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Positional isomers of sulfated oligosaccharides obtained from agarans and carrageenans: preparation and capillary electrophoresis separation

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Abstract—Partial reductive hydrolysis was used to produce oligosaccharide alditols from repetitive sulfated galactans obtained from four Rhodophyta species: κ-carrageenan (from *Kappaphycus alvarezii*), θ-carrageenan (*Gigartina skottsbergii*—alkali-treated λ-carrageenan), agarose 6-sulfate (*Gracilaria domingensis*), and pyruvylated agarose 2-sulfate (*Acanthophora spicifera*—alkali-treated pyruvylated agaran sulfate). Each hydrolyzate was submitted to anion-exchange and gel-filtration chromatography, and the isolated oligosaccharide alditols were identified by 1D and 2D NMR spectroscopy and by ESI mass spectrometry. The positional isomers of the sulfated oligosaccharide alditols were then completely resolved by capillary electrophoresis in a borate buffer. Attempts to correlate the availability of the hydroxyl groups for borate complexation with the relative migration of the oligosaccharides are presented.

Keywords: Capillary electrophoresis; Oligosaccharide alditol structure; Positional isomers; Borate complexation; Carrageenans; Agarans

1. Introduction

Galactans biosynthesized by red seaweeds (Rhodophyta) are essentially constituted of disaccharide repeating units of 3-linked β-D-Gal*p* and 4-linked α-Gal*p*, and in many cases, the latter residue appears as 3,6-AnGal*p*. These galactans are classified in accordance with the enantiomeric configuration of the α-units, which are L- in agarans and D- in carrageenans.[1] Most of these polysaccharides are found as anionic polymers with varying degrees of sulfation. Furthermore, some algal species produce carrageenans or agarans with an almost idealized sulfation pattern, for example, *Kappaphycus alvarezii* (Gigartinales) produces mainly κ-carrageenan (alternating 3-linked β-D-Gal*p* 4-sulfate and 4-linked 3,6-An-α-D-Gal*p*).[2–9] The use of partial hydrolysis methods on repetitive agarans and carrageenans has been an attractive way to obtain oligosaccharides with a specific sulfation positioning in relatively high yields.[10–13] These oligosaccharides can be utilized as useful standards for analytical techniques in the study of complex algal galactans, such as carrageenans with hybrid sulfation patterns and D,L-hybrid galactans.[14–17]

Red seaweed galactans are also potential sources of different positional isomers and/or diasteroisomers of sulfated oligosaccharides. Therefore, a technique capable of resolving complex mixtures of isomeric oligosaccharides is desirable for the study of carrageenan and agaran-derivative oligosaccharides. For this purpose, capillary electrophoresis (CE) has proved to be a powerful technique that allows rapid separations with high resolution and sensitivity.[18] CE methods have been developed to separate acidic oligosaccharides derived from chondroitin sulfate,[19] hyaluronan,[20,21] heparan sulfate,[22] κ-carrageenan,[23] and pectins.[24] Mixtures of isomers of oligosaccharides containing sialic acid residues[25] and positional isomers of sulfated

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monosaccharides[26] have also been successfully resolved by the use of CE. In addition, a large number of CE separations of carbohydrates have been carried out using borate buffers. These separations are based on the fact that borate anions form negatively charged complexes with polyhydroxy compounds, thus increasing the selectivity of their separation.[27–40]

We now describe the preparation and NMR/MS characterization of oligosaccharide alditols produced from four repetitive red seaweed galactans (two carrageenans and two agarans). Furthermore, we demonstrate CE separation of positional isomers of sulfated oligosaccharide alditols using a borate buffer. Attempts to correlate hydroxyl groups availability to form borate complexes with the relative migration of the oligosaccharides are presented.

2. Experimental

2.1. Extraction and processing of the galactans

Selected strains from tetraspores of *Kappaphycus alvarezii* were obtained in the laboratory and cultivated in the sea (Ubatuba, São Paulo, Brazil). Plants were then collected, washed with running tap water, sun dried, milled, and extracted with water (1.5% w/v) at 65 °C. Insoluble residual material was removed by centrifugation, and the supernatant was dialyzed and freeze-dried. The extracted polysaccharides were submitted to alkaline treatment[41] giving κ-carrageenan as the main polysaccharide.

Samples of the tetrasporic phase of *Gigartina skottsbergii* (Gigartinales) were collected in Bahia Camarones (Chubut Province, Argentina), and the extraction of λ-carrageenans, as well as their alkaline treatment, were carried out as previously described.[42] Alkaline treatment of λ-carrageenan renders θ-carrageenan.

Agarose 6-sulfate was obtained from *Gracilaria domingensis* (Gracilariales) as previously described.[43] The powdered alga was submitted to an aqueous extraction (90 °C, 4 h), and the soluble agarose 6-sulfate was precipitated with ethanol (3 v), followed by dialysis and freeze-drying.

