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Solid State ^{13}C-NMR Spectroscopy of Red Seaweeds, Agars and Carrageenans

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ABSTRACT

The ^{13}C CP-MAS spectra of kappa-, iota-, lambda-carrageenans, partly desulfated kappa-carrageenan (furcellaran) and different agaroses, isolated or in situ *in algae, are reported. Comparison of spectra obtained from these polysaccharides in their solid state and in solution is also presented.*

INTRODUCTION

Nowadays, nuclear magnetic resonance spectroscopy (NMR) is a widely used technique for the structural study of polysaccharides and, recently, Usov (1984) reviewed its application for the analysis of red seaweed cell-wall polysaccharides. Although NMR spectroscopy (^{13}C, ^{1}H) is helpful in the primary structure elucidation, it also permits the characterization of conformations and gels formed by seaweed polysaccharides.

To record high resolution NMR spectra (^{13}C, ^{1}H) of algal cell-wall polysaccharides, extraction and purification of the molecules are first required. Unfortunately, such long and tedius processes affect the types of molecules isolated (Wen & Craigie, 1984; Lahaye, 1986) and thus the NMR information obtained does not describe the exact structure of all

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the polysaccharides present in the algal cell-wall. Therefore, a method permitting the characterization of molecules *in situ* in the algal cell-wall would be more beneficial. In that respect, the use of cross-polarization-magic-angle-spinning ^{13}C-NMR spectroscopy (CP-MAS ^{13}C-NMR) which allows the characterization of polysaccharides in their solid state, was investigated. A review describing this recent technique and its potential in carbohydrate chemistry has been published by Pfeffer (1984). We now report on the CP-MAS ^{13}C-NMR spectra of agars and carrageenans either as extracted polysaccharides or *in situ* in algae, and attempt the correlation of the observed chemical shifts with those reported in the literature from their solution spectra.

MATERIALS AND METHODS

Kappa- and iota-carrageenans were purchased from Sigma (Saint Louis, MO, USA); Lambda-carrageenan, extracted from *Gigartina canicu-lata,* and agarose were gifts from Marine Colloids (Rockland, Maine, USA); agarose oligomers were prepared as described (Rochas *et al.,* 1986); furcellaran was a gift from Litex Company (Copenhagen, Denmark); *Hypnea musciformis,* the sample studied by Greer *et al.* (1984), was collected from St Lawrence Bay, Barbados, West Indies; *Gracilaria compressa* and *G. domingensis* were the samples studied by Duckworth *et al.* (1971) and were collected from the Caribbean island of Barbados. The cold water extract agar from *G. compressa* was obtained as described by Lahaye *et al.,* 1986. *G. eucheumoides* was collected on Hainan island, People's Republic of China; *G. crassissima* was the sample studied by Lahaye *et al.* (1988) and was collected at Point Sable, St Lucia, West Indies; and *G. bursa-pastoris* agar, extracted at pH 11.0, was a gift from Dr M. Doty. The latter preparation was essentially composed of charged polysaccarides with a sulfate content of 9·23% estimated on the dry weight basis of polysaccharides using the turbidimetric method used by Craigie *et al.* (1984). The major fraction of these polysaccarides was eluted with 1 M NaCl solution from DEAE-Sephadex A 50 when fractionated as described (Ji *et al.,* 1985; Fig. 1(A)) and had a ^{13}C-NMR spectrum with chemical shifts characteristic of agar composed essentially of agarobiose repeating units (Fig. 1(B); G, A) or agarobiose substituted by methoxyl (Fig. 1(B); G′) or sulfate groups (Fig. 1(B); A″, G″) on carbon 6 of the β-*D*-galactopyranose residue (Nicolaisen *et al.,* 1980; Usov *et al.,* 1983).

Commercial samples were used as they were. Air dried seaweeds were broken into small pieces (granules, sticks, or both, of about 1–2 mm), the

