Journal of Applied Polymer Science

A Comprehensive Study on the Size Exclusion Chromatography of Kappa-Carrageenan for the Identification of After-Peaks

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ABSTRACT: Aqueous size exclusion chromatography (SEC) of polysaccharides in general and carrageenans in particular is complicated by a number of factors. The chromatograms of carrageenans which are sulfated anionic natural polymers contain a number of after-peaks depending on the occlusion, adsorption, or association of various ionic species either naturally present or evolved during their processing. A systematic SEC analysis of after-peaks appearing in the chromatograms was made to identify the species responsible for their formation. The five after-peaks constantly appearing in the aqueous (0.1*M* $NaNO_3$) SEC of kappa-carrageenan are attributed to sulfate, chloride, and nitrate anions whereas the first three and the fourth are due to divalent cations, mostly, and the fifth appears to result from the unknown impurities. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: size exclusion chromatography; kappa-carrageenan; after-peaks

Received 17 July 2011; accepted 22 March 2012; published online
DOI: 10.1002/app.37758

INTRODUCTION

Carrageenans are sulfated natural anionic polymers that comprise the main structural polysaccharides in red seaweed. Carrageenans are composed of alternating $\alpha(1 \rightarrow 3)$ and $\beta(1 \rightarrow 4)$ linked D-galactose residues. The three primary forms of kappa, lambda, and iota (κ, λ, ι) carrageenans are based on the modification of the disaccharide repeating unit resulting from the occurrence of the ester sulfate, or anhydride formation in the four-linked residue. The repeating units of the idealized structure of κ, λ, ι carrageenans are shown in Figure 1. κ- and ι-carrageenan have the ability to form thermo-reversible gels upon cooling of hot aqueous solutions containing various salts. λ-Carrageenan does not jellify as it is soluble in cold water.[1]

For the last two decades numerous papers have been published on the carrageenans due to their nonfood applications and versatile applications in food industry. The earliest studies on carrageenans have focused primarily on the identification of gelation mechanism, investigation of morphological properties, and the effect of counter ions on the gelation and rheological properties.[2–4]

Recent studies of carrageenans have focused primarily on the identification of nonfood application areas,[5,6] interaction with high energy radiation, explanation of the degradation mechanism,[7] and the effect of molecular weight on the toxicological behavior.[8,9] Carrageenan oligomers were shown to elicit marker enzymes of the defence metabolism in Rubus cells and protoplast and also to have anti-HIV activity.[5,6,10] It has also been reported that the degree of sulfation and molecular weight are the important factors that have influenced the anti-HIV activity of κ-carrageenan (KC). Due to frequent use of the carrageenans within, for example, the food and pharmaceutical industry, extensive toxicological evaluation has also been carried out. The major problem that has been identified involves the possibility of causing lesion for the low molecular mass (<20,000 g/mol) fractions. Degraded carrageenans cause ulcerative colitis in rats and guinea pigs and is used in experimental models to study the effects of pharmacological and therapeutic agents.[11–13]

Due to the importance of the molecular weight realized in the final application of polysaccharides, many attempts have been made in recent years for the controlled degradation of carrageenans to achieve targed molecular weights.[14] Understanding the significant effects of high energy radiation on controlling the molecular weight and distribution of carrageenans has therefore once more been the subject of extensive studies since this technique is known to be the most direct one in scissioning the main chain of polysaccharides as compared to conventional techniques.[7,15,16]

When the molecular weight averages and distribution of polymers are considered, size exclusion chromatography (SEC) is the most useful method to be used. In aqueous size exclusion

