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Assessment of methanolysis for the determination of composite sugars of gelling carrageenans and agarose by HPLC

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Abstract

The characterization of the main composite sugars of commercial gelling red algae galactans (agarose, iota and kappa carrageenans) by methanolysis and separation of the methyl glycosides produced by high performance liquid chromatography is described. The methanolysis (methanolic hydrochloric acid strength, temperature, and reaction time) was optimized in order to release monosaccharides in near quantitative yield. The results were compared to those obtained by (*1*) gas chromatography of the alditol acetates of the neutral sugars released by acid hydrolysis and (*2*) specific colorimetric determination of the acid-labile 3,6-anhydrogalactosyl residue. Conditions such as methanolic 0.125 M HCl for 1 h at 85°C were sufficient to release all of the galactosidic and 3,6-anhydrogalactosidic bonds for iota carrageenan without apparent degradation of the anhydrogalactosyl unit. However, with the same conditions, the yields of 3,6-anhydrogalactosyl residues were 80 and 70% for kappa carrageenan and agarose, respectively. These yields were not improved by stronger conditions. At the opposite extreme, under very mild methanolysis conditions such as methanolic 0.01 M HCl at 100°C for 1 h, agars and gelling carrageenans were well differentiated by the respective determination of agarobiose- and carrabiose-dimethyl acetal which are well-separated on octadecyl reversed phase HPLC columns with water as eluent.

Keywords: Carrageenans; Agarose; NMR; MS; Methanolysis; HPLC

1. Introduction

Agars and carrageenans are complex galactans extracted from red seaweeds and are used as food additives for their gelling and thickening properties. They are built on the

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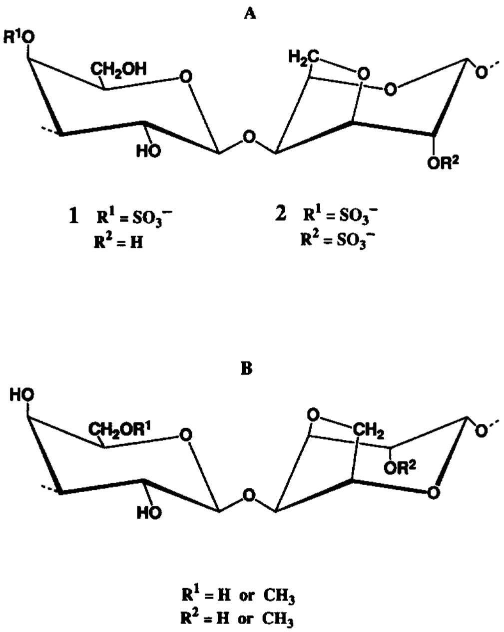

Fig. 1. (A) Carrabiose, the basic repeating unit (1) of kappa carrageenan and (2) of iota carrageenan. (B) Agarobiose, the basic repeating unit of agarose.

repetition of 1,3-linked β-D-galactopyranose alternating with 1,4-linked α-galactopyranose [1,2]. The latter sugar is in the L and D configuration in agars and carrageenans, respectively [3], and often occurs as 3,6-anhydrogalactose. The basic repeating disaccharides in agars and gelling carrageenans are referred to as agarobiose and carrabiose, respectively [3] (Fig. 1). The sulfation pattern of carrabiose is the basis of carrageenan classification [2] and mainly three types of carrageenans are industrially used (lambda, kappa, and iota). Other substitutions of carrageenan by methyl ether or pyruvate acetal have also been reported [4]. Agars are also often modified by methyl ethers, sulfate esters, and/or pyruvate acetal [5,6], and like carrageenans are hybrid polysaccharides composed of several differently substituted repeating units. Agarose consists of the least substituted agar polysaccharide.

