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Negative-ion electrospray ionisation–mass spectrometry (ESI–MS) as a tool for analysing structural heterogeneity in *kappa*-carrageenan oligosaccharides

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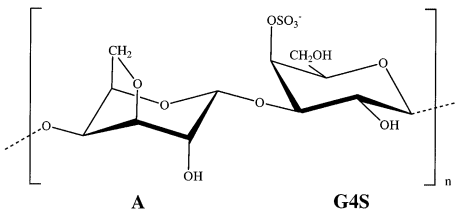
Abstract

Oligosaccharides, enzymically produced from *kappa*-carrageenan, have been investigated by electrospray ionisation mass spectrometry (ESI–MS). The technique was used without prior derivatisation of the oligosaccharide originally obtained by size-exclusion chromatography (SEC). The structure of the oligosaccharides was mainly 4-sulphated neocarrabiose (**A-G4S**) with an increasing length ranging from di- to dodecasaccharides. However, in the larger oligosaccharides, structural motifs deviating from the perfect alternating **A-G4S** structure were detected, i.e. (**A2S-G4S**). Although resulting in reduced signal intensity, samples to which NaCl was added also gave rise to reliable mass spectra. Desulphation was induced at elevated cone voltages and in acidic or alkaline salt solutions.

Keywords: Electrospray ionisation mass spectrometry; ESI–MS; ESI–MS/MS; Sulphated oligosaccharides; *Kappa*-carrageenan; *Kappa*-carrageenase; Neocarrabiose 4-sulphate

1. Introduction

Carrageenan is a collective term for a group of polysaccharides found in red algae. They consist of alternating 3-linked β-D-galactopyranose (**G**-units) and 4-linked α-D-galactopyranose (**D**-units). In the gelling family, the **D**-units are of the 3,6 anhydro form (**A**). Both units might be sulphated giving the economically important *kappa*- (**A-G4S**) (Scheme 1) or *iota*-(**A2S-G4S**) carrageenan.[1] Analysis of their structures can be performed by GC–MS after derivatisation of monosaccharides obtained by acidic hydrolysis.[2] Recently, developed methods based on reductive hydrolysis are non-destructive for 3,6-anhydrogalactose, and hence both monosaccharides in the agar or carrageenan backbone may be determined.[3,4] Linkage and sulphate positions have been determined up to the size of tetra-



A **G4S**

−[→4)-α-3,6 anhydro-D-Gal*p*-(1→3)-β-D-Gal*p*-4-sulphate-(1→]$_n$

Scheme 1.

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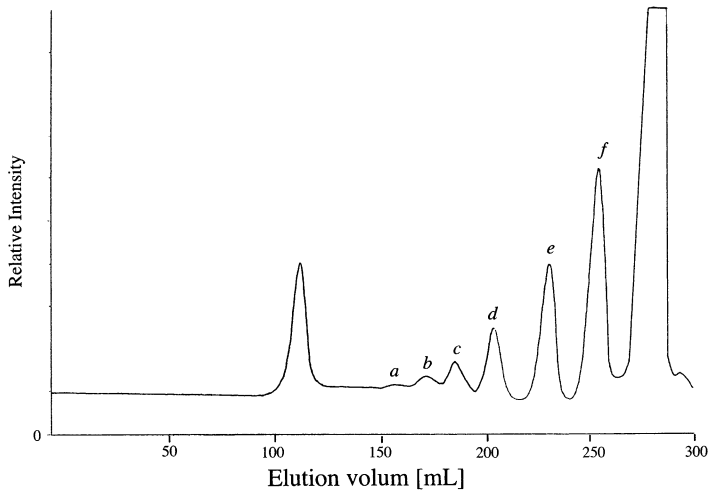


Fig. 1. Fast preparative scale size-exclusion chromatography (Superdex 30 eluted with 50 mM $(NH_4)_2CO_3$) of oligosaccharides enzymic produced from *kappa*-carrageenan. The sample was solubilised in the eluent. The different fractions are (a) dodecasaccharide (b) decasaccharide (c) octasaccharide (d) hexasaccharide (e) tetrasaccharide and (f) disaccharide.

saccharides.[5] The use of ^{13}C NMR in combination with specific carrageenolytic enzymes has permitted detection of minor structures in the samples.[6,7] Enzymic resistant fractions, as well as oligosaccharides representative of the main repeating backbone, have been purified from enzymic hydrolysates by size-exclusion chromatography (SEC) and analysed by ^{13}C and ^{1}H NMR.[8]

The technique of HPLC–ESI–MS is one of the most exciting developments of recent times in analytical methodology.[9] ESI has been recognised as a powerful ionisation technique for the introduction of oligosaccharides into an MS. ESI–MS is recognised widely in glycobiology as a tool for structural analysis of carbohydrates.[10,11] Its main advantage over NMR spectroscopy is the low-detection limit, which makes detection of heterogeneity easier. A variety of thermolabile, polar and non-volatile compounds present in a complex matrix can be separated, characterised and quantified unambiguously. Several ESI–MS studies have been performed on derivatised oligosaccharides.[12–14] However, by the use of ESI–MS chromatographic separation and derivatisation may be omitted.[15]

The present study provides details on the use of ESI–MS and ESI–MS/MS for identification and characterisation of oligosaccharides of the neocarrabiose type, **(A-G4S)**$_n$, enzymically produced from *kappa*-carrageenan. The present work show that ESI–MS, in combination with SEC, is a promising method for investigation of sulphate substitution patterns in carrageenans.

