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Isolation of κ-carrageenan oligosaccharides using ion-pair liquid chromatography—characterisation by electrospray ionisation mass spectrometry in positive-ion mode

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Abstract—Oligo-κ-carrageenans participate as elicitors in the cell–cell recognition process in marine plants. Analytical methods can be usefully applied to gain insight into the biochemistry of these biological processes. Therefore, enzymatically digested oligomers of κ-carrageenans have been separated and isolated on a Spherisorb ODS1 (250×4 mm i.d., particle size 5 μm) column using ion-pair liquid chromatography coupled with an evaporative light scattering detector. Heptylamine (5 mM, pH 4) has been selected as the ion-pairing agent and MeOH as the organic modifier in a gradient mode. Overloading the column with 1 mg of the mixture, the chromatographic mechanism presented adequate stability. The mobile phase of each isolated oligomer was evaporated and the residue was infused into an electrospray ionisation mass spectrometry (ESIMS) in positive-ion mode with 4:1 MeCN–water as mobile phase. Each ESIMS spectrum presented ions consisting of the oligomer attached with a number of heptylammonium ions depending on the molecule size. In addition, the different m/z values permitted direct detection of the oligomers in ESIMS positive-ion mode. The analytical method developed separated the oligomers up to dotriacontasaccharide.

Keywords: κ-Carrageenan; Ion-pair liquid chromatography; Electrospray ionisation mass spectrometry; Evaporative light scattering detector; Sulfated oligosaccharides

1. Introduction

Carrageenans are sulfated galactans occurring in the cell wall of marine red seaweeds (*Rhodophyta*). They are major components of the matrix involved in the building up of the cell-wall architecture[1,2] and they mediate cell–cell recognition in host–pathogen interactions.[3,4] The exceptional physico-chemical properties of carrageenans are widely exploited as thickening and gelling agents in various structural and functional applications.[5–7] This large family of hydrocolloids share the same backbone structure, which consists of a linear chain of 3-linked, β-D-galactose (**G**-unit) and 4-linked α-D-galactose (**D**-unit). Carrageenans are classified according to the number and the position of sulfated ester, and by the occurrence of 3,6 anhydro-bridges in the α-linked residues (**A**-unit) found in gelling carrageenans. For example, the three most industrially exploited carrageenans, namely *kappa*- (κ, **A-G4S**) (Fig. 1), *iota*- (ι, **A2S-G4S**)

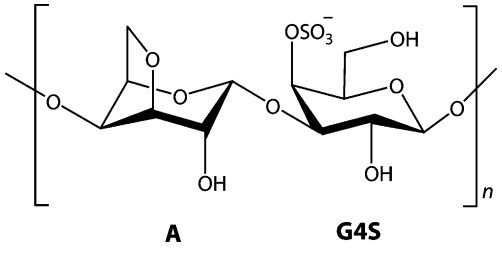

Figure 1. Neocarrabiose $(\mathbf{A\text{-}G4S})_n$, the repeating unit of oligo-κ-carrageenans.

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and *lambda*- (λ, **D2S6S-G2S**) carrageenans, are distinguished by the presence of one, two and three esters sulfate groups per repeating disaccharidic unit, respectively.

However, carrageenans have very heterogeneous chemical structures, depending on the algal sources, the life stages and the extraction procedures of the polysaccharides. This structural complexity is attributed to the occurrence of a mixture of carrageenans in extracts as well as to the combination of ideal carrabiose motives in purified carrageenans giving rise to a hybrid or copolymer chain.[1,8,9] The most classical copolymers of carrageenan are those found in native or unprocessed κ- and ι-carrageenan chains that usually contain fractions of their biosynthetic precursors named *mu*- (μ, **D6S-G4S**) and *nu*- (ν, **D2S6S-G4S**) carrageenans, respectively.[10,11] Other carrabiose combinations have been also demonstrated such as the κ/ι-(**A-G4S/A2S-G4S**) carrageenan hybrids (or κ2-carrageenan) in several species of the *Gigartinacae* family[12–14] and the κ/β-(**A-G4S/A-G**) carrageenan copolymer found in *Furcellaria* sp. and *Euchema gelatinae*.[15,16] Another layer of complexity is also reached when methyl or pyruvate groups are taken into account to describe carrageenan structures.[8,9]

The chemical and spectrometric methods that have been developed until now and applied to the structural analysis of carrageenans, usually lead to the determination of linkages and averaged composition.[1,8,17] However, the fine description of the organisation of carrabiose units as well as other chemical groups along carrageenan chains is still an unsolved problem. The pioneering work by Yaphe's group[12,15,18] suggests that enzymes could be very helpful tools for a better understanding of carrabiose sequences in carrageenans. Indeed, carrageenan hydrolases or carrageenases offer the advantage of fragmenting carrageenan molecules by the specific disruption of the β-(1 → 4) glycosidic linkages in aqueous conditions without drastic chemical treatment that may interfere in the solving of the native structure. Carrageenases are also specific to a given class of carrageenan that is, κ-carrageenases degrade κ-carrageenans but are inactive on ι-carrageenan.