Usual methods for the determination of the main constituents of polysaccharides generally involve acid hydrolysis under conditions wherein 3,6-anhydrogalactosyl residues of agars and carrageenans are rapidly degraded to 5-hydroxymethyl-furfural. To overcome this problem, various alternative chemical approaches have been proposed such as mercaptolysis [7], formolysis [8], or bromine oxydation during hydrolysis [9]. However, none of these techniques yielded satisfactory results. Colorimetry [10] was the only suitable procedure to quantitatively measure the 3,6-anhydrogalactosyl residues, but this technique is tedious and does not give any information on the other substituted residues. More recently, double hydrolysis reduction- and reductive hydrolysis-methods [11] have been proposed. The double hydrolysis reduction-procedure involves a preliminary mild acid hydrolysis under conditions sufficient to cleave all of the 3,6-anhydrogalactosidic bonds without significant further degradation of the labile 3,6-anhydrogalactosyl residue. The reductive hydrolysis-method uses the acid-stable 4-methylmorpholine-borane to reduce the 3,6-anhydrogalactosyl residues as they are released during acid hydrolysis. These improved analytical methods have been applied successfully to carrageenans as well as to some agars. However, the results reported were expressed in molar percentage only.

Methanolysis of agars and carrageenans has also been proposed [12,13], but although it usually protects the sensitive reducing-end sugars from degradation by formation of ethers, when it is performed on these galactans under usual conditions (1–2 M methanolic hydrogen chloride), it partially destroys 3,6-anhydrogalactose dimethyl acetal [12]. By contrast, very mild methanolysis conditions preferentially cleave the 3,6-anhydro-galactosyl bonds leading to high yields of the dimethyl acetals of agarobiose [14] and carrabiose [15]. These acetals, as their corresponding acetates, have similar retention times in gas–liquid chromatography and their separation, together with related sugars, proved more effective by high performance liquid chromatography [16] (HPLC). Since no extensive research on methanolysis of red algal polysaccharides has been performed, except qualitative investigations [16,17], we have explored the quantitative release of the main methyl glycosides under various methanolysis conditions and their fractionation by HPLC on a C_{18} reversed phase column.

2. Experimental

Materials.—D-Galactose and 6-*O*-methyl D-galactose, used as standards, were from Sigma. Dimethyl L-tartrate was from Fluka and silver carbonate from Merck. Materials studied were commercial agarose, commercial kappa and iota carrageenans from *Euchema cottonii* and *Euchema spinosum*, respectively. Agar from *Gracilaria eucheumoides* was a laboratory prepared sample [6].

General methods.—The 3,6-anhydrogalactose content in agarose, carrageenans, and purified methyl-glycosides was determined by the resorcinol colorimetric method [10]. The galactose and 6-*O*-methyl galactose contents in polysaccharides were estimated by gas–liquid chromatography of their alditol acetates after hydrolysis with 2 M H_2SO_4 (2 h, 100°C) [18]. The alditol acetates were analysed on a Delsi DI 200 fitted with a DB-225 coated fused-silica capillary column (30 m × 0.32 mm i.d., 0.15-mm film thickness, J & W Scientific) running at 220°C with H_2 as carrier gas and *myo*-inositol as internal standard. The polysaccharides sulfate content was determined after 2 M CF_3CO_2H (2 h, 100°C) hydrolysis and ion-pair chromatography on C_{18} phase with tetrabutylammonium as the counter ion. Detection was by inverse UV spectrophotometry (λ 269 nm, 0.05 AUFS).

Electron (EI) and chemical ionization (CI) mass spectrometry (MS) were performed with a Nermag R10-10C mass spectrometer using an electron energy of 70 eV. Ammonia was used as CI reagent gas. EI and CI mass spectra of purified compounds were recorded after either their transfer on direct insertion probe or GLC–MS of their trimethylsilyl derivatives [12] using a Delsi DI 700 chromatograph fitted with a OV-1 coated capillary column (25 m × 0.22 mm i.d., 0.2-mm film thickness) temperature programmed from 110 to 250°C at 10°C/min using He as carrier gas.

^{1}H Nuclear magnetic resonance spectra were recorded at 30°C on a Bruker ARX 400 operating at 400.13 MHz. The samples were freeze-dried and dissolved twice in deuterium oxide (D_2O) (99.9%, Aldrich) and then in 0.5 mL of 100% D_2O (Sigma). The HOD resonance was set to 4.70 ppm.

