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Composition of carrageenan blends inferred from ^{13}C-NMR and infrared spectroscopic analysis

T. Turquois*, S. Acquistapace, F. Arce Vera & D. H. Welti

NESTEC Ltd, Research Centre, Vers-chez-les-Blanc, P.O. Box 44, CH-1000 Lausanne 26, Switzerland

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Carrageenan blends mainly composed of kappa, iota and lambda carrageenan were investigated by ^{13}C-NMR and infrared (IR) spectroscopy. ^{13}C-NMR was shown to be a powerful tool for quantifying the kappa-iota ratio in a carrageenan blend. The technique, however, was not helpful for identifying lambda carrageenan. Consequently, IR spectroscopy was used to achieve a semi-quantitative determination of lambda carrageenan. By combining both techniques, the carrageenan composition of several commercial samples was established. © 1997 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Carrageenans are sulphated galactans extracted from many species of red algae, the Rhodophyceae (Rees, 1962). They are composed of D-galactose (G) residues linked alternately with α-(1→3) and β-(1→4) linkages. These sulphated galactans are classified according to the presence of 3,6-anhydrogalactose (AG) on the 4-linked residue and the position and the number of sulphate groups (Rees, 1969; McCandless & Craigie, 1979). The most important types of carrageenans are the kappa (Fig. 1a), iota (Fig. 1b) and lambda carrageenan (Fig. 1c). Kappa and iota contain the 3,6-anhydrogalactose unit and are gelling polymers, but lambda carrageenan with only galactose residues, is a thickening polymer. Carrageenans are frequently used as components of ice cream stabilizer blends. Stabilizers are incorporated in ice cream formulations to improve the stability and eating texture of the product. Stabilizers give a thick body to the ice cream and so contribute to the creaminess of the product. During distribution, storage in the retail chain and consumer use, ice cream products can be exposed to considerable temperature fluctuations. Stabilizers are claimed to protect the ice cream from such temperature shock by both retarding ice crystal growth and maintaining product integrity even under conditions of partial melting.

To achieve a better understanding of the influence of carrageenan blend composition on their functionality as ice cream stabilizers, it has been decided to carry out a fundamental study. However, before investigating the functionality of carrageenan blends in ice cream, the carrageenan blend analysis methodology itself had to be elaborated. Usual methods for determination of the main constituents of polysaccharides involve hydrolysis under conditions wherein 3,6-anhydrogalactose residues of carrageenans are rapidly degraded. To overcome this problem various chemical approaches were proposed such as mercaptolysis (Araki & Hirase, 1953), formolysis (Brasch *et al.*, 1984), bromine oxydation during hydrolysis (Anderson *et al.*, 1968), double hydrolysis reduction and reductive hydrolysis (Stevenson & Furneaux, 1991) or methanolysis (Duckworth & Yaphe, 1971; Lahaye *et al.*, 1986). However, these techniques did not yield satisfactory results. Furthermore, in a blend they cannot distinguish from which type of carrageenan the monosaccharides are released. In the present work, the determination of the carrageenan composition with the help of ^{13}C-NMR and IR spectroscopy is described.

MATERIALS AND METHODS

Materials

Kappa, iota and lambda carrageenan were commercial samples supplied by FMC/Litex. Commercial blends were obtained from different suppliers: FMC/Litex, Shemberg, Systems Bio-Industries (SBI) and Hercules.

NMR spectroscopy

Samples (40mg/ml) were prepared using H_2O/D_2O in a weight ratio 80/20 in WILMAD 513-7PP 10mm

*Author to whom correspondence should be addressed.

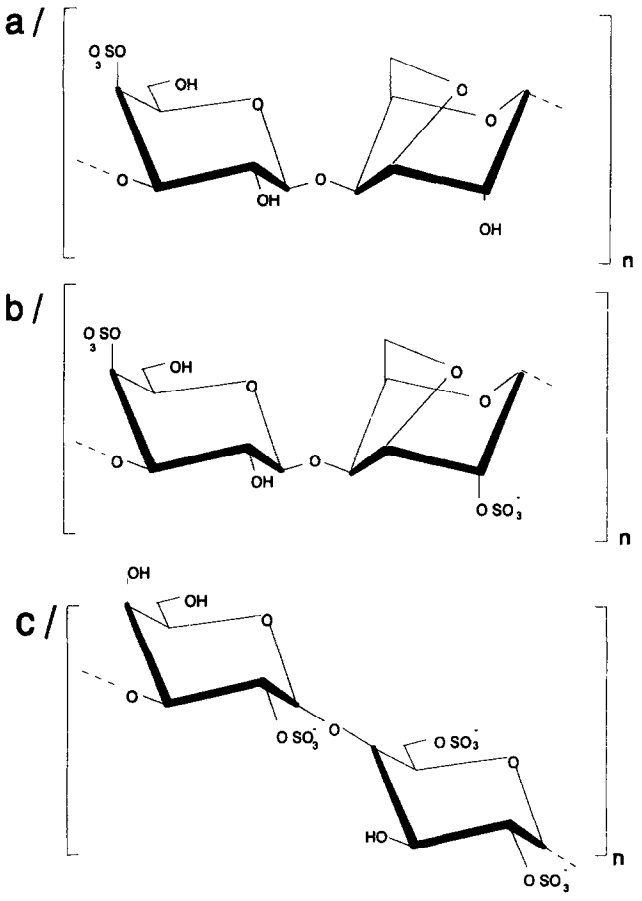

Fig. 1. Idealized structure of galactan repeating units: (a) kappa carrageenan; (b) iota carrageenan; (c) lambda carrageenan.