The extraction and alkaline treatment of the *Acanthophora spicifera* (Ceramiales) agaran sulfate was as previously described.[13] This process renders a repetitive agaran with sulfate groups mainly at C-2 of the β-D-Gal*p* unit with a minor amount of 4,6-*O*-linked pyruvic acid acetal that could be described as pyruvylated agarose 2-sulfate.[44]

2.2. Production and purification of the oligosaccharides

Samples of agarose 6-sulfate (360 mg), κ- (400 mg), and θ-carrageenans (400 mg) were partially depolymerized by partial reductive hydrolysis.[10] Each sample was dissolved in water (30 mL), the solution was heated to 60 °C, and 2.70 g of 4-methylmorpholine·borane (MMB) complex (97% Sigma–Aldrich) was then added, followed by 2 M CF_3COOH (10 mL). The mixtures were maintained at 65 °C for 8 h, and the acid was then evaporated by co-distillation with water. For θ-carrageenan four extra hours at 65 °C were necessary to complete the partial hydrolytic process. Each hydrolyzate was then resuspended in water (~4 mL) and applied to a DEAE-Sephadex A-25 (Cl^-) column (2.5 × 12 cm × 33 mL/h). Elutions were carried out with water and then with a continuous NaCl gradient (0–0.15 M) utilizing a Pharmacia Biotech pump P-1 for flux and gradient control. Anion-exchange fractions were desalted by aqueous elution on a BioGel P-2 column (1.5 × 100 cm × 20 mL/h). The column eluents were analyzed for carbohydrates by the phenol–sulfuric acid method.[45]

Agarobiitol 2^2-sulfate and $4^2,6^2$-*O*-(1-carboxyethylidene)-agarobiitol were produced and purified as previously described from the alkali-treated agaran sulfate of *A. spicifera*.[13]

2.3. Nuclear magnetic resonance spectroscopy

For NMR experiments, a portion of each dried sample (2–8 mg) was exchanged with deuterium by repeated evaporations in D_2O, and then dissolved in 99.99% D_2O (0.35 mL). NMR spectra were obtained with a Bruker Advance DRX 400 spectrometer equipped with a 5-mm inverse probe. 1D ^1H, ^{13}C, DEPT and 2D ^1H, ^1H COSY, TOCSY and ^1H, ^{13}C HMQC spectra were obtained at a base frequency of 100.63 MHz for ^{13}C and 400 MHz for ^1H nuclei. Chemical shifts are reported relative to an internal acetone standard at 2.225 and 30.20 ppm for ^1H and ^{13}C NMR spectra, respectively. ^{13}C NMR DEPT spectra were obtained at $\theta z = 135°$, where *C*H and *C*H_3 signals appear in a positive phase with *C*H_2 in a negative phase. For ^1H NMR experiments, the DO*H* signal was suppressed by low-power irradiation during relaxation. NMR experiments were performed at 25 °C for **KA**, **TA**, **TB**, **GA**, and **WB**, 40 °C for **WA**, and 50 °C for **KB**.

2.4. Electrospray-ionization mass spectrometry

The ESIMS equipment used was a Micromass Quattro LC–MS/MS triple quadrupole mass spectrometer. Data acquisition and processing were performed using Maslynx 3.2 software. Samples were diluted in 7:3 CH_3CN/H_2O at 1 mg/mL and introduced into the spectrometer by a syringe pump (KD Scientific Inc.). N_2 was used as nebuliser (83 L/h) and desolvation gas (309 L/h). The source was operated at 150 °C with a desolvation temperature of 250 °C. The pressure in the analyzer

was 1.1×10^{-5} mbar and 2.0×10^{-5} mbar in the gas cell. To provide parent ions, the positive-ion mode parameters were 5.0 kV and 50 V (capillary and cone, respectively). Negative-ion mode experiments were performed using 4.0 kV for the capillary and 100 V for the cone.

2.5. Capillary electrophoresis

CE analyses were carried out using a Beckman P/ACE 5010 equipment with a fused silica external coated capillary tube (50 cm × 50 μm i.d.). Samples (0.5 mg/mL) were introduced onto the capillary by hydrodynamic loading (3 s, 0.5 psi), using nitrogen gas. All analyses were performed in the normal polarity using voltages of 10–15 kV and temperatures ranging from 25 to 50 °C. The separation buffers were prepared from stock solutions of disodium hydrogen phosphate (200 mM), SDS (200 mM) and sodium tetraborate (100 mM). The buffer pH was adjusted by the addition of 3 M NaOH. The absorbance of the capillary eluant was monitored by UV at 214 nm by indirect detection, using *p*-toluenesulfinic acid (10 mM) as the detectable co-ion. Before each run, the capillary was conditioned by washing it with 0.1 M NaOH for 1 min, followed by a 3-min rinse with running buffer.

