Analysis of carrageenans using capillary electrophoresis

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A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy
at the University of York

Department of Chemistry
University of York

December 2000

Abstract

This thesis reports the use of capillary electrophoresis (CE) for the analysis of carrageenans, anionic polysaccharides extracted from red seaweeds and widely used in the food industry for their gelling and thickening properties. The three main types, *kappa*, *iota* and *lambda*, differ in the number of sulfate groups and the presence or absence of a 3,6-anhydro bridge in the disaccharide residue repeat unit. CE separates analytes according to their charge to frictional coefficient ratios, therefore it is suitable to separate these biopolymers. In order to detect polysaccharides in CE, our approach consisted in derivatising the reducing ends of the saccharides by reductive amination with a fluorophore, 1-aminopyrene-3,6,8-trisulfonate (APTS). This allowed sensitive detection by laser induced fluorescence.

Method development gave optimal conditions for separation using a polyvinyl alcohol coated capillary and a 25 mM ammonium acetate, pH 8.0 background electrolyte. The effects of changes of both instrumental parameters (temperature, injection mode, field strength) and the composition of the BGE (concentration and pH) are reported, and explained in terms of the physical chemistry of the BGE and the biopolymers.

The conditions of the derivatisation reaction were studied in order to minimise degradation due in particular to acid catalysis and to reduction of the reacting sites occurring in competition with derivatisation. Characterisation of the derivatised carrageenans by SEC-MALLS-RI was performed and showed that the extent of degradation occurring during the labelling reaction was a maximum of 40 % for *kappa* and 20 % for *iota* and *lambda*. The presence of the label APTS in excess and its reaction with the reagents during the labelling reaction produces peaks interfering with those from the carrageenan. A sample clean-up was therefore required before injection onto CE. A comparison was made of a range of clean-up procedures (centrifugation, dialysis, preparative SEC) to remove side products of the reaction and salts and to concentrate the carrageenans.

Various seaweed extracts were analysed, including standards of carrageenans not available commercially. This study revealed that carrageenans are complex structures, and often

occurring as hybrids between sub-types. CE has the ability to characterise these hybrids, unlike spectroscopic methods which detect individual residues.

When using actual food products, preliminary steps such as defatting and dialysis were found to be necessary to allow satisfactory detection of carrageenans. Finally the strategy for sample purification, derivatisation, clean-up and separation was successfully applied to additive mixtures used as raw materials in the food industry and to finished products (jelly, dairy products). CE has proved to be a fast and sensitive method to identify and provide semi-quantitative information on carrageenans present in such mixtures.

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Symbols and abbreviations

AA	ammonium acetate
A_c	corrected normalised peak area
AcO^-	acetate ion
AcOH	acetic acid
A_n	normalised peak area
APTS	8-aminopyrene 1,3,6-trisulfonic acid
BGE	background electrolyte
CBQCA	3-(4-carboxybenzoyl)-2-quinolinecarboxylaldehyde
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	isoelectric focusing
CMC	carboxymethyl cellulose
CZE	capillary zone electrophoresis
D	diffusion coefficient
DE	degree of esterification
DMSO	dimethyl sulfoxide
DP	degree of polymerisation
e	elementary charge
E	electric field strength
ε	permittivity of the medium
EKI	electrokinetic injection
EOF	electroosmotic flow
F_e	electrical force
F_f	frictional force
ζ	zeta potential
HS	heptane sulfonate
IS	internal standard
ITP	isotachophoresis
η	viscosity

θ	temperature in °C
I	current
I_{sep}	current during the separation
I_{inj}	current during the injection
I_p	polydispersity
k	Boltzmann constant
k_1, k_2, k_3	rate constants for hydrolysis
l	length to the detector
L	total length of the capillary
LBG	locust bean gum
LIF	laser induced fluorescence
LOD	limit of detection
m	mass
M	molar mass
MEKC	micellar electrokinetic capillary chromatography
M_i	molar mass of species i
M_n	number average molecular weight
M_w	mass average molecular weight
MWCO	molecular weight cut-off
μ	electrophoretic mobility
μ_{ep}	electrophoretic mobility
μ_{eo}	electroosmotic mobility
μ_{obs}	observed mobility
n	total number of macromolecules
n_i	number of macromolecules of molecular weight M_i
N	efficiency
PI	pressure injection
PVA	polyvinyl alcohol
q	charge of the analyte
r	radius of the analyte
RFU	relative fluorescence units
v	velocity
R_c	resistance of the total capillary

R_s	resolution
R_{sz}	resistance of the sample zone
RMT	relative migration time
RSD	relative standard deviation
σ^2	peak variance
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
t	time
T	absolute temperature in K
TBA	tetrabutylammonium
THF	tetrahydrofuran
T_m	transition midpoint temperature
T_o	helix onset temperature
t_m	migration time
TRIS	tris(hydroxymethyl)aminomethane
V	voltage
V_{inj}	injection voltage
V_r	recovered volume
V_{sep}	separation voltage
V_{sz}	potential difference across the sample zone
V_c	potential difference across the capillary
z	charge number

Acknowledgements

I would like to thank my academic supervisor, David Goodall, for his support and help throughout the this work, and particularly during the writing up period. Special thanks go to Matthew-Alan Roberts, my industrial supervisor from Nestlé Research Center in Lausanne, for his help and support.

I am also grateful to Jacques Prodolliet, who initiated the project in Nestlé, and Claudia Hischenhuber, both of whom welcomed me into their groups and showed interest in the application of the method to food products. I thank everybody in the Carbohydrate Team for their help, particularly Christelle for her support and her assistance in transfer of data between Lausanne and York. Many thanks to Paulo Fernandes from Friskies France for supplying the molecular weight standards.

Particular mention should also go to Patrick Boulenguer and Mireille Amat for providing me with carrageenan standards and fractions, and helping me do the SEC-MALLS measurements at SKW in Baupte, France.

Thanks to all my friends and colleagues at York for their help. I would like to mention for their constant support Elif and Deborah, and also Ahtziri, who is probably the only sociologist to know what carrageenans are.

Finally, I would like to thank my family for their constant support during my studies, and Stéphane for putting up with me and my PhD.

Chapter 1: Introduction

1.1 Introduction to hydrocolloids

Foodstuffs generally consist of two main parts: (i) a continuous phase containing the main product (e.g. milk, vegetable, meat), together with water, aromas and colorants; (ii) a discontinuous phase containing non miscible products such as particles, fats and gases. The presence of texturising agents such as hydrocolloids prevents these components in the discontinuous phase from sedimenting or creaming (Rizzotti 1994). Hydrocolloids are water-soluble, hydrophilic macromolecules which modify the rheological properties of foodstuffs. If no interaction occurs between molecules, thickening is due to an increase in viscosity. A gelation phenomenon appears if the molecules can form a 3-dimensional network. Gelation properties are of interest in the development of low-fat products where biopolymers are used to provide the texturing function, i.e. mouthfeel, usually fulfilled by fats.

Two classes of hydrocolloids can be distinguished: proteins (e.g. gelatin) and polysaccharides from vegetable or bacterial origin. Texturising agents which are digested in the gastrointestinal tract are classified as nutrients, such as gelatin and starch, whereas others are considered as additives and in the European Community are given a code number. The main polysaccharides used in the food industry are presented in Table 1.1.

Name	Code	Origin	Function
sodium alginate	E 401	brown seaweeds	T, G
agar	E 406	red seaweeds	T
carrageenan	E 407	red seaweeds	T, G
locust bean gum (LBG)	E 410	seeds	T
guar gum	E 412	seeds	T
gum arabic	E 414	tree exudate	T
xanthan gum	E 415	fermentation	T
pectin	E 440	cell wall	G
carboxymethylcellulose	E 466		T

Table 1.1. The main polysaccharides used as texturising agents in the food industry. T, thickener; G, gelling agent.

Carrageenans are sulfated linear polysaccharides of alternating 1,3-linked β-D-galactopyranose residues and 1,4-linked α-D-galactopyranose residues (see Figure 1.1). The differences between carrageenans are due to the position and the number of sulfate groups and the presence of the anhydro bridge. *Kappa* (κ) and *iota* (ι) carrageenan contain anhydrogalactose units, in which the sugar molecule is forced from the $^4C_1$ chair conformation into the $^1C_4$ chair conformation. Other carrageenan sub-types include *mu* (μ) and *nu* (ν), which are respectively the biological precursors of *kappa* and *iota*. *Mu* and *nu* do not contain an anhydro bridge, but an additional sulfate ester at C_6 which disappears when the intramolecular bridge is formed (see Figure 1.2). This conformation allows *iota* and *kappa* to form helices. They are therefore gelling agents, whereas *lambda* carrageenan, composed only of galactopyranose units, is a thickener. *Lambda* (λ) is the precursor of *theta* (θ). *Xi* (ξ) has a similar structure to *lambda*, except for the lack of complete sulfation at C_6 on the 1,4-linked units.

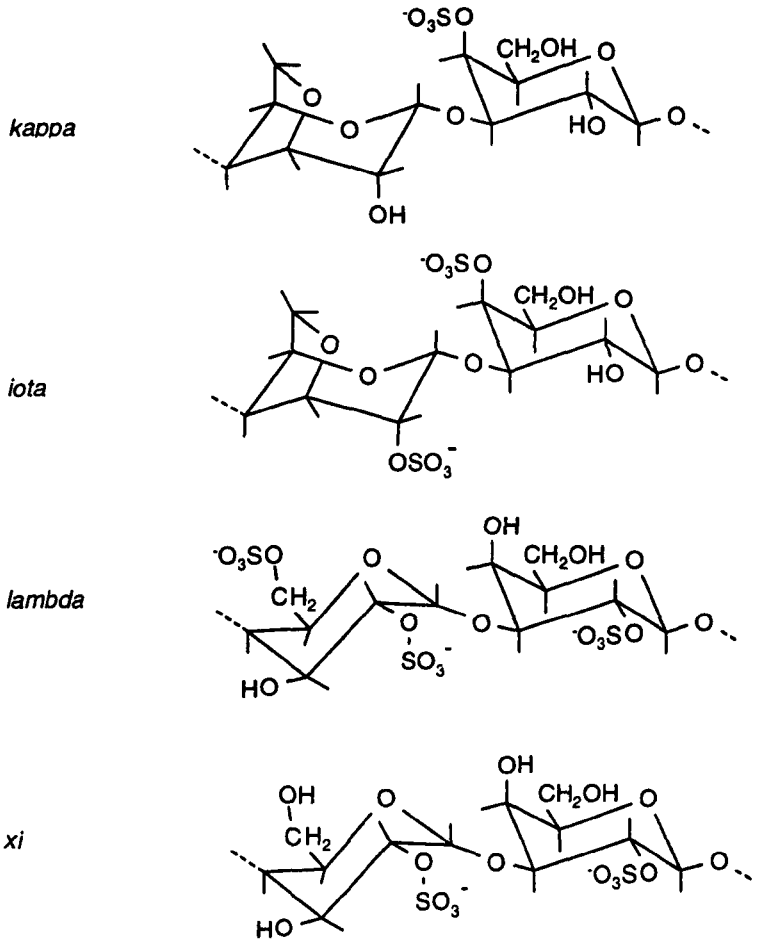

Figure 1.1. Structure of the main carrageenan sub-types (*kappa, iota, lambda, xi*).

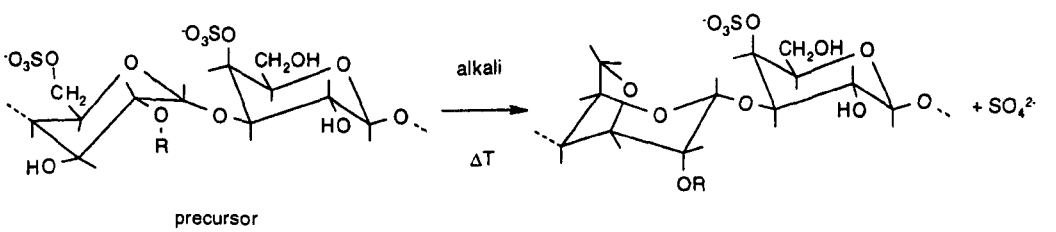

Figure 1.2. Scheme of the conversion of precursor carrageenans. *Mu, kappa* : R = H; *nu, iota* : R = SO_3^-.

Carrageenan structure is usually investigated by NMR spectroscopy, which gives information not only on the substituents of the hydroxyl groups, but also on the linkage (Usov 1998). Substituents other than sulfate have been identified, such as methyl, pyruvate and glycosyl (monosaccharide) groups. A new nomenclature has been proposed by Knutsen

et al. (1994) to describe the chemical structure of red algal galactans (including carrageenan and agar groups). This system is based on the letter codes D (4-linked α-D-galactopyranosyl), G (3-linked β-D-galactopyranosyl), DA (4-linked 3,6-anhydro-α-D-galactopyranosyl) and S (ester sulfate) for carrageenans and allows a convenient description of hybrid molecules and sequences found in NMR (Knutsen *et al.* 1992). In this system, *kappa*'s disaccharide is designated by G4S-DA, and *lambda* G2S-D2S,6S.

In solution gelling carrageenans undergo conformational modifications depending on their concentration, the chemical environment and the temperature. Carrageenans are extracted from the cell wall of various species of red seaweeds (*Rhodophyta*) found in Asia, North Africa, South America and Northern Europe. The characteristics of the product depend on the source, the extraction process and the season of harvesting. The species currently grown for the production of carrageenans are *Eucheuma* species and the major zone of production is the Philippines. The different genera and species are shown in Table 1.2, as well as the carrageenan fractions they provide.

Genera	Species	Origin	Main fractions
Eucheuma	*cottonii*	Philippines	κ + μ
	gelatinae	Philippines	
	isiforme	US	
	denticulatum (spinosum)	Indonesia, Philippines	ι and ± ν
Gigartina	*pistillata*	Morocco	λ, κ and μ
	stellata	France, Spain	λ, κ and μ
	radula	Chile, US	λ + μ and κ
	acicularis	Morocco	λ + μ and κ
	canaliculata	Mexico	ξ
	chamissoi	Chile	ξ
Chondrus	*crispus*	US, Canada, France	λ, κ and μ
	ocellatus	Korea	κ + μ
Hypnea	*muciformis*	Senegal, Brazil	κ and μ
Iridaea	*flaccida*		λ + κ + μ

Table 1.2. Principal species of red seaweeds used in the industry for the production of carrageenans (Doublier *et al.* 1992, Glicksman 1983)

Two processes for extracting carrageenans are referred to in the literature. These are the traditional extraction and the alternative process producing Philippine Natural Grade (PNG) carrageenan, which is suitable for gelling carrageenans sub-types *iota* and *kappa* (Tye 1994). The exact conditions vary from one manufacturer to the other. The traditional extraction process consists in boiling the raw seaweed in alkali in order to dissolve the polysaccharides into the extraction liquor. After filtration, carrageenans can be recovered by precipitation using isopropyl alcohol. Another possibility called gel press processing takes advantage of the fact that *iota* and *kappa* carrageenans form gels with potassium ions, and uses the coagulation with potassium chloride; water is subsequently squeezed from the coagulum. In both alcohol and gel press processing, the extracts are dried and ground. Unlike the traditional process, the Philippine process does not destroy the cellulosic membrane of the seaweed, and is performed in the solid state of the polysaccharide. It consists of several washes at moderate temperatures, first with alkali to stabilise and increase the content in gelling carrageenan via conversion of the precursor, then with fresh water in order to remove impurities. The carrageenan remains associated with the cellulosic plant matrix in the finished product. This latter process is less expensive than the traditional one, and can be performed at the harvesting site. Phillips (1996) investigated the chemical nature of PNG carrageenan and found that it conformed to the Joint Expert Committee for Food Additives of the United Nations Food and Agricultural Organisation (JECFA) and European Community (EC) specifications, and was chemically identical to traditionally extracted carrageenan, with the exception of the acid insoluble matter (10-14 % w/w in PNG, 0.1 % w/w in traditionally processed *kappa* carrageenan). According to the EC specifications the acid insoluble matter in carrageenan should not represent more than 2 % w/w. This insoluble fraction was characterised by NMR and X-ray diffraction and found to be cellulose present in normal algal cell walls (Phillips 1996). Nowadays, PNG carrageenan is also called Processed Eucheuma Seaweed (PES), and is designated with the code E 407a to differentiate it from traditionally extracted carrageenans.

The principal food issue with carrageenans is that low molecular weight oligomers have been hypothesised to be toxic if released during digestion (Ekström 1985). Carrageenan fragments of M_w below 100 kDa are designated by the term polygeenan. Since low M_w fragments are characterised by low viscosity, the JECFA has specified for regulatory purposes that the viscosity at 75 °C of a 1.5 % w/w solution of food grade carrageenan,

intact and filtered, has to be greater than 5 mPa s (Phillips 1996).

Chemical degradation has been carried out on carrageenans in both ordered and disordered states to compare their respective apparent molecular weights. Hydrochloric acid solutions of different pH values were used in order to mimic the gastric conditions (Ekström & Kuivinen 1983; Karlsson & Singh 1999). It was found that *kappa* carrageenan is more sensitive to hydrolysis than *iota* carrageenan. *Iota* retains the double-helical structure at the investigated temperature (37 °C), whereas under these conditions *kappa* is in a coil conformation. The molecular weight distribution was shifted towards lower values after hydrolysis for 6 h at 37 °C and pH 1, with more than 30 % material with M_w below 30,000 kDa (weight average) for *kappa* carrageenan. Ekström investigated the degradation in simulated gastric juice containing pepsin (Ekström 1985). After treatment for 2 h at pH 1.2, nearly 90 % of the material had $M_w < 100$ kDa, and 25 % in the M_w distribution had $M_w <$ 20 kDa. Degradation of carrageenans during digestion produces low M_w material and depends on the acidity and the time spent in the stomach medium. Another research group performed degradation of carrageenans in 0.1 M HCl at 37 °C (Hjerde *et al.* 1998). The molecular weight was estimated by size exclusion chromatography (SEC) coupled to refractive index (RI) and low angle laser light scattering (LALLS). When the reaction was performed on *kappa* carrageenan in the disordered state, M_w decreased from 4.1×10^5 to 4000 after hydrolysis for 4 h. A rate constant of $(31 \pm 2)\times10^{-3}$ h^{-1} for cleavage of glycosidic linkages was found. In the studies reported by Ekström, *kappa* carrageenan was in the coil state, and *iota* in the helical state. Capron *et al.* investigated the stability of *kappa* and *iota* carrageenans in the helical state in an artificial stomach, because these gelling carrageenans are almost always in the helical state when ingested in food products (Capron *et al.* 1996). They found that the degradation was much reduced, and that under the most drastic conditions (6 h at pH 1.2) 20 % of *kappa* carrageenan had $M_w < 100$ kDa. *Iota* carrageenan was found to be slightly more resistant to hydrolysis than *kappa*. This publication demonstrates the importance of the conformational state of the carrageenans used as a reactant for degradation studies.

A recent paper reported the effect of non degraded *kappa* carrageenan given as a liquid or a gel to rats, in order to assess its influence on colon cancer (Corpet *et al.* 1997). The criterion to assess the effect of carrageenans was the number of aberrant crypt foci (ACF).

Carrageenan was found to have no influence on healthy rats when given as a 10 % jelly replacing water for 8 days. This showed that carrageenan jelly does not initiate colon cancer. Cancer was initiated in a different group of rats, which were given either a liquid solution (0.25 %) or a solid gel (2.5 %) of *kappa* carrageenan for 100 days. Administration of carrageenan as a solution did not change significantly the growth of the ACF, in comparison to the administration of water as a control. The rats fed with the carrageenan gel showed a higher ACF multiplicity than the rats fed with the liquid solution, suggesting that carrageenan given as a jelly does affect the growth of ACF, and potentially promotes colon cancer in rats. Currently, there is no hard evidence or consensus opinion which could suggest toxicity; however the issue persists as a consumer safety topic and therefore is of analytical relevance.

1.2 Techniques for carrageenan analysis

Various methods dealing with the analysis of carrageenans in food have been reported in the literature. A comprehensive review has been recently published by Roberts & Quemener (1999). These methods include spectroscopic techniques, immunoassays and separative techniques and are presented below.

1.2.1 Colorimetric methods

Being polyanions, carrageenans are possible candidates for complexation with cationic dyes. After binding, the spectral properties of the dye are modified and the amount of polysaccharide can be deduced from a spectrophotometric measurement. Usually the absorbance maximum is shifted to shorter wavelengths, and the phenomenon is called metachromasy; a number of dyes have been tested for metachromasy with alginic acid, agar and carrageenans (Güven & Güvener 1985). Since the effect depends on the number of sulfate groups, colorimetry gives slightly different responses for the carrageenan sub types. However, this method does not differentiate between *iota*, *lambda*, and *kappa* carrageenans and allows determination of the total amount of carrageenan present.

Yaphe and Arsenault reported the use of resorcinol to measure 3,6-anhydrogalactose (1965). The polysaccharide is firstly hydrolysed and 3,6-anhydrogalactose is degraded into 5-hydroxymethyl-2-furaldehyde. This latter compound complexes resorcinol, and the absorbance of the complex is measured at 550 nm. The method was able to detect 3,6-anhydrogalactose in the range 0-0.25 µmol. However, the method showed a sensitivity to fructose slightly higher than that to 3,6-anhydrogalactose. This is an important drawback for food containing fructose either as a monosaccharide or in oligomers such as sucrose or raffinose, which will require preliminary clean-up to remove these small sugars.

Yabe *et al.* reported the use of alcian blue as a complexing agent for carrageenans (1991). The dye-polysaccharide complex precipitated, and was washed and redissolved in ethanolamine for its absorbance to be measured at 615 nm. No precipitate was observed when applying the method to other thickening agents. The limit of detection was 0.05 %

when carrageenans were spiked in jelly and salad dressing.

A recent advance in quantification by a colorimetric method is that using methylene blue (Soedjak 1994). The improvement in comparison to other methods is that the use of very dilute solutions of dye and polysaccharide makes the complex soluble. No precipitation or centrifugation are therefore required, and the absorbance can be measured directly. Methylene blue showed two maxima in the visible region, at 610 nm and 664 nm. Upon complexation with carrageenans the absorbances at these maxima decreased, and an absorbance appeared at 559 nm and increased with the concentration of polysaccharide. The absorbance varied linearly with the polymer concentration. Different slopes were observed for the various types of carrageenans, correlating with the number of the sulfate esters. The method was tested on other hydrocolloids and food components such as organic acids, salts, emulsifiers, proteins, and carbohydrates. Carboxylated anionic polysaccharides (alginate, carboxymethyl cellulose, xanthan and gum arabic) showed a complexing ability for methylene blue with different reactivities. Phosphate buffer in the pH range 1.75-12.0 was found to efficiently inhibit the complexation of the dye with carboxylates, but not with sulfates. Thus this method can provide the measurement of carrageenans when performed in phosphate, and the measurement of carboxylate-containing hydrocolloids by difference when performed in water. The high dilution factor used for this analysis prevented interferences from other compounds. Its suitability was tested in milky media of increasing content in milk. The relationship between concentration and absorbance was linear, with slope varying with the proportion of milk.

1.2.2 Spectroscopic methods

NMR spectroscopy has been recently shown to provide more than structural information about carrageenans; Turquois *et al.* (1996) used this technique in conjunction with infrared spectroscopy to determine relative proportions of carrageenans in commercial blends. *Iota* and *kappa* carrageenans gave one peak per carbon atom in ^{13}C NMR, whereas the spectrum obtained for *lambda* showed broad peaks unsuitable for quantitation. However, a method was developed which used infrared spectroscopy to determine the proportion of *lambda* carrageenan. In this technique, the sulfate groups of the carrageenans are detected at 1250 cm^{-1}, and galactose-4-sulfate at 845 cm^{-1}. The ratio of amounts of *kappa* and *iota*

carrageenans is determined by the ratio of absorbances at 845 and 1250 cm^{-1}. The presence of *lambda* induced an increase of the absorbance at 1250 cm^{-1}. The ratio of absorbances was modified, but a good correlation was retained with the ratio of *iota* and *kappa* carrageenans. The relative ratio of amounts of *kappa* and *iota* was therefore first determined by NMR, then IR spectroscopy performed a mixture of *iota* and *kappa* with this ratio. Comparison of the ratio of absorbances at 845 and 1250 cm^{-1} yielded a semi-quantitative estimate of *lambda* carrageenan.

1.2.3 Immunoassays

Haines and Patel have reviewed the use of receptor binding assay methods, both by enzyme linked immunosorbent assay (ELISA) and enzyme-linked lectin assay (ELLA), for the analysis of gums and thickeners (1997). For carrageenans specific antibodies were used as targets and an ELISA and a dot-blot assay were developed (Williams *et al.* 1994). Antibodies are produced by animals exposed to the polysaccharide of interest. The reported assays concerned *kappa* carrageenan and proved to be sensitive and specific to this particular carrageenan sub-type, with no cross-reaction to other thickeners. This technique allowed the analysis of *kappa* carrageenan with a limit of detection in the range 0.001-0.01 % w/w in foodstuffs. The determination of *kappa* carrageenan in infant food was presented. It was reported that no cross-reaction occurred with a variety of other thickeners, including other carrageenans (Haines & Patel 1997). Another paper was presented by Arakawa *et al.* (1991) using a sandwich ELISA for the determination of *kappa* carrageenan. *Kappa* carrageenan could be quantified in the range 16 to 256 ng mL^{-1}, and the assay was successfully applied to a jelly and a custard pudding. In this paper cross-reactions were investigated only with six other thickeners, but no mention of other carrageenan sub-types was made.

1.2.4 Gas chromatography

A comprehensive study of the use of GC for structural characterisation of carrageenans was reported by Stevenson & Furneaux (1991). GC requires hydrolysis of polysaccharides into their monosaccharide constituents, followed by derivatisation of these monosaccharides to give volatile compounds. The key point when hydrolysing carrageenans is to avoid

degradation of the anhydrogalactose residues, which are very sensitive to hydrolysis. Two protocols for depolymerisation were optimised, the double hydrolysis-reduction procedure and the reductive hydrolysis. Double hydrolysis-reduction involved a preliminary hydrolysis in 0.1 M trifluoroacetic acid (TFA) at 80 °C, during which the glycosidic linkages of the anhydrogalactosyl residues were cleaved and the sulfate groups are removed. Then the mixture was reduced in aqueous sodium cyanoborohydride, and submitted to a second hydrolysis in 2.0 M TFA at 120 °C, reduction, and acetylation. Reductive hydrolysis consisted of hydrolysis in the presence of the reducing agent 4-methylmorpholine borane, this latter reagent being added three times to the reaction mixture containing an initial concentration of 2.4 M TFA. These two procedures were applied to various carrageenan and agar samples and were found to give good agreement on the analysis of released monosaccharides, with equal amounts of galactose and anhydrogalactose found in *iota* and *kappa* carrageenans. Reductive hydrolysis is less time consuming and was found to be more convenient than the double hydrolysis-reduction procedure. In the same publication, the authors investigated methylation for the determination of linkage and substitution patterns of sugar components of extracts from red algae. The polysaccharides were transformed into their triethylammonium salts by dialysis, and subsequently methylated in DMSO. The resulting polymers were submitted to reductive hydrolysis and acetylated before analysis in GC. This procedure produces partially methylated alditol acetates, and allows the determination of substituted sites and linkage positions of the monosaccharide rings. *Iota*, *kappa*, and *lambda* carrageenans available commercially from Sigma were analysed. *Iota* and *kappa* presented expected residues, corresponding to their ideal structure. *Lambda* was found to display residues typical for the *xi* structure. In addition, enzymatic analysis showed that it also contained substantial amounts of 4,6-pyruvate ketal group on 3- linked galactosyl residues. The methods reported in this paper were shown to be able to identify carrageenan sub-types in pure samples. However, these methods were not applied to food products.

1.2.5 HPLC

Methanolysis of *iota* and *kappa* carrageenans and agar has been reported (Quemener *et al.* 1995). Constituent sugars were released as methyl glucosides and separated using reversed phase HPLC. Conditions for methanolysis were carefully investigated in order to optimise the yield of monosaccharides. The crucial point when depolymerising carrageenans is that

the anhydrogalactose ring is easily degraded in acid conditions. Under mild conditions (0.125 M methanolic HCl), the dimethyl acetals of the dimers carrabiose and agarobiose were released. Increasing the methanolysis time or the acid concentration led to a decrease of these dimer derivatives, but also a decrease in the yield in the 3,6-anhydrogalactose derivatives. *Iota* was found to show a different behaviour to *kappa* and agar towards methanolysis, with a quantitative release of anhydrogalactose after 1 h solvolysis in 0.125 M methanolic HCl. In all cases the polysaccharides were incompletely depolymerised, and calculations had to include carrabiose and agarobiose, respectively. Recovery of anhydrogalactose after methanolysis of *kappa* and agar were 80 and 70 % respectively, suggesting partial destruction of this monosaccharide residue. The increased stability in *iota* carrageenan was attributed to stabilisation by sulfate on O_2 of the 3,6-anhydrogalactose residue.

In a later paper, the same group investigated the effect of variation of pH of the reaction mixture after methanolysis (Quemener & Lahaye 1998). It was found that adjusting the pH to 6-7 prevented further degradation of 3,6-anhydrogalactose dimethylacetal during methanol evaporation. In this paper, the authors also investigate high performance anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) as an alternative to GC (Stevenson & Furneaux 1991) for the analysis of alditols. Reductive hydrolysis / HPAEC-PAD and methanolysis / RP-HPLC methods were used to analyse various samples of carrageenans and agars. They were found to agree on all samples investigated, with the exception of *lambda*. This was explained by the fact that mild methanolysis conditions were not sufficient to desulfate and depolymerise *lambda* carrageenan. Methanolysis is less time consuming since it does not require derivatisation of the monosaccharides after depolymerisation.

Further uses of methanolysis were reported by Quemener *et al.* (2000) for the quantitative analysis of *iota* and *kappa* carrageenans in food. The 3,6-anhydrogalactose content was used to quantify gelling carrageenans in food samples such as home-made liver pâté, yogurt and gelling milk, in the presence of other hydrocolloids such as pectins, alginate, xanthan or galactomannans. The 3,6-anhydrogalactose content of the standards used in the preparation of the food products was determined; the authors give average values for analysis performed in cases where these standards are not available. Since this method is based on 3,6-

anhydrogalactose, it is not suitable for determination of *lambda* carrageenan or of *kappa* and *iota* in the presence of agar.

1.2.6 Electrophoretic methods

Pechanek *et al.* reported a separation method for carrageenans and other thickeners in food using electrophoresis on cellulose acetate membranes as an alternative to polyacrylamide or agarose gel electrophoresis (1982). Following removal of fats, proteins and degradation of starch, the samples were applied onto the membrane, subjected to electrophoresis and stained for detection with Schiff's reagent (for reducing sugars) and toluidine blue (for anionic sugars). Polysaccharides could be identified by their migration time, staining properties and peak shape. *Iota* and *kappa* gave two peaks each. Carrageenans were stained by toluidine blue and methylene blue and the mass limit of detection was found to be 95 ng. Recovery for carrageenans was found to be 90-100 % in various food products including puddings and ice cream.

Another staining procedure for carrageenans was developed by Marrs; this consisted in complexation of carrageenans to a water-soluble protein followed by staining with a protein-sensitive dye. The method was used in the separation of the three types of carrageenans on cellulose acetate strips in barium acetate buffer (Marrs 1986). The mobilities found for the carrageenans varied inversely with the number of sulfate groups per residue, with *kappa* migrating the fastest; this mobility order was attributed to complexation of the sulfate esters with barium.

1.3 Capillary electrophoresis

1.3.1 Historical background

Capillary electrophoresis (CE) is a relatively recent separation technique, which is based on the different migration behaviours exhibited by ionic species in the presence of an electric field. Pioneering work was done by Tiselius in 1937; he separated serum proteins in free solution in quartz tubes. Subsequently, various forms of electrophoresis were developed, including paper electrophoresis and polymer and agarose gel electrophoreses, widely used in the field of proteins, DNA and RNA. Tiselius was awarded the Nobel Prize for his studies in 1948. Further improvement was made to the technique by Hjerten (1967), who used capillary tubes of internal diameter < 3 mm and built the first capillary electrophoresis instrument. The use of electrolytes in this type of tubes generated high currents, thus increase in temperature and mixing of the analytes by convection. In order to reduce these phenomena, the tube was continuously rotated around its axis. Hjerten demonstrated separation of small molecules (inorganic ions, nucleotides) as well as macromolecules (proteins) and microorganisms. In the early 1980s, Jorgenson & Lukacs were the first researchers to use 75-100 μm fused silica capillaries and to demonstrate the ability of CE to provide high efficiency separation under high electric fields. Nowadays, capillaries are available in very small dimensions (typically 20-100 μm internal diameter, 375 μm outer diameter) and lengths from 20-100 cm. The external surface of the capillary is coated with polyimide in order to make it more flexible. In most commercial instruments the capillary is thermostatted, either by pulsed air or by circulation of cooling liquid, which allows the use of high voltages (up to 30 kV) and a wide range of ionic strengths. CE has reached the same degree of miniaturisation and automation as HPLC. Fig. 1.3 shows a schematic diagram of a CE instrument. Separation takes place in the capillary preliminarily filled with the separating medium (background electrolyte, BGE). The sample can be injected electrokinetically, by applying a low voltage (1 to 10 kV) between the sample vial at the inlet and a BGE vial at the outlet, or by pressure injection, which entails applying a pressure difference (typically 0.5 psi) between inlet and outlet vials. During separation, the ends of the capillary and the electrodes are dipped into BGE vials, and the separation voltage is applied between these two vials. The analytes migrate towards the detector, where they are

finally detected. The whole system is monitored by a computer, where analyses are programmed and results are processed.

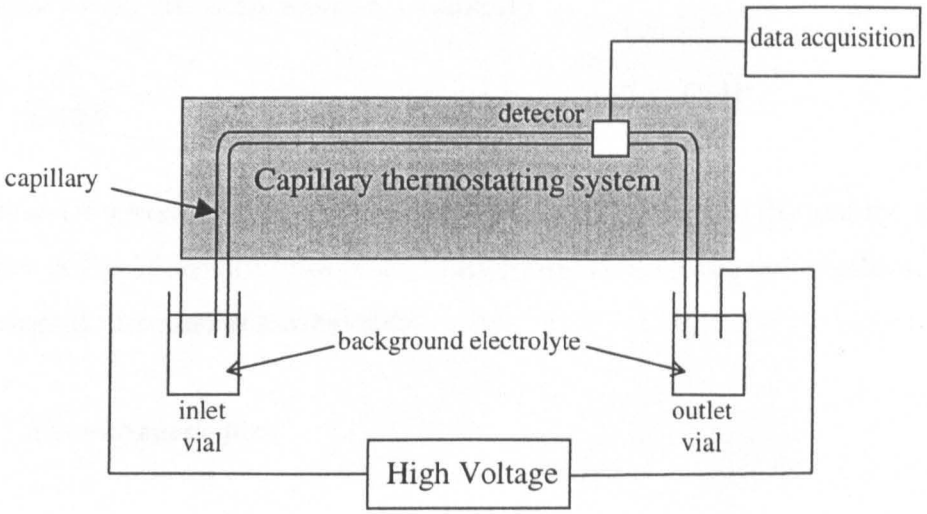

Figure 1.3. Schematic diagram of a CE instrument.

1.3.2 Capillary zone electrophoresis

1.3.2.1 Basic principles

CE separates analytes according to their mobilities. The mobility of a given analyte depends on its chemical environment, in particular the pH of the BGE. In the absence of an electroosmotic flow (EOF), the electrophoretic mobility μ_{ep} is given by the following equation:

$$\mu_{ep} = \frac{v}{E} \qquad [1.1]$$

where v is the velocity of the analyte and E is the field strength. SI units for these quantities are m s^{-1} and V m^{-1} respectively.

During the electrophoretic process, a steady state is reached, where the moduli of the electrical force F_e and the frictional force F_f have the same value. For a spherical ion, these forces are described by equations 1.2 and 1.3:

$$F_e = qE \qquad [1.2]$$

$$F_f = -6\pi\eta r v \qquad [1.3]$$

where q is the charge of the analyte, η is the viscosity of the BGE and r the radius of the analyte.

Setting $|F_e| = |F_f|$ and using equation 1.1 leads to

$$\mu_{ep} = \frac{q}{6\pi\eta r} \qquad [1.4]$$

Equation 1.4 predicts that the higher the charge and the smaller the ion, the larger the mobility. A simplification is often made, which consists in assuming that mobility scales with the charge to size ratio of a compound.

1.3.2.2 Electroosmotic flow

Electroosmosis is the movement caused by an electric field of an uncharged liquid in contact with a surface of fixed charge. This bulk movement is a consequence of the interface existing between these two phases, described using Stern's model of the double layer and displayed in Fig. 1.4.

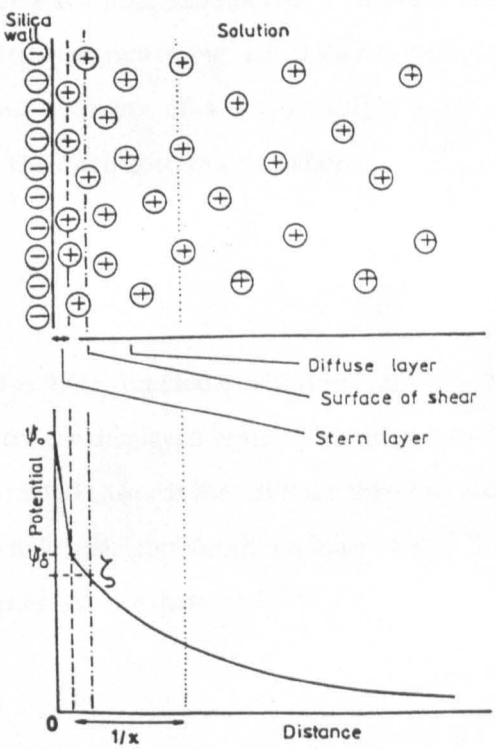

Figure 1.4. Schematic representation of the structure of the double layer according to Stern's theory (from Foret *et al.* 1993).

The silica surface of the capillary is partly charged at pH > 2, due to deprotonation of the silanol groups Si-O-H. In order to maintain local electroneutrality, counterions are attracted, a fraction of which are adsorbed and form the so-called Stern layer. The rest of these ions are mobile and form a diffuse double layer called Gouy-Chapman or Debye-Hückel layer. This double layer is the origin of a difference in potential between the silica surface and the diffuse layer, where the potential is zero. Within the Stern layer the potential decreases linearly with the distance from the surface. In the diffuse layer the potential decreases exponentially with distance from the surface. The potential at the surface of shear is called the zeta (ζ) potential, and determines the mobility of the electroosmotic flow through the Helmoltz-Smoluchowski equation

$$\mu_{eo} = \frac{\zeta \varepsilon}{4\pi\eta} \qquad [1.5]$$

where ζ is the zeta potential, ε the permittivity of the medium and μ_{eo} the electroosmotic mobility. The electroosmotic flow transports all liquid across the capillary towards the cathode. Since the driving force for this phenomenon is located all along the capillary, the profile of the EOF is plug-like, as shown in Fig. 1.5, unlike that of pressure driven systems such as HPLC. The observed mobility of a given analyte is the algebraic sum of its electrophoretic mobility and the electroosmotic mobility.

$$\mu_{obs} = \mu_{eo} + \mu_{ep}$$

Considering the usual case of an EOF directed towards the cathode, the resulting migration behaviours of different analytes are displayed in Fig. 1.5. Neutral molecules migrate at the EOF velocity. Cations are attracted towards the cathode, therefore they migrate faster than the EOF, whereas anions, whose electrophoretic mobility would lead them towards the anode, are retarded and migrate slower than the EOF.

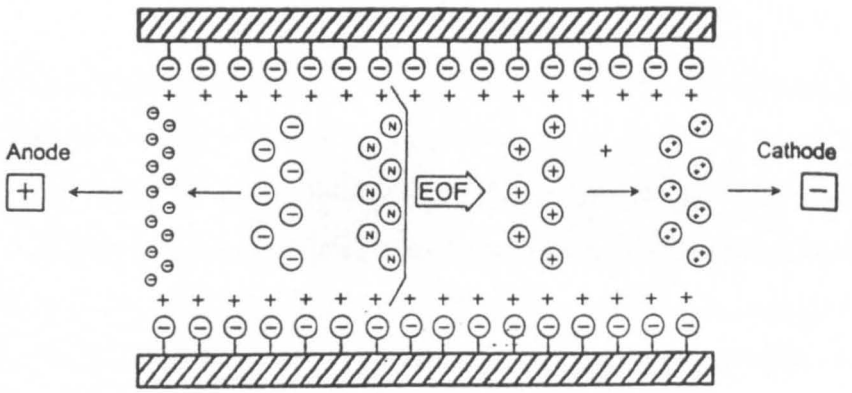

Figure 1.5. Electroosmotic flow and analyte migration in a fused silica capillary (from Foret *et al.* 1993).

The zeta potential is affected by the pH of the electrolyte solution and by its ionic strength. The higher the pH, the larger the charge of the surface and the higher the zeta potential and the EOF. In contrast, an increase in ionic strength leads to a increased balancing of the charges at the surface, and a decrease in zeta potential and in EOF.

The EOF is a feature of CE which can be modified (e.g. via the pH of the BGE) and, if necessary, suppressed, depending on the type of compounds to be separated. Use of cationic surfactants can for example reverse the EOF. Polymer-coated capillaries are commercially available and display no EOF.

Since the analytes migrate at different velocities, the signal recorded by the detector depends on this velocity. The slower the velocity of the analyte, the longer it will be detected at the detector window. Therefore, the areas of the analyte peaks are corrected for this effect by calculating the normalised area, defined as area divided by migration time.

