LATEST UPDATE ON THE WORK OF MARINALG INTERNATIONAL TO MEASURE THE MOLECULAR WEIGHT DISTRIBUTION OF CARRAGEENAN AND PROCESSED EUCHEUMA SEAWEED

As of 2018, the feasibility of measuring Low Molecular weight Tail (LMT) remains limited based on available technology. Since its previous work done from 2003-2015, the Marinalg Technical WG has considered additional publications[1,2], including those indicating polysaccharide analysis using carbohydrate gel electrophoresis (PACE), which relies on derivation of the reducing ends of sugars with a fluorophore, followed by electrophoresis under optimized conditions in polyacrylamide gels, may be a potential solution. However, EFSA's specification requires that small sugar units with Mw less than 50kD from CGN and PES must not exceed 5% of the total CGN profile. As the technology involved in PACE is not adequate for detecting this Mw, it presents reason to doubt the success of this method to determine LMT.

Several publications show gel analysis of hydrolyzed polysaccharides, and separation up to a degree of polymerization (DP) of 20 on the gel. However, EFSA specifies that precise oligomer resolution must reach DP 100 or higher. Additionally, while enzymatic treatment is intended to separate one oligosaccharide from another, this may not always occur, as oligosaccharides may stick to the heaviest molecule. It is uncertain whether methods exist to address these challenges.

In addition, developing a validated method for measuring LMT would logically require a standard within the 50kD Mw range to which the sample could be compared. However, it should be noted that standards of mono- and polysaccharides do not exist above DP 4.

Marinalg has reached the stage in this work where its analytical findings on method development should be discussed with EFSA. The failure to develop and validate an analytical method to measure the LMT using the best available technologies and recognized experts in this field needs to be addressed.

Following is a report previously submitted to EFSA in 2017 which illustrates the previous work of Marinalg International to measure the LMT of CGN and PES.

[1] Goubet et al. (2002) Polysaccharide Analysis Using Carbohydrate Gel Electrophoresis: A Method to Study Plant Cell Wall Polysaccharides and Polysaccharide Hydrolases. Anal Biochem 300, 53-68.

[2] Goubet et al. (2011) Carbohydrate gel electrophoresis. Method in Molec Biol 715, 81-92.

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

SUMMARY

In its 2003 review of carrageenan, the European Commissions' Scientific Committee on Food ("SCF") concluded "… that *if feasible* (emphasis added), a molecular weight limit of not >5% below 50 kDa should be introduced into the specification, in order to ensure that the presence of any degraded carrageenan is kept to a minimum." As a result of the 2003 SCF review, the European Commission (EC) published a new specification for carrageenan (E407, "CGN") and Processed Eucheuma Seaweed (E407a, "PES") in the European Purity Criteria. The specification required that CGN and PES for use in food must not contain more than 5% molar mass with molecular weight less than 50,000 Daltons (50 kDa). Contrary to the conclusion of the SCF, the EC established the specification without demonstration or proof of feasibility.

In response, the trade association Marinalg International (Marinalg) established a technical working group (WG). Since 2003, the WG has worked diligently to determine the feasibility to develop and validate an analytical method to qualify and quantify the low molecular weight tail (LMT) of CGN and PES.

Having evaluated best available technology, and having enlisted the support of experts, external and internal to the industry, after twelve years of planning, experimentation, and analysis (2003 to 2015), the Marinalg Technical Working Group has been unable to develop a validated analytical method in response to this new specification. The WG's accuracy and reproducibility targets were set to achieve a consistent relative standard deviation (RSD) of 15% or less on triplicate sample analysis, and including spiking and recovery validation within 95 – 105% of added LMT to CGN and PES samples.

Marinalg has tested several methods based on size exclusion chromatography (SEC) and light scattering (LS), and none have given reliable and reproducible results. Below 50 kDa, the signal to noise ratio when light scattering is used for molecular weight determination is too low for the accuracy required by the EU specification. An embodiment of SEC/LS has been adopted by the Japan Ministry of Health for measuring this specification, but the methodology is flawed, because pullulan standards were used to calibrate the SEC columns (Uno, *et al*, 2001). Pullulan is not an appropriate model for CGN for this purpose because the two polymers have different molecular shapes in solution, CGN being much more linear than pullulan. Also, CGN is a polyelectrolyte and its apparent hydrodynamic volume will vary with the solvent quality, e.g. ionic strength, of the eluent. These shape and physical chemistry differences result in significant molecular size differentials for the same weight-average molecular weight (Mw), particularly with these hydrated molecules. This, in turn, impacts the data from SEC, as this chromatographic separation is based on molecular size and shape. That is, the column elution times for pullulan and CGN at the same Mw and under the same conditions are not identical. The resulting Mw for CGN using pullulan standards was between 1.5 – 2.5 times higher than those methods using light scattering.

To correct this deficiency, ten CGN molecular weight standards were prepared for the molecular weight (Mw) range of 600 kDa to 12 kDa. The polydispersity indexes (PDI, Mw/Mn) of the Mw standards between 600 kDa and 73 kDa were all less than 2.0 (1.3 - 1.8) indicating fairly sharp molecular weight profiles. The PDI values of the lower molecular weight standards (56 kDa to 12 kDa) are in the range 3.5 – 5.0, too polydisperse for column calibration and measurement of the LMT. The eight pullulan standards covering the same molecular weight range all had PDI values close to 1.2. Attempts to produce CGN standards with PDI values close to 1.2 failed (as anticipated). No further work is planned on CGN standards.

Spiking and recovery experiments, using poligeenan (PGN) as a surrogate for the LMT, failed completely with recoveries well outside acceptable limits. Two kappa-CGNs with different Mw and %<50 kDa were each separately powder-blended with 0-15% PGN and profiled by the routine SEC/LS/RI protocols at four

different locations. The measured Mw and %<50 kDa values for the CGN/PGN blends were far from the calculated values based on detailed sample purities. These results endorsed the position that our SEC/LS/RI methodology needs further technology advances.