2. Results and discussion

*Analysis of **A-G4S**-type carrageenan oligosaccharides.*—Mixtures of oligosaccharides obtained by enzymic degradation were separated by SEC and analysed by ^{1}H NMR[8] (data not shown). Spectroscopic data and the comparison of actual retention times of the fractions with those of commercial standards indicated a series of 4-sulphated neocarrabiose oligosaccharides **(A-G4S)**$_n$ ($n = 1–6$) as initially shown on the chromatogram in Fig. 1.

Positive and negative ion ESI–MS analysis of these oligosaccharide fractions were performed. The best sensitivity was obtained when the mass spectrometer was operated in negative mode, as expected due to the presence of sulphate anion groups. For this reason, the results in this study are restricted to the formation of anions. The ability to form multiply charged ions, as well as availability of different counter ions, explain the presence of the different peaks in the mass spectra. The number of charges formed depends on the size of the oligosaccharides and the number of sulphate groups present.

Fig. 2 shows the mass spectra of the different oligosaccharide fractions enzymically pro-

duced from *kappa*-carrageenan $(\mathbf{A\text{-}G4S})_n$. For the disaccharide ($n = 1$), the base peak is found at m/z 403, which corresponds to the single charged anion $[\mathbf{A\text{-}G4S}]^-$. Minor amounts of $[(\mathbf{A\text{-}G4S})_2\mathrm{Na}]^-$ and $[(\mathbf{A\text{-}G4S})_2\mathrm{H}]^-$ anions are found at m/z 829 and 807, respectively. These ions are clusters formed by ion/neutral reactions, and are not tetrasaccharides. This result is also supported by ^{1}H NMR spectroscopy[16] in that the spectrum of the disaccharide fraction has no signals from contaminating tetrasaccharides or other carbohydrates (data not shown). In the mass spectrum of the tetrasaccharide (Fig. 2(b)), the base peak is found at m/z 394, which corresponds to the doubly charged anion $[(\mathbf{A\text{-}G4S})_2]^{2-}$. When one of the sulphate groups adds Na^+ or H^+, peaks at m/z 811 or 789 are observed, respectively. The peak found at m/z 709 corresponds to a tetra-saccharide with only one sulphate group, $[\mathbf{(A\text{-}G)(A\text{-}G4S)}]^-$ or $[\mathbf{(A\text{-}G4S)(A\text{-}G)}]^-$. The position of the single

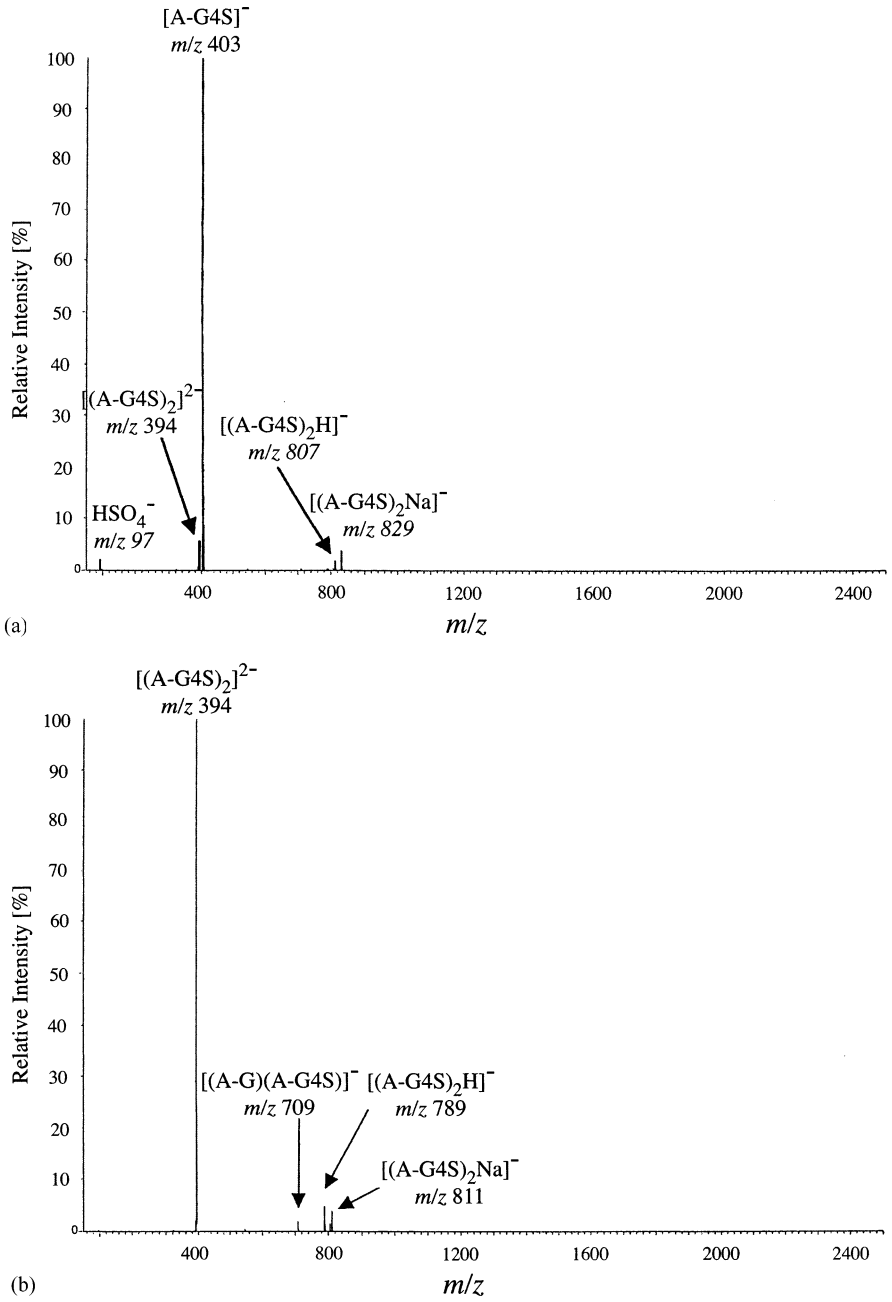
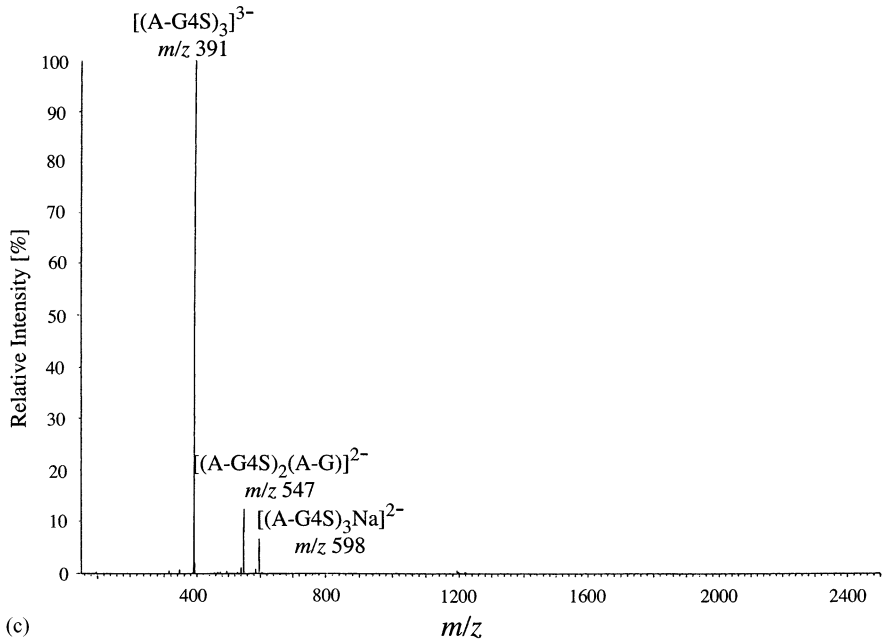