Pyrex glass NMR tubes. ^{13}C experiments were performed at 90.56MHz on a Bruker AM-360 narrow bore NMR spectrometer, equipped with a process controller, Aspect 3000 computer and variable temperature control system. Fourier transform ^{13}C-NMR spectra were obtained using the 10mm broadband multinuclear probe head at about 80°C with a spectral width of 10416Hz at a resolution of 0.636Hz per point. All ^{13}C-NMR measurements were performed in conditions of near-quantitative analysis, which required an estimation of the spin-lattice relaxation times, T_1, of the different components by preliminary inversion–recovery experiments. Spectral conditions were generally as follows: pulse width (PW) 12μs, corresponding to approximately 80° pulse angle; acquisition time (AQ) 0.393s; relaxation delay (RD) 2.80s; number of scans (NS) variable according to the desired signal to noise ratio (S/N) of the spectrum, but usually 15000–20000; and acquired data points 8K, zero filling to 32K total data points before the Fourier transformation. Chemical shifts are given relative to a frequency determined as zero with a sample that contained 3-trimethylsilyl-tetradeuteropropionic acid sodium salt (TSP) in the same solvent mixture, measured at the same temperature setting (indirect external standard). In truly quantitative analysis, the ^{13}C spectra should be recorded without the nuclear Overhauser effect (nOe). This avoids the risk of unequal Overhauser enhancements, but it significantly reduces the S/N and, thus, the precision of the signal integration. In the case of carrageenans, all the carbons in the molecules bear at least one directly bound proton and are, thus, likely to experience a nearly full nOe. It has been checked on well characterized samples of carrageenans in known ratios, that within the attainable precision of the measurement the nOe does not affect the ratios between the integrals of the different signals. It was, therefore, judged advantageous to improve the S/N ratio by using full composite pulse decoupling throughout. The precision of the measurements depends upon the time allowed for the data acquisition. With an overnight acquisition, the noise level of the spectra is such that an error of ±3% of the total carrageenan quantity (not of the individual signal areas) must be accepted. This means that the determination of minor components (below 10%) becomes somewhat problematic.

Infrared spectroscopy

Films for IR analysis were obtained by drying, in polyethylene moulds at 45°C, 1ml of an aqueous solution containing 10mg of polysaccharide or mixture of polysaccharides. The spectra were recorded on a Perkin-Elmer 1600 FTIR spectrophotometer under standard conditions. The absorbance of bands in the IR spectrum were calculated using the most probable base line (Fig. 2) and Equation 1 or Equation 2 (Colthup *et al.*, 1964).

$$A = \log T_b/T_p \quad (1)$$

$$A = A_p - A_b \quad (2)$$

Symbols refer to transmittance (T) and absorbance (A) of base line (b) and peak (p).

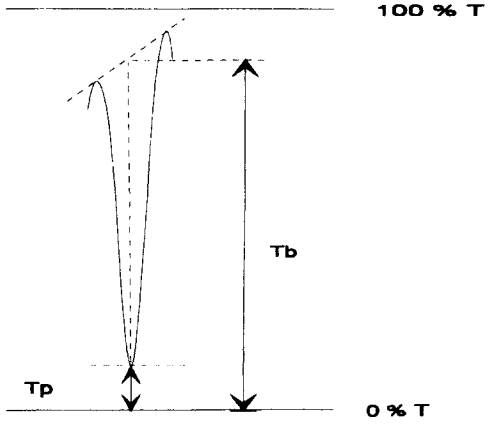

Fig. 2. Base line method for determining transmittance.

RESULTS AND DISCUSSION

^{13}C-NMR study

^{13}C-NMR of the starting polysaccharides

The spectra of kappa and iota carrageenans (Fig. 3a, b) consist of 12 signals, corresponding to the 12 carbon atoms of the repeating disaccharide unit. These spectra were performed at about 80°C in order to decrease the line widths by reducing the viscosity of the samples. The assignment of these spectra was done by comparison with those of methyl-β-D-galactopyranoside (Gorin & Mazurek, 1975) and methyl-3,6-anhydro-α-D-galactopyranoside (Balza *et al.*, 1977), which are two monomers considered as models for the units of the carrageenans. Complementary information was provided by ^{13}C-NMR analysis of carrageenan oligosaccharides (Rochas *et al.*, 1980; Greer *et al.*, 1985). The chemical shifts of kappa and iota carrageenans are given in Table 1. It should be noted that these assignments are based on close similarity with the literature values. They are not necessarily unequivocal for all carbon atoms. However, for the anomeric carbons, which are evaluated for quantitative analysis, the assignments are indisputable. The presence of sulphate groups results in specific modifications of the ^{13}C-NMR spectrum of the parent polysaccharide (Usov, 1992). The substituents usually give rise to a substantial downfield shift of the nearest carbon atom resonance (α-effect) and to moderate upfield shifts of the