1.3.3 Other modes of CE

Several techniques have been developed in order to adapt CE to different types of analytes. In all cases the composition of the BGE is modified, and in some cases the capillary is filled with packing material of a gel network.

1.3.3.1 Capillary Gel Electrophoresis (CGE)

CGE was introduced by Karger and co-workers in 1987 for the separation of proteins and nucleic acids (Cohen *et al.* 1987, Cohen *et al.* 1988). Two types of sieving materials can be used: a gel network polymerised inside the capillary (chemical gel, non replaceable and fragile), or solutions of linear polymer above the entanglement threshold (viscous solution, replaceable). The presence of a sieving material in the capillary adds an extra criterion for separation, which is the size of the molecule. A typical polymer used for CGE is polyacrylamide, but other synthetic polymers and derivatised polysaccharides have been reported. CGE is widely used in the field of charged biopolymers, mainly nucleic acids.

1.3.3.2 Micellar Electrokinetic Chromatography (MEKC)

MEKC was introduced by Terabe and coworkers in 1984 to allow the separation of uncharged analytes by CE. Surfactants are amphiphilic molecules containing a hydrophilic and a hydrophobic part. Above the critical micellar concentration they form spherical micelles, which are aggregates in which the hydrophobic parts are orientated towards the interior of the micelle, while the hydrophilic groups are in contact with the aqueous phase of the BGE. The most commonly-used surfactant is sodium dodecyl sulfate (SDS). Being charged, micelles show a migration behaviour under an electric field. The separation is based on the distribution of analytes between the aqueous phase and the pseudostationary micellar phase present in the BGE. The extent of interaction (solubilisation, ion-pairing) of analytes with the micelle is then reflected on their migration behaviour. Neutral species as well as charged species can thus be analysed in the same run, and differentiated according to their different hydrophobic characters.

1.3.3.3 Capillary Electrochromatography (CEC)

In CEC the capillary is packed with a chromatographic material which can retain analytes as in traditional chromatography. The main difference with MEKC is that the phase in CEC is a true stationary phase, held in the capillary by frits located at both ends. In CEC electroosmosis occurs in a similar way to CZE, therefore EOF takes place and generates a chromatographic separation driven by a flow with a flat profile. Applications of CEC are

separation of small molecules. CEC is not a very commonly used technique, due to the difficulty of column preparation and possible increased adsorption of analytes in the stationary phase due to the higher surface area in comparison to other CE modes.

1.3.3.4 Isotachophoresis (ITP)

ITP is based on the separation of ions according to their mobilities. The main difference with CZE is the use of a discontinuous BGE consisting of a leading electrolyte injected before the sample, and a terminating electrolyte injected after the sample. The mobility of the coion in the leading electrolyte is higher than that of the analyte ion of interest, and the mobility of the coion in the terminating electrolyte is smaller. Under separation conditions, to have a constant current across the capillary, the different ions present in the capillary form individual zones according to their mobility and all zones migrate at the same velocity. The local field is constant in each zone, and increases on moving across the boundary from higher to lower mobility ions. The ITP electropherogram consists of a series of steps, and not peaks as usually observed for CE techniques. All sample components succeed each other sandwiched according to their mobility between the leading electrolyte and the terminating electrolyte. The height of the step is a qualitative property which serves to identify a particular component. The time between steps is proportional to the amount of analyte present. In order to use UV detection in ITP, compounds should have significantly different absorbances.

1.3.3.5 Capillary Isoelectric Focusing (CIEF)

Unlike other CE techniques, CIEF is based on the separation of proteins according to differences in their isoelectric points (pI) rather than in their electrophoretic mobilities (Hjerten & Zhu 1985). Isoelectric focusing has been used in slab gel electrophoresis. In CIEF no gel is necessary; the capillary is filled with an electrolyte containing carrier ampholytes. The anodic end of the capillary is placed in an acidic solution, and the cathodic end is placed in a basic solution. Under an electric field, the ampholytes migrate in the capillary to the position where they have a net zero charge and generate a pH gradient. Sample analytes can be injected at either end of the capillary or can be mixed with the ampholyte and fill the capillary, in which case a lower limit of detection will be achieved. A

very large number of components must be present in the carrier ampholyte in order to allow resolution in the desired pH range. In order to reach a steady state and achieve focusing, no EOF should be present in the capillary. Under these conditions proteins migrate through the medium until they reach a region where they become neutral, and stop migrating. Zones of analytes are thus focused, and since all compounds are neutral, the current drops in the capillary. For detection the zones can be mobilised towards the detector by application of pressure or reversal of the electrolytes at the capillary ends in order to generate electrophoretic migration of the whole pH gradient.

1.3.4 Use of CE in food analysis

The use of CE in food analysis has been reviewed by Sørensen *et al.* (1999). The majority of compounds analysed in food by CE are small molecules including ions (CZE), sugars (CZE, MEKC), carboxylic acids (MEKC), vitamins (CZE, MEKC), peptides (CZE, MEKC) and proteins (CZE, CIEF, MEKC).

1.4 Capillary electrophoresis of sugars

Over the last ten years, capillary electrophoresis has been extensively explored for the development of methods in carbohydrate analysis (Suzuki & Honda 1998, El Rassi 1999). From the viewpoint of detection, a carbohydrate is not an ideal molecule. The basic building block, a sugar ring, has OH and CH groups, neither of which absorbs any light in the UV region down to 190 nm. The lack of a chromophore and fluorophore hampers direct monitoring of carbohydrates by UV/Vis absorption and fluorescence. Normally derivatisation is required to provide easily detectable molecules. Derivatising agents which add charged groups convert the carbohydrates, most of which are neutral, into ionic derivatives which can be separated by CE. In most cases the CE technique used for carbohydrates or their derivatives is CZE, since they are hydrophilic compounds.

1.4.1 Direct UV detection

A few types of carbohydrates possessing groups with higher molar absorption than simple

sugars can be directly detected. For example unsaturated acidic disaccharides released from glucosaminoglycans can be detected at 232 nm (Linhardt & Pervin 1996). Also some polysaccharides can be directly detected; examples are pectins (192 nm) (Zhong *et al.* 1997) and hyaluronic acid (185 nm) (Hayase *et al.* 1997), which contain carboxyl groups. The complexation of borate with the hydroxyl groups shifts the equilibrium towards the open-chain form, thus improving the detectability via enhancement of the proportion of chromophoric free carbonyl groups. Direct detection of the reducing -CHO terminal group in mono and oligosaccharides has been performed at 195 nm, taking advantage of borate complexation. Peaks were very broad at ambient temperature due to anomerisation, and the sensitivity was low (millimolar range) (Hoffstetter-Kuhn *et al.* 1991).

1.4.2. Indirect optical detection

This method consists in adding a chromophore or a fluorophore to the background electrolyte, whose mobility is similar to the compounds of interest. When performing the separation the analytes displace the chromophore and thus induce negative peaks. An important drawback is that very high pH background electrolytes are required to induce charge on otherwise neutral sugars. This is likely to bring about early ageing of the capillary and degradation of the analytes. Therefore, the potential of indirect detection for saccharide analysis is limited. Also, indirect detection suffers from a lack of sensitivity and the presence of system peaks.

Klockow *et al.* (1994) reported the analysis of monosaccharide composition in fruit juices with indirect UV using sorbate as buffer medium. The concentration limit of detection (LOD) was around 0.2 mM for glucose, fructose and sucrose. The performances of different absorbing BGEs were investigated by Lee & Lin (1996). Soga & Serwe (2000) reported recently the successful determination of mono- and disaccharides in food and beverages using 2,6-pyridinedicarboxylate as absorbing electrolyte, with a limit of detection of 12 to 16 mg L^{-1} (80 µM) for fructose, glucose and saccharose.

Other studies used indirect laser induced fluorescence (LIF). Garner & Yeung (1990) reported the use of coumarin (excitation at 442 nm) for analysis of monosaccharides, with

an amount LOD of 2 fmol, and mentioned that the strategy fits best for resolution of acidic sugars, which carry an intrinsic charge and thus displace more fluorophore. Richmond & Yeung (1993) analysed polysaccharides such as dextran, amylopectin, amylose and carrageenan with indirect LIF using fluorescein (excitation at 488 nm), in the picogram range in some cases.

1.4.3. Derivatisation followed by optical detection

Precolumn derivatisation is representative of the methods of conversion of carbohydrates to species with both a chromophore (or fluorophore) and a charge. Different labels have been proposed based on three main reaction types: reductive amination with a primary amine, reaction with CBQCA (3-(4-carboxybenzoyl)-2-quinolinecarboxylaldehyde) (Liu *et al.* 1991) and condensation with PMP (1-phenyl-3-methyl-5-pyrazolone) (Honda *et al.* 1989). The different tags have been recently reviewed by Hase (1996). Corresponding schemes for the reactions are shown in Fig. 1.6.

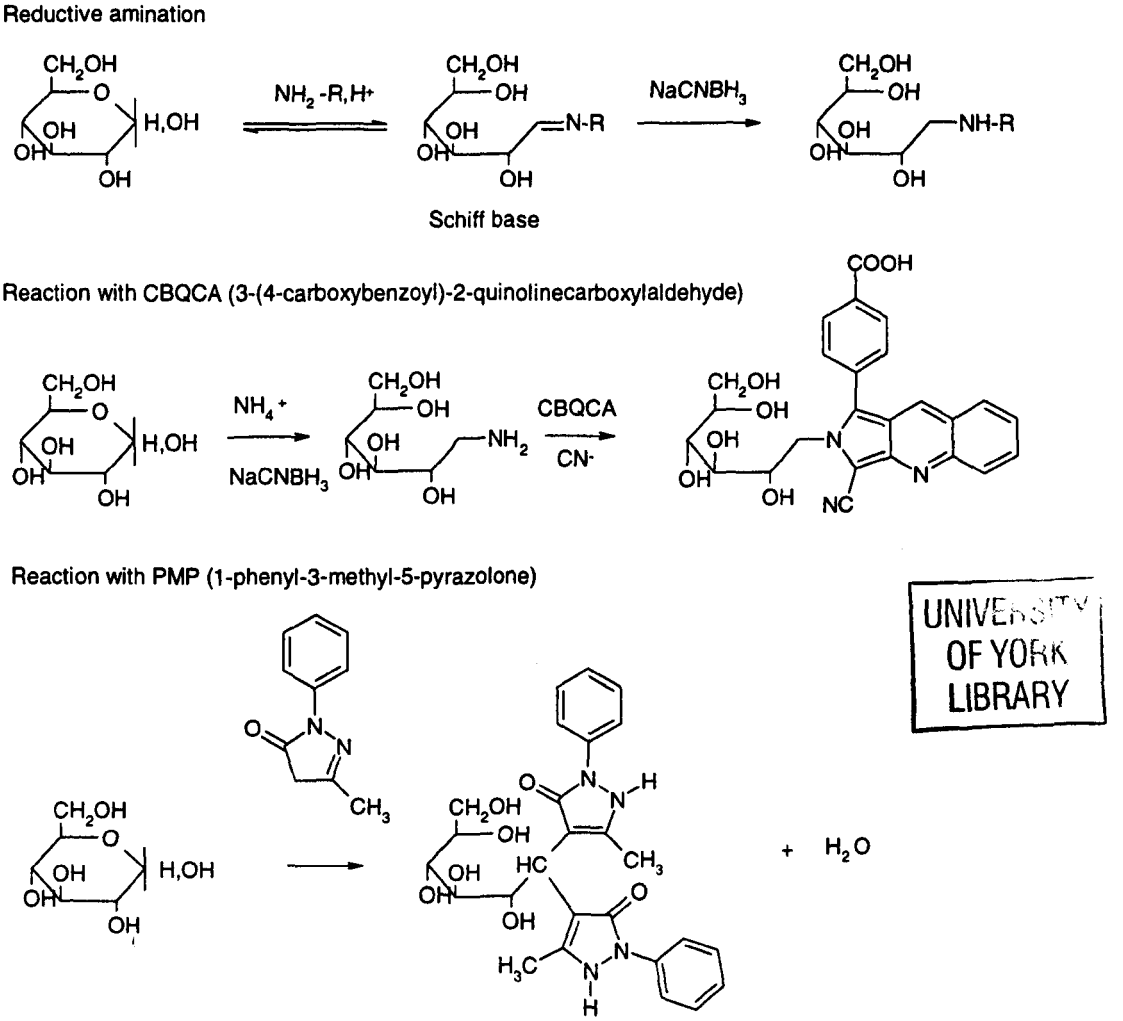

Figure 1.6. Reaction schemes for derivatisation reactions of carbohydrates.

The most widely used reaction scheme is based on the reductive amination of the carbonyl group with a primary amine. In most cases the investigated sugars are mono- or oligosaccharides, a particular field of interest being the complex oligosaccharides cleaved from glycoproteins (Dwek 1996).

2-aminopyridine was introduced as the first label for carbohydrates in CE by Honda *et al.* (1992). Aminobenzoic acid derivatives (Grill *et al.* 1993), amino naphthalene derivatives (Chiesa & Horvàth 1993), 2-aminoacridone (AMAC) (Greenaway *et al.* 1994), 8-aminopyrene 1,3,6-trisulfonic acid (APTS) (Evangelista *et al.* 1995) have been used to label mono-, oligo-, and polysaccharides (Roberts *et al.* 1998). APTS-labelled oligosaccharides could be collected and analysed by matrix-assisted laser desorption / ionisation time-of-flight

mass spectrometry (MALDI-TOF MS) (Suzuki *et al.* 1997).

In most cases these reactions work only for reducing sugars. An example of sugar for which the method is not applicable is sucrose, since it does not have a reducing end. An exception is reductive amination using the labels 4-aminobenzonitrile and 4-aminobenzoic acid, which were reported to be introduced to ketoses (Grill *et al* 1993, Schwaiger *et al* 1994).

Some derivatising agents carry hydrophobic groups and allow application of MEKC to the corresponding derivatives. For example, PMP derivatives may be separated by differences in their solubility in SDS micelles. The PMP derivatives more easily solubilised (of smaller degree of polymerisation) are more strongly retarded. AMAC is another hydrophobic label. The use of MEKC with a taurodeoxycholate/borate buffer improves the resolution between AMAC-derivatives of sugars and prevents interference from the label, since it is more strongly incorporated in the micelle than the labelled analyte.

Amongst the commercially-available detection methods, laser-induced fluorescence (LIF) is the most sensitive for CE, with reported limits of detection lower than 10^{-13} M. This compares with 10^{-5} to 10^{-6} M for direct and indirect detection (Swinney & Bornhop 2000). The use of fluorophores to derivatise carbohydrates is therefore the most appropriate methodology.

1.5. Size exclusion chromatography with multiple angle laser-light scattering and refractive index detection (SEC-MALLS-RI)

As with all polymers, the physico-chemical properties of polysaccharides depend on their molecular weight. Natural polymers such as carrageenans consist of molecules of different lengths, and are therefore polydisperse. The parameters describing this distribution are number-average molecular weight (M_n), weight average molecular weight (M_w) and polydispersity (I_p), defined below for a polymer consisting of n macromolecules of individual molecular weights M_i and concentrations c_i.

$$M_n = \frac{\sum n_i M_i}{\sum n_i}$$

$$M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i}$$

$$I_p = \frac{M_w}{M_n}$$

One of the techniques used to determine these parameters is size exclusion chromatography, also called gel permeation chromatography (GPC), in which separation of compounds is based on their size. The stationary phase consists of a gel of defined pore size. The volume of the column can be divided into three parts. V_g is the volume occupied by the gel itself, which is unaccessible to solutes. V_i is the internal pore volume. V_o is the total exclusion volume, or dead volume, accessible to all molecules including the largest ones which cannot enter the pores. The smaller the molecule, the more accessible is the volume inside the pores of the sieving material, and the longer the migration time. The retention volume V_R of a solute will therefore lie between V_o and V_o+V_i.

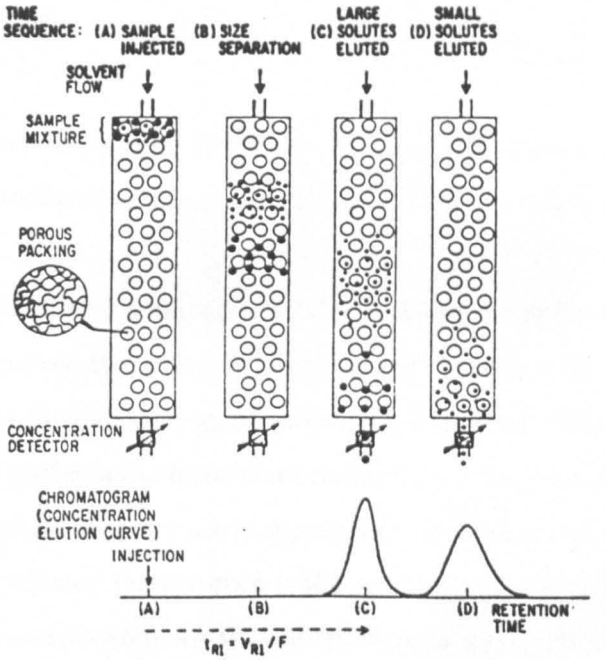

Figure 1.7. Principle of SEC (from Yau *et al.* 1979).

When fractions of narrow molecular weight range are available, the column can be calibrated using these fractions as external standards. In the case of polysaccharides, standards consist normally of dextran fractions. However, since polysaccharide shapes differ from one type to the other, this determination gives a value of the molecular weight in equivalent dextran molecular weight (Harding *et al.* 1991).

Multi-angle laser light scattering is a detection method based on the interaction of polymer with light according to its shape and size. Light scattering is an absolute method for determining the molecular parameters described above. Thus, in SEC-MALLS-RI, SEC realises size-based separation of the molecules of different sizes present in a polydisperse sample, while refractive index measures the mass concentration and MALLS detection provides the molecular weight of the solute. This combination allows the characterisation of polymers without the use of external standards.

1.6 Aims of the thesis

The main aim of this thesis is to investigate the use of capillary electrophoresis for identification and quantitation of carrageenans in raw materials and food products.

The different sub-types differ by the number of their sulfate substituents, therefore CZE is the best mode to separate them. As far as detection is concerned, direct and indirect detection were not considered to be sensitive enough. Moreover, indirect detection is not selective and would probably suffer from interferences due to components of the matrix. The derivatisation approach seems particularly appropriate, because the reaction is specific for aldehydes, and laser induced fluorescence is the most sensitive detection mode for CE. Using derivatisation with a fluorophore, we will therefore develop a capillary electrophoresis method to separate carrageenans standards and use this method to investigate the reaction conditions in order to optimise the derivatisation yield. Subsequently, the protocol will be applied and implemented for the analysis of carrageenans in representative food samples.

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Chapter 2: Development of a CE method to separate carrageenans

2.1 Introduction

2.1.1 Carrageenan structure and conformation in solution

X-ray diffraction studies have shown that both *iota* and *kappa* carrageenans have a double helical conformation in the solid state (Millane *et al.* 1988). Structures derived from fibre diffraction for these two biopolymers are shown in Figure 2.1.

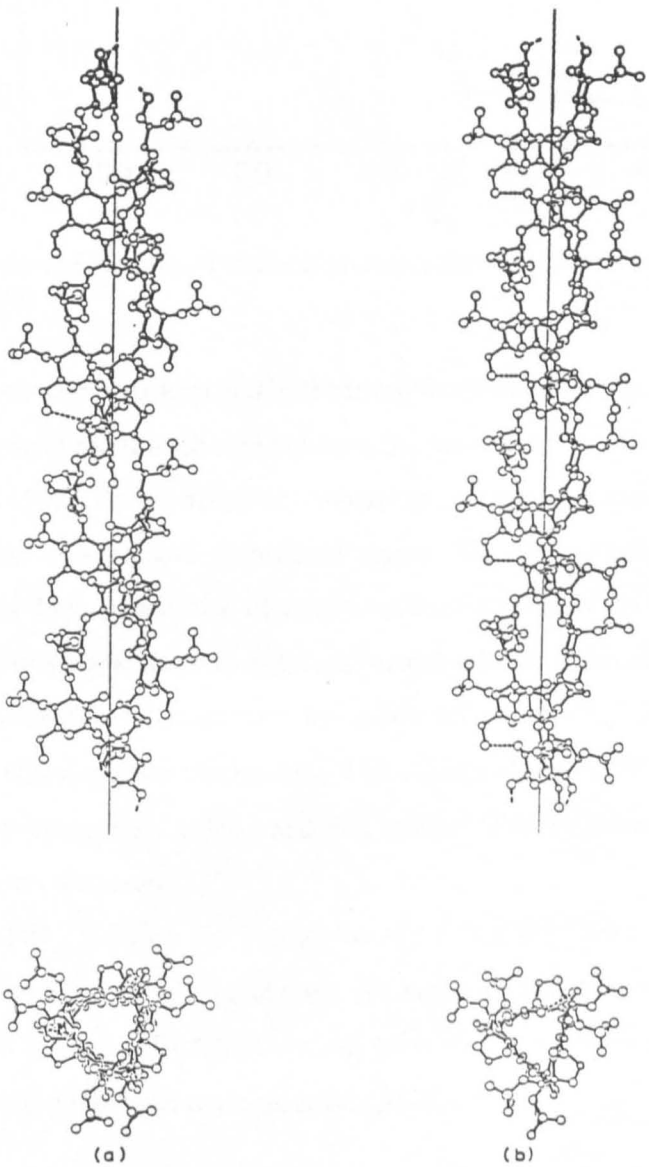

Figure 2.1. Structures of (a) the double helix structure for *kappa* carrageenan and (b) *iota* carrageenan, from Millane *et al* 1988.

In solution these carrageenans undergo conformational transitions depending on the chemical environment and the temperature. Examples of the order-disorder transition monitored by optical rotation are presented in Figure 2.2 for *iota* and *kappa*.

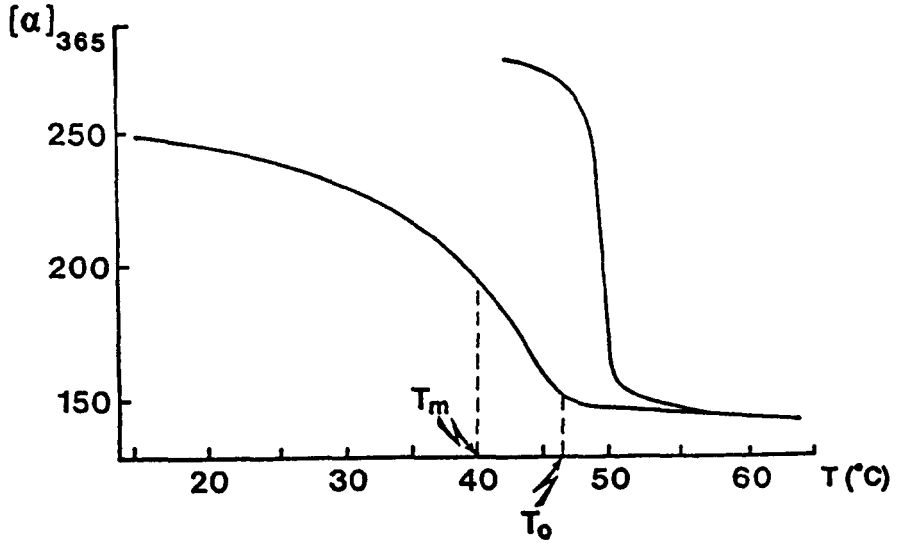

Figure 2.2. Variation of the optical rotation of a carrageenan solution with the temperature (from Piculell 1995)

Conformational change is characterised by the transition midpoint temperature, T_m, at which half of the total optical rotation change between the disordered and the ordered states has occurred (Piculell 1995). At this midpoint temperature, the solution contains equal numbers of residues in the ordered and disordered states. The helix onset temperature, T_o, corresponds to the first appearance of conformational change when the temperature is decreased. Ordering is a precursor to aggregation and gelation. *Lambda* carrageenan does not undergo a conformational transition, and is non gelling. Gelling carrageenans, and in particular kappa, show specific interactions with cations (Norton 1990). Cations can be classified into two categories, gelling and non gelling. The increase of stability of the ordered state follows the series:

$Rb^+ > Cs^+ > K^+ > NH_4^+ > Ca^{2+} > Sr^{2+} > Mg^{2+} >> Na^+ > N(CH_3)_4^+ >> Li^+$

Anions generally have less effect than cations. An exception is iodide, which in the case of *kappa* carrageenan favours helix formation and prevents aggregation, allowing studies on the pure helical state (Grasdalen and Smidsrød 1981).

The physical properties of carrageenans in solution have been extensively studied in different research groups using various techniques including light scattering (Vanneste *et al.* 1994),

infrared spectroscopy (Norton *et al.* 1983a), stopped flow polarimetry (Norton *et al.* 1983b, Norton *et al.* 1983c), NMR (Nerdal *et al.* 1993), cryo transmission microscopy (Borgström *et al.* 1996), gel permeation chromatography coupled to multi-angle laser light scattering (GPC-MALLS) (Viebke *et al.* 1995) and field flow fractionation coupled to multi-angle light scattering (FFF-MALLS) (Wittgren *et al.* 1998). In order to model the behaviour of carrageenans and its dependence on salt type and concentration, Piculell and his group have extended Manning's polyelectrolyte theory (Manning 1978). Their Poisson-Boltzmann cell model (PBCM) assumes that the polyelectrolyte is an infinitely long charged rod of finite radius included in a cylindrical cell, whose diameter is a function of the biopolymer concentration and the proportions in the helix state. They studied successively the effects of changes in polysaccharide concentration (Nilsson *et al.* 1989a), salt concentration (Nilsson and Piculell 1989b), solvent (Nilsson and Piculell 1990) and the specificity of the binding site of the counterion (Nilsson and Piculell 1991).

There has been some controversy about the nature of the carrageenan helix, in particular, whether it is a single or a double helix. Goodall *et al.* have studied the kinetics of the salt-induced ordering of carrageenans by stopped-flow polarimetry (Goodall & Norton 1987). For both *iota* and *kappa* carrageenans, an order of reaction of 2 was found for the conformational ordering reaction, and an order of reaction of 1 was found for the reverse reaction. These results support double helix formation. The same conclusion was reached by Piculell's group when modelling the transition (Nilsson *et al.* 1989a) and by Hjerde *et al.* when investigating the rate of acid degradation of carrageenans in the ordered and disordered states, via measurement of the specific viscosity and determination of the number of reducing end groups (Hjerde *et al.* 1996). Viebke *et al.* performed sonication on carrageenans to obtain samples of different molecular weights, and investigated them with GPC-MALLS (Viebke *et al.* 1995). Selection of electrolyte composition was used to control the desired state (helix or coil). All groups concluded that the results were best described in terms of a double-stranded structure. Viebke, Piculell and Nilsson have also recently shown that gel network formation occurs at a suprahelical level (Viebke *et al.* 1994), as shown in Figure 2.3.

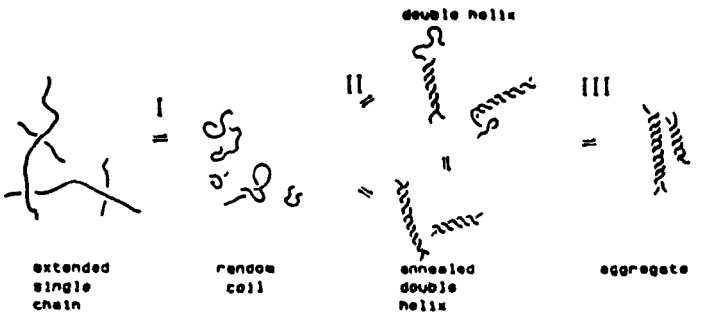

Figure 2.3. Ordering and gel formation (from Goodall & Norton 1987).

2.1.2 CE of polysaccharides

There have only been a small number of publications which have dealt with the analysis of native polysaccharides in CE (Zhong *et al.* 1997, Roberts *et al.* 1998, Stefansson & Novotny 1994a, Sudor & Novotny 1997, Stefansson 1999a, Stefansson 1999b). Samples of intact polysaccharide can be both polymolecular and polydisperse, and the differences in mobility between large molecules (DP >100) of different degree of polymerisation are too small to allow resolution of individual polymer chain lengths in free solution CE, normally called capillary zone electrophoresis (CZE). Most studies concentrate on polysaccharide hydrolysates obtained by enzymatic (Liu *et al.* 1992), acidic (Stefansson & Novotny 1994b) or ultrasonic treatment (Hong *et al.* 1998). To improve resolution in CZE, several strategies have been described in the literature; these include modifications of the label as well as of the background electrolyte. Other reports have used capillary gel electrophoresis (CGE) with either sieving or gel buffers in order to improve separation efficiency. In all cases the sugars were derivatised with the labels CBQCA, ANTS (8-aminopyrene 1,3,6-trisulfonic acid), or APTS to make them suitable for detection in CE.

i) Capillary zone electrophoresis

The mobility of a polyelectrolyte is independent of its size because it is proportional to the ratio of charge to frictional coefficient and both parameters scale in the same way with DP.

This holds for a free-draining coil, the conformational model normally used to describe hydrodynamic properties of polyelectrolytes such as DNA. One way to improve separation consists in derivatisation with a chromophore of suitable size and charge. This approach of derivatisation was developed recently in the case of DNA and is called End-Labeled Free-Solution Electrophoresis (ELFSE) (Mayer *et al.* 1994). Sudor and Novotny applied this strategy to carbohydrates, using ANTS (z = -3) and aminoquinoline (slightly positively charged at the investigated pH) to derivatise low molecular weight carrageenans (Sudor & Novotny 1995). The effect of the fluorophore was always stronger for the smaller oligosaccharides and decreased with increasing DP. They verified the ELFSE theory, and showed that changing the label size or charge influenced dramatically the separation and in particular the migration order. Further investigations have been performed on heparins (average M_w 15000 Da) with a synthesised label, maltoheptaosyl 1,5-diamino naphthalene (Sudor & Novotny 1997). One of the two amino functions carried by this label reacts with the carbohydrate of interest, the other is linked to an oligosaccharide; this modifies substantially the derivative's frictional properties. The patterns resulting from the separation of oligomers could be successfully interpreted and predicted with the aid of this theory. A limitation arose when the sample presented not only polydispersity in size, but also in shape. In another study Stefansson and Novotny (1994a) investigated and modelled the interaction between additives and charged and neutral polysaccharides initially converted to their CBQCA derivatives. The mobility of heparins was studied in presence of charged additives such as TRIS, ethylene diamine and spermine, whereas that of modified celluloses was studied in the presence of SDS micelles. A Langmuir type adsorption profile for the dependence of mobility on concentration was observed.

Recently, intact polysaccharides such as pectins (Zhong *et al.* 1997) and carrageenans (Roberts *et al.* 1998) have been investigated in CZE. In the case of pectins, detected in their native state using UV absorbance, the authors demonstrate the possibility of quantitation of native pectins as well as the determination of their degree of esterification from migration times in phosphate buffer. Derivatisation of carrageenans with APTS allowed their detection with LIF and separation of *iota* and *kappa* carrageenans in citrate buffer. Further studies showed that some low DP carrageenan species could also be separated and characterised using CE-mass spectrometry (Hau & Roberts 1999); here an ammonium acetate buffer was used due to its inherent volatility.

ii) Capillary gel electrophoresis

Novotny's group have conducted extensive investigations of the behaviour of polysaccharides in CGE. They showed that oligomers obtained by degradation of poly(galacturonic acid) and labelling with CBQCA could be separated up to the 80-mer using polyacrylamide gel (Liu *et al.* 1992a). The use of gels with high polyacrylamide concentration and low mesh size allowed the resolution of oligosaccharides derived from hyaluronic acid up to DP 30 (Liu *et al.* 1992b). Very high concentrations were required compared to the usual gels for DNA analysis. These gels are not easy to synthesise inside a capillary. An alternative to these is the use of sieving media consisting of a polymer added to the running buffer. Studies using entangled matrices of linear polyacrylamide were reported for the separation of oligo- and polysaccharides. In one particular study Sudor and Novotny investigated the separation of polydextran standards derivatised with CBQCA (Sudor & Novotny 1993). The macromolecules migrated through the network by reptation, and exhibited an unusual feature of two peaks per analyte. The authors performed CE under pulsed field conditions to overcome this phenomenon, and successfully separated the standards according to their size, including a sample of molecular mass as large as 2×10^6 Da.

When investigating the migration of hyaluronate oligomers in CGE, positively charged additives including protonated aminodextran were used also in a sieving medium of linear polyacrylamide (Stefansson *et al.* 1997). Hyaluronic acid was enzymatically degraded and derivatised with APTS. Oligomers up to a molecular mass of 7×10^4 Da could be resolved individually. The degree of complexation between aminodextran and hyaluronic acid was controlled by adjusting the ionic strength of the medium. Hyaluronic acid was also investigated in its intact state in CGE (Hong *et al.* 1998). Individual oligomers up to DP 190 (M_w 80 kDa) could be detected. The conditions were similar to those described in the previous paper dealing with degraded hyaluronic acid and aminodextrans. Each oligomer showed two peaks, a main peak and a satellite peak whose height depended on the pH. The use of urea (denaturing agent) suppressed appearance of the satellite peaks, which were consequently attributed to different conformations of the polymer. CE-LIF and SEC coupled to low angle laser light scattering (LALLS) were used simultaneously to determine the pattern after enzymatic or ultrasonic degradation of the samples. SEC-LALLS of the same

sample gave much higher molecular weights (256 kDa). Two possible explanations were proposed for the differences between the CE and SEC results. One was that the fluorescence of the derivatised polymers could decrease with increasing molecular weight; the other was that higher M_w chains could be less reactive than low M_w species for the labelling reaction, due to decreased accessibility of the reducing terminus. This suggests a problem arising from derivatisation of high DP molecules for CE, leading to potential underestimates of M_w. This problem will be reviewed and discussed in more detail in the case of carrageenans in Chapter 3.

Stefansson showed that negatively charged polysaccharides form aggregates under certain conditions in polymer sieving media, resulting in additional features (shoulders or sharp peaks) which migrate at velocities lower than expected (Stefansson 1999a, Stefansson 1999b). This phenomenon is called segregation and was first described for DNA (Mitnik *et al.* 1995). Above a certain field strength the polyelectrolyte chains are polarised, and local instabilities resulting from this polarisation lead to aggregation and phase separation. The electric field threshold for segregation increased when using a higher ionic strength or a cationic additive such as aminodextran, and this was accounted for by extra screening of the charges present on the polymer. A lower value of the electric field was sufficient to induce segregation when using higher concentrations or higher molecular weights for either the analyte (hyaluronic acid) and the sieving medium (linear polyacrylamide) (Stefansson 1999a). In a recent publication Stefansson investigated alginic acid (Stefansson 1999b), which also presented aggregation phenomena. The study concentrated on the structure of this polysaccharide. Alginic acid is built up of two major monomers, guluronic and mannuronic acid. The calcium ion is well known for its properties of complexation of alginic acid according to the egg-box model (Grant *et al.* 1973). It is a better complexing agent for guluronic acid than for mannuronic acid, and this was reflected in the migration behaviour of alginic samples containing varying proportions of guluronic acid. Since the average guluronate block length was known, the mobility could be correlated with the length of the guluronic acid sequence. This shows that CE has the potential to provide information on the actual structure of alginic acid.

2.1.3 Aim of this chapter

As seen in Figure 1.1, carrageenan monomers differ from one another in the number of their sulfate residues and for *lambda*, the absence of a 3,6-anhydro bridge in the 1,4-linked galactose ring in the disaccharide residue. Our aim is to find electrophoretic conditions to separate and thus identify them without preliminary depolymerisation, in contrast to the techniques of GC and HPLC discussed in Chapter 1. Since the principle of separation in CE is velocity differences in the presence of an electric field, CE should be a convenient method to separate these biopolymers which have charges -1, -2, -3 per disaccharide residue for *kappa*, *iota*, and *lambda* respectively. It is important to note that individual DP components of a given carrageenan cannot be resolved in CZE because their molecular weights are too high. Sieving media such as the ones used in CGE, which separate more according to size or shape than charge, could possibly be used to separate these components. Our choice of capillary zone electrophoresis, building on earlier work done in the University of York (Roberts *et al.* 1998), is based on the differences in charge per residue unit.

2.2 Experimental

2.2.1 Materials

Chemicals

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). For fluorescent conjugation a trisodium 8-aminopyrene-1,3,6-trisulfonate (APTS) label was obtained from Beckman and sodium cyanoborohydride from Aldrich. Ammonium acetate, tetrahydrofuran and glycerol (87% v/v in water) were purchased from Merck, Darmstadt, Germany. Stock solutions of ammonium acetate (0.5 M and 1 M) were prepared and further diluted in high purity water (Millipore MilliQ) to the desired concentration. Sodium azide (0.015 % in ammonium acetate stock solution, 0.0003 % in carrageenan solution) was used as an anti-microbial agent. Acetic acid (pure or 20 % v/v in water) and ammonium hydroxide (2 or 0.5% v/v in water) were used to adjust the pH.

2.2.2 Derivatisation protocol

As pointed out in the introductory chapter, a commonly used strategy consists of derivatising carbohydrates with a fluorophore and then detecting them by LIF. A derivatisation protocol adapted from the Beckman technical note was used. A generalised derivatisation scheme as well as the formula of APTS are displayed in Figure 2.4.

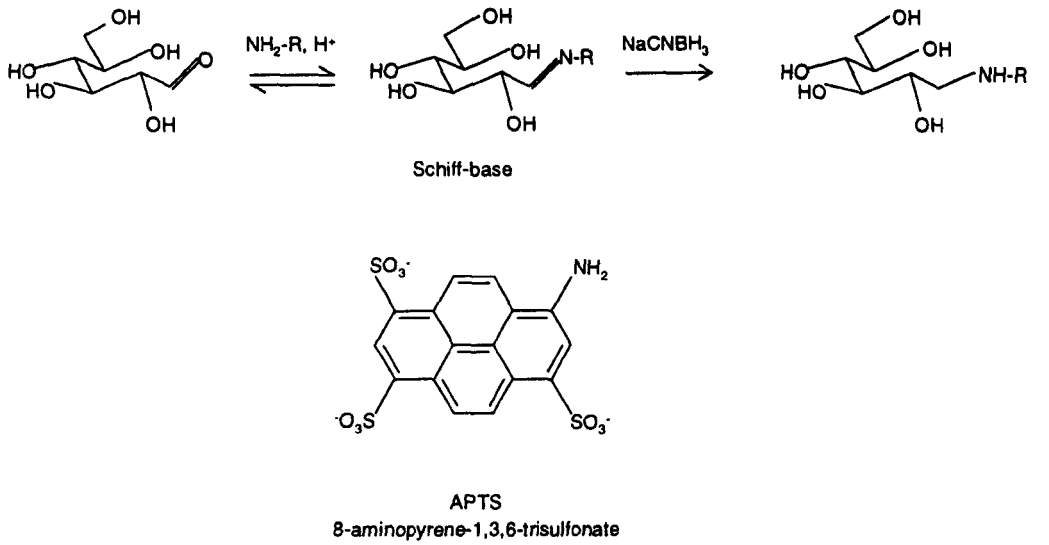

Figure 2.4. Derivatisation scheme and formula of APTS

For the preparation of carrageenan solutions, the sample was slowly added to water containing 3 mg L^{-1} sodium azide under stirring at room temperature. The solution was then stirred for 30 min at 75 °C. The concentrations of the stock solutions were 6.5 mg mL^{-1} for *kappa* and 5.2 mg mL^{-1} for *iota* and *lambda* respectively. For derivatisation, 8 µL of these solutions were dispensed into an Eppendorf tube, followed by the addition of 2 µL 75 % v/v acetic acid, 2 µL APTS reagent (0.2 M in 15 % v/v acetic acid) and 2 µL reducing agent (1 M sodium cyanoborohydride freshly dissolved in tetrahydrofuran). The reaction mixture was heated at 55 °C overnight, then quenched through addition of 100 µL HPLC grade water. 55 °C is above the helix-coil transition temperature for both *iota* and *kappa* carrageenans (Norton 1990). When submitted to derivatisation, the polysaccharides are thus in the coil state, which is believed to facilitate the reaction.

2.2.3 Filtration step

This procedure has two advantages : firstly, removing the bulk of the unreacted label and its degradation products; secondly, removing ions and small molecules such as cyanoborohydride, thus desalting the reaction medium whilst carrageenans remain above the filter (Roberts *et al.* 1998). Micro-concentrators with a 30 kDa molecular weight cut-off filter were purchased from Amicon (Gillingham, UK). This procedure is shown in Figure 2.5. This filtration procedure also presents the advantage of concentrating and desalting the

sample in a single step. Half the derivatised amount was added to a microcentrifuge vial. The level was filled up to 300 µL with HPLC grade water. After vortexing, the samples were centrifuged for 15 min at 6,000 rpm on a microcentrifuge. This step was repeated twice more. The final volume was adjusted with water to 20 µL. The sample was then further diluted in HPLC grade water for injection.

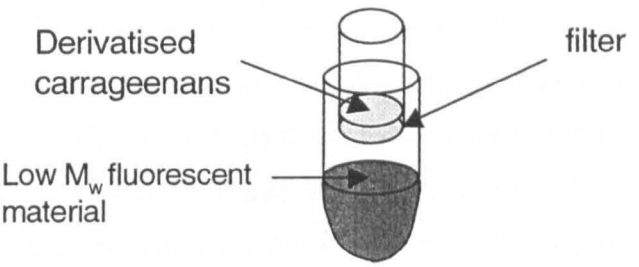

Figure 2.5. Centrifugation step.