Fig. 2. ESI–MS mass spectra of the oligosaccharides from *kappa*-carrageenan using 50 vol% methanol in water: (a) disaccharide; (b) tetrasaccharide; (c) hexasaccharide; (d) octasaccharide; (e) decasaccharide; (f) dodecasaccharide.



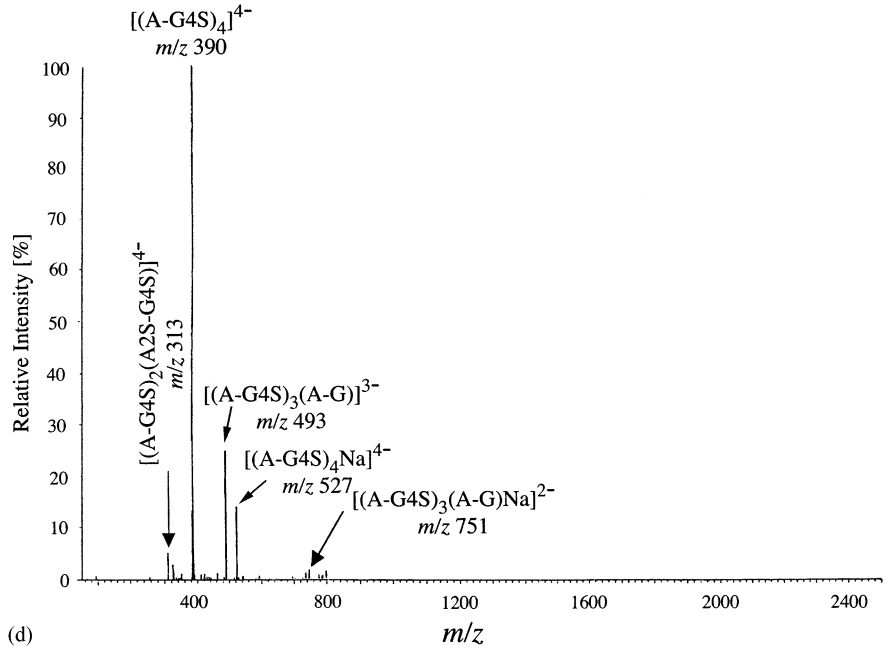


Fig. 2. (*Continued*)

sulphate group is not determined at this stage. Formation of this desulphated tetrasaccharide can take place in the mass spectrometer or it can be formed prior to injection, Desulphation that takes place inside the MS instrument can be due to high cone voltage or to chemical reactions catalysed or caused by the mobile phase. Experiments performed using different cone voltages (5–70 V) and different salts (NaCl, NaI, LiCl, CH_3COONa, $(NH_4)_2CO_3$, $CaCO_3$, Na_2SO_4, $CaCl_2$) in the sample showed that ions at m/z 709 are formed inside the mass spectrometer caused by both the above parameters (data not shown). Formation of desulphated oligosaccharides in the mass spectrometer can principally take place by an intramolecular rearrangement reaction followed by expulsion of SO_3, or by an ion/neutral reaction with water. The latter is catalysed by acid and base. Collision induced dissociation (CID) of the $[\mathbf{A\text{-}G4S}]_n$ should then eliminate SO_3 if the former reaction was