Methanolysis procedure.—Methanolic HCl (2 mL of 0.01, 0.125, 0.25, or 0.5 M; prepared from instant methanolic HCl kit [19]), with dimethyl L-tartrate (2 mg/mL) used as internal standard [19], were added to 10 mg of dry sample. Methanolysis was carried out in Reacti-vial with stirring for various times at 85°C, except for 0.01 M (100°C). After cooling, neutralization with Ag_2CO_3 and centrifugation (5000*g* for 5 min), the supernatant solution was concentrated (40°C, under vacuum), and the dry residue was disolved in water (2 mL). The resulting methyl glycosides were analyzed by high performance liquid chromatography on a Merck Superspher end-capped C_{18} cartridge (25 × 0.4 cm) thermostated to 15°C and equipped with a Merck C_{18} guard cartridge [20]. Eluent was ultrapure water at a flow rate of 0.7–0.8 mL/min. The HPLC system used involved a Waters 590 programmable pump, an Erma ERC-3310 degasser, a Touzart/Matignon pulsation dumper, a Valco C 6 W injection valve equipped with 10-μL loop, an Erma ERC-7510 differential refractometer, and a Shimadzu CR 4 A integrator.

Semi-preparative HPLC of agarose and of iota carrageenan methanolysates (0.125 M methanolic HCl, 16 h, and 0.01 M methanolic HCl, 30 min, respectively) were carried out on a R Sil C_{18} H L column (5 μm, 25 × 1 cm, Alltech Associates) using ultrapure water as eluent at a flow rate of 2.5 mL/min.

3. Results and discussion

Identification of methanolysis products.—HPLC analysis. Preliminary experiments showed that HPLC of the 0.01 M HCl (100°C, 3 h) methanolysate of agarose and carrageenans yielded several peaks (Fig. 2) corresponding to the methyl glycosides released, the majority of them being common to the three samples analyzed. Peak 2 corresponded to methyl α- and β-galactopyranosides which coeluted and peaks 3 and 4 to methyl α- and β-galactofuranosides, respectively [19,20]. The main compounds eluting under peaks 5, 6, 9, 10, 12, 13, and 16 (Fig. 2) were purified by semi-preparative HPLC in order to use them, after identification, for quantitative estimation.

MS and ^{1}H NMR analysis. The mass spectrometry data of the unknown compounds are summarized in Table 1. The CI mass spectra of the methyl glycosides (purified from agarose methanolyzate), eluted as peaks 5 and 6, exhibited an $(M + NH_4)^+$ ion of m/z 194. Fragmentation by EI did not yield the m/z 75 basic ion corresponding to the dimethyl acetal group $[CH(OCH_3)_2]$ at C-1 of aldopyranose [23]. The chemical shifts and coupling constants of these methyl glycosides are reported in Table 2. Peak 5 corresponded to 3,6-anhydro-α-L-galactopyranoside as the chemical shifts and coupling constants corresponded to published values [21,22]. From mass-spectral data and the particular NMR chemical shifts measured, peak 6 was attributed to 3,6-anhydro-β-L-galactopyranoside. Varying proportions of 1-*O*-methyl-3,6-anhydro-α- and -β-galactose were always present in the methanolysates of agarose, iota and kappa carrageenans, irrespective of the methanolysis conditions used. These results suggest that the pyranose ring of part of the 3,6-anhydrogalactose molecules is not opened during methanolysis.

The CI mass spectra of the methyl glycosides eluted as peaks 9 and 10 exhibited an $(M + NH_4)^+$ ion of m/z 388 which was consistent with agarobiose- and carrabiose-dimethyl acetal, respectively. It is important to note the ability of this chromatographic system to well separate agarobiose- and carrabiose-dimethyl acetals, thus allowing a facile differentiation between agar and carrageenan polysaccharide structures.

The CI mass spectra of the methyl glycosides eluted as peaks 12, 13, and 16 exhibited $(M + NH_4)^+$ ions of m/z 356, 226, and 402, consistent with methyl agarobiose, 3,6-anhydrogalactose dimethyl acetal, and 6-*O*-methylated agarobiose dimethyl acetal, respectively. Fragmentation by EI of the methyl glycoside eluted as peak 13 yielded the m/z 75 ion for the dimethyl acetal group. These results were confirmed by the GC–EI mass spectrum of the Me_3Si derivative of this compound which yielded fragments with intensities relative to m/z 75 consistent with 2,4,5-tri-*O*-trimethylsilyl-3,6-anhydrogalactose dimethyl acetal [24]. The 6-*O*-methylated agarobiose dimethyl acetal nearly disappeared after strong methanolysis conditions and was always absent from the chromatogram of iota carrageenan known to be free of 6-methyl galactose.

