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Separation of high-molecular-mass carrageenan polysaccharides by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

A method is described for the separation and quantitation of unhydrolyzed carrageenan polysaccharides (molecular mass approx. $3 \cdot 10^5$ rel. mol mass) by capillary electrophoresis. Various carrageenan samples were first derivatized with the fluorescent reagent, trisodium 8-aminopyrene-1,3,6-trisulfonate (APTS), in both laboratory and commercial blend samples. Microcentrifuge filters ($3 \cdot 10^4$ rel. mol. mass) were then employed to separate derivatized samples into low (eluate) and high (retentate) molecular mass fractions which could each be assayed by CE with laser-induced fluorescence detection. Eluate fractions contained small amounts of kappa and iota species which could be efficiently separated by capillary zone electrophoresis (CZE) using a buffer with an anti-convective additive. Separation of species contained in the retentate fraction (kappa, iota, lambda) could be achieved in under 5 min by CZE using a citrate buffer, pH 3.0. The influence of the pre-filtration step, field strength and temperature on peak efficiency and resolution are studied. Quantitative aspects are evaluated and the method then applied to real commercial samples of food additive mixtures. © 1998 Published by Elsevier Science B.V.

Keywords: Food analysis; Polysaccharides; Carrageenan; Aminopyrene-1,3,6-trisulfonate

1. Introduction

The present study will demonstrate the separation of derivatized but intact, high-molecular-mass carbohydrates which are important additives and components of food products. The analysis of carbohydrates in general by capillary electrophoresis (CE) has been reviewed by Novotny and Sudor [1]. Much of the reported work involves the analysis of oligosaccharides of small degree of polymerisation (DP; generally <50), almost all of which are digests of larger polysaccharides. Digestions are usually carried out by acid, heat or enzymatic treatment. Liu et al. [2] reported the separation of oligosaccharides produced from an autoclave digestion of high-molecular-mass polygalacturonic acid. This study used capillary gel electrophoresis (CGE) with gels having a small pore size prepared using polyacrylamide with high percentage of total monomer {18% T, 3% C; T= g acrylamide+g *N,N*-methylenebisacrylamide (Bis)/100 ml solution; C=g Bis/% T}. Oligosaccharides were measured by their fluorescence using an argon-ion laser line following a derivatization procedure with the probe 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBCQA). Sudor and Novotny have also reported the analysis of hydrolysed kappa (κ)-carrageenan after labelling with either 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) or 6-aminoquinoline derivatives [3]. The fluorescence was measured using a helium–cadmium laser source and collecting the emission (>495 nm) with standard optical components. Both of the re-

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sulting conjugates were analyzed by CE using linear polyacrylamide coated capillaries and experiments were performed with and without a dynamic sieving buffer. A significant improvement in resolution was observed between low DP fragments with the dynamic sieving buffer. Of particular recent interest is the high resolution separation of an enzymatically treated sample of corn amylopectin. Not only was baseline resolution achieved for the enzymatic products but also different branch positions could be distinguished [4].

The analysis of large DP polysaccharides in food substances, formulations, or ingredients has received some attention to date; however, robust methods only exist for a small number of analytes one might wish to study in foods [4]. The laboratory headed by Novotny has reported the characterisation of a few derivatized polysaccharides such as cellulose and heparin derivatized with a fluorescent tag at the reducing terminus. In another study, high DP polydextrans were separated using CE with entangled polymer buffers and pulsed field conditions (see also Ref. [1]). Novotny has also recently referred to methods development for charged hyaluronic, alginic and polygalacturonic acids using ''appropriately chosen entangled polymer webs'' [4]. All of the examples to date from this laboratory involve important characterisation of the polysaccharides; however, separations from other potentially interfering biopolymers have not been demonstrated.

Both the characterisation and separation of large pectin molecules (DP 200–1000) was recently achieved by CZE [5]. Samples used were from commercially available lemon pectin that was enzymatically treated in order to control the degree of esterification. This step did not alter the DP of the molecules under study. The degree of esterification (DE) of the sample pectins directly influenced the migration time, and measurement of pectin mobility could therefore be used for the quantification of DE. The resolution of the method was determined by separation of a three component laboratory preparation containing 31.1, 47.2, and 70.3% DE pectins. Analysis times are less than 20 min. The method has recently been extended to provide rapid measurements of both DE and the DE distribution of pectin samples [6]. It is important to note that no sample derivatization is necessary, which greatly reduces method complexity.

The aim of the present study is to demonstrate the separation of derivatized but intact, high-molecular-mass, carrageenan polysaccharides which are important additives used in the food industry. Their current uses and emerging applications have been recently reviewed [7]. These polysaccharides are harvested from various species of seaweed by industrial scale extraction, precipitation and fractionation techniques. The three principle molecular types which are obtained after processing have been termed kappa (κ)-, iota (ι)- and lambda (λ)-carrageenans. Kappa and iota forms are gelling while lambda is a non-gelling, thickening agent. These additives give textural properties and protective effects to a wide range of food products such as frozen desserts, chocolate, cottage cheese, whipped cream, instant breakfasts, yoghurt, jellies, pet foods, relishes, sauces, syrups, etc. [8,9].

At present there is a lack of adequate analytical techniques to determine the amounts, the polydispersity, and the purity of these important components of modern food products. The range of techniques applied to this problem include colorimetric staining with Alcian blue (total carrageenan determination) [10], immunological detection [11], sodium dodecyl sulphate–polyacrylamide slab-gel electrophoresis [12], ^{1}H nuclear magnetic resonance (NMR) [13], ^{13}C-NMR [14], as well as high-performance liquid chromatography (HPLC) [15] and high-performance anion-exchange chromatography [16] methods that include the acid hydrolysis of carrageenans and subsequent analysis of the monosaccharide profile. Each of these techniques have advantages and disadvantages and normally have to be used in combination to accurately determine carrageenan type and amount. Furthermore, most of the techniques mentioned above are of very low sensitivity or specificity for the high-molecular-mass carrageenans of interest to the food industry. CE seems to offer the possibility to analyze the intact polysaccharide, without any subsequent loss in molecular information which may occur during degradation. This method may also offer advantages in sensitivity when combined with laser-induced fluorescence (LIF) detection. Furthermore, CE is very amenable to automation and is a

recognised to be a rapid technique with low running costs [17].

The term ''carrageenan'' is used to name a class of galactan polysaccharides that occur as intercellular matrix material in numerous species of red seaweed (marine algae of the class *Rhodophyta*). It is a hydrocolloid consisting mainly of the sulfate esters of galactose and 3,6-anhydo-galactose copolymers. Carrageenan is a linear polysaccharide with a primary structure of alternating α-(1→3)- and β-(1→4)-linked galactose residues. Thus, the repeating units are disaccharides. In addition, the galactose units linked β-(1→4) in this general structure often occur as 3,6-anhydro-D-galactose. All carrageenans are polyanions, with sulfate ester groups present on some or all galactose units. Commercial carrageenans are usually sold as κ-, ι- or λ-carrageenans. These are normally not pure forms, but contain varying amounts of the other carrageenan types, the exact amount depending on the weed source and extraction procedure [18]. These commercial products are frequently diluted with sugars for standardisation purposes and mixed with food grade salts required for obtaining gelling or thickening characteristics.