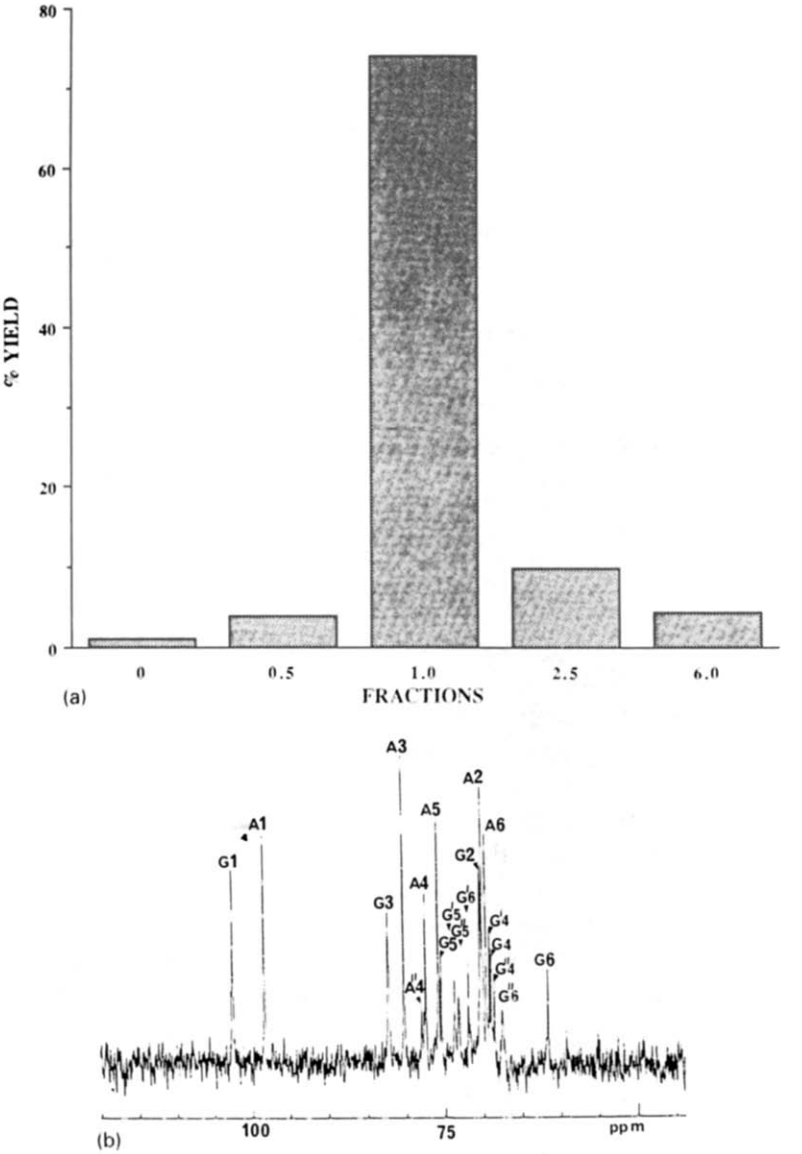

Fig. 1. (a) Yield as percentage of total carbohydrate recovered in the fraction of *Gracilaria bursa-pastoris* agar eluted from DEAE-Sephadex A 50 with (0) distilled water, 0·5, 1·0, 2·5 M NaCl and 6·0 M urea. (b) ^{13}C-NMR spectrum at 80°C of the *Gracilaria bursa-pastoris* agar fraction eluted with 1·0 M NaCl from DEAE-Sephadex A 50. A and G refer to carbons belonging to the anhydrogalactose and galactose residue, respectively, in the agarobiose repeating unit; A″ and G″ refer to carbons belonging to the anhydrogalactose and galactose-6-sulfate, respectively, in the 6-*O* sulfated agarobiose repeating unit; G′ refers to carbons of the 6-*O* methyl galactose in the 6-*O* methylated agarobiose repeat unit. Numbers associated to the above letters refer to specific carbons in each residue. 3200 transients were accumulated from a 40 g l^{-1} D_2O agar solution on a Bruker WH 400 spectrometer operating at 100·6 MHz. Chemical shifts are expressed in part per million (ppm) relative to internal DMSO and converted to values relative to TMS (conversion constant 39·6).

size of which did not affect the quality of the CP-MAS ^{13}C-NMR spectra. The solid-state spectra consisted of 1000–3000 scans recorded on a CXP 200 Bruker with a spinning of 3·900 kHz and contact and repetition times of 5 ms and 3 s, respectively. Chemical shifts were measured from hexamethyl benzene and converted to values related to TMS.

Simulated CP-MAS ^{13}C-NMR spectra were obtained using the program Panic from the Bruker company. Only two parameters were used: chemical shift and peak width. The chemical shifts used were those observed on the high resolution ^{13}C-NMR spectra of carrageenans and agaroses solutions as cited in the text. A peak width of 90 Hz which was that of the well resolved signals of C-1 of carrageenan or C-6 of every sample on the solid-state ^{13}C-NMR spectra, was fixed for all the signals.

RESULTS AND DISCUSSION

Correlations have often been attempted between the chemical shifts observed on ^{13}C-NMR spectra of solutions and solids, but most of them were unsuccessful (Pfeffer *et al.*, 1983) for at least three reasons: firstly, polysaccharides can either be amorphous or crystalline; secondly, they can adopt different conformations or organizations in their solid and liquid states (Hewitt *et al.*, 1986); and thirdly, they can have different level of hydration. These three particular factors affect the solid-state ^{13}C-NMR chemical shifts and thus render difficult the correlation with shifts observed on solution ^{13}C-NMR spectra. Since the crystallinity of agaroses and carrageenans is low, it is legitimate to correlate, in a first approach, chemical shifts observed on ^{13}C-NMR spectra of solutions and solids.

Carrageenans and carrageenophytes

The CP-MAS ^{13}C-NMR spectra of kappa- and iota-carrageenans show six signals (Figs 2 and 3) and, for both polysaccharides, there is a good agreement between the chemical shifts of peaks 1, 2, 5 and 6 observed on the solid-state spectra and those on the solution spectra (Table 1; Greer *et al.*, 1985; Rochas *et al.*, 1980). However, because of the above reasons, slight variations are observed for the chemical shifts of peaks 1 and 6.