A discrepancy was found between the elution order of these different methyl glycosides and that of the respective standards chromatographed by Valiente et al. [17] under similar conditions (Lichrosorb RP_{18} column, water as eluent). It was particularly important for methyl 3,6-anhydro-α-L-galactose, 3,6-anhydrogalactose dimethyl acetal, and 3,6-anhydro-2-*O*-methyl-galactose dimethyl acetal (cf. below). Their respective retention times relative to methyl α-D-galactopyranoside were 4.6, 2.9, and 3.1 min for these authors while we obtained 4.1, 7.7, and 22 min. In reversed-phase partitioning, the elution order of the compounds is related to their increasing hydrophobic nature. The more numerous the methyl groups, the later the compound will elute. Our results are in good agreement with the expected order.

Calibration of the HPLC system.—Calibration of purified anhydroglycoside aqueous solutions. The purity of the main methyl glycosides relative to 3,6-anhydrogalactose (dimethyl acetals of 3,6-anhydrogalactose, agarobiose, and carrabiose), controlled by analytical chromatography, were in all cases > 99.5%. In order to use them as standards for HPLC analysis, their respective concentrations in aqueous solutions were determined by the specific resorcinol colorimetric method [10] and compared to values obtained by gravimetry for 3,6-anhydro-D-galactose dimethyl acetal and for carrabiose dimethyl acetal. The estimation of the amount of purified agarobiose dimethyl acetal was determined by colorimetry only. As the colorimetric method allows the estimation of 3,6-anhydrogalactose but not directly anhydroglycosides, the anhydroglycoside : 3,6-anhydrogalactose molar mass ratio was taken into account in the calculation of the concentrations of these methyl glycosides. Under these conditions, the reproducibility of the colorimetric method determined by analysing three sets of duplicate aliquots was good with coefficients of variation for the mean of means of 2.5 and 3% for 3,6-anhydro-D-galactose dimethyl acetal and carrabiose dimethyl acetal, respectively. As gravimetric values fitted well with the respective colorimetric mean values (coefficients of variation of 2.2 and 3.5% for carrabiose dimethyl acetal and 3,6-anhydro-D-galactose dimethyl acetal, respectively), the accuracies of the colorimetric determinations and of the calculation mode were consistent.

For HPLC quantification purposes, the relative response factor (RRF) values of these purified methyl glycosides to the internal standard (dimethyl-L-tartrate) were determined. The RRF values obtained for the dimethyl acetals of agarobiose, carrabiose, and 3,6-anhydrogalactose were very close to each other (Table 3) as was expected from refractive index detection. Indeed, this mode of detection is well known to give similar response for compounds of a homologous series, provided that the measurements are based on peak areas [25]. Consequently, a mean RRF value of 0.76 was given to 1-*O*-methyl-3,6-anhydro-α- and β-galactopyranosides. Methanolysis of D-galactose, the other major sugar in agars and carrageenans, yields four methyl galactosides (α- and β-pyranosides and furanosides) chromatographed in three peaks under our conditions [20]. The determination of the RRF value of D-galactose was based on the area of all these peaks and varied between 0.73 ± 0.03 after 0.01 M methanolic HCl treatment at 100°C for reaction times varying from 1 to 24 h (six assays) and 0.71 after 1 M methanolic HCl treatment at 85°C for 16 h. Thus, a mean value of 0.72 was taken for all the subsequent methanolysis conditions used. Lastly, the mean RRF value ($n = 3$) for the 6-*O*-methyl-D-galactose derivatives determined after 1 M methanolic HCl treatment at 85°C for 16 h was 0.73 ± 0.02 when the calculation was based on the four well separated peaks and 0.90 ± 0.03 when it was based on the two major peaks (relative retention times of 0.49 and 0.51, Table 3). This second value was used to estimate the very low 6-*O*-methyl galactose content in kappa carrageenan.