The structures of the repeating units of the three principle carrageenan types, kappa, iota and lambda, are depicted in Fig. 1. As can be seen, these three forms differ discretely in their charge per residue and should therefore be amenable to electrophoretic separation. They exist naturally in states of very high DP and also with a high degree of polydispersity [9,19]. They have previously been characterised by size-exclusion chromatography (SEC) as having molecular mass ranges from $3 \cdot 10^5$ rel. mol. mass to $5 \cdot 10^5$ rel. mol. mass (weight average) [20].

The number and positions of the ester sulfate groups have a dramatic effect on the tertiary structure and the subsequent interactions for the different carrageenan species. Kappa is sulfated only at C4 in the 1,3-linked galactose ring, while iota has an additional sulfate at C2 in the 1,4-linked-3,6-anhydro-D-galactose ring. These structures allow the formation of double-helical segments that can form gel structures at the appropriate conditions of salt concentration and temperature [8,9,21]. λ-Carrageenan is seen to differ from ι- and κ-carrageenan

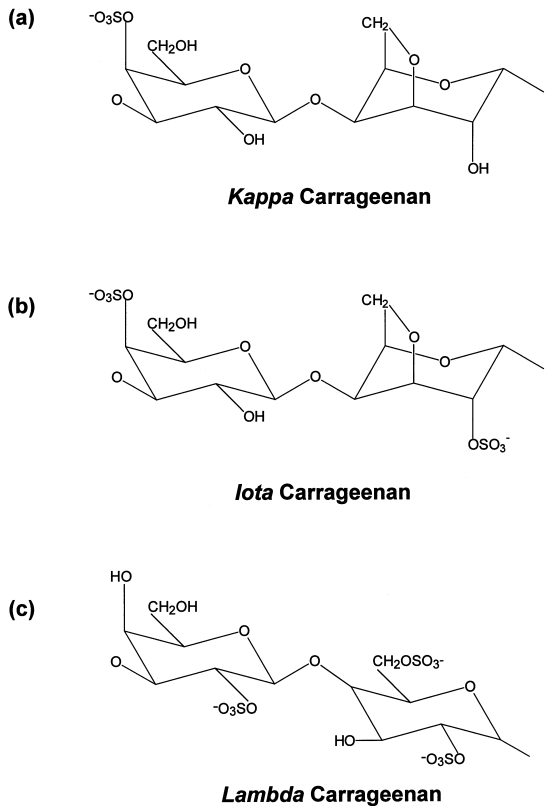

Fig. 1. Structures of the disaccharide repeat residues of the three principal carrageenans used in the food industry; (a) kappa, (b) iota and (c) lambda.

in several respects. Firstly, the level of sulfation is higher, with a charge of −2.7 per disaccharide residue on average. Secondly, the β-(1→4)-D galactose ring is not conformationally locked in the –3,6-anhydro form, and carries sulfate groups at C2 and C6. Finally, sulfation in the α(1→3)-D-galactose residue is at C2. Removal of the anhydro bridge confers a kinking effect which inhibits double helix formation [22]. λ-Carrageenan does not undergo conformational ordering, and hence does not form gels [8,9,19]. Due to the change in primary and tertiary structure, the electrophoretic mobility of lambda could be expected to be different from the value predicted by comparing its charge per residue with those of kappa and iota. For this reason, the present initial work concentrates on the separation of κ- and ι-carrageenans; however, the mobility of

lambda is also characterised. It will be shown that the presence of this biopolymer can also be qualitatively determined in real food samples.

2. Experimental

2.1. Materials

Iota (Type V from *Eucheuma spinosa*), kappa (Type III from *Eucheuma cottonii*), and lambda (Type IV) Irish Moss carrageenan standards were obtained from Sigma (St. Louis, MO, USA). Samples of food additives used throughout the industry, which were known to contain carrageenans, were obtained from various suppliers. For fluorescent conjugation a trisodium 8-aminopyrene-1,3,6-trisulfonate (APTS) label was obtained from Beckman (Fullerton, CA, USA) and sodium cyanoborohydride from Aldrich (Gillingham, UK). Micro-concentrators with a $3 \cdot 10^4$ rel. mol. mass cut-off filter were purchased from Amicon (Gillingham, UK). All salts used to prepare electrophoresis buffers were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were of the highest purity.

2.2. Sample preparation and conjugation with APTS

For the preparation of carrageenan solutions, the sample was slowly added to water containing 0.05% (w/w) sodium azide as an anti-microbial agent under stirring at room temperature. The solution was then stirred for 30 min at 75°C and at 90°C for 15 min. Blend samples were prepared in a similar fashion, the only difference being stirring for 1–2 h rather than 15 min at 90°C to ensure adequate solubilization.

For derivatization, 0.2 mg of solid carrageenan (standards), 5 μl of 2 mg ml^{-1} carrageenan (calibration solutions), or 5 μl of 0.5 mg ml^{-1} with some of the blend sample was mixed with 10 μl acetic acid–water (15:85, v/v). In each case, the sample was then mixed with 2 μl reconstituted labelling dye (APTS). The mixture was heated to 95°C for 2 min before addition of 2 μl of 1 *M* sodium cyanoborohydride and then derivatized at 95°C for 3 h in sealed Eppendorf tubes. Forty-six μl of CE-grade water was added to the reaction mixture to stop reaction. The samples were stable when stored at −20°C for at least three months. The quenched samples derivatized from solid carrageenan were diluted 40-fold using CE-grade water before being microcentrifuged.

Micro-centrifuge filters were introduced here to remove excess derivatizing reagent. Since most of carrageenan samples have molecular masses well above $3 \cdot 10^4$ rel. mol. mass, this was chosen as the cut-off value. Twenty μl of quenched sample derivatized from solid carrageenan (or 20 μl of 40-fold diluted quenched sample from carrageenan solution) was pipetted into a $3 \cdot 10^4$ rel. mol. mass microconcentrator, and 300 μl CE-grade water added. The mixture was centrifuged at 6000 rpm for 20 min, the process taking low-molecular-mass materials through the $3 \cdot 10^4$ rel. mol. mass filter. The centrifuge separation was repeated twice using 300 μl additions of CE-grade water. About 10 μl of solution containing the carrageenan remained above the filter, and this sample retentate was recovered by placing the sample reservoir upside down in a new vial, then spinning at 4000 rpm for 4 s, thereby transferring the concentrated retentate into the vial. The volume of retentate was measured by withdrawal into a pipette with 1-μl step graduations and made up to 20 μl before analysis.

