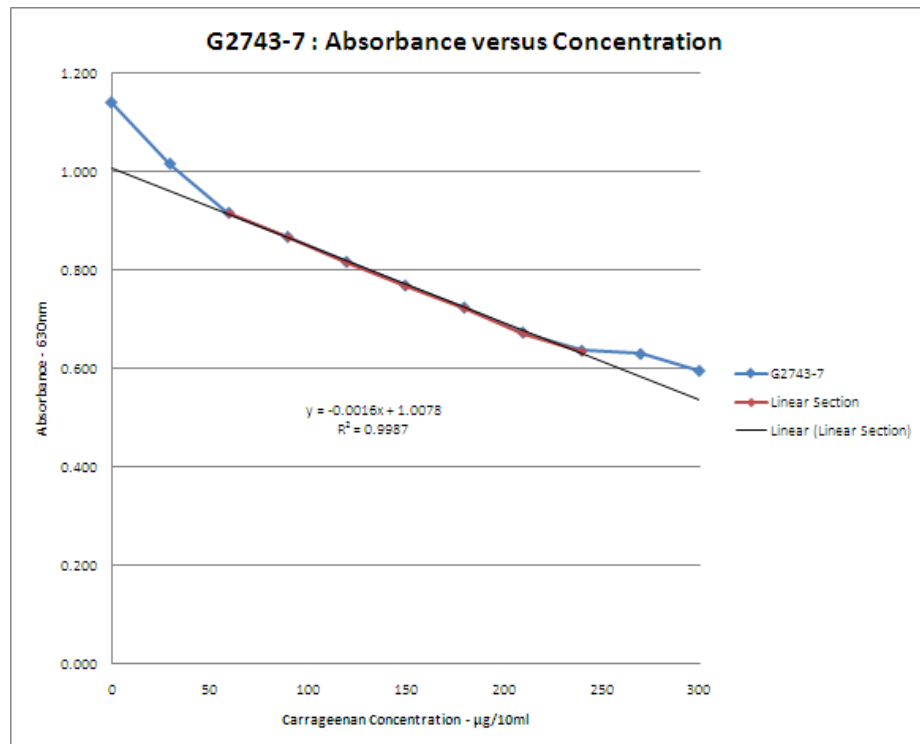


Figure 7



Carrageenan standard G2407-149 (73k Da) was prepared at 2.5% concentration by the method already detailed but a gel was formed. This gel was melted by heating to 85C and the solution applied hot to both centrifuge tubes (100k Da and 30k Da porosities). The solution was centrifuged for 1h at 2,000rpm. Both tubes had filtrate, and both retentates had gelled. The filtrates were measured for carrageenan

concentration using the standard toluidine blue chart. This concentration of 2.5% was necessary in order to yield filtrate concentrations above 0.05% for SEC/MALS analysis. The fact that the retentate gelled means that passage of the lower molecular weight fraction of carrageenan as permeate may have been incomplete, such carrageenan fraction being impeded from entering the membrane by the gel on the membrane surface. However, it would remain reasonable to use the permeate data as to the efficacy of the membrane.

Carrageenan standard G2743-7 (12k Da) was prepared at 0.25% concentration by the method already detailed with no gel formation. The solution was applied at 20°C to both centrifuge tubes (100k Da and 30k Da porosities). The solution was centrifuged for 1h at 2,000rpm. Both tubes had only filtrate with no liquid retentate. The filtrates were measured for carrageenan concentration using the standard toluidine blue chart. This concentration of 0.25% yielded filtrate concentrations above 0.05% for SEC/MALS analysis.

Detailed results of the filtrations are in Table 3.

Table 3 – Analytical Results – Marinalg Ultrafiltration

Carrageenan	Solution Conc. (%)	Membrane (k Da)	Mw of Cgn (k Da)	Cgn Conc. in Filtrate (%)	Cgn Through Membrane (%)
G-2407-149	2.50	30	73	0.089	3.6
G-2407-149	2.50	100	73	0.182	7.3
G-2743-7	0.25	30	12	0.056	22.4
G-2743-7	0.25	100	12	0.096	38.5

The four filtrates detailed in the above table were freeze dried to concentrate the carrageenan, re-dissolved in water, and analyzed for molecular weight profile by SEC/MALS.

7.3% of the carrageenan standard at Mw 73k Da passed through the 100k Da porosity membrane and 3.6% through the 30k Da porosity membrane. The molecular weight profile of the 7.3% through the 100k Da porosity membrane had 94% with Mw = 1.9k Da, and 5% with Mw = 32k Da (as determined by LS). The molecular weight profile of the 3.6% through the 30k Da porosity membrane had 93% with Mw = 0.9k Da, and 6% with Mw = 12k Da.

38.5% of the carrageenan standard at Mw 12k Da passed through the 100k Da porosity membrane and 22.5% through the 30k Da porosity membrane. The molecular weight profile of the 38.5% through the 100k Da porosity membrane had 95% with Mw = 1.6k Da, and 5% with Mw = 10k Da. The molecular weight profile of the 22.5% through the 30k Da porosity membrane was inconclusive, probably due to the carrageenan concentration in the filtrate being too low.