Recently (2015), a method for the measurement of the LMT of PES has been investigated and is generating data of similar accuracy as currently demonstrated for CGN. Round-robin testing of one PES and two CGN samples at three different locations that use three different combinations of SEC/LS/RI equipment indicated that the RSD values for the LMT results were in the range 11-25%, still too high for method validation. Although individual RSD values between 10-15% were measured for all three samples, the overall results were far from consistent. Also, significant levels of starch and nitrogen (most likely hydrolyzed protein) were detected in the LMT of all three samples, and these components are being investigated, and hopefully quantified.

To achieve the desired molecular separation, the Marinalg Technical Working Group evaluated membrane (ultra-filtration) separation technology. The results using membranes with manufacturers' reported porosities of 30 kDa and 100 kDa clearly indicate that higher porosity membranes in the nominal range of 300 – 500 kDa would be necessary to be permeable to the specified 50 kDa CGN molecules. This will require significant advancements in membrane manufacturing. No further work is planned using ultrafiltration at this time.

The Working Group (WG) re-examined SEC/LS methods by Marinalg members and other scientists (Uno, *et al*, 2001, and Spichtig & Austin, 2008) to determine if any opportunities for improvement had been overlooked, but none were forthcoming. Recent new research work has confirmed this position (Blakemore, *et al*, 2014).

Certainly SEC/LS has many practical attributes that make it desirable for the targeted use. However, there is little justification for investing heavily in resources to further method development unless there is a high probability that fundamentals like baseline drift and signal to noise in the LMT region can be improved. At this time there is low probability that these short comings will be eliminated with the current state-of-the-art equipment, and development of methods to produce consistent and accurate results for preparing certificates of analysis.

The necessary technology advances to consistently and accurately measure the LMT of CGN by a validated method does not exist currently. The necessary technological advances needed include (a) equipment advances to improve the signal/noise ratio and cut-off accuracy, (b) development of a suitable means for validation by spiking and recovery, and (c) development of a suitable means to remove, quantify and confirm the non-CGN components (starch, protein) in the LMT.

Marinalg has reached the stage in this work where its analytical findings on method development should be discussed with EFSA. The failure to develop and validate an analytical method to measure the LMT using the best available technologies and recognized experts in this field needs to be addressed.

William R. Blakemore, Chairman
Marinalg Working Group on Carrageenan Molecular Weight
President / Principal
Celtic Colloids Inc.
Topsham, ME 04086, USA
August, 2015.

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

LIST OF ABBREVIATIONS

CGN	Carrageenan
Da	Daltons
d-CGN	Degraded carrageenan
EC	European Commission
LALS	Low angle light scattering
LFI	Leatherhead Food International
LMT	Low Molecular Weight Tail
LS	Light scattering
Marinalg	Marinalg International
Mn	Number-average molecular weight
Mw	Weight-average molecular weight
PES	Processed Eucheuma Seaweed
PGN	Poligeenan
PDI	Polydispersity Index
RALS	Right angle light scattering
RI	Refractive index
RSD	Relative standard deviation
SCF	Scientific Committee for Food
SEC	Size exclusion chromatography
S/N	Signal to noise ratio
UF	Ultrafiltration
WG	Marinalg Technical Working Group

CONTENTS

FULL REPORT

1.0 INTRODUCTION

The following report describes the results of work by Marinalg International (Marinalg) to develop and validate a test method to determine the low molecular weight tail (LMT, % below 50,000 Daltons, 50 kDa) of carrageenan (CGN) and Processed Eucheuma Seaweed (PES). The work is described in the chronological order as performed since 2003 to August, 2015.

In its 2003 review of CGN, the European Commission's Scientific Committee on Food ("SCF") concluded "… that *if feasible* (emphasis added), a molecular weight limit of not >5% below 50 kDa should be introduced into the specification, in order to ensure that the presence of any degraded carrageenan is kept to a minimum." As a result of the 2003 SCF review, the European Commission (EC) published a new specification for CGN (E407) and PES (E407a) in the European Purity Criteria. The specification required that CGN and PES for use in food must not contain more than 5% molar mass with molecular weight less than 50,000 Daltons (50 kDa). Contrary to the conclusion of the SCF, the EC established the specification without demonstration or proof of feasibility. Since that time the Marinalg Technical Working Group (WG) has worked diligently to determine the feasibility to develop and validate an analytical method for determining the low molecular weight tail (LMT) of CGN and PES.

Typical measurement of weight-average molecular weight (Mw) or number-average molecular weight (Mn) involves separation of the molecules by size, for example by chromatography, in combination with concentration measurement of each molecular fraction, for example by refractive index, and measurement of molecular weight of each molecular fraction, for example directly by light scattering, or indirectly by the application of molecular weight standards. The chromatography conditions have to ensure full solubility of the test material, and avoid any gelation or molecular aggregation, which, in turn, necessitates the use of ionic buffers, high temperatures, and low test material concentrations. As CGNs are naturally polydisperse, they have molecular weight profiles covering a wide range of molecular weights. For example, CGN with a Mw of 600 kDa may have individual molecules between about 20 kDa to 1,000 kDa, this range occurring during natural plant growth synthesis.

Another method would be to try to separate the molecules at some specific size, and measuring the concentration below a certain Mw. This involves the application of membranes of various porosities and the use of osmotic pressure with or without added force (e.g. centrifugation) to accelerate equilibrium. This also requires full solubility and avoidance of gelation and molecular aggregation.

2.0 DEFINING THE GOALS AND OBJECTIVES OF THE EXPERIMENTAL PROGRAM

Before the WG had adequate time to determine the feasibility of measuring the new specification, it was prematurely 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 CGN 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 August 2015, the WG still has not been able to develop a method for molecular weight distribution measurement that is sufficiently accurate and reproducible to yield a validated method. Our accuracy and reproducibility targets were set to achieve a relative standard deviation (RSD) of 15% or less on triplicate sample analysis, and including spiking and recovery validation within 95 – 105% of added LMT to CGN samples.