2.3. Electrophoresis conditions

CE experiments were carried out on an automated CE apparatus (P/ACE 5500, Beckman) equipped with a LIF detection system (Beckman LIF detector with P/ACE laser module 488: 488 nm ex, 520 nm em). The background electrolyte (BGE) used in CZE was a citrate buffer, prepared by titrating 25 m*M* trisodium citrate ($Na_3C_6H_5O_7 \cdot H_2O$) with 1 *M* citric acid to pH 3.0. All capillaries (47 cm×50 μm I.D.) were first conditioned by rinsing for 30 min with a 0.1 *M* NaOH solution prior to experimental use. Between runs the capillary was washed for 2 min with water and 3 min with BGE solution.

Initial experiments showed that an electrokinetic injection of APTS-derivatized carrageenan samples led to much higher sensitivity than that observed by pressure injection. Carrageenans are often present in

food samples in very low concentrations and, therefore, all experiments were carried out in this mode. Samples were loaded by electrokinetic injection (5 s at −10 kV) after first injecting water hydrodynamically (3.3 kPa) for 2 s; a third washing injection for 1 s at −1 kV from water was made immediately after loading the sample. Electrophoretic separations were carried out at reverse polarity (outlet anodic) and a potential difference of −30 kV unless otherwise noted. For the analysis of lower DP components found in the micro-concentrator eluates, the Beckman Carbohydrate Kit was used according to the manufacturer's instructions: CHO-coated capillary (47 cm×50 μm I.D.), voltage −30 kV, current 20 μA, pressure injection at 3.3 kPa, temperature 20°C, Beckman gel buffer.

3. Results and discussion

3.1. CE of APTS-derived carrageenans

All of the carrageenans are negatively charged across a broad pH range (>pH 1) [23], due to the strongly acidic nature of the ester sulfate groups and should therefore migrate towards the anode under conditions of low electroosmotic flow (EOF). As discussed in the literature [24,25], at low pH the ionization of the surface silanol groups on uncoated fused-silica capillaries is suppressed, and the EOF approaches zero. Indeed, with the citric acid buffer used in the following experiments, the EOF could not be detected with coumarin 334 as a neutral marker [26]. In these experiments, normal polarity was used and run times were up to 21 min; this implies the electroosmotic mobility (μ_{eo}) is less than $8.5 \cdot 10^{-10}$ m^2 V^{-1} s^{-1}. Under these conditions the apparent mobility observed in the following electropherograms can be considered for the purpose of calculations to be the electrophoretic mobility. The label at the reducing terminus (charge −3) is not considered to induce a significant mobility shift because of the large size and overall high charge of the biopolymer (charge>−200) [3].

After conjugation to the fluorescent label APTS, unfiltered carrageenans were observed under electrophoretic conditions, as shown in Fig. 2. It is clear from these electropherograms that a possible separation between kappa and iota could be obtained; however, significant interference with low-molecular-mass compounds was observed. Many of these compounds could be observed in a blank reaction where no carrageenan is added (data not shown). Other peaks which were not observed in the blank reaction might have been derived from the hydrolytic breakdown of carrageenans under the conditions of the labelling reaction. Although the peak heights can be quite large for these compounds, their relative concentrations compared to the parent carrageenans are certainly very small. As the APTS-labelling protocol is an end-labelling procedure with attachment to the reducing end of the carbohydrate chain, a small oligosaccharide will have the same amount of label as would a large polysaccharide on a molar basis. Therefore, the LIF signal for the same mass will be many-fold higher with peaks from small DP species than with peaks from large DP species.

In order to empirically determine that the compounds responsible for the interfering peaks were of a very different molecular mass range than the parent carrageenan molecules, derivatized sample solutions were passed through a micro-concentrator (microcentrifuge filter). A $3 \cdot 10^4$ rel. mol. mass cut-off filter was chosen here to separate the APTS-derived carrageenan samples into two size ranges. The low-adsorption characteristics of the membrane and other component parts of the device, together with an inverted recovery spin, combine to yield unusually high recovery rates – typically >95% of the sample (manufacturer's data). After centrifugation, both eluates and retentates were collected and analyzed electrophoretically.

3.2. Eluate analysis

Under the previously described conditions (−30 kV, citrate buffer, pH 3.0, uncoated fused-silica capillary) a good separation of eluate components was not achieved via CZE (electropherogram not shown). An excellent separation was achieved, for the various eluate components, by using a commercially available buffer recommended for carbohydrate separations. This buffer has an anti-convective additive which improves peak efficiency. These results are shown in Fig. 3. The labelling reagent, APTS, was clearly identified as the first peak by

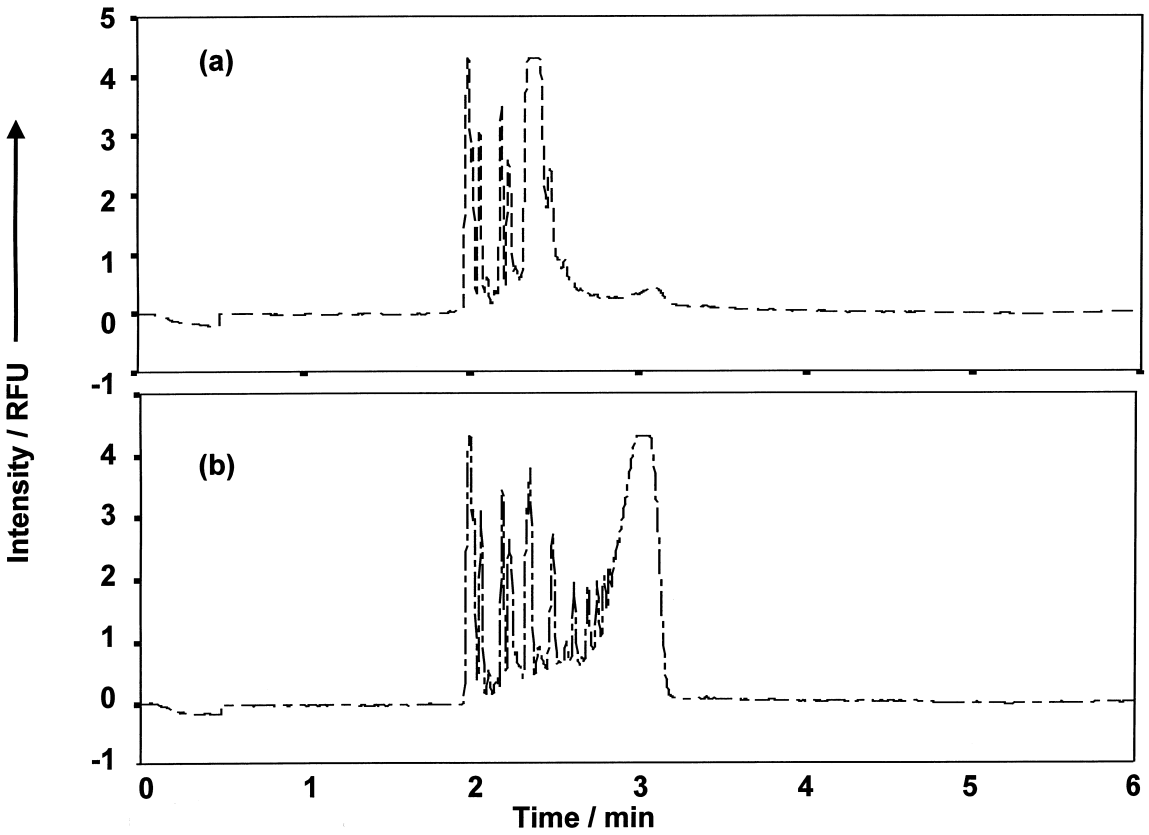

Fig. 2. Electropherograms of (a) APTS–κ-carrageenan and (b) APTS–ι-carrageenan before centrifugation. CE conditions: capillary 47 cm×50 μm, voltage −30 kV, current 100 μA, electrokinetic injection 5 s at −10 kV, LIF detection (488 nm ex, 520 nm em), temperature 50°C, buffer 25 m*M* trisodium citrate, adjusted to pH 3.0 with 1 *M* citric acid.