Hence, the analysis of carrageenan mixtures and enrichment of the minor fractions (enzymes resistant fractions) becomes a very accessible perspective. More recently, Knutsen and Grasdalen have observed that the κ-carrageenase of *Alteromonas carrageenovora* is able to accommodate in its active site some carrabiose motives that differ from the κ-carrabiose sulfating patterns.[16,19] Thus, analysis of the degradation products could lead to a description of the carrabiose motives distribution along the carrageenan chains.[20] Nevertheless, in an attempt to describe routinely the detailed carrageenan structure with enzymes, further progress is needed in the understanding of the mode of action of these catalysts as well as some methodological developments for an in-depth analysis of their degradation products.

In this context, nuclear magnetic resonance (NMR) spectroscopy (both ^{1}H and ^{13}C NMR) is one of the most powerful techniques applied to the characterisation of carrageenan oligomers and polymers, and advances in that field have been reviewed recently.[21]

Electrospray ionisation mass spectrometry (ESIMS) is another spectrometric emerging method that has strong analytic potential.[22–25] Unlike NMR, the amount of specimen analysed is very low and the mass spectrometer (MS) can be coupled directly to liquid chromatography (LC), enabling on-line analysis of complex structures. However, the LC/ESIMS coupling applied to the characterisation of poly-sulfated oligo-carrageenans has not yet been reported because separation by LC of highly ionic saccharides is achieved using very alkaline and/or high salt concentration,[26–28] and such conditions are incompatible with an on-line injection of the fractions in the mass spectrometer. Indeed, analytical methods based on ion exchange and porous graphitic carbon columns separated the oligo-κ-carrageenans, but entailed the difficulty of coupling the LC system with the mass spectrometer.[29]

Ion-pair reversed phase liquid chromatography (IP-RPLC) offers a different retention mechanism that requires a smaller salt concentration than for the more classical LC purification procedures already proposed. The IP-RPLC/ESIMS coupling has been successfully applied to the characterisation of several sulfonated saccharide compounds using volatile ammonium ions as pairing agent.[30,31]

In the present work, we have tested the performance of IP-RPLC coupled to an evaporative light scattering detector (ELSD) applied to fragmented κ-carrageenan with enzymes. The IP-RPLC/ESIMS coupling has been also undertaken in order to define the limitations of this analytical procedure in the precise case of carrageenan oligomers.

2. Results and discussion

2.1. Selection of ion-pairing agent

The principle of ELSD,[32] as well as the isolation of the oligomers, restricted us to volatile ion-pairing agents. Alkylammonium formate salts under certain concentrations are sufficiently volatile[33] and due to the polyanionic nature of oligo-κ-carrageenans, a low concentration of these salts is sufficient to observe an increase in retention time. Therefore, triethyl, pentyl and heptylammonium formate were tested, in a constant concentration of 5 mM, at a pH value of 4 in 15% of MeCN in isocratic mode. The concentration of the ion-

pairing agent remains constant even when an organic modifier is involved, despite the fact that the latter can modify the pH value.[34]

Triethyl and pentylammonium formate gave a poor resolution, as in the case of the sulfobutyl ether of β-cyclodextrin.[30] In order to have adequate selectivity, we ought to increase the concentration of the ion-pairing agent. However, the sensitivity of ESIMS is reduced when amine concentration in the HPLC eluent is increased. Consequently, low concentrations of the ion-pairing agent are desirable.[35] Heptylamine was selected as the ion-pairing agent because of its higher selectivity at a smaller concentration and because this concentration level does not pose volatility problems for the ELSD. To obtain selectivities with pentylamine at the same level as with heptylamine, a concentration of up to 10 times the concentration of the latter is needed. Alkylamines with a longer alkyl chain were not tested as they would pose volatility problems for the ELSD.

In order to study the effect of concentration of the heptylammonium ion on the selectivity of the oligo-κ-carrageenans, different concentrations of the above ion-pairing agent have been examined. Figure 2 depicts the separation of the oligomers of κ-carrageenan in a concentration of 3.0, 3.4 and 4.0 mM of heptylammonium formate. A small change in the former affects the retention and the selectivity of these oligomers. Linear regression analysis (Fig. 3) proved that the retention factor (k) is in a linear correlation with the concentration of the ion-pairing agent.[36]

In our case, the concentration of the ion-paring agent was kept constant because a variation in the content of the mobile phase in organic modifier would create a variation in the concentration of the ion-pairing agent in the stationary phase.[36]