3.0 METHODS EVALUATED

3.1 SIZE EXCLUSION CHROMATOGRAPHY & LIGHT SCATTERING

3.1.1 DESCRIPTION

Any chromatographic method comprises four steps; sample preparation, separation, detection, and data reduction. Each of these steps, if performed inappropriately, may adversely affect to accuracy and precision of the results. Each of these four steps has multiple complex components, which require lengthy development times to balance equipment and method protocols to yield consistent results.

The methods studied in detail from 2003 to 2008 were all based on Size Exclusion Chromatography (SEC). SEC is used to separate the CGN molecular size distribution in the flow stream exiting the columns so that narrow molecular weight fractions can be available for measurement and analysis. Note that this separation is by hydrated 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 CGN concentration determination, and light scattering and/or intrinsic viscosity for molecular weight determination. Some instruments include chemical detectors to ensure that only CGN is being measured in the flow stream. The Japanese Ministry of Health method (GPC/ICP; gel permeation chromatography / inductively coupled plasma, measuring sulfur) is one such method (Uno, *et al*, 2001).

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 development and validation. Six laboratories participated in this preliminary study, Degussa Corporation (now Cargill Inc.); Danisco A/S (now DuPont Nutrition and Health); 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 CGN 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.

3.1.2 RESULTS FOR SEC/LS

Despite all the effort to impose technical discipline, inter-lab reproducibility of the LMT was poor. In all cases, detectors downstream of the SEC columns were unable to effectively measure polymer concentration and molecular weight in the range represented by the LMT.

The interlaboratory results with respect to Mw, LMT and recovery (mass measured versus mass injected) were poor on all samples, with wide differences in LMT assay. For example, one sample with six test results from six different test locations had the following data:

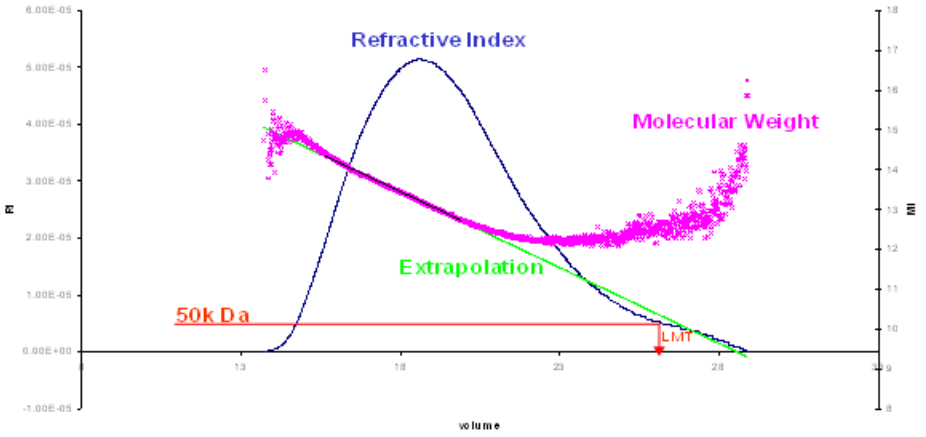
Location	1	2	3	4	5	6
Location	NEWI	San-Ei	Viscotek	Degussa	PSS	Danisco
Mw	676 kDa	1,637 kDa	939 kDa	1,030 kDa	1,084 kDa	868kDa.
LMT	0%	1%	2%	1%	25%	0%
Recovery	69%	-	96%	-	50%	50%
Conc^{n}	1.0mg/ml	0.1mg/ml	1.0mg/ml	0.5mg/ml	1.0mg/ml	1.0mg/ml
Buffer	$LiNO_3$/EDTA	$NaNO_3$	$LiNO_3$/EDTA	$LiNO_3$/EDTA	LiBr/NaN_3	$LiNO_3$/NaN_3
Temp.	70ºC	50ºC	70ºC	60ºC	65ºC	40ºC
Detection	MALS/RI	Pullulan/RI	LALS/RALS/RI	MALLS/RI	MALLS/RI	MALLS/RI

Only the data from locations 3 (Viscotek, SEC/LALS/RALS) and 4 (Degussa, SEC/MALLS) offered method potential. Locations 1(North East Wales Institute), 5 (Polymer Standards Services) and 6 (Danisco) were excluded based on low recoveries, and location 5 additionally due to an impossibly high LMT result (on both statistical and functional bases). Location 2 was excluded due to the high Mw (from use of pullulan standards – see description of these standards in section 3.1.3.1.1).

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

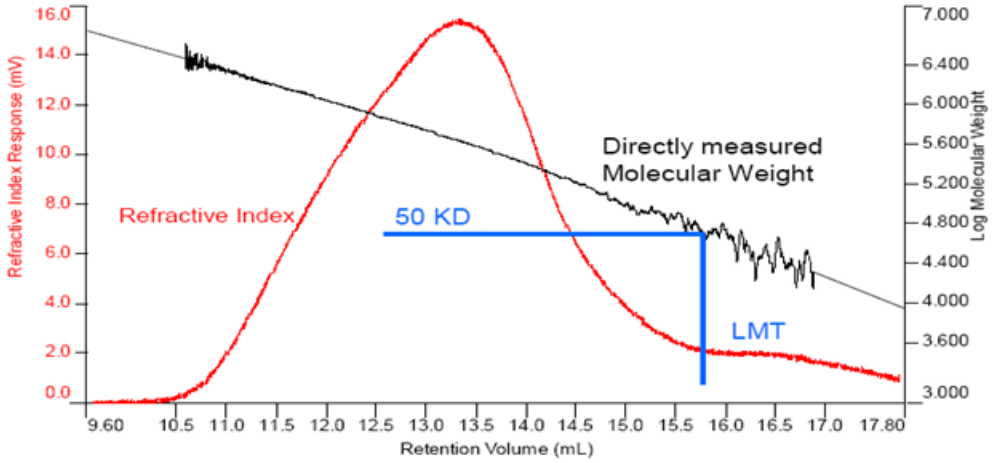
(Fig. 1). The LS signal goes non-linear (called the "swish" by some) well before the LMT has exited the column. In addition, several test locations experienced drifting baselines. This led to variable recoveries (input of CGN to the SEC column did not equal the CGN in the effluent stream). These added problems make the LMT estimates even more unreliable.