In the solid-state, kappa- and iota-carrageenans adopt helical conformations (Anderson *et al.*, 1969), and thus their CP-MAS ^{13}C-NMR spectra are those of helices. In contrast, their high resolution ^{13}C-NMR spectra represent molecules under conditions (temperature, ionic con-

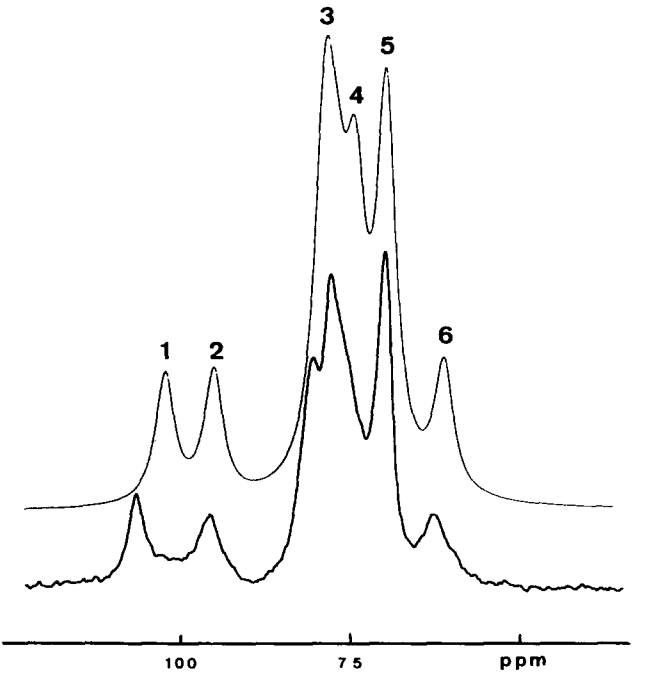

Fig. 2. 50·3 MHz CP-MAS ^{13}C-NMR spectra of kappa-carrageenan: top, simulated from chemical shifts published by Rochas *et al.* (1983); bottom, experimental spectrum.

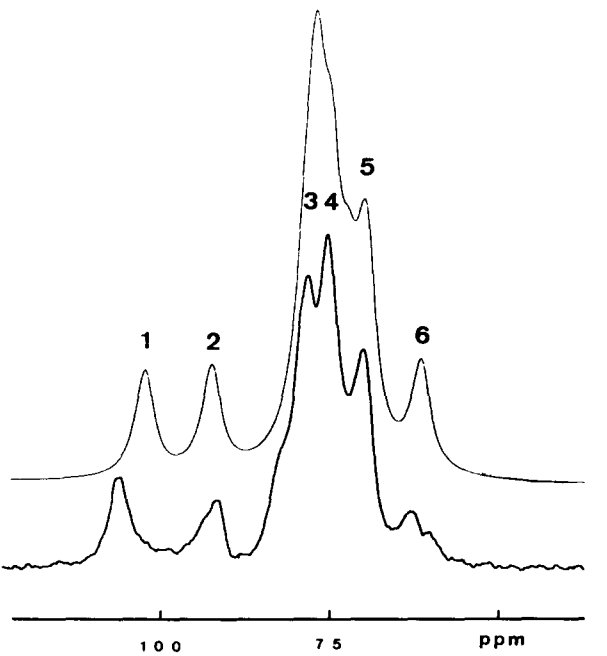

Fig. 3. 50·3 MHz CP-MAS ^{13}C-NMR spectra of iota-carrageenan: top, simulated spectrum from chemical shifts published by Greer *et al.* (1985); bottom, experimental spectrum.

TABLE 1
Solution (80°C) and Tentative CP-MAS ^{13}C-NMR (room temperature) Chemical Shifts Assignments (ppm) of Kappa- and Iota-carrageenan

Carbon[a]	*Peak*	*Solid*	*Solution*
Kappa-carrageenan[b]			
1G	1	106·6	102·2
1A	2	95·8	94·9
3A, 3G, 4A	3	80·8	78·9, 78·7, 78·0
5A, 5G, 4G	4	78·3	76·5, 74·5, 73·8
2A, 2G, 6A	5	70·4	69·8, 69·6, 69·55
6G	6	63·5	61·1
Iota-carrageenan[c]			
1G	1	106·4	102·2
1A	2	92·1	92·1
4A, 3A, 3G, 5A	3	78·7	78·3, 77·9, 77·2, 76·9
5G, 2A	4	75·8	75·0, 74·9
4G, 6A, 2G	5	70·8	72·2, 69·9, 69·4
6G	6	63·5	61·3

[a]G represents the galactopyranose and A, the anhydrogalactopyranose residues; the number refers to the number of the carbon atom in each residue.
[b]From Rochas *et al.* (1983).
[c]From Greer *et al.* (1985).

centration) where they are in the coil state (Rochas *et al.*, 1980). Thus, because of these different conformations, the torsion angles of the glycosidic linkages differ between the solid and solution states of these polysaccharides and result in the shift of the resonance of carbons involved in these linkages (1G, 3G, 1A, and 4A; Table 1; Hewitt *et al.*, 1986). The conformational effect is particularly important for the peak of 1G of both kappa- and iota-carrageenans since one observes a downfield shift of 4·4 ppm of this signal between the solution and solid-state ^{13}C-NMR spectra. However, and probably because of the rigidity of the anhydrogalactose residue, the conformational effect on the chemical shift of carbon 1A is small (0·9 ppm downfield) for kappa-carrageenan and absent for iota-carrageenan. Similarly, and as expected from the rigidity of this 4-linked anhydro-sugar, no confirmational effect was observed on the chemical shift of 4A in both polysaccharides. However, a downfield shift of 3 ± 2 ppm of the 3G signal, as observed for 1G, would explain the differences observed for peaks 3 and 4 between the simulated spectra and the solid-state spectra, as well as the differences of intensity observed on the kappa- and iota-carrageenans solid-state spectra.