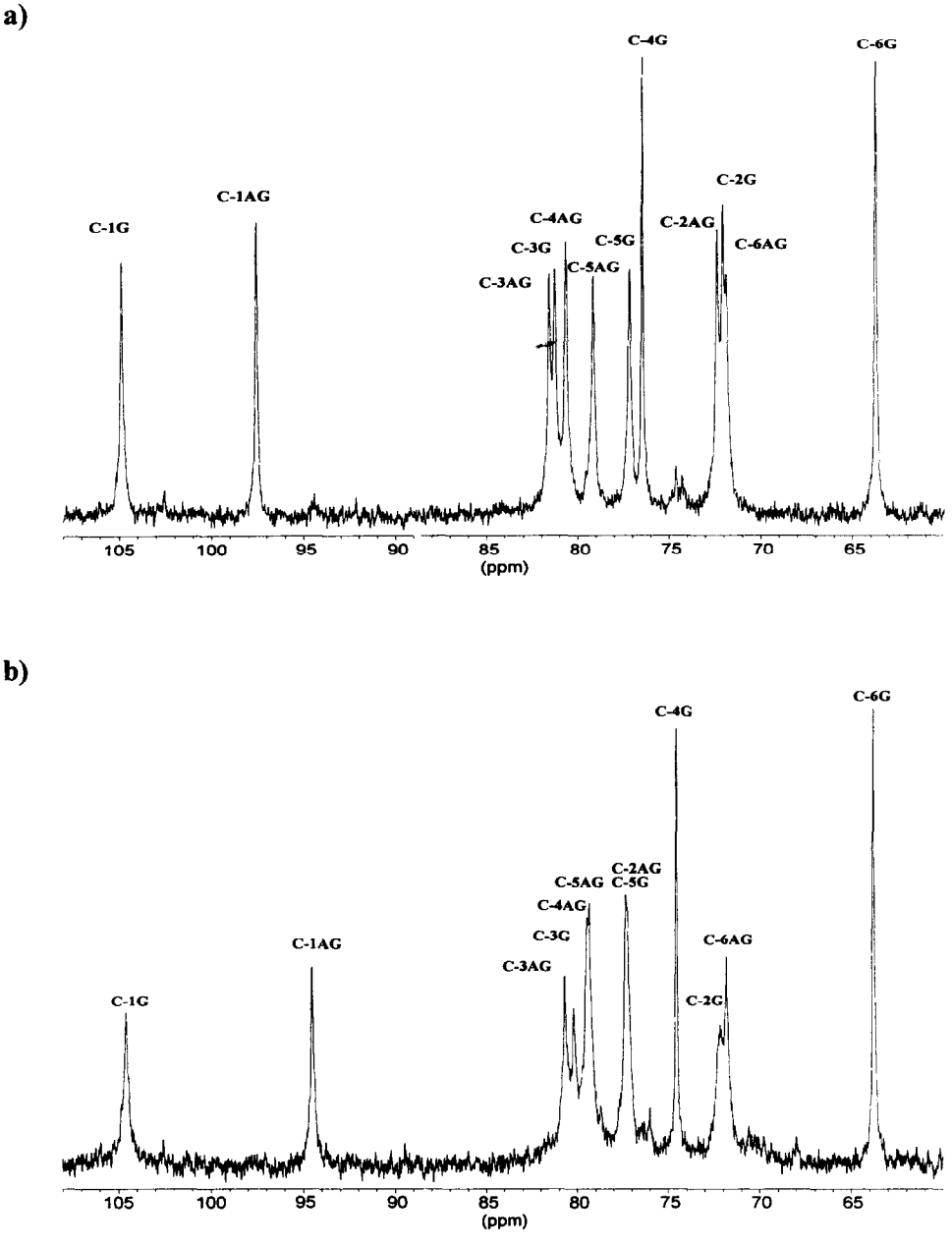

Fig. 3. ^{13}C-NMR spectra at about 80°C in H_2O/D_2O of: (a) kappa carrageenan; (b) iota carrageenan.

Table 1. ^{13}C chemical shifts of kappa and iota carrageenans obtained at about 80°C relative to TSP indirect external

Sample	^{13}C chemical shifts (ppm)						
	Solvent	C-1	C-2	C-3	C-4	C-5	C-6
Kappa carrageenan	H_2O/D_2O						
Galactosyl unit		104.86	72.06	81.26	76.47	77.16	63.75
Anhydro-3,6-D-galactosyl unit		97.58	72.30	81.57[a]	80.65[a]	79.15	71.87
Iota carrageenan	H_2O/D_2O						
Galactosyl unit		104.59	71.80	80.18	74.55	77.32	63.78
Anhydro-3,6-D-galactosyl unit		94.52	77.32	80.67[b]	79.33[b]	79.33	71.80

[a,b] Assignment is not unequivocal and may have to be reversed.

signals of the neighbouring carbon atoms in the monosaccharide residue (β-effect). The influence of sulphate groups is illustrated by the ^{13}C-NMR spectrum of iota carrageenan (Fig. 3b), which, compared to kappa carrageenan, has one more sulphate on the C-2 carbon of the AG unit (Fig. 1). The comparison of the ^{13}C chemical shifts of kappa and iota carrageenans (Table 1) shows the downfield shift of the C-2AG ($\Delta\delta = +5.07$ppm) and upfield shifts of the C-1AG ($\Delta\delta = -3.06$ppm), C-4AG ($\Delta\delta = -1.32$ppm) and C-3AG ($\Delta\delta = -0.90$ppm). Moreover, the iota carrageenan spectrum contains a very characteristic signal at 74.55ppm attributed to C-4G. This signal is absent from the spectra of other red algae galactans. It may be interpreted as the result of specific interaction between two sulphate groups belonging to adjacent monosaccharide residues in the iota carrageenan molecule.

In view of the later quantitative evaluation of the carrageenan mixtures, the spin–lattice relaxation times of the individual carbons should be approximately known, because quantitative spectra acquisition means that the waiting time between the radiofrequency pulses should be long enough that the system has time to come back to thermal equilibrium before it is disturbed by the next pulse. In the case of iota carrageenan, a rough relaxation time measurement revealed approximate T_1 values of less than 500ms for all carbon nuclei. Thus, an interpulse delay of about 3s should guarantee sufficient recovery.

In comparison with the methods available earlier, the structural analysis of red algae galactans (kappa and iota carrageenans) is greatly simplified by ^{13}C-NMR spectroscopy applied to hot aqueous solutions of these materials (Usov *et al.*, 1980). However, this technique has not been a significant aid in identifying lambda carrageenan. Native lambda carrageenan generally yields very viscous solutions even when hot, probably due to their high molecular weights and possibly also due to interactions with minor components, such as proteins, in the samples (King & Lautherbach, 1987). The resulting ^{13}C-NMR spectra are generally composed of very broad envelopes, rather than clearly defined signals and cannot, therefore, be quantitatively evaluated.

^{13}C-NMR of a known carrageenan blend

A carrageenan blend made directly from the individual polysaccharides was studied by ^{13}C-NMR at about 80°C under conditions of quantitative analysis. Figure 4 shows the spectrum of a mixture of kappa, iota and lambda carrageenan in proportions of 60/14/26%. By subtracting the spectra of kappa and iota carrageenan, it can be shown that this spectrum is essentially the sum of kappa and iota spectra (Fig. 3a, b). As expected, no narrow signals of lambda carrageenan are detected. Valuable structural information may be obtained not only from the resonance positions in the ^{13}C-NMR spectra, but also from the integrated signal intensities.

