

**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

INTRODUCTION

The Marinalg Working Group on Molecular Weight Determination (William Blakemore FMC, Chairman; Dr. Harris Bixler, SIAP, Secretary; Arne Graff Anderson, CPKelco; Dr. Joop de Vries / Dr. Graham Swarn, Danisco; Dr. Patrick Boulenguer, Degussa) has been carrying out experiments since April, 2003 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 for these hydrocolloids to augment the 5 cps water viscosity “if feasible”.

The purpose of the new specification is to have better control over the amount or proportion of the very low molecular weight tail of carrageenan and PES used in food products. Marinalg believes that there is no toxicological evidence to support the establishment of the specification.

Poligeenan has demonstrated adverse toxicological effects when fed under stressful conditions and in large quantities to certain rodents. Poligeenan, a substance used in medical imaging, uses carrageenan as a starting material. The carrageenan raw material is deliberately acid hydrolysed at high temperatures for extended periods of time to produce a finished product with a average molecular weight (Mw) of about 10,000 – 20,000 Daltons.

By its very nature, carrageenan comprises a broad range of molecular weight components. Therefore, animals fed carrageenan in chronic and sub-chronic feeding studies ingest this broad spectrum of molecular weight that makes up carrageenan. There is neither toxicological nor epidemiological evidence to indicate that consumption of carrageenan imparts an adverse toxicological effect. Hence, the effect of these very small amounts of naturally-occurring lower molecular components in carrageenan is moot, because studies show that consumption of carrageenan does not cause adverse toxicological effects.

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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 March 2008, the Working Group or others still have 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.

MARINALG EXPERIMENTAL WORK

OVERVIEW

The methods studied to date in detail have all been based on size exclusion chromatography (SEC) followed by concentration and molecular weight detection in the stream exiting the chromatography columns. SEC is used to spread out the carrageenan molecular size distribution in the flow stream exiting the columns. Note that this separation is by molecular size and not molecular weight, so physical models are 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.

These are highly developed commercial research instruments of great technical sophistication. Nevertheless, none met the most important objective of the Working Group. Six laboratories participated in this study, Degussa; Danisco; Viscotek, 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

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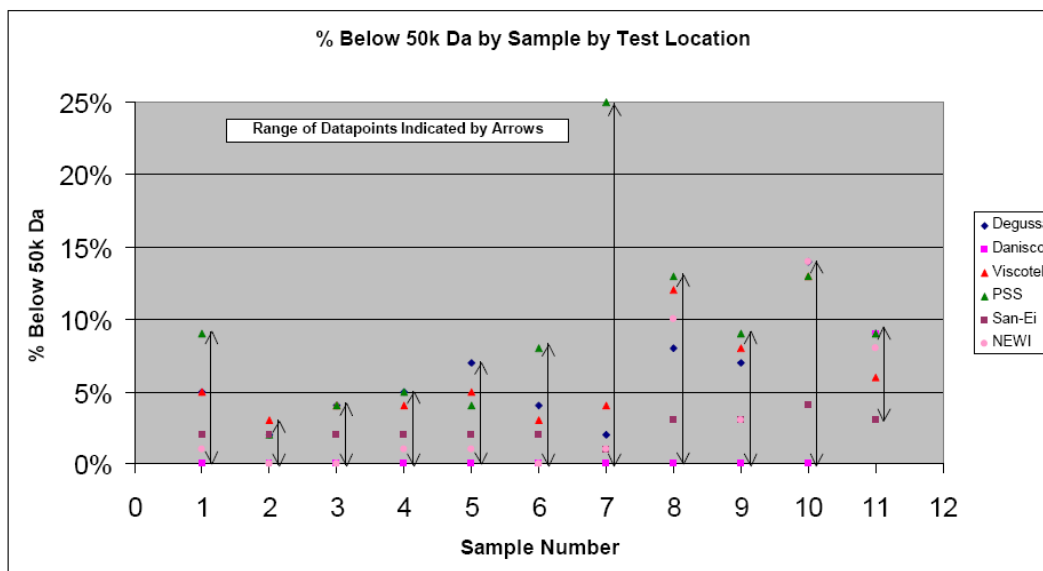
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concentration, eluent type and concentration, etc.) were recorded for each lab and approved by the Working Group. Eleven different commercial carrageenan and PES samples, representing different sulfated polygalactose types (nominally kappa, lambda and iota) made by five different producers, were tested by all laboratories under "Round Robin" conditions.