2.2.4 Experimental conditions : instrumental parameters

All experiments were performed on a Beckman P/ACE 5000 CE system equipped with a LIF detector connected to an Ar-ion laser (488 nm ex, 520 nm em). The data collection rate was 5 Hz and rise time 1 s. The rise time represents the response time for the detector electronics to a rapidly varying signal. Polyvinyl alcohol coated capillaries with low electro-osmotic flow (EOF) were used throughout the study; these were supplied by Beckman, and are described as "eCAP™ N-CHO coated capillaries". Such capillaries often lead to improvements in reproducibility due to suppression of EOF and removal of analyte-wall interactions. The capillaries had an internal diameter of 50 µm, and were cut to 67 cm length. The length to the detector was 60 cm. Except in the study investigating the effect of temperature, the capillary was thermostatted at 25.0 °C. The background electrolyte (BGE) used for all investigations concerning the effects of variation of instrumental parameters was 25 mM ammonium acetate at its natural pH, 6.8. The capillary

was rinsed for 2 min with water, then for 2 min with the BGE between runs. A pre-separation process was performed before sample injection for 2 min using the same voltage as the separation to condition the capillary. Before and after each sample injection water was injected electrokinetically for 1 s at -1 kV (field strength 1.5×10^3 V m^{-1}). This procedure cleaned the capillary inlet end, avoided contamination and enhanced electrokinetic injection.

2.2.4.1 Injection mode

Both electrokinetic injection (EKI) and pressure injection (PI) are available on the P/ACE 5500 instrument. Since carrageenans are charged solutes, both modes should be applicable to these polysaccharides. The two injection modes were investigated using a sample mixture containing equal masses of both *iota* and *kappa* at concentration 0.16 mg mL^{-1}. For EKI the sample was injected for 3 s at -10 kV (field strength 1.5×10^4 V m^{-1}). For PI the sample was injected for 10 s at a pressure of 0.5 psi. The same analysis was repeated 10 times with each injection mode. Different sample vials (containing the same solution) were used for EKI and PI. Separation was performed using a voltage of 30 kV (field strength 4.5×10^4 V m^{-1}).

2.2.4.2 Injection time

The effect of variation of injection time was investigated using EKI (1.5×10^4 V m^{-1}) applied for 2, 3, 4, 6, 8, 10 s on a mixture of derivatised *iota* and *kappa* carrageenans of concentration 0.77 mg mL^{-1} for both *iota* and *kappa*. Separation was performed using a voltage of -30 kV.

2.2.4.3 Field strength

The field strength was varied in the range from 0.75 to 4.5×10^4 V m^{-1}. The investigated sample was a mixture of the three carrageenans at concentrations of 0.06 mg mL^{-1} for both *iota* and *lambda* and 0.076 mg mL^{-1} for *kappa*. The sample was injected for 3 s electrokinetically at -10 kV. The experiment was performed in a new capillary, in which migration times were particularly stable.

2.2.4.4 Temperature

Different capillary thermostatting temperatures (17.5, 25, 35, 42.5, 50.0 °C) were investigated using a 25 mM ammonium acetate BGE. The investigated sample was a mixture of concentration 0.77 mg mL^{-1} for both *iota* and *kappa*. Separation was performed using a voltage of -30 kV. The sample was injected for 3 s electrokinetically at -10 kV.

2.2.5 Experimental conditions : background electrolyte modifications

As far as instrument, method and capillary are concerned, all experiments studying the BGE were performed using the conditions described in Section 2.2.4. The capillary was thermostatted at 25 °C. The capillary was rinsed for 2 min with water, then for 2 min with the BGE between runs.

2.2.5.1 Ionic strength

Different ionic strengths for ammonium acetate were investigated. Solutions were prepared from a stock solution of 500 mM ammonium acetate in MilliQ water. Glycerol (8.7% v/v) was added to all solutions in order to minimise the current. For all concentrations the measured pH values were approximately 6.8. Separation was performed using a voltage of -30 kV. Except in the case of the highest concentration of ammonium acetate (400 mM) the resulting current was below the maximum value allowed for the system (250 µA). For 400 mM ammonium acetate the separation was performed under constant current conditions (250 µA) rather than under constant voltage conditions, corresponding to 22 kV instead of the normal voltage of 30 kV. A mixture of *iota* (0.08 mg mL^{-1}) and *kappa* (0.11 mg mL^{-1}) carrageenans was analysed in triplicate with each buffer solution. The sample was injected for 3 s electrokinetically at -10 kV.

2.2.5.2 pH of the BGE

The background electrolyte used was 25 mM ammonium acetate, with pH value adjusted through the addition of either pure or aqueous acetic acid (20% v/v) for acidic pH values,

or of ammonium hydroxide solution (2% v/v) for basic pH values. In the case of the pH 9 solution, the concentration of total ammonium species ($NH_3 + NH_4^+$) was 42 mM. Buffers with total concentrations of 25 mM in ammonium (pH 9) or acetate (pH 5) were also prepared by mixing 25 mM ammonium acetate with a 25 mM solution of either acetic acid or ammonium hydroxide solution. The same BGE compositions were initially investigated with glycerol as an anti-convective agent, as described previously in this section for ionic strength investigations. Since the current values were not particularly high, glycerol was not used when repeating the pH investigations. A mixture of *iota* (0.03 mg mL^{-1}) and *kappa* (0.04 mg mL^{-1}) carrageenans was analysed in triplicate with each buffer solution. The sample was injected electrokinetically for 3 s at -10 kV. Separation was performed using a voltage of -30 kV.

2.2.5.3 Addition of tetrabutylammonium ion

Appropriate volumes of ammonium acetate and tetrabutylammonium (TBA) hydroxide stock solutions were mixed to produce BGE solutions of a constant concentration of 25 mM in ammonium acetate and the following increasing concentrations of TBA : 0.05, 0.1, 0.5, 1, and 5 mM. The pH of these solutions was measured and increased with the concentration of TBA hydroxide. In the case of the highest concentration 5 mM, the pH value was 8.8. In all cases the current was 25 µA and was stable over each run. The sample was a mixture of *iota* (0.08 mg mL^{-1}) and *kappa* carrageenan (0.11 mg mL^{-1}) and was injected for 3 s electrokinetically at -10 kV. Separation was performed using a voltage of -30 kV.

2.3 Results and discussion

2.3.1 Separation and migration order of the carrageenans

Under the acidic BGE conditions used by Roberts *et al.*, the electro-osmotic flow mobility was very low (less than 8.5×10^{-10} m^2 V^{-1} s^{-1}) and had little influence on analyte migration time, t_m. Due to the inherent link between buffer pH and EOF mobility, method development strategies for an uncoated capillary would have to take into account large variations in migration time and the possibility of reversal of migration direction. Coated capillaries showing low EOF over a wider range of pH were therefore more appropriate to investigate the effect of pH on the separation of carrageenan sub-types. A coated capillary also has the advantage of making resulting methods more robust across a range of experimental conditions. All of the subsequent studies reported here refer to separations performed in coated capillaries.

If the electrophoretic mobilities of carrageenans were based on their sulfate hemiester content, their migration times should follow the order *lambda* < *iota* < *kappa*; however, in previous work lambda was shown to have a migration time intermediate between *kappa* and *iota* (Roberts *et al.* 1998). Using Manning's theory, which takes into account counterion condensation, the resulting charges for *lambda* and *iota* were found to be almost exactly the same (Manning 1978). In the present study, derivatives of *lambda* carrageenan gave a profile consisting of a main peak appearing before that of *iota*, and a small broad shoulder resembling the signal obtained previously in citric acid. This peak profile was observed in both citric acid and ammonium acetate BGEs, and the relative heights of the two parts varied with the reaction batch. As can be seen in Figure 2.6, the observed mobility order in ammonium acetate BGE is in accordance with the expected *lambda* > *iota* > *kappa*. Different batches of carrageenan standards from Sigma were used in the two studies. *Lambda* is generally not pure when extracted from seaweeds, and the presence of another species in different proportion could explain the variations observed between the two studies.

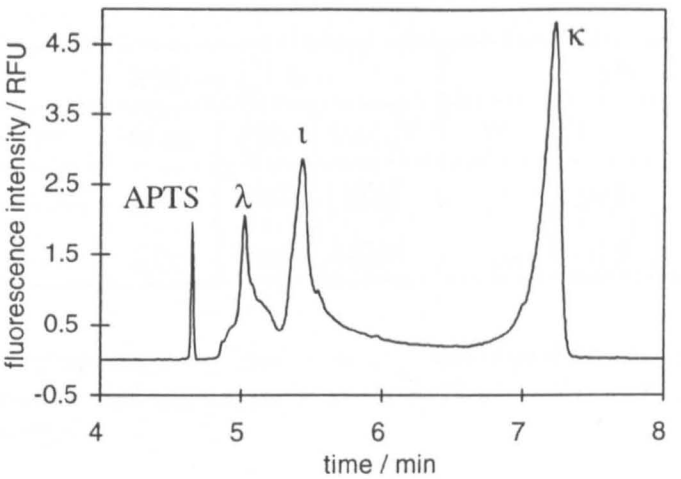

Figure 2.6. Carrageenan separation in 25 mM ammonium acetate, pH 6.8. Field strength: 4.5×10^4 V m^{-1}; temperature: 25 °C ; sample : mixture of *iota*, *lambda* and *kappa* carrageenans, concentration 0.06 mg mL^{-1} for each; injection: EKI: 3 s at 1.5×10^4 V m^{-1}.

2.3.2. Injection mode

The electrokinetic mode injects charged analytes in amounts proportional to their mobilities, μ, since the driving force is the electric field applied between the sample vial and the outlet BGE vial. In contrast, pressure injection injects a fixed sample volume.

A series of experiments were performed using a 25 mM ammonium acetate solution as the background electrolyte (pH = 6.8), this represents the same conditions as Fig. 2.6. The same analysis was repeated 10 times with each injection mode. Different samples were used for EKI and PI. The relative standard deviation (RSD) is reported in Table 2.1 for migration time, t_m, and normalised peak area, A_n, (peak area / migration time) in the case of *iota* and *kappa* carrageenans. Calculations were made with and without an internal standard (IS), with APTS chosen to act as a standard, to determine the utility of absolute versus relative determinations. In the case of measurements with the IS, the RSD is on the ratio t_m (analyte) / t_m (IS) or A_n (analyte) / A_n (IS).

Carrageenan	RSD on t_m / %				RSD on A_n / %			
	PI	PI-IS	EKI	EKI-IS	PI	PI-IS	EKI	EKI-IS
iota	0.04	0.03	0.03	0.03	7.3	6.5	5.3	2.1
kappa	0.07	0.06	0.04	0.04	7.0	6.9	7.1	1.3

Table 2.1. RSD values for normalised area (A_n) and migration time (t_m) in the case of pressure injection (PI) and electrokinetic injection (EKI) without and with APTS as an internal standard (IS).

From the area of the peaks in each mode, one can deduce the increase in sensitivity due to EKI. The signal obtained per second of injection in EKI was 106 times higher for APTS, 68 times for *iota* and 58 times for *kappa*. This trend is in accordance with the expected scaling between A_n and μ, with μ proportional to t_m^{-1}.

The repeatability of the migration time, measured at the peak maximum, is seen to be very high, with RSDs in all cases better than 0.1 %. The use of APTS as an IS does not significantly improve the RSDs for migration time.

When using EKI, the injected amount showed a systematic decrease with the number of injections, whereas with PI variations in peak area with injection number were random, as shown in Fig. 2.7 for *kappa*. A possible explanation of this is sample depletion, which is always a possibility when carrying out EKI using small volumes of sample. An estimate for our case of 20 µL and figures reported for the enhancement of EKI signal relative to the PI signal is 10 % depletion for 10 injections, which is of the same order of magnitude as observed. An alternative explanation might be that despite the injection of water before and after the sample, the sample vials could have been contaminated with salts from the BGE, which would induce an increase in ionic strength. This would result in an increased conductivity, and therefore, a systematic decrease in injected amount with injection repetition. Whilst there is little difference between RSDs in normalised peak areas when comparing PI with and without the IS, use of the IS in EKI allowed us to improve peak area RSDs for carrageenans from 5-7 % to 1-2 %. These results suggest that the use of APTS as an internal standard for area reduces the variance in peak area due to injection. In

summary, EKI is more appropriate than PI because of increased sensitivity and better repeatability. If the sample concentration and volume are not too low, one can repeat the analysis using EKI without fear of depletion of the sample affecting the repeatability.

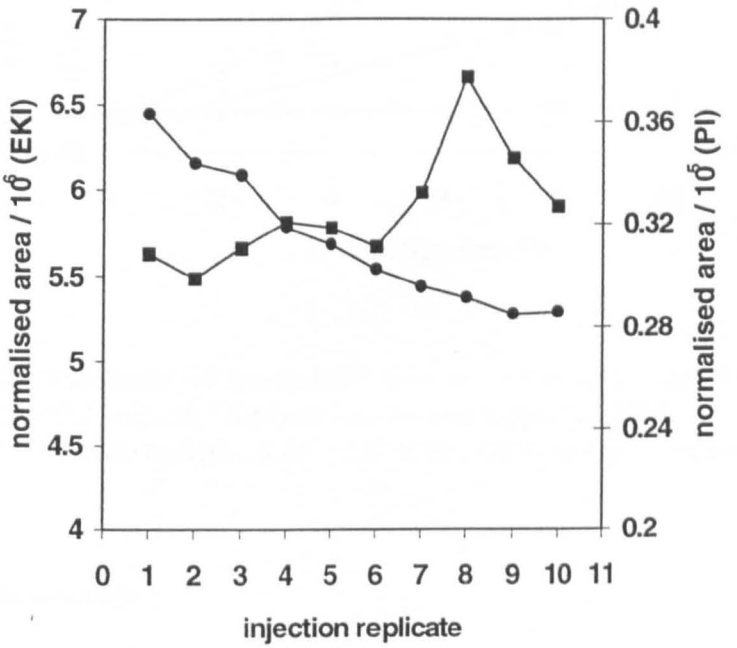

Figure 2.7. Peak area for *kappa* for repeated (n = 10) electrokinetic and hydrodynamic injections. Sample : mixture of concentration 0.16 mg mL^{-1} for both *iota* and *kappa* ; EKI (●): 3 s at 1.49×10^4 V m^{-1} ; PI (■): 10 s at 0.5 psi ; field strength : 4.48×10^4 V m^{-1}.

2.3.3 Injection time for EKI

When injected by EKI, the analytes migrate from the vial into the capillary according to their mobilities. As previously discussed in Section 2.3.2, this results in an injection bias between the different compounds. The main effect of an increase in injection time should be an increase in peak area. As displayed in Figure 2.8, a linear increase of area versus injection time was observed, showing different slopes for the different analytes. Above 6 s, increasing the injection time led to a broadening of peaks and a decrease in efficiency and consequently resolution.

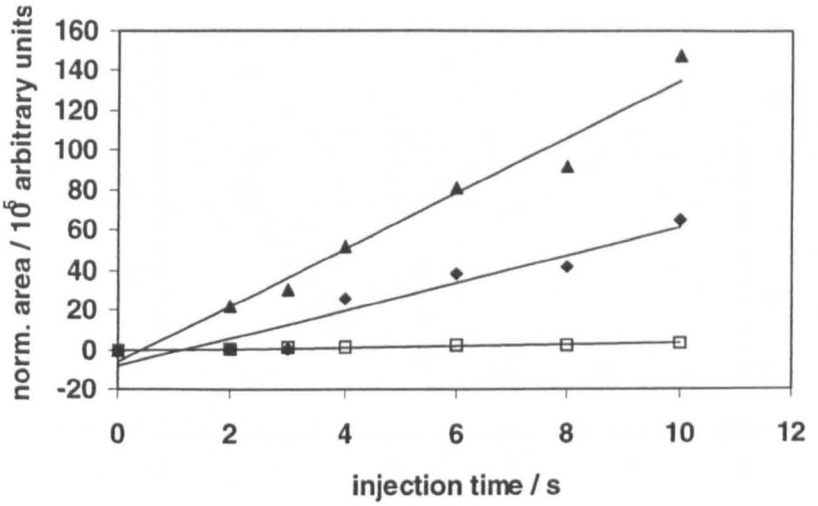

Figure 2.8. Variations of normalised area with injection time. Sample: mixture of concentration 0.77 mg mL^{-1} for both *iota* (♦) and *kappa* (▲). EKI : 2, 3, 4, 6, 8, 10 s at -10 kV. Separation field strength : 4.48×10^4 V m^{-1}. Other symbol : APTS (□).

2.3.4. Field strength

Using a run temperature of 25.0 °C, the field strength was varied in the range 0.75 to 4.5 $\times 10^4$ V m^{-1}. The main purpose was to estimate the effect on resolution, but the experiments allowed us to check whether there was a linear dependence (Ohm's law) between field strength, *E*, and current, *I*. The relationship was indeed linear over the whole range (Fig. 2.9), with a very satisfactory correlation coefficient $R^2 = 0.9997$. This shows that the maximal value of field strength (4.5×10^4 V m^{-1}) can be applied in our system without excessive electrical heating of the capillary (Joule heating).

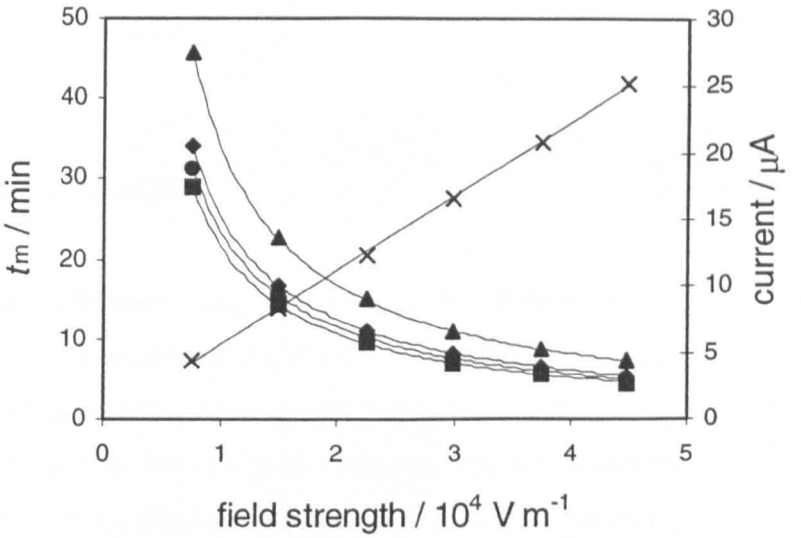

Figure 2.9. Effect of field strength on current (Ohm's law plot) and migration time. Field strengths 0.75, 1.5, 2.25, 3.0, 3.75, 4.5 × 10^4 V m^{-1} ; sample : mixture of *iota* (◆, 0.06 mg mL^{-1}), *lambda* (●, 0.06 mg mL^{-1}) and *kappa* (▲, 0.076 mg mL^{-1}) ; injection as in Fig. 2.6. Other symbols : APTS (■), current (×).

Migration times are found by regression to be inversely proportional to field strength, as expected from the following relationship

$$t_m = \frac{l}{v} = \frac{l}{\mu E} \qquad (2.1)$$

where v is the velocity of the analyte and l the length to the detector.

The separation of two analytes is described in terms of resolution and efficiency. A way to characterise the separation of two samples 1 and 2 is to calculate the resolution defined by

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where t_i is the migration time of analyte i and w_i is its peak width at the baseline. Gaussian peaks are baseline resolved when when $R_s \geq 1.5$.

The number of theoretical plates is a measure of the peak efficiency and is defined by the following equation

$$N = \frac{l^2}{\sigma^2} \qquad (2.2)$$

where σ^2 is the peak variance.

Resolution and efficiency were not affected by changes in electric field as far as carrageenans are concerned, and the efficiency of the APTS peak reaches a maximum at 2.2 $\times 10^4$ V m^{-1}. A test analysis (injecting APTS alone) was performed to check if the presence of carrageenans had an effect on these variations. The profile and efficiency for the APTS peak were similar in the absence or the presence of polysaccharides (see Figure 2.10). Our experiments were done with the default values of rise time (1 s) and data collection rate (5 Hz).

When diffusion is the only source of peak variance, $\sigma^2 = 2Dt_m$.
Since

$$t_m = \frac{l}{v} = \frac{lL}{\mu V}$$

one can substitute expressions for t_m and σ, and obtain the following equation for N (Kenndler 1998).

$$N = \frac{V}{2}\frac{\mu}{D}\frac{l}{L} = Vz\frac{e}{2kT}\frac{l}{L} \qquad (2.3)$$

where V is the applied voltage, D the diffusion coefficient, l the length to the detector, L the total length of the capillary, z the charge number, e the elementary charge, k the Boltzmann constant, and T the absolute temperature.

Thus, efficiency should scale with field strength for APTS which is a small charged molecule.
Substituting values for the fundamental constants, setting the temperature equal to 298 K

(25 °C) and assuming l=L, leads to an approximate expression correlating the efficiency to the charge of the analyte in a simple way (Kenndler 1998).

$$N \approx 20zV$$

This equation leads to theoretical values of efficiency for APTS increasing from 3×10^5 to 1.8×10^6 in the range 5 to 30 kV. The maximum observed value for APTS was 3.5×10^5 at 15 kV.

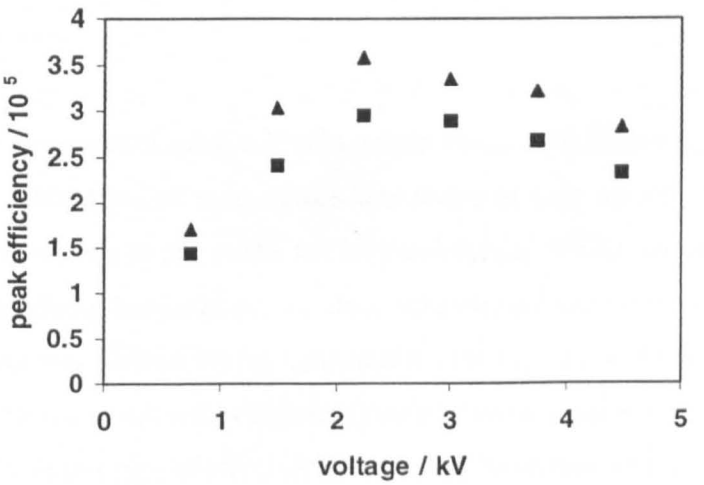

Figure 2.10. Variation of peak efficiency with applied voltage for APTS in the presence (■) and absence (▲) of carrageenans in the sample. Conditions as in Figure 2.9. Concentration of APTS unknown in the retentate mixture.

A simulation program (Reijenga & Kenndler 1994) was used to vary the value of the rise time and see its effect on peak efficiency for a small charged molecule, adenosine diphosphate (ADP^{2-}) analysed in conditions (current, temperature, capillary diameter and length, pH) similar to ours. The variation of peak efficiency with field strength is also influenced by the rise time. When using a fast rise time (0.1 s) the efficiency was found to scale with field strength, with a value of 4.4×10^5 for the peak efficiency at 30 kV. The peak variance is then mainly due to diffusion. In contrast, using a rise time of 1 s caused this rather than diffusion to be the main contribution to peak variance. In this latter case, the efficiency reached a maximum value of 2.5×10^5 for a voltage of 10 kV.

Efficiencies of the carrageenan peaks were found to be independent of field strength, with mean values 7, 5 and 12×10^3 for *lambda*, *iota* and *kappa* respectively and a random

variation over the range studied. Because carrageenans are large molecules whose diffusion should be very limited, polydispersity is the key factor as far as peak width and efficiency are concerned (Roberts *et al.* 1998). Since resolution between *iota* and *kappa* was also found to be invariant with field strength (R_s = 6), the maximum value 30 kV, yielding the shortest analysis time, was chosen as a standard value of the applied voltage for carrageenan analysis. *Lambda / iota* resolution was constant (R_s = 1.5) across the full field strength range.

2.3.5. Temperature

Separations were investigated using a 25 mM ammonium acetate BGE at 17.5, 25.0, 35.0, 42.5 and 50.0 °C. Variation of temperature was found to have no significant effect on efficiencies and resolution of the peaks for *iota* and *kappa*. Whilst the migration times decreased with increasing temperature, the electropherogram had the same appearance as in Fig. 2.6. Current was plotted versus temperature (see Fig. 2.11). At 50 °C the current reached 38 μA, which was still well within acceptable limits to avoid deviation from a linear Ohm's law plot. With this type of electrolyte, there is no deviation from a linear plot up to 50 °C. The key factor determining how the current and analyte migration time change with temperature is the viscosity, η, of the BGE. Relationship expected are $I \propto \eta^{-1}$ and $t_m \propto \eta$. Using the following parametrisation of η as a function of θ (Atkins 1990), the viscosity was calculated for the different values of temperature.

$$\log \frac{\eta_{20}}{\eta} = \frac{1.37023(\theta - 20) + 8.36 \times 10^{-4}(\theta - 20)^2}{109 + \theta}$$

where θ is the temperature in °C and η is in kg m^{-1} s^{-1}. The values for $I \times \eta$ and t_m / η are shown in Table 2.2

θ / °C	η	$I \times \eta$	t_m (APTS) $\times \eta^{-1}$	t_m (*iota*) $\times \eta^{-1}$	t_m (*kappa*) $\times \eta^{-1}$
17.5	10.7	2.16	3.81	4.59	7.09
25	8.90	2.14	3.9	4.75	7.36
35	7.19	2.11	4.03	4.93	7.73
42.5	6.23	2.09	4.13	5.10	8.08
50	5.46	2.08	4.24	5.29	8.33
RSD / %		1.6	4.3	5.6	6.6

Table 2.2. Values of $I \times \eta$ and t_m / η and % RSDs for the temperatures investigated. Units were 10^{-4} kg m^{-1} s^{-1} for η, 10^{-8} A kg m^{-1} s^{-1} for $I \times \eta$ and 10^{5} kg^{-1} m s^{2} for t_m / η.

The data presented were found to conform to the expected relationships. This proved that carrageenan did not undergo any conformation changes when varying the temperature in the range 17.5 – 50 °C. This is consistent with carrageenans being in the coil state in 25 mM ammonium acetate over this range of temperature, as reported in the literature for *kappa* (Piculell 1995, Rochas & Rinaudo 1980).

Injected amounts in EKI were found to increase with temperature, with an average increase in normalised peak area of 50 % for all analytes on changing from 25 to 50 °C. This can be explained as follows. The potential difference between the inlet and outlet electrodes is divided between the sample vial and the capillary according to their resistances. As the temperature increases, the resistance of the solution in the capillary decreases, hence the field strength in the sample vial increases in magnitude, leading to a larger amount injected. In going from 25 to 50 °C, the average increase in peak area was 50 %.

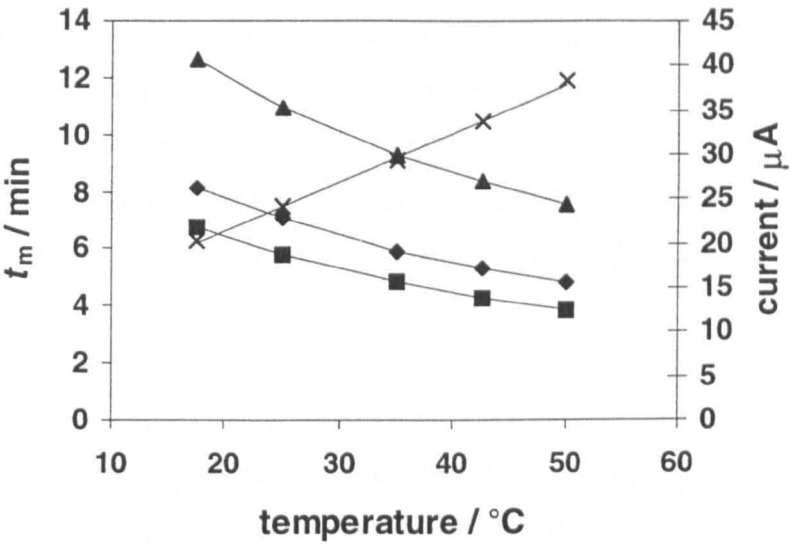

Figure 2.11. Effect of temperature on current and migration time. Temperatures : 17.5, 25.0, 35.0, 42.5, 50.0 °C; sample : mixture of concentration 0.77 mg mL^{-1} for both *iota* (♦) and *kappa* (▲); field strength and injection as in Fig. 2.6. Other symbols : APTS (■), current (×).

Migration times show a decrease with increasing temperature, as shown in Figure 2.11, enabling carrageenan separation within 7.6 min at 50 °C versus 10.9 min at 25 °C in a 67 cm-long capillary. Injection repeatability was investigated at 50 °C and showed results similar to those at 25 °C. Whilst these results suggest benefits in terms of shorter analysis times and better sensitivity by working at higher temperature, a run temperature of 25 °C was used during method development because of concerns about possible degradation of the capillary coating at 50 °C.

2.3.6 Ionic strength

The current was found to increase linearly with concentration of ammonium acetate. Considering the upper limit of 250 μA for the CE apparatus, this would restrict the BGE concentration to 250 mM. Addition of 10 % v/v of an 87 % v/v solution of glycerol in water was used to decrease the current. At this concentration, glycerol was expected to increase the solution viscosity by 31 % (James & Lord 1992), thereby allowing investigation of ammonium acetate concentrations up to approximately 330 mM. In the case of 400 mM,

separation was performed at a constant current of 250 μA in the capillary, corresponding to 22 kV instead of the normal voltage of 30 kV. An additional benefit of addition of glycerol is in diminishing peak broadening effects of convection and Joule heating. The main disadvantage of its use, though, originates from the same phenomenon: increase in viscosity increases migration times, and thus, analysis duration. On average the migration time increased by the expected value of 30 % when 10 % v/v of 87 % v/v aqueous glycerol was added to the background electrolyte.

At low BGE concentration (10, 18, 25 mM), peak shapes were similar to those observed in Fig. 2.6 for all analytes, and resolution was maintained between them. Above 25 mM, degradation of peak shape began to occur, diminishing the resolution of *lambda* and *iota* from $R_s = 1.5$ to unacceptable values.

At low and intermediate concentration (10-50 mM), an increase in concentration caused a slight increase in migration time, probably through ionic strength effects on mobility (Survay *et al.* 1996, Li *et al.* 1999). The most striking effect is the increase in area, as shown in Fig. 2.12. This is a consequence of the use of electrokinetic injection, and the explanation is analogous to that given in the previous section when discussing the effect of change of temperature, with BGE concentration determining the resistance in the capillary in this case. During EKI, the injection voltage V_{inj} is split between the sample zone, across which the potential difference is V_{sz}, and the capillary filled with BGE, across which the potential difference is V_c. Thus, $V_{inj} = V_{sz} + V_c$, and

$$I_{inj} = \frac{V_{inj}}{R_{sz} + R_c}$$

where R_{sz} and R_c are the resistances of the sample zone and of the capillary, respectively, and I_{inj} the current during the injection.

The injected amount scales with the potential difference V_{sz}. Therefore the normalised area, A_n, is also proportional to V_{sz} and

$A_n \propto V_{sz} \propto R_{sz} I_{inj}$

From these equations it follows that

$$A_n \propto \frac{R_{sz} V_{inj}}{R_{sz} + R_c}, \text{ and } \frac{1}{A_n} = K \times \left(1 + \frac{R_c}{R_{sz}}\right)$$

where K is a constant for a fixed value of V_{inj}. During separation, when the high resistance sample vial is replaced by a low resistance BGE vial, V_c is equal to the applied voltage V_{sep} and $R_c = V_{sep} / I_{sep}$, where I_{sep} is the current measured by the instrument. Therefore, A_n is linked to the experimentally-measured value I_{sep} by the following equation:

$$\frac{1}{A_n} = K \times \left(1 + \frac{V_{sep}}{R_{sz}} \frac{1}{I_{sep}}\right)$$

It should be noted that the modulus of the voltage is used with this and the previous equations. $1/A_n$ was plotted versus $1/I_{sep}$ for the different analytes. V_{inj} was -10 kV and V_{sep} was -30 kV. From the ratio of the slope to the y-axis intercept, K is eliminated and one can deduce the resistance of the sample and compare its order of magnitude to that of the BGE. The ratios slope / intercept were found to be 20×10^{-6}, 33×10^{-6} and 11×10^{-6} A for APTS, *iota* and *kappa* respectively. Taking an average value of 20×10^{-6} A leads to a resistance of 1.5 MΩ for the sample. During the separation at -30 kV in 25 mM BGE, the current was 21 μA, which corresponds to a resistance of 1.5 MΩ. This shows that the sample and capillary resistances are comparable, and that the model gives a realistic account of the effect of variation of BGE concentration on injected amount. The same calculation was used with the variations due to temperature. For the same range of variation of I, the same relative increase in area was observed. The ratios slope / intercept were found to be slightly higher than in the BGE concentration study, which may be due to a different salt concentration in the sample used.

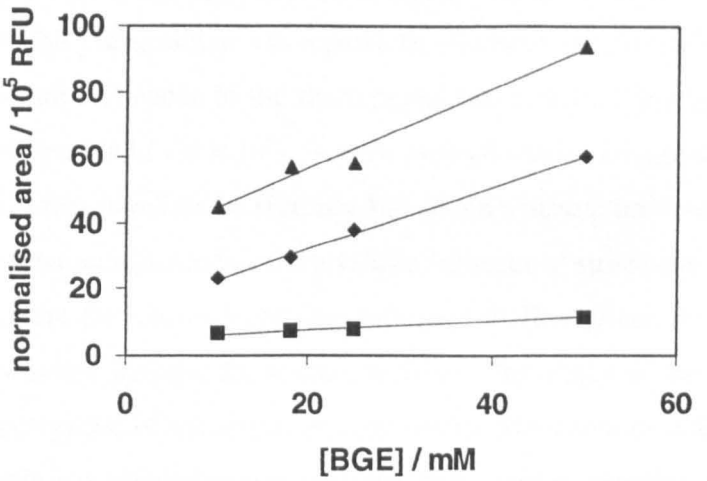

Figure 2.12. Variation of normalised area with concentration of the BGE. Ammonium acetate concentrations 10, 18, 25, 50 mM in 91.3 / 8.7 v/v water / glycerol ; sample : mixture of *iota* (◆, 0.08 mg mL^{-1}) and *kappa* (▲, 0.11 mg mL^{-1}) carrageenans analysed in triplicate with each solution ; field strength and injection as in Fig. 2.6. Other symbol : APTS (■).

Repeatability for migration time was satisfactory for this range of BGE concentration (better than 0.1 %), as shown in Table 2.3.

[BGE]	10 mM		18 mM		25 mM		50 mM	
RSD / %	t_m	area	t_m	area	t_m	area	t_m	area
APTS	0.00	28.5	0.03	6.5	0.06	10.0	0.03	5.3
iota	0.05	26.8	0.03	9.7	0.09	16.0	0.04	2.4
kappa	0.1	29.1	0.05	10.6	0.1	19.1	0.55	3.5

Table 2.3. Relative standard deviations / % ($n = 3$) for migration time and area for different concentrations of the BGE.

Above 50 mM BGE concentration, the *kappa* peak shape changed dramatically. The helix-coil transition temperatures for *kappa* in solutions of ammonium ion concentration 25 and 50 mM are 19 and 27 °C, respectively (Rochas & Rinaudo 1980). Since the temperature of the CE run is 25 °C, this suggests that the peak profile degradation can be attributed to the salt-induced conformational change of *kappa* carrageenan from the random coil to the helix form. At the highest concentrations studied (200 and 400 mM) a "spiking" effect was

observed, with very high and sharp peaks appearing in addition to the smaller normal ones (see Figure 2.13). This phenomenon was repeatedly observed at high BGE concentration, but the migration time behaviour of the sharp peaks was erratic. The efficiency of these peaks was very high indeed (~ 4×10^6). Achievement of sharp carrageenan peaks in the present study was often found to be reproducible within a series; however, a number of factors seemed to have an influence on the presence / absence of spikes and their migration time. For this reason, the use of high concentration BGE solutions to generate high efficiency peaks was not pursued for routine analysis. The origin of the sharp peaks is presumed to be aggregation of *kappa* carrageenan helices (Hermansson 1989, Hermansson *et al.* 1991). In addition to intermolecular repulsions between the polyanions being screened at high ionic strength, cation-specific effects have been documented with *kappa* carrageenan (Piculell 1995). The potassium ion is particularly effective for inducing aggregation, and it might be expected that spikes would appear at even lower concentration if ammonium acetate were replaced by potassium acetate as BGE.

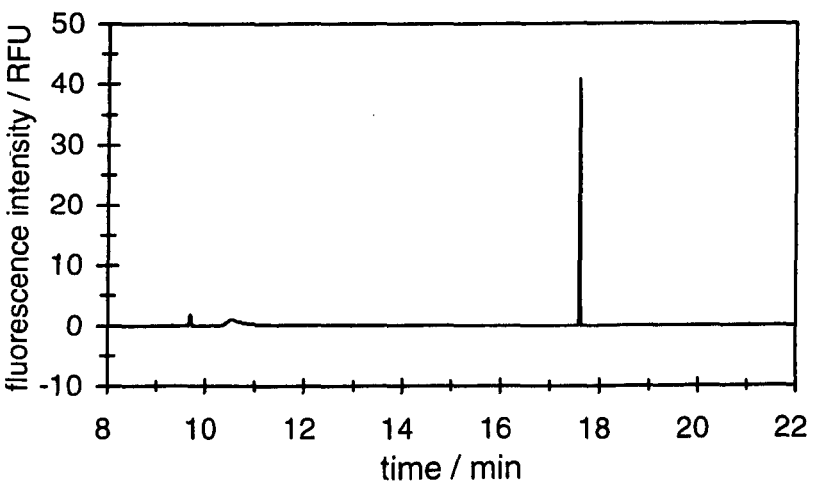

Figure 2.13. Separation of carrageenans in 400 mM ammonium acetate BGE containing 8.7 % v/v glycerol. Constant current applied 250 μA ; other conditions as in Fig. 2.12.

A concentration of 25 mM was selected as optimum for the ammonium acetate BGE, as it gave good repeatability, and the highest normalised peak area without peak profile degradation. As already noted, the value for the helix-coil transition temperature in 25 mM NH_4^+ for *kappa* is 19 °C (Rochas & Rinaudo 1980); that for *iota* was extrapolated to be 5 °C from values reported at higher concentrations (Norton 1990). Therefore, at 25 °C, both

carrageenans should be in the coil state during the CE analyses.

2.3.7 pH

The principal electrolyte used was 25 mM ammonium acetate; as the pH (6.8) is outside the buffer range of the weak acid and weak base components, this is in fact not a buffer solution. To check the effect of moving the pH into the buffering ranges, the pH was adjusted through the addition of either pure or aqueous acetic acid (20 % v/v) for acidic pH values or of ammonium hydroxide solution (2 % v/v) for basic pH values. Using concentrated reagents in this way did not change the overall salt concentration, and the current was approximately 26 μA for all pH values. In the case of the pH 9 solution, the total concentration of ammonium species ($NH_4^+ + NH_3$) was 42 mM. In the case of pH 4, the total concentration of acetate species ($CH_3COO^- + CH_3COOH$) was calculated to be 34 mM.

A mixture of *iota* and *kappa* carrageenans was analysed in triplicate with each BGE solution, allowing evaluation of the relative standard deviation in each case. Migration times remained similar across the pH range tested, with a slight trend for increase with increasing pH. A maximum of 3 % increase was observed in going from pH 5 to 9. The sulfate ester groups present on the carrageenan repeating unit are fully ionised in this range of pH. A possible explanation for this behaviour is the presence of a slight electro-osmotic flow. An increase in pH increases the EOF (whose direction is towards the inlet electrode), and consequently decreases the net mobility of the solutes and leads to longer migration times.

Table 2.4 shows data for RSDs as a function of pH. The most significant observation is that repeatability in normalised area was optimal at pH 8. Migration time repeatability for triplicate injections was good across the whole pH range tested. Relative standard deviations were < 0.1 % for migration times and < 2 % for normalised peak area at pH 8. When using APTS as an internal standard (cf. Table 2.4) these values were lowered for both migration time and normalised areas. The values obtained for RSDs on relative migration time were 0.02 % for *iota* and 0.05 % for *kappa*; for relative normalised area they were 0.4 % for *iota* and 0.8 % for *kappa*. Comparison with results in Table 2.1 shows that the RSDs on relative normalised area are significantly better at pH 8 than at pH 6.8.

pH	pH 5.0		pH 6.0		pH 6.7		pH 8.0		pH 9.0	
	t_m	A_n	t_m	A_n	t_m	A_n	t_m	A_n	t_m	A_n
APTS	0.13	5.0	0.04	2.7	0.10	2.6	0.06	0.8	0.04	2.2
iota	0.11	3.4	0	4.8	0.11	2.9	0.05	1.3	0.06	2.7
kappa	0.16	2.5	0.04	6.2	0.04	2.6	0.06	1.5	0.07	3.0

Table 2.4. Relative standard deviations /% ($n = 3$) for migration time and area for different pH values of the BGE.

No effect of pH on resolution between *iota* and *kappa* was observed: the value was $R_s \sim 4$ for all pH values. The peak efficiency was slightly improved at low buffer pH for APTS and *kappa* but showed no trend for *iota*. A problem observed at pH 4.0 - 4.5 was a large increase in the standard deviation of integrated peak area, most probably due to an increased degradation rate of the capillary coating. A peak shape change appeared at pH 9, with a fronting shoulder appearing on the *iota* peak. However, this did not significantly affect the peak integration. Considering the fact that resolution does not vary with pH, a pH of 8 was considered optimal due to the best values of repeatability. It is also advantageous to be working above pH 7 because degradation of carrageenans is lower than at acidic and neutral pH values (Piculell 1995).

2.3.8 Tetrabutylammonium as counterion

Tetrabutylammonium is a large cation. Tetramethylammonium was investigated in previous studies for its potential ability as a counterion to prevent carrageenans forming double helices (Norton 1990). The effect of TBA in the separating medium was investigated for different concentrations ranging from 0.05 mM to 5 mM. The addition of TBA (hydroxide form) to a 25 mM ammonium acetate solution increased the pH to 8.8. It is known from the pH study that no dramatic changes occur due to change of pH alone in the range 5 to 9.