comparison with fresh standards. The next three peaks, as well as the fifth peak shown in Fig. 3, were all found in a blank reaction (identical reaction conditions with only APTS and no carrageenan) as well as in reactions containing κ-, ι- and λ-carrageenan samples. Derivatized λ-carrageenan samples displayed exactly the same spectrum of peaks as the blank reaction, thereby indicating that no carrageenan components smaller than $3 \cdot 10^4$ rel. mol. mass were present after the conjugation reaction. The bottom electropherogram in Fig. 3 is similar to these. A new peak (peak 4) is observed only when ι-carrageenan is present. When κ-carrageenan is present in the derivatization reaction a new series of peaks appear at slightly longer migration times than in the iota or blank reaction peaks, which is consistent with the migration order predicted from the charge per residue, as well as to the observed order seen in the electropherograms of unfiltered samples shown in Fig. 2.

The κ-carrageenan eluate electropherogram, shown in the inset of Fig. 3, is particularly noteworthy since a series of separated components appear very close to one another, forming a type of molecular ladder. These results are reminiscent of separated DP series which have been previously reported for various hydrolysed polysaccharides [3,4,27]. Carrageenans are known to hydrolyse quite easily under acidic conditions [23] and, therefore, the appearance of a small amount of low DP carrageenans most probably originates from acid hydrolysis due to the presence of acetic acid during the reaction step with APTS. Fortunately, these components are easily separated from the parent compound with the use of

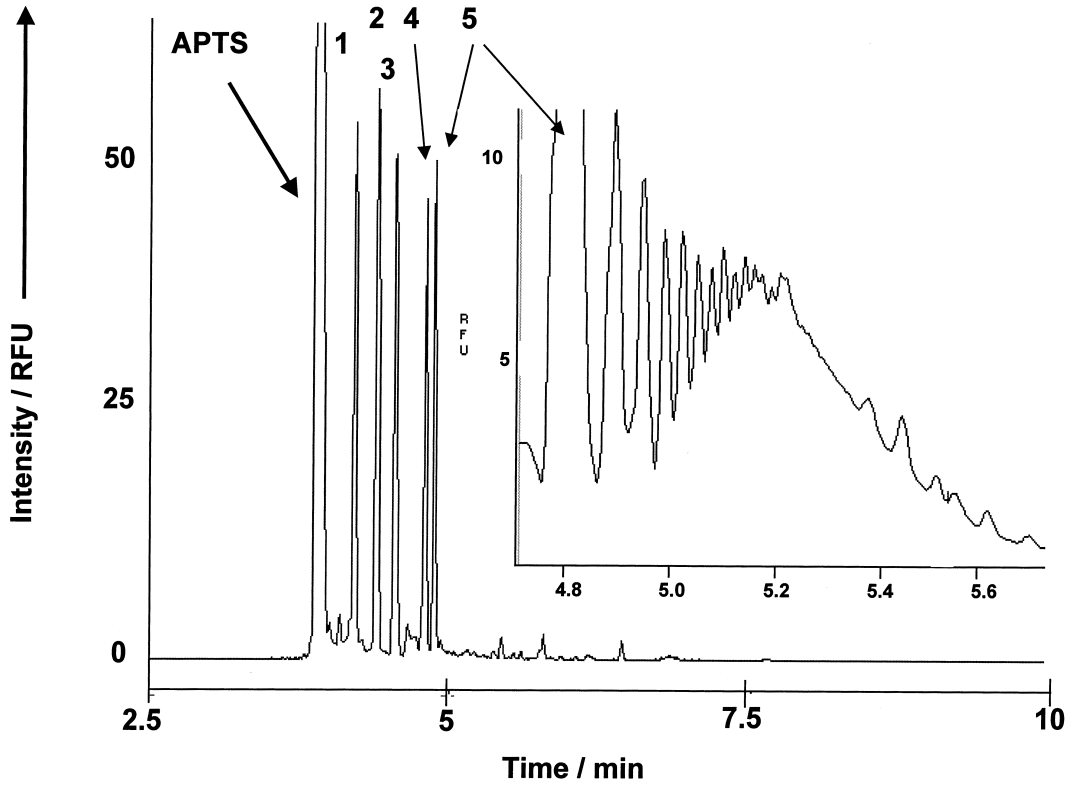

Fig. 3. Analysis of eluate ($3 \cdot 10^4$ rel. mol. mass filter) from iota (bottom) and kappa (inset) carrageenan labelling reactions. CE conditions: capillary 47 cm×50 μm, voltage −30 kV, current 20 μA, pressure injection 2 s, LIF detection (488 nm ex, 520 nm em), temperature 20°C, Beckman Carbohydrate Gel Buffer.

a micro-centrifuge filtration step prior to CE analysis. Furthermore, the degradation rates within the temperature range used here have been previously shown to be significantly higher for κ- than for ι-carrageenan [23]. This is consistent with our results (Fig. 3) with overall peak areas from new peaks in the eluate fraction higher for κ- than for ι-carrageenan.

3.3. Retentate analysis

As shown in Fig. 4, the electropherograms of APTS-derivatized κ-, ι- and λ-carrageenans are relatively free of any interfering peaks after treatment with the centrifuge filter. In comparison with Fig. 2, it can be clearly seen that most of the labelling reagent has been removed and that the principal peaks observed correspond to κ-, ι- and λ-carrageenans. A small amount of APTS is still present after filtration; however, this is quite useful as a mobility standard to guard against any shift in migration time that might occur with the age of a capillary. Furthermore, one can use the peak area of APTS to qualitatively monitor filtration efficiency. It should also be noted that, at the end of the filtration step, the retentate is contained in a much reduced volume, thereby concentrating the sample and improving the detection limits for the technique. This can be seen by noting the increase in the relative fluorescence intensity scales between Figs. 2 and 4.