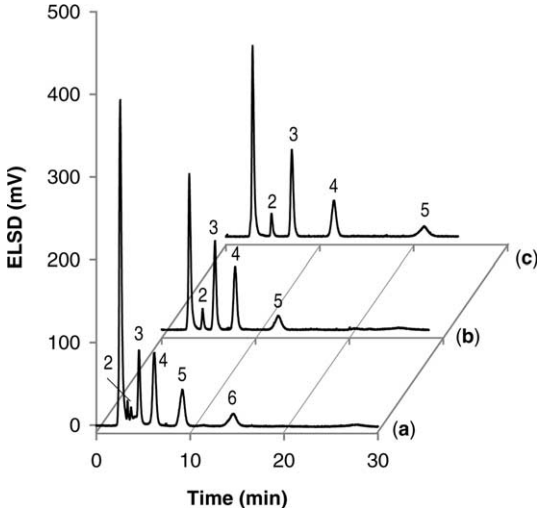

Figure 2. Chromatograms of oligo-κ-carrageenans in 15% of MeCN in a concentration of (a) 3.0 mM; (b) 3.4 mM; (c) 4.0 mM of ion-pairing agent (heptylammonium formate, pH 4) in the mobile phase in isocratic mode.

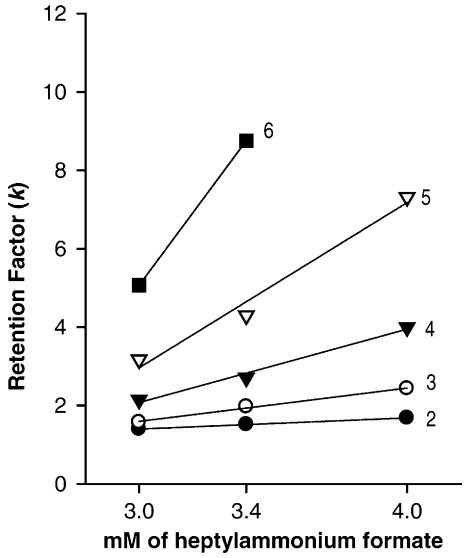

Figure 3. Variation of the retention factor (k) of oligo-κ-carrageenans in 15% of MeCN as a function of the concentration of ion-pairing agent (heptylammonium formate, pH 4) in the mobile phase. Lines represent linear regression.

2.2. Selection of organic modifier

The k values as well as selectivities of solutes separated by ion-pair approaches with reversed-phase systems depend markedly on the mobile phase composition and in particular on the organic solvent selected.[37] Two typical organic modifiers (MeOH and MeCN) have been studied in the presence of heptylammonium formate (5 mM, pH 4) as the ion-pairing agent.

Figures 4 and 5 depict the chromatograms of oligo-κ-carrageenans with MeOH and MeCN as organic modifiers, respectively, in isocratic mode in ion-pair liquid chromatography.

An enhancement of the ELSD response can be noted when MeCN was used as an organic modifier. This is due to the modification of the scattered light from residual microparticles, after the nebulisation–vaporisation process, in which the size changes with the nature of the mobile phase.[32]

Acetonitrile was found to be more eluting than MeOH, as 15% of the former was needed to elute the first four oligomers while 30% of the latter was needed to elute the same peaks with the same ion-pairing concentration. The eluting strength of MeCN in comparison to MeOH can be seen in Figure 6, which depicts the logarithm of the retention factor (k) of oligo-κ-carrageenans as a function of the volume fraction of organic modifier in the mobile phase. The slope for the first five peaks was greater for MeCN than for MeOH, confirming the higher elution strength of MeCN.

The different solvent behaviour between MeCN and MeOH could be partially attributed to their chemical nature. The dielectric constant (ε) is known to be an

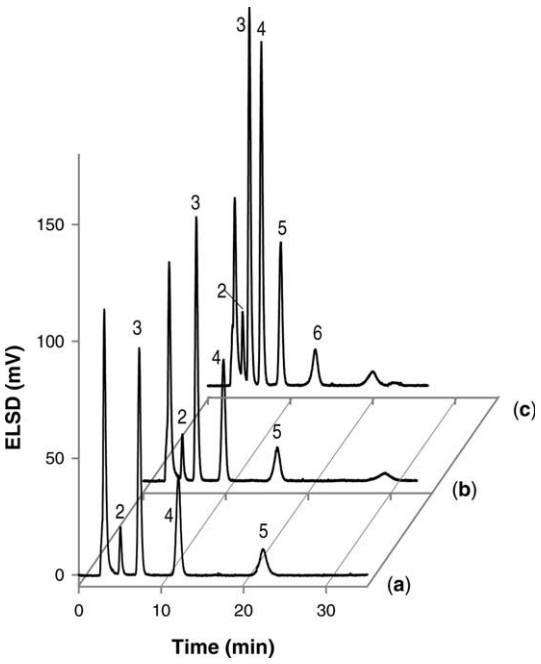

Figure 4. Chromatograms of oligo-κ-carrageenans in 5 mM of ion-pairing agent (heptylammonium formate, pH 4) in a concentration of (a) 30%; (b) 32%; (c) 35% of MeOH in the mobile phase in isocratic mode.