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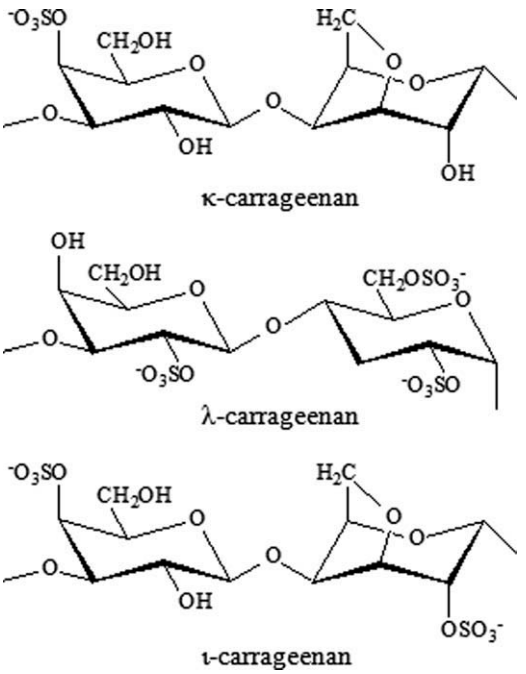

Figure 1. The idealized structure of kappa (κ), lambda (λ), and iota (ι) carrageenans.

chromatography, however, many factors affect the reliable determination of molecular weight of polyelectrolytes. In aqueous SEC, the separation mechanism is the same as organic SEC systems for nonionic, hydrophilic polymers. However, for anionic, cationic, and/or hydrophobic polymers nonsize-exclusion effects have been observed to take place complicating the data analysis.*

To make a reliable analysis with aqueous SEC systems, the nonsize-exclusion effects must be understood. Ionic interactions between polymer and packing materials (ion exclusion, ion inclusion, and ion exchange), adsorption on packing materials (by hydrogen bonding and by hydrophobic interactions), intramolecular electrostatic effects, and solvophobic association can be mentioned under this category.

In our studies, we observed that κ-carrageenans give five distinct after-peaks in aqueous SEC when eluted by using hydroxylated methacrylate columns. Similar peaks that have been observed and reported in literature by a number of researchers are referred to as "after-peak" or "impurity" in the molecular weight determination studies and even by the producers of the column systems[17,18] and in many studies these after-peaks are not even mentioned at all.[19,20] Only one study performed by Karlsson and Singh has mentioned about the first and the second after-peaks which were also observed in this study. They have used the first after-peak for the determination of free sulfate groups and the latter for following the desulphation and depolymerization of KC during the acid hydrolysis.[13] However, nothing was mentioned about the other after-peaks observed in SE chromatogram of KC in their study.

*Waters Corporation Technical notes "Water Soluble Polymers" Rev. 21199.

In our studies, especially on marine-based polysaccharides the main interest is focused in controlling the molecular weights of polysaccharides by degrading them to targed molecular weights using ionizing radiation. In our comprehensive SEC studies of pristine and degraded carrageenans, we have constantly and reproducibly observed distinct after-peaks in the chromatograms of this polymer and decided to elaborate on this behavior more closely. In this article, we report the identification of after-peaks and characterization of species giving rise to their formation.

MATERIALS AND METHODS

Materials

κ-carrageenan (KC) was purchased from Aldrich (lot no: 19003TA). KC samples having different molecular weights were obtained by irradiating KC in air at ambient temperature in a Gamma Cell ^{60}Co irradiator with the dose rate of 0.07 kGy/h. The irradiation doses together with the weight ($\overline{M}_w$) and number ($\overline{M}_n$) average molecular weights of κ-carrageenan samples used in this study are given in Table I. The salts used for the identification of after-peaks LiCl, $LiNO_3$, Li_2SO_4, NaCl, $NaNO_3$, Na_2SO_4, KCl, KNO_3, $Ca(NO_3)_2$, and $MgCl_2$, were of Analar grade and all obtained from Merck.