In contrast, there is a good agreement between the peak 5 observed on the experimental and simulated solid-state spectra of kappa- and iota-carrageenans. Indeed, this is because the chemical shifts of carbons occurring in this peak (Table 1) are not sensitive to a conformational change since the carbons are not involved in the glycosidic linkage.

The conformation of the CH_2OH group differs in the solution and solid-state of kappa- and iota-carrageenan and thus affects the chemical shift of 6G on the CP-MAS ^{13}C-NMR spectrum. This carbon resonates 2·4 and 2·2 ppm downfield, respectively, as compared to the chemical shift observed on their respective solution spectra, and have a chemical shift that suggests a gauche-*trans* conformation of the CH_2OH group (Pfeffer, 1984; Hewitt *et al.*, 1986; Horri *et al.*, 1987).

The relative change in intensity of peaks 3, 4 and 5 between the spectra of kappa- and iota-carrageenans can be explained by the NMR substitution rules applied to a sulfation (Usov, 1984). Indeed, iota-carrageenan can be considered as a kappa-carrageenan with the hydroxyl linked to carbon 2A substituted by a sulfate group. With respect to the effect of this substitution observed on the solution spectrum of iota-carrageenan, the 2A resonance on CP-MAS ^{13}C-NMR spectrum of iota-carrageenan is attributed 5·4 ppm downfield compared to that of kappa-carrangeenan spectrum with respect to that of kappa-carrageenan (Figs 2 and 3).

The CP-MAS ^{13}C-NMR spectrum of the alga *Hypnea musciformis* (Fig. 4), is intermediate to that of kappa- and iota-carrageenans (Greer *et al.*, 1984). This result was expected since this alga synthesizes a hybrid of kappa- and iota-carrageenans (Greer *et al.*, 1984). Furthermore, from results reported below, and although the signal for the carboxyl group is not observed, it is probable that the asymmetrical wide signal centered at 33·6 ppm is due to the methyl of a pyruvate group (Gorin *et al.*, 1982). Pyruvic acid has alreay been described as a covalently linked substituent group in carrageenans (Hirase & Watanabe, 1972; DiNinno *et al.*, 1978, 1979) but its presence in *Hypnea musciformis* carrageenan remains to be established. The very wide resonance centered at 33·0 ppm could correspond to CH_2 groups of lipids and/or pigments.

The CP-MAS ^{13}C-NMR spectrum of furcellaran presented six resonances with chemical shifts identical to those of kappa-carrageenan (Fig. 4). However, in contrast to the kappa-carrageenan spectrum, the resonance at 78·4 ppm is of lower intensity to the advantage of that of the signal at 70·4 ppm which is increased. In fact, furcellaran can be considered as a partially desulfated kappa-carrageenan, and thus, due to the presence of the unsubstituted hydroxyl on 4G, this carbon is expected to resonate upfield (Usov *et al.*, 1980), and in this case a shift of 7·7 ppm is observed.

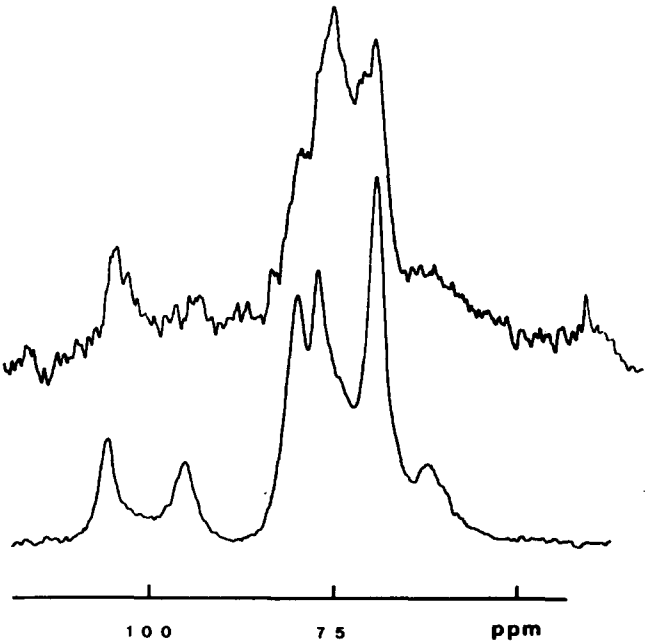

Fig. 4. 50·3 MHz CP-MAS ^{13}C-NMR spectra of *Hypnea musciformis* (top) and furcellaran (bottom).