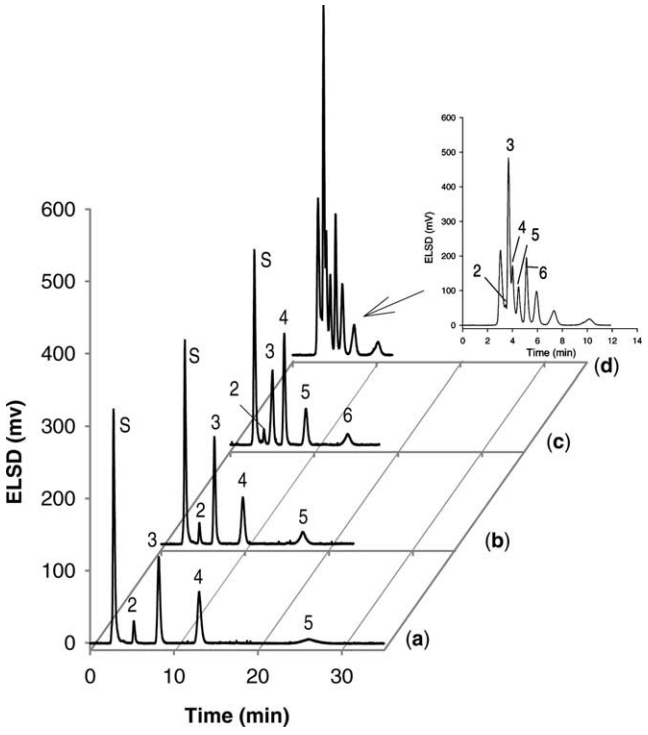

Figure 5. Chromatograms of oligo-κ-carrageenans in 5 mM of ion-pairing agent (heptylammonium formate, pH 4) in a concentration of (a) 15%; (b) 17%; (c) 19%; (d) 21% of MeCN in the mobile phase in isocratic mode.

important parameter in defining the ability of a solvent to solvate ions.[37] Since the ability of a solvent to disperse

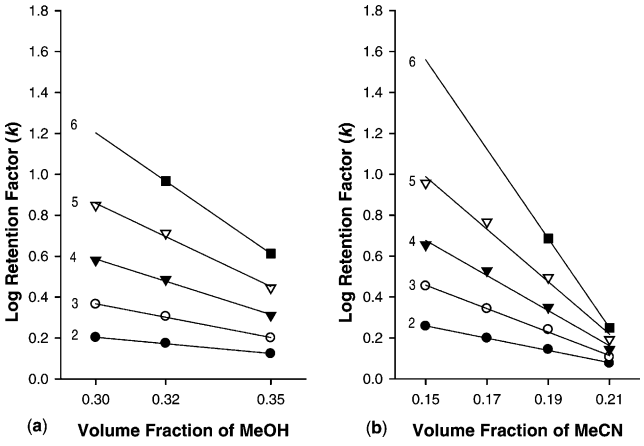

Figure 6. Variation of the retention factor (k) of oligo-κ-carrageenans in 5 mM of ion-pairing agent (heptylammonium formate, pH 4) as a function of the organic modifier (a) MeOH; (b) MeCN; in the mobile phase. Lines represent linear regression.

electrostatic charges via ion dipole interactions is inversely related to the dielectric constant, MeOH ($\varepsilon = 32.3$) tends to exhibit more pronounced solvation effects for the heptylammonium ion than MeCN ($\varepsilon = 38.8$).

In addition, MeOH is a solvent, which can potentially solvate the ion-pair complex (oligo-κ-carrageenan-heptylamine) *via* hydrogen bonding and result in a favourable extraction process whereas MeCN does not.[37] This explains the fast elution of oligo-κ-carrageenans in the latter solvent.

In isocratic mode and at a constant concentration of ion-pairing agent, the elution of the most charged oligosaccharides required a high concentration of organic modifier. But in this case, the less charged oligo-κ-carrageenans co-elute. Considering the forementioned discussion, we selected a gradient of MeOH, while keeping the ion-pairing agent constant.

2.3. Separation of oligo-κ-carrageenans

Figure 7 presents the separation of the oligo-κ-carrageenans in a gradient mode of MeOH. Numbers above the peaks represent $(\mathbf{A\text{-}G4S})_n$ of the neocarrabiose unit. The smallest one that was separated was a $(\mathbf{A\text{-}G4S})_2$ and the largest one a $(\mathbf{A\text{-}G4S})_{16}$. Despite the fact that Spherisorb ODS1 is a stationary phase with a significant number of free sinalol groups, its influence on peak tailing is negligible due to the neutralisation of the sinalol groups by the heptylammonium ion.