The effect of variation of TBA concentration was investigated using a mixture of *iota* and *kappa* carrageenans. TBA had a positive effect on carrageenan peak efficiency and thus on *iota* - *kappa* resolution, but presented the disadvantage of lengthening APTS migration time,

thus decreasing its resolution from *iota* (see Figure 2.14). The relationship between APTS migration time and TBA concentration indicates that, at 5 mM, APTS would be detected after *kappa*. APTS is an anion carrying 3 negative charges and can interact with the cations tetrabutylammonium or ammonium according to the following reactions:

$APTS^{3-} + TBA^{+} \rightleftarrows [APTS\text{-}TBA]^{2-}$

$APTS^{3-} + NH_4^{+} \rightleftarrows [APTS\text{-}NH_4]^{2-}$

The variation of the APTS peak migration time induced by TBA between 0 and 1 mM was 0.7 min, whereas that observed when varying the NH_4^+ BGE concentration from 18 to 50 mM was 0.5 min. This suggests a more efficient ion-pairing of APTS with TBA than with ammonium, which would reduce the average charge and thus lengthen its migration time. Since APTS is a substituted pyrene, one can suspect hydrophobic interactions occurring between the aromatic rings and the alkyl chains of TBA, whereas interaction with NH_4^+ is purely ionic.

Up to 1 mM, a slight tendency of *iota* migration time to shift towards that of *kappa* is noticeable too. At 5 mM only one peak was detected, whose migration time corresponds to *kappa*, so it appeared that there has been a strong effect on *iota* mobility between 1 and 5 mM TBA, which caused the *iota* peak to slow down and shift to the same time as the *kappa* peak. As far as carrageenans are concerned, the use of Manning's theory showed that *iota* undergoes counter-ion condensation, but not *kappa* (Roberts *et al.* 1998). At high concentration of the large TBA counterion, the charge/size ratio of *iota* is probably reduced and its migration in the capillary is similar to that of *kappa*.

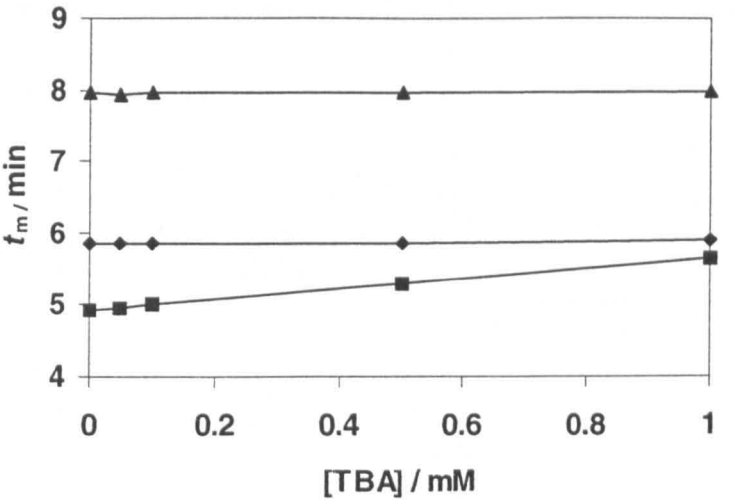

Figure 2.14. Variations of migration times with concentration of TBA as an additive in ammonium acetate BGE. BGEs : 25 mM in ammonium acetate containing increasing concentrations of TBA : 0.05, 0.1, 0.5, 1.5 mM ; Sample : mixture of *iota* (◆, 0.08 mg mL^{-1}) and *kappa* carrageenan (▲, 0.11 mg mL^{-1}) ; EKI : 3 s at -10 kV; Separation field strength 4.5×10^4 V m^{-1}. Other symbol: APTS (■).

2.4 Fast separation using optimised BGE conditions

To obtain a shorter run time, an experiment was set up using a shorter capillary (47 cm), the highest temperature and voltage which could be used in the CE apparatus (50 °C and -30 kV respectively), together with the ammonium acetate BGE at optimal concentration and pH (25 mM and pH 8 respectively). Figure 2.15 shows that separation of the three carrageenans is obtained in less than 3 min with satisfactory resolution and efficiency. An improvement in comparison to previous studies using citrate buffer (Roberts *et al.* 1998) is noticeable, in particular concerning the separation of *iota* and *lambda*.

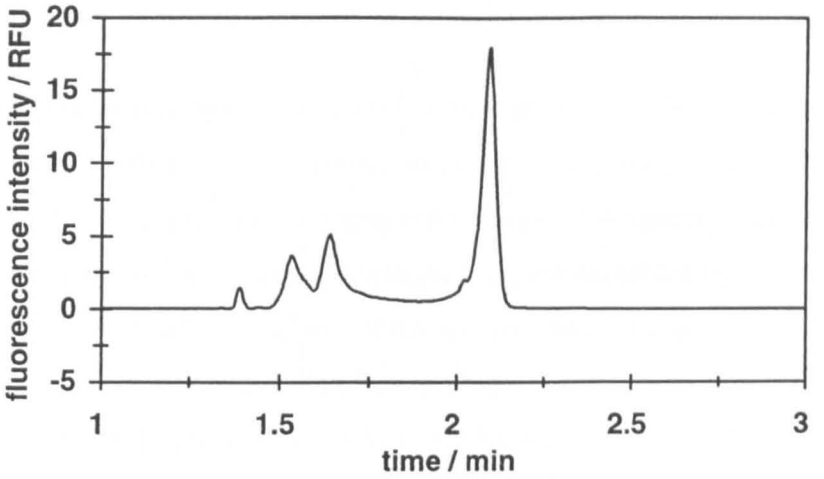

Figure 2.15. Separation of carrageenans in optimal conditions. Buffer : 25 mM ammonium acetate, pH 8.0 ; capillary length 47 cm, 40 cm to the detector ; Separation field strength : 6.38×10^4 V m^{-1} ; Temperature 50 °C ; Current 56.5 µA ; Sample : mixture of concentration 0.06 mg mL^{-1} for *iota*, *lambda* and *kappa* ; EKI, 3 s at -10 kV.

2.5 Conclusions

As far as injection is concerned, these studies have shown that electrokinetic injection is more appropriate than pressure injection because of increased sensitivity and better repeatability. In routine analysis, the same run is repeated a maximum of 3 times. If the sample volume is not too low, sample depletion does not occur and the variations in area are random. The use of APTS as an internal standard significantly improves the repeatability of experiments. The linear relationship between injection time and injected amount was important to study, as future analyses may require higher sensitivity to the analyte. During this initial study a 3 s EKI was used in order to maintain the highest resolution. Concerning temperature, 25 °C was used in most of the experiments reported in this thesis, but the use of 50 °C in routine analysis would provide shorter analysis times and a better sensitivity. Migration times became very long (up to 45 min) at low values of field strength. Since resolution between *iota* and *kappa* was good enough ($R_s = 4$) even at high field strength, 30 kV was chosen as a standard value of the applied voltage for carrageenan analysis. *Lambda / iota* resolution was constant ($R_s = 1.5$) across the full field strength range.

Variation of BGE concentration was shown to have a dramatic effect on separation and efficiency. We used 25 mM as a convenient concentration giving good repeatability and in general adequate sensitivity. The values of helix-coil transition temperature for *iota* and *kappa* in 25 mM NH_4^+ are 5 °C and 21 °C respectively. The analyses were thus performed on carrageenans in the coil state. Above 50 mM ammonium BGE concentration, changes were observed on the *kappa* peak, with the appearance of sharp thin peaks. This correlated well with a change in conformation for *kappa* carrageenan, since the helix-coil transition temperature in 50 mM ammonium was reported to be 27 °C. Higher concentrations of ammonium lead to more dramatic peak shape changes, with a "spiking" effect occurring at 400 mM. This spike is believed to originate from the presence of aggregates of *kappa* carrageenan. Considering the fact that resolution does not vary with pH, a range of 6.5-8 was chosen to be optimal due to the best values of repeatability and efficiency being found in this range.

Addition of glycerol increases solution viscosity, and thereby, offers an efficient protection

against peak broadening effects of convection and Joule heating. The main disadvantage of its use, though, originates from the same phenomenon : increase in viscosity increases migration times, and thus, analysis duration. We decided to use glycerol only when needed, that is to say, in the case of high ionic strength buffers, where the current for a purely aqueous BGE would exceed the limit value of 250 µA at an applied voltage of -30 kV.

Optimal values for both physical and chemical parameters affecting the separation were determined in this study. An experiment was carried out using all these optimal values, i. e. 50 °C for temperature, -30 kV for voltage (field strength 6.7 $\times 10^4$ V m^{-1}), ammonium acetate concentration 25 mM and pH 8. A 47 cm PVA coated capillary was used. Separation of the three carrageenans was achieved in less than 3 min with satisfactory resolution and efficiency. An improvement in comparison to previous studies using citrate buffer is noticeable, in particular concerning the separation of *iota* and *lambda*.

This method development was very important since it was the first step in this thesis work, and all subsequent experiments used ammonium acetate to separate carrageenans. Some modifications were made later in order to improve robustness of the method, but the BGE remained 25 mM ammonium acetate at pH 8.0. In particular, the temperature used was 25 °C, to avoid the possibility of thermally-induced degradation of the capillary coating at higher temperatures.

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Chapter 3: Investigation of sample preparation for CE-LIF of carrageenans

3.1 Introduction

3.1.1 Reductive amination

The advantage of derivatisation, especially with fluorophores, is the tremendous increase in sensitivity that it offers in comparison to other methods of optical detection such as direct or indirect UV. Jackson reported the first reductive amination of sugars for fluorescence detection (Jackson 1990). Use of acetic acid was found necessary for a satisfactory derivatisation yield: it was used at a concentration of 1.3 M, resulting in a pH value of 2.3. Other work on the reactivity of sodium cyanoborohydride has shown that below pH 4 the reactivity of this reducing agent is increased, and the compound can reduce not only the Schiff base (imine), but also reducing ends (aldehyde) (Borch *et al.* 1972). This suggests that use of acidic conditions could possibly prevent saccharide labelling. At pH 6-7 the reduction of imines proceeds faster and reduction of aldehydes is negligible. However, acidic conditions do not seem to be a problem with many carbohydrates since other authors have reported the use of even stronger acids as catalysts when derivatising monosaccharides and oligosaccharides cleaved from glycoproteins with APTS (Evangelista *et al.* 1996a; Evangelista *et al.* 1996b; Chen *et al.* 1998a). It should be emphasised that this method is not applicable to non reducing sugars such as sucrose.

However, recent publications seem to indicate non-negligible disadvantages. Chmelik *et al.* used size exclusion chromatography (SEC) coupled to fluorescence detection for derivatives of dextrans with 8-aminonaphtalene-1,3,6-trisulfonic acid (ANTS) and showed that the average molecular weight of the derivatised fraction was lower than that of the initial material (Chmelik *et al.* 1997). The authors attributed this to the lower reactivity of the higher molecular weight chains. This confirms observations made by Mort *et al.* when using CE and HPLC to investigate the yield of derivatised galacturonic acid polymers (Mort *et al.* 1996). Pfaff *et al.* used a Box-Behnken experimental design to investigate effects of the derivatisation conditions on both the labelling yield and the molecular weight distribution of malto-oligosaccharides derivatised with 2-aminonaphthalene-6,8-disulfonic acid (ANDS) (Pfaff *et al.* 1999). A decisive criterion was the ratio between the signals of derivatised

maltose (DP 2) and maltoheptaose (DP 7). When hydrolysis occurs, this ratio is abnormally high. As a compromise between high peak areas and limited hydrolysis the authors chose the conditions leading to high peak areas together with a minimum area ratio between DPs 2 and 7, thus minimising the carbohydrate degradation during derivatisation. Their results contrast with earlier work by O'Shea *et al.*, who reported a similar labelling efficiency for all DPs >2 with ANTS (O'Shea *et al.* 1997).

3.1.2 Acid-catalysed degradation of carrageenans

The degradation of carrageenans has been widely studied, linked in with investigations of their conformations (single or double helix). Singh *et al.* reported modelling of the hydrolysis of carrageenans at 35, 45 and 55 °C. Compounds were monitored using a SEC-MALLS-RI system (Singh *et al.* 1994a). Since no data is available concerning the hydrolysis of the two different types of bonds linking rings in the main chain in *kappa* carrageenan, the authors supposed the rate of cleavage to be the same for both bonds. They also mentioned the fact that the results should be valid in the pH range from 2 to 3. Karlsson & Singh investigated the rate of desulfation in order to check its influence on molecular weight measurements (Karlsson & Singh 1999). They discovered that, unlike dextran sulfate, carrageenans undergo little desulfation at pH 2, the main effect observed being hydrolysis with rate constants of 4.4×10^{-4} h^{-1} at 35 °C and 5.4×10^{-3} h^{-1} at 55 °C. When monitoring the reaction by gel strength and viscosity the same group showed a good correlation of these results with those obtained with SEC (Singh & Jacobsson1994). These two techniques can therefore be used to investigate *kappa* degradation. At 55 °C the variations of M_w with time were found to be best described using a double exponential, whereas for reaction at 35 °C a simple first order equation was more appropriate. The authors attributed these results to different hydrolysis rates for the hydrolysis of the two bonds in *kappa* carrageenan, one of them being predominantly hydrolysed at low temperature and the other reacting significantly at higher temperature. In contrast, Myslabodski *et al.* (1996) quoted several early references showing evidence that only one of the two glycosidic linkages is hydrolysed, the glycosidic bond between C1 of the anhydrogalactose unit and C3 of the galactose unit. The anhydro bridge was believed to be the reason for the fragility of *kappa* carrageenan. In the case of *iota* carrageenan the anhydrogalactose ring is stabilised by an extra sulfate, and this could explain the much lower rate of hydrolysis found for *iota*. Since *lambda* carrageenan

completely lacks such a bridge in its structure its hydrolysis rate is lower than for *iota* and *kappa*. Table 3.1 summarises values for the reaction rate constants from all authors.

θ / °C	Singh & Jacobsson1994[1]	Myslaboski *et al.* 1996 [1]	Karlsson & Singh 1999 [1]	Singh & Jacobsson 1994 [2]	Singh *et al.* 1994 [2]
35	1.45×10^{-6}	1.8×10^{-6}	7.4×10^{-6}	0.0023	0.0039
45	8.35×10^{-6}			0.00742	0.0117
55	2.13×10^{-5}		9.0×10^{-5}	0.0147	0.0206

Table 3.1. Reaction rate constant k / min^{-1} for the hydrolysis of *kappa* carrageenan in HCl at pH 2.
[1] Simplified equation $1 / M_w(t) = 1 / M_w(0) + (k_1 \times t / m)$ (Tanford 1961).
[2] First order exponential rate equation $M_w(t) = M_w(0) \times \exp(-k_2 t)$. $M_w(0)$ is the initial M_w at $t = 0$, $M_w(t)$ is the M_w after t min of hydrolysis and m in g mol^{-1} is the molecular weight of the monomer ($m = 392$ for (Myslabodski 1996) and 192 for (Singh & Jacobsson 1994)).

The kinetics of the degradation were investigated at different temperatures and monitored both by viscosity measurements and number of reducing-ends (Hjerde *et al.* 1996). In both the ordered and disordered states, different rates were calculated from the two sets of data obtained by viscosity and number of reducing ends. The key parameter used to compare the hydrolysis behaviour is the ratio of these two (rate for viscosity / rate for reducing ends). This ratio increased by a factor of 20 in going from the ordered to the disordered state. The conclusion of this study was that carrageenans are much more susceptible to hydrolysis in the disordered than in the ordered state. Furthermore, *iota* was found to be 5-6 times less sensitive to hydrolysis than *kappa*. This was attributed to the stabilisation of the anhydrogalactose residue through the presence of an extra sulfate group.

Initial conditions for derivatisation, as used in Chapter 2, were 55 °C and 2.25 M acetic acid. The presence of sodium ions originating from the reducing agent and APTS result in an ionic strength of 0.23 M. According to Norton (1990), under these conditions of temperature, salt type and ionic strength, *kappa* carrageenan should be in the disordered state. Neglecting any buffering effects of other species present in the solution, the calculated pH is found to be 2.2. Using the values from Table 3.1 for rate constants at pH 2 and 55 °C, a calculation using the first order exponential rate equation with $t = 60$ min yields $M_w(60) / M_w(0) = 0.414$ using $k_2 = 0.0147$ min^{-1}. This corresponds to a decrease in molecular weight

of 60 %. With $k_2 = 0.0206$ min^{-1}, one finds a loss of 70 %. At pH 2.2, the pH used in our work, we would expect hydrolysis to be less by a factor reflecting the difference in pH between 2.0 and 2.2.

3.1.3 Purification methods

Another side-effect of labelling reactions is the formation of degradation products of the label which are still fluorescent and can interfere with the derivatives of interest (Chen *et al.* 1998a; Chen *et al.* 1998b). The purities of both APTS and of the solvent used (THF) were found to be crucial for the sensitivity of oligosaccharide detection. The authors also mentioned that the quality of the tube used for derivatisation can play a role. Moreover, as seen from Fig. 3.1, several peaks are present in the electropherogram of the reaction blank at short migration times. Since N-linked oligosaccharides or maltodextrin ladders only carry the charge provided by APTS, their migration behaviour is very different from that of the label. In contrast, carrageenans, which carry sulfate groups on each repeat unit, have a more similar electrophoretic behaviour to APTS and their peaks (particularly for *lambda* and *iota*) appear in the same migration time window as the peaks present in the reaction blank. Moreover, since carrageenans are polysaccharides, the fraction of reducing ends, which are the reacting sites for the fluorophore, is much smaller than in the case of oligosaccharides, and sensitivity is an even more important issue than in the case of small sugars. Therefore, when dealing with negatively charged polysaccharides, one should use a preliminary physical procedure to separate them from small molecules giving parasite peaks.

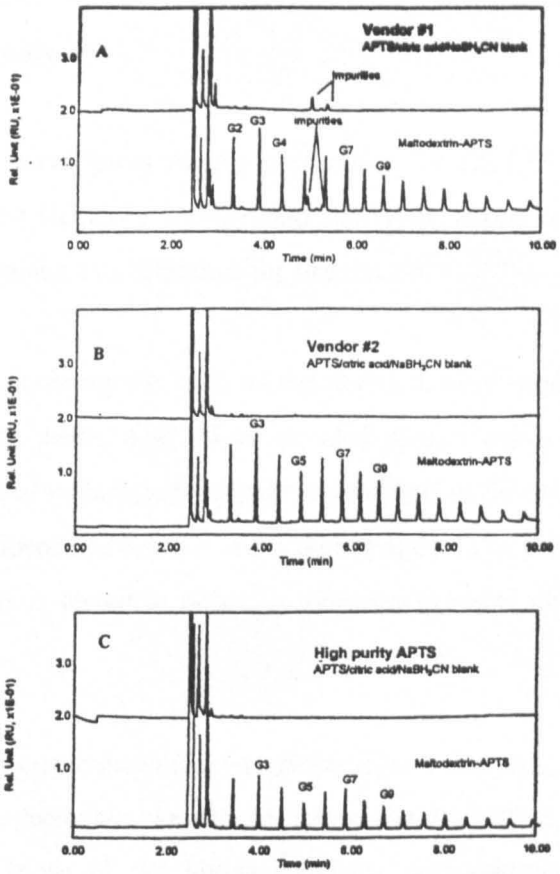

Figure 3.1. Separation of APTS-derivatised maltodextrin oligomers by CE, showing the blank reaction (Chen *et al.* 1998a).

Mort *et al.* used scavenger beads carrying aldehyde groups able to react with the excess of ANTS label (Mort *et al.* 1998). Since all oligosaccharides were small enough to enter the bead pores, their distribution was not affected by the procedure. This protocol was efficient but some degradation products of ANTS, which do not carry any reactive amino group, could not be eliminated. In another publication Mort's group removed the excess of reagent by dialysis before submitting derivatives of galacturonic acid oligomers to enzymatic digestion (Zhang *et al.* 1996). Another efficient cleaning protocol is that used by Roberts *et al.* in their study of carrageenans by CE (Roberts *et al.* 1998). It consists in using microcentrifuge filters of defined molecular weight cut-off to give a size-based separation of high M_w derivatised carrageenans from the low M_w products of degradation of the label. Stefansson has used precipitation in ethanol after derivatisation to clean derivatised polysaccharides (Stefansson 1999a; Stefansson 1999b). A key issue when using all of these procedures is not only the efficiency of removal of small molecules, but also the recovery of the high molecular weight fraction.

3.1.4 Aim of this chapter

The present chapter investigates sample preparation for CE-LIF in the case of *kappa*-carrageenan. Amongst the three carrageenan sub-types, *kappa* is the most sensitive to hydrolysis and was chosen as a reference for this reason.

In a first attempt to increase the yield of the reaction, citric acid is investigated as an alternative catalyst to acetic acid. More detailed studies using acetic acid are made concerning the effects of variation of concentration and pH of the catalyst, the reaction time and the combined influence of catalyst and reducing agent. The performances of different purification protocols is tested in order to minimise eventual degradation and loss of material.

Some of the samples used are molecular weight fractions of *kappa* carrageenan provided by SBI (now SKW). The molecular weights for native and derivatised carrageenans of these fractions as well as those of the Sigma standards are determined by size exclusion chromatography coupled to multiple angle laser light scattering and refractive index detectors (SEC-MALLS-RI). These measurements are performed at SKW in Baupte, France.

Modifications to sample preparation and injection methods are carried out throughout the investigations, and lead to significant improvements in the robustness of the method required for its ultimate application to complex matrices of food samples.

3.2 Experimental

3.2.1 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. The molecular weight standards were provided by SKW (Baupte, France), and had M_w values 69, 120, 205, 256, 369 and 569 kDa. The carrageenan oligomers were from Dextra (Reading, UK). For fluorescent conjugation a trisodium 8-aminopyrene-1,3,6-trisulfonate (APTS) label was obtained from Molecular Probes (Eugene, USA) and Lambda Fluoreszenztechnologie (Graz, Austria) and sodium cyanoborohydride (powder and solution in THF) from Aldrich (Gillingham, UK). Methylene blue was obtained from Sigma. Micro-concentrators with a 30 kDa molecular weight cut-off (MWCO) filter were purchased from Millipore (Gillingham, UK), as well as the 13 mm 0.025 micron cellulose nitrate / acetate membranes used for dialysis. Dialysers using 10-100 µL volumes of sample (Slide-A-Lyzer® MINI Dialysis Units, MWCO 10,000, regenerated cellulose) were purchased from Pierce (Rockford, USA). Dialysers handling a larger amount of sample (1 mL) were obtained from Spectrum (Rancho Dominguez, USA). These Spectra/Por® DispoDialyzers were in cellulose ester, with a MWCO 50,000. The gel used for preparative size exclusion chromatography (SEC) was Bio-Gel P10, obtained from Bio-Rad (Hercules, USA). The polyacrylamide beads have hydrated sizes between 90 and 180 µm, with a fractionation range from 1,500 to 20,000 Da, suitable for the purification of proteins and polypeptides. Ammonium acetate was purchased from Merck (Darmstadt, Germany). Stock solutions of ammonium acetate (0.5 M) were prepared and further diluted in ultrapure water (generated either by an Elgastat UHQ II or by a MilliQ unit) to desired concentration. Sodium azide (0.015 % in ammonium acetate stock solution, 0.002 % in carrageenan solution) was used as an anti-microbial agent. Ammonium hydroxide (2 % v/v in water) was used to adjust the pH. All buffer solutions were filtered through filters (0.45 µm) before use.

3.2.2 Experimental conditions for CE

As described earlier in Section 2.2.4, all experiments were performed on a Beckman P/ACE

5000 CE system equipped with a LIF detector connected to an Ar-ion laser (488 nm ex, 520 nm em). Polyvinyl alcohol "eCAP™ N-CHO" coated capillaries supplied by Beckman were used. The internal diameter of these capillaries was 50 µm. The capillary was thermostatted at 25.0 °C. The background electrolyte used for all investigations was 25 mM ammonium acetate, pH 8.0, and the separation voltage was -25 kV. At -30 kV some electric arcing took place, and sometimes the instrument would abort the run automatically. Therefore, -25 kV proved more reliable. Between runs, the capillary was rinsed for 2 min with water, then for 2 min with the BGE. Data collection rate was 20 Hz unless otherwise specified.

3.2.3 Derivatisation protocols

3.2.3.1 Protocol 1: Derivatisation with different catalysts and catalyst concentrations

The *kappa* carrageenan fractions of M_w 69, 120, and 539 kDa of were investigated. For each M_w, a 1 mg mL^{-1} carrageenan solution was freshly dissolved by heating to 65 °C. The solutions were left to cool down to room temperature before dispensing 8 µL for derivatisation. The following were then added: 2 µL of derivatising solution (0.2 M APTS), 2 µL of catalyst of required concentration to have the final desired concentration (0.3, 0.6, 1.0, 2.25 M for acetic acid ; 0.15 and 0.6 M for citric acid) and 2 µL of reducing agent (1 M sodium cyanoborohydride in THF, commercial solution). After an overnight incubation (15 h) at 55 °C the reaction was quenched by addition of water up to 100 µL. The resulting reaction mixtures were lyophilised and the volume made up to 100 µL before dispensing half of the total volume for filtration. This 50 µL volume was transferred to a Microcon concentrator and, after dilution in water to give a total volume of 300 µL, centrifuged for 15 min at 6,000 rpm in a microcentrifuge. This operation was repeated twice more, and the retentate was recovered by turning the filter upside down and spinning for a few seconds. The volume was then adjusted to 100 µL and further diluted in ultrapure water to give a final carrageenan concentration of 0.005 mg mL^{-1}. The usual 30 kDa cut-off filter allowed easy filtration of the 120 kDa and 539 kDa fractions, but the lowest one (69 kDa) did not filter fast enough: it appeared that the pores were clogged by the biopolymer. Attempts to use a lower M_w cut-off (3 kDa, 10 kDa) were not much more successful. Only the reactions catalysed by 0.3 M acetic acid and 0.15 M citric acid were filtered and are referred to in this study. Retentates from the 69 kDa sample still contained a lot of low M_w material, as

evidenced by their yellow colour signifying the presence of APTS and byproducts.

This experiment was repeated with the fraction of *kappa* of M_w 256 kDa for 2.25 M acetic acid, 0.15 M and 0.3 M citric acid. For each catalyst conditions two reactions were carried out, one with an aqueous solution of reducing agent, the other with $NaCNBH_3$ dissolved in THF.
The volume was made up to 100 µL after incubation and the whole reaction medium was filtered with a micro-concentrator. Sample was diluted to 0.002 mg mL^{-1} for injection.

All these samples were investigated using a 57 cm × 50 µm PVA coated capillary; EKI was performed for 3 s at -10 kV. Before and after each sample injection, water was injected electrokinetically for 1 s at -1 kV.

3.2.3.2 Protocol 2: Derivatisation with low concentrations and buffered solutions of acetic acid

The fraction of *kappa* of M_w 256 kDa was derivatised with low final concentrations of acetic acid : 0.001, 0.01, 0.1 and 0.63 M. Other conditions were the same as in Protocol 1 except that the stock solution of APTS had a concentration of 0.04 M and the total reaction volume was increased to 50 µL. After filtration, the volume of all retentates was made up to 100 µL, resulting in a *kappa* carrageenan concentration of 0.08 mg mL^{-1}, and injected on the CE instrument without further dilution. This series of samples was investigated using a 57 cm × 50 µm PVA coated capillary; EKI was performed for 3 s at -10 kV. Before and after each sample injection, water was injected hydrodynamically for 3 s at 0.5 psi.

In order to compare the actual and calculated pH of the reaction mixtures, one thousand fold-scaled up mixtures (total volume 50 mL) containing all components except the labelling agent APTS were prepared to reproduce the reaction conditions for each acetic acid concentration. The pH of the mixtures was measured before and, in the case of the 0.6 M acetic acid concentration, after incubation in a water bath at 55 °C.

Using Protocol 2, derivatisations were also performed on the fraction of M_w 256 kDa using acetic acid/acetate buffer solutions of concentration 0.1 M in acetate species, and of defined

pH values 4.0 and 4.8. A derivative was prepared under the same experimental conditions but with acetic acid 0.6 M resulting in pH 2.5, to have a comparison with previous experiments. The sample was diluted to a concentration of 0.08 mg mL^{-1} before analysis using a 47 cm × 50 µm PVA coated capillary; EKI was performed for 3 s at -10 kV. Before and after each sample injection, water was injected hydrodynamically for 3 s at 0.5 psi. Samples derivatised with non-buffered conditions (acetic acid concentrations 2.2 and 0.001 M) were reinjected under these conditions for direct comparison.

3.2.3.3 Derivatisation of mono- and oligosaccharides

Solutions of standard oligosaccharides of *kappa* (the disaccharide neocarrabiose-4-*O*-sulfate, the tetrasaccharide neocarratetraose-$4^{1,3}$-di-*O*-sulfate, the hexasaccharide neocarrahexaose-$4^{1,3,5}$-tri-*O*-sulfate) were prepared. The concentrations were 1.25 mM for the disaccharide and the tetrasaccharide, and 1.35 mM for the hexasaccharide. A solution of label of concentration 0.04 M was used. The appropriate volumes containing 40 nmol of sugars were dispensed in Eppendorf tubes for derivatisation with a two-fold excess of APTS. Derivatisation used 0.7 M acetic acid and was performed in a total volume of 50 µL overnight at 55 °C. The volume was increased to 100 µL after reaction but these solutions were not filtered. They were diluted 100 times, resulting in a concentration in oligosaccharide of 8 µM. These standards were injected hydrodynamically for 3 s at 0.5 psi on a 57 cm × 50 µm PVA coated capillary.

Glucose, fructose and sucrose were derivatised according to Protocol 1. Sucrose does not carry any site suitable for derivatisation, but occurs in foodstuff. If hydrolysed, it could produce monosaccharides. Stock solutions of sugars were prepared with a concentration of 0.125 M, and use of 8 µL of sugar solution resulted in an excess of 2.5 in sugar over APTS. As described for the *kappa* oligomers, the volume was made up to 100 µL. Sucrose and fructose solutions were diluted by a factor of 2000, and the glucose solution was diluted × 8000. Diluted solutions were injected hydrodynamically for 3 s at 0.5 psi on a 57 cm × 50 µm PVA coated capillary; capillary temperature was 50 °C. Temperature did not play a significant role on the value of the relative migration time as checked with sucrose in a separate experiment performed at 25 °C.

3.2.3.4 Investigation of the effect of variation of reaction time

A series of identical reaction mixtures using the standard conditions (2.25 M acetic acid, Protocol 1) was incubated for increasing periods of time (1, 2, 3, 6, 22 h). Sample treatment was the standard microfiltration performed until no liquid eluted from the filter. The retentate volume was then minimal (2 µL), and water was added to bring the volume to 100 µL. Before electrokinetic injection the sample was diluted by a factor of 20, giving a *kappa* carrageenan concentration of 0.004 mg mL^{-1}. CE conditions were identical to those described in Section 3.2.3.1.

3.2.3.5 Protocol 3: improved conditions limiting degradation

Following all investigations a new protocol was designed, which reduces hydrolysis of carrageenans. The desired amount of freshly dissolved carrageenan was dispensed in an Eppendorf tube. To this were added 2 µL of labelling agent (0.04 or 0.2 M), 10 µL of acetic acid 3.0 M (resulting in 0.6 M final concentration), 2 µL of 1 M sodium cyanoborohydride (freshly dissolved in THF), and water to bring the total volume to 50 µL. The solution was vortexed and incubated for 1 h at 55 °C.

3.2.3.6 Combined influence of the reducing agent and the catalyst acetic acid

In this series of experiments 16 µL of a 1 mg mL^{-1} solution of the standard *kappa* carrageenan from Sigma were dispensed for derivatisation in Eppendorf tubes. The different mixtures tested are reported in Table 3.2. The total volume was set to 60 µL in order to result in an acetic acid concentration of 0.6 M. Minor volume variations resulting from these investigations should not influence the reaction. The sodium cyanoborohydride solution was freshly prepared from the powder form for each addition.

Experiment	First hour at 55 °C	Second hour at 55 °C
1	- 2 µL 0.04 M APTS - 2 µL of 1 M $NaCNBH_3$ freshly dissolved in THF - 8 µL 4.5 M acetic acid - 32 µL water	reaction mixture removed from incubator and kept in the fridge
2	same as 1	no addition, reaction duration extended.
3	same as 1	- 2 µL of 1 M $NaCNBH_3$ freshly dissolved in THF
4	no reducing agent added	- 2 µL of 1 M $NaCNBH_3$ freshly dissolved in THF
5	no APTS added	- 2 µL 0.04 M APTS - 2 µL of 1 M $NaCNBH_3$ freshly dissolved in THF
6	no APTS and no reducing agent added	- 2 µL 0.04 M APTS - 2 µL of 1 M $NaCNBH_3$ freshly dissolved in THF

Table 3.2. Design of experiments testing the combined effect of reducing agent and catalyst

All reaction mixtures were dialysed on floating membranes as a purification step (see Section 3.2.4.1), and their resulting volumes were measured before injection onto the CE instrument (see Table 3.5 in Section 3.3.4). A 37 cm × 50 µm coated capillary was used. Before and after each sample injection, water was injected hydrodynamically for 1 s at 0.5 psi. Pressure injection at 0.5 psi was used for 3 s.

3.2.4 Post-derivatisation sample clean up

3.2.4.1 Preparation of samples treated with drop-dialysis and centrifugation

Ten replicates of the 256 kDa *kappa* sample (8 µg) were derivatised according to Protocol 3 with an APTS concentration of 0.04 M. Sample 1 was centrifuged three times for 15 min each time. Extra centrifugation was then performed until no further liquid passed through the membrane. This resulted in a recovery volume of 2 µL. Samples 2, 6, 7 and 8 were centrifuged three times for precisely 15 min each time. This resulted in different retentate

volumes in the range of 10-14 µL. The volume of all retentates was made up to 100 µL. Sample 5 was kept as a reference. No treatment was made and it remained the intact reaction mixture. Samples 3, 4, 9 and 10 were dialysed using the drop-dialysis method. Times chosen were 6 h (sample 3) and 13 h (samples 4, 9 and 10). Dialysis volumes were small in the cases of 3 and 4, for which the dialysis took place in a Petri dish, whereas the other two samples were dialysed in a 400 mL beaker. After purification, the volume of the carrageenan solution was increased to 100 µL by addition of water.

All analyses were performed in a 47 cm × 50 µm PVA coated capillary thermostatted at 25 °C; conditions for EKI were 3 s at -10 kV. For pressure injection 0.5 psi was applied for 10 s. In both injection modes water was injected hydrodynamically for 3 s at 0.5 psi before and after each sample injection.

Samples 6, 7 and 8 were injected hydrodynamically in triplicate directly from the final 100 µL solution. The carrageenan concentration in the sample solution was 0.08 mg mL^{-1}. They were also investigated using EKI. For that purpose the samples were diluted 20 times (i) in water (ii) in a 50 µM solution of sodium heptane sulfonate, resulting in a concentration of 0.004 mg mL^{-1} for each solution.

3.2.4.2 Preparation of samples treated with commercial dialysers and floating membranes

Triplicate derivatives of *lambda* and *iota* standard carrageenans, and 9 replicates of *kappa* standard were prepared ; each time a volume of 32 µL of 1 mg mL^{-1} carrageenan solution was derivatised according to Protocol 3 using a solution of 0.04 M APTS.

For each carrageenan type, one total reaction mixture was transferred to a Slide-A-Lyzer mini dialysis unit. Slide-A-Lyzer mini dialysis units are very small plastic tubes, the flat bottom of which consists in a membrane. The advantage with these is that they are shut and held in a floating buoy, and can be exposed to successive dialysis solutions to improve dialysis, unlike the floating membranes. They were left for 24 h floating in a 500 mL beaker of ultrapure water with 4 changes of dialysing solution.

One of the *kappa* replicates was dialysed for 1 h 30 min on a floating membrane as described

in Section 3.2.4.1. Its volume increased to 105 µL. Then 30 µL of this solution was dialysed identically, lyophilised and redissolved in 30 µL ultrapure water for direct comparison.

The same experiment was repeated using a 1 mM solution of ammonium acetate to dialyse 25 µL of *lambda*, *iota*, and *kappa* reaction mixtures for 1 h on floating membranes. The resulting drops were integrally transferred to a second membrane and dialysed for another hour against a fresh solution of the same salt. These solutions were lyophilised for 4 hours and redissolved in 25 µL before CE injection. A third dialysis was performed for an extra hour, this time without lyophilisation, and the resulting solutions were injected to test whether there was any improvement in removal of fluorescent material.

These samples were investigated using a 57 cm × 50 µm PVA coated capillary; PI was performed for 3 s at 0.5 psi. The data collection rate was 5 Hz. Before and after each sample injection, water was injected hydrodynamically for 3 s at 0.5 psi.

3.2.4.3 Preparation of samples treated with SEC

Preparation of the columns

A 0.05 M stock solution of disodium carbonate (0.1 M in Na^+) was prepared and used as eluent throughout the experiment. The gel phase (2 g) was mixed with 30 mL of eluent and handled according to the supplier's recommendations. After gentle swirling, the gel was left to swell for 4 h. Then the supernatant was carefully removed with a Pasteur pipette, and sodium carbonate solution was added to reach the same volume as previously and the mixture shaken gently. The gel was left to settle for 15 min; the operation was usually repeated twice, to ensure efficient removal of any fine particles present in the gel. When the gel was ready it was shaken again and a portion of the suspension immediately transferred with a plastic pipette to a 1 mL syringe, with outlet blocked with a piece of cotton wool (see Fig. 3.2). Flexible tubing was attached to the outlet, and an adjustable clip positioned here. Another piece of tubing of small internal diameter was attached after the section with the clip in order to ensure a small volume for each eluting drop (~25 µL). While the gel was settling, some more suspension was added to fill up the syringe to the desired height. It was rinsed with several column volumes of eluent. Precautions were taken not to let the gel dry. When the gel column was not in use for separation, the clip was used to stop flow through

the tubing, and the top of the syringe covered by plastic film to prevent evaporation.

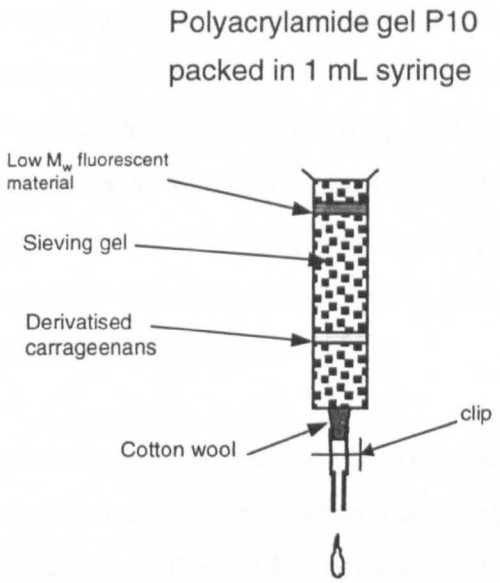

Figure 3.2. Preparative column for SEC, based on a syringe.

Sample treatment

Derivatives of *kappa* (60 µg), *iota* (60 µg) and *lambda* (100 µg) were prepared according to Protocol 3, with a concentration of APTS of 0.2 M. After incubation, 10 µL of the reaction mixtures was dispensed on the top of the column. Eluent was added constantly. The flow rate was about 50 µL min^{-1}. Output from the column over the first 3 min was collected together, and after that all drops were collected separately, giving a volume of 25 µL per fraction. Fractions were injected directly onto the CE capillary using the experimental conditions described in Section 3.2.4.2.

3.2.5 Methylene blue method

Spectroscopic measurements were performed in 1 mL disposable polypropylene cuvettes according to the protocol described by Soedjak (1994). The absorbance of the carrageenan-methylene blue complex was measured at 559 nm using a double-beam UV-Vis spectrophotometer (UVIKON 810, Kontron). A stock solution of carrageenan of concentration 0.25 mg mL^{-1} was prepared. The calibration curve was made as follows: 0, 20, 40 or 60 µL of stock solution (corresponding to 0, 5, 10 or 15 µg of carrageenan) was

dispensed in the cuvette; water was added to bring the volume up to 900 µL; 100 µL of methylene blue of concentration 0.41 M were finally added and the cuvette was shaken. Bubbles were removed and the absorbance measured for each solution in the series of increasing amount of carrageenan. The absorbance was zeroed using two cuvettes of ultra pure water. The reference was the mixture containing 900 µL of water and 100 µL of methylene blue. This solution had an absorbance of 0.420 at 559 nm, which was thus the y-intercept of the calibration curve of absorbance versus carrageenan concentration. The equation of this calibration line was calculated and used for determination of the amount of carrageenan present in the samples investigated.

This spectroscopic method was applied to estimate the recovery of microcentrifugation. In this experiment three replicates of 50 µg of intact *kappa* standard carrageenan were dispensed on three Microcon filters. The volume was made up to 300 µL, and the samples were centrifuged for 15 min at 6,000 rpm. After each spin one of the tubes was removed from the centrifuge and spun upside down to recover the retentate. 300 µL were added to the remaining filter(s) and the operation was repeated twice. This gave a representative sample for each centrifugation step. The volume was made up to 50 µL, and 10 µL of each retentate (theoretically corresponding to 10 µg carrageenan) were analysed by methylene blue complexation. The whole experiment was repeated with the fraction of carrageenan of M_w 69 kDa, which was thought to be closer in size to actual derivatives having undergone possible hydrolysis. In this case, the calibration curve was also done with the 69 kDa solution.