The spectrum of lambda-carrageenan shows only four major signals (Fig. 5) at 102·8, 93·2, 76·4, and 65·8 ppm, the latter signal having many shoulders. A comparison with literature values for lambda-carrageenan chemical shifts is impossible since they have not been yet reported. Two minor signals at 176·3 and 27·3 ppm (not represented) are attributed to the carboxyl and methyl groups, respectively, and thus suggest the presence of pyruvate groups in *Gigartina caniculata* lambda-carrageenan. No NMR spectra of crude lambda-carrageenan have yet been published because the high viscosity of the solution prevents a well resolved spectrum being obtained. A simple way to obtain such a spectrum is to reduce the viscosity and consequently the molecular weight of the polymer or family of polysaccharides. Although hydrolysis with a weak acid has been used with success (Rochas, 1982), we used ultrasonication to reduce the viscosity of the lambda-carrageenan solution and obtained a good spectrum (Fig. 5). The chemical shift of the major signals on the solution spectrum is in agreement with that observed on the solid-state spectrum. However, due to the low resolution of the latter spectrum, no precise comparison nor assignment were attempted. Nevertheless, one can observe the complexity of the spectrum reflecting the structural diversity of the polysaccharide or

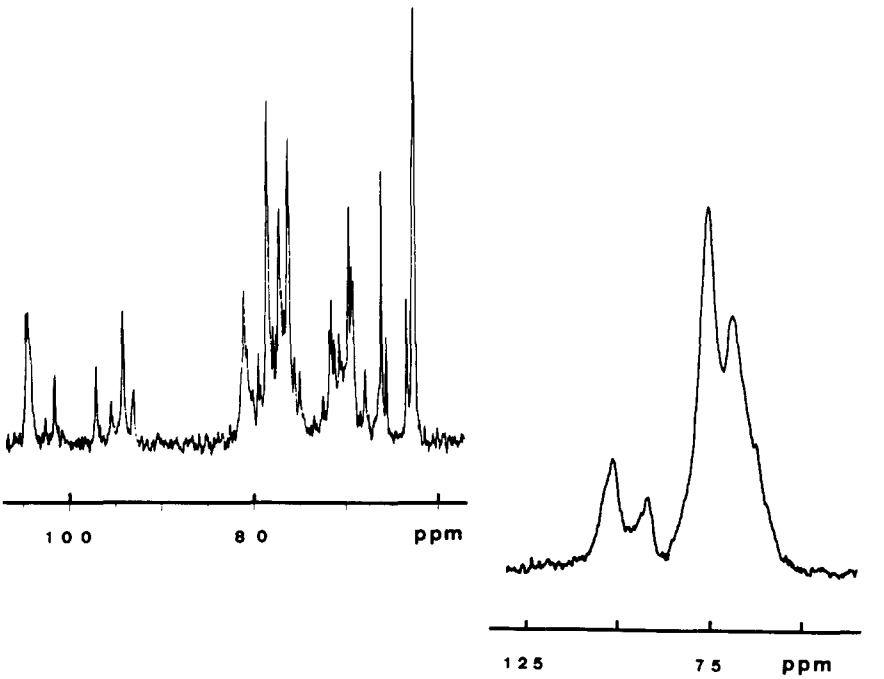

Fig. 5. 50·3 MHz CP-MAS ^{13}C-NMR spectrum at 80°C of sonicated lambda-carrageenan recorded on a Bruker AM 300 from a 40 g l^{-1} D_2O solution (top) and ^{13}C-NMR spectrum of lambda-carrageenan (bottom).

family of polysaccharides. Several anomeric carbons observed in the range of 90–105 ppm, demonstrate the presence of several types of repeating structure in lambda-carrageenan.

Agarose and agarophytes

The solid-state ^{13}C-NMR spectrum of agarose (Fig. 6) shows resonances with chemical shifts in good agreement with those observed on the solution spectrum (Table 2), except for the signal 6G which is explained as for carrageenans, because of conformational effects on the CH_2OH group.

The CP-MAS ^{13}C-NMR spectra of agarose oligomers (Fig. 7) have signals with chemical shifts in good agreement with those observed on their solution spectrum (Table 2) except, again, for 6G which adopts a gauche-*trans* conformation in the solid-state.

The comparison of CP-MAS ^{13}C-NMR spectra of agarose and agarose oligomers demonstrates a very good agreement of the chemical shifts (Figs 6 and 7; Table 2) if one considers the presence of the