When ion-pair chromatography is compared with the anion-exchange mechanism,[29] the former was able to separate the oligosaccharides of κ-carrageenan up to $(\mathbf{A\text{-}G4S})_{16}$ while the anion-exchange mechanism separated only up to $(\mathbf{A\text{-}G4S})_9$. In addition, the method with the proper modifications (data not shown) is able to separate the oligosaccharides of ι-carrageenan in a smaller

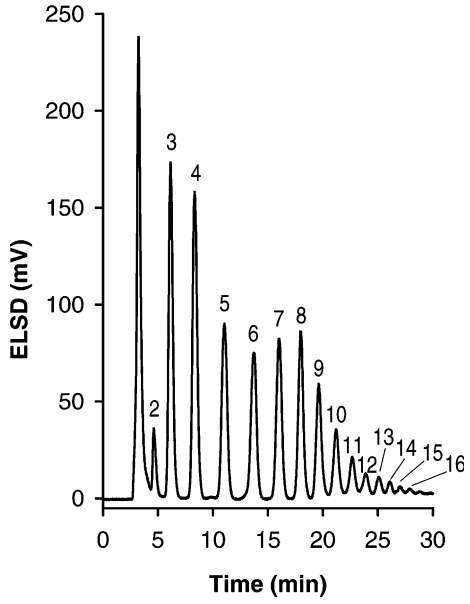

Figure 7. Separation of oligomers of κ-carrageenan.

concentration of ion-pairing agent than that used for the oligo-κ-carrageenans. We propose that the fact that the repeating unit of oligo-ι-carrageenans is more charged than that of κ-carrageenan, increases the possibility of reacting with the ion-pairing agent, and therefore a smaller concentration is needed.

2.4. Isolation and characterisation of oligomers

Figure 8 depicts the chromatograms of oligo-κ-carrageenans [**A-G4S**]$_n$ injected at concentrations of 5000, 25,000 and 50,000 ppm (0.1, 0.5 and 1.0 mg of the mixture injected, respectively).

Only the first eight oligomers were collected and characterised. The rest of the oligomers were not characterised because, when decreasing the ion-pairing agent or increasing the organic modifier, they were co-eluted.

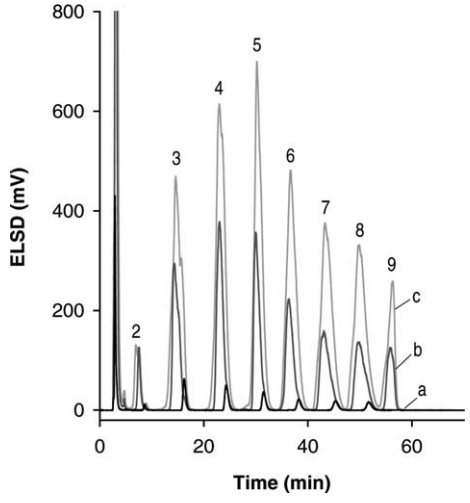

Figure 8. Chromatograms of oligo-κ-carrageenans as a function of the quantity of oligo-κ-carrageenans injected. (a) 0.1 mg; (b) 0.5 mg; (c) 1.0 mg of oligo-κ-carrageenans mixture injected.

Negative-ion ESIMS analysis for oligo-κ-carrageenans up to dodecasaccharide (**A-G4S**)$_6$ has been described elsewhere.[25] In our case, we applied it just to verify the oligomers of the first three peaks (peak number 2, 3, 4 in Fig. 8). Further identification in negative-ion mode with the same experimental conditions as in the positive-ion mode was not possible.

Our analysis is limited to positive-ion mode. In contrast to a previous report that used a mixture of MeOH–water to analyse the oligomers,[25] we selected a mixture of 4:1 MeCN–water, which due to its higher conductivity and lower surface tension,[38] required lower capillary potentials.

Table 1 presents the ions identified in the oligo-κ-carrageenans, while Figure 9a–c stand, respectively, for the peak numbers 2, 5 and 9 of Figure 8 corresponding to the tetrasaccharide, decasaccharide and octadecasaccharide.

All the oligosaccharides gave ions with the heptylamine molecule present. This phenomenon can be explained by the fact that the heptylamine molecules formed salts with the sulfate groups, and the ions detected were formed by the excess of the heptylamine groups that were not removed during the collisional desolvation steps in the electrospray source.

Table 1. Ions identified for the oligo-κ-carrageenans in the positive ion-mode ESIMS