Derivatives treated by centrifugation were also analysed using the methylene blue method. A blank reaction belonging to this particular derivatisation set was centrifuged and used as the reference. The derivatives consisted in a calibration curve (20, 40, 60, 80, 100 µg) of *kappa* carrageenan (from Sigma), derivatised according to Protocol 3, with 0.2 M APTS. They were centrifuged 3 times for 10 min at 6,000 rpm and their volume was made up to 100 µL with water. For analysis with methylene blue, 10 µL of each retentate including that of the blank reaction were dispensed in cuvettes and analysed as described above.

3.2.6 Characterisation of the working range

For determination of the working range for *kappa* Sigma standard, a serial dilution method was used. Protocol 3 was used to derivatise 2 µg of carrageenan, using an initial concentration of 0.2 M APTS. After incubation the reaction mixture was filtered three times for 10 min at 6,000 rpm. The recovered volume was 40 µL, resulting in a carrageenan concentration of 0.05 mg mL^{-1}. This sample was serially diluted, giving concentrations of 0.01, 0.003, and 0.0015 mg mL^{-1}.

For determination of the lowest derivatisable amount, decreasing amounts of *kappa* carrageenan 10, 1, 0.1, 0.05, 0.01 µg were derivatised and treated by centrifugation in the conditions described above. The volumes varied between 10 and 30 µL. Retentates were directly injected without dilution.

These samples were investigated using a 57 cm × 50 µm PVA coated capillary; PI was performed for 3 s at 0.5 psi. The data collection rate was 10 Hz. Before and after each sample injection, water was injected hydrodynamically for 3 s at 0.5 psi.

3.2.7 SEC-MALLS-RI on native and derivatised carrageenan samples

Approximately 12-13 mg of sample were weighed in vials containing 5 mL of high purity water with 0.03 % sodium azide. After vigorous stirring at room temperature, the solutions were heated and stirred at 65-70 °C for 30 min. They were subsequently left to cool down to room temperature. For derivatisation, 40 µL (100 µg) of these solutions were dispensed into Eppendorf tubes, followed by the addition of 2 µL APTS reagent (0.2 M), 6 µL 5.0 M acetic acid, and 2 µL reducing agent (1 M sodium cyanoborohydride freshly dissolved in tetrahydrofuran). Four replicates of each sample were prepared. The reaction mixtures were heated at 55 °C for 1 h. The reaction mixtures were dispensed into microcentrifuge vials (Microcon 30 kDa molecular weight cut-off filters) and the volume brought up to 300 µL with high purity water. After vortexing, the samples were centrifuged for 10 min at 6,000 rpm in a microcentrifuge. This step was repeated three times more. The resulting volumes of the samples varied between 60 and 157 µL. For injection into the SEC system, the concentrations of the native carrageenans were 2.5 mg mL^{-1}, and the concentrations of the

derivatives approximately 1 mg mL^{-1}. Native carrageenans were diluted by a factor 2.5 in 0.2 M lithium chloride. Derivatised fractions were injected as such. The two replicates having the volume closest to 100 µL were mixed for injection in SEC-MALLS-RI. Therefore, the concentration of the injected derivatives was about 1 mg mL^{-1}. The other two replicates were not used.

The chromatographic system consisted of a Waters 515 pump, a Gilson column oven set to 60 °C, a Gilson injector with a sample loop volume of 100 µL, and two identical columns (TSK GMPWXL) in series. The eluent from the column passed through a MALLS detector (Dawn DSP-F laser photometer from Wyatt Technology, equipped with a laser of wavelength 623.8 nm), and a refractive index detector (Merck RI 71). The eluent was 0.1 M lithium nitrate containing 1 g L^{-1} EDTA, with a flow rate of 0.6 mL min^{-1}. Injection volume was set to 100 µL for all samples. Data were processed by the MALLS software (Astra, Wyatt Technology), which provided number average molecular mass M_n and mass average molecular mass M_w. Values of 0.115 mL g^{-1} and 2.62×10^{-3} mol mL g^{-2} were used for *dn/dc* and the virial coefficient A_2, respectively (Lecacheux *et al.* 1985; Hoffmann *et al.* 1996).

3.3 Results and discussion

3.3.1 Effect of variation of concentration and pH of acetic acid as catalyst

Increasing concentrations of acetic acid were used to derivatise different fractions of *kappa* carrageenan (69 kDa, 120 kDa, 539 kDa) under conditions described in Protocol 1. Acetic acid concentrations were 0.3, 0.6, 1.0, 2.25 M, the last one corresponding to 12.8 % v/v acetic acid in the reaction mixture as used in the CE method development in Chapter 2. Variation of acetic acid concentration was found to have little effect on the yield, as shown in Fig. 3.3 for the sample of M_w 120 kDa.

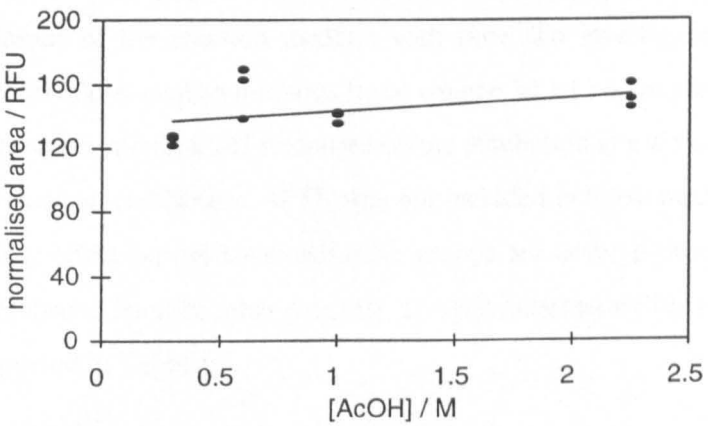

Figure 3.3. Normalised peak area for APTS-derivatised 120 kDa *kappa* carrageenan as a function of acetic acid concentration. Sample concentration 0.005 mg mL^{-1}.

Values of pH were calculated for these concentrations assuming that acetic acid (AcOH) is weakly dissociated. Then $[AcO^-] = [H^+]$ and

$$K_a = \frac{[AcO^-][H^+]}{[AcOH]} = \frac{[H^+]^2}{[AcOH]} \qquad (3.1)$$

and

$$pH = -\log[H^+] = \frac{pK_a - \log[AcOH]}{2} \qquad (3.2)$$

With $pK_a = 4.75$, the values calculated with this equation vary between pH = 2.6 for [AcOH] = 0.3 M and 2.2 for [AcOH] = 2.25 M. This study did not cover a wide range in pH. In order to determine the influence of pH on the reaction, the use of lower concentrations of acetic acid (0.001, 0.01, 0.1 and 0.63 M) was investigated for derivatisation of the fraction of *kappa* of M_w 256 kDa, which lies in the middle of the molecular weight range available. When derivatising in 14 µL as described in Protocol 1, the solution dries out very rapidly. We found that an increase in the reaction volume to 50 µL, whilst resulting in a significant dilution, gave a 30 % increase in normalised area of the CE peak. Therefore, in Protocol 2 used here, the reaction volume was increased to 50 µL.

Cyanoborohydride liberates cyanide, which is the conjugate base of a weak acid (pK_a = 9.21), and can therefore accept protons from acetic acid. This process can consequently change the conditions of the reaction medium with time. To investigate precisely the influence of pH, 1000 fold-scaled up mixtures (total volume 50 mL) were prepared for each acetic acid concentration and their pH measured before incubation in a water bath at 55 °C to reproduce the reaction conditions. APTS was not included in these mixtures, but this should not have any effect on pH since sulfonate groups are never protonated and also APTS is less concentrated than the other reagents. The pH values as well as the areas of the derivatives are reported in Table 3.3.

[AcOH]/ M	Calculated pH	Measured pH	Normalised area/RFU
0.63	2.5	2.5	413
0.1	2.9	3	223
0.01	3.4	3.9	62
0.001	3.9	8.4	5.2

Table 3.3. pH values of reaction mixtures before incubation, using different acetic acid concentrations. Normalised peak area of 256 kDa *kappa* carrageenan after derivatisation under these conditions.

These solutions were not buffered, therefore the pH varied tremendously when mixing low concentrations of acetic acid with the reducing agent solution. The observed decrease in H^+ concentration is equal to 1×10^{-4} M for [AcOH] = 0.001 M, and 3×10^{-4} M for [AcOH] = 0.01 M and [AcOH] = 0.1 M, showing that a similar amount of protons is consumed by the

reducing agent. Higher concentrations of acetic acid are not affected by this consumption of protons, and the pH remained unchanged.

With [AcOH]=0.001 M the normalised area was 80 times lower than with 0.63 M. This suggests either that reaction using 0.001 M AcOH was extremely slow, or that hydrolysis was not occurring.

When investigating the scaled-up volume synthetic mixtures, production of hydrogen bubbles was observed to cease after about one hour. This suggested that cyanoborohydride reacts with acetic acid for a limited period of time. We could shorten the reaction time to an hour since probably no Schiff's base reduction can take place after the cyanoborohydride has been consumed in the reaction with acetic acid. The reactions involved during the derivatisation process are written below (Borch *et al.* 1972):
with the Schiff base

$$3(\text{-CH = NR}) + H^+ + CNBH_3^- + 3H_2O \longrightarrow H_3BO_3 + HCN + 3(-CH_2NHR)$$

with the aldehyde

$$3(\text{-CH = O}) + H^+ + CNBH_3^- + 3H_2O \longrightarrow H_3BO_3 + HCN + 3(-CH_2OH)$$

with acid

$$H^+ + CNBH_3^- + 3H_2O \longrightarrow H_3BO_3 + HCN + 3H_2$$

In the case of 0.63 M acetic acid, the pH of the reaction mixture was measured after incubation. It was found to be 3.4. In an attempt to understand the increase from the original pH of 2.5, we assumed that, after an hour, the cyanoborohydride (of concentration 0.04 M in the mixture) had reacted on a 1 to 1 basis with H^+ (from acetic acid) to form HCN and acetate. Then the pH of the solution is described by the following equation:

$$pH = pK_a + \log\frac{[AcO^-]}{[AcOH]} = 4.75 + \log\frac{0.04}{0.59} = 3.58 \qquad (3.3)$$

This is close to the observed value, and shows that the reaction between acetic acid and

cyanoborohydride occurs within an hour. When HCN is in excess over acetic acid, the pH of the mixture is governed by an equation analogous to Eq. 3.3 but with HCN as the acid. This explains why a pH of 8.4 was observed in the reaction mixture with 0.001 M AcOH (see Table 3.3).

Derivatisations were also performed using acetic acid/acetate buffer solutions of concentration 0.1 M and of defined pH values 4.0 and 4.8. The normalised peak area for the reaction performed at pH 4.8 was 30 times lower than that of the pH 2.5 derivatised on the same day as a reference. This is only slightly higher than that for the lowest concentration mentioned above ([AcOH] = 0.001 M). When plotting the log of normalised area *vs* pH, the correlation between the three points of pH 2.5, 4.0 and 4.8 was good ($r^2 = 0.992$) and the slope found was -0.69.

3.3.2 Effect of citric acid

Replacement of acetic by 0.15 M citric acid caused a dramatic effect on the yield, with a significant increase in normalised peak area of 18 % for 120 kDa and 280 % for 539 kDa. However, increase of the citric acid concentration from 0.15 M to 0.6 M seemed to induce complete degradation of the carrageenan, with no material migrating at *kappa* migration time. The pattern of the electropherogram looked very much like a ladder, as shown in Fig. 3.4, and is investigated in Section 3.4. This series of samples was lyophilised, which removed acetic acid but not citric acid, then they were redissolved and kept at -18 °C. Derivatisation with 0.6 M citric acid was repeated with the 256 kDa fraction, with immediate filtration, and the same degradation occurred. Therefore it can be asserted that degradation happened during, and not after incubation. If hydrolysis is almost total in the case of 0.6 M citric acid it may occur for lower concentrations but to a lesser extent, and simply reduce the molecular weight of the carrageenan.

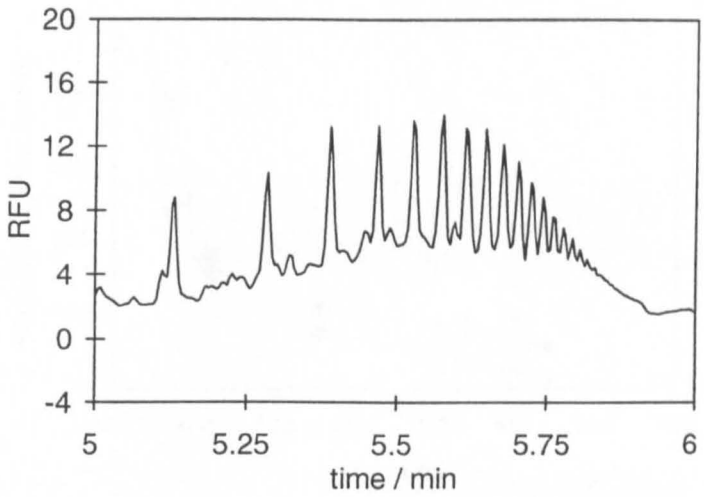

Figure 3.4. Electropherogram of APTS-derivatised *kappa* carrageenan oligomers, the product of derivatisation of the 539 kDa *kappa* fraction in the presence of 0.6 M citric acid. CE conditions : 57 cm × 50 µm PVA coated capillary; EKI: 3 s at -10 kV.

Having several fractions derivatised under the same conditions (69, 120, 256, and 529 kDa) allowed us to compare normalised peak areas using acetic and citric acid as catalysts, and to correlate the results with the molecular weight of the samples. Standards conditions were 2.25 M acetic acid or 0.15 M citric acid; because the effect of acetic acid concentration was not significant in the range studied we used the 0.3 M acetic acid data for the 69 kDa derivative value since 2.25 M acetic acid data was not available for this M_w sample. In Fig. 3.5 normalised peak area is plotted as a function of M_w: the mass concentration was identical for all samples. The normalised area is seen to decrease in an approximately inverse relationship with increasing molecular weight. This is consistent with the fact that the number of reducing ends, thus the fluorescence signal, should be inversely proportional to the molecular weight. Correlating the logarithm of the normalised peak area versus the logarithm of the M_w in the range 69 to 539 kDa (acetic acid catalysed derivatisation) leads to a slope of -1.3 and a correlation coefficient $r^2 = 0.94$.

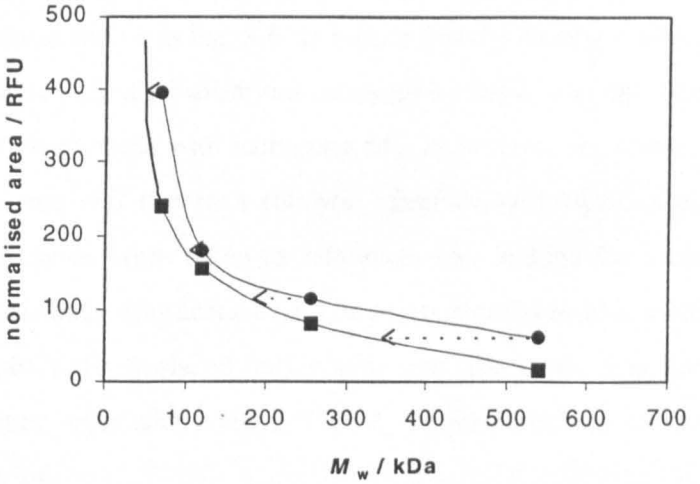

Figure 3.5. Variation of normalised area with M_w for APTS-derivatised *kappa* carrageenan with reaction catalysed by (■) 2.25 M acetic and (●) 0.15 M citric acid. Sample concentration 0.005 mg mL^{-1}.

Migration times seemed to depend on the M_w as well, but the slight differences might be caused by run-to-run variations. Consequently it was decided to use APTS as an internal standard for migration time, in order to allow direct comparison of the migration behaviour of the different M_w fractions injected one after the other. The relative migration time (*RMT*), defined below, was calculated for all of the derivatives.

$$RMT = \frac{t_m(kappa)}{t_m(\mathrm{APTS})} \tag{3.4}$$

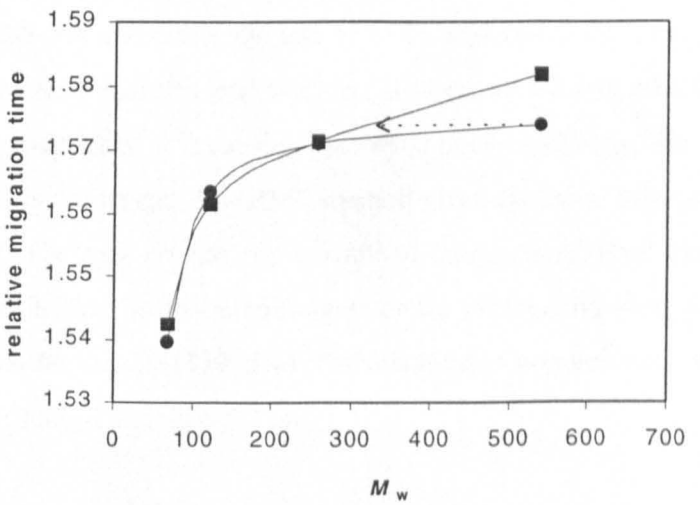

Figure 3.6. Variation of relative migration time with M_w for APTS-derivatised *kappa* carrageenan with reaction catalysed by (■) 2.25 M acetic and (●) 0.15 M citric acid. Sample concentration 0.005 mg mL^{-1}.

The resultant curve is shown in Fig. 3.6. In Figs. 3.5 and 3.6 curves were plotted through the points representing the derivatisations catalysed by acetic acid (■). The area decreases and RMT is seen to increase with increasing M_w. In order to determine if the observed increase in peak area was due to a catalytic effect on derivatisation or to a possible hydrolysis, these curves were taken as reference, i.e., making the assumption that no hydrolysis occurred when using acetic acid. The points representing the same M_w derivatised with citric acid (●) were displaced horizontally onto the acetic acid curve, in order to estimate their actual molecular weight. The M_w values found for different samples are reported in Table 3.4.

	Molecular weight / kDa			
Nominal	69	120	256	539
Estimated, area method	40	100	170	300
Estimated, RMT method	50	150	250	300

Table 3.4. Estimated molecular weights of samples derivatised with 0.15 M citric acid as catalyst, referenced to the corresponding derivatives produced in 2.25 M acetic acid as standards at nominal M_w.

Results from Table 3.4 show that the use of citric acid induces a decrease in molecular weight in comparison to that of acetic acid; this is especially evident from the area method. This is consistent with citric acid causing hydrolysis of carrageenans. The results obtained by the two methods are spread. The RMT method gives systematically higher values than the area method. The area reflects the amount of carrageenan of all molecular weights present in the distribution. Both methods agree on the fact that the most dramatic effect is seen on the highest M_w sample (539 kDa). The relationship between migration time and M_w is discussed more fully in Sections 3.4 and 3.5.

Additional experiments made with the 256 kDa fraction investigated the effect of citric acid combined with the possible influence of the solvent in which sodium cyanoborohydride is prepared (see Section 3.2.3.1). Derivatisation was performed with 0.15 and 0.3 M citric

acid, plus 2.25 M acetic acid as a reference. In each case, two experiments, one with an aqueous solution, the other with an organic solution (THF) of reducing agent, were carried out. The results are displayed in Fig. 3.7. These experiments confirmed that derivatisation with citric acid as catalyst gives a product with a larger signal than does derivatisation with acetic acid. In this set of experiments, normalised area increased with increase in citric acid concentration. When incorporating results from the previous set of experiments, the concentration for highest yield with citric acid was found to be 0.3 M; larger concentration (e.g. 0.6 M) induced complete hydrolysis. The results correlate with acid strength and calculated pH values. Citric acid has a lower pK_a ($pK_{a1} = 3.14$) than acetic acid ($pK_a = 4.75$). Using equation 3.2, the pH of the reaction media using 2.25 M acetic acid, 0.15 M and 0.3 M citric acid are respectively 2.2, 2.0 and 1.8. From Fig. 3.7 it can be seen that aqueous $NaCNBH_3$ gave a lower reaction yield than a THF solution of the reducing agent, consequently organic solutions of sodium cyanoborohydride were used in subsequent experiments. More details about the use of sodium cyanoborohydride are reported in Section 3.3.4.

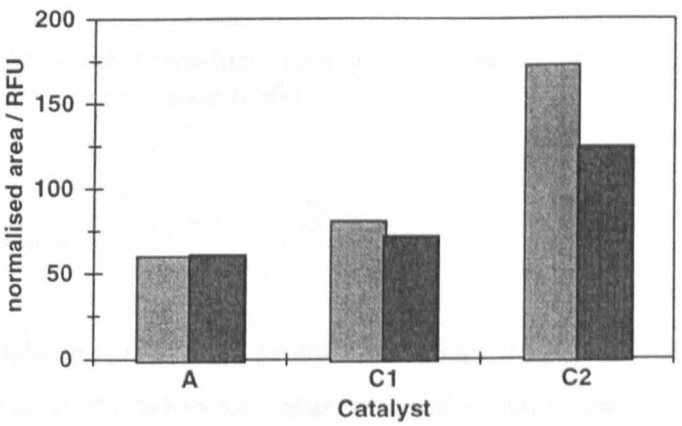

Figure 3.7. Variations of normalised areas for 256 kDa *kappa* carrageenan derivatised with (A) 2.25 M acetic acid, (C1) 0.15 M citric acid and (C2) 0.3 M citric acid, using THF or water as solvent for $NaCNBH_3$. THF: light grey bar; water: dark grey bar. Sample concentration 0.002 mg mL^{-1}.

3.3.3 Effect of variation of reaction time

A series of identical reaction mixtures using the standard conditions (2.25 M acetic acid) was incubated for increasing periods of time (1, 2, 3, 6, 22 h). The result is shown in Fig.

3.8. Electrokinetic injection was not very reproducible, as shown by the range of three injection replicates at each reaction time point. However, it is evident from the slope of the line that increase of reaction time beyond 1 h has little influence on the derivatisation yield. We concluded that we could reduce the reaction time if needed. This was in agreement with scaled-up experiments investigating the pH of the reaction medium and reported in Section 3.3.1.

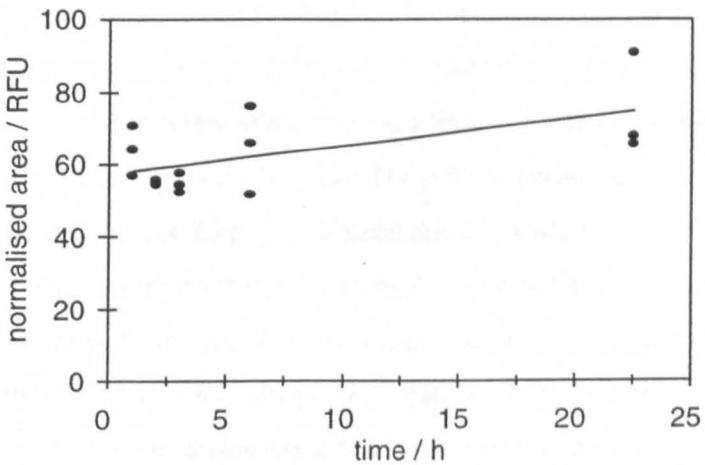

Figure 3.8. Variation of normalised area with incubation time for 256 kDa *kappa* carrageenan. Sample concentration 0.004 mg mL^{-1}.

3.3.4 Reducing agent

Some authors, including Novotny's group, used water to prepare the reducing agent solution (Hong *et al.* 1998). When the 1 M solutions of sodium cyanoborohydride reducing agent were prepared in water and in THF, both of them were saturated. It should be noted that the solubility of the salt was found to decrease on aging. A clear 1 M solution could be prepared only from a new bottle of salt. A major satellite peak was observed in the electropherogram following use of the freshly dissolved reducing agent, but not when using the commercial 1 M solution, which contains stabilisers. The fluorescent material giving this peak was supposed to originate from a side product of the label. This suggests a loss of efficiency in the reducing power of the compound when stabilised. We suspect efficient reactions occurring between cyanide and the aromatic ring and/or the sulfonate substituents.

All the satellite peaks appear after that of APTS, which could be explained by either a reduction in charge or an increase in size.

A systematic study was done on the effects of acetic acid as catalyst and sodium cyanoborohydride as reducing agent. In order to assess the actual effect of each reagent the reaction time was lengthened to 2 h, the first hour devoted to a particular influence and the second to derivatisation itself. Only a small proportion of acid is consumed during the reaction process, therefore no acid was added for the second step when it was present in the first hour of the reaction. On the other hand, when required, the reducing agent solution was freshly prepared when it had to be added a second time. All details are reported in Section 3.2.3.6, Table 3.2, in the experimental section. The standard protocol is actually represented by Exp. 1 (1 h incubation) but Exp. 2 (2 h incubation) should be the reference for direct comparison of all other variations in the Protocol. Exp. 3 is designed to provide information on the reactivity of the reducing agent, in particular relative to the reduction of the Schiff's base formed when APTS reacts with the polysaccharides. Exp. 4 and Exp. 6 investigate the effect of the presence of acetic acid in the absence of reducing agent, that is to say, the pure hydrolysis of polysaccharides. In the case of Exp. 4 APTS is added, whereas it is not in Exp. 6. Exp. 5 investigates the actual reductive hydrolysis (in the absence of APTS) in the first step.

The resulting electropherograms were processed and normalised areas were calculated for all samples. To take into account the fact that the recovered volume varied from one sample to another the normalised areas had to be further corrected. For convenience areas were multiplied by the recovered volume and divided by 100, effectively representing the fluorescence signal that would be obtained if the sample had been recovered in 100 µL. The resulting values are reported in Table 3.5.

$$\text{corrected area} = \frac{\text{normalised area} \times \text{recovered volume}}{100} \qquad (3.5)$$

Experiment	volume recovered / μL	normalised area / RFU	corrected area / RFU
1	112	47.6	53
2	125	57.7	72
3	114	58.6	67
4	120	199.2	239
5	123	21.9	27
6	119	228.9	272

Table 3.5. Normalised and corrected areas for experiments 1-6 investigating the effect of cyanoborohydride on the reaction. Details for each experiment are described in Table 3.2 in the experimental Section 3.2.3.6.

The RSD on dialysed samples, to be reported in Section 3.3.6.3, was found to be 3 %, therefore all differences between the values of corrected area are significant. There was a 35 % increase in area when the sample was left to incubate for an extra hour. This is in contrast with earlier results reported in Section 3.3.3, where the derivatisation yield was found to be essentially unchanged with increase in reaction time. The present derivatives (Exp. 1-6) were dialysed and injected hydrodynamically, whereas the previous samples derivatised for 1-22 h were centrifuged and injected by EKI. Comparison of the two sample clean-up protocols followed by the two injection modes is reported in Section 3.3.6.4, and indicates that dialysis followed by PI is more reliable. Therefore, derivatisation yield probably does increase with longer reaction time, as seen here for Exp. 2. Addition of fresh reducing agent after one hour (Exp. 3) resulted in a slight decrease in the yield (7% in comparison to Exp. 2). An increase in the yield due to the reduction of more Schiff bases might have been expected. Results in fact show that the amount of $NaCNBH_3$ used in the first hour was sufficient, and adding more of it resulted in a decrease of the number of available reducing ends and prevented further reaction with the label. This experiment confirms that $NaCNBH_3$ has the ability to destroy the reacting sites of the sugars under our reaction conditions at pH 2.5. This is in agreement with the reported increased reactivity of cyanoborohydride below pH 4, pointed out in the introduction to this Chapter (Section 3.1.1). Exp. 4 and Exp. 6 both showed tremendous increases in yield, 230 and 280 %, respectively. The values are especially meaningful because they show the "pure" effect of acetic acid. The lower value obtained for Exp. 4 is probably due to consumption of acetic

acid in the derivatisation process of all reducing ends produced by hydrolysis. When $NaCNBH_3$ was left to react with the polysaccharides at the same time as hydrolysis occurs it destroyed reacting sites on the sugars, converting aldehyde into alcohol (Exp 5). The value of corrected area in Exp. 5 (27 RFU) should be compared to that of Exp. 6 where hydrolysis was occurring without reduction (272 RFU). The effect of the reducing agent is seen to be tremendous, eliminating 90 % of the reducing ends possibly derivatisable.

3.3.5 Comparison of APTS-derivatives of *kappa* oligomers and small neutral sugars

Di-, tetra-, and hexa- saccharide oligomers of *kappa* carrageenans were derivatised, as were three small neutral sugars, glucose, fructose and sucrose. Direct comparison of their relative migration times reported in Table 3.6 shows a clear difference in their electrophoretic behaviour, the *kappa* oligomers migrating faster than the neutral sugars. As far as desulfation is concerned our results suggest that little desulfation occurs with *kappa* carrageenan. In the series of oligomers arising from the degradation with citric acid (Fig. 3.4), no peak is present at the relative migration time typical for neutral mono- and disaccharides. Thus, small oligomers of DP 1, 2 and 3 should not lose their sulfate group under the standard derivatisation conditions.

Charged oligomers		Neutral oligomers	
***kappa*-disaccharide**	1.18	**Glucose**	1.33
***kappa*-tetrasaccharide**	1.23	**Fructose**	1.33
***kappa*-hexasaccharide**	1.32	**Sucrose**	1.34*

Table 3.6. Relative migration times (with respect to APTS, free label) of oligomers.
* This indicates hydrolysis.

Fructose gave normalised areas ten times lower than that of glucose, which is consistent with its being a ketone and not an aldehyde, and therefore not as easy to derivatise by reductive amination. The fact that sucrose displays a peak at a similar migration time to that of the monosaccharides suggests a small amount of hydrolysis of this particular disaccharide to glucose and fructose.

3.3.6 Sample treatments

Initially all experiments used microcentrifugation to remove undesired fluorescent material from the retentate. However, it was found by rinsing the membrane with water after spinning down the retentate that some derivatised *kappa* carrageenan was still present on the membrane filter. This was also observed when working with other polysaccharides. Therefore, the use of other means of salt and label removal was investigated. Biologists working in protein crystallisation currently use beads covered with a membrane for desalting purposes, but trials made with coloured solutions convinced us that this would induce a minimum of 30 % loss of starting material during manipulation, especially when fixing the membrane onto the bead. Another option used in protein desalting (Marusyk & Sergeant1980) and DNA desalting (Bruin *et al.* 1995) is the so called "drop dialysis". It consists in dispensing the sample solution (50-100 µL) onto a cellulose acetate / nitrate membrane of desired pore size (0.05 or 0.025 micron) floating on water in a Petri dish. Fig. 3.9 shows the process with APTS diffusing in a concentrated stream down to the bottom of the receptacle. The membranes can be ordered in different sizes: the smallest diameter (13 mm) was used here. Large biopolymers diffuse much slower than the salts present and this allows a quick desalting of the sample, which after 20 minutes to an hour can be drawn off the membrane using a pipette. DNA and carrageenans have rod-like conformations and are therefore even less likely to diffuse through the membrane than neutral biopolymers of similar M_w, which adopt random coil conformations with smaller hydrodynamic radii. The dialysis behaviour of oligomers of alginate and *kappa* carrageenan has previously been investigated using membranes of different MWCO (Knutsen *et al.* 1993). It was found that for DP > 6 the rigid conformation of these small molecules prevented them from diffusing through membranes of theoretically appropriate MWCO range.

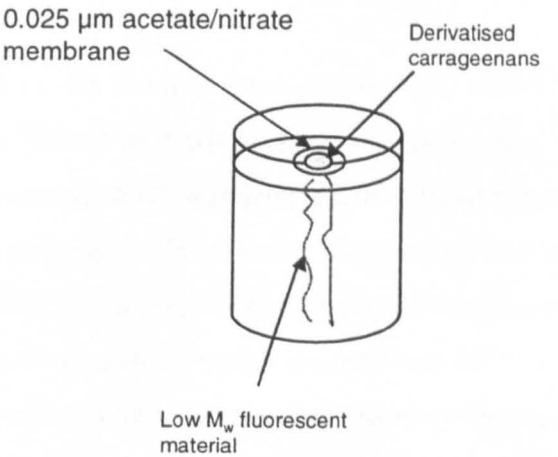

Figure 3.9. Schematic diagram of the dialysing process using floating membranes.

The repeatabilities of centrifugation and dialysis were investigated and compared. Other dialysis devices were used and experiments were carried out using preparative SEC.

3.3.6.1 Centrifugation and EKI: Protocol 1

Three derivatisation replicates of the *kappa* fraction of M_w 256 kDa were prepared, centrifuged and injected according to Protocol 1 with 2.25 M acetic acid, which was the concentration used previously in method development. The sample concentration for EKI was 0.004 mg mL^{-1}. Each derivatisation replicate was injected 3 times, an average value for normalised area was calculated for each of them separately (intra-sample repeatability), and the relative standard deviation was calculated with these values (inter-sample repeatability). The RSD between reaction replicates was found to be 24 %, which was high. When the second half of the reaction medium was filtered and investigated using an identical procedure, it showed the same relative variations between samples (replicate 1 > replicate 3 > replicate 2). All values were systematically higher than the first filtration batch, and the RSD reached 37 %. Using APTS as an internal standard was attempted as described in the previous chapter, but whilst this method improved intra-sample injection RSD, inter-sample repeatability on relative normalised areas was still high (22 %). APTS, being a small molecule, is not filtered identically on different membranes and the normalised area of carrageenan divided by that of the label showed the same discrepancies as did the absolute

value of normalised area. To be considered as an internal standard, APTS should be totally removed and then added to the carrageenan sample in known amount before injection.

Further derivatisations of this fraction were performed in triplicate, this time the total reaction mixture being filtered as a whole. Care was also taken in these filtrations to centrifuge the samples until no eluate was detected, in order to minimise the volume of the retentate and thus, the presence of salts. In these conditions the inter-sample reproducibility was found to be 9 % which is satisfactory considering all the intermediate steps required. This value of the RSD allows us to consider as significant the variations observed when replacing acetic with citric acid, investigating the effects of varying reaction time and the concentration of acetic acid. A sample derivatised with 0.04 M APTS showed a signal within the range of the 0.2 M APTS-derivatised replicates. The repeatability of the reaction with 0.04 M was investigated and the RSD found to be 4 %. APTS being an expensive label, all the following derivatisations reported in this Chapter and some in Chapter 4 were performed with 0.04 M solutions of APTS (Protocol 3).

3.3.6.2 Milder centrifugation - Effects of heptane sulfonate as a sample additive for electrokinetic injection

The reproducibility of a milder centrifugation method (>10 µL recovery) described in Section 3.2.4.1 was estimated on samples 6, 7 and 8. The relative standard deviation was calculated for each sample (injected hydrodynamically 3 times) and for the three derivatives. The highest intra-sample RSD was found to be 3.1 %, and the inter-sample RSD was 1.2 %. These results are satisfactory.

In Section 3.3.6.1 the repeatability of the 2 µL retentate method was investigated by EKI on diluted samples, therefore it was estimated on samples 6, 7 and 8 as well. We investigated the use of sodium heptane sulfonate (HS) as a sample additive at low concentration, in order to control the ionic strength and produce a constant injected amount. HS is a low-mobility additive used in isotachophoresis and to aid stacking of anionic analytes (Jones & Jandik 1992). It has a high critical micelle concentration, and at

concentrations below this value of concentration it increases the ionic strength of the sample without influencing the migration, since it is a slow moving ion. With heptane sulfonate the intra-sample RSD for EKI was below 3 % for 2 of the replicates, and 35 % for the last one. Inter-sample RSD was found to be 34 %, which was high, considering that HS should provide a constant ionic strength for all samples, and thus more uniform conditions for EKI. With water repeatability was worse for individual injections (RSDs 13, 4, and 17 %) and the inter-sample RSD value was 45%. The variations of the average normalised areas matched the variations of retentate volume. This was expected since the higher the retentate volume, the higher the residual ionic strength and the lower the injected amount in electrokinetic mode.

3.3.6.3 Repeatability of the drop-dialysis method

Slight modifications were made to the drop-dialysis method reported for DNA and proteins, especially concerning the time. Carrageenans are high-M_w molecules which have lower diffusion coefficients than the salts and fluorophore, therefore they can be left on the membrane a long time without loss. When dialysis was performed in a Petri dish (samples 3 and 4), there were little volume variations for the sample drop, but in a beaker the volume of samples 9 and 10 were increased to 250 and 220 µL respectively. Their normalised areas were corrected by a factor 2.5 and 2.2, respectively. Results are shown in Table 3.7.

Sample	volume /µL	norm. area	RSD / %	corrected norm. area
3	100	86.2	2.2	86.2
4	100	92.9	2.1	92.9
9	250	37.2	2.4	93
10	220	41.3	0.6	90.9

Table 3.7. Results for the 4 samples purified by the drop dialysis method. Sample concentration 0.08 mg mL^{-1}, 10 s hydrodynamic injection.

Individual RSDs were lower than 3 %. After correction due to the difference in concentration the average area was calculated to be 90.7, with an RSD of 3.5 %. This value of inter-sample repeatability was excellent, showing that this method is amenable to the

production of reliable results. It also shows that the duration of the dialysis does not have a significant effect on the recovery, confirming that carrageenans cannot pass through the membrane barrier. Fig. 3.10 shows superimposed electropherograms of a reaction mixture and a dialysed sample of the same concentration. This suggests that the loss on the filter is minimal and that dialysis is the softest method to clean up carrageenans after derivatisation.

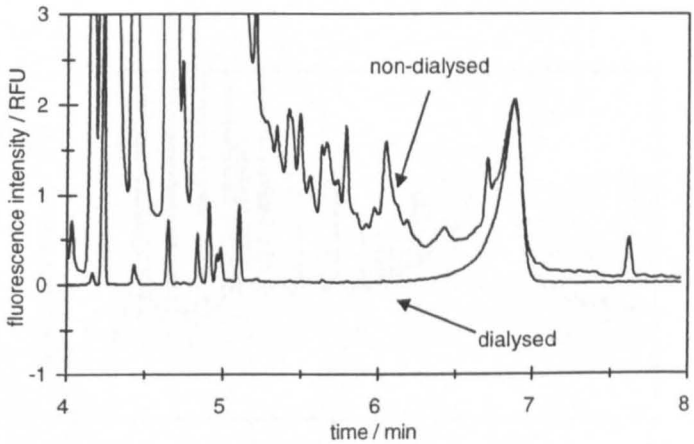

Figure 3.10. Superimposed electropherograms of a reaction mixture before and after dialysis. Sample concentration in both cases 0.4 mg mL^{-1}. CE conditions: 57 cm × 50 µm PVA coated capillary; PI: 3 s at 0.5 psi; data collection rate: 5 Hz.

The possibility of performing several dialyses on the same sample was studied. Experiments are described in Section 3.2.4.2. When dialysing with pure water and lyophilising, it was found that the carrageenan had been degraded, as shown in Fig. 3.11. This electropherogram was cleaner than the one obtained after only one dialysis. The peak for *kappa* was present but smaller than expected, flat at the top and showed a degradation pattern consisting of supposed oligomer peaks. After extensive dialysis with pure water carrageenans are partially in their acid form, protons having replaced some of the metal cations present originally, and can undergo autohydrolysis (Hoffman *et al.* 1996) during the lyophilising process. Therefore, if extensive dialysis is to be used, one must use salt solutions of low concentration as an alternative to pure water, so that carrageenans remain associated with cations. The three carrageenan types were investigated using successive dialyses with 1 mM ammonium acetate. Three dialyses are necessary to clearly identify *iota* and *lambda*. Fig 3.12 shows the electropherogram of *lambda* after two dialyses, lyophilisation and an extra

dialysis. All these successive steps are time consuming and increase the risk of loss of sample. They cannot be performed routinely. The samples shown in Figures 3.10, 3.11 and 3.12 were derivatised with 0.04 M APTS. Use of 0.2 M APTS in subsequent studies was preferred, in order to maximise the derivatisation yield. This resulted in an increase in number and area of the satellite peaks, and made the removal of these fluorescent material by dialysis more difficult.

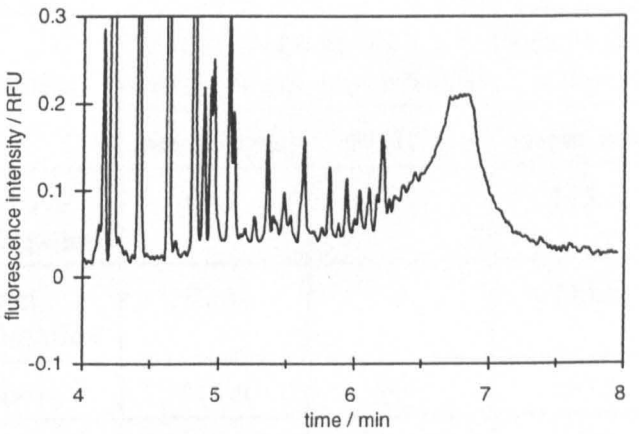

Figure 3.11. Electropherogram of *kappa* carrageenan treated successively with two dialyses with ultrapure water, lyophilisation and redissolved in ultra-pure water. Sample concentration 0.3 mg mL^{-1}. Other experimental conditions as in Fig. 3.10.

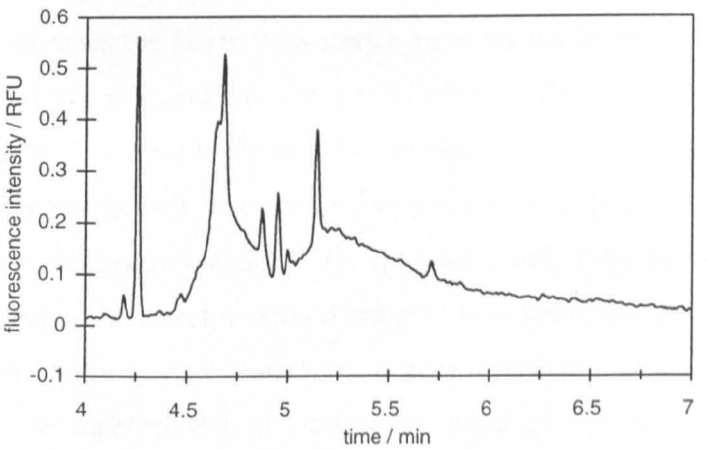

Figure 3.12. Electropherogram of *lambda* carrageenan treated successively with two dialyses with 1 mM ammonium acetate, lyophilisation and one dialysis with 1 mM ammonium acetate. Sample concentration 0.7 mg mL^{-1}. Other experimental conditions as in Fig. 3.10.