RESULTS

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 this technical discipline, inter-lab reproducibility of the LMT was shown to be poor (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).

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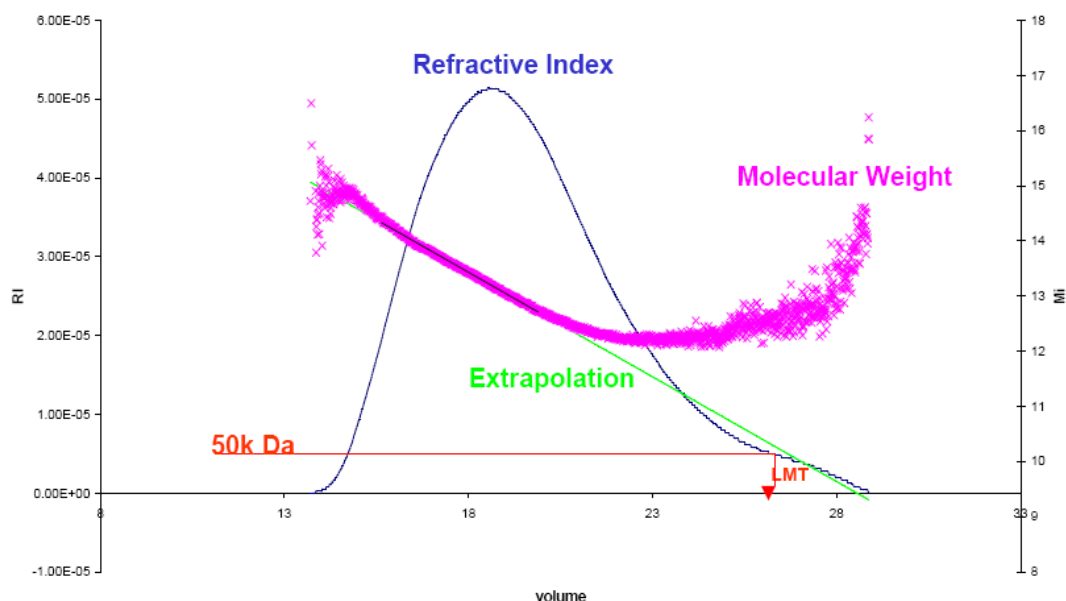
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Several test locations have experienced drifting baselines, and variable recoveries (measured concentration versus actual), both of which make data interpretation even more complex and unreliable.

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 (Figure 3).

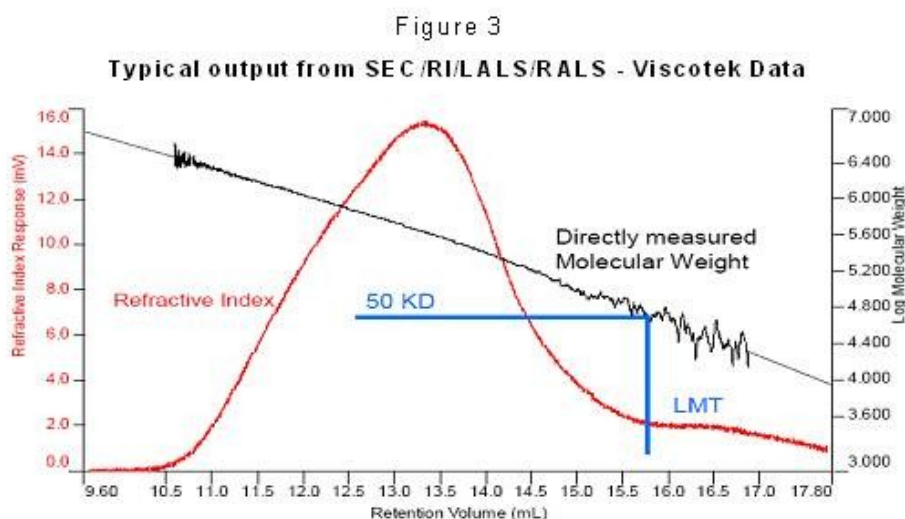
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DISCUSSION

Various fitting and extrapolation routines (Zimm, Debye, Berry) are being used in the MALS detection systems 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 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

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below about 10,000 Da by any of the light scattering techniques, 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, achieved 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 viscosity helps to maximize sample solution concentration without overloading 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, a lambda type that is known to be a more rigid rod in solution than the kappa and iota types. Furthermore there was fairly good correlation for M_w versus water viscosity (Fig. 4), except for the one aberrant sample already noted.