The electropherograms in Fig. 4 show that the iota sample contains a small amount of kappa and, in turn, kappa contains a small amount of iota. In order to calculate the amount of these impurities kappa and iota peak areas were compared. By running three replicate analyses on three separate derivatization reactions for each carrageenan, it was estimated that there is a 3% impurity of iota in kappa and a 4%

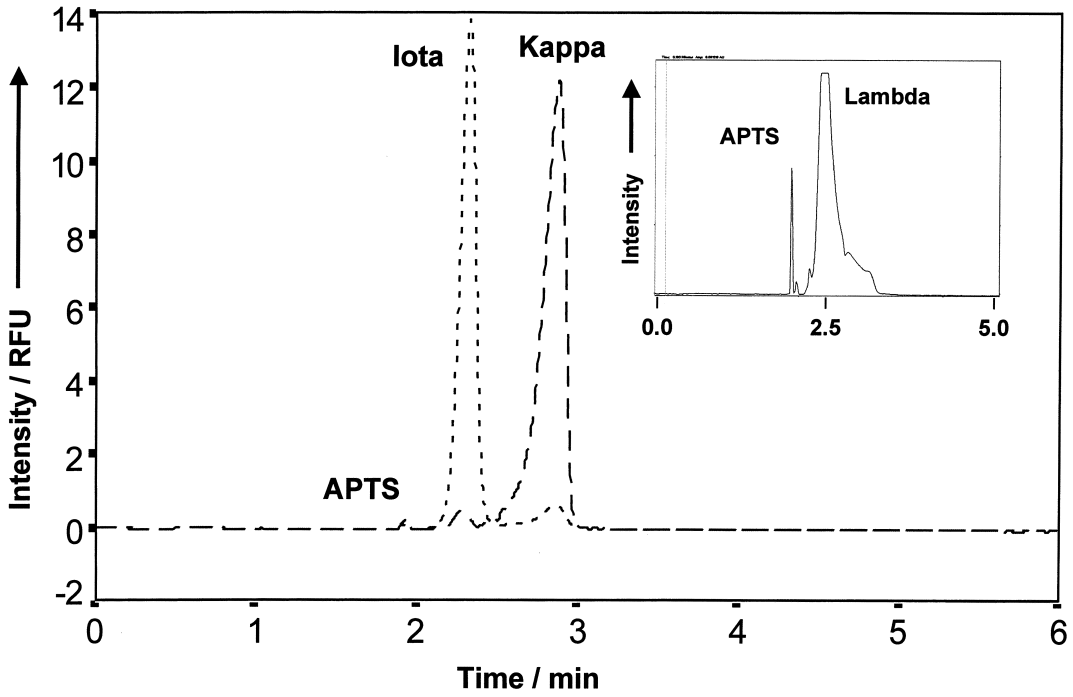

Fig. 4. Electropherograms of separate retentates from APTS-derivatised ι- and κ-carrageenans after centrifugation. CE conditions: capillary 47 cm×50 μm, voltage −30 kV, current 100 μA, electrokinetic injection 5 s at −10 kV, LIF detection (488 nm ex, 520 nm em), temperature 50°C, buffer 25 m*M* trisodium citrate, adjusted to pH 3.0 with 1 *M* citric acid.

impurity of kappa in iota. This is similar to the roughly 6% impurities which have been noted in previous studies [23,28]. An electropherogram from a 50:50 (w/w) mixture of the kappa and iota standards which were derivatized together, and then subsequently analyzed by CZE, is shown in Fig. 5. The migration times and peak shapes remained the same as when run separately. Under these conditions, the two carrageenan fractions could be easily separated and quantitatively determined in under 5 min.

Derivatized λ-carrageenan had an intermediate mobility, as shown in the inset of Fig. 4, but could not be resolved from APTS-derivatized ι-carrageenan. The observed peak shape was broad with additional complex structure not present in either κ- or ι-carrageenan. A qualitative explanation of this has to do with two effects. Firstly, the primary structure of lambda is known to be somewhat different than that of kappa or iota as discussed in Section 1. The different frictional coefficient could lead to a migration time out of the expected order. Secondly, counter-ion condensation must be accounted for. Manning's theory [29] shows that counter-ions will condense (non-specific binding) onto a poly-ion until $\xi_{eff}=\xi_c$, where ξ is the charge density; $\xi_c=1$ when the poly-ion and counter-ion both have unit charge, and ξ_{eff} is the effective charge density (in water at 298 K, $\xi=0.714\ \text{nm}/b$, where b is the average spacing between charges on the polyelectrolyte chain, and $b=l/z$, where l is the residue length and z is the charge per residue. $l=1.0$ nm for the random coil conformation of carrageenans [30]). Calculations of the various parameters for kappa, iota and lambda with $z=-1$, -2 and -2.7, respectively give: $b=1.0$, 0.50, 0.37 nm; $\xi=0.71$, 1.43, 1.93; $\xi_{eff}=0.71$, 1.0, 1.0; $z_{eff}=-1$, -1.4, -1.4. This shows that counter-ions condense onto iota and lambda, but not kappa, and that the charge per residue of iota and lambda is reduced to an effective charge of -1.4. Since mobility scales with effective charge, this helps explain the observed behaviour of the carrageenans, where lambda and iota have similar migration times, and the ratio of migration times between kappa and iota is 1.3, close to the value of