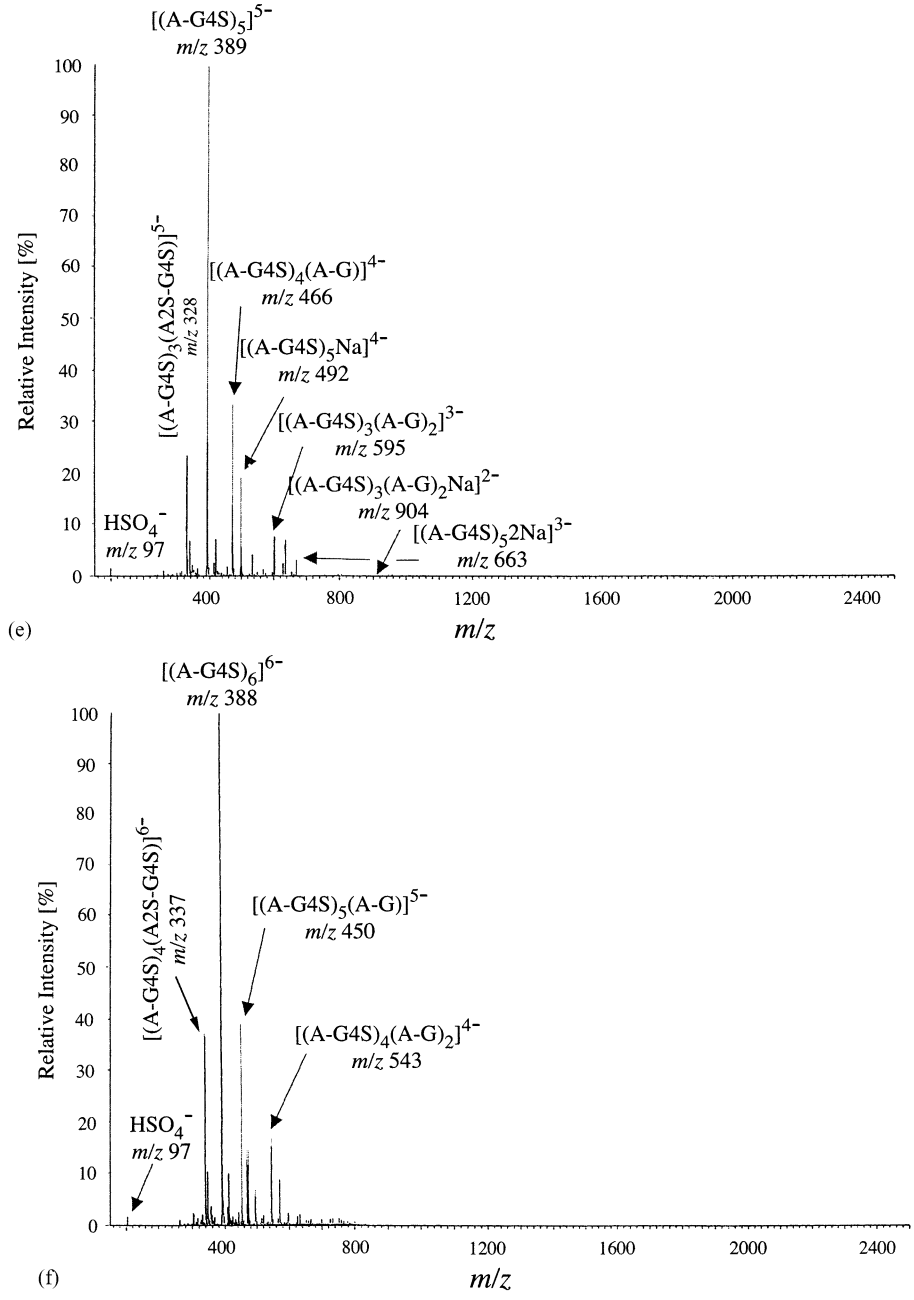


Fig. 2. (*Continued*)

the one taking place, but this is not observed (Fig. 3). In addition, a significant increase of m/z 709 was observed when $(NH_4)_2CO_3$ or $CaCO_3$ was present in the sample. The CID mass spectra of the **[A-G4S]**$_n$ ($n = 1$, 2 and 3) are shown in Fig. 3. There are two main daughter ions formed when **[A-G4S]**$_n$ is collisionally activated. These ions are formed by cleavage of the glycoside bond followed by migration of the hydrogen from or to the charged daughter. We have not studied the mechanisms of the fragmentation in detail, but it suggests that there is an elimination reaction and the charge is located on the sulphate group. Assignment of the different daughter ions by collision-induced dissociation of **[A-G4S]**$_n$ ($n = 1$, 2 and 3) are shown in Scheme 2.