Quantification of methanolysis products. The above response factors were used to monitor the methanolysis kinetics from the products separated by HPLC as a function of the solvolysis conditions. The yields of 3,6-anhydrogalactose methyl derivatives released with time from agarose, kappa and iota carrageenans in methanolic 0.125 and 0.25 M HCl, are shown in Fig. 3. In methanolic 0.125 M HCl, large amounts of carrabiose and agarobiose dimethyl acetals (14.6, 17.0, and 16.4% of kappa and iota carrageenan and agarose dry weight, respectively) were detected after 30 min of solvolysis, attesting to the incomplete depolymerization of the polysaccharides. The content of carrabiose dimethyl acetal decreased sharply between 1 to 3 h solvolysis, while that of 3,6-anhydrogalactose dimethyl acetal remained constant for kappa carrageenan (Fig. 3A) or increased slightly for iota carrageenan (Fig. 3B). Thus, as the methanolysis end products of this dimer are, namely, 3,6-anhydro-D-galactose dimethyl acetal and methyl D-galactose, these results mean that the 3,6-anhydrogalactose derivatives were partially destroyed after 1–2 h of methanolysis. This degradation was even more important with methanolic 0.25 M HCl since, under these conditions, its content decreased dramatically after just 1 h of solvolysis. Weight percentages of individual sugars in the polymer were obtained by applying, to the respective methyl glycosides measured, factors which take into account their methylation and the conversion monomer–polymer. Thus, for example, the amounts of 3,6-anhydrogalactose and D-galactose in carrageenans are equal to: 3,6-anhydrogalactose = (methyl 3,6-anhydrogalactopyranoside × 0.82) + (carrabiose dimethyl acetal × 0.39) + (3,6-anhydrogalactose dimethyl acetal × 0.69); D-galactose =