Figure 1: Typical output from SEC/RI/MALLS – Degussa / Cargill Data



Initially the WG thought that the Viscotek triple detector method (refractive index, low angle light scattering and intrinsic viscosity) was giving promising results. However, further testing indicated that these same issues applied, but to a much lesser degree (Fig. 2).

Figure 2: Typical output from SEC/RI/LALS/RALS – Viscotek / Malvern Data



3.1.3 DISCUSSION SEC/LS

Various fitting and extrapolation routines (Zimm, Debye, Berry) are used in the MALS detection system's software to determine Mw where there is acceptable signal to noise ratio (S/N). These data are then further extrapolated by some routine method (usually linear) into the region of poor S/N. For all of the CGN 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. 1, green line). This type of extrapolation is subject to significant error. This can be seen in Fig. 1 where the extremely small LMT region is shown graphically. Clearly, any shift in baseline or green line extrapolation will have a mathematically significant effect on the very small

LMT region calculated for commercial CGN being used in foods. It is estimated that it is virtually impossible to determine the molecular weight of SEC-spread samples below about 10 kDa by any of the light scattering techniques.

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 1 & 2), thus reducing, but not eliminating the impact of extrapolation techniques. Also, the inclusion of a viscometer by Viscotek to measure an eluate's intrinsic viscosity helps to optimize sample solution concentration to avoid 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 WG's experience with SEC/LS does not detract from its use as a valuable research tool. The technique is widely used for estimating polymer molecular structure in food and industrial applications. However, a higher level of accuracy is required for determining regulatory compliance with the LMT specification.

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-CGN components as part of the LMT. No steps were taken to remove the salts and inherent impurities. Other inaccuracies occurred due to inconsistent measurement of cut-off-points and incomplete dissolution of the CGN (e.g. gelation and aggregation) leading to poor SEC separation. SEC column selection and previous uses of columns also contribute to variable results. Note that method development was confined to CGN extracts only and that many commercial CGN products are blends of two or more CGN extracts.

The measurement of the LMT in PES must involve an additional process step to quantitatively extract the CGN from the PES without degradation. Although such extraction would appear to be relatively straightforward, significant effort would be necessary to ensure that the extraction was carried out consistently and without degradation of the CGN. The optimum conditions (pH, temperature, time, shear, etc.) for extracting CGN from PES would need to be developed and validated for PES produced from each of the permitted seaweeds (*E. cottonii* and *E. spinosum*). This work has been put "on hold" until a validated method for CGN has been developed.

3.1.3.1 COLUMN CALIBRATION TO AUGMENT LIGHT SCATTERING

3.1.3.1.1 BACKGROUND

Column calibration involves preparing a calibration curve of exit time from the SEC column versus molecular weight for a set of standards with a very narrow molecular weight distribution (Mw/Mn < 1.2) (Mw/Mn 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, 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 Mw from 5.3 – 760 kDa that are commercially available from Showadenko (Shodex). Pullulan is a glucan polysaccharide polymer consisting of maltotriose units connected by α-1,4 glycosidic bonds, these triose units connected to each other by a α-1,6 glycosidic bonds. Pullulan is a linear molecule with a single repeating structure (triose) that is readily cleaved between these trioses by the enzyme pullulanase. This

enzymatic Mw reduction results in fractions with low polydispersity (PDI, Mw/Mn) of about 1.2, which in turn gives sharp elution peaks from SEC columns. This method has been tested on commercial CGNs, and the results have been reported in the scientific literature by Japanese scientists (Uno, *et al*, 2001). No correction was applied in this work for the differences between pullulan and CGN molecular sizes versus molecular weights, so validation of the LMT values reported by Uno remains in question.

Having a set of degraded carrageenan standards would be preferable and the WG explored the preparation of such a set (Table 1). It should be noted, however, that, as CGN has multiple repeating units, producing CGN standards with PDI <1.2 will be very difficult, if not impossible. From past experience, PDI values would be expected to be about 1.5 at best, and, more likely, closer to 2.0, and outside the range of PDI needed for LMT accuracy.

It has to be emphasized that the term “degraded carrageenan” (d-CGN) is being used here to mean carrageenan that has been deliberately reduced in Mw to produce test sample standards over a wide range of Mw, the lowest Mw sample being “poligeenan” (PGN), as defined by the US Adopted Names Council, rather than “carrageenan”. Also note that five samples in the middle of the range do not fit the definitions of either CGN or PGN. These d-CGN standards were prepared and used for analytical purposes only.

CGN must not be confused with “poligeenan” (PGN), also identified in some literature as “degraded carrageenan”. PGN is manufactured from CGN by deliberate acid-hydrolysis at ~pH1 and ~90ºC for up to 6 hours. This reduces the Mw from the commercial CGN range of 200,000 – 800,000 Da to the PGN range of 10,000 – 20,000 Da. CGN and PGN are two completely different and separate materials, with different toxicological properties, specifications and applications. PGN is used primarily in clinical diagnostics to suspend barium sulfate for X-ray imaging purposes. PGN has no functional utility in food.

The difficulties in obtaining d-CGN standards led the WG to explore the application of a technology referred to as "universal calibration", a physical model for converting a pullulan calibration curve to a CGN calibration curve (Grubisic, *et al*, 1967). The model takes into consideration size and shape differences for the two polymers when their molecular weights are the same. Preliminary work applying this technique to the Uno data did not demonstrate any promise of success, most likely due to the extreme differences between CGN and pullulan, and consequently was not pursued further.