3.3.6.4 Comparison between microcentrifugation and drop dialysis, and pressure and electrokinetic injection

Samples 1, 2, 3 and 4 were injected one after the other directly by pressure injection (PI) from the 0.08 mg mL^{-1} solutions. EKI was also performed on 20 fold dilutions as described above for samples 6, 7 and 8. Results are given in Table 3.8.

Sample	Treatment	PI (10 s) of 0.08 mg mL^{-1} sample in aqueous solution		EKI (3 s) of 0.004 mg mL^{-1} sample in HS solution	
		norm. area	RSD / %	norm. area	RSD / %
1	extensive centrifugation	60.7	2.2	115.3	5.7
2	mild centrifugation	92.1	1	235.5	7.1
3	dialysis	118.6	0.7	163.5	33.7
4	dialysis	127.6	1.6	212.8	5.2

Table 3.8. Results of EKI and PI injections (n=3) of derivatised 256 kDa *kappa* carrageenan purified by different sample treatments

Bearing in mind that peak areas depend tremendously on the salt content of each sample, these results show again the lower intra-sample repeatability of EKI with respect to PI, especially when samples of high ionic strength are injected. The absolute amounts injected hydrodynamically are lower when the sample is purified by the centrifugation method than when using the dialysis method, as evidenced by a reduction in peak area of 50 % for the extensive microcentrifugation and 25 % for the milder one. This series of experiments suggests that the microconcentrator method induces a systematic loss on the membrane, and that the lower the volume of the retentate, the larger the amount staying on the membrane. Fig. 3.13 shows the superposition of electropherograms of samples 4 (dialysed) and 1 (centrifuged).

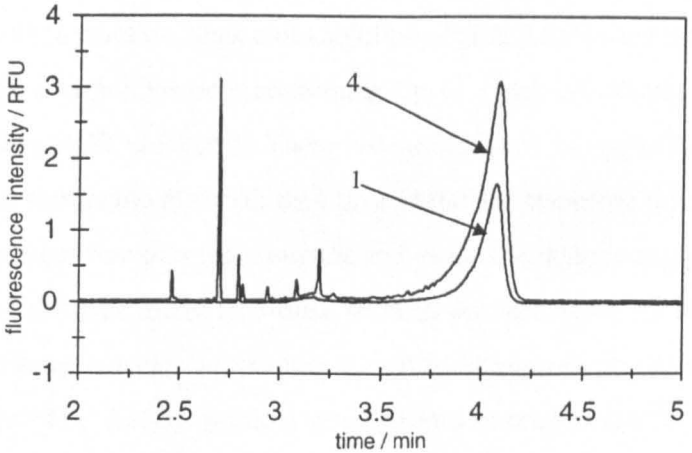

Figure 3.13. Superimposed electropherograms of dialysed (4) and centrifuged (1) reaction mixtures of APTS-derivatised 256 kDa *kappa* carrageenan. Sample concentration 0.08 mg mL^{-1}. CE conditions: 47 cm × 50 µm PVA coated capillary; PI: 10 s at 0.5 psi.

The membranes of microconcentrators are made of regenerated cellulose, and so are the membranes used for dialysis. The difference of behaviour is mainly due to the procedure, mild in the case of dialysis but strong for centrifugation. The centrifugal force scales with mass, driving the biopolymers onto the membrane with more force than small ions. This could block them inside the pores and create aggregates which would not be recovered. In the case of dialysis carrageenans undergo a softer process, where diffusion in the concentration gradient across the membrane is the only method by which the biopolymer would access the pores.

3.3.6.5 Commercial dialysers

Following promising experiments obtained with floating membranes, further dialysis experiments were made with commercial devices, Slide-A-Lyzer MINIs. These dialysers have nominal molecular weight cut-off limits specified for proteins, whereas the membranes are of defined pore size. A MWCO of 10,000 Da was chosen, which was the highest available. Initial experiments investigating carrageenan standards from Sigma were unsuccessful with these minidialysers. Fluorescent material remained with carrageenans in the device in larger proportion than with drop dialysis; this could be assessed visually, since the colour inside the dialyser remained yellow. Increasing the dialysis time and the

temperature did not yield any improvement. In an attempt to understand the origin of this unsatisfactory result, a reaction blank and a solution of pure APTS were dialysed using both these devices and a larger version containing up to 1 mL of sample (DispoDialyzers Spectra/Por®, with a MWCO 50,000). These last devices are thin, approximately 8 cm-long tubes made of the membrane material; they float vertically, therefore the surface available for exchange is greater. Samples from outside and inside the dialysis bag were analysed by CE. Only APTS itself was found to diffuse through the membrane. In both dialysers the impurities present in the commercial product as well as the byproducts of the reaction stayed inside the dialyser. The dialysis process resulted in a concentration of these interfering species with respect to APTS inside the dialysis bag. This shows that the commercial dialysers are not appropriate for our requirements. The structures and molecular weights of these impurities are unknown, but we surmise that they are condensed or aggregated polyaromatic rings of larger dimensions than APTS and therefore cannot pass the barrier. The reason why they actually are filtered through the membrane in the microcentrifugation process is that centrifugal force is applied. When only *kappa* is to be detected, drop dialysis is sufficient and avoids loss of biopolymer material. In other cases one should use microcentrifugation to be able to detect other carrageenans, being aware of the eventual losses inherent to this method.

3.3.6.6 Preparative SEC

Bio-Gel P10 was chosen because it had a fractionation range from 1,500 to 20,000 Da. It was expected to exclude derivatised carrageenans, whose M_w should be above 20,000 even after any hydrolysis occurring during derivatisation. Sodium carbonate was used in the eluent because a concentration of 0.1 M in sodium is commonly used in SEC (Viebke *et al.* 1995); under these conditions *kappa* carrageenan is in the coil state at room temperature. In initial experiments *kappa* carrageenan was loaded onto the column and the collected fractions were analysed by spectroscopy with the methylene blue method. A yellow band containing all fluorescent material migrated very slowly and required more than 30 min to reach the outlet of the column. Collection was stopped when this band eluted. Qualitative measurements were carried out on early fractions to determine if carrageenans actually eluted. Since the absorbance of the different fractions showed variations from the blank, notably a maximum at elution time 6 min, the experiment was repeated for *kappa*, *iota*, and

lambda and the fractions were injected in CE. For each type, the fractions of interest containing significant amounts of carrageenan eluted between 6 and 10 minutes elution time. Reconstituted samples were prepared by mixing the individual drops from the 4 min time window between 6 and 10 min; these had total volumes of 200 µL and were injected in CE. The electropherograms of *kappa* and *iota* are shown in Figures 3.14 and 3.15.

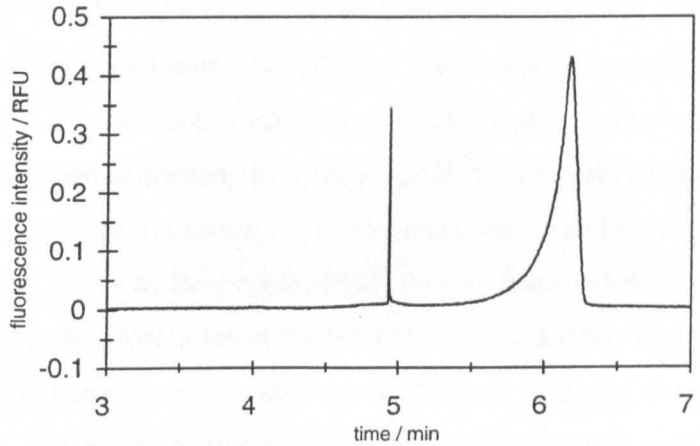

Figure 3.14. Electropherogram of reconstituted sample of APTS-derivatised *kappa* carrageenan standard purified by preparative SEC. Sample concentration 0.06 mg mL^{-1}; data collection rate 10 Hz. Other experimental conditions as in Fig. 3.10.

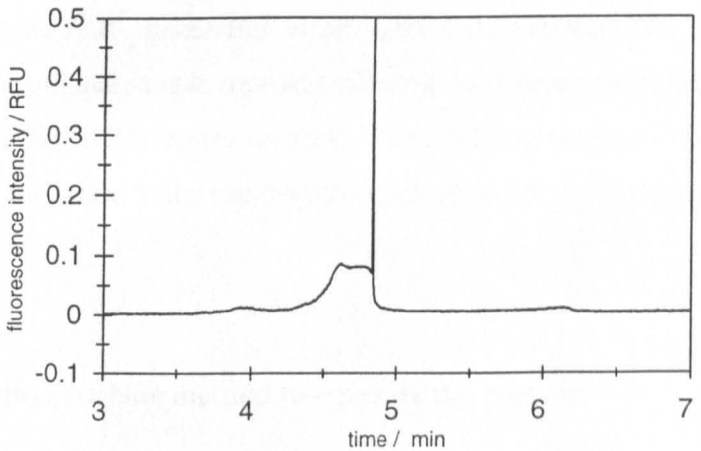

Figure 3.15. Electropherogram of reconstituted sample of APTS-derivatised *iota* carrageenan standard purified by preparative SEC. Sample concentration 0.06 mg mL^{-1}; data collection rate 10 Hz. Other experimental conditions as in Fig. 3.10.

The purified reconstituted samples are seen to be very clean, all small molecular weight

fluorescent molecules having been retained in the column much longer than the carrageenans. However, there was a spike in all electropherograms, approximately at the same time for each. It was present in all fractions, even those containing no carrageenans, but to a lesser extent than in the fractions which contained carrageenans, where the height of the spike varied in proportion to the amount of carrageenan present in the fraction. At 0.1 M Na^+, the transition temperature of *kappa* carrageenan is 11 °C (Piculell 1995, Rochas & Rinaudo1980) and that of *iota*, 31 °C (Norton 1990). Therefore a proportion of *iota* carrageenan may have been in the helical state during the preparative SEC. In 25 mM ammonium acetate the transition temperature for *iota* is estimated to be to 5 °C (Chapter 2, Section 2.3.6). When performing the analysis at 25 °C, any ordered *iota* species (double helices or aggregates) should transform to the disordered, random coil form in 25 mM ammonium acetate. Work by Bergström (1991) showed that the helical state of *iota* has a lower conductivity, therefore a lower mobility than the coil state. *Kappa* was in the coil state, and the peak shape observed was as usual. The peak shape for iota was broader than usually observed. The migration time expected for *iota* corresponds to the first part of the peak ($t_m = 4.57$ min). The spike has a slightly longer migration time than *iota*. A contribution to the peak shape is a destacking effect due to the ionic strength in the sample (100 mM Na^+, 50 mM CO_3^{2-}) being higher than that in the BGE (25 mM NH_4^+, 25 mM acetate). The carbonate ion undergoes acid-base reaction with ammonium when migrating from the sample zone to the BGE, generating bicarbonate and ammonia. The destacking causes broadening of the original sample zone and there will be transient isotachophoresis until the carrageenan and the HCO_3^- zones separate. Since the carrageenans undergo very little diffusion during migration in the capillary the peak shape of *iota* is retained.

3.3.7 Use of methylene blue method to estimate the recovery

Spectroscopy using complexation with methylene blue is a very sensitive method for carrageenan analysis (Soedjak 1994). The complexation process is subject to interferences from salts or other hydrocolloids present in the solution. To avoid competition from other polysaccharides, for example alginates, Soedjak recommends the replacement of water with phosphate buffer, which prevents complexation of other polysaccharides but does not affect

carrageenans. In our experiments on pure carrageenans, analysis was carried out in water. In the case of retentates of derivatised carrageenans, the reference was a blank retentate to see if there was any influence of residual salts or APTS. No significant difference between the absorbance of this blank retentate and that of water was observed.

The absorbance values for the carrageenan solutions of different concentrations were measured, followed by the absorbances of the samples of interest. The linear equation of the calibration curve was determined, the actual amount of carrageenan present in the cuvette was deduced from its absorbance value, and the recovery was calculated. All complexes were prepared the same way, therefore no correction for dilution was needed.

centrifugation time	recovery, *kappa* standard	recovery, 69 kDa *kappa*
1 × 15 min	86 %	100 %
2 × 15 min	91 %	98 %
3 × 15 min	94 %	106 %

Table 3.9. Recovery of intact Sigma standard and 69 kDa carrageenan fraction after 1, 2, and 3 spins performed at 6,000 rpm for 15 min. Correlation coefficient for the calibration curve was 0.9997.

theoretical / µg	found / µg	recovery / %
2	1.8	90
4	3.9	98
6	6.2	104
8	8.9	111
10	11.1	111

Table 3.10. Recovery as a function of mass of *kappa* standard derivatised.

These experiments suggest that recovery is satisfactory whether handling intact or derivatised carrageenans. A very good correlation, $r^2 = 0.998$, was found for the calibration curve of the derivatised carrageenans analysed by complexation. This method could be used

to check derivatised samples in cases where unexpectedly low values were found in CE, to assess if the problem was related to recovery or to reaction itself.

Results from the methylene blue method are different from those obtained previously when comparing centrifugation and dialysis, which suggested that a significant amount of material was trapped on the filter during centrifugation. Differences could be due to the fact that methylene blue complexation detects the total amount of carrageenan present, whereas CE-LIF detects fluorescent end groups and is therefore biased towards the low M_w part of the distribution. Further discussion of this point will be given later in Section 3.5. Some reservations must also be pointed out concerning the absolute values found. Additional experiments compared the slopes of the calibration curves with the 69 and 539 kDa fractions. The slope was found to be higher for the 69 kDa than for the 539 kDa fraction (0.0392 and 0.0355 μg^{-1} mL respectively). Similarly, probably because derivatives are hydrolysed, the slope was found to be higher for the derivatised than for the intact *kappa* carrageenan standard (0.0340 versus 0.0306 μg^{-1} mL). In this particular case the difference in slope could be due to the presence of salts. Other experiments performed in phosphate buffer showed a 25 % lower value of the slope. Use of the calibration curve prepared with intact carrageenan may therefore give an overestimate of the mass in the case of the derivatised material. The ratio of the slope of the native to that of the derivatised *kappa* carrageenan is 0.0306 / 0.0340 = 0.90. One can consider this value as a correcting factor and multiply the recovery values reported in Table 3.10, thus reducing them all by 10 %. The results are still acceptable, with 100 % recovery for the highest amounts derivatised and 80 % for the lowest amount derivatised. The differences between the native 69 and 539 kDa fractions might be accounted for by a different water content of each sample, though they were obtained via fractionation of the same original *kappa* carrageenan sample. However, this series of experiments shows that the methylene blue method is very sensitive to the ionic environment of the polysaccharide and possibly also to its molecular weight.

3.3.8 Estimation of the yield

The *kappa* carrageenans oligomers of low DP were derivatised. Direct comparison of these oligomers and a *kappa* polymer is not possible using electrokinetic injection (EKI) in CE because of the large differences in ionic strength between the retentate of *kappa* polymer

and the intact oligomer reaction mixture. To make sure both oligomer and polymer are injected in the same ionic strength conditions, mixtures of neocarrahexaose-4^{1,3,5}-tri-*O*-sulfate (*kappa* trimer) and 256 kDa *kappa* were prepared and diluted in different media in order to estimate the relative peak areas and thus, amounts of oligomer and polymer. The addition of an electrolyte such as heptanesulfonate improves the reproducibility of EKI. The media investigated were ultra-pure water, 50 µM ammonium acetate, and 50 µM HS. The hexamer concentration was 4 nM, and the mass concentration of the polymer was 0.004 mg mL^{-1}.

The concentration of labelled sites, thus the yield of the derivatisation reaction on the polymer, can be deduced from the ratio of the normalised areas. Since EKI injects analytes in proportion to their electrophoretic mobility, it does not exactly reflect the proportions present in the sample solution. The faster migrating trimer is injected in larger amount than the polymer. Therefore one has to correct these areas with the ratio of the mobilities of the analytes, given by the inverse ratio of their migration times.

$$[\text{labelled sites}] = \frac{A_n(kappa)}{A_n(\text{trimer})} \times \frac{\mu_{\text{trimer}}}{\mu_{kappa}} \times [\text{trimer}]$$

$$[\text{labelled sites}] = \frac{A_n(kappa)}{A_n(\text{trimer})} \times \frac{t_{kappa}}{t_{\text{trimer}}} \times 4 \times 10^{-9}\,\text{M}$$

Medium	RSD / %	[labelled sites] / 10^{-8} M
water	2.6	2.4
50 µM HS	1.2	2.4
50 µM AA	0.9	2.3

Table 3.11. Estimation of the concentration of APTS-derivatised molecules in 256 kDa *kappa* carrageenan of concentration 0.004 mg mL^{-1} diluted in different media. Samples were injected in triplicate.

The concentration was found to be independent of the medium and equal to 2.4 ×10^{-8} M. Since the molecular weight of this fraction is known, as well as its mass concentration in the

sample, one can calculate an approximate value and compare it with 2.4×10^{-8} M.

$$[\text{labelled sites}] = \frac{0.004 \text{ g L}^{-1}}{256 \times 10^{3} \text{ g mol}^{-1}} = 1.6 \times 10^{-8} \text{M}$$

The comparison is satisfactory since both values are of the same order of magnitude. This is not proof that no degradation occurs, since probably both hydrolysis and reduction of the aldehyde groups happen at the same time. It is reassuring to know that we do not detect a significantly larger or smaller amount of carrageenans than what is supposed to be present. The value used for M_w is a mass average molecular mass and will be higher than the number average molecular mass M_n, which is the average relevant for comparison with the CE results. Further discussion of the molecular mass averages will be given in Section 3.5.

3.3.9 Determination of the working range

There are two ways of determining the minimum detectable concentration in the case of derivatives. The value obtained by dilution reflects more the performance of the detector, and is the minimal concentration or amount of the species of interest loaded on the capillary. The value obtained when derivatising decreasing amounts of material shows the limitations of the protocol itself, and is the minimal absolute amount that can be handled. In each case this minimal value gave a detector signal equal to three times the noise level (IUPAC 1993). In the case of *kappa* carrageenan, the minimal concentration detectable after successive dilutions was found to be 1.5 µg mL^{-1}. As far as derivatisation was concerned, the minimum detectable mass of carrageenan sample derivatised was 0.1 µg. Since the volume of this particular retentate was 18 µL, this results in a concentration of 5 µg mL^{-1}. This is higher than the value obtained by dilution. The reaction is therefore the limiting factor in the method. This value of 0.1 µg means that when derivatising 10 µg of starting material, one should be able to determine 1 % of carrageenan. However, the present determination was done using the carrageenan alone. In the presence of other components the viscosity of the sample is modified and filtration is difficult.

3.4 Study of the *kappa* oligomer ladder

The use of 0.6 M citric acid as a catalyst caused substantial degradation of *kappa* polysaccharide, and produced a ladder of oligosaccharides. These oligomers were detected in the retentate, though most of this low molecular weight material had probably gone through the membrane. They were not detected in the eluate, probably due to the dilution in 300 µL resulting in very low signals hidden amongst those of the blank.

Values for relative migration times were obtained from the electropherogram of the 120 kDa *kappa* carrageenan derivatised in the presence of 0.6 M citric acid as catalyst shown in Fig. 3.4. The series of RMTs is similar to that of the 539 kDa sample derivatised under the same conditions. One peak was identified as neocarrahexaose-tetra-*O*-sulfate (trimer of regular *kappa* carrageenan). The relative migration time values included this peak and the 17 following ones.

Assuming that the mobility of all derivatives is proportional to their charge to size ratio, a simple equation for the migration behaviour is the following:

$$\mu = C \frac{n+3}{n \times m + m_{APTS}} \qquad (3.6)$$

where n is the number of residues, μ the electrophoretic mobility of the oligomer with n residues, C a proportionality constant, m the mass per residue, and m_{APTS} the increase in mass resulting from the labelling reaction. It follows that

$$\mu = C \frac{1+\frac{3}{n}}{m+\frac{m_{APTS}}{n}} = \frac{C}{m} \frac{1+\frac{3}{n}}{1+\frac{m_{APTS}}{m}\frac{1}{n}}$$

If t is the migration time of the oligomer and RMT the relative migration time, since $t \propto 1/\mu$ and RMT = t / t_{APTS} it follows that

$$RMT = K \frac{1+\frac{m_{APTS}}{m}\frac{1}{n}}{1+\frac{3}{n}} = K \frac{1+a\frac{1}{n}}{1+b\frac{1}{n}}$$

where K, a and b are parameters which can be tuned in order to fit the experimental data; a and b reflect the relative size and charge, respectively, of APTS and the *kappa* carrageenan residue.

Two options are possible when investigating the ladder. One is to consider that hydrolysis degrades the biopolymer down to the level of the disaccharide repeat unit; then the values to be taken for n are 3, 4, ...20. This is the assumption made by Sudor and Novotny when investigating the migration of *kappa*-carrageenan oligosaccharides derivatised with different fluorophores, following comparison with the migration time of D-glucose-6-sulfate (Sudor & Novotny 1995). Their assumption is consistent with the observations mentioned by Myslabodski *et al.* (1996). In our case we have *kappa* oligomer standards and can identify one of the peaks as the trimer, corresponding to 3 *kappa* repeat units. Alternatively one can suppose that the chain is hydrolysed at any linkage between monosaccharide units, resulting in a series of oligomers containing 6, 7 ...23 monosaccharide (galactose or anhydrogalactose) units. The equation above is then modified and two equations have to be considered depending on whether the monosaccharide residue carrying the reducing end is sulfated or not, which corresponds to values of n = 6, 8, 10 etc... or n = 7, 9, 11 etc, respectively. Options considering degradation to the monosaccharide or to the disaccharide were applied; experimental data were processed using non-linear least squares fits to functions. The best fit was obtained using the disaccharide model and is reported here. The molecular weight of the *kappa* disaccharide unit is m = 385.31. The ratio m_{APTS}/m was calculated to be 438.39 / 385.31 = 1.14. This was set as the initial value of a for the non-linear regression fitting, but varying this starting value did not have any influence on the final value of the parameter. Since K is the value for $n \rightarrow$ infinity, and the maximum value of t_r found earlier for 539 kDa *kappa* carrageenan is 1.582, 1.6 was taken as the initial value of K. Different levels of fitting can be used: one can choose to fix the value of b to 3 (model 1), or optimise it simultaneously with a and K (model 2). The results are summarised in Table 3.12.

Model 1	Model 2
$RMT = K \dfrac{1 + a\,\frac{1}{n}}{1 + 3\,\frac{1}{n}}$	$RMT = K \dfrac{1 + a\,\frac{1}{n}}{1 + b\,\frac{1}{n}}$
$K = 1.551$ $a = 2.091$ $r^2 = 0.999$	$K = 1.541$ $a = 1.348$ $b = 2.107$ $r^2 = 1.000$

Table 3.12. Modelling of the variation of RMT with DP, using the disaccharide as repeat unit.

Allowing b to vary did not yield a significant improvement, and $b = 3$ better reflects the charge carried by APTS than does $b = 2.107$. a represents the ratio of the frictional coefficient of APTS to that of *kappa* carrageenan, of which we can give an approximate value using the size of the molecules. An approximate of length for APTS is 1.1 nm. A value of 1 nm was used for the *kappa* carrageenan disaccharide (Knutsen *et al.* 1993). This leads to a value of a of 1.1, which is closer to the value found by Model 2. Fig. 3.16 shows the fitting in the case of the disaccharide repeat unit for $b = 3$, $K = 1.551$ and $a = 2.091$. The high values found for the correlation coefficient are promising and suggest that CE could give not only identification of carrageenans but also molecular weight information.

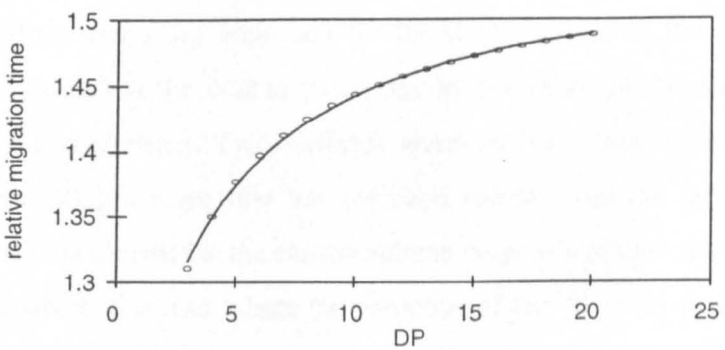

Figure 3.16. Fit of experimental relative migration times (o) with model 1 (–).

3.5 Molecular weight measurements by SEC-MALLS-RI

A typical chromatogram showing the mass concentration obtained from the RI response and the molecular mass values (M) from MALLS is shown in Fig. 3.17.

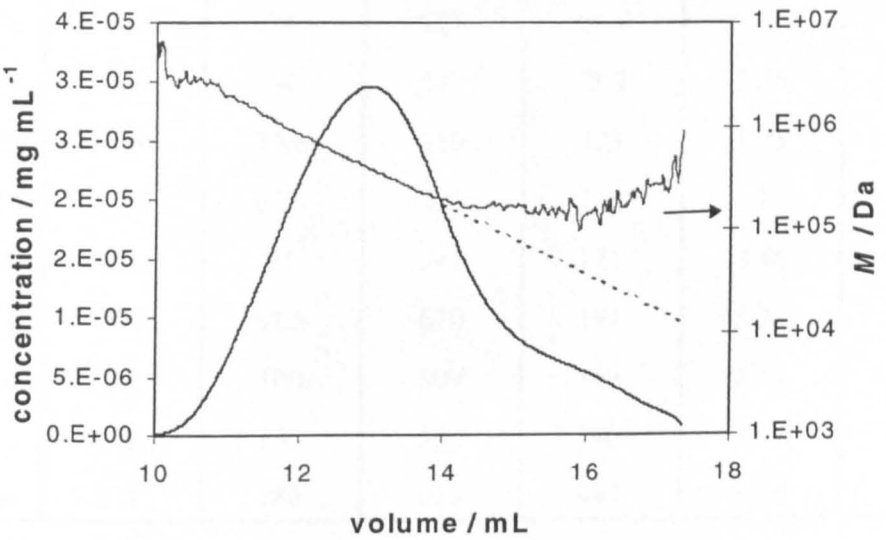

Figure 3.17. Results from RI and MALLS detector responses in SEC elution for *kappa* carrageenan, Sigma standard, non derivatised. Linear extrapolation (- -) used in calculating M at high elution volume.

As observed in previous work (Hoffmann *et al.* 1996), the plot of log M versus V was not linear for very long and short chains, of which typically very little material is present. However, the short chains are important for the determination of the number average molecular mass, which is the crucial parameter to determine in this study concerning derivatisation of carrageenans. Two methods were used to process the data. The first consisted in calculating a regression line for each sample from the raw MALLS data, extrapolating the data obtained in the elution volume range where the maximum amount of material eluted, which was also where the response of the MALLS detector was most reliable. Thus, each sample had its own calibration for M versus elution volume. With this calibration, average values M_n, M_w and the polydispersity index, $I_p = M_w / M_n$, were obtained. M_w was not significantly different from that calculated by the Astra software, but M_n was significantly lower. The regression line was slightly different from fraction to fraction, and the slope of the line obtained for each derivatised sample was slightly different

from that obtained for the corresponding native sample. The results from the method using individual calibrations are reported in Table 3.13.

	M_n / kDa		M_w / kDa		$I_p = M_w / M_n$	
sample	**native**	**derivatised**	**native**	**derivatised**	**native**	**derivatised**
69 kDa	22	24	62.9	64.4	2.86	2.68
120 kDa	42.4	34	117	73.3	2.76	2.15
205 kDa	108	77.8	189	123	1.75	1.58
256 kDa	129	67.9	231	142	1.79	2.09
366 kDa	138	91	342	171	2.48	1.88
539 kDa	204	82.5	670	191	3.28	2.31
kappa	162	100	509	193	3.14	1.93
iota	156	130	552	290	3.54	2.23
lambda	231	188	913	497	3.95	2.64

Table 3.13. Molecular masses and polydispersity index obtained using individual calibrations for native and derivatised carrageenan fractions.

The second method used a common calibration of M versus elution volume for all *kappa* samples. This regression line was obtained by averaging data in the linear range of the plots of log M_w as a function of elution volume for all fractions of native samples of *kappa* carrageenans. The sample of lowest M_w, 69 kDa, was excluded because its curve hardly presented any straight fragment, probably because its M_w was not in the fractionation range of the column material, and also because the response of the MALLS detector is not satisfactory in the low molecular weight range, where this sample has a significant proportion of material. These linear sections of the curves were the ones used previously to deduce the individual calibrations and are shown in Fig. 3.18. This figure also shows the master plot which was deduced from them. Values for M_n, M_w and I_p obtained with this general calibration are reported in Table 3.14.

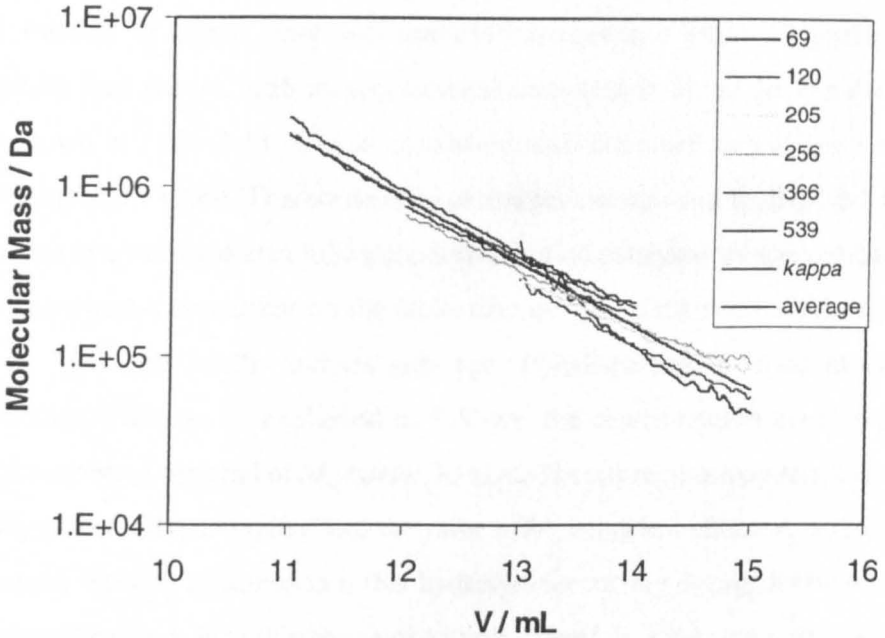

Figure 3.18. Log-linear plots of M versus V for the non-derivatised *kappa* samples. The straight line is the calibration deduced in averaging all samples except 69 kDa.

	M_n / kDa		M_w / kDa		$I_p = M_w / M_n$	
sample	**native**	**derivatised**	**native**	**derivatised**	**native**	**derivatised**
120 kDa	57.2	52.8	118	97.6	2.06	1.85
205 kDa	92.7	73.1	226	146	2.44	2.00
256 kDa	102	75.3	254	157	2.49	2.08
366 kDa	122	87.2	354	186	2.9	2.13
539 kDa	163	93.4	577	212	3.54	2.27
kappa	128	86.9	497	211	3.88	2.43

Table 3.14. Molecular masses and polydispersity index obtained using an average calibration for *kappa* samples.

Table 3.15 shows the values for the ratio M_n (derivatised) / M_n (native) calculated using both the individual and general calibrations. Different values were obtained by the two methods. Using either method, all carrageenan samples were found to be partially hydrolysed. The larger the molecular weight, the more significant was the hydrolysis, reaching up to 40 %

(as calculated using the average calibration method) in the case of the highest molecular weight fraction of *kappa*. *Iota* and *lambda* carrageenans show a significantly lower degradation than *kappa*, with an approximate reduction in M_n of 20 % calculated from results shown in Table 3.13. This is consistent with literature data (Hjerde *et al.* 1996, Myslabodski *et al.* 1996). This series of experiments explains our findings in CE-LIF, that the increase in normalised area following derivatisation catalysed by acids of different pK_as or concentrations is dependent on the molecular weight of the initial carrageenan sample (Sections 3.3.1 and 3.3.2), and its sub-type. Polydispersity is found to decrease on derivatisation. This can be explained as follows: the centrifugation used as purification procedure removes material of M_w below 30 kDa. Therefore, this may result in an increase of the M_n value for the derivative, and the value of M_w being less affected, the polydispersity is decreased. Another explanation is that hydrolysis occurring during derivatisation affects high M more than low M within the same sample, therefore it decreases the value of M_w to a larger extent than that of M_n. $I_p = M_w/M_n$ is consequently reduced after derivatisation.

	$\frac{M_n(\text{derivatised})}{M_n(\text{native})}$	
sample	**(a) individual calibration**	**(b) average calibration**
69 kDa	1.09	
120 kDa	0.80	0.92
205 kDa	0.72	0.79
256 kDa	0.53	0.74
366 kDa	0.66	0.71
539 kDa	0.40	0.57
kappa	0.62	0.68
iota	0.83	
lambda	0.81	

Table 3.15. Ratios of number average molecular masses of the derivatised and the corresponding native samples calculated using (a) individual (b) general calibration plots of M versus V.

Since the polydispersity is narrower in the case of the derivative and the highest degradation

observed is 40 % (for the 539 kDa fraction), one can estimate that the hydrolysis occurring during derivatisation can in the worst case induce an increase in 40 % reducing ends, thus potential labelling sites for derivatisation. Therefore, our labelling strategy can lead to an overestimate of the carrageenan material of 40 % in the case of *kappa* carrageenan. As far as *iota* and *lambda* are concerned, the hydrolysis occurs to a lesser extent, as reported in the literature (Hjerde *et al.* 1996) with an increase in reducing ends of approximately 20 %.

Using the general calibration, the molecular mass distribution was calculated (Wyatt Technology software guide 1998; Billingham 1977). Fig. 3.19 shows this distribution for *kappa* Sigma standard.

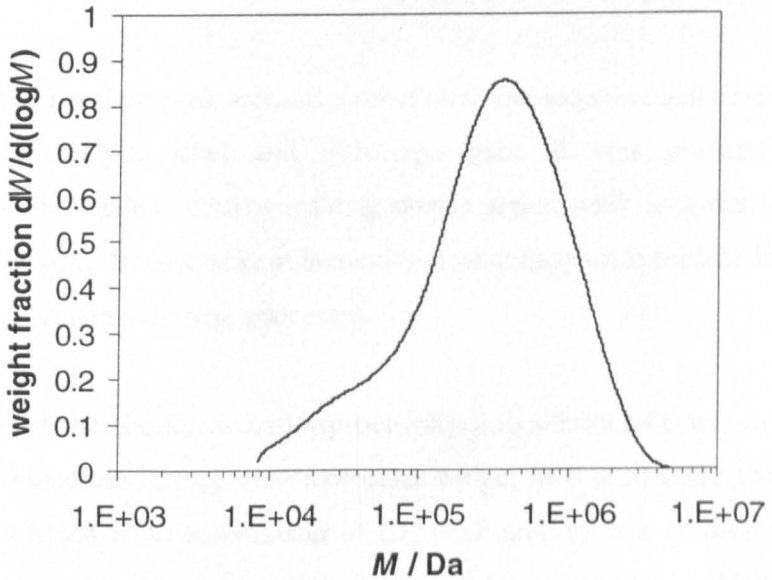

Figure 3.19. Molecular mass distribution in native *kappa* carrageenan.

3.6 Summary

Investigation of the derivatisation conditions confronted us with the possible hydrolysis of carrageenans in the presence of an acid as catalyst. Use of citric acid at a concentration higher than 0.3 M brought about complete loss of the main *kappa* peak, the signal consisting then of an oligosaccharide ladder. By derivatising *kappa* standards of different M_w, a simple method correlating molecular weight with relative migration time or peak area proved that citric acid catalysis caused a decrease in M_w in comparison to acetic acid catalysis. Working with dilute (< 0.1 M) or buffered solutions of acetic acid in order to limit hydrolysis was not possible, since the yield was not satisfactory. Therefore, the concentration of acetic acid used was set to 0.6 M with a reaction time of 1 h to limit hydrolysis, in place of the 2.25 M concentration and 15 h reaction time used in previous work (Chapter 2).

By examining normalised peak areas as a function of the sequence and time of addition of reagents acid catalyst, label and reducing agent, it was proved that sodium cyanoborohydride is able to destroy reacting sites in sugars, while hydrolysis by acetic acid creates reacting sites. It must be kept in mind that what happens in the labelling protocol is a competition involving all these processes.

All results suggest that the derivatisation process has a significant influence on the molecular weight of carrageenans, though the molecular weight of a derivatised 256 kDa *kappa* carrageenan inferred from comparison of CE peak area to that of derivatised *kappa*-hexasaccharide in comparison was within 50 % of that expected from calculation.

Drop dialysis gave better results for *kappa* carrageenan in terms of recovery, in comparison to purification by centrifugation. However, the removal of interfering material was not sufficient for correct identification of *lambda* and *iota*, since fluorescent byproducts of reaction migrated in the time window of these two carrageenans. Consequently, microcentrifugation remained the method of choice, despite the disadvantages pointed out here. The procedure was slightly modified, performing three spins of exactly 15 or 10 minutes. No effort was made to minimise the volume of the retentate, since pressure injection was used and was not affected by ionic strength. EKI, though more suitable for

sensitivity purposes, induces bias in the analysis. When the sample is not clean, the low M_w fluorescent byproducts are injected in larger proportion and provide greater interference. Pressure injection is more appropriate to compare samples and does not cause sample depletion.

The protocol recommended for carrageenans when working with APTS is as follows:
- required volume of 1 mg mL^{-1} (can be increased to 2.5 mg mL^{-1}) solution of carrageenans
- 10 µL 3.0 M acetic acid
- 2 µL 0.2 M APTS in water
- 2 µL 1 M $NaCNBH_3$ in THF
- water to fill up to 50 µL
- incubation for 1 h at 55 °C
- microcentrifugation

A limited number of experiments were performed with preparative SEC; unlike the other methods, they showed a total removal of the interfering fluorescent byproducts. No data concerning repeatability or comparison with the other methods are available, but this seems a very promising strategy to detect all macromolecules after derivatisation. Lyophilisation may be required in order to reconcentrate the sample, and some adjustments (sample quantity) may be needed to adapt it to true food samples.

Investigations using SEC-MALLS-RI were performed at SKW on native and derivatised carrageenans. They allowed us to estimate the decrease in M_n and M_w undergone by carrageenans during the labelling reaction using 0.6 M acetic acid. Hydrolysis was found to increase with increasing M_w of the initial material, yielding a maximum degradation of 40 % for the highest M_w fraction of 539 kDa *kappa* carrageenan. This means that, assuming all reducing ends are labelled, the APTS derivatisation leads to an overestimate of 40 % of the *kappa* carrageenan present initially in the sample. Hydrolysis was found to affect *iota* and *lambda* carrageenan to a lesser extent, with only 20 % decrease in M_n. When determining carrageenan amounts in samples, a calibration curve is produced under the same conditions, using the corresponding carrageenan Sigma standard. This should correct for the hydrolysis, provided that the molecular weight of the standard is in the same range of M_w as that of the sample. The molecular weight of commercial carrageenans are very similar, as reported for *kappa* by Hoffmann *et al.* (1996), so the present derivatisation method should provide good semi-quantitative results for carrageenans.

From this series of hydrolyses one can deduce the rate constant of carrageenan using the formula $1 / M_w(t) = 1 / M_w(0) + (k_1 \times t / m)$ (cf. Table 3.1), with m the molecular mass of the disaccharide. Results for M_w(native) and M_w(derivatised) are taken as $M_w(0)$ and $M_w(t)$ respectively, with t the reaction time of 60 min. Averaging the values of k for the 6 fractions gave $k_1 = (1.6 \pm 0.3) \times 10^{-5}$ min^{-1}. The good fit of data for all carrageenan fractions, reflected in the relatively low standard deviation, supports use of the formula for variation of M_w with time. The relationship with $1/M_w$ varying linearly with time shows that the lower the value of M_w, the slower the change in M_w. This is a reflection on the rate of hydrolysis being dependent on the rate for bond cleavage, and the more bonds there are in the chain, the faster the breakdown. Table 3.16 gives a comparison with results from previous work, discussed in Section 3.1.2.

Author	Hydrolysis medium	pH	k_1 / min^{-1} (pH)	k_1 / min^{-1} (2.5)
present work	0.6 M acetic acid	2.5[a]	1.6×10^{-5} (2.5)	1.6×10^{-5}
Singh & Jacobsson 1994	0.012 M HCl 0.088 M LiCl	2	4.3×10^{-5} (2.0)[b]	1.4×10^{-5}
Hjerde *et al.* 1996 [c]	0.1 M HCl 0.1 M LiI	1	1.2×10^{-3} (1.0)	4.0×10^{-5}

Table 3.16. Rate constant for acid-catalysed hydrolysis of *kappa* carrageenan, with present work compared to literature values.
[a] pH measured at ambient temperature. The pK_a of acetic acid is not significantly different at 25 °C and 55 °C, therefore pH = 2.5 was also used when correcting the values of the rate constants found in the literature for different pHs.
[b] Rate constant corrected for change in mass per residue. The authors assumed m = 192, i.e. the monosaccharide. We have shown that cleavage occurs at the disaccharide repeat unit, with m = 385. Thus the value given in the original paper, and in Table 3.1, has been multiplied by a factor 2.
[c] The value of k_3 at 55 °C was calculated using the Arrhenius equation with the activation energy and pre-exponential factor given in the paper. The authors used the formula $1 / M_n (t) = 1 / M_n(0) + (k_3 \times t / m)$, with k' the rate constant for bond cleavage. The equation for M_w is then related to this as follows: $1 / M_w(t) = 1 / M_w(0) + (k_3 \times t / 2m)$. Thus $k_1 = k_3 / 2$

The first thing to note is the very good agreement between the rate constant deduced in the present work and values reported in the literature. Such differences as there are could in part be due to differences ionic strength. Hjerde *et al.* (1996) showed that the rate constant increased as the ionic strength decreased. This is as expected for a reaction involving ions of opposite charge, in this case the anionic polysaccharide and H^+ as catalyst. In making the comparison between our value and results of previous workers, we assume that there is no contribution to the catalysis from acetic acid - i.e. the reaction is subject to specific rather than general acid catalysis. Finally, the pH in our reaction mixture increased during the run (due to consumption of acid by the CN^- breakdown product of cyanoborohydride), therefore the value of k reported in Table 3.16 is probably an underestimate of the true value at pH 2.5.