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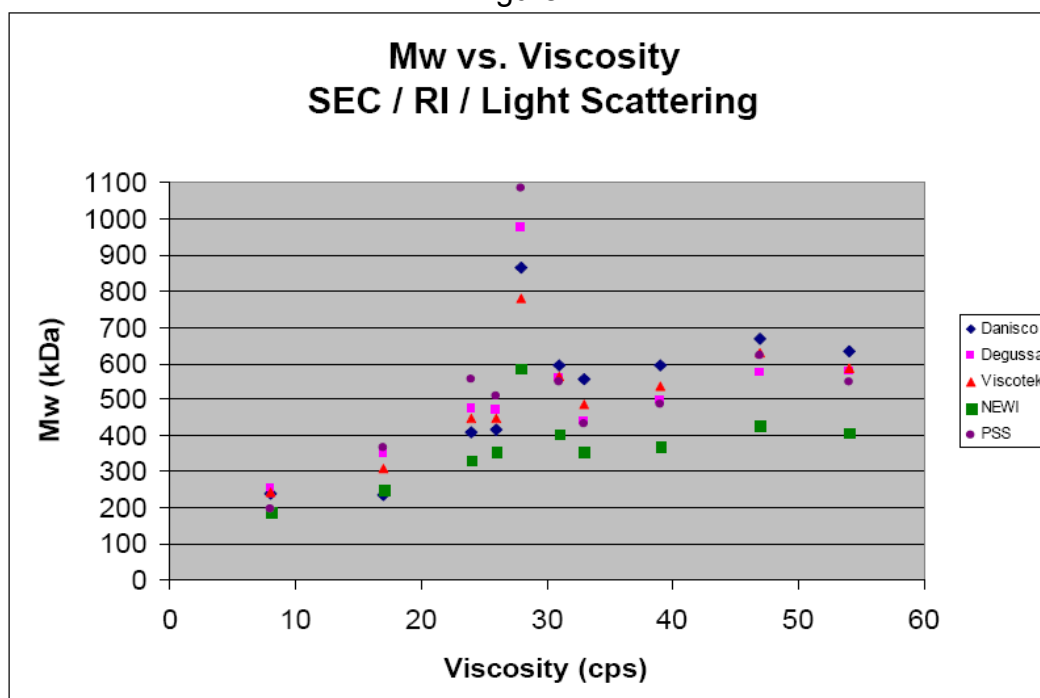
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Figure 4



The causes of these inconsistencies and poor correlations when using SEC/LI 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 measurement of non-carrageenan components as part of the LMT (e.g. salts, proteins, other carbohydrates), inaccurate and inconsistent measurement cut-off points, poor SEC separations (e.g. gelation, aggregation), column selection and efficiency, eluate and elution characteristics (e.g. concentration, viscosity), and signal to noise ratios of the measuring equipments.

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CONCLUSIONS (SEC-LS METHODS)

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 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 SEC/light scattering is probably not possible.

ALTERNATE METHODS

CARRAGEENAN STANDARDS

While the work to date with light scattering has led to frustrating conclusions, it pointed in a direction of potentially more promise which has recently been explored by Nestle researchers (Spichtig, V. and Austin, S. 2008. Determination of the low molecular weight fraction of food-grade carrageenans. Journal of Chromatography B, 861 (2008) 81-87. The Working Group had given consideration to this method before it became aware of the Nestle work. Now that the Nestle work has been published, the method seems less promising than previously thought.

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Ideally, the 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 ($M_w/M_n < 1.2$) (polydispersity index or PDI). The molecular weight of the polymer standards is 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 samples of carrageenan used in the Round Robin SEC study reported above.

For water soluble hydrocolloids, the most widely used standards are eight pullulans ranging in M_w from 5,300 to 760,000 Daltons with PDIs meeting the above polydispersity criterion. These 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, Food Additives and Contaminants, 18, No. 9, pp763-772, 2001). No correction was applied in this work for the differences between pullulan and carrageenan sizes versus molecular weights, so validation of the LMTs reported by Uno remains in question. Of course, having a set of low polydispersity carrageenan standards could eliminate the shortcomings of using pullulan standards for column calibration, but preliminary attempts to prepare carrageenan standards in the laboratory by both Nestle and the Marinalg Working Group has been disappointing. PDIs ranged from 1.3 to 2.6 with most in the range of 1.6 to 1.7, and as will be explained below were too polydisperse for reliable column calibration in the range of the LMT. The overall conclusions were similar to those reported earlier for the Viscotek data. The method appears to be reproducible with respect to repeat LMT measurements on the same sample in the same laboratory, but several hurdles stand in the way of it being developed into a universal validated method.