A related technology referred to as "polydisperse or broad standard calibration" was also investigated (Malawer, *et al*, 1980). For this purpose, a very broad molecular weight distribution CGN is prepared as a standard that has relatively high concentrations of CGN in the low and high molecular weight tails and spans the range of Mw of interest. Again, physical modeling and computer analysis is employed to convert SEC exit time to a CGN molecular weight. Further review of this technique did not indicate any promise of success, and consequently was not pursued further.

3.1.3.1.2 PREPARATION / ANALYSIS OF CARRAGEENAN STANDARDS

In 2009/2010 d-CGN standards were prepared by the deliberate degradation of kappa carrageenan extracted from a single batch of *K. alverezii* 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. 3. The trendlines are logarithmic with excellent correlations.

Table 1 – Analysis of Degraded Kappa Carrageenan Standards

Reference	Viscosity (mPa s^{-1}) (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 3

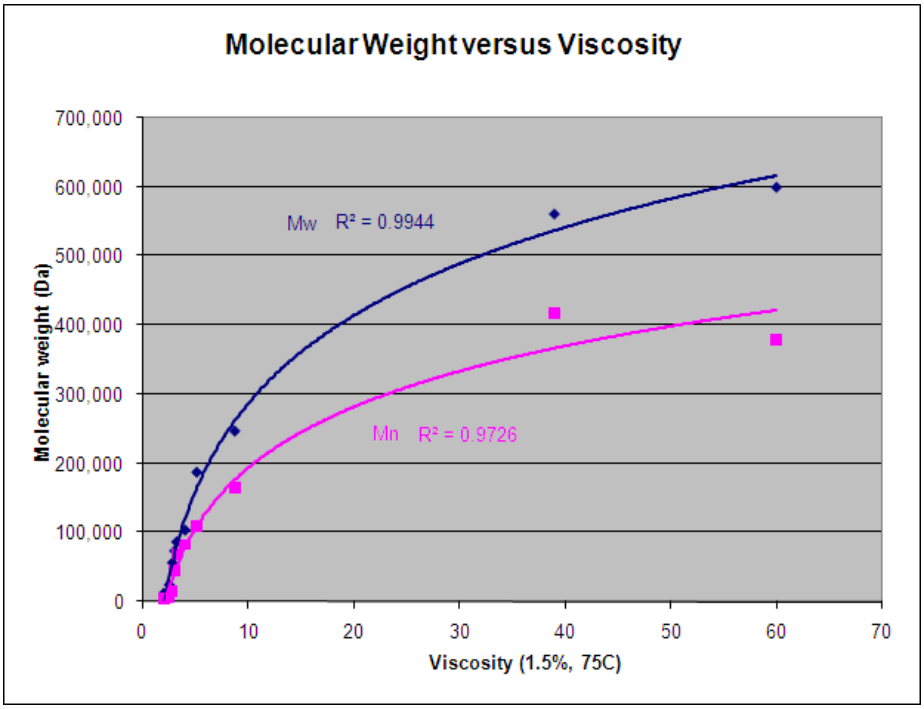


Table 1 shows that, although the PDI values of the seven highest Mw standards are considered "excellent" for CGN, the PDI values of the lower three Mw standards are in the range 3.5 – 5.0, significantly higher than the pullulan equivalents, and too polydisperse for the accurate application of "universal or polydisperse column calibration". It seems doubtful at this time lower PDI values can be obtained for low Mw d-CGNs with the current technology being used to make the d-CGN standards. Consequently, no further work is planned for the preparation of CGN standards.

3.1.3.1.3 SPIKING AND RECOVERY EXPERIMENTS

Attempts were made to try to measure the LMT of a commercial kappa-CGN deliberately blended / spiked with known levels of "LMT" derived from PGN. However, measurements of the total LMT in these synthetic blends at the Cargill, DuPont, San-Ei, and Viscotek facilities were all inconsistent with and far removed from the calculated values.

Two well-characterized kappa-CGNs and one PGN sample were both powder-blended at 0%, 5%, 10%, and 15% PGN. The kappa-CGNs had Mw values of (A) 585 kDa (75.0%, 5.42%) and (B) 258 kDa (85.0, 6.87%) and the PGN sample tested at 12.5 kDa (89.0%, 95.1%). Sample purities and %<50 kDa are shown respectively in parentheses. The powder blends tested at Viscotek produced the following results for spiking and recovery analysis (Table 2).

Table 2 – Results of Spiking and Recovery Experiment at Viskotek

		Measured		Calculated	
CGN	PGN (%)	Mw (kDa)	<50 kDa (%)	<50 kDa (%)	Recovery (%)
A	0	585	5.42	5.42	-
A	5	556	8.70	10.69	81.4
A	10	567	9.82	15.86	61.9
A	15	523	13.95	20.94	66.6
B	0	258	6.87	6.87	-
B	5	253	8.91	11.48	77.6
B	10	245	12.50	16.06	77.8
B	15	245	11.45	20.63	55.8

All the recovery values for the %<50 kDa were well outside acceptable ranges (e.g. 100+/-5%) and low. In addition, the measured Mw values were inconsistently higher than the calculated values. Similar recovery failures were produced at the other test locations. The Viscotek data presented above was the best of the four test locations.

As previously mentioned, having a consistent Mw cut-off point is essential for accurate and consistent molecular weight profiling and measurement of the LMT. The Gaussian distribution of the molecular weight profile heavily skews the LMT of CGN towards 50 kDa. The bulk of the LMT is between 40-50 kDa, with less between 30-40 kDa, and less between 20-30 kDa, this continuum resulting in minimal / inconsequential amounts of LMT below 20 kDa. However, PGN normally has 95%+ below 50 kDa and 50%+ below 10 kDa. This means that recovery of PGN after spiking is extremely sensitive to the molecular weight cut-off point, which, in turn, is difficult to keep constant due to the poor S/N as previously explained. This means that PGN is most likely not a good surrogate for the LMT of CGN for this method, and may explain the poor results.