Generally, the intensity of every signal depends not only on the amount of the corresponding carbon atoms in the sample, but also on their relaxation times and nuclear Overhauser enhancement. Taking into account these factors, the proportions of integrated intensities of signals may be used as an analytical tool. The correctness of the data obtained was proved by chemical methods for several oligo- (Hamer *et al.*, 1977) and polysaccharides (Usov & Arkhipova, 1981). Thus, on the carrageenan blend investigated, the integration of C-1AG signals at 97.56 and 94.47ppm arising from kappa and iota carrageenan, respectively, gives an experimental kappa–iota ratio of 80/20% which corresponds well to the theoretical kappa–iota ratio of 81/19%.

Thus, ^{13}C-NMR spectroscopy of carrageenan blends, under conditions of quantitative analysis, allowed the kappa–iota composition to be determined, while the presence of lambda carrageenan was not detected. In the hope of achieving ^{13}C-NMR detection of lambda carrageenan, acid hydrolysis (H_2SO_4, adjusted to pH 3.0 at 80°C for 60, 120 and 180 min) was carried out, which should decrease the molecular weight of lambda carrageenan and thus reduce its viscosity. However, no quantitative determination of the three carrageenan types could be obtained in this way. This was probably due to the partial degradation of the kappa and iota carrageenan anhydrogalactose residues during the hydrolysis. The quantification of lambda carrageenan by ^{13}C-NMR was, therefore, abandoned and a complementary method based on IR spectroscopy was developed.

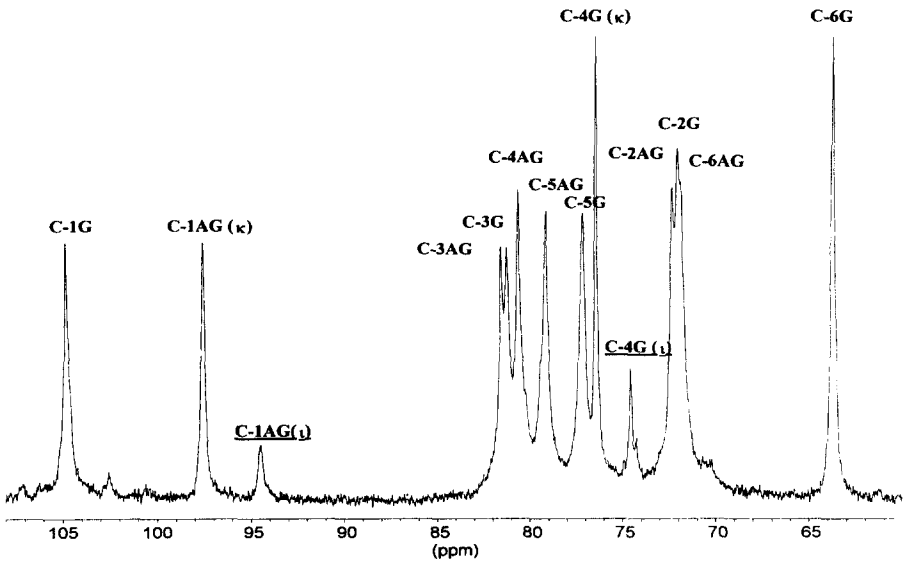

Fig. 4. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of a kappa–iota–lambda carrageenan blend in a ratio 60/14/26%.

Infrared spectroscopy study

Infrared spectroscopy of the starting polysaccharides

Infrared spectroscopy has been widely used to characterize kappa, iota and lambda carrageenan (Stancioff & Stanley, 1969; McCandless *et al.*, 1973, 1982; Dawes *et al.*, 1977; Whyte *et al.*, 1985; Rochas *et al.*, 1986). The IR technique is rapid, non-destructive and requires only a few milligrams of sample. The absorbance of IR bands provides information (Fig. 5a–c) on the presence in polymers of: 3,6-anhydrogalactose, at 930cm^{-1}; sulphate groups at 1250 and 1370cm^{-1}; galactose-4-sulphate at 845cm^{-1}; galactose-2-sulphate at 830cm^{-1}; galactose-6-sulphate at 820cm^{-1}; and 3,6-anhydrogalactose-2-sulphate at 805cm^{-1}. Finally, the absorbance band at 2920cm^{-1}, due to the C-H content, represents a good reference for the total sugar content.

The IR spectra of kappa, iota and lambda carrageenan are shown in Fig. 5a–c, respectively. All these spectra display an absorbance band at 1250cm^{-1} due to the total sulphate content. As expected, the absorbance of this band is much higher for lambda than for kappa and iota carrageenan. The structural features of each carrageenan (Fig. 1), in terms of sulphate position, give rise to other characteristic IR absorbance bands. Thus, the IR spectra of kappa and iota carrageenan (Fig. 5a, b) display one band at 845cm^{-1} arising from the galactose-4-sulphate (Fig. 1a, b). Iota carrageenan has one more sulphate on 3,6-anhydrogalactose-2-sulphate (Fig. 1b). This structural difference is illustrated by the band at 805cm^{-1} (Fig. 5b). The main structural feature of lambda carrageenan is to contain three types of sulphate groups. In the IR spectrum of lambda carrageenan (Fig. 5c), the absorbance at 830 and 820cm^{-1} are attributed to galactose-2-sulphate and to galactose-6-sulphate, respectively.