Oligosaccharide (**A-G4S**)$_n$		m/z Found
Tetrasaccharide, ($n = 2$)	$[(\mathbf{A\text{-}G4S})_2+(C_7H_{15}NH_3^+)_3-H_2O]^+$	1119
	$[(\mathbf{A\text{-}G4S})_2+(C_7H_{15}NH_3^+)_3]^+$	1137
	$[(\mathbf{A\text{-}G4S})(\mathbf{A\text{-}G})+(C_7H_{15}NH_3^+)_2-H_2O]^+$	924
	$[(\mathbf{A\text{-}G4S})_2+(C_7H_{15}NH_3^+)_4+SO_3]^+$	1332
Hexasaccharide, ($n = 3$)	$[(\mathbf{A\text{-}G4S})_3+(C_7H_{15}NH_3^+)_4]^+$	1638
	$[(\mathbf{A\text{-}G4S})_3+(C_7H_{15}NH_3^+)_4-H_2O]^+$	1620
Octasaccharide, ($n = 4$)	$[(\mathbf{A\text{-}G4S})_4+(C_7H_{15}NH_3^+)_6-H_2O]^{2+}$	1119
	$[(\mathbf{A\text{-}G4S})_4+(C_7H_{15}NH_3^+)_5-H_2O]^+$	2122
	$[(\mathbf{A\text{-}G4S})_4+(C_7H_{15}NH_3^+)_5]^+$	2140
	$[(\mathbf{A\text{-}G4S})_3(\mathbf{A\text{-}G})+(C_7H_{15}NH_3^+)_5-H_2O]^{2+}$	1021
Decasaccharide, ($n = 5$)	$[(\mathbf{A\text{-}G4S})_5+(C_7H_{15}NH_3^+)_7]^{2+}$	1379
	$[(\mathbf{A\text{-}G4S})_5+(C_7H_{15}NH_3^+)_6]^+$	2641
Dodecasaccharide, ($n = 6$)	$[(\mathbf{A\text{-}G4S})_6+(C_7H_{15}NH_3^+)_8-H_2O]^{2+}$	1620
	$[(\mathbf{A\text{-}G4S})_6+(C_7H_{15}NH_3^+)_8]^{2+}$	1629
Tetradecasaccharide, ($n = 7$)	$[(\mathbf{A\text{-}G4S})_7+(C_7H_{15}NH_3^+)_9-H_2O]^{2+}$	1871
	$[(\mathbf{A\text{-}G4S})_7+(C_7H_{15}NH_3^+)_9]^{2+}$	1880
Hexadecasaccharide, ($n = 8$)	$[(\mathbf{A\text{-}G4S})_8+(C_7H_{15}NH_3^+)_{10}-H_2O]^{2+}$	2122
	$[(\mathbf{A\text{-}G4S})_8+(C_7H_{15}NH_3^+)_{10}]^{2+}$	2131
Octadecasaccharide, ($n = 9$)	$[(\mathbf{A\text{-}G4S})_9+(C_7H_{15}NH_3^+)_{11}]^{2+}$	2382
	$[(\mathbf{A\text{-}G4S})_9+(C_7H_{15}NH_3^+)_{12}]^{3+}$	1626

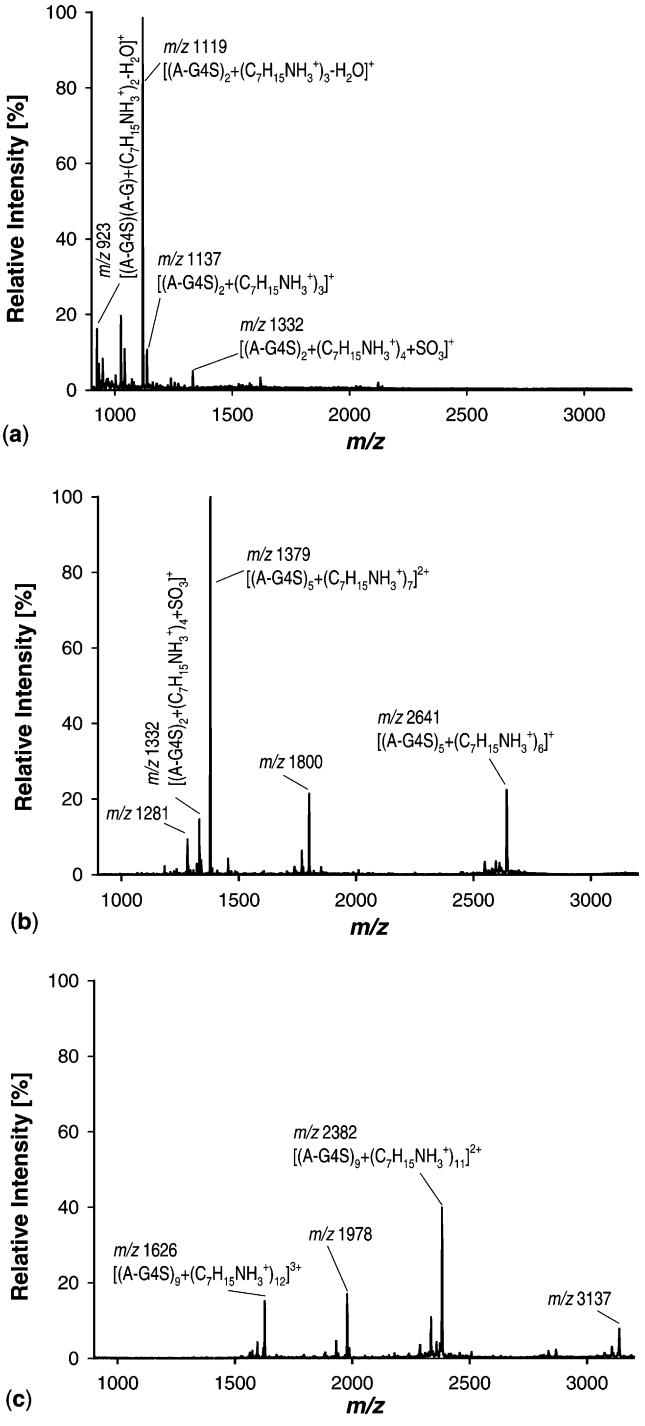

Figure 9. Positive ESIMS spectra of the isolated oligosaccharides of κ-carrageenan: (a) tetrasaccharide; (b) decasaccharide; (c) octadecasaccharide.