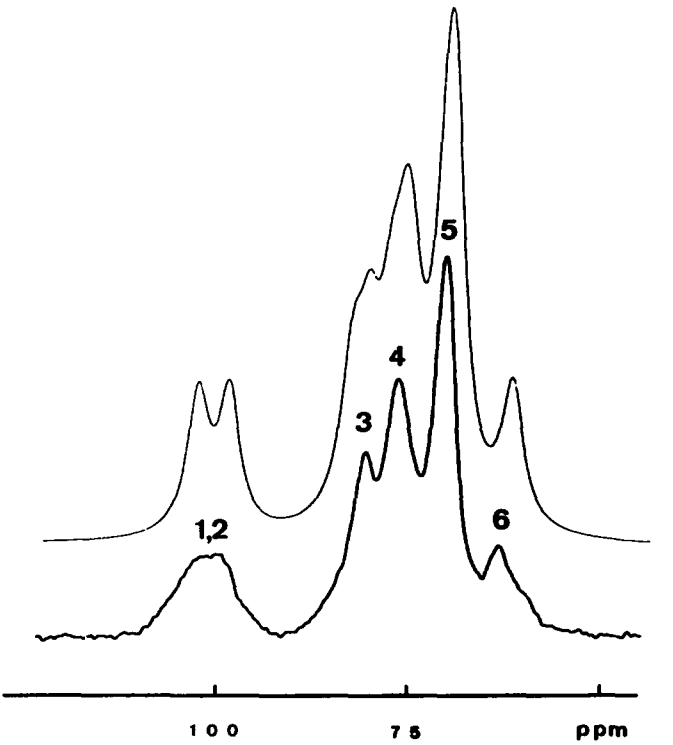

Fig. 6. 50·3 MHz CP-MAS ^{13}C-NMR spectrum of agarose: top, experimental spectrum; bottom, simulated spectrum from chemical shifts published by Rochas *et al.* (1986).

particular reducing end and non-reducing end repeating units in oligomers (Rochas *et al.*, 1986) which affect the relative intensity of some signals or induce the presence of others. For example, the signal for 1Gβ is only seen on the spectrum of oligomers of low degree of polymerization (DP). There is, however, a small chemical shift difference (2·2–1·8 ppm) for the signal of 1G, 1A, and 1Gβ which are at 99·5–99·9 ppm in oligomers and at 101·7 ppm for the polymer. In contrast to carrageenans, there is a total agreement between the chemical shifts on the simulated spectrum and those on the solid-state spectrum of agarose. There is also a good agreement between the solid-state spectra of oligomers and agarose polymer. Although oligomers of low DP in solid-state and agarose in solution are random coils and agarose in solid-state is in helical conformation (Arnott *et al.*, 1974), the similarity of the chemical shifts observed on the CP-MAS and solution ^{13}C-NMR spectra suggests that the mean state of agarose coils in solution is close to the helical conformation.

TABLE 2
Solution and Tentative CP-MAS ^{13}C-NMR Chemical Shifts Assignments (ppm of Agarose Oligomers DP2 and DP4 and Agarose

Carbon[a]	*Sample*	*Peak*	*Solid*[b]	*Solution*[c]
1G, 1A, 1Gβ	DP2	1	99·5	102·7, 98·7–98·5, 97·1
	DP4		99·9	102·7, 98·7[d]–98·5, 97·1
	Agarose		101·7	102·4, 98·3
1Gα	DP2	2	93·2	93·1
	DP4		93	93·1
3G, 3A	DP2	3	80·8	82·9–81·4, 81·3–80·4
	DP4		81·4	82·9[d]–81·4, 81·3[d]–80·4
	Agarose		81·2	82·2, 80·1
4A, 5G, 5A	DP2	4	76·7	77·7[e]–70·4, 75·7–75·5, 77·8–75·9
	DP4		77·0	77·7–70·4[d,e], 75·7–75·5[c], 77·8[c]–75·9
	Agarose		76·9	77·4, 75·6, 74·7
2G, 2A, 6A, 4G	DP2	5	70·8	71·8–70·6, 70·1–70, 69·7–69·45, 75·7–75·5
	DP4		70·9	71·8[d]–70·6, 70·1–70[d], 69·7[d]–69·45, 69·2–69·1[d]
	Agarose		70·9	70·3, 69·9, 68·8, 69·4
6G	DP2	6	63·5	61·8
	DP4		63·9	61·8
	Agarose		63·5	61·4

[a]See legend of Table 1 for carbon nomenclature, α and β refer to the anomeric configuration.
[b]At room temperature.
[c]DP2 and DP4 at 30°C and agarose at 80°C from Rochas *et al.* (1986).
[d]Minor signal.
[e]Signal probably overlapping that of peak 6 on the solid-state spectrum.

The cold extract agar from *Gracilaria compressa* and the alga itself yield similar spectra (Fig. 8) with six well defined resonances at 176·0, 102·5 (composed of two unresolved signals), 80·5, 75·7, 71·2, 33·5 (in a large peak centered at 26·0 ppm) and shoulders observed upfield of the resonance at 71·2 ppm. As said above for carrageenan and agarose, the signal at 176·0 ppm is attributed to the carbon of the carboxyl in a pyruvate group; the large peak centered at 102·5 ppm is attributed to carbons 1A and 1G; the signals between 80·5 and 71·2 ppm represent resonances of the other carbons except that of 6G which appears as a shoulder of the signal at 71·2 ppm. The large signal centered at 26·0 ppm is attributed to the methyl carbon of a pyruvate group and also probably