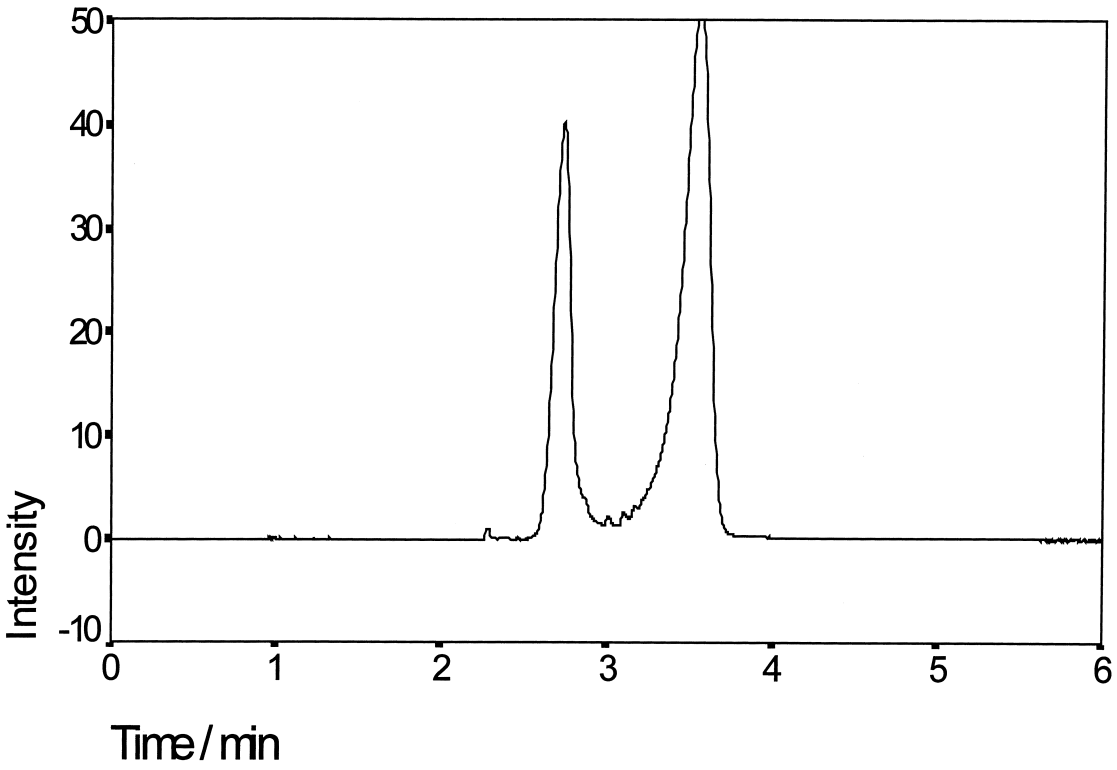

Fig. 5. Electropherograms of retentates from APTS-derivatised mixture (1:1, w/w) of ι- and κ-carrageenans after treatment by $3 \cdot 10^4$ rel. mol. mass micro-centrifuge filter. CE conditions as in Fig. 4.

1.4 estimated from this simple application of polyelectrolyte theory. Although there remains a problem for the analysis of λ-carrageenan when in the presence of iota and kappa, the migration time at the peak maximum is slightly different, and because of additional difference in peak shape, this molecular type can be unambiguously determined in a qualitative sense, as will be shown later.

One noticeable feature of all of the carrageenan retentate peaks is their broad distribution, which has also been noted for other large macromolecular structures [31] as well as for other large polysaccharides [5] that have been analyzed by CZE. The reasons for this are that the molecules and/or the macromolecular structures themselves are polydisperse. Resolution into individual molecular peaks for polymers with DPs of several hundred or a thousand, like the carrageenans, is not currently possible in CZE with existing technology. The broad peak distributions on the one hand increase the resolution requirements in CZE, but on the other hand can also be used to obtain information on the distribution of molecular types that may exist in real samples. It was shown previously for liposome vesicle populations that peak distributions observed in CZE correlated well with laser-light scattering data detailing their size distribution [31]. Similar information from CZE for polysaccharides could prove very useful to food technologists who must formulate films, gels and solutions of ever increasing precision in order to maintain consistent, stable and better tasting products [7].

3.4. Method optimization

To investigate the potential of the CE method to quantify kappa/iota ratios in mixtures in a wide range of compositions, solutions with total carrageenan concentration ~2 mg ml^{-1} were prepared by mixing appropriate volumes of aqueous stock

solutions of the individual carrageenans at 2 mg ml^{-1} each. Residual levels of the other carrageenan in each sample (Fig. 4), found as discussed previously to be 3% iota in kappa, 4% kappa in iota, mean that actual mass concentration ratios of the minor to the major carrageenans in the mixtures for the nominal ratios of 0.01, 0.10, 0.25, 1.00 are: set 1: kappa/iota, 0.055, 0.146, 0.296, 1.030; set 2: iota/kappa, 0.040, 0.128, 0.273, 0.973. The mixtures were derivatized, purified by centrifugation, and separated by electrophoresis as before. A log–log plot of the ratio of normalised peak areas, A/t (peak area/migration time) as a function of ratio of mass concentration, c, in the original mixture is shown in Fig. 6. With a the minor and b the major component, the ordinate and abscissa of Fig. 6 are $\log([A/t]_a/[A/t]_b)$ and $\log(c_a/c_b)$, respectively. The graph has slope and 95% confidence limits of 1.14 ± 0.18. The experimental data is in satisfactory agreement with the theoretical slope of 1.00, suggesting that the method can be used to quantitate carrageenans even in samples where the concentrations might be quite different.

Since the conformational states of carrageenan polysaccharides are a function of the temperature [21,23] the migration behaviour of carrageenans was studied with the capillary thermostatted to 25, 37 and 50°C, the maximum temperature possible with the current instrumentation. The transition midpoint temperature, T_m, from an ordered (double helix) to a disordered (random coil) state increases with increasing salt concentration [32]. Interpolation of literature data from alkali halide solutions [19] to $[Na]^+ = 0.075$ *M*, the sodium ion concentration in the citrate buffer used for CE measurement, suggests T_m values of <9°C and approx. 30°C for κ- and ι-carrageenan, respectively. Thus both should be in the random coil form across the temperature range studies. Electropherograms (data not shown) confirmed that there was no change in the peak shape with temperature. A slight reduction in efficiency manifests itself as a similarly-sized reduction in the kappa-iota resolution which is shown in Fig. 7. What is more dramatic is the reduction in migration time (Fig. 7), when a 50°C separation with a resolution (R_S) of 3.2 is achieved in under 2.5 min. To our knowledge, CE provides the only technique which is capable of performing analysis of large intact polysaccharides in this time frame.

The efficiencies, *N*, in terms of plate number, for

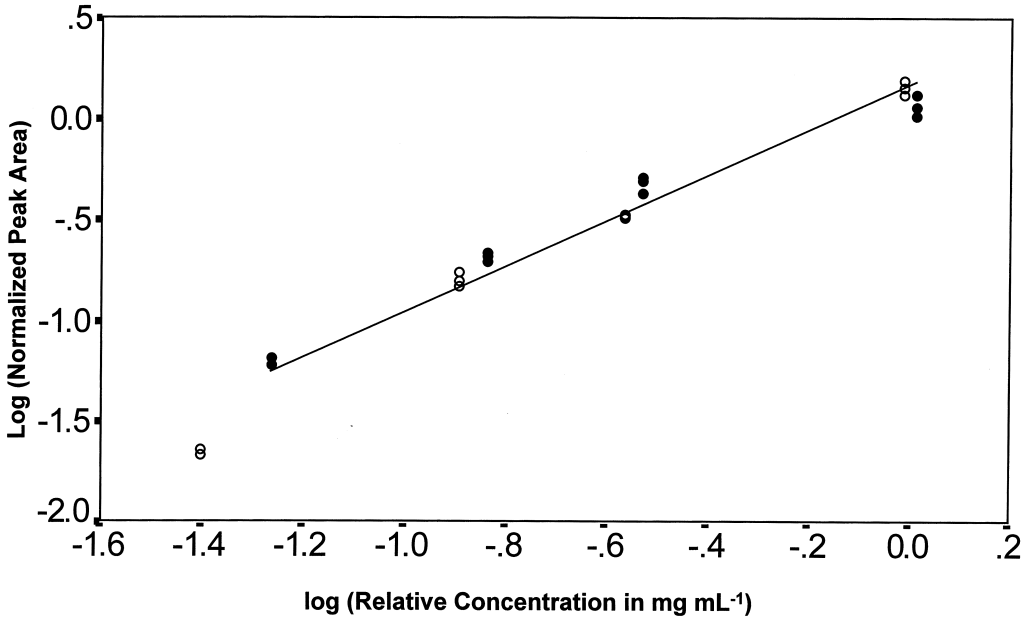

Fig. 6. Log–log plot of normalised peak area as a function of the ratio of mass concentrations of carrageenans in mixtures: (●) kappa as minor component in iota, (○) iota as minor component in kappa. CE conditions as in Fig. 4.