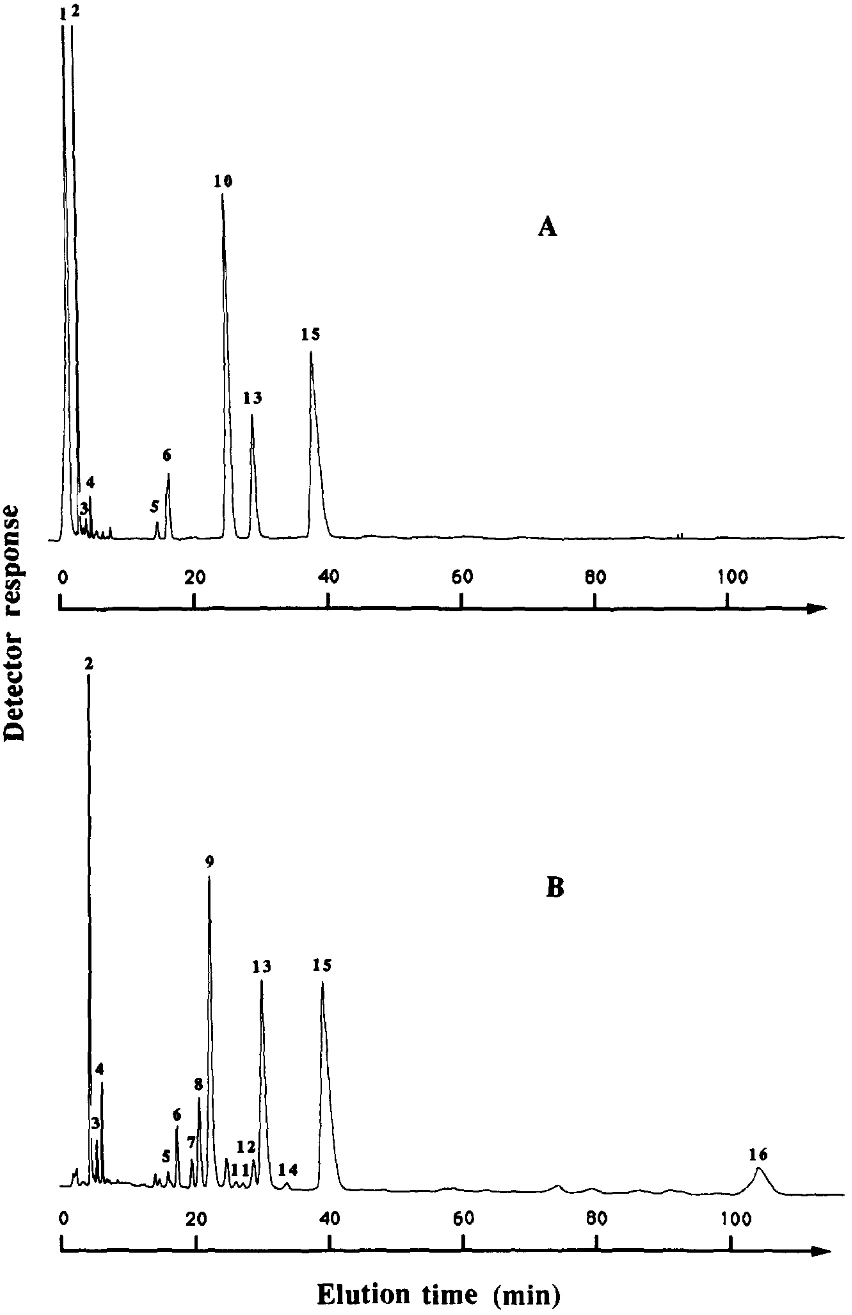

Fig. 2. HPLC of methanolysis products (A) from iota carrageenan and (B) from agar: 1, salts (sulfates); 2, methyl α- and β-D-galactopyranosides; 3, methyl α-D-galactofuranoside; 4, methyl β-D-galactofuranoside; 5, methyl 3,6-anhydro-α-galactopyranoside; 6, methyl 3,6-anhydro-β-galactopyranoside; 7, methyl 6-*O*-methyl-α-D-galactopyranoside; 8, methyl 6-*O*-methyl-β-D-galactopyranoside; 9, agarobiose dimethyl acetal; 10, carrabiose dimethyl acetal; 11, methyl 6-*O*-methyl-α-D-galactofuranoside; 12, methyl agarobiose; 13, 3,6-anhydrogalactose dimethyl acetal; 14, methyl 6-*O*-methyl-β-D-galactofuranoside; 15, dimethyl-L-tartrate (internal standard); 16, 6-methylated agarobiose dimethyl acetal.

Table 1

Essential ions obtained by EIMS (70 eV) and CIMS (NH_3) from purified methyl glycosides

HPLC peak number	EIMS (m/z 75 ion)	CIMS (molecular ion)	Peak assignment
5, 6	–	194	methyl 3,6-anhydrogalactose
9	+	388	agarobiose dimethyl acetal
10	+	388	carrabiose dimethyl acetal
12	n.d. [a]	356	methyl agarobiose
13	+	226	3,6-anhydrogalactose dimethyl acetal
16	+	402	6-*O*-methylated agarobiose dimethyl acetal

[a] Not determined.

Table 2

Chemical shifts and coupling constants of methyl 3,6-anhydro-α-L- and β-L-galactopyranosides

Proton	Methyl 3,6-anhydro-α-L-galactopyranoside		Methyl 3,6-anhydro-β-L-galactopyranoside	
	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)
1	4.815	$J_{1,2}$ 2.6	4.549	$J_{1,2} \sim 0$
2	3.987	$J_{2,3}$ 5.4	3.996	$J_{2,3}$ 4.8
3	4.323	$J_{3,4} \sim 0$	4.260	$J_{3,4} \sim 0$
4	4.445	$J_{4,5}$ 1.9	4.396	$J_{4,5} \sim 0$
5	4.383	$J_{5,6\text{endo}} \sim 0$	4.396	$J_{5,6\text{endo}} \sim 0$
6 endo	4.201	$J_{5,6\text{exo}}$ 3.0	4.169	$J_{5,6\text{exo}}$ 2.3
6 exo	4.012	$J_{6\text{endo},6\text{exo}}$ −10.7	3.923	$J_{6\text{endo},6\text{exo}}$ −10.1
CH_3	3.500		3.382	

Table 3

Relative response factors and relative retention times of 3,6-anhydrogalactose and galactose derivatives to the internal standard (dimethyl-L-tartrate)

Methyl Glycoside	Relative response factor	Relative retention times
Methyl D-galactosides	0.72 ± 0.02 [a]	0.10; 0.13; 0.15 [b]
Methyl 2-*O*-methyl-D-galactoside	n.d. [c]	0.22
Methyl 3,6-anhydrogalactosides	n.d.	0.41; 0.46
Methyl 6-*O*-methyl-D-galactosides	0.73 ± 0.02 [d] 0.90 ± 0.03 [e]	0.49; 0.51; 0.67; 0.87
Agarobiose dimethyl acetal	0.75 ± 0.01	0.56
Carrabiose dimethyl acetal	0.74 ± 0.01	0.68
3,6-Anhydrogalactose dimethyl acetal	0.80 ± 0.01	0.77
2-*O*-Methyl 3,6-anhydrogalactose dimethyl acetal	n.d.	2.24
6-*O*-Methyl agarobiose dimethyl acetal	n.d.	2.80

[a] Determined on all the three corresponding peaks.