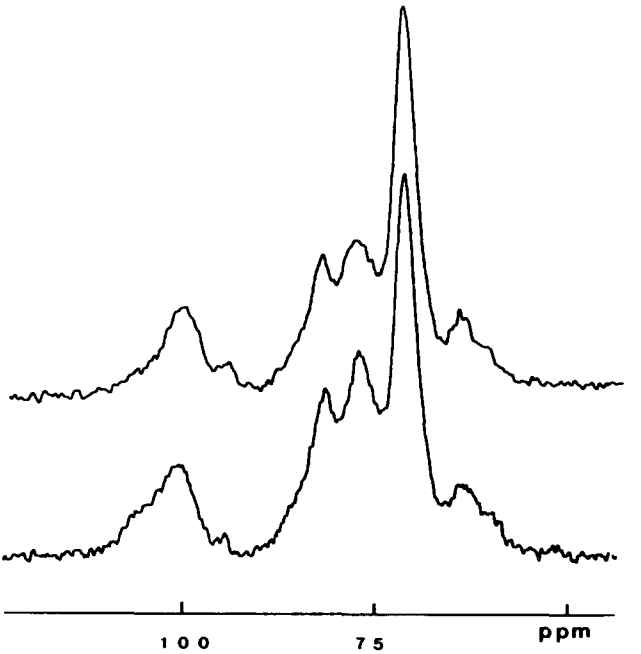

Fig. 7. 50·3 MHz CP-MAS ^{13}C-NMR spectra of agarose oligomer DP2 (top) and agarose oligomer DP4 (bottom).

to CH_2 groups of lipids and/or pigments. A similar spectrum was obtained from *G. domingensis* (Fig. 8) except that the concentration of pyruvate groups is lower as observed from the signal intensity at 176·0 and 26·0 ppm. The presence of pyruvate on these agars is in agreement with previously published data (Duckworth *et al.*, 1971; Lahaye, 1986).

In contrast, the solid-state spectrum of *G. bursa-pastoris* agar suggests the lack of pyruvate group (Fig. 9) which is in agreement with results obtained from the solution spectrum (Fig. 1). However, a large resonance centered at 60·0 ppm is observed which, from the agarose spectrum, does not correspond to 6G, unless the latter carbon is affected by a substituent group. The presence of a methoxyl group on 6G is demonstrated by the dipolar dephasing spectrum (Fig. 9) where almost all the signals of the original solid-state spectrum disappeared except that at 60·3 ppm. This particular pulse sequence markedly attenuates the protonated carbons resonance except that of methyl groups (Pfeffer *et al.*, 1983), and was used here to identify the methoxyl signal on the spectrum of *G. bursa-pastoris*. However, according to chemical shifts observed for 6-*O*-methylated agarose solution spectra, the presence of a methoxyl on 6G would shift the methylene carbon signal to the 71–72

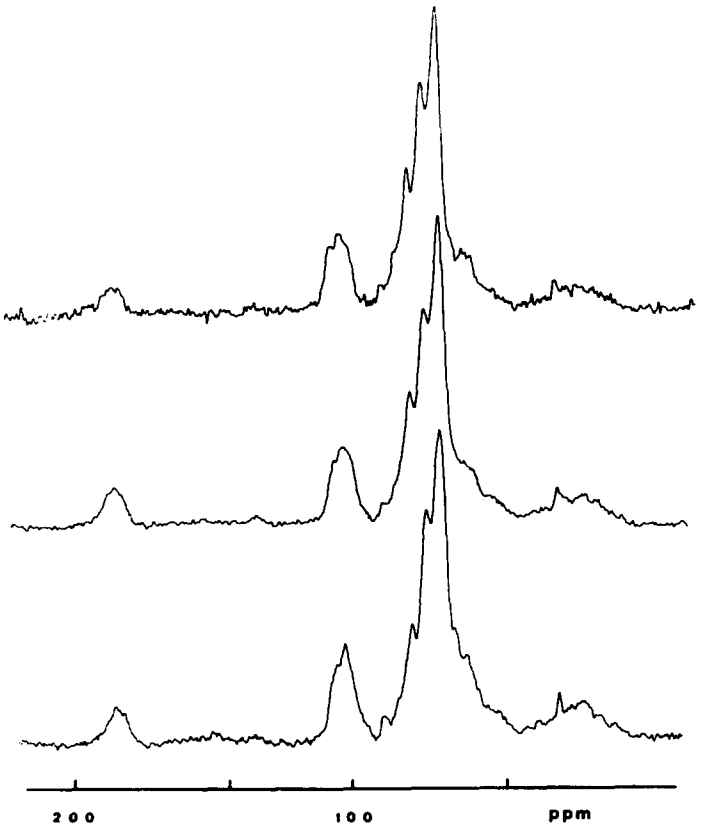

Fig. 8. 50·3 MHz CP-MAS ^{13}C-NMR spectra of *Gracilaria domingensis* (top), *G. compressa* (center) and of the cold water extract agar from *G. compressa* (bottom).

ppm region (Nicolaisen *et al.*, 1980). Therefore, the signal overlapping that of the methoxyl on the solid-state spectrum may not be attributed to 6G substituted by O-CH_3 but rather to 6G substituted by a sulfate group. The latter substituted carbon is known to resonate on solution spectra of sulfated agarose at 67·5 ppm (Usov *et al.*, 1983) and further work is needed to clearly establish the chemical shift of such carbon on a solid-state spectrum.