The earlier expectation of success with the Viscotek method was refuted 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/LS technology is not feasible.

3.1.4 SEC/LS WORK OF OTHERS

The only additional relevant publication using SEC/LS to analyze CGN is by Spichtig and Austin of Nestle Research Centre in Lausanne, Switzerland (Spichtig & Austin, 2008). This research work essentially confirms the conclusions of the Marinalg WG with respect to measuring the LMT of CGN using SEC/LS. Their method developed using HPSEC/RI to measure the LMT is an excellent research tool for the relative comparison of similar CGN 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 CGN samples with Mw 535 kDa to 889 kDa respectively, is higher than the most probable LMT range determined by the WG for CGNs of comparable Mw. This suggests that their LMT data may include components other than pure CGN, but confirmation would require the measured recovery data from the SEC/RI spectra which were not reported.

The suggestion by the authors 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 making dessert jellies at pH values at or above 4.0 had little or no effect on Mw or LMT values. This endorses the well-established position to avoid processing CGN at low pH for long times at high temperatures (Blakemore & Harpell, 2010).

3.1.5 RECENT WORK USING SEC/RI/LALS/RALS

Recent work (Blakemore, *et al*, 2014) has confirmed that the Viscotek / Malvern method applying SEC/RI/LALS/RALS continues to fall well short of the accuracy and precision of LMT measurement needed for regulatory specification and compliance. In this study, a typical commercial CGN used in infant formulations was fully analyzed independently four times by the same external laboratory (Viscotek / Malvern, Germany) and same analyst over the two-year timeframe of a piglet feeding study (Weiner, *et al*, 2015). The four identical powder samples were taken from the same triple-sealed box stored at room temperature in a dry location.

The data are shown in Table 3.

Table 3: Analysis of Carrageenan Test Material – Lot 90303011

Molecular Weight Profile	Sample 1	Sample 2	Sample 3	Sample 4	Mean	STD DEV
Sample Date	June 2011	January 2012	January2 013	May 2013	-	-
Mw (kDa)	702	732	664	729	707	32
Mn (kDa)	243	343	226	308	280	55
PDI (Mw/Mn)	2.9	2.1	2.9	2.4	2.6	0.4
LMT (%)	3.4	0.3	3.9	1.0	2.2	1.8

The LMT data range from 0.3% to 3.9%, with an average of 2.2% and standard deviation of 1.8%. These analyses confirm the earlier conclusion that the method is not accurate enough for regulatory specification and compliance.

Processed Eucheuma Seaweed (PES, E407a) is manufactured by a much simpler and lower cost process than used for CGN (E407) (Blakemore & Harpell, 2010). The CGN in PES remains as an integral component of the seaweed plant structure and these cellulosic solids (8-15%) remain in the final product. PES requires high thermal treatment to extract the CGN into solution, which remains cloudy with the insoluble cellulosic particulates, and limits the feasible applications for PES.

Very recently in 2015, attempts have been made to develop a method protocol to measure the Mw and LMT of PES. In the case of PES, the CGN needs to be consistently and quantitatively extracted from the PES without degradation and then profiled by the method developed for CGN. The extraction protocol was carried out in LiNO3/EDTA buffer at pH7 at 80ºC for 90 minutes to produce (after centrifugation) CGN concentrations that could be injected directly into the SEC columns. This avoids having to isolate the CGN and subsequent re-dissolution. A kappa-CGN and an iota-CGN were included with this PES sample and tested at three laboratory locations by Malvern/Viscotek (SEC/LALS/RALS/RI), Cargill (SEC/MALLS/RI), and DuPont (SEC/MALLS/RI). System equipment components (e.g. columns, detectors, temperatures, buffer solutions, solution concentrations, etc.) were different at all locations. Results are not yet finalized, but to date we have the following conclusions.

The method developed for PES appears to be producing Mw and LMT data with the same level of consistency and accuracy as determined currently for CGN extracts.

At Malvern/Viscotek, triplicate runs using triplicate preparations (9 data points) resulted in LMT values for the PES at 6.25+/-1.61% (RSD 25%), for the iota-CGN at 5.11+/-0.60% (RSD 12%), and for the kappa-CGN at 2.50+/- 0.28 (RSD 11%).

Cargill produced similar data for all three samples, both Mw and LMT, and including one data set for the PES sample at 11% RSD. Although most of the data were close between testing locations, there were several outliers. The RSD values for the LMT test method need to be lowered towards 10% (less than 15%) for the method to be considered for validation and subsequent specification enforcement. This level of RSD has been achieved on an individual data set basis at one test location, but has yet to be achieved consistently on repeat sample runs and at all test locations.

The experiments at DuPont are incomplete at this time. However, it is noted that the LMT being measured is not all CGN. Evidence has been found that the LMT comprises significant levels of both starch (detected by NMR) and nitrogen (most likely hydrolyzed protein, and detected by Kjeldahl method). The initial detected levels of these two components would reduce the LMT of the PES sample from about 6% to about 3%, the iota-CGN from about 5% to about 3%, and the kappa-CGN from about 3% to about 2%. Attempts to accurately measure these non-CGN LMT components are underway.

3.1.6 CONCLUSIONS FOR SEC/LS

The Marinalg Working Group concludes that SEC/LS as currently practiced cannot measure the LMT with the accuracy needed. In reaching its conclusions, the WG 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 International Specialty Products and Dr. Maguerite Rinaudo at Centre de Recherchessur les Macromolecules Veetales) who have been involved in developing and using SEC/light scattering for a variety of research purposes. While some indicated that the WG's goal might be achieved, it was made clear that this program by the WG would be breaking new ground. Through this process, consensus was gradually reached that the current equipment employing light scattering and the attendant software would not measure the LMT specification with sufficient accuracy to survive the necessary validation protocols.