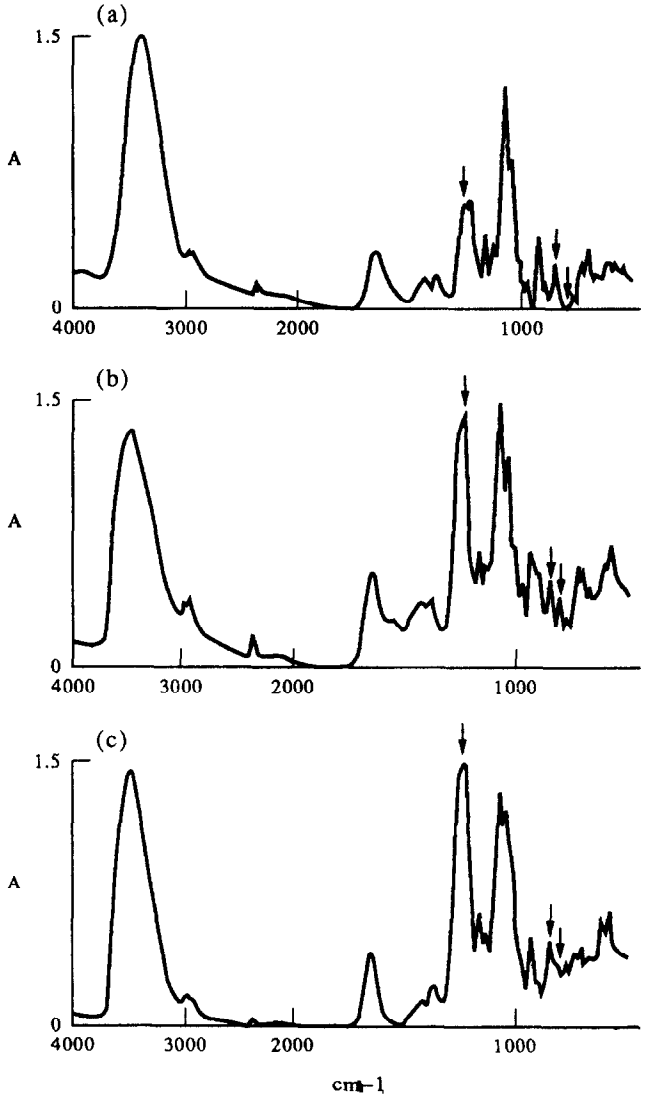

Fig. 5. Infrared spectra of: (a) kappa carrageenan; (b) iota carrageenan; (c) lambda carrageenan.

IR spectroscopy of known carrageenan blends

Figure 6 shows the experimental variation of the ratio of absorbances at 845/1250cm^{-1} with the kappa–iota composition. This experimental variation fits with the theoretical one obtained by using the ratio of absorbances of unblended kappa and iota carrageenan in the proportions required. A linear correlation is obtained. This correlation line, therefore, may be used to determine the composition of kappa–iota blends.

As the primary structure of lambda carrageenan is depleted of galactose-4-sulphate, it is not possible to determine the ratio of absorbances at 845/1250cm^{-1}. Thus, the theoretical correlation between the ratio of absorbances at 845/1250cm^{-1} and the kappa–iota–lambda composition cannot be established. However, as shown in Fig. 6, the addition of lambda carrageenan to a kappa–iota blend gives a ratio of absorbances at 845/1250cm^{-1}, drastically lower than the ones provided by a kappa–iota blend without any lambda carrageenan (the total sulphate content being strongly increased by the lambda carrageenan contribution).

Consequently, the determination of kappa–iota–lambda carrageenan composition can only be achieved by using both ^{13}C-NMR and IR spectroscopy. Firstly, the ^{13}C-NMR study allows the kappa–iota ratio to be determined. Secondly, IR spectroscopy, by determination of the ratio of absorbances at 845/1250cm^{-1}, allows one to distinguish whether the sample is a pure kappa–iota blend or if it contains lambda carrageenan molecules. In the case of a pure kappa–iota blend, the absorbance ratio at 845/1250cm^{-1} will be comparable to the one provided by the correlation line representing the ratio of absorbances at 845/1250cm^{-1} as a function of the kappa–iota composition. Otherwise, the ratio at 845/1250cm^{-1} will be much lower (Fig. 6). In this way, a semi-quantitative determination of lambda carrageenan in the blend can be achieved. Lambda carrageenan must be added to a kappa–iota blend having the same ratio as the one determined by ^{13}C-NMR investigation, in order to obtain the same ratio of absorbances at 845/1250cm^{-1} as the one displayed by the unknown commercial blend.

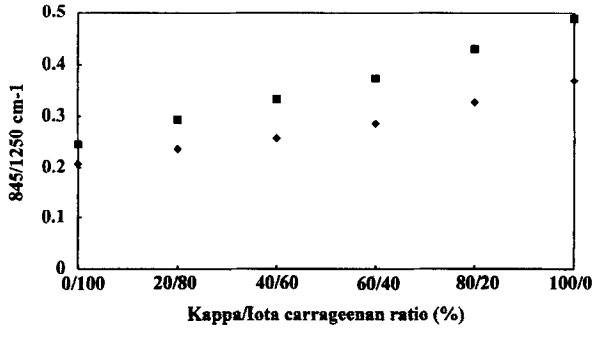

Fig. 6. Variation of the ratio of absorbances at 845/1250cm^{-1} with the kappa-iota carrageenan ratio of blends: ■ without lambda carrageenan; ◆ with 30% lambda carrageenan.

^{13}C-NMR and IR analysis of commercial carrageenan blends

^{13}C-NMR of sample no. 1

The ^{13}C-NMR spectrum (Fig. 7) of the commercial carrageenan blend shows the presence of biological precursors at 66.5ppm, near 70.3, 100.4 and 107.0ppm (Wong & Graigie, 1978). At 72.9 and 102.5ppm, floridean starch is detected. Floridean starch is a reserve polysaccharide of red seaweeds and various species may differ considerably in its content (Meeuse *et al.*, 1960). Floridean starch is mainly a branched α-glucan similar to amylopectins of plants (Ozaki *et al.*, 1967), sometimes resembling animal glycogen in its degree of branching (Whyte, 1971). The absence of any linear fraction of the amylose type was considered, formerly, as the main difference between floridean starches and the real starches of plants. However, amylose was also detected in several seaweeds (McCracken & Cain, 1981). In the anomeric region, the integration of C-1AG signals arising from kappa and iota carrageenan provides a kappa-iota ratio of 73/27%. IR analysis allowed us to detect about 30% of lambda carrageenan. Consequently, a kappa–iota–lambda composition of 51, 19 and 30% is obtained.