SEC Analysis

SEC of the κ-carrageenans was performed on a Waters chromatograph equipped with Waters 1525 binary HPLC pump, Waters 2414 refractive index detector and two waters ultrahydrogel columns (Ultrahydrogel 2000 and 1000). Elution was carried out by using 0.1M $NaNO_3$ as the mobile phase at a flow rate of 0.8 mL/min. The temperature of the columns and the detector were both maintained at 45°C. A universal calibration curve was constructed by using polyethylene oxide standards taking the K and a constants as $K = 6.9 \times 10^{-3}$ mL/g and $a = 0.81$ for PEO.[21] The K and a constants used for κ-carrageenan were $K = 5.98 \times 10^{-3}$ mL/g and $a = 0.90$.[22] The carrageenan concentration used was 0.2%.

RESULTS AND DISCUSSION

SEC of PEO in Aqueous Solutions

Before investigation of the chromatographic behavior of κ-carrageenan, we first examined the after-peak formation in the SE chromatograms of a neutral and narrowly distributed poly(ethylene oxide). The SE chromatograms of PEO (M_{peak} : 553,000)

Table I. The Weight ($\overline{M}_w$) and Number ($\overline{M}_n$) Average Molecular Weight of κ-Carrageenan Samples Used in This Study

Name	Irradiation dose (kGy)	$\overline{M}_w$	$\overline{M}_n$	$\overline{M}_w/\overline{M}_n$
KC	0.0	507,250	145,200	3.49
KC-1	2.5	325,100	112,000	2.90
KC-2	10.0	174,100	77,700	2.24
KC-3	20.0	123,400	60,000	2.06
KC-4	50.0	65,600	39,000	1.68
KC-5	100.0	39,850	27,000	1.48

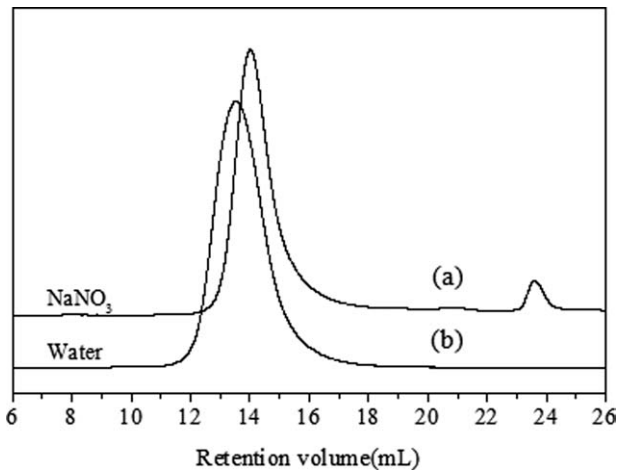

Figure 2. The size exclusion chromatograms of PEO (M_{peak}: 553,000 g/mol) obtained in hydroxylated methacrylate columns. The eluents are (a) 0.1*M* $NaNO_3$ solution and (b) HPLC grade ultrapure water.

obtained in hydroxylated methacrylate columns when 0.1*M* $NaNO_3$ solution and HPLC grade ultrapure water were used as the eluents are given in Figure 2. As can be seen from the figure, there is no peak other than the polymer peak of PEO observed in the chromatogram when the eluent was pure water. However, in the chromatogram of the same polymer, there exists another peak following the polymer peak when the eluent is 0.1*M* $NaNO_3$ solution. This peak is named as the "salt peak." This is an unavoidable result when a nonionic and hydrophilic polymer is analyzed in a salt solution. The salt peak observed in the SE chromatogram of PEO is the result of the concentration gradient created throughout the run by the added salt. Keeping the salt concentration as low as possible and/or adjustment of the pH to a polymer specific value to minimize the dissociation tendency are two ways of minimizing the salt peak. Although, PEOs have no salt peak when eluted in pure water, it is important to note this behavior in 0.1*M* $NaNO_3$ solution since PEOs were used to construct the universal calibration curve of KC.