We observed that when increasing the molecular weight of the oligomer analysed, more heptylammonium ions were attached to it. In detail, while the tetrasaccharide (**A-G4S**)$_2$ to octasaccharide (**A-G4S**)$_4$ presented adducts mainly with one charge, the decasaccharide (**A-G4S**)$_5$ to hexadecasaccharide (**A-G4S**)$_8$ presented adducts with two charges, and the octadecasaccharide (**A-G4S**)$_9$ with three charges. The same phenomenon has been reported for the analysis of dextran polysaccharides during direct infusion in positive-ion ESIMS ion trap.[39]

Desulfated oligosaccharides were detected in the mass spectra of the tetrasaccharide and octasaccharide. Ekeberg et al.[25] have already reported this fact and they attributed the phenomenon to an intramolecular rearrangement reaction followed by expulsion of SO_3 or by an ion/neutral reaction with water. However, we could not verify this hypothesis when using collision induced dissociation (CID) of the [**A-G4S**]$_n$.

In the mass spectrum of the tetrasaccharide, an extra sulfated ion was present. Extra sulfated oligosaccharides have previously been observed.[25,40] The phenomenon can be attributed to the inclusions of ι-carrageenan.[40]

All ESIMS spectra, except (**A-G4S**)$_5$ and (**A-G4S**)$_9$, presented adducts with the loss of a water molecule. The latter has been recently observed for xylo-oligosaccharides[41] in MS/MS spectrum, a phenomenon attributed to a loss of water from the reducing end.[42] Therefore, it is possible to have in source fragmentation of the oligo-κ-carrageenans resulting in the loss of a water molecule.

In view of the fact that the oligo-κ-carrageenans gave adducts with different m/z values, we infused the mixture directly into the ESIMS together with the ion-pairing agent without prior separation. Figure 10 presents the mass spectrum of the mixture obtained. All oligosaccharides responded mainly with the adduct with the general formula: $[(\mathbf{A\text{-}G4S})_n + (C_7H_{15}NH_3^+)_x]^{(x-n)+}$ where n is the degree of polymerisation, x are the heptylamines attached to the oligomer and $(x-n)+$ is the charge of the adduct.

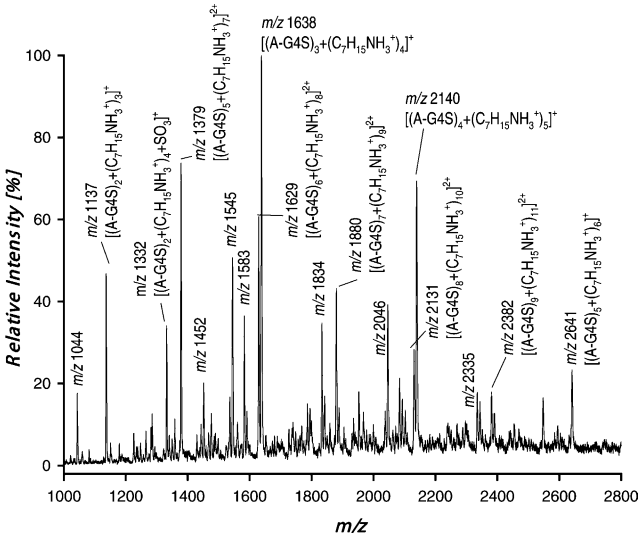

Figure 10. Positive ESIMS spectrum of the mixture of the oligosaccharides.

3. Concluding remarks

Ion-pair chromatography permitted the separation of oligomers of κ-carrageenan up to $(\mathbf{A\text{-}G4S})_{16}$. The scale-up purification also demonstrated that the method is stable for the first eight oligomers without any extra addition of ion-pairing agent. The small concentration of ion-pairing agent permitted their detection in the positive-ion ESIMS. The molecules attached to the oligomers gave adducts with different m/z values in the ESIMS in positive-ion mode. Their charge state seemed to be dependent on the molecule size of the oligomer.

4. Experimental

4.1. Oligomers of κ-carrageenan

Oligo-κ-carrageenans were produced according to Rochas and Heyraud[43] using recombinant κ-carrageenase from *Alteromonas carrageenovora*.[44] They were prepared by enzymatic digestion with κ-carrageenases.