The desulphation reaction is catalysed by acid or base. By increasing the cone voltage, the kinetic energy of the ions will increase, and hence add sufficient energy upon colli-

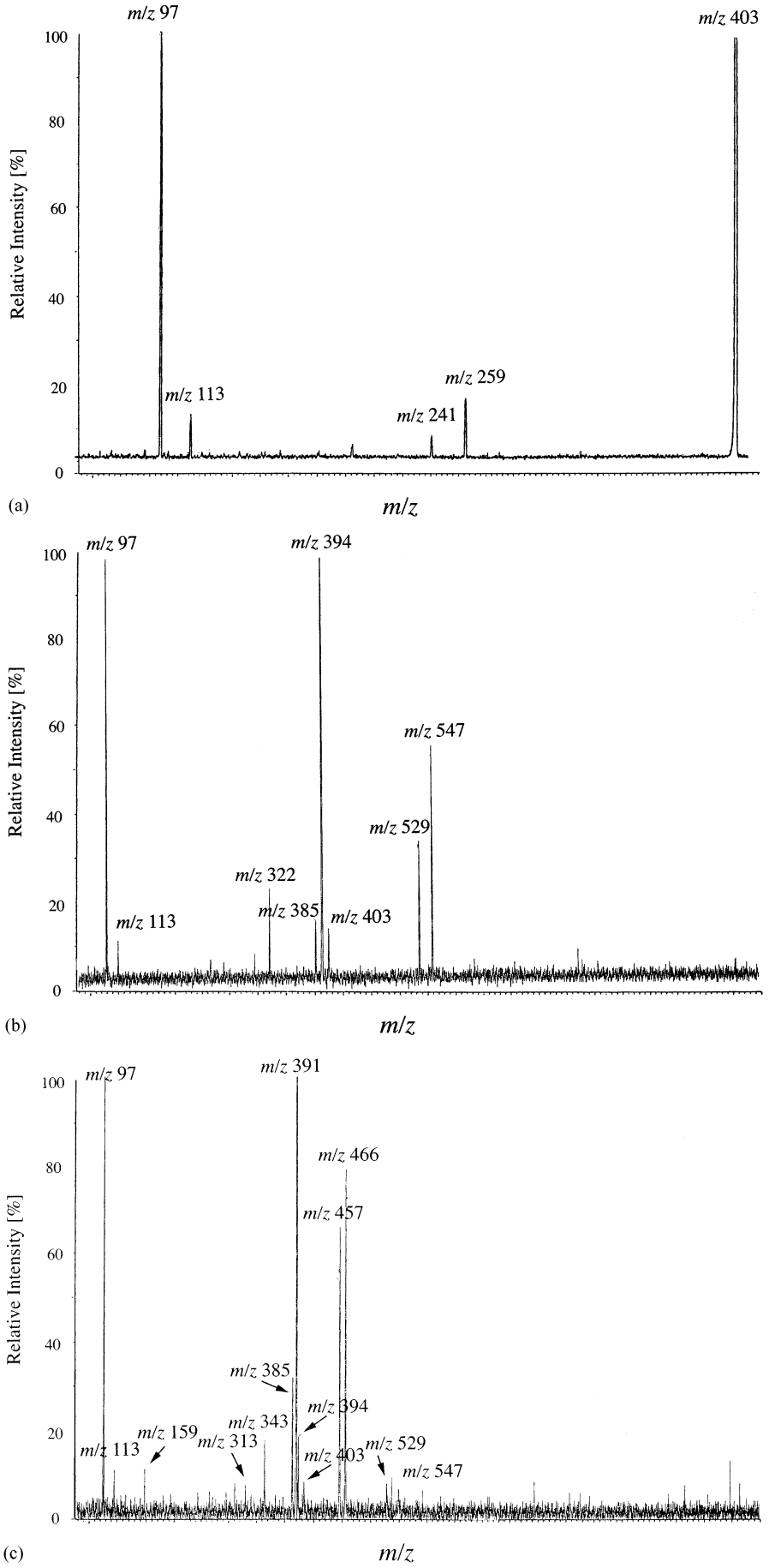
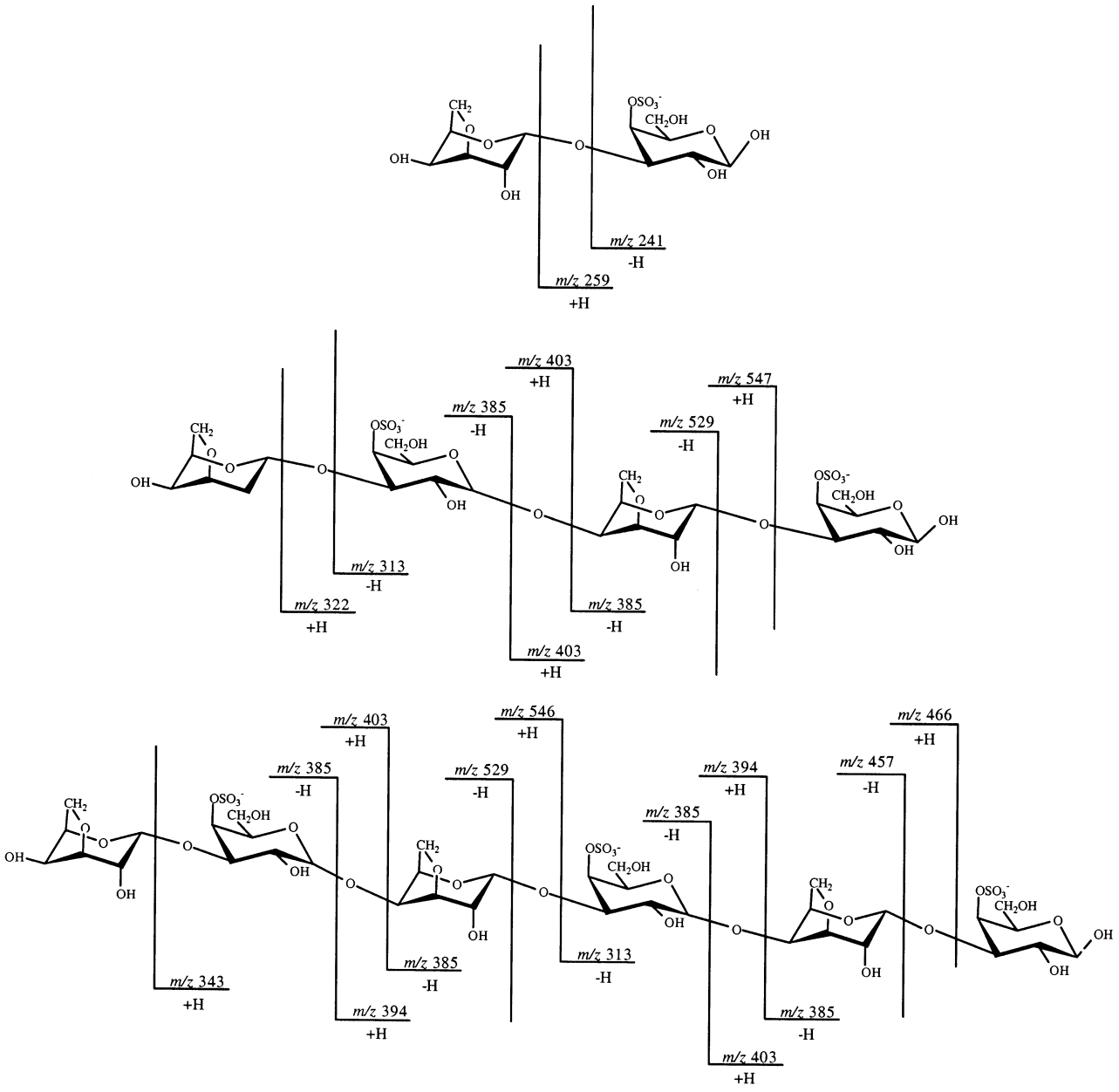