6.4 DISCUSSION & CONCLUSIONS FOR ULTRAFILTRATION

In theory, both carrageenan standards at 73k Da and 12k Da should have passed through the 100k Da porosity membrane. However, the actual carrageenan levels passing through this membrane were only 7.3% and 38.5% respectively. This means that 92.7% of the carrageenan with Mw 73k Da had molecular size above the nominal 100k Da, as did 61.5% of the carrageenan with Mw 12k Da. This endorses the fact that the carrageenan molecule is significantly larger in size in solution than the spherical proteins used to calibrate these membranes. The difference in shape further aggravates this relationship.

The results clearly indicate that higher porosity membranes are needed for the present purpose. This runs counter to the Leatherhead conclusion, but since the Leatherhead project has been closed the differences will have to go unresolved. The membrane cut-offs would have to be in the nominal range of the order 200-400k Da (as determined with protein standards) in order to match up with carrageenan having molecular weight of 50k Da. Unfortunately, membranes with such porosities are not currently commercially available. Also, it may not be feasible to manufacture such membranes with consistent carrageenan porosity.

7.0 OVERALL CONCLUSION

The following table (Table 4) overviews the work to date and the identified technical challenges.

As can be seen in Table 4 it remains clear at this point in time that successfully measuring the EC molecular weight specification is not currently feasible.

Table 4 – Marinalg Work Overview and Challenges

Analytical Method		Need for Carrageenan Standards	Identified Challenges	Conclusion, Applicability of the Tested Analytical Method
Separation Technique	Detection Method			
SEC	LS/RI	No	Signal to noise ratio needs significant improvement to accurately quantify LMT. Pullulan standards do not provide satisfactory calibration due to the significant differences in physical and chemical properties between pullulan and carrageenan / PES.	Not feasible, LMT measurement would be feasible if advances can be achieved to improve signal to noise ratio.
	Viscosity/RI	Yes		
	RI	Yes		
	ICP	Yes		Not feasible, LMT measurement not accurate enough due to poor signal to noise ratio and lack of quality carrageenan standards with low PDI.
Ultrafiltration	LS/RI	No	Membrane porosity in the nominal range of 200-400k would be necessary	Not feasible, current membrane manufacturing techniques cannot produce the required porosity.
	Viscosity/RI	Yes		
	RI	Yes		
	ICP	Yes		

The Marinalg Working Group plans to re-examine its work on SEC/LS methods and recent work of some other scientists (e.g. Uno, Annex VIII, Spichtig, Annex VII) to determine if any opportunities for improvement have been overlooked. Certainly SEC/LS has many practical attributes that makes it desirable for the present use. However, new ideas for increasing the probability of successfully preparing standards in adequate quantities to be adopted for routine testing are needed and Marinalg cannot guarantee success. There is little justification for investing a lot of money on method development unless there is a high probability that fundamentals like baseline drift and signal to noise ratio in the LMT can be improved.

Marinalg will explore new methods as technologies advance, and are willing to investigate new technologies, if any promising ones emerge.

ANNEXES

- Annex I Summary of Round Robin #1 and #2
- Annex II Sample Information
- Annex III Weiner, M. L., D. Nuber, W. R. Blakemore, J. F. Harriman and S. M. Cohen, 2007, "A 90-day dietary study on kappa carrageenan with emphasis on the gastrointestinal tract", *Food Chem. Toxicol.* **45**, 98-106".
- Annex IV Leatherhead Report No. 863, October, 2005, by Titoria, P.M. *et al.*, "Recovery of low molecular weight carrageenan fractions by ultrafiltration through semi-permeable membranes – a feasibility study", Leatherhead Food International, U.K.
- Annex V Beattie, I. A., W. R. Blakemore, E. T. Dewar, and M. H. Warwick, 1970. "A study of orally-administered degraded carrageenan in the baboon". *Fd. Cosmet. Toxicol.*, **8**, 257-266.
- Annex VI MacIntosh, F. C., 1941, "A colorimetric method for the standardization of heparin preparations", *Biochem. J.*, **35**, 776.
- Annex VII Spichtig, V. and Austin, S., 2008. "Determination of the low molecular weight fraction of food-grade carrageenans", *Journal of Chromatography B*, **861**, 81-87.
- Annex VIII Uno, Y., *et al*, 2001. "Molecular weight distribution of carrageenans studied by a combined gel permeation / inductively coupled plasma (GPC/ICP) method", *Food Additives and Contaminants*, **18**, 763-772.
- Annex IX Benoit, H, *et al*, 1966. "Etude par chromatographie en phase liquid de polystyrenes lineaire et ramifies de structures connue", *J. Chim. Phys.*, **63**, 1507-1514.
- Annex X Malawer, E.G. and Montana, A.J., 1980. "Introduction to size exclusion chromatography", *Journal of Polymer Science: Polymer Physics Edition*, **18**, 2303-2305.
- Annex XI Grubisic, Z., *et al*, 1967. "A universal calibration for gel permeation chromatography", *Polymer Letters*, **5**, 753-759.