^{13}C-NMR of sample no. 2

The investigation of the commercial blend by ^{13}C-NMR (Fig. 8) allowed the biological precursors at 66.8, 70.3, 100.4 and 107.0ppm to be detected. Some signals in ^{13}C-NMR spectrum show a splitting (or rather two lines with a small shift difference), especially the anomeric carbons of the 3,6-anhydrogalactose residue of each carrageenan. This effect was observed earlier and was attributed to irregularly sulphated hybrid structures (Usov *et al.*, 1980). A similar phenomenon was noted for an incompletely sulphated kappa carrageenan called furcellaran, or Danish agar (Fig. 9). However, a large amount of glucose was detected in the latter sample, which may also play a role in creating different molecular environments for a part of the carrageenan subunits. The signals of the glucose itself, identified by comparison with the spectrum of genuine glucose in D_2O at the same temperature, are also split into two subsignals.

The combined ^{13}C-NMR and IR spectroscopy measurements suggest a kappa–iota–lambda composition of 56, 24 and 20%, respectively, for this sample.

^{13}C-NMR of sample no. 3

From the ^{13}C-NMR analysis (Fig. 10), this blend, also showing structural irregularities, appears as a kappa carrageenan containing about 5% of iota carrageenan (S/N insufficient for accurate quantification). The iota carrageenan structures can be dispersed in the chains of kappa carrageenan, or they can be present as pure iota

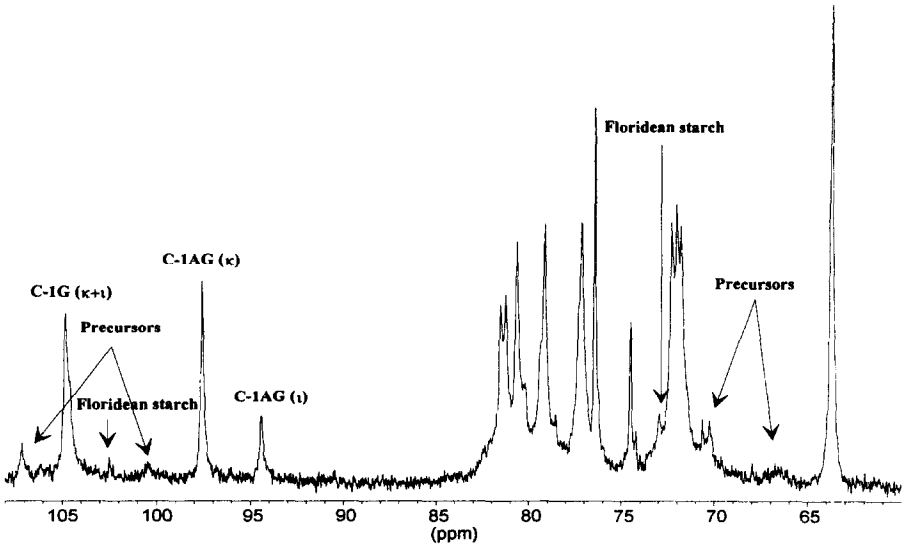

Fig. 7. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of sample no. 1.

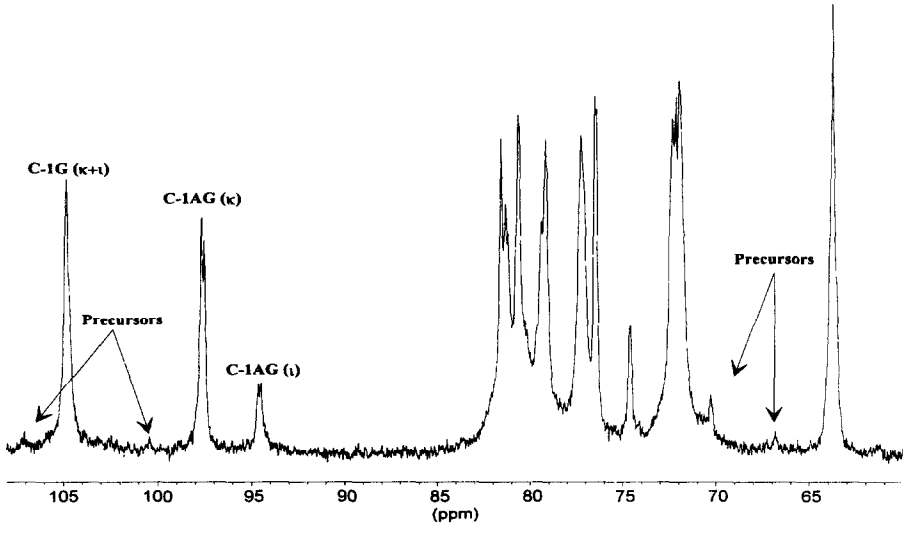

Fig. 8. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of sample no. 2.

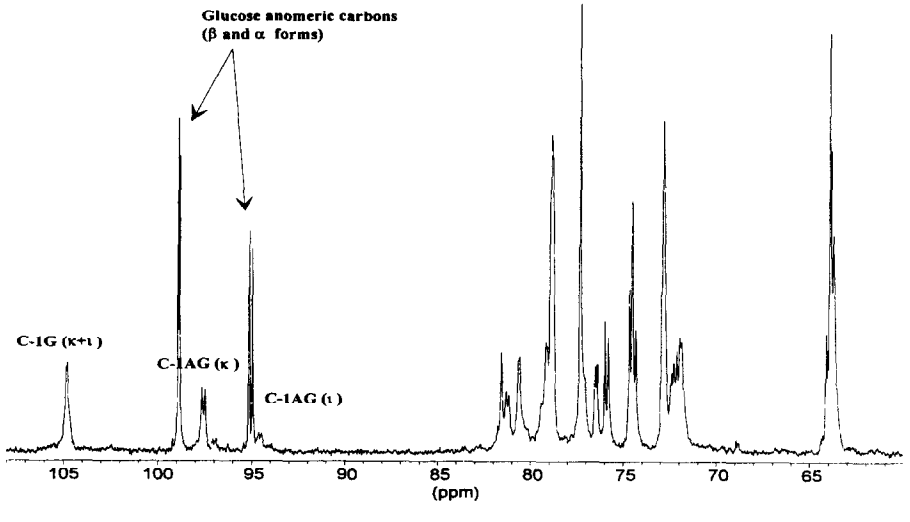

Fig. 9. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of furcellaran.