SEC of κ-Carrageenans

The SE chromatograms of KC samples having different molecular weights are given in Figure 3. As can be seen from the figure, all KC samples have five positive after-peaks following the main peak of the polymer. These after-peaks do not overlap with the main peak significantly until an average molecular weight of 40,000. However, below that molecular weight, these after-peaks start overlapping with the small molecular weight fractions of the polymer. These after-peaks were either not given or not explained at all by many researchers in their carrageenan-related molecular weight determination studies. In fact, observation of these peaks also in the chromatogram of purified KC samples point out that they are not impurities. On the other hand, change in the relative ratios of these peaks in the chromatograms of the KC samples purchased from different manufacturers and disappearance of some of them in the SE chromatograms of Na^+-KC samples obtained by exchanging the K^+ ion in KC structure with Na^+ ions prove that these after-peaks are caused by the ions associated with the KC structure. The chromatograms of Na^+-KC prepared by dialyzing KC against NaCl and nondialyzed KC samples are given in Figure 4.

To convert KC used in this study into the Na^+ form, the KC solution (%1 w/w) had been dialyzed against 0.2*M* NaCl solution for 4 days. The SE chromatogram of the sample, dried before removal of excess NaCl by washing, was given in Figure 4 and named as "dialyzed but not washed KC." As can be seen from the chromatogram of the dialyzed but not washed KC sample, the number of the after-peaks and their relative ratios to the main polymer peak are different from those of the original KC sample. Intensities of the second (II) and the third (III) after-peaks in the chromatogram of dialyzed but not washed sample are greater than those of original KC sample. This increase in the intensity was the result of excess NaCl adsorbed by the polymer. It is interesting to note that the fourth (IV) after-peak completely disappeared in the dialyzed sample. The SE chromatogram of the KC sample (Na^+-KC), washed with 90% 2-isopropanol/10% water mixture twice, is also given in Figure 4. The significant decrease in the intensities of the second and the third peaks and disappearance of the fourth and

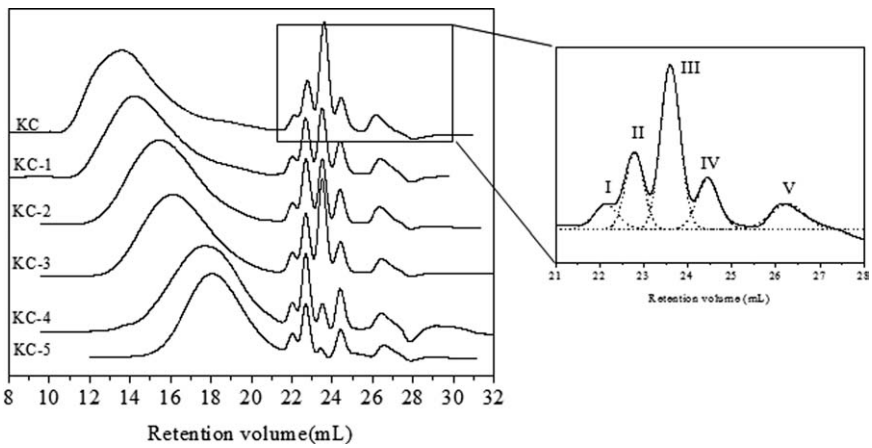

Figure 3. The size exclusion chromatograms of KC samples having different molecular weights as listed in Table I.

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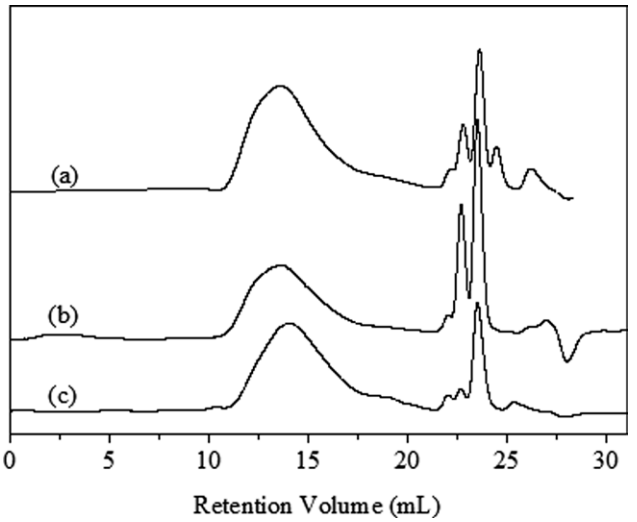

Figure 4. The size exclusion chromatograms of (a) original KC, (b) dialyzed but notwashed KC, and (c) dialyzed and washed KC.

the fifth peaks after washing indicates that ion exchange process took place during the dialysis and washing stages which confirms the idea that the after-peaks are caused by the ions associated with KC structure.