4.2. Column dimensions, chromatographic support and eluents

The column used was a Spherisorb ODS1 (250×4 mm i.d., particle size 5 μm) from Phase Sep (Düren, Germany). Dead volume was determined by injecting $NaNO_3$ with 3:7 MeCN–water as mobile phase. It corresponded to the very first peak of each chromatogram.

Eluent constituents were as follows: Deionised water was obtained by an Elgastat UHQ II system (18 MΩ) from Elga (Antony, France), MeCN and MeOH from J. T. Baker (Noisy le Sec, France). Heptylamine ($C_7H_{15}NH_2$), pentylamine, Et_3N and formic acid were from Fluka (St. Quentin Fallavier, France). All reagents were of analytical grade.

Data acquisition of the chromatograms was performed using EZChrom Elite Client/Server software, version 2.5, Scientific Software (Pleasanton, CA, USA).

The ion-pairing agents were a mixture of alkylamine (triethyl, pentyl or heptylamine) and formic acid. They were prepared by imposing the alkylammonium concentration value and pH value of 4. The formate ion concentration and the ionic strength of each eluent were calculated by PHoEBus,[45] an application program help for buffer studies. For the preparation of ion-pairing agent heptylammonium formate (20 mM of heptylammonium ion, pH 4), 0.283 mL of formic acid and 0.745 mL of heptylamine were diluted into a volume of 250 mL. The pH value was checked with a Beckman pH meter (Model F10, Gagny, France).

4.3. Pumps and detectors

The liquid chromatographic apparatus consisted of a Thermoseparation (Les Ulis, France) model P4000 inert quaternary gradient pump, a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 20 μL sample loop and an ELSD system (Sedere, Alfortville, France) Model Sedex 75. The ELSD settings were as follows: photomultiplier range, 10 (7 for the isolation part); evaporative drift tube temperature, 47 °C; nebulisation gas pressure, 3.5 bar.

4.4. Isolation of oligomers

The mixture of oligo-κ-carrageenans was injected in a concentration of 50,000 ppm (1 mg of oligo-κ-carrageenans injected). The gradient program was as follows: eluent A: 20 mM of heptylammonium formate, pH 4; eluent B: MeOH; eluent C: water. Gradient program: 0–25 min, 25% of A, 20–32% of B, 55–43% of C; 25–60 min, 25% of A, 32–39% of B, of 43–36% of C. Flow rate 1 mL min^{-1}.

The oligo-κ-carrageenans were collected using a passive split with a ratio of 1:10. The mobile phase of each peak was evaporated under N_2 atmosphere at room temperature and then 0.5 mL of 4:1 MeCN–water were added to the residue.

4.5. Analysis of oligomers

All samples of hydrolysates of κ-carrageenans were injected at a concentration of 1220 ppm. Experimental conditions: Eluent A: 20 mM of heptylamine/formic acid, pH 4; eluent B: MeOH; eluent C: water. The gradient program was as follows: 0–30 min, 15% of A, 27–47% of B, 58–38% of C. Flow rate 1 mL min^{-1}.

4.6. Mass spectrometry

Electrospray ionisation mass spectrometry (ESIMS) was used to obtain the mass spectra of the oligosaccharides containing the molecular ions. The ESIMS system used was a Quatro Ultima triple quadrupole mass spectrometer (Micromass Ltd, Manchester, UK) equipped with a pneumatically assisted electrospray ionisation source. Data acquisition and processing were performed using MASSLYNX 4.0 software. The analyte (200 ppm of mixture of oligo-κ-carrageenans with 1 mM of heptylammonium formate for the direct ESIMS analysis) was introduced into the mass spectrometer via the ESI probe with a Harvard Apparatus pump 11 (Harvard Apparatus, Massachusetts, USA) with a flow-rate of 5 μL min^{-1}. The molecular weight of oligo-κ-carrageenans was calculated with Macisotopes software, ver. 1.2.

ESIMS conditions were as follows: N_2 was used as both nebulising gas and desolvation gas at flow rates of

50 and 500 L/h, respectively. The electrospray capillary voltage was 2.75 kV and the cone voltage was 32 V. The RF-Lens 1 was set at 0.0, the aperture was set at 0.0 and the RF-Lens 2 at 1.0. The source operated at a temperature of 130 °C and the desolvation temperature at 150 °C. The LM-resolution 1 and HM-resolution 1 were both set at 12.5, while the ion energy 1 was at 1.1 eV. The entrance and exit of the collision cell were both set at 30, while the collision energy was set at 0 eV. The LM-resolution 2 and HM-resolution 2 were both set at 15.0 while the ion energy 2 was set at 1.5 eV. The multiplier was set at 650. The pressure in the analyser was 8.5×10^{-6} and 1.0×10^{-4} mbar in the gas cell. Full scan mode was used and both positive and negative ionisation modes were tried.

The mass scan range was 300–4000 amu, for 1 min total scan time, with 3 s scan time and 0.1 s inter-scan time. All mass spectra were smoothed twice using Savitzky Golay algorithm with 0.75 Da peak width.

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