It is possible to get an estimation of the extent of hydrolysis in our experiments in citric acid, where the duration of derivatisation was 15 h (Section 3.2.3.1). The pH value is 1.7 in 0.6 M citric acid. Using the value of rate constant corrected for pH and a time of 15 h gave a calculated final M_n of 2.1 kDa for the derivative, which represents a number average DP of

5. This is likely to be an underestimate, since the pH will increase on formation of HCN during the 15 h of the reaction, so oligomers of higher DP could still be present. The electropherogram indeed showed the presence of a ladder, studied in detail in Section 3.4, where the DPs of the peaks were found to vary between 3 and 20.

Ionic strength may have played a key role in the experiment reporting the influence of the acid and the reducing agent (Section 3.3.4). The reaction rate has been shown to decrease with increasing ionic strength (Hjerde *et al.* 1996). The reducing agent is the main source of ions in the reaction mixtures in our experiments. In Exp. 4 and Exp. 6, where cyanoborohydride was added only after an hour, a dramatic increase in normalised peak area was observed, indicative of substantial hydrolysis during the first 60 min. Hydrolysis by acetic acid during this time is favoured by both the low ionic strength and constancy of pH in the absence of the reducing agent.

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Chapter 4: Analysis of carrageenans in polysaccharide mixtures and food samples

4.1 Introduction

4.1.1 Occurrence of carrageenans in food

Carrageenans are used as such for their physical properties, but also in conjunction with other hydrocolloids such as locust bean gum (LBG) or with proteins such as casein (milk protein) or whey proteins (Fernandes 1996) in dairy products. Table 4.1 summarises the different uses of carrageenan in food applications (Glicksman 1983).

Use	Function	Type	Concentration /% w/w
frozen desserts, ice cream	stabilisation, meltdown control	κ	0.01-0.03
custards	gelation	κ, κ + ι	0.2-0.3
whipped cream	stabiliser	λ	0.15-0.5
dairy dessert	bodying, suspension	κ + LBG	0.2-0.5
dessert gels	gelation	κ + ι, κ + ι + LBG	0.5-1.0
pet-food (canned)	fat stabilisation, thickening, suspending	κ + LBG	0.2-1.0
condensed milk	stabilisation	κ + ι + λ	0.01
salad dressing	emulsion stabilisation	ι	0.4-0.6

Table 4.1. Use of carrageenans in the food industry

When industrialists buy carrageenans as raw materials, they buy a product which presents the physico-chemical properties required for a particular application (e.g. aqueous matrix, dairy food). Since the content in each carrageenan sub-type can vary from batch to batch (more or less *kappa* precursor, for example), a certain amount of sugars is added to standardise the properties of the mixture, as well as salt (in particular containing potassium, which increases the proportion of ordered carrageenan and stability towards acid degradation), so that the properties remain the same for different batches of the same product. Some of the properties specified on the product sheet are rheology of the gel

formed, pH, and average particle size.

Since in general the different types of carrageenans are present simultaneously in seaweed extracts, as shown in Table 1.2, and they are mixed with other polysaccharides in foodstuffs, analytical methods should ideally be able to identify carrageenan sub-types as well as non-carrageenan polysaccharides.

4.1.2 Isolation of polysaccharide in food matrices

Pechanek *et al.* (1982) described protocols for the removal of fats, enzymic degradation of starch and precipitation of proteins in various finished products. Polysaccharides were recovered by precipitation in ethanol before electrophoresis on cellulose acetate strips. For carrageenans recovery was greater than 90 %.

Two pretreatment methods were investigated by Quemener *et al.* (2000) for extraction of carrageenan from food samples (liver pâté, custard) prior to analysis by methanolysis / RP-HPLC. One of the pretreatment methods involved homogenisation with a mixer, removal of lipid with hexane, followed by lyophilisation and grinding. The other pretreatment method consisted of extraction in water at 90-100 °C, centrifugation, concentration steps repeated 4-5 times, precipitation in ethanol, filtration and drying. Both sample preparation methods gave recoveries above 80 %. The second procedure is the simplest, since it does not include fat removal and gives satisfactory results without interferences from the matrix components in the chromatogram. However, the yield from precipitation with ethanol was found to depend on the carrageenan concentration. This is a real drawback of the method, since the concentration is not known in many of the food samples presented for analysis.

4.1.3 Aim of this chapter

In the present chapter, the protocols developed in Chapter 2 for capillary electrophoresis conditions and in Chapter 3 for sample preparation have been applied to standards of carrageenan sub-types provided by SKW. These include *kappa*, *iota*, and their precursors *mu* and *nu*, *lambda*, and *xi*. SKW provided us with other seaweed extracts to test the utility

of our electrophoretic method. Synthetic mixtures of carrageenans and other hydrocolloids were also investigated using the Sigma standards *iota*, *kappa* and *lambda*. Finally, analyses were carried out on commercial carrageenan raw materials, which are mixtures, and finished products, including an aqueous matrix and a dairy product. From the M_w measurements performed in Section 3.3.5, we deduced that the method could yield a maximum error in the estimation of 40 %, due to partial hydrolysis of the carrageenans during derivatisation. Derivatisation is not the only potential source of error, and this chapter investigates the effects of the sample matrix on the analysis. Several steps have to be added to the protocol when it is required to isolate carrageenans from other components of the food matrix, in particular fats and sugars.

4.2 Experimental

4.2.1 Materials

The carrageenan standards and the reagents used in this study were identical to those listed in Chapter 3. Sodium cyanoborohydride was purchased from Aldrich (Gillingham, UK) as a powder. APTS was obtained from Molecular Probes (Eugene, USA). Commercial carrageenan mixtures and blends for ice cream were from Nestlé suppliers. Gum arabic, xanthan, alginate, agar and LBG were from Sigma. Carboxymethyl cellulose (CMC) and pectin from apple were from Fluka (Buchs, Switzerland). Furcellaran was from FMC (Philadelphia, US). Seaweed extracts were a gift from Patrick Boulenguer and Mireille Amat from SKW (Baupte, France). All filtration and dialysis devices were as in Chapter 3, Section 3.2.1.

4.2.2 Experimental conditions for CE

As described earlier in Section 3.2.2, all experiments were performed on a Beckman P/ACE 5000 CE system equipped with a LIF detector connected to an Ar-ion laser (488 nm ex, 520 nm em). Polyvinyl alcohol "eCAP™ N-CHO" coated capillaries of internal diameter 50 µm and various lengths, supplied by Beckman, were used. The capillary was thermostatted at 25.0 °C. The background electrolyte used for all investigations was 25 mM ammonium acetate, pH 8.0, and the separation voltage was -25 kV. Between runs, the capillary was rinsed for 2 min with water, then for 2 min with the BGE.

4.2.3 Derivatisation protocol

Stock solutions of samples were prepared by weighing the powders into vials containing 5 mL of high purity water with 0.002-0.03 % sodium azide. After vigorous stirring at room temperature, the solutions were heated and stirred at 65-70 °C for 30 min. They were subsequently left to cool down to room temperature. The desired volume of freshly dissolved carrageenan was dispensed into an Eppendorf tube. The following were then added : 2 µL of labelling agent APTS (0.04 or 0.2 M), 10 µL of 3.0 M acetic acid or 6 µL

of 5.0 M acetic acid (resulting in a final concentration of 0.6 M in either case), 2 μL of 1 M sodium cyanoborohydride freshly dissolved in THF, and the required volume of water to give a total of 50 μL. The solutions were vortexed and incubated for 1 h at 55 °C.

4.2.4 Sample clean up

Centrifugation

The reaction mixtures were dispensed into microcentrifuge vials (Microcon 30 kDa molecular weight cut-off filters). The volume was brought up to 300 μL with high purity water. After vortexing, the samples were centrifuged for 10-15 min at 6,000 rpm in a microcentrifuge. This step was repeated two or three times more. The volume of the retentate was adjusted to 100 μL before injection.

Dialysis

Drop dialysis was performed as described in Section 3.9. Other dialyses performed before derivatisation used commercial dialysis bags.

4.2.5. Calibration curves and carrageenan mixtures

Stock solutions of carrageenans of concentration 2.5 mg mL^{-1} were prepared as described in Section 4.2.3. For calibration, derivatives of increasing masses of *kappa* (10, 25, 100 μg) and *iota* (25, 50, 75, 100 μg) were prepared. Mixtures (M1-M9) were prepared with the compositions listed in Table 4.2.

Mixture	mass *iota* / µg	mass *kappa* / µg	mass *lambda* / µg
M1	10	90	0
M2	90	10	0
M3	30	70	0
M4	70	30	0
M5	50	50	0
M6	30	70	10
M7	33	33	33
M8	20	50	30
M9	25	15	60

Table 4.2. Composition of prepared mixtures of *iota*, *kappa*, and *lambda* carrageenans.

Samples were derivatised according to the protocol described in Section 4.2.3 with a concentration of APTS of 0.2 M. Reaction mixtures were centrifuged as described in Section 4.2.4. All these samples were investigated using a 57 cm × 50 µm PVA coated capillary; the separation voltage was -25 kV (anodic detection); pressure injection was performed for 3 s at 0.5 psi. The data collection rate was 10 Hz. Before and after each sample injection, water was injected hydrodynamically for 3 s at 0.5 psi.

4.2.6. Study of other polysaccharides

Individual derivatives

Concentrations for the stock solutions were 1.0 mg mL^{-1} for gum arabic, carrageenans, alginate and furcellaran. For pectin, xanthan, agar, LBG and CMC the concentration was 0.2 mg mL^{-1}. Agar and LBG were never completely dissolved. For derivatisation, 8 µL were dispensed into Eppendorf tubes and derivatised according to the protocol described in Section 4.2.3 with a concentration of 0.04 M for APTS. The reaction mixtures were centrifuged as described in Section 4.2.4. All these samples were investigated using a 47 cm × 50 µm PVA coated capillary; the separation voltage was -25 kV (anodic detection); EKI was performed for 3 s at -10 kV. The data collection rate was 20 Hz. Before and after each

sample injection, water was injected hydrodynamically for 3 s at 0.5 psi.

Mixtures of equal amounts of kappa carrageenan with other polysaccharides

For each biopolymer, a stock solution of concentration 1 mg mL^{-1} was made up. Mixtures of *kappa* carrageenan with gum arabic, carrageenans, alginate and furcellaran were prepared by dispensing 16 µL of *kappa* (16 µg) and 16 µL of the other polysaccharide (16 µg) into an Eppendorf tube after the solutions had cooled to room temperature. Pectin, xanthan, agar, LBG and CMC solutions were pipetted when still warm, and 40 µL (8 µg) were mixed with 8 µL of *kappa* (8 µg). A mixture of *kappa*, *iota*, and *lambda* (16 µg of each) was also derivatised. As a reference, derivatives of *kappa* carrageenan (8 and 16 µg) were also prepared. To all sugars were added successively 2 µl of a 0.04 M APTS solution, 8 µl of a 4.5 M acetic acid solution, 2 µl of 1 M sodium cyanoborohydride ($NaCNBH_3$) and water to give a total of 60 µl (resulting in a concentration of 0.6 M in acetic acid). Incubation was performed for an hour at 55 °C, followed by the drop dialysis treatment performed for about an hour in a beaker containing ~ 150 mL water. The volumes of the drops were carefully measured after recovery. In the case of the mixtures of *kappa* carrageenan with CMC and gum arabic, these could only be recovered partially. For these two samples the membrane sank below the surface of the water in the beaker during removal of the drop by pipette, causing sample loss by dilution into the very large volume.

Mixtures of *kappa* with apple pectin, agar, LBG, xanthan, CMC, *iota*, furcellaran, alginate and gum arabic were investigated using a 47 cm × 50 µm PVA coated capillary; the separation voltage was -25 kV (anodic detection); pressure injection was performed for 6 s at 0.5 psi. Mixtures of *kappa* with *lambda*, and *iota* and *lambda*, were injected on the same capillary shortened to 37 cm after breakage. The voltage was reduced from -25 kV to -20 kV, to allow separation to occur at the same field strength (5.3×10^4 V m^{-1}). Under these conditions the current was exactly the same as in the 10 cm-longer capillary (32 µA). The data collection rate was 20 Hz. Before and after each sample injection, water was injected hydrodynamically for 3 s at 0.5 psi.

Mixtures of kappa carrageenan with guar, LBG and CMC

The composition of the samples is reported in Table 4.3. A calibration curve (2, 5, 15, 25 μg) was also prepared. The powders were not completely dissolved; it was assumed that all the carrageenan had gone into solution. Before dispensing 40 μL of the solutions for derivatisation, the solids were allowed to settle out. Derivatisation was performed according to the protocol described in Section 4.2.3, with a concentration of 0.2 M for APTS. Reaction mixtures were filtered. The retentate volumes recovered for the synthetic mixtures were larger than for the *kappa* standards. Volumes were adjusted to 50 μL for the derivatives of the calibration. For the mixtures they were measured and ranged from 175 to 225 μL, resulting in theoretical concentrations in the range 0.02 - 0.05 mg mL^{-1}. As a comparison, the concentration of carrageenan at the lowest point of the calibration corresponded to 0.04 mg mL^{-1}. Experimental conditions for separation were the same as in Section 4.2.5.

sample	G1	G2	C1	C2
guar / % w/w	68	64		
LBG / % w/w	21	31	78	71
CMC / % w/w			12	23
kappa / % w/w	11	5	10	6
total polysaccharide concentration / mg mL^{-1}	1.8	1.9	1.8	2.4

Table 4.3. Composition and total concentration of *kappa* carrageenan and other polysaccharides in mixtures imitating ice cream blends.

4.2.7 Study of seaweed extracts, commercial carrageenan samples and food samples

Stock solutions of concentration 2.5 mg mL^{-1} were prepared from all samples in powder form. 40 μL (representing 100 μg) were derivatised according to the protocol described in Section 4.2.3 with 0.2 M APTS, and were centrifuged 3 or 4 times according to the method in Section 4.2.4.

In the case of the seaweed extracts the volume of the retentates varied between 75 and 127 µL. The derivatives were injected, without adjustment of the volume, into a 67 cm × 50 µm PVA coated capillary. Pressure injection was performed for 10 s at 0.5 psi. The data collection rate was 20 Hz.

Sample preparation for the jelly consisted of a simple dilution of the jelly dessert in which the carrageenans were already in an aqueous matrix. Two solutions of concentrations 5 g L^{-1} and 10 g L^{-1} respectively were prepared. They were stirred at 55 °C until dissolution was complete. Then 40 µL of these stock solutions (respectively 2 and 4 mg) were dispensed for derivatisation. Increasing amounts of *iota* and *kappa* carrageenan (10, 20, 50 and 100 µg) were derivatised simultaneously, to provide a calibration curve. The final volume after centrifugation was brought up to 100 µL by addition of water.

In the case of the dairy desserts, sample preparation included fat removal. A suspension of the dessert (15 g in 100 mL) was prepared in dioxan and stirred at 60 °C for ~ 30 min. This suspension was then filtered using a membrane filter (Millipore type FH, pore size 0.5 µm, 47 mm diameter) and washed twice with 50 mL dioxan and once with 50 mL ethanol on the filter. The filter precipitate was left to dry overnight in a dessicator under vacuum. This procedure removed the fat and the liquid phase of the dessert and reduced the mass of the sample by 80 to 90 %. The precipitate was then redissolved in water at 55 °C. In order to remove particles, the solution was centrifuged at 7,000 rpm for 10 min. The resulting supernatant solutions were subsequently dialysed overnight using commercial devices (in one case a Slide-A-Lyzer® MINI Dialysis Unit, in the other case a Spectra/Por® DispoDialyzer) in 500 mL beakers of ultrapure water. In order to test the two types of dialysers, both dialysed and undialysed samples were derivatised. The volumes of the samples were measured to allow correction for any dilution occurring during dialysis. After derivatisation the volume of the retentates varied between 30 and 60 µL. Solutions were injected without further dilution into a 57 cm × 50 µm PVA coated capillary. Pressure injection was performed for 3 s at 0.5 psi for the derivatives of the carrageenan mixtures, and 10 s for the derivatives of the dairy desserts. The data collection rate was 10 Hz.

4.3 Results and discussion

4.3.1 Synthetic mixtures

4.3.1.1 Determination of carrageenan composition in carrageenan mixtures

In order to test the possibility of quantifying carrageenan sub-types, mixtures of various proportions of *kappa*, *iota*, and *lambda* were prepared. *Lambda* carrageenan cannot be correctly integrated because of its peak profile, which shows two peaks (see Fig. 3.12), and the presence of a satellite peak of APTS, but increasing amounts of *kappa* and *iota* carrageenans were derivatised for calibration, as described in Section 4.2.5. Fig. 4.1 shows the electropherogram of mixture 5.

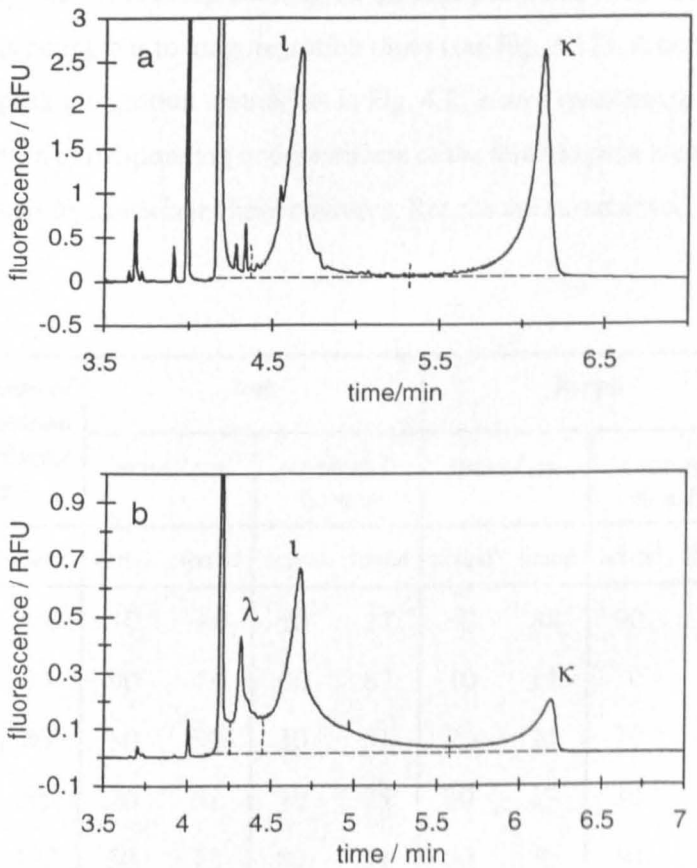

Figure 4.1. Electropherogram of an APTS-derivatised mixtures. (a) mixture of *iota* (1.0 mg mL^{-1}), and *kappa* (1.0 mg mL^{-1}) carrageenans (M5); (b) mixture of *lambda* (1.2 mg mL^{-1}), *iota* (0.5 mg mL^{-1}), and *kappa* (0.3 mg mL^{-1}) carrageenans (M9). CE conditions : 57 cm × 50 µm PVA coated capillary ; pressure injection : 3 s at 0.5 psi.

The calibration curves for normalised areas, A_n, of *iota* and *kappa* versus mass derivatised gave satisfactory regression coefficients of values 0.989 and 0.997, respectively. Equations found were the following:

for *iota*, $A_n / 10^5 = (0.724 \pm 0.045) \times (\text{mass} / \mu g) - 1.4 \pm 2.7$

for *kappa*, $A_n / 10^5 = (0.615 \pm 0.025) \times (\text{mass} / \mu g) - 1.9 \pm 1.3$

Linear regression allowed determination of the absolute amounts of these two carrageenans present in the mixtures. When only *iota* and *kappa* were present, the proportion of the total amount found could also be calculated. Peaks were integrated drawing a horizontal baseline, setting the limits at the minima between peaks, and dropping verticals. In the case of *iota* - *kappa* carrageenan mixtures, the signal almost reached the baseline between the two carrageenans (Fig. 4.1). When *lambda* was present, especially in mixtures where it was the main species (Fig. 4.2), it induced a tailing on the *iota* peak, due to the double peak profile of *lambda* and its tailing out to long migration times (see Fig. 3.12). A consequence of this, when using the peak integration method as in Fig. 4.2, is an overestimate of the *iota* peak area. There will be a corresponding underestimate of the *lambda* peak area, although we do not attempt to quantify *lambda* in these mixtures. Results are summarised in Tables 4.4 and 4.5.

sample	total mass of carrageenan derivatised / µg		*iota*				*kappa*				|deviation| /% w/w
			mass / µg		content / % w/w		mass / µg		content / % w/w		
	actual	found	actual	found	actual	found	actual	found	actual	found	
M1	100	106	10	18	10	17	90	88	90	83	7
M2	100	85	90	74	90	87	10	11	10	13	3
M3	100	63	30	32	30	51	70	31	70	49	21
M4	100	68	70	51	70	75	30	17	30	25	5
M5	100	142	50	72	50	51	50	70	50	49	1

Table 4.4. Absolute amounts and compositions found for artificial mixtures of *iota* and *kappa* carrageenans

Adding the amounts found for each component of the mixture gave a value for the recovery

of the mixtures. This apparent recovery varied from 63 to 142 % in the case of derivatised mixtures of *iota* and *kappa*. This is not realistic, and most probably a physical effect must account for these results. One of the reasons may be a different viscosity for each sample, according to the content of each of the carrageenans, which leads to variations in amount injected into the capillary. This could explain the differences observed between the measured and true values. If viscosity is the reason for the discrepancy in absolute mass determinations, the contents are more likely to match the expected values, since pressure injection has no influence on the relative amounts injected for each species. Table 4.4 shows that the proportions found for *iota* and *kappa* are close to the actual values. An exception is the mixture M3, where an excessive proportion of *iota* was found. On average, the root mean square deviation from the actual values for the proportion was found to be 6 %. The root mean square deviation for the total amount found is ± 17 µg for 100 µg. The higher RSD for mass than for proportion is consistent with the explanation suggested above.

Iota and *kappa* carrageenan standards from Sigma contain a small fraction of each other, which has been estimated in previous work to be a maximum of 10 % (Hjerde 1996). In our CE measurements there is a small peak of *iota* in *kappa* and vice versa. The content of each carrageenan in the other was estimated by peak height to be 4 %. Taking into account these values means that a 90 : 10 (*iota* / *kappa*) mixture is actually 87 :13, whilst 70 : 30 is corrected to 69 : 31, and 50 : 50 remains after correction 50 : 50. This cannot explain the discrepancy observed for mixture M3, but is in agreement with the trend observed for the 90 : 10 mixtures M1 and M2.

sample	**total mass of carrageenan derivatised /µg**	***iota* mass /µg**		***kappa* mass /µg**		***iota* / *kappa* ratio**	
		derivatised	found	derivatised	found	derivatised	found
M6	110	30	33	70	41	0.4	0.8
M7	100	33	40	33	21	1.0	1.9
M8	100	20	37	50	41	0.4	0.9
M9	100	25	31	15	11	1.7	2.8

Table 4.5. Absolute amounts and ratios found for *iota* and *kappa* carrageenans in prepared mixtures of *iota*, *kappa* and *lambda* carrageenans

In the case of the mixtures containing *lambda* carrageenan, not only did the masses found for *iota* and *kappa* vary from the true values, but also the relative proportions of *iota* and *kappa* were significantly different from those expected. The presence of *lambda* influences the viscosity of the solution and also biases the determination of *iota*, because (as discussed before) part of the *lambda* peak co-migrates with *iota*. The values of the *iota / kappa* ratios are shown in Table 4.5 for samples M6-M9. They are systematically higher than expected, which means a systematic overestimation of *iota* with respect to *kappa*.

4.3.1.2 Mixtures of kappa carrageenan with other hydrocolloids, 1:1 ratio

Individual polysaccharides

In order to test the possibility of interference with carrageenans, other polysaccharides were derivatised and analysed in a series including *iota*, *kappa* and *lambda* carrageenans, according to the protocol described in Section 4.2.6. After the usual recovery by fast spinning, the centrifugation filter was rinsed in order to recover possible remaining polysaccharide material. The solutions were then injected into the CE capillary. In the case of carrageenans, alginate and gum arabic some derivatised polysaccharide material was still found in the rinsing solution, but it gave a very low signal. In contrast, in the case of pectin, most material had not been removed in the first retentate, and significantly more material was detected in the rinsing solution. The electropherogram of the rinsing solution is shown in Fig. 4.2d. Nothing was detected in the rinsing solutions in other cases.

Alginate, gum arabic, pectin and furcellaran gave peaks, but LBG, agar, xanthan and CMC did not. LBG is a neutral polysaccharide, therefore the only charges present are those of APTS end groups, which could not induce a sufficient mobility to reach the detection window in our analysis time. Agar carries only a small proportion of sulfate groups, therefore its mobility is also probably too small to be detected in our conditions. An unknown is the molecular weight of these polymers. If significantly higher than carrageenans, there would be insufficient end groups for derivatisation. CMC and xanthan are negatively charged polysaccharides. For xanthan, M_w and M_n were found to be 6.5×10^6 Da and 1.9×10^6 Da, respectively (Runyon *et al.* 1996). This is significantly higher than the

molecular weight found for carrageenans (Section 3.5).

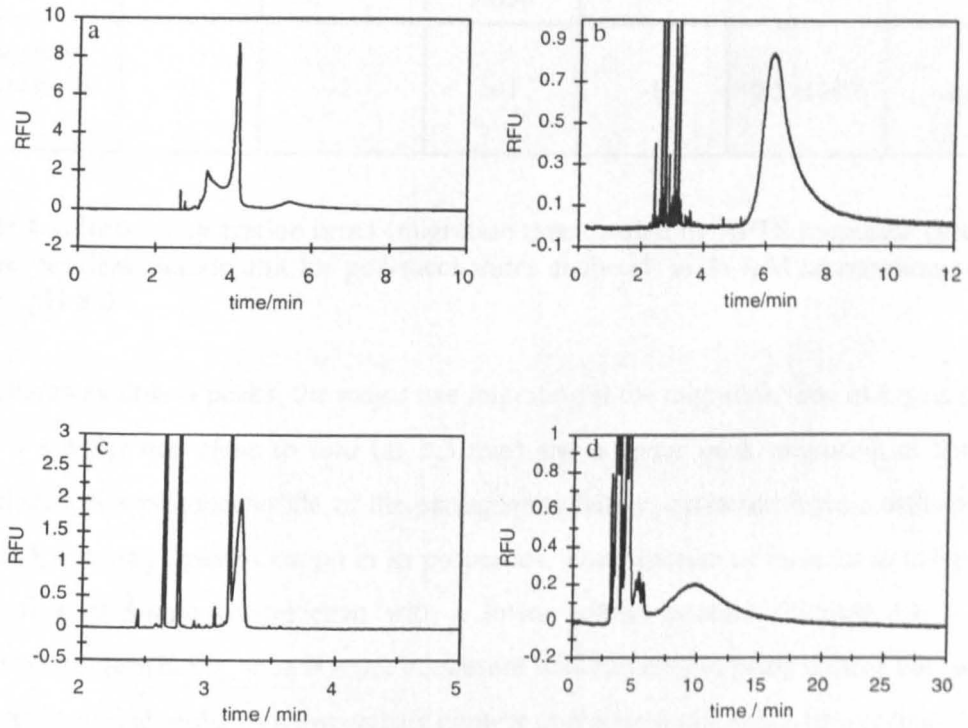

Figure 4.2. Electropherograms of APTS-derivatised (a) furcellaran (0.08 mg mL^{-1}); (b) gum arabic (0.08 mg mL^{-1}); (c) alginate (0.08 mg mL^{-1}); (d) pectin. CE conditions : 47 cm × 50 µm PVA coated capillary ; EKI : 3 s at -10 kV.

Fig. 4.2 shows electropherograms of furcellaran, gum arabic, alginate and pectin. As in previous work (Section 3.3.2) the relative migration times were calculated as the ratio between the migration time of the polysaccharide and that of APTS. These values are reported in Table 4.6. The charge per disaccharide is also reported in the table. In the case of pectin, the degree of esterification (DE) was 70-75 %. Therefore per disaccharide the charge is 2 ×-1 ×(0.25 to 0.30), which gives a charge in the range -0.5 to -0.6. Gum arabic is a complex polymer consisting of a galactose backbone chain with branches containing arabinose, rhamnose, glucuronic acid and 4-O-methyl glucuronic acid. On average the repeat unit (~40 monosaccharides) carries a maximum of 12 charged monosaccharides (Stephen 1990). The average charge per disaccharide is therefore 2 × -1 × 12 / 40 = -0.6.

	iota	alginate	furcellaran	*kappa*	pectin	gum arabic
RMT	1.196	1.226	1.264, 1.548, 2.056	1.560	1.802	2.330
charge per disaccharide	-2	-2	>-1	-1	-0.5 to -0.6	-0.6

Table 4.6. Relative migration times (migration time divided by APTS migration time) and charge per disaccharide unit for polysaccharides analysed in 25 mM ammonium acetate buffer, pH 8.0.

Furcellaran exhibits 3 peaks, the major one migrating at the migration time of *kappa* (at 4.1 min), a smaller one close to *iota* (at 3.3 min) and a minor peak migrating at 5.4 min. Furcellaran is a polysaccharide of the carrageenan family, extracted from a different red seaweed, but very close to *kappa* in its properties. The structure of furcellaran is reported to be that of *kappa* carrageenan with a lower sulfate content (Piculell 1995). The electropherogram in Fig. 4.2a is more consistent with furcellaran being a three component mixture, including probably a low-sulfate content component migrating slower than *kappa*. Alginate is composed of mannuronic and guluronic acid residues, both of which carry a negative charge at pH 8.0. Therefore, it migrates in the carrageenan time window and gives a good quality single peak between *iota* and *kappa* but close to *iota*. The intense, thin peak observed in the electropherogram of alginate is present in the blank reaction and is due to an APTS impurity. The gum arabic peak has a good shape, and its low charge per disaccharide explains why its RMT is greater than that of *kappa*. The branched structure gives gum arabic a high frictional coefficient. This accounts for the higher RMT than for pectin, which has approximately the same charge per disaccharide. Pectin gave a very broad and late peak, with a low signal (by peak height) in comparison to the other polysaccharides. The migration behaviour of pectins is related to their degree of esterification (DE), and studies by Zhong *et al.* on native pectins detected by UV have shown that, in CZE, the mobility correlates with DE (Zhong *et al.* 1997; Zhong *et al.* 1998).

From Table 4.6, one can deduce the possible interferences of polysaccharides with carrageenans. Alginate has a RMT close to *iota* and the peaks of these two polysaccharides would probably not be fully resolved if they were mixed. Furcellaran, being from the

carrageenan family, will interfere with both *iota* and *kappa*, as shown in the following study for the latter.

Mixtures of polysaccharides with *kappa* carrageenan

Dialysis was previously shown to produce samples with good recovery and reproducibility (Section 3.3.63), therefore it was used to investigate the possible effects of other polysaccharides on carrageenan derivatisation and recovery. Unfortunately, peaks for *lambda*, *iota* and the alginates migrate too close to the satellite peaks of APTS to be satisfactorily integrated. *Kappa* migrates slower and its peak is distinct from the satellite peaks. Therefore, 1:1 w/w mixtures of *kappa* and of various individual polysaccharides were derivatised and dialysed as described in Section 4.2.6. Reference derivatised samples of *kappa* carrageenan were also prepared. For direct comparison they were injected under the same conditions as the mixtures. As discussed in Section 4.3.1, peaks were integrated using a horizontal baseline and taking care to choose the same limits for peak start and peak end. In order to be compared, the areas were normalised with migration time and corrected for variations of volume, by normalisation to 100 µL, as explained in Section 3.3.6.3. Recovered volumes V_r, corrected areas for *kappa*, A_c, and relative migration times RMT for *kappa* are reported in Table 4.7 for mixtures with other carrageenans, furcellaran, alginate, and arabic gum (mixed with 16 µg of *kappa* carrageenan). Table 4.8 gives values from mixtures with pectin, agar, LBG, xanthan and CMC (mixed with 8 µg *kappa* carrageenan).

Polysaccharide mixed with *kappa*	V_r / µL	A_c(*kappa*)	A_c(*kappa*)/ A_c(standard)	RMT
47 cm capillary				
none	**105**	**40.1**		**1.574**
iota	168	44.5	1.11	1.568
furcellaran	191	51.2	1.28	1.573
alginate	186	38.9	0.97	1.568
gum arabic [1]	60	11.8	0.29	1.572
37 cm capillary				
none	**105**	**68.1**		**1.492**
lambda	187	70.3	1.03	1.495
iota and *lambda*	260	82.7	1.21	1.496

Table 4.7. Results of quantitative analysis of *kappa* carrageenan in 1:1 w/w mixtures with other carrageenans, alginate and arabic gum. Total mass derivatised 16 µg *kappa* + 16 µg of the other component, including in the mixture with *iota* and *lambda* (16 µg of each). Sample purification by drop dialysis.
[1] partially recovered

Polysaccharide mixed with *kappa*	V_r / µL	A_c(*kappa*)	A_c(*kappa*)/ A_c(standard)	RMT
47 cm capillary				
none	**100**	**18.4**		**1.571**
apple pectin	160	17.2	0.93	1.568
agar	171	19.0	1.03	1.570
LBG	120	19.0	1.03	1.566
xanthan*	127	21.1	1.15	1.592
CMC*[1]	40	7.2	0.39	1.575

Table 4.8. Results of the derivatisation of 8 µg *kappa* carrageenan with apple pectin, agar, LBG, xanthan and CMC. Sample purification by drop dialysis.
* Xanthan and CMC mixtures were injected on a different day to the others and in particular the 8 µg *kappa* standard.
[1] partially recovered.

The mixtures of *kappa* carrageenan with CMC and gum arabic were only partially recovered, as discussed in Section 4.2.6 because the membrane sank in the beaker while pipetting the solutions. Therefore a significant fraction of sample was lost. This explains why the results for corrected areas do not match the expected values in these two cases.

The reported values for the peak areas of *kappa* carrageenan found in most polysaccharide mixtures are in good agreement with those of the standards. This means that one can be confident about the quantification of *kappa* carrageenan in 1:1 w/w mixtures with other polysaccharides, when the clean-up procedure is drop dialysis. Considering all mixtures fully recovered, an average of A_c (*kappa*) /A_c (standard) is 1.02 ± 0.07. This corresponds to an RSD of 7 %. When excluding xanthan (injected on a different day from the standard), the average for the area ratio is 0.99 ± 0.04. This corresponds to a standard deviation of 4 %, which is close to the reproducibility of the drop-dialysis protocol, 3 %, determined previously (Section 3.3.6.3).

Alginate and xanthan do not seem to have an influence on the detectable quantity of *kappa* carrageenan. Aggregates appeared in the xanthan-*kappa* carrageenan mixture (Fig. 4.3a), giving rise to spikes, which could be integrated separately and subtracted from the area of interest. If there is an interaction, it does not modify *kappa*'s behaviour during the derivatisation process or its migration in the capillary. There may be an interesting effect of xanthan on the relative migration time of *kappa*. The value of RMT increases to 1.590, in comparison with the average 1.570 found. This would require further investigations. No overlap with the *kappa* peak was noticed except for pectin (see Figure 4.3b). In this case the peak of carrageenan was integrated with the pectin peak as baseline. Agar and LBG did not give any signal, as observed previously.

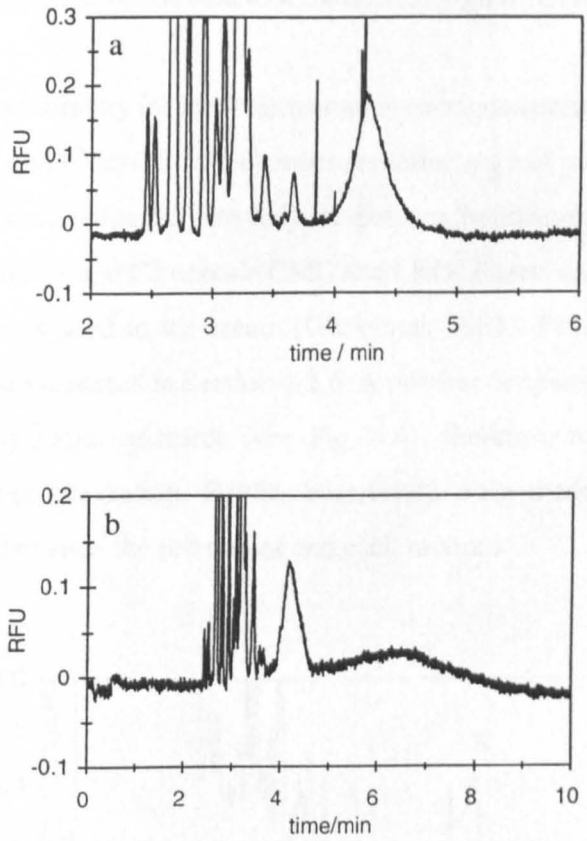

Figure 4.3. Electropherogram of an APTS-derivatised mixture of (a) *kappa* carrageenan (0.06 mg mL^{-1}) and xanthan (0.06 mg mL^{-1}); (b) *kappa* carrageenan (0.05 mg mL^{-1}) and pectin from apple (0.05 mg mL^{-1}). CE conditions : 47 cm × 50 µm PVA coated capillary ; pressure injection : 6 s at 0.5 psi.

Results in Table 4.7 clearly indicate that both *iota* and furcellaran, which belong to the carrageenan group, contain a proportion of *kappa* carrageenan. This is concordant with observations from the *iota* and furcellaran standards.

The shortening of the capillary following its breakage had some noticeable consequences. As expected, and shown in Table 4.7, the relative migration times were lower, though consistent within the set. The same injection time (6 s) resulted in a larger amount injected (nearly 70 %). Finally, there was a significant change in the peak shape. In the long capillary, *kappa* peaks were broad and slightly tailing. In the 37 cm capillary the shape was changed into the usual fronting shape obtained for *kappa*. This last observation suggests degradation of the coating in the 47 cm capillary prior to breakage.

4.3.1.3 Mixtures of kappa carrageenan with an excess of guar, CMC and LBG

Blends used in the food industry for ice creams contain carrageenans mixed with locust bean gum, CMC, guar and emulsifiers. Synthetic mixtures reflecting real proportions determined previously at Nestlé were prepared with only the polysaccharide components. G1 and G2 contain guar and LBG, C1 and C2 contain CMC and LBG. *Kappa* carrageenan was added because this sub-type is used in ice cream (Glicksman 1983). Precise composition and experimental details are reported in Section 4.2.6. A number of spikes were present on the *kappa* peak in the synthetic mixtures (see Fig. 4.4), therefore their areas had to be substracted for correct integration. Further corrections were made to account for the difference in volume between the references and each mixture.

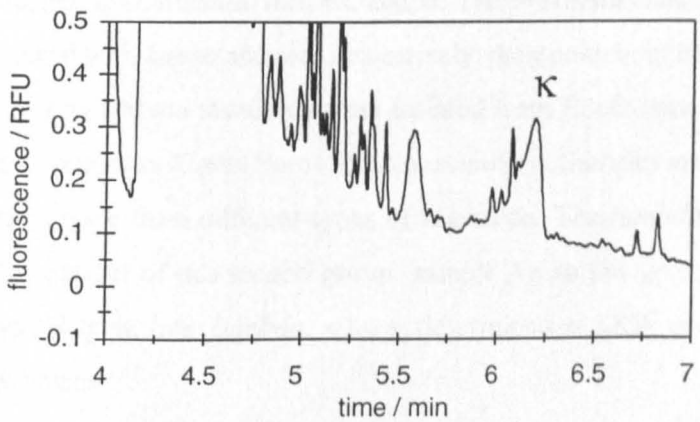

Figure 4.4. Electropherogram of an APTS-derivatised mixture of *kappa* carrageenan (0.04 mg mL^{-1}), CMC, and LBG (C1). CE conditions : 57 cm × 50 µm PVA coated capillary ; pressure injection : 3 s at 0.5 psi.

sample	G1	G2	C1	C2
actual / % w/w	11	5	10	6
determined / % w/w	19	7	16	5
ldifferencel / % w/w	8	2	6	1

Table 4.9. Quantification of *kappa* carrageenan in polysaccharide mixtures containing guar and LBG (G1 & G2) and LBG and CMC (C1 & C2).

The results concerning estimation of *kappa* in the mixtures are summarised in Table 4.9. The values found for *kappa* are in general higher than expected, but they are in the correct range. This proves that the presence of other polysaccharides does not significantly affect the derivatisation and identification of *kappa* carrageenan. The average difference from the true value is + 3.4 % w/w. The presence of guar, LBG, and CMC leads to overestimation of *kappa* carrageenan.