Considering the basic principles of size exclusion chromatography, one can immediately conclude that the after-peaks are due to small-sized species excluded from the carrageenan chains. Their ionic forms have already been confirmed from our dialysis studies, now the question is the identification of species giving rise to the formation of these after-peaks. Either the counter-ions associated with the dissociated sulfate groups on the carrageenan chains or impurity ions adsorbed on the chains are being released with the consequent effect of appearance as after-peaks.

To investigate the separation performance of the columns which are filled with microgels made of hydroxylated methacrylates towards various anions and cations that are most likely to be present in carrageenans, their 1×10^{-3} *M* salt solutions were injected to the chromatograph under conditions identical to polymer injection. The SE chromatograms of these salt solutions will be discussed below.

SE Chromatography of Various Salts

Figure 5 shows the chromatograms of 1×10^{-3} *M* salt solutions obtained in hydroxylated methacrylate columns. The after-peaks observed in the chromatogram of the original KC sample were given again for a direct comparison. Despite their relatively small sizes, it is clear from the chromatograms of salt solutions given in Figure 5 that anions and cations of these salts were separated as a result of the ion inclusion and exclusion effects in this column system by giving positive and negative peaks.

When two sulfate containing salts, namely Na_2SO_4 and Li_2SO_4 were injected the corresponding chromatograms give neatly positive peaks appearing at 22.2 mL retention volume. The first after-peak of KC appears at exactly this retention volume. The chromatograms of chloride containing salts namely, NaCl, LiCl, KCl, and $MgCl_2$ give positive peaks consistently at 22.8 mL retention volume which overlaps with the second after-peak of KC. The chromatograms of $NaNO_3$ and $Ca(NO_3)_2$ on the other hand give positive peaks at 23.6 mL, this volume corresponding exactly to the third after-peak of KC which is due to nitrate anion. We are, therefore, now able to assign the first three after-peaks(I, II, and III) of KC to be related to the presence of sulfate, chloride, and nitrate anions associated within the polymer structure. Other interesting features observed from these chromatograms are the following. The behavior of cations seems to be more complicated. K, Ca, and Mg ions elute at 24.4 mL irrespective of their counterions. This retention volume again corresponds directly to the fourth (IV) after-peak of KC, which was totally removed when the polymer was dialyzed by NaCl. Therefore, the fourth after-peak of carrageenan is most probably due to the presence of divalent cations adsorbed by the polymer. Three monovalent cations investigated in this work tend to show negative peaks at around 23.6 mL though it is very small for Na, more distinct for Li and K ions. Since the effect of Na