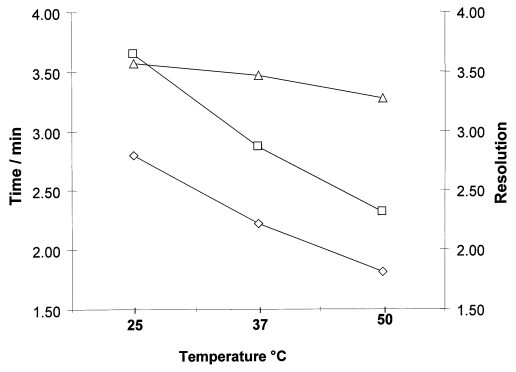

Fig. 7. Temperature effects on κ–ι-carrageenan separations. (□) Kappa migration time (min), (◇) iota migration time (min), (△) kappa–iota resolution. CE conditions as in Fig. 4 except for temperature.

ι- and κ-carrageenan retentates, as well as the resolution between the two peaks, were studied as a function of field strength over the range $1–6\cdot10^4$ V m^{-1}. Values for theses parameters were calculated according to standard equations [33], and averaged $N=2.3\cdot10^3$ for iota and $5.7\cdot10^3$ for kappa. The efficiency for each peak showed little variation with field strength, which was as expected with polydispersity as the key factor in determining peak width. The resolution decreased only slightly, from $R_S=3.9$ to 3.7, as the field strength was increased. It was observed that the migration time for κ-carrageenan, the slowest migrating carrageenan, decreased from 24.1 to 3.4 min with an increase in field strength. From these experiments, it was clear that κ- and ι-carrageenans could be separated at all field strengths with adequate resolution and, therefore the highest field strength conditions were chosen for use in further analysis.

The repeatability of the method, as measured by relative standard deviation (R.S.D.) in peak area and migration time, varied somewhat with the field strength. Between $1.1\cdot10^4$ and $3.2\cdot10^4$ V m^{-1} the area R.S.D. was approx. 4% while it ranged between 6 and 10% for higher field strengths. The highest field strength tested provided reasonable R.S.D.s (5.8 and 7.1%) for quantitation as well as very fast separation times, which was considered optimal for measurement of carrageenans. Migration times measured at the peak maxima were very reproducible, considering the polydisperse nature of the sample, and generally varied between 0.1 and 0.25% with no correlation with field strength. In only one case was the migration R.S.D. greater than this. Although most of the parameters examined in these studies deteriorated slightly at higher field strengths, the large increase in speed of analysis was considered to offset this minor disadvantage. Therefore, highest field strengths were used throughout this study and in subsequent analyses.

To investigate the possibility for using the method in quantitative analysis, peak areas normalized for differences in migration times were studied as a function of concentration of the carrageenans. In the first set of experiments, κ- and ι-carrageenans were mixed 1:1 (v/v) after treatment by micro-centrifuge filters. The initial solution (3.2 mg ml^{-1}) was serially diluted by 2, 10, 40, 100, 200, 400, 800, 1600 in water. The calibration of normalized peak area vs. dilution followed the equation $\log y=(0.90\pm0.10)\log x-(1.59\pm0.16)$ where y is the normalized peak area and x the dilution of the original solution. Within 95% confidence limits, these data show a linear dynamic range varying over two-orders of magnitude in concentration and a limit of detection (qualitative) for either ι- or κ-carrageenan of approx. 0.3 μg ml^{-1}, which represents a substantial improvement over other existing techniques such as slab-gel electrophoresis and NMR. It should be noted that the high sensitivity of LIF is needed for this type of analyte due to the end-labelling derivatization procedure which drastically limits the label:analyte ratio in terms of mass.

3.5. Commercial sample analysis

It was important to establish early on that the CZE method under development was capable of measuring differences in carrageenans found in commercial food additives used in industry. After processing, such products can have a wide range of small sugars, stabilisers and salts added to insure consistency in functionality, even when sold only as carrageenans. In blend samples the measurement difficulties would be expected to increase due to additional large polysaccharides, emulsifiers, colorants, etc. present as a complex mixture.

Several commercial samples of ''κ''-carrageenan,

along with laboratory standards, were labelled with APTS and then examined by CZE after purification as previously described. Three examples from this study are shown in Fig. 8. The first two examples (samples 1 and 2) had migration time ratios (kappa:APTS=1.5) that unambiguously confirmed their kappa authenticity when compared with the standards. Furthermore, it could be observed that these samples actually had a higher level of purity than the standard since no second carrageenan peak was seen. Sample 3 was surprising as it appeared to contain little or no κ-carrageenan at all. The migration time ratio (lambda:APTS=1.22) of this sample confirmed that it was largely λ-carrageenan. In this case of suspected miss-labelling, the CZE results were confirmed by preparing a highly concentrated (6 mg ml^{-1}), heated (75°C) solution of a kappa standard and the ''kappa'' commercial sample under question, and then observing the gelling behaviour of each upon cooling. The ''kappa'' commercial sample 3 remained a liquid when cooled down to 4°C whereas the standard formed a complete unpourable gel. As kappa is a strong gelling additive and lambda is only a thickening additive this result could clearly confirm the CZE determination. Many cases of authenticity issues would not have such a total difference in carrageenan type; however, this example serves to demonstrate and confirm the applicability of the CZE method for the qualitative determination of additive quality and authenticity.

A number of hydrocolloid blend samples known to contain carrageenans were also analyzed and one example will illustrate use of the CZE method. This sample had been previously studied by HPLC, slab-