4.3.2 Seaweed extracts

Two groups of seaweed extracts were provided by SKW. Samples in the first group were standards, extracted from specific seaweeds, whose extraction products are well-known. These included *kappa*, *iota*, *lambda*, *mu*, *nu*, and *xi*. The precursors *mu* and *nu* cannot be isolated and are mixed with *kappa* and *iota* respectively; their content in the extract is in the range 20-30 %. *Kappa* and *mu* standards were isolated from Eucheuma cotonii, *iota* and *nu* from Eucheuma spinosum. *Xi* was from Gigartina acicularis. Samples in the second group of seaweed extracts were from different types of seaweeds. The *lambda* standard was a fraction (A2) of an extract of this second group (sample A). In this group of samples the content in sub-type (*kappa*, *iota*, *lambda*, *xi*) was determined at SKW and provided to us after the CE experiments.

Carrageenan standards of *iota*, *kappa* and *lambda* from Sigma were derivatised in this series of experiments for comparison, since they were used as references throughout this thesis work. Electropherograms of the derivatised standards provided by SKW and of *lambda* from Sigma are displayed in Fig. 4.5 a-g.

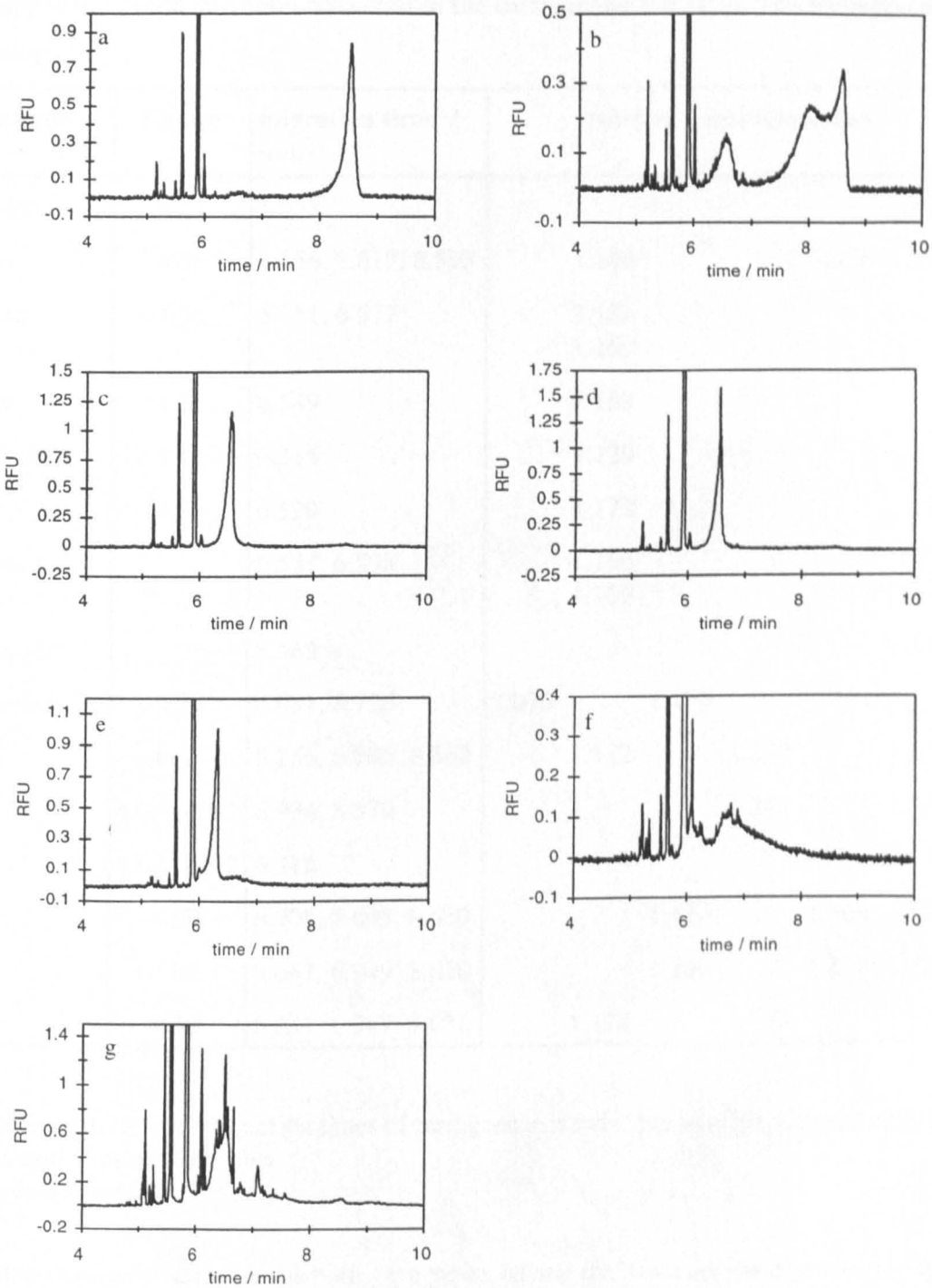

Figure 4.5. Electropherograms of APTS-derivatised carrageenan standards. (a) *kappa* (0.9 mg mL^{-1}), (b) *mu* (0.9 mg mL^{-1}), (c) *iota* (1.1 mg mL^{-1}), (d) *nu* (1.3 mg mL^{-1}), (e) *lambda* (1.2 mg mL^{-1}), (f) *lambda* from Sigma (1.1 mg mL^{-1}), *xi* (1.2 mg mL^{-1}). *Xi* was derivatised and injected separately from other seaweed extracts. CE conditions : 57 cm × 50 µm PVA coated capillary ; pressure injection : 10 s at 0.5 psi.

The relative migration times as defined in Chapter 1 were calculated to enable precise

comparison of the migration behaviour of the carrageenan sub-types, and are reported in Table 4.10.

sample	Figure	migration time[a] / min	relative migration times
kappa	4.5a	8.539	1.524
mu	4.5b	6.556, 8.013, 8.593	1.166 1.426 1.529
iota	4.5c	6.551, 6.577	1.164 1.169
nu	4.5d	6.549	1.163
lambda	4.5e	6.315	1.129
xi	4.5g	6.520	1.172
iota [b]		6.581, 6.598	1.166 1.169
kappa [b]		8.569	1.538
lambda [b]	4.5f	6.081, 6.726	1.076 1.190
A	4.6A	6.256, 6.905, 8.567	1.122 1.238 1.536
A1	4.6A1&A2	6.934, 8.570	1.241 1.534
A2	4.6A1&A2	6.315	1.129
B	4.6B	6.708, 7.695, 8.630	1.185 1.360 1.525
C	4.6C	6.667, 6.949, 8.630	1.180 1.322 1.521
D	4.6D	6.651, 6,947, 8.631	1.178 1.230 1.528

Table 4.10. Relative migration times of carrageenan standards and other seaweed extracts.
[a] measured at peak maxima
[b] Sigma standards

Kappa and *iota* carrageenans both gave peaks having the same appearance whether they were from Sigma or from SKW. Both *iota* samples showed a peak split at the top. *Lambda* from Sigma exhibited two peaks. This sample exhibited different peak ratios according to the derivatisation procedure, as mentioned in Chapter 2. In all cases its peak area is smaller than that of the other carrageenans; this can be accounted for because the molecular weight of the native material is higher (see Section 3.5) and its hydrolysis rate is lower than the other two carrageenans. Peak area differences are more noticeable using the optimised

derivatisation conditions than when using the procedure described in Chapter 2. Because of the soft extraction conditions used, *lambda* is usually extracted with *kappa*'s precursor *mu*, which could explain the second part of the peak. Stevenson & Furneaux (1991) reported the composition of *lambda* carrageenan from Sigma, which is extracted from *Gigartina acicularis* and *pistillata*. This *lambda* was found to be a hybrid or mixture of *lambda* and *xi*, with 1.8 % pyruvate residues. This means that a pyruvate ketal is present in 1 in 6 disaccharide units, as 4,6-pyruvate ketal group on 3-linked galactosyl residues. Pyruvate brings a COO^- group, therefore an additional charge to the residue. This means that the true structure of this carrageenan sub-type presents less sulfate groups than its theoretical formula, and that additional substituents can modify its frictional coefficient and charge in comparison to expectations. This helps in understanding its electrophoretic behaviour. The *lambda* standard provided by SKW gave one main peak, with a migration time lower than *iota*. This is consistent with *lambda* carrying more charge than *iota*. This sample is much purer than that provided by Sigma.

The extract containing the precursor *mu* gave 3 peaks. *Kappa* was present as the major component, with some *iota*. In this sample, *iota* was not removed, whereas it had been from the pure *kappa* standard. The precursor *mu* migrated faster than *kappa*, as expected, since it carries one extra sulfate group. The new peak added a shoulder on the normal *kappa* peak, which is consistent with the fact that precursors are hybrid molecules, with the 1,4-linked ring partly sulfated at C6, partly in the 3,6-anhydrogalactose form.

Nu carrageenan displayed a peak at a migration time almost identical to that of *iota*. The peak of the precursor is not separated from that of *iota*. When zooming on the peak, one notices a difference in peak shape, since the top of the peak is much thinner in the case of the *nu* sample. Because of counterion condensation, *iota* and *nu* residues could have the same net charge per residue. The *iota* residue contains an anhydrogalactose bridge, which could be responsible for a difference in its frictional coefficient, and induce a slightly different mobility.

Xi carrageenan was more viscous than the other carrageenans, and its electropherogram shows residual peaks of small M_w fluorescent material. The migration behaviour of *xi* was similar to that of *iota*, as expected from its structure carrying two sulfate groups. In

mixtures containing *iota* and *xi*, *iota* will be overestimated.

The electropherograms of other extracts are shown in Fig. 4.6. Sample A1 and A2 are fractions of sample A. The electropherograms of samples A1 and A2 displayed specific parts of the electropherogram of sample A. The patterns displayed by these samples are different from these given by the synthetic carrageenan mixtures studied in Section 4.3.1. Some material migrates at a time intermediate between those of *iota* and *kappa* carrageenans, and is deduced to be a hybrid. The hybrid nature of carrageenans has been demonstrated by Bellion *et al.* (1982). Boulenguer and Amat found that the composition in repeat units of sample B was 65 % *kappa*, 27 % *iota*, and 8 % *lambda*. Similarly, sample C was found to contain 55 % *kappa*, 15 % *iota*, and 30 % *lambda*. The electropherograms showed completely different distributions of the peak areas, with more material present as a hybrid in sample B than in sample C. The *kappa* peak area is greater in C than in B, implying the presence of a greater proportion of chains containing a majority of *kappa* units in sample C. None of the samples A-C gave a peak having the migration time expected for *iota*, suggesting that pure *iota* chains did not exist in these samples. Sample C resembles more the artificial mixtures of standards than does sample B, though the fluorescence signal does not go as close to the baseline between the *kappa* and *iota* peaks as would be expected from mixtures of the two standards (cf. Fig.4.1a). The composition of sample D was 50 % *kappa*, 15 % *iota* and 35 % *xi*. Its electropherogram showed three main peaks; from these, *kappa* was the only clearly identified sub-type. The first peak has *iota* peak shape, but a slightly longer migration time. The peak at the intermediate migration time could be a hybrid.

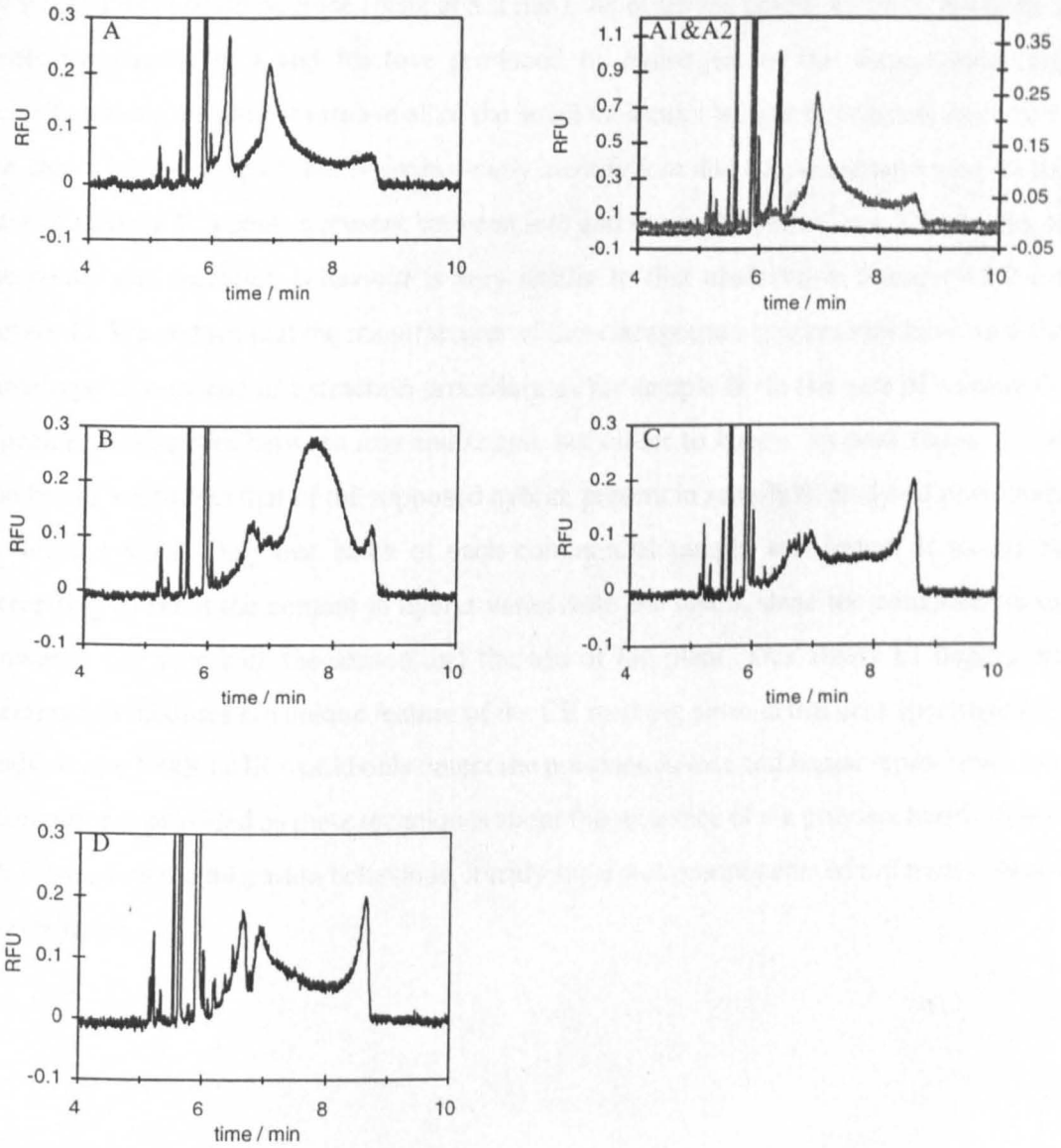

Figure 4.6. Electropherograms of APTS-derivatised seaweed extracts. A (1.2 mg mL^{-1}), superposition of A1 and A2 (1.0 and 1.2 mg mL^{-1}, respectively), B (1.3 mg mL^{-1}), C (1.0 mg mL^{-1}), D (0.8 mg mL^{-1}). CE conditions : as in Fig. 4.5.

4.3.3 Carrageenan blends containing sucrose

Various commercial carrageenan mixtures from suppliers were tested with our method, in order to determine what type of carrageenans they contain. Electropherograms shown in Fig. 4.7 were obtained from two commercial carrageenan samples used in the preparation of dairy desserts, with and without use of dialysis (using Slide-A-Lyzer® MINI Dialysis Units) before derivatisation. The dialysis procedure was found to be very successful and

eliminated most of the sucrose (peak at 5.2 min). As observed before in 3.3.5, this peak is probably glucose or / and fructose produced by hydrolysis of the disaccharide. The centrifugation step did not remove all of the small molecular weight fluorescent material in the case of these samples, but one can clearly identify *iota* and *kappa* carrageenans. In the case of mixture F, a peak is present between *iota* and *kappa*, closer to *iota*. This profile of the peaks and migration behaviour is very similar to that observed in Section 4.3.2 for sample D. We deduce that the manufacturer of this carrageenan mixture may have used the same type of seaweed or extraction procedure as for sample D. In the case of mixture G, a peak is also present between *iota* and *kappa*, but closer to *kappa*. Its peak shape, round and broad, resembles that of the supposed hybrid, present in sample B, analysed previously in Section 4.3.2. Only one batch of each commercial sample was tested. It would be interesting to see if the content in hybrid varies with the batch, since the composition of seaweeds can vary with the season and the age of the plant. This ability to fingerprint carrageenan mixtures is a unique feature of the CE method, since in this case spectroscopic methods like NMR or IR would only detect the presence of *iota* and *kappa* repeat units. No information is provided by these techniques about the sequence of the polysaccharide. Since CE is based on the migration behaviour, it truly separates components of different charge-to-size ratios.

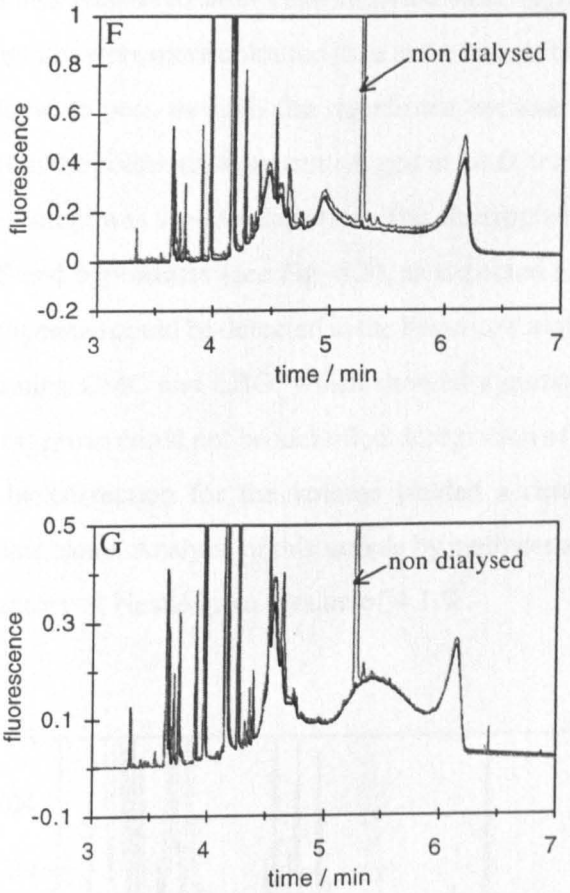

Figure 4.7. Electropherograms of two APTS-derivatised commercial carrageenan mixtures, treated with and without dialysis before derivatisation. (a) F (non dialysed 1.3 mg mL^{-1}, dialysed 2.5 mg mL^{-1}), (b) G (non dialysed 1.3 mg mL^{-1}, dialysed 1.8 mg mL^{-1}). CE conditions : as in Fig. 4.4.

4.3.4 Blend containing other polysaccharides and emulsifiers (mono- and diglycerides), used for ice cream

Real blends were derivatised within the series studied in Section 4.3.1. Polysaccharide components of these blends are carrageenan, LBG, CMC and guar. Carrageenan is a minor component of this blend. No carrageenan sub-type or content is provided by the manufacturer. Non-polysaccharide components are mono- and diglycerides used as emulsifiers. These blends did not completely dissolve. We assumed that the carrageenans had gone into solution after the time allowed to solubilise the standards. The solutions were dispensed warm, in contrast to the other samples, because the viscosities of these mixtures

were higher. The volumes recovered after centrifugation were higher than for the Sigma standards, and the retentates were more coloured than in the case of the standards, testifying to the difficulty for liquid to pass through the membrane because of the high viscosity. Before pressure injection the solutions were centrifuged at 14,000 rpm in order to remove particles, and the supernatant was used for injection. The electropherogram displayed a lot of peaks due to APTS and byproducts (see Fig. 4.8), as expected from the colour of the retentates. *Kappa* carrageenan could be detected in the blend containing guar and LBG, but not in the blend containing CMC and LBG, which showed a complex electropherogram where even *kappa* carrageenan could not be identified. Integration of the *kappa* peak in the first blend, followed by correction for the volume yielded a content of 5 % w/w of *kappa* carrageenan in this blend. Analysis of this sample by methylene blue complexation in the carbohydrate laboratory at Nestlé gave a value of 4.1 %.

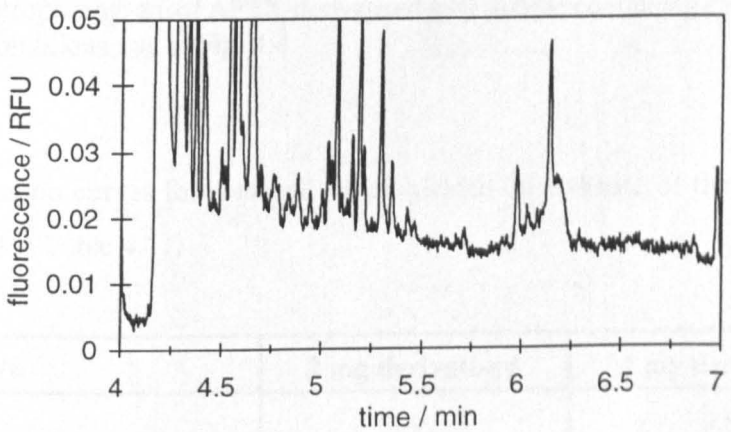

Figure 4.8. Electropherogram of APTS-derivatised blend containing carrageenans (concentration in blend 1.5 mg mL^{-1}). CE conditions : as in Fig. 4.4.

4.3.5 Food samples

4.3.5.1 Aqueous matrix : jelly

The electropherogram of the jelly showed two peaks corresponding to *iota* and *kappa*, and a narrow peak at an intermediate migration time (see Fig. 4.9). The migration time of this

peak corresponded to that of glucose, due to its sucrose content. Not all of the sucrose was eliminated by microcentrifugation.

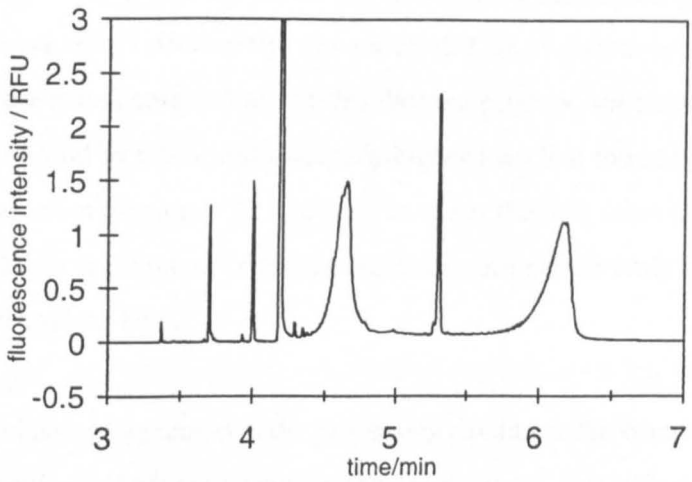

Figure 4.9. Electropherogram of APTS-derivatised jelly sample containing carrageenans (4 mg mL^{-1}). CE conditions : as in Fig. 4.4.

Using the calibration curves for *iota* and *kappa* yielded an estimate of the carrageenan content reported in Table 4.11.

content / % w/w	**2 mg derivatised**	**4 mg derivatised**
iota	0.92	0.88
kappa	0.94	0.89
total carrageenan	1.86	1.78

Table 4.11. Content of *iota* and *kappa* carrageenans in the jelly.

The values found for the two reaction replicates were very similar. *Iota* and *kappa* carrageenans were found to be present in equal amounts in this sample. The determination by CE was repeated, and a value of 1.4 % was found, with a slightly different composition (44 % *iota*, 56 % *kappa*). The content in carrageenan was also determined by the methylene blue method described in Chapter 3. Since the slope is different for the different carrageenan sub-types, a 50/50 w/w mixture of the two carrageenans was used as calibration. The total

carrageenan content was found to be 0.85 % w/w. When measuring the content of carrageenan in the blend, it was found to be 51 %, thus the total content of blend in the jelly is calculated to be 0.85 % / 0.51 = 1.67 %. Using the blend as calibrating material, the jelly was measured to contain 1.56 % of this raw material. These values are consistent with each other, and with the actual composition of this finished product, supposed to be 1.28 % in blend. The value found by CE is systematically higher than that found by methylene blue. The study performed in Section 4.3.1.1 allows us to say that the value (1.7 % on average) found here by CE for the content of the carrageenans are significantly above the content determined by methylene blue.

The stability of gelling carrageenans under processing conditions has been reported by Marrs (1998). The recommended pH for a water-based dessert is 4.1. It was shown that pH played a key role in the stability of carrageenans when processing them at 80 °C for 15 min. Ingredients in the jelly included 1 % carrageenan, 18 % sucrose, 0.32 % potassium citrate, 0.35 % citric acid. Experiments carried out on *kappa* at pH 3.6 in the study by Marrs showed that there was more than 30 % of material with M_w < 100 kDa, which would not meet the standard for viscosity (see Chapter 1). Jellies containing *kappa* carrageenan were also processed at higher temperatures, in the range 85-140 °C, for 10-30 s. After processing, the content of material with M_w < 100 kDa was found to be within the range of native carrageenans, except in the case of the highest temperatures (130 and 140 °C), where it reached 20 and 35 %, respectively. Under appropriate conditions of pH and temperature, hydrolysis does occur, but within acceptable limits.

In the particular case of the jelly analysed here, the range of conditions (pH, temperature and duration) possible during processing were disclosed. (For reasons of confidentiality these conditions cannot be given in the thesis). Using this information and the parameters given in the paper by Hjerde *et al.* (1996), as already described in Section 3.6, the extent of degradation of *iota* and *kappa* carrageenans was estimated, starting with a M_n value of 150 kDa, as determined in Section 3.2.7 for native carrageenans. We found that M_n for *iota* could be reduced to as low as 50 kDa, and that of *kappa* to 30 kDa. Therefore, a very plausible reason for the overestimation by our CE method is that there was a higher content in reducing ends in the sample following the processing than in the unprocessed calibrating material. An advantage of analysis of carrageenans in this jelly by CE is that it provides

information on the relative amounts of *iota* and *kappa* carrageenans and on the hydrolysis undergone by the carrageenans during the processing. However, accurate quantification is hardly possible in these conditions, when the processing conditions are unknown. Moreover, since the hydrolysis of *iota* proceeds slower than that of *kappa*, it is likely that when degradations occurs, estimation by CE will lead to an overestimate of *kappa* and an underestimate of *iota*.

4.3.5.2 Milk matrix : dairy dessert

As discussed in the previous section, the commercial carrageenan samples were found to contain sucrose, which gave a narrow glucose peak between those of *iota* and *kappa*. Peaks due to sucrose appeared thus in all CE electropherograms of derivatised food samples. It should be noted that as well as being in the additive mixture, sucrose is frequently also an ingredient. Its peak did not interfere significantly with the integration of the *kappa* and *iota* peaks in the case of the jelly sample. Centrifugation did not clean the derivatised dairy dessert samples sufficiently to enable identification of *iota* or *lambda*, probably due to the high viscosity of the sample and the presence of ingredients such as proteins. In the dairy food samples, lactose was also present giving a sharp, intense peak on the leading edge of the *kappa* peak, and making it very difficult to identify *kappa*. To minimise the interference of small sugars in the electropherogram and their consumption of APTS, dialysis was performed on the food product before labelling.

Fig. 4.10 shows the electropherograms of a dessert sample, non dialysed and dialysed with Slide-A-Lyzer® MINI Dialysis Units. This dialyser was found to be more efficient than the larger size one (Spectra/Por® DispoDialyzer). This shows that dialysis performed before labelling is a helpful procedure for detecting *kappa* carrageenan in finished dairy products. An estimation of the content of this dessert is 0.03 % w/w *kappa* carrageenan. The recipe indicates ~ 0.5 % w/w in blend, containing not only *kappa* carrageenan, but also *iota*, possibly other subtypes, and sucrose. Therefore, the value found is probably an underestimate of *kappa* carrageenan content in this product.

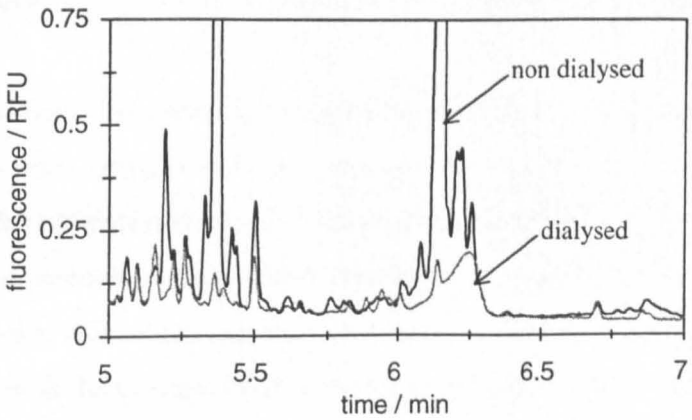

Figure 4.10. Electropherogram of APTS-derivatised dairy dessert sample containing carrageenans (non dialysed 1.6 mg mL^{-1}, dialysed in Spectra/Por® DispoDialyzers 2.9 mg mL^{-1}, dialysed in MINI Dialysis Units 2.9 mg mL^{-1}). CE conditions : 57 cm × 50 µm PVA coated capillary ; pressure injection : 10 s at 0.5 psi.

4.4 Conclusions

The study performed on synthetic mixtures showed that we can identify the main carrageenan sub-types, and that in the case where only gelling carrageenans are present, our method gives a fair estimate of the relative amounts of *iota* and *kappa*. The normalised areas cannot be related precisely to the absolute amounts present in the mixture, most probably due to differences in viscosities in samples containing different proportions of biopolymer. When it is present in the mixture, *lambda* carrageenan leads to an overestimation of *iota*. However, because of their different properties, the subtypes should seldom be found in equivalent proportions. In most cases, *iota* and *kappa* are expected to be the dominant species. *Lambda* is present in small proportions in certain seaweeds containing mainly the gelling fractions. Moreover, since *lambda* gives a lower peak area per unit mass derivatised, it should not interfere significantly in the quantification of *iota*. An appropriate standard and more investigations are needed for the analysis of this carrageenan subtype.

The study of the artificial mixtures with other hydrocolloids showed that quantitative recovery of *kappa* carrageenan can be obtained when using a 1:1 w/w ratio without significant interference apart from other carrageenans. Determination of *iota* carrageenan could be hindered if alginate occurred in the sample. The RSD was found to be 4 % on the area of all mixtures relative to the standard containing the same mass of derivatised *kappa* standard. In solutions containing a minor proportion of *kappa* carrageenan, the estimate was of the correct order of magnitude, as well as in the case of the ice cream blend. However, the clean up procedure was not able to remove efficiently all low M_w fluorescent material. It would probably be helpful to perform a preparative SEC clean up as described in Section 3.3.6.6 to improve the separation of carrageenans from low M_w fluorescent material. The issue of variable viscosity affecting the volume injected may not be completely solved by preparative SEC clean up, since hydrocolloids will most probably be eluted with carrageenans. The same problem was encountered with the dairy sample even after fat removal. Pre-labelling dialysis was shown to be very efficient for removing small sugars such as lactose and sucrose. In experiments not reported here, maltose and lactose standards were derivatised and gave the same migration time, so one would expect interference from maltose with the *kappa* peak to be similar to that observed with lactose. Since they do not

carry negative charges, higher DP maltodextrins would migrate later than maltose, and would interfere less with the *kappa* peak. This holds for other types of neutral oligosaccharides used in food (e.g. inulin). The labelling reaction is less efficient with non reducing sugars, but their eventual degradation products could give peaks, as mentioned for sucrose, which gives a peak at the migration time of glucose.

The seaweed extracts provided by SKW helped us understand peak profiles observed in commercial mixtures. Of particular interest are *iota-kappa* hybrid structures, which yielded migration behaviour intermediate between that of *iota* and *kappa*. Unlike NMR and IR techniques, which detect individual repeat units of carrageenan sub-types, capillary electrophoresis accounts for the average charge / size of each chain, and can thus tell if the mixture contains pure *kappa* and *iota* types, as in the case of the jelly, or hybrid chains as in the case of the seaweed extracts and the commercial additive. The analysis of the jelly showed that the processing of the product could have a significant effect in decreasing M_n of the carrageenans, and hence lead to their overestimation by CE using standards of undegraded biopolymers.

This chapter shows that capillary electrophoresis gives information about composition of samples in *iota* and *kappa* carrageenans. Spectroscopic and other chromatographic techniques give information about the content in repeat units or monosaccharide types. Since it does not require preliminary hydrolysis, capillary electrophoresis provides information about the behaviour of molecules of the polymer and thus, the distribution of repeat units in these chains, and the presence and proportion of hybrids. Another technique for identification of hybrids involves the use of specific enzymes, *iota* carrageenase or *kappa* carrageenase and fractionation, prior to NMR (Greer & Yaphe 1984, Knutsen *et al.* 1995). Methylene blue complexation gives the total amount of carrageenans present in a sample. A mixture of carrageenan standards with the appropriate composition should be used for calibration with methylene blue, in order to determine the absolute amount of carrageenan present in the sample. The use of CE in combination with other spectroscopic methods (IR, NMR, colorimetry) would be the best way to characterise the sample.

4.5 References

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Chapter 5: Conclusion and future work

5.1 Capillary electrophoresis

The work reported here used an ammonium acetate BGE, which proved to be suitable for the separation of carrageenan sub-types according to their charge-to-size ratio and gave good results in term of repeatability of the amount injected. It should be noted that the peaks appeared to be split at the top in some cases, and that all carrageenans displayed fronting peak shapes. In CE, the mobility of the BGE is crucial to obtain good peak shapes. Since we use carrageenans and coated capillaries with the EOF suppressed, there are no acidic functional groups either on the analytes or the capillary wall, and buffering action is not required. It could then be possible to investigate the use a dilute solution of native *kappa* carrageenan as the BGE, which would have exact matching mobilities for the derivatised carrageenan to be analysed. This should remove any contribution from electromigration dispersion and improve the peak shape.

A crucial feature of the CE method reported in this thesis is injection. In Chapter 3, we found that pressure injection is more reliable than electrokinetic injection, even though it injects smaller amounts and therefore has a higher limit of detection. However, in both EKI and PI, the amount injected depends on the viscosity of the sample, which varies dramatically with the content and type of hydrocolloids and emulsifiers present in finished products. An improvement could be obtained using higher temperatures both for separation and injection, so that the viscosity would be reduced in the sample. Separation is not affected by an increase in temperature, as shown in Chapter 1. To have injection performed at higher temperature one would need the sample to be thermostatted, for example at 50 °C, which is usually the maximum temperature allowed for commercial capillary electrophoretic systems.

5.2 Sample preparation

This thesis has investigated in detail the effects of the different parameters influencing the derivatisation reaction and the sample clean-up required to isolate the polysaccharides from the fluorescent material coming from the unreacted label. We have reached a compromise

in which carrageenans are partially hydrolysed, but sufficiently derivatised. The hydrolysis has been characterised by SEC and was found to occur to a maximum extent of 40 % in the case of *kappa* carrageenan, and of 20 % for *iota* and *lambda*. The principal potential error introduced by the CE method when determining carrageenans is in the range 20 - 40 %; however corrections are made for this by using of a calibration curve of the corresponding standard derivatised under the same conditions. Improvement could still be done to the derivatisation protocol, in order to reduce further the hydrolysis due to the presence of acetic acid. Hjerde *et al.* (1996) report that hydrolysis rates decreased with increased ionic strength. Adding a salt during the labelling reaction would thus decrease the hydrolytic effect of the acid catalyst. Another beneficial effect of a high ionic strength is that it screens charges, so it would favour the reaction between carrageenans and APTS, which are both anionic species and thus normally repel each other. One would have to be careful in the choice of the salt, particularly the cation, so that carrageenans would still be in the coil state during reaction at 55 °C. Lithium or sodium ions would probably be appropriate choices, because the concentrations required to induce helix transition are higher than 0.5 M at the reaction temperature (Piculell 1995, Rochas & Rinaudo 1980).

An important point of the derivatisation reaction is the yield as a function of M_w. Coupling a fluorescence detector to an SEC instrument would allow comparison of the distribution of the derivatised fraction with that of the whole carrageenan material. This would also help understand the peak shape and maybe gain additional M_w information from the electropherogram.

Another improvement in sample preparation is the clean-up by preparative SEC. Few experiments were performed with this procedure, because it was only developed during the final stage of the project. The SEC protocol is very promising, since it provided very clean samples of carrageenans. One experiment not reported used preparative SEC for a finished dairy product, and showed the same efficiency in clean-up. However, the signal from the derivatised carrageenans was close to the noise, due to the dilution occurring during the procedure. Lyophilisation may be required in the case of true samples, which contain low amount of carrageenans. The recovery of the procedure should also be estimated, probably using the methylene blue method.

5.3 Analysis of real mixtures

The different mixtures analysed in Chapter 4 showed the advantages and the limitations of our approach. Derivatisation followed by CE-LIF can identify carrageenan sub-types according to their charge-to-size ratio. *Iota* and *kappa* are well defined gelling fractions and usually do not present problems when they are pure and present together. However, other carrageenan sub-types can also be present in commercial carrageenans. *Lambda* is often a hybrid structure containing repeat units from *xi* or *kappa*, and sometimes substituents other than sulfates. This results in a migration behaviour closer to *iota* and can give a peak with a broad shoulder. Another important issue is the presence in some seaweeds of a less sulfated form of non gelling carrageenan, *xi* carrageenan. This carrageenan, having a similar migration time to that of *iota* will directly interfere with *iota*. Amongst the precursors of the gelling carrageenans, only *mu* could be shown to display a different electrophoretic behaviour (hybrid containing both the disulfated, precursor repeat units and the anhydrogalactose containing, *kappa* repeat units). In general the precursors should not be found in commercial mixtures of *iota* and *kappa*, due to the extraction procedures which are designed to increase the content in gelling carrageenans, and thus convert precursors into *kappa* and *iota*. However, precursors can appear in fractions of non gelling carrageenans like *lambda* or *xi*, extracted under milder conditions. Commercial mixtures were found to contain hybrid molecules, which cannot be differentiated from *iota*, *kappa* and *lambda* by other methods like NMR or IR. Our CE method, which does differentiate species, would be a useful tool for the investigation of seaweed extracts, and could potentially provide information on the polysaccharides linked in with the species and the age of the plant.

Interferences from other thickeners should be limited, since they usually carry different substituents. One particular case is that of furcellaran, which is a polysaccharide from the carrageenan family and shows material migrating at *iota* and *kappa* migration times. Another polyelectrolyte is alginate, which could interfere with *iota*, but it is usually not found in mixtures with carrageenans.

The polysaccharides used in the food industry in conjunction with carrageenans are locust bean gum, guar, and CMC. Being less charged than carrageenans, they do not display any

peaks, but they increase the viscosity and hamper the microcentrifugation sample clean up. So do emulsifiers and any type of matrix in food products. An improved clean-up procedure for these complex matrices could use preparative SEC, following the promising results obtained with the standards. Water or ammonium acetate solution could be used as eluent for this protocol, since they would avoid any aggregation of carrageenans. Lyophilisation may be required because of the dilution occurring during the process. The other polysaccharides will probably be eluted in the same fractions as the carrageenans, and increasing the temperature, as mentioned in Section 5.1, would help to reduce the viscosity for injection onto CE.

The analysis of the jelly showed that CE could detect the occurrence of degradation during the processing of the dessert, creating more reducing ends than in the calibrating material. Such degradation cannot be detected by spectroscopic methods. This can be assessed by comparison of CE results with results obtained by the methylene blue method. In the case of the jelly, it could be directly concluded that CE lead to an overestimation because the matrix of this particular dessert was water and did not influence the injection.

Dialysis performed before derivatisation proved to be a efficient procedure to remove small sugars present in excess, such as sucrose and lactose present in commercial mixtures and desserts. These saccharides not only consume APTS but also interfere in the electropherogram (especially neutral disaccharides, which co-migrate with *kappa*). This preliminary dialysis step can be conveniently performed overnight using the commercial dialysers.

By implementing the suggestions given here, especially the sample clean-up required to detect the early peaks of *iota* and *lambda*, our method should be sensitive enough to be applied to dairy desserts. Used in conjunction with methylene blue, it could give a fair idea of the material present in the sample.

5.4 Other detection methods

Our approach was derivatisation followed by LIF detection in order to have the maximum sensitivity using a commercial instrument. This led us to investigate the derivatisation reaction required for this type of detection. Other types of detectors, which would not require derivatisation, could potentially be used for carrageenans. One could consider using a refractive index detector in order to detect carrageenans in their native state. RI is widely used following size exclusion chromatography and has been reported for CE (Bruno *et al.* 1991). The difficulty in building such a detector is the size of the cell used for detection in CE (pathlength of 50 microns on the capillary).

Another type of detection used for sugars is amperometric detection. Because it is based on oxidation, the different carrageenan subtypes are likely to have different responses. Amperometric detectors have been developed and reported for CE for the analysis of small molecules. They require high precision in the alignment of the electrode with the capillary, but are extremely sensitive. A potential drawback of this detection is that it uses high pH BGEs and could induce depolymerisation of carrageenans.

Recent developments have been reported in the area of chemiluminescence detection for HPLC by an american company, Antek Instruments (Homan & Borny 2000). This type of detection exists for sulfur and may be used for carrageenans. Coupling of this type of detection to CE is under investigation.

These various detection approaches would simplify the method and allow detection of native carrageenans. However, the issues of overlapping carrageenans and the presence of hybrids, for which no standard is available, will remain the same as in CE-LIF. No single analytical method is yet able to provide satisfactory information on carrageenans at the levels present in complex food samples.

A challenge for the future is to have all techniques necessary for carrageenan characterisation in a single laboratory and combine their results for identification and quantification.

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