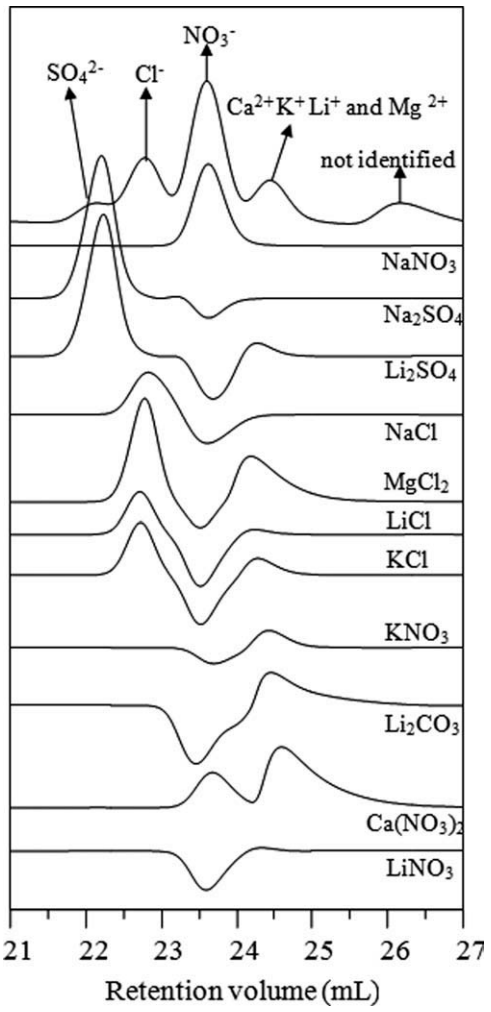

Figure 5. The chromatograms of 1×10^{-3} *M* salt solutions obtained in hydroxylated methacrylate columns.

ions especially is rather small at this retention volume and nitrate appearing at the same volume is very strongly positive, we think we are correct in assigning the positive peak of 23.6 mL to be coming exclusively from nitrate.

The column system used in this study was hydroxylated methacrylate column. This column packing materials contains small amount of weakly anionic groups. The anionic character of the column packing material creates repulsive (exclusion) forces at low ionic strength of the eluent when the sample has anionic character. This results with the sample exclusion from the pores and a lower elution volume than theoretically expected. Conversely, the cationic samples are retained by ionic adsorption and/or inclusion and elute later.

It is known that ion exclusion is observed when the sample and the packing material have the same charge. Ionic repulsions prevent the sample to permeate into the pores and the result is a lower elusion volume than expected.

In the case of ion inclusion, the polyelectrolyte charge is opposite of the column packing material. The smaller molecules are retarded by the larger molecules of the same charge from leaving the pores. This effect is known as the Donnan membrane effect. Here, membrane is the interface between the interstitial volume and the pore volume. Since only the small molecules and the counter ions are able to pass through the membrane, this effect causes an increase in the elution volumes of the low molecular weight fractions with the increase in the concentration of the injected sample.

In our opinion, the separation of various anions and cations as observed in this work is mainly due to the ion-dipole interactions between the corresponding ions and the column filling material, hydroxylated methacrylates mediated by the eluent. Observation of the divalent sulfate anion as the first after-peak is the result of its stronger exclusion from the pores when compared with monovalent chloride and nitrate anions. These experimental results also indicate that the inclusion behavior for the cations is not a distinctive property for their separation in this column material.

Although the instrument we used is not an ion chromatograph, both the differences in the sizes and interactions with the column material of various ions provided enough difference in their retention volumes making their relative separation possible by SEC.

CONCLUSIONS

Working with aqueous SEC is not as easy as working with organic SEC because of the effects like ion exclusion, ion inclusion, hydrogen bonding, etc. The evaluation of data from the chromatograms of polysaccharides is even more difficult due to natural origin of these polymers. The difficulty and complication of interpreting molecular weight averages of marine-based polysaccharides is mainly due to the presence of a number of after-peaks appearing in their SEC chromatograms. They were assumed to be due to some ionic impurities but never tried to be explained by the researchers working with these polymers and especially carrageenans. A systematic analytical approach displayed in this work helped us to identify these after-peaks in the case of kappa carrageenan. We attribute the five after-peaks always observed in aqueous SEC of this polymer as due to sulfate, chloride, nitrate anions for the first three and the fourth to monovalent and divalent metallic ions, K and Ca being the most likely ones. The fifth after-peak was not identified and assumed to be due to impurities. This work shows that with a careful examination of the after-peaks observed in the aqueous SEC of carrageenan with hydroxylated methacrylate column system, it is possible to have an insight into the type of ionic species existing in κ-carrageenan.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support provided by the Hacettepe University Research Funding through 02 G 105 Infrastructure Project. OG appreciates the support of the Academy of Sciences of Turkey and the International Atomic Energy Agency through Research Contract No. 14475/R02.