[b] Correspond to α- or β-pyranosyl and furanosyl forms.

[c] Not determined.

[d] Determined on all the four corresponding peaks.

[e] Determined on the first two main peaks.

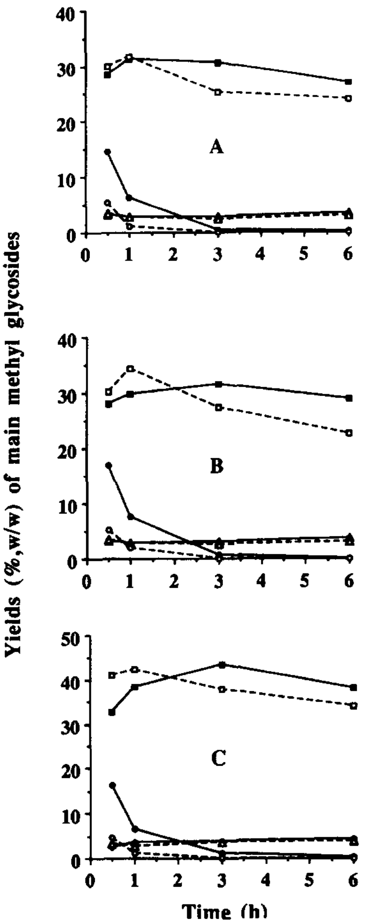

Fig. 3. Yields of the main methyl glycosides relative to 3,6-anhydrogalactose and released with time (A) from kappa carrageenan, (B) from iota carrageenan, and (C) from agar in methanolic 0.125 M HCl (———) and methanolic 0.25 M HCl (- - - - -): ■, □ 3,6-anhydrogalactose dimethyl acetal; ●, ○ agarobiose or carrabiose dimethyl acetal; ▲, △ methyl 3,6-anhydro-α- and β-galactopyranosides.

(methyl D-galactopyranoside × 0.9) + (carrabiose dimethyl acetal × 0.43). Best yields of 3,6-anhydrogalactose was obtained from carrageenan samples for a 0.5–1 h solvolysis with methanolic 0.125 and 0.25 M HCl, whereas the maximum amount of this methyl glycoside from agarose was obtained after 1–3 h solvolysis in methanolic 0.125 M HCl and 0.5–1 h in methanolic 0.25 M HCl (Table 4). Compared to colorimetric determinations, anhydrogalactose recoveries of 100, 80, and 70% were obtained from iota and kappa carrageenan and agarose methanolysis, respectively. D-Galactose and 6-*O*-methyl-D-galactose amounts, determined by methanolysis, were close to reference GC determinations, attesting to the nearly complete cleavage of agarose under these conditions. It is clear that the poor anhydrogalactose recovery in the case of the agarose sample corresponds to a partial destruction of the released 3,6-anhydro-L-galactose. The results

Table 4
Yields (% dm) of main sugars released from commercial agarose and carrageenans by methanolysis at 85°C under various conditions and by reference methods (see text)