One issue with using standards for column calibration is related to how close the numerical value at the top of the elution peak is to the measured M_w . They are

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close to being equal with the pullulan standards with polydispersity indices of 1.1 to 1.2. Pullulan standards give very sharp elution curves. However, it is not the case with polydispersity indices in the 1.6 to 1.7 range. For one of the Marinalg carrageenan standards the Mw was 246,000 Da and the Mn 149,500 Da for a polydispersity index of 1.6. The molecular weight at the top of the peak was 193,000 Da. This represents an error of over 20%. Any use of carrageenan standards must use the molecular weight at the elution peak, and not the Mw (average) to be an improvement over the SEC methods reported above.

Adopting a method using carrageenan standards would demand that such standards be readily available for all involved as is the case with the commercial pullulan standards. This means that the carrageenan standards would have to be made in bulk, and made accurately and reproducibly. No such protocols are close to being successfully developed.

Austin, et al suggested in their paper that the LMT specification might be moved from 5% to 8%. All of their measured LMTs were less than 8%; whereas only half of the sample LMTs were less than 5%. Since there is little basis in fact toxicologically for the EC numerical LMT specification as it is, industry should resist the adoption of an expensive, inaccurate method on the basis that if the LMT is set high enough all commercial samples seem to pass. Either an economical, accurate method can be developed or the long employed water viscosity limit should be continued as a molecular composition health safety criterion.

The Nestle work on jellies did confirm prior experimental work and conclusions with respect to controlling carrageenan degradation during food processing and storage, as has been detailed over the years in the JECFA dossiers.

UNIVERSAL CALIBRATION

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, Z. et al, Polymer Letters, 5, pp753-759, 1967). The model takes into consideration size and shape differences for the two polymers

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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. However, it must continually be kept in mind that the Uno method is extremely expensive and technology intensive. It could be a significant problem for smaller companies that make perfectly safe carrageenan as determined by the long used, reliable and inexpensive water viscosity purity criterion.

A related technology referred to as "polydisperse or broad standard calibration" is also under investigation (Malawer, E.G. and A.J. Montana, Journal of Polymer Science: Polymer Physics Edition, 18, pp2303-2305, 1980). 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 Mw 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 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.

ULTRAFILTRATION

One technology currently being assessed is the potential use of membranes to separate the LMT from the rest of the carrageenan. This potential method would have to be combined with the colorimetric measurement of very low concentrations of carrageenan or some other equivalent method. The primary issue for development of this technology would be the production of membranes with consistent cut-off at 50k Da.

Recent work carried out at Leatherhead Food International has shed some light on this potential technology. (Titoria, P.M., Marrs, W.M., Haynes, C.L., and Franco, I. Recovery of Low Molecular Weight Carrageenan Fractions by Ultrafiltration Through Semi-permeable Membranes - A feasibility Study. Research Report No. 863, October 2005.)

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The carrageenans were separated into permeate and retentate using Vivaspin membrane devices, with nominal cut-off points at 100kDa and 50kDa. The fractions were analysed for Mw using SEC.

The primary conclusions were as follows.

- These membrane types are capable of separating carrageenans into permeate and retentate fractions, but not characterized by a sharp cut-off point in terms of molecular size.
- The membranes used (100kDa and 50kDa) are not suitable for this application, primarily because the nominal cut-off Mw points were characterized for globular proteins, and not carrageenan, these hydrocolloids having significantly different molecular size to weight ratios. Membrane products with lower nominal porosities in the 30kDa to 5kDa range were recommended.
- The movement through the membrane is kinetic in nature, the longer chain lengths taking much longer time to pass through the membrane. This means that care has to be taken to achieve equilibrium, if complete equilibrium is actually possible within practical time limits.
- It is clear that this technology needs significant advances before becoming a realistic option to measure the LMT.

CONCLUSIONS (ALTERNATE METHODS)

The Marinalg Working Group will continue to explore these alternate methods, but success seems very remote.

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.

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