REFERENCES

1. Te Nijenhuis, K. Thermoreversable Networks: Viscoelastic Properties and Structure of Gels. Advances in Polymer Science, 1st ed.; Springer Verlag: Berlin, **1997;** Vol. 130, pp 203–209.
2. Morris, E. R.; Rees, D. A.; Robinson, C. *J. Mol. Biol.* **1980,** *138*, 349.
3. Smidsrod, O.; Gransdalen, H. *Carbohydr. Polym.* **1982,** *2*, 270.
4. Aranilla, C. T.; Yoshii, F.; dela Rosa, A. M.; Makuuchi K. *Radiat. Phys. Chem.* **1999,** *55*, 127.
5. Yamada, T.; Ogamo, A.; Saito, T.; Watanabe, J.; Uchiyama, H.; Nakagawa, Y. *Carbohydr. Polym.* **1997,** *32*, 51.
6. Yamada, T.; Ogamo, A.; Saito, T.; Uchiyama, H.; Nakagawa, Y. *Carbohydr. Polym.* **2000,** *41*, 115.
7. Relleve, L.; Nagasawa, N.; Luan, L. Q.; Yagi, T.; Aranilla, C.; Abad, L.; Kume, T.; Yoshii, F.; dela Rosa, A. *Polym. Degrad. Stab.* **2005,** *87*, 403.
8. Tobacman, J. K. *Environ. Health Perspect.* **2001,** *109*, 983.
9. Tobacman, J. K.; Wallace, R. B.; Zimmerman, M. B. *Med. Hypotheses* **2001,** *56*, 589.
10. Patier, P.; Potin, P.; Rochas, C.; Kloareg, B.; Yvin, J. C.; Lienard, Y. *Plant Sci.* **1995,** *110*, 27.
11. Delahunty, T.; Recker, L.; Hollander, D. *Food Chem. Toxicol.* **1987,** *25*, 113.
12. Marcus, A. J.; Marcus, S. N.; Marcus, R.; Watt, J. *J. Pharm. Pharmacol.* **1989,** *41*, 423.
13. Karlsson, A.; Singh, S. K. *Carbohydr. Polym.* **1999,** *38*, 7.
14. Şen, M.; Yolaçan, B.; Güven, O. *Nucl. Instrum. Methods Phys. Res. Sec. B* **2007,** *265*, 429.
15. Nagasawa, N.; Mitomo, H.; Yoshii, F.; Kume, T. *Polym. Degrad. Stab.* **2000,** *69*, 279.
16. Kume, T.; Nagasawa, N.; Yoshii, F. *Radiat. Phys. Chem.* **2002,** *63*, 625.

17. Ueda, K.; Itoh, M.; Matsuzaki, Y.; Ochial, H.; Imamura, A. *Macromolecules* **1998,** *31*, 675.
18. Knutsen, S. H.; Sletmoen, M.; Kristensen, T.; Barbeyron, T.; Kloareg, B.; Potin, P. *Carbohydr. Res.* **2001,** *331*, 101.
19. Villanueva, R. D.; Mendoza, W. G.; Rodrigueza, M. R. C.; Romero, J. B.; Montano, M. N. E. *Food Hydrocolloids* **2004,** *18*, 283.
20. Meunier, V.; Nicolai, T.; Durand, D.; Parker, A. *Macromolecules* **1999,** *32*, 2610.
21. Kurata, M.; Tsunashima, Y. In Polymer Handbook; Brandrup, J., Immergut, E. H., Eds.; John Wiley and Sons: New York, **1989;** p VII/22.
22. Lai Vivian, M. F.; Cheng-yi, L.; Wei-Ling, H.; Ting-Jang, L. *Food Chem.* **2000,** *68*, 319.