		Agarose			Kappa Carrageenan			Iota Carrageenan	
		D-galactose	6-*O*-Methyl-D-galactose	3,6-Anhydro-L-galactose	D-galactose	6-*O*-Methyl-D-galactose	3,6-Anhydro-D-galactose	D-Galactose	3,6-Anhydro-D-galactose
HCl (M)	Time (h)								
0.125	0.5	34.1 ± 1.0	8.2 ± 1.1	30.8 ± 1.2	32.1 ± 2.0	1.3 ± 0.2	28.0 ± 1.9	31.9 ± 1.4	28.6 ± 1.6
	1	34.4 ± 1.6	10.1 ± 0.9	31.8 ± 1.5	32.1 ± 0.8	1.5 ± 0.1	26.3 ± 1.0	31.4 ± 2.5	25.7 ± 2.0
	3	36.7 ± 1.9	12.2 ± 0.5	33.4 ± 0.8	30.9 ± 0.1	1.4 ± 0.1	23.6 ± 0.4	32.6 ± 1.6	24.4 ± 1.6
0.25	0.5	35.5 ± 1.9	10.9 ± 0.9	32.6 ± 1.8	31.8 ± 1.0	1.3 ± 0.1	25.6 ± 1.4	31.9 ± 2.1	25.6 ± 1.7
	1	35.8 ± 1.4	11.8 ± 0.2	31.9 ± 0.7	32.7 ± 1.8	1.5 ± 0.1	24.6 ± 1.6	35.3 ± 2.6	26.6 ± 2.3
0.5	3	37.4	13.7	22.4	35.4	2.0	17.2	29.2 ± 1.1	12.2 ± 0.2
References methods		35.5 ± 0.2	12.5 ± 0.3	46.4 ± 2.4	30.9 ± 0.7	1.5 ± 0.1	35.9 ± 1.3	29.2 ± 0.1	27.2 ± 1.7

obtained under still stronger conditions confirmed this hypothesis. Indeed, the 3,6-anhydrogalactose yields determined after treatment of iota and kappa carrageenans and agarose for 3 h with methanolic 0.5 M HCl were still lower (12.2, 17.2, and 22.4%, respectively). These values are in agreement with those previously determined under similar conditions [12,13].

Mild methanolysis of agar from Gracilaria eucheumoides. Agarose very often contains small amounts of 3.6-anhydro-2-*O*-methyl-L-galactose. In order to detect this sugar, the agar of *Gracilaria eucheumoides* known to be rich in such sugar [6] was subjected to methanolysis under optimal conditions. The HPLC peak identified to 3,6-anhydro-2-*O*-methyl-L-galactose dimethyl acetal by the characteristic $(M+NH_4)^+$ ion of m/z 240 and the m/z 75 ion observed by CIMS and EIMS, respectively, had a relative retention time of 2.24. Unfortunately, the partial overlap of an unknown peak of RRT 2.13 ($(M+NH_4)^+$ ion of m/z 208 by CIMS and no m/z 75 ion by EIMS) precluded its quantitative determination. Methyl 2-*O*-methyl-L-galactose was also identified by the characteristic $(M+NH_4)^+$ ion of m/z 212 observed by CIMS and the absence of the m/z 75 ion on the EI mass spectrum, under the HPLC peak eluted at a RRT 0.22. Additional work is required to quantitatively measure these derivatives in agar.

4. Conclusions

HPLC analysis of methyl glycosides released from galactans of red algae under very mild methanolysis conditions such as methanolic 0.01 M HCl at 100°C for 1 h may be used to differentiate between agars and gelling carrageenans by the respective determination of agarobiose- and carrabiose-dimethyl acetal which are well-separated on C_{18} reversed phase with water as eluent. Stronger conditions such as methanolic 0.125 M HCl for 1 h at 85°C release, quantitatively, the main composite sugars of iota carrageenan. However, under these conditions, the depolymerization was incomplete and necessitated taking into account the carrabiose dimethyl acetal and 3,6-anhydro-α- and β-methyl galactose amounts for 3,6-anhydrogalactose content determination. Under the same conditions, the yields of 3,6-anhydrogalactose were 80 and 70% for kappa carrageenan and agarose, respectively. The apparent correlation of these recovery values with the content of 6-*O*-methyl-D-galactose in the polysaccharide — the higher the level of 6-*O*-methyl-D-galactose, the lower the recovery of 3,6-anhydrogalactose is — is inconsistent with the almost total release of this methyl glycoside. Still stronger methanolysis conditions were efficient for complete cleavage of all glycosidic bonds, but the recoveries were not improved because of a greater destruction of 3,6-anhydrogalactose dimethyl acetal. The different behavior of iota carrageenan compared to kappa carrageenan or agar towards methanolysis may be explained by the temporary stabilization of the 3,6-anhydrogalactose residue by sulfate on O_2. Finally, it may be emphasized that the relative response factors of the different methyl glycosides were quite constant under our analysis conditions. This property, which is characteristic of differential refractometric detection, may be useful for their quantification as these compounds are not commercially available.

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