3.2 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 "CGN" in solution less than 50k Da (the LMT). However, it was hoped that the "CGN" in the permeate was in some way proportional to the total LMT.

3.2.1 ULTRAFILTRATION WORK BY LEATHERHEAD FOOD INTERNATIONAL

This work titled "Recovery of low molecular weight carrageenan fractions by ultrafiltration through semi-permeable membranes – a feasibility study" (Leatherhead, 2005, "LFI") was published in October, 2005, and pre-dates the Marinalg work on UF. At that time, this report was restricted to LFI's members, so the WG did not learn about it until they had decided to try UF.

Two semi-permeable membrane units (Vivaspin), with cut-offs of 100 kDa and 50 kDa, were used to generate permeate fractions and their molecular weight profiles determined using size exclusion chromatography (HPSEC). The CGN used was kappa-CGN from "Eucheuma cottonii" (*Kappaphycus alvarezii*), and several samples of deliberately hydrolyzed "d-CGN" were made from this starting CGN. 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, CGNs 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 hydrated CGN 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 CGN 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 4.

Table 4 – Analytical Results – Leatherhead Ultrafiltration

Carrageenan	Mw	Membrane	Cgn Filtered	Cgn Filtered
	(kDa)	Cut-off (kDa)	(%)	(kDa)
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 (100 kDa and 50 kDa) were too high and that lower cut-off membranes should be investigated. To our knowledge, no additional work has been carried out at LFI.

3.2.2 ULTRAFILTRATION WORK BY MARINALG INTERNATIONAL

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

3.2.2.1 PROCEDURES AND RESULTS

Dilute solutions of two of the d-CGN standards (detailed in previous section) with Mw of 73 kDa (G-2407-149) and 12 kDa (G-2743-7) were centrifuged through Millipore centrifuge tubes fitted with nominal membrane porosities of 30 kDa and 100 kDa. The centrifugates were analyzed for d-CGN concentration using the toluidine blue method (Beattie, et al, 1970; MacIntosh, 1941) and molecular weight profile by SEC/MALS.

The initial concentrations of the d-CGN 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 d-CGN standards using the toluidine blue method for absorbance versus CGN concentration and these are shown in Figures 4 & 5. The linear ranges were used to determine the d-CGN concentration in the test filtrates.

Figure 4

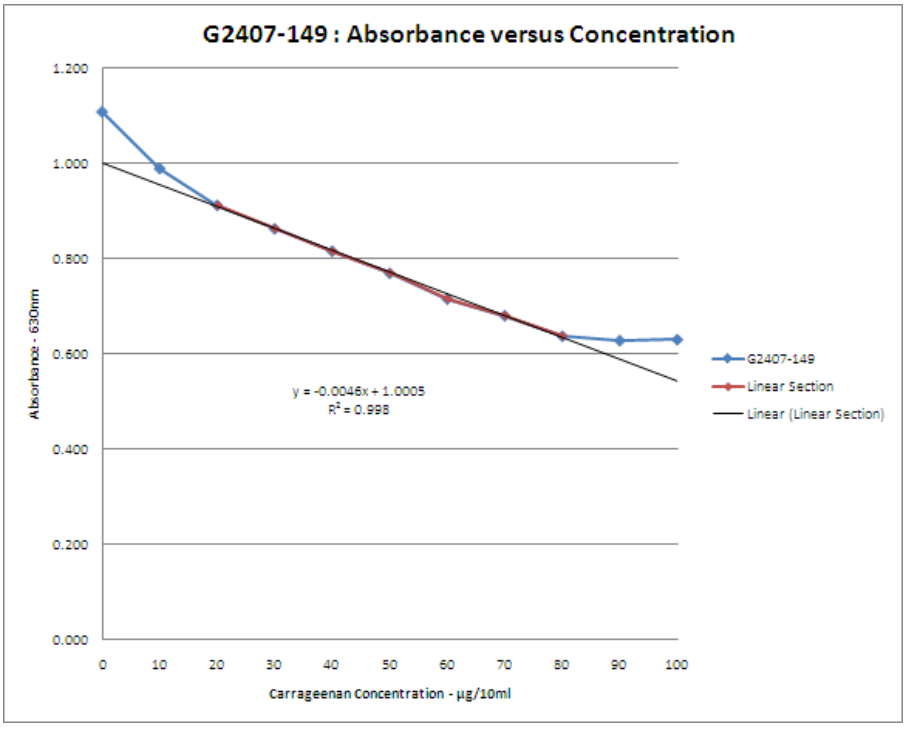
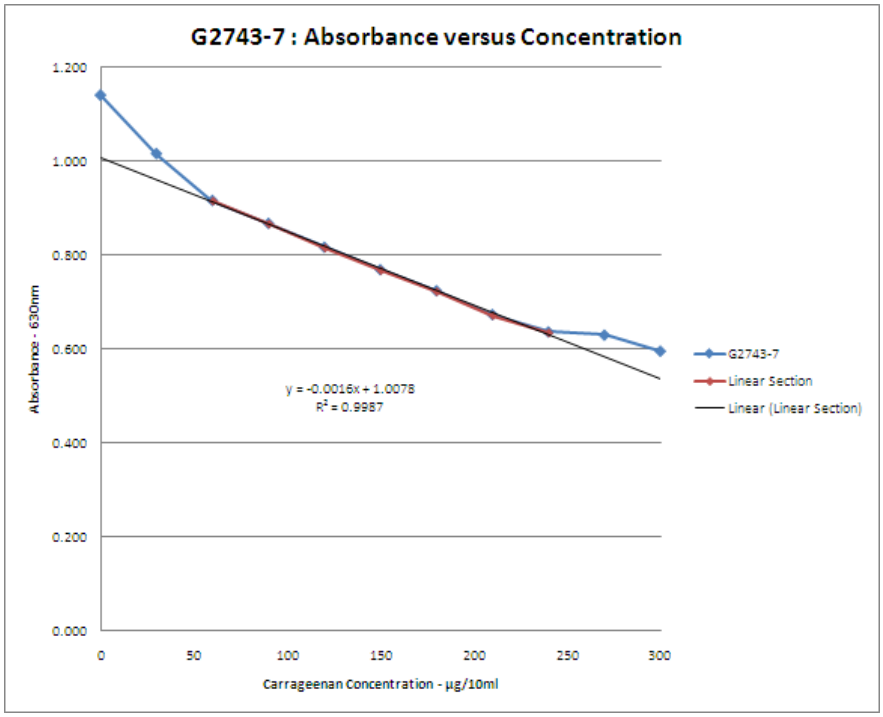