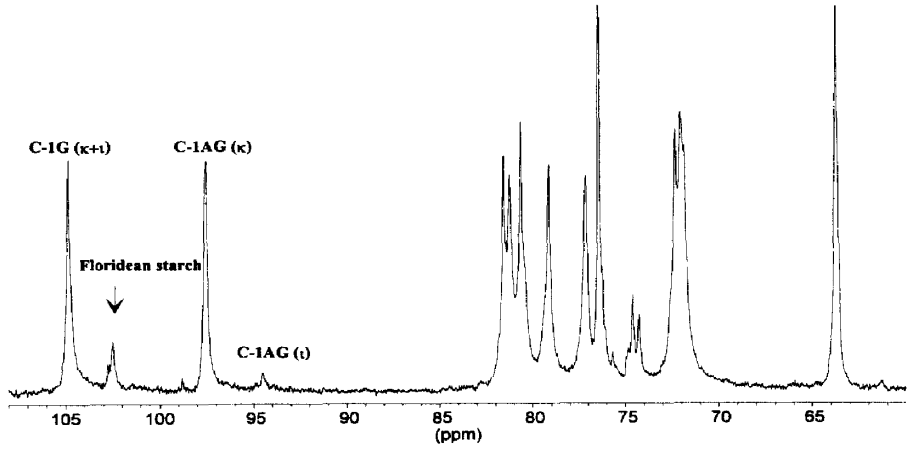

Fig. 10. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of sample no. 3.

carrageenan chains. Those two cases cannot be distinguished spectroscopically. Another feature of this commercial product is its contamination by about 10% floridean starch (signal at about 102.6ppm). No lambda carrageenan was detected in this sample by IR spectroscopy.

^{13}C-NMR of sample no. 4

The ^{13}C-NMR (Fig. 11) and IR spectra of this sample show the characteristics already observed with the commercial sample no. 3 provided by the same supplier. Thus, this sample can be considered as a kappa carrageenan contaminated by about 5% iota carrageenan and 5% floridean starch. The same difficulty of iota carrageenan quantification applies. No lambda carrageenan was detected by IR spectroscopy.

^{13}C-NMR of sample no. 5

As shown for the ^{13}C-NMR spectrum (Fig. 12), the gel strength of this sample was standardized with sucrose, represented by 12 narrow lines. The sucrose was identified by comparison with the spectrum of genuine sucrose in D_2O, at the same temperature. From the spectroscopic analyses, it was established that this commercial product is composed of 90% kappa and 10% iota carrageenan. The accuracy of ^{13}C-NMR determination is limited by the overlap of C-1′ signal of α-D-glucose and C-1AG signal of iota carrageenan.

^{13}C-NMR of sample no. 6

As already mentioned for the sample no. 2, the ^{13}C-NMR spectrum of this sample (Fig. 13) shows the presence of kappa and iota carrageenan which are probably irregularly sulphated (splitting of anomeric signals, but also of other carbon signals). From the anomeric ^{13}C-NMR signals, the proportion of kappa and iota is estimated at 55 and 45%, respectively. Moreover, at 95.1 and 98.9ppm, glucose signals (α and β forms) are detected. Note that both glucose anomeric carbon signals are split into two parts with slightly different shifts. This may tentatively be interpreted as the result of a binding of part of the glucose molecules to the carrageenan macromolecules. Adding some more glucose slightly increased the integral ratio glucose C-1 (β)/C-1AG (κ) and reduced the shift differences between the signal pairs of glucose and of both carrageenans.

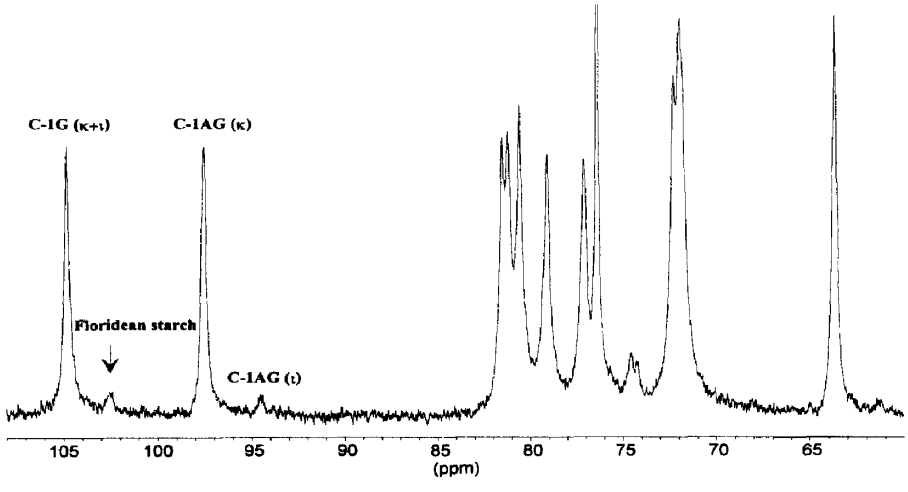

Fig. 11. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of sample no. 4.