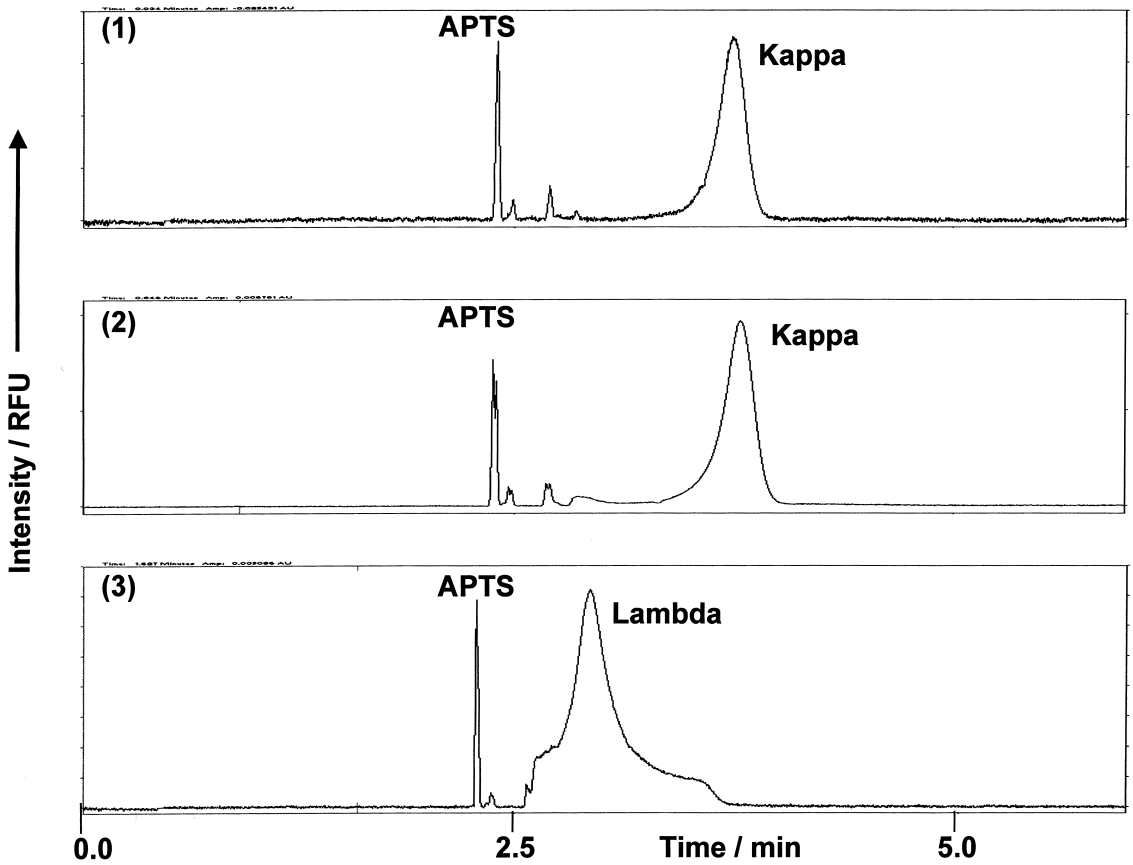

Fig. 8. Authenticity testing of commercial samples labelled ''κ''-carrageenan. All samples were obtained from well-known commercial vendors of food ingredients. CE conditions as in Fig. 4.

gel electrophoresis, immunoassay, and NMR (NRC internal study). From these studies it was known that the sample contained large amounts of locust bean gum and guar, about 20% by weight of carrageenans, as well as various non-polysaccharide emulsifying agents, small sugars and salts. The pre-analysis filtration process used here should separate most of the non-polysaccharide agents based on the size of the molecules as well as desalt the sample. Locust bean gum and guar are uncharged polysaccharides and after APTS labelling would have very low mobility in CZE. The electropherogram of this sample, shown in Fig. 9, displays little evidence of anything other than APTS, its breakdown products, or the carrageenans and demonstrates the good selectivity of the mobility window used in the current method. By comparing the migration time ratios of the two observed carrageenan peaks to standards it could be determined that the sample contained both λ- and κ-carrageenan. It is interesting to note that both slab-gel electrophoresis and NMR are fairly insensitive to λ-carrageenan and, therefore, this sample had only been classified as having κ-carrageenan. In the case of the CZE method the resolution between lambda and the other carrageenans is not optimal; however, the sensitivity for APTS-derivatized λ-carrageenan seems to at least be equal to the others. Therefore, CZE–LIF may allow the speciation of carrageenans that was not possible with previous methods.

4. Conclusions

It has been demonstrated that capillary electrophoresis provides a simple, rapid method for the quantitative detection and separation of κ- and ι-carrageenans. Furthermore, λ-carrageenan, although not baseline resolved from kappa and iota, could be clearly identified by its migration time and peak shape. This method was also shown to be capable of assessing the purity of polysaccharide food additives

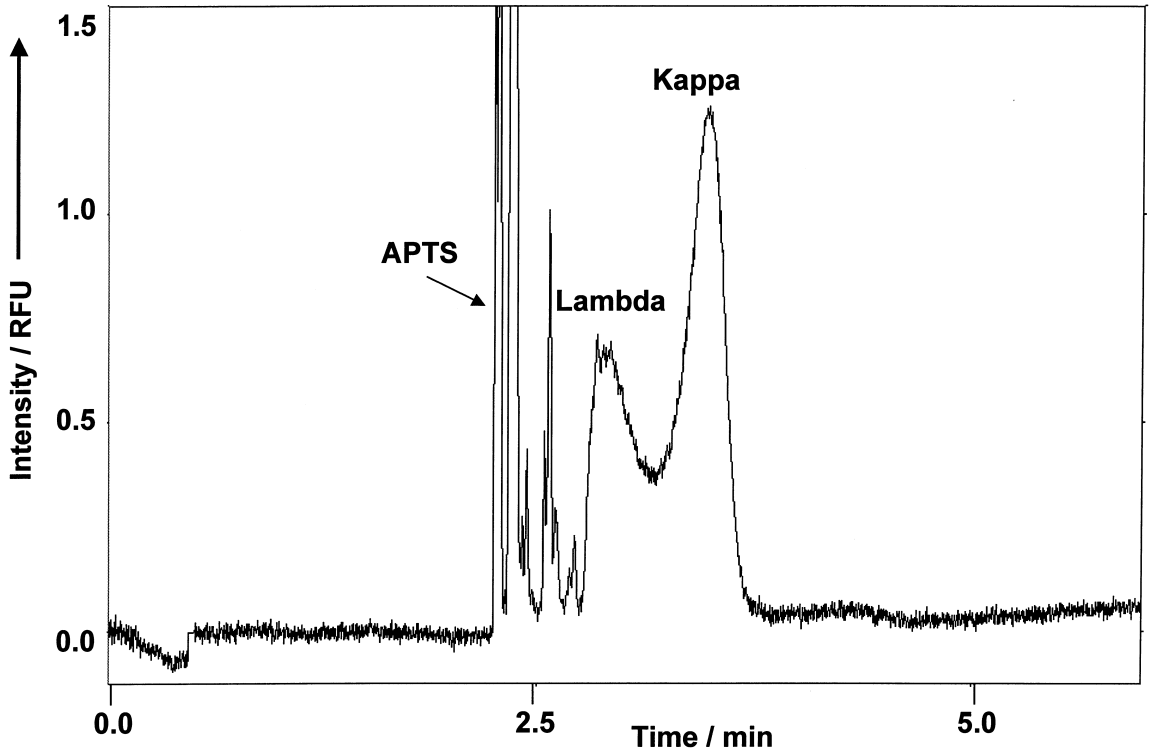

Fig. 9. CZE analysis of a commercial blend sample containing carrageenans, locust bean gum and various emulsifiers. CE conditions as in Fig. 4.

found in commercial samples. It is clear that the use of CE has a great deal of potential in the industrial analysis of these anionic biopolymers as well as providing a research tool for probing their properties and functions in food products.

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