Fig. 3. Collision induced dissociation mass spectra of (a) $[\mathbf{A\text{-}G4S}]^-$, $([\mathbf{A\text{-}G4S}]_2)^{2-}$ and $([\mathbf{A\text{-}G4S}]_3)^{3-}$.



Scheme 2.

sions to initiate the desulphation reaction. This is supported by an increased amount of desulphated ions in the mass spectra upon an increase of the cone voltage as well as by adding an increased amount of acidic or basic salts.

Desulphated oligosaccharides were observed in the mass spectra obtained from $(\mathbf{A\text{-}G4S})_n$ for $n = 2$, 3, 4, 5 and 6. In the mass spectrum of the hexasaccharide (Fig. 2(c)), the base peak was found at m/z 391. A peak at m/z 547 corresponded to double charged desulphated hexasaccharide anions carrying only two sulphate groups. The relative amounts of desulphated ions increased in proportion to the size of the oligosaccharide. For the octasaccharide (Fig. 2(d)) the peaks found at m/z 493 and m/z 751 in the mass spectrum corresponded to triple and double charged desulphated ions $[(\mathbf{A\text{-}G4S})_3(\mathbf{A\text{-}G})]^{3-}$ and $[(\mathbf{A\text{-}G4S})_3(\mathbf{A\text{-}G})\mathrm{Na}]^{2-}$, respectively. In the mass spectra of the decasaccharide and dodecasaccharide (Fig. 2(e and f)), peaks corresponding to oligosaccharides with two sulphate groups less than expected for *kappa*-carrageenan were observed. Ions at m/z 595 and 904 in Fig. 2(e)

corresponded to $[(\mathbf{A\text{-}G4S})_3(\mathbf{A\text{-}G})_2]^{3-}$ and $[(\mathbf{A\text{-}G4S})_3(\mathbf{A\text{-}G})_2\mathrm{Na}]^{2-}$, respectively. These desulphated structures are most likely not representative of sequences occurring in the starting carrageenan substrate.

The chromatographic fractions of oligosaccharides produced from *kappa*-carrageenan assigned as octa-, deca- and dodecasaccharides also contained small amounts of oligosaccharides with a shorter chain length in addition to the major ones. Sulphated neocarrabiose oligosaccharides consisting of one less neocarrabiose unit, but with an extra sulphate group co-eluted with the regular ones above. This demonstrates that an extra sulphate group strongly influences the hydrodynamic volume and hence the retention times in SEC. The shorter oligosaccharides were assigned to $[(\mathbf{A\text{-}G4S})_2(\mathbf{A2S\text{-}G4S})]$, $[(\mathbf{A\text{-}G4S})_3(\mathbf{A2S\text{-}G4S})]$ and $[(\mathbf{A\text{-}G4S})_4(\mathbf{A2S\text{-}G4S})]$, respectively. As indicated, the exact positions of the extra sulphate groups have not been determined, but are attributed to inclusions of *iota*-carrageenan, a common impurity in commercial *kappa*-carrageenan.[6] These molecules are observed as multiply charged anions (Fig. 2(d, e and f)). For example, in the mass spectrum of the octasaccharide (Fig. 2(d)) the peak at m/z 313 corresponds to $[(\mathbf{A\text{-}G4S})_2(\mathbf{A2S\text{-}G4S})]^{4-}$ ions. It should be noted that these oversulphated oligosaccharides are representative of sequences in the starting material. Hence, the '*iota*' content in this '*kappa*' material does not occur in black noise distribution.