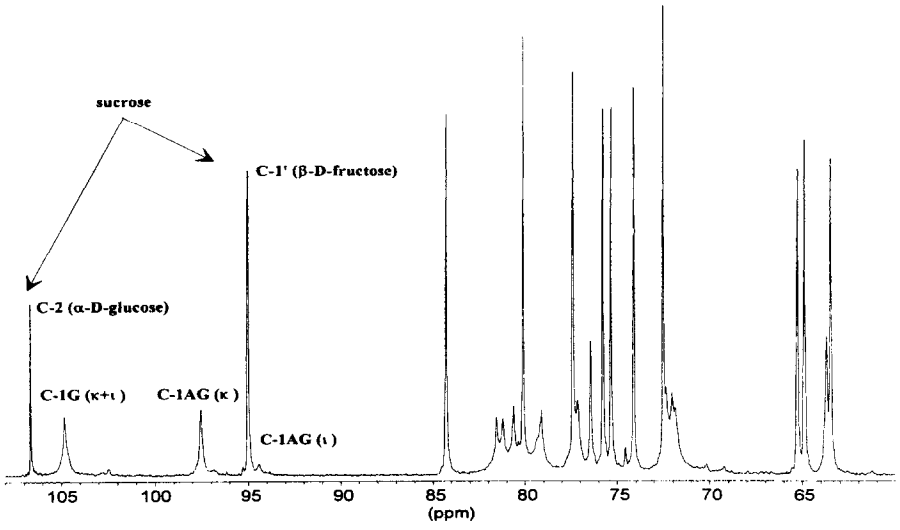

Fig. 12. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of sample no. 5.

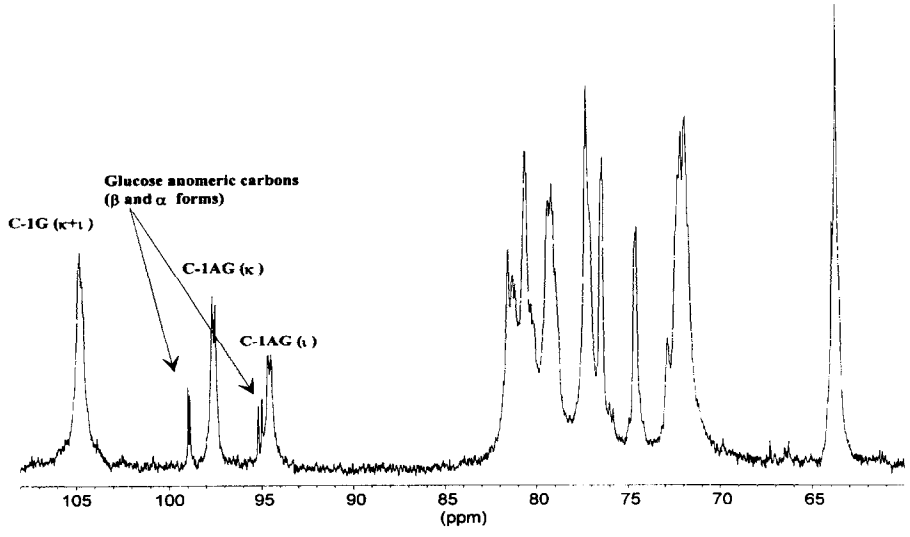

Fig. 13. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of sample no. 6.

The latter phenomenon may be a concentration effect. These results are preliminary and do not permit final conclusions, but it seems that the carrageenan(s) or other components of the commercial products have the capability of intermolecular association with the glucose, and that depending on the concentration ratio a free and a bound form of glucose may coexist, possibly in slow chemical exchange. Again, IR spectroscopy did not detect any lambda carrageenan in this sample.

^{13}C-NMR of sample no. 7

The ^{13}C-NMR (Fig. 14) and IR spectra of this sample show characteristics already observed with the commercial sample no. 6 provided by the same supplier. This product contains only kappa and iota carrageenan macromolecules, irregularly sulphated, in a ratio of about 58/42%, as estimated from the C-1AG integrals (after subtracting the interfering α-glucose signal). The main difference to the previous sample is the higher glucose content.

CONCLUSIONS

^{13}C-NMR and IR spectroscopy measurements were used successfully to determine the carrageenan composition of several commercial samples. The kappa and iota carrageenans and irregularly sulphated hybrid structures could be distinguished. Additional information, e.g. on the presence of floridean starch, glucose, sucrose as well as the detection of biological precursors, was simultaneously obtained. The precision of quantification of all the components in blend samples depends on their composition and on the achievable signal/noise ratio of the NMR spectra as well as a sufficient line separation.

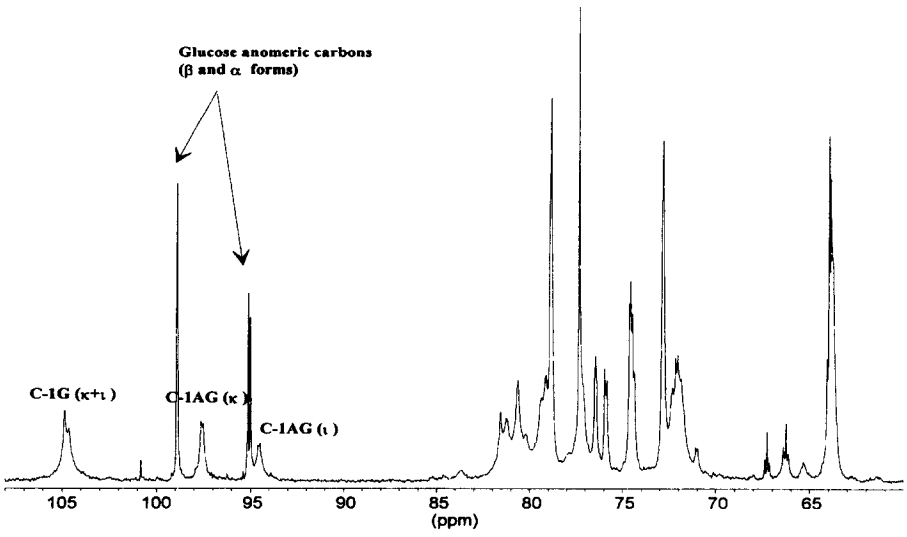

Fig. 14. ^{13}C-NMR spectrum at about 80°C in H_2O/D_2O of sample no. 7.

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