Figure 5



D-CGN standard G2407-149 (73 kDa) 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 (100 kDa and 30 kDa porosities). The solution was centrifuged for 1h at 2,000rpm. Both tubes had filtrate, and both retentates had gelled. The filtrates were measured for d-CGN 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 d-CGN as permeate may have been incomplete, such d-CGN 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.

D-CGN standard G2743-7 (12 kDa) 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 (100 kDa and 30 kDa porosities). The solution was centrifuged for 1h at 2,000rpm. Both tubes had only filtrate with no liquid retentate. The filtrates were measured for CGN 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 5.

Table 5 – Analytical Results – Marinalg Ultrafiltration

Carrageenan	Solution Conc.	Membrane	Mw of Std	Std Conc. in Filtrate	Std Through Membrane
	(%)	(kDa)	(kDa)	(%)	(%)
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 d-CGN fraction, re-dissolved in water, and analyzed for molecular weight profile by SEC/MALS.

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

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

3.2.4 DISCUSSION & CONCLUSIONS FOR ULTRAFILTRATION

In theory, both d-CGN standards at 73 kDa and 12 kDa should have passed through the 100 kDa porosity membrane. However, the actual d-CGN levels passing through this membrane were only 7.3% and 38.5% respectively. This means that 92.7% of the d-CGN with Mw 73 kDa had molecular size above the nominal 100 kDa, as did 61.5% of the d-CGN with Mw 12 kDa. This endorses the fact that the CGN/d-CGN/PGN molecules are 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 LFI conclusion, but since the LFI project has been closed the differences are unresolved. The membrane cut-offs would have to be in the nominal range of the order 300 – 500 kDa (as determined with protein standards) in order to match up with d-CGN having molecular weight of 50 kDa. Unfortunately, membranes with such porosities are not currently commercially available. Also, it may not be feasible to manufacture such membranes with consistent CGN/d-CGN/PGN porosity.

4.0 OVERALL CONCLUSION

The following table (Table 6) gives an overview of the work to date and the identified technical challenges.

As can be seen in Table 6 it remains clear at this point in time that successfully measuring the LMT molecular weight specification is not currently feasible.

Table 6 – Marinalg Work Overview and Challenges

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

Until there is a change in the purity criteria for carrageenan (E407) and PES (E407a), the Marinalg WG will continue to monitor advances in current technologies, such as SEC/LS and ultrafiltration, and explore new technologies as they become relevant to the measurement of the LMT of CGN and PES.

William R. Blakemore, Chairman
Marinalg Working Group on Carrageenan Molecular Weight
President / Principal
Celtic Colloids Inc.
Topsham, ME 04086, USA
August, 2015.

5.0 **REFERENCES**

Beattie IA, Blakemore WR, Dewar ET, Warwick MH (1970). A study of orally-administered degraded carrageenan in the baboon. Fd. Cosmet. Toxicol., **8**, 257-266.

Blakemore, W. R. and Harpell, A. R. (2010). Chapter 5: Carrageenan, In Food Stabilisers, Thickeners and Gelling Agents, Ed. Alan Imeson, Wiley-Blackwell, Blackwell Publishing Ltd.

Blakemore WR, Davis SR, Hroncich MM, Vurma M (2014). Carrageenan Analysis: Part 1: Characterisation of the carrageenan test material and stability in swine-adapted infant formula. Food Additives & Contaminants: Part A, DOI: 10.1080/19440049.2014.955536.

Commission Directive 2004/45/EC of 16 April, 2004. Official Journal of the European Union, 20.4, 2004, L113/20, 21.

Grubisic Z, Rempp P, Benoit H (1967). "A universal calibration for gel permeation chromatography", Polymer Letters, **5**, 753-759.

Leatherhead Report No. 863, (2005). Titoria PM, Marrs WM, Haynes CL, Franco I. Recovery of low molecular weight carrageenan fractions by ultrafiltration through semi-permeable membranes – a feasibility study, Leatherhead Food International, U.K.

MacIntosh FC, (1941). A colorimetric method for the standardization of heparin preparations. Biochem. J., **35**, 776.

Malawer EG, Montana AJ, (1980). Introduction to size exclusion chromatography, Journal of Polymer Science: Polymer Physics Edition, **18**, 2303-2305.

Scientific Committee on Food. Opinion of the Scientific Committee on Food on Carrageenan (expressed on March 5, 2003). SCF/CS/ADD/EMU/199, Final, 21 February 2003.

Spichtig V, Austin S, (2008). Determination of the low molecular weight fraction of food-grade carrageenans. Journal of Chromatography B, **861**, 81-87.

Uno Y, Omoto T, Goto Y, Asai I, Nakamura M, Maitani T (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.

Weiner MI, Ferguson HE, Thorsrud BA, Nelson KG, Blakemore WR, Zeigler B, Cameron MJ, Brant A, Cochrane L, Pellerin M, and Mahadevan B (2015). An infant formula toxicity and toxicokinetic feeding study on carrageenan in preweaning piglets with special attention to the immune system and gastrointestinal tract. Food and Chemical Toxicology, **77**, 120-131.