Investigation of a kappa-*carrageenase hydrolysate.*—During desalting of the hydrolysate, the disaccharide co-eluted with NaCl and was removed. The most abundant ions in the mass spectrum (Fig. 4) of the desalted hydrolysate were observed at m/z 394 and 391. These ions corresponded to $[(\mathbf{A\text{-}G4S})_2]^{2-}$ from the tetrasaccharide and $[(\mathbf{A\text{-}G4S})_3]^{3-}$ from the hexasaccharide, respectively. In addition, ions from the remaining oligosaccharides were also detected. Signals arising from a high-molecular weight fraction (enzyme resistant molecules) were not observed in this experiment. The results indicate that the enzymic treated solution may be injected directly into the ESI–MS instrument without chromatographic separation and analysed for its oligosaccharide content, as long as the salt content is kept low.

The effect of residual salts in the sample.—The concentration of NaCl was found to be an important parameter in the ionisation mechanisms. Therefore ESI–MS was performed on samples containing different concentrations of NaCl in addition to an equal amount of tetrasaccharide. The total-ion chro-

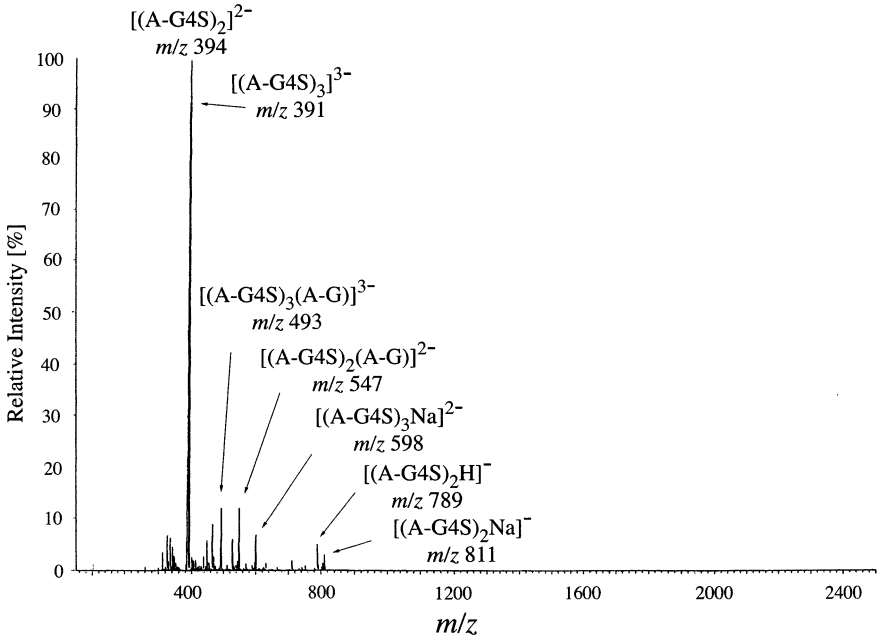


Fig. 4. The ESI mass spectrum of the enzymic hydrolysate of *kappa*-carrageenan after desalting on Pharmacia HiPrep.

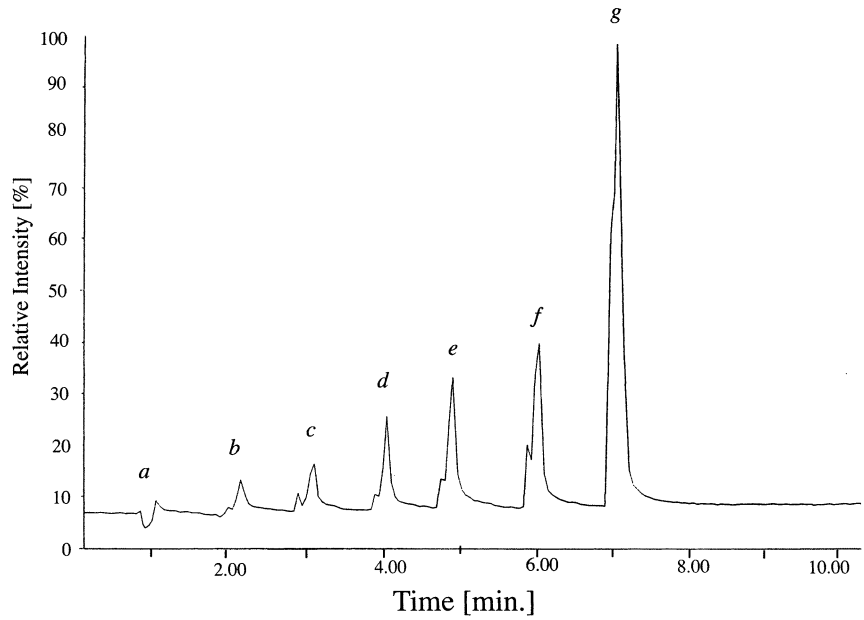


Fig. 5. Total ion current (TIC) of 0.1 mg/mL tetrasaccharide ([**A-G4S**]$_2$) with different concentrations of NaCl. (a) 0 mg/mL; (b) 0.1 mg/mL; (c) 0.5 mg/mL; (d) 1.0 mg/mL; (e) 2.0 mg/mL; (f) 4.0 mg/mL; and (g) 10.0 mg/mL. Samples were injected with 1-min intervals.