The spectrum of *G. crassissima* (Fig. 10) is not markedly different from that of agarose (Fig. 6), except the intensity of the resonance at 81·2 ppm which is smaller than that at 75·9 ppm in regard to the agarose spectrum. Such a difference can be explained by the presence in this agar of a high concentration of repeating units having the anhydrogalactose residue replaced by *L*-galactose-6-sulfate (Lahaye *et al.*, 1988).

Finally, the spectrum of *G. eucheumoides* (Fig. 10) is very different

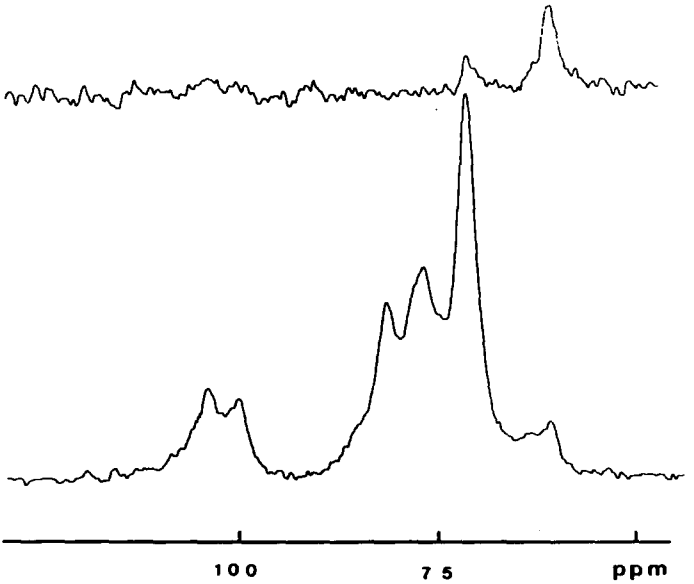

Fig. 9. 50·3 MHz CP-MAS ^{13}C-NMR spectra of *Gracilaria bursa-pastoris* agar: top, spectrum with a dipolar dephasing of 40 μs; bottom, spectra without dephasing.

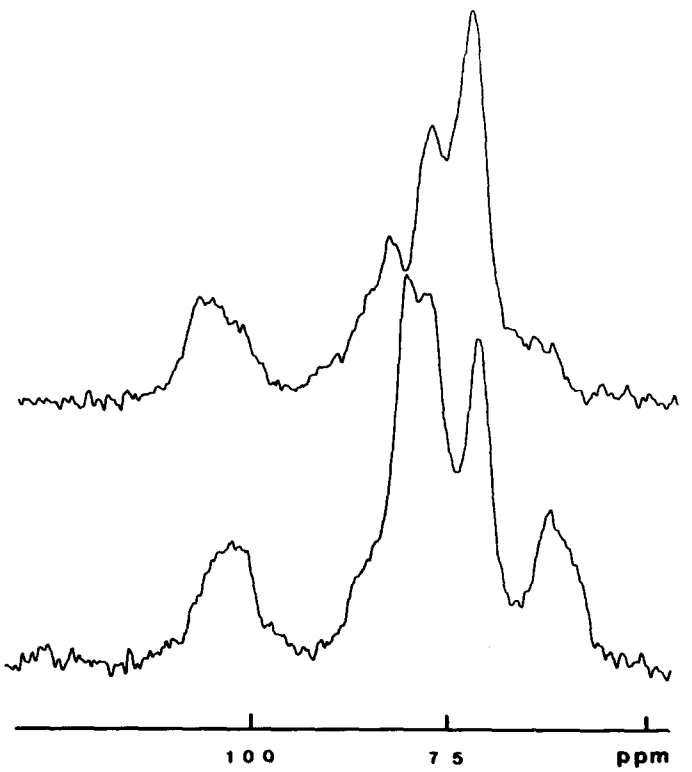

Fig. 10. 50·3 MHz CP-MAS ^{13}C-NMR spectra of *Gracilaria crassissima* (top) and *G. eucheumoides* (bottom).

from that of agarose in regard to the relative intensity of the three signals between 81 and 87 ppm. These peaks result from the strong downfield shift of the methoxyl substituted 2A carbon (Lahaye *et al.*, 1986). The highly asymmetrical signal at 61·7 ppm is attributed to 6G and carbon of the methoxyl group. The presence of this substituent group is confirmed as in the case of *G. bursa-pastoris* (see above), from the dipolar dephasing spectrum and is attributed to the signal at 60·4 ppm.

CONCLUSION

Although the resolution of solid-state ^{13}C-NMR spectra is very much lower than that of solutions, this non-destructive technique is unique in allowing the *in situ* characterization of the major algal cell-wall polysaccharides. It is therefore possible to differentiate agarophytes and carrageenophytes without the need of tedious polysaccharide extractions and offers an alternative to the pyrolysis method (Helleur *et al.*, 1985) in a rapid screening for agar or carrageenan producing algae. Furthermore, in addition to the identification of these galactans, some of their substituents such as methoxyl and pyruvate groups can be characterized. The present results also suggest that agars and carrageenans are in helical conformation in the algal cell-wall because the CP-MAS spectra of the extracted polysaccharides were similar to those of the algae from which they were extracted. It also appears possible to analyze living algal tissue, and one can imagine analyzing agarose and carrageenan gels which it is actually impossible to realize by high resolution NMR spectroscopy.

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