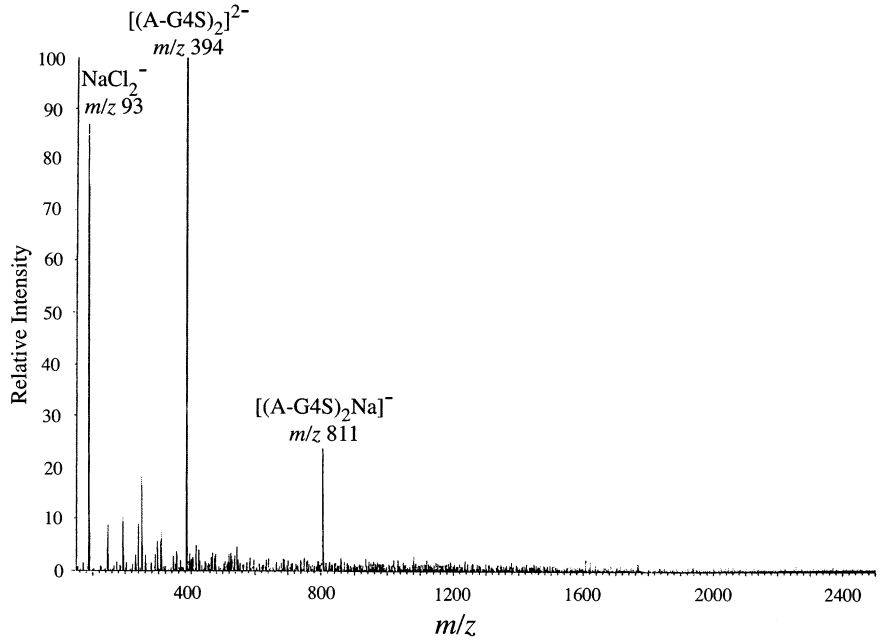


Fig. 6. The mass spectrum of tetrasaccharide-containing 10 mg/mL NaCl.

matograms (TIC) are shown in Fig. 5. The chromatogram with the largest area was obtained when no salt was present. These results can be explained by the formation of soluble salts of the oligosaccharides in the presence of NaCl. In this way, salts interfere with the formation of anions. Despite their lower intensity, the different major peaks occurring in the mass spectra can be identified (Fig. 6).

3. Concluding remarks

The results presented are innovative regarding the analysis of carrageenan oligosaccharides and carrageenans in general. Extra sulphation (**A2S**) as single '*iota*' inclusions were observed. In the mass spectra peaks from individual molecules could be identified. In NMR spectroscopy resonances from different

oligosaccharides may overlap and their individual identities may not be identified. By conventional methods based on GC of derivatives of carrabiose fragments, the largest oligosaccharides to be detected corresponded to four sugar units only.[3–5]

Furthermore the ESI–MS analysis requires a small amount of sample. By applying this technique, the distribution of the different oligosaccharides on a time scale may be investigated and hence kinetic data during an enzymic hydrolysis can be obtained. Combined with collision induced dissociation mass spectrometry, this methodology is an important addition to existing analytical methods for the study of sulphate sequence in carrageenans. However, care should be taken to avoid substitution reactions to take place due to high cone voltage or catalysed by the salt present in the sample.

4. Experimental

Production of oligosaccharides.—Commercial *kappa*-carrageenan, from alkali treated *Kappaphycus alwarezii*, was obtained from Copenhagen Pectin Factory (Genuvisco X-6913). *Kappa*-carrageenan was solubilised in 100 mM NaCl and 5 mM $NaHCO_3$ and degraded by *kappa*-carrageenase[17] at ambient temperature. Size-exclusion chromatography was performed by a LC pump (Pharmacia High Load P50) connected to a column (Pharmacia XK-26/70) filled with Pharmacia Superdex 30 eluted with 50 mM $(NH_4)_2CO_3$ at ambient temperature. The detector used was a refractive index detector (Shimadzu RID-6A). The fractions were pooled and freeze dried.

Desalting.—The salt content in the hydrolysate was reduced by solubilising in 50 mM $(NH_4)_2CO_3$ and applied to Pharmacia HiPrep 26/10 (Gel height 100×26 mm I.D.) eluted with the same solvent using the chromatographic system as described above. The fractions separated from the NaCl peak were pooled and freeze dried.

Instrumental.—Electrospray ionisation mass spectrometry (ESI–MS) was used to obtain the mass spectra of the oligosaccharides containing the molecular ions. The ESI–MS system used was a Waters 2690 (Massachusetts, USA) mobile phase pump coupled to a Quatro LC–MS/MS triple quadrupole mass spectrometer (Micromass Ltd, Altrincham, UK) equipped with a pneumatically assisted electrospray ionisation source. Data acquisition and processing was performed using a MASLYNX 3.1 software. The analyte (1.0 mg/mL) was introduced into the mass spectrometer directly via the LC injection loop. The volume of the injection loop was 10 μL. The LC conditions were: mobile phase: 50 vol% MeOH–water, flow rate 0.2 mL/min. ESI–MS conditions were as follows: N_2 was used as both drying gas and nebulising gas at flow rates of approximately 1000 L/h and 80 L/h, respectively. The cone voltage was set to 50 V and the electrospray capillary was at 4 kV. The source was operating at a temperature of 100 °C and the desolvation temperature at 180 °C. The pressure in the analyser was 6.7×10^{-6} mbar and 3.7×10^{-5} mbar in the gas cell. Full scan mode was used and both positive and negative ionisation modes were tried. The mass scan range was 50–2500 amu.

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