3. Results

3.1. Oligosaccharide sources

Carrageenans found in the red seaweeds *K. alvarezii* and *G. skottsbergii* have already been extensively studied to determine their primary structures and sulfate substitution patterns. *K. alvarezii* produces after alkaline treatment mainly κ-carrageenan [→3)-β-D-Gal*p* 4-sulfate-(1→4)-3,6-An-α-D-Gal*p*-(1→],[2–9] while the tetrasporic phase of *G. skottsbergii* synthesizes, as its main water-soluble polysaccharide, λ-carrageenan [→3)-β-D-Gal*p* 2-sulfate-(1→4)-α-D-Gal*p* 2,6-disulfate-(1→].[42] Under alkaline treatment, λ-carrageenan was transformed into θ-carrageenan via cyclization of α-D-Gal*p* 2,6-disulfate units, giving rise to those of 3,6-An-α-D-Gal*p* 2-sulfate. The aqueous soluble polysaccharide from *A. spicifera* has been studied recently in terms of both its structure[13,44] and antiviral activity.[44] After alkaline treatment, this galactan had a repetitive agarose-type backbone [→3)-β-D-Gal*p*-(1→4)-3,6-An-α-L-Gal*p*-(1→] with sulfate groups mainly at C-2 of the β-D-Gal*p* unit and a 4,6-*O*-linked pyruvic acid acetal content of ~10%. Guimarães[43] demonstrated that the polysaccharide extracted from *G. domingensis* corresponds to an agaran mainly constituted by the following disaccharide repeating unit, [→3)-β-D-Gal*p* 6-sulfate-(1→4)-3,6-An-α-L-Gal*p*-(1→].

Considering the repetitive nature of these galactans and the specificity of partial reductive hydrolysis (cleavage of the 3,6-anhydrogalactosidic bonds), application of this hydrolytic method to these polysaccharides should yield only disaccharide alditols with sulfate at specific positions. With this aim in mind, κ- and θ-carrageenans, agarose 6-sulfate and *A. spicifera* agaran were submitted to partial reductive hydrolysis.

3.2. Preparation of the oligosaccharide alditols

After standard partial reductive hydrolysis,[10] the κ-carrageenan hydrolyzate was directly applied on a DEAE-Sephadex A-25 (Cl^-) column, first eluted with water and then with a continuous gradient of NaCl from 0 to 0.15 M. ^{13}C NMR analysis of the water-eluted fraction showed only characteristic signals of 4-methylmorpholine (4-MMB byproduct) at 63.6, 53.0, and

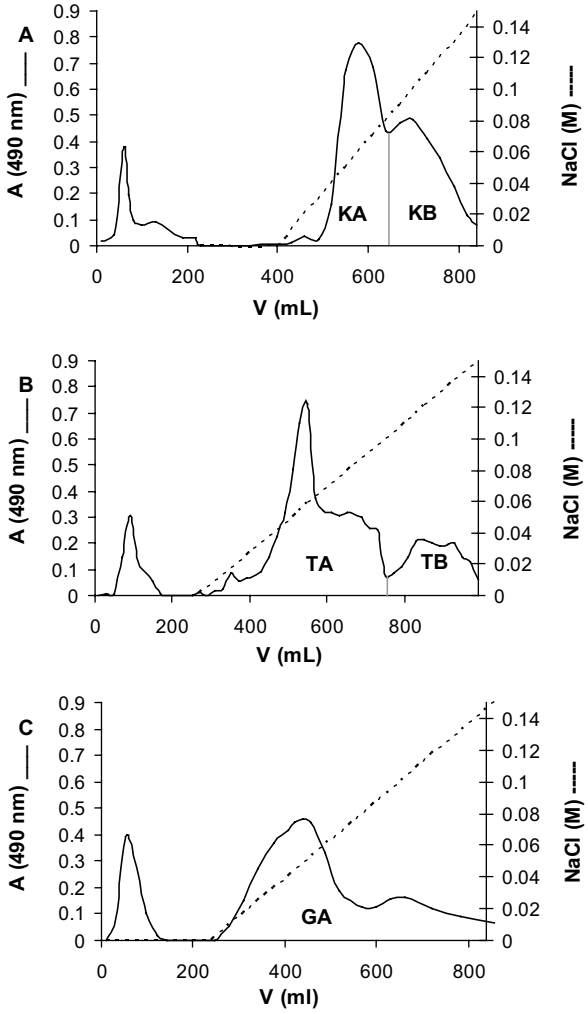


Figure 1. Elution profiles of anion-exchange chromatography on a DEAE-Sephadex A-25 column (2.5 × 12 cm × 33 mL/h) of the hydrolyzates from κ-carrageenan (A), θ-carrageenan (B), and agarose 6-sulfate (C).

43.0 ppm.[13] The relatively high absorbance of this fraction (Fig. 1A) was from the interference caused by the byproduct.

The NaCl gradient was divided into several fractions, and preliminary analysis showed the presence of two carbohydrate components: fractions **KA** and **KB** (Fig. 1A). The desalted fractions were analyzed by ESIMS spectrometry and NMR spectroscopy, incorporating 1D ^{1}H, ^{13}C, and DEPT and 2D ^{1}H, ^{1}H COSY, TOCSY and ^{1}H, ^{13}C HMQC procedures.

Fraction **KA** had ^{13}C resonances characteristic of a disaccharide alditol. Complete ^{13}C and ^{1}H assignments were achieved with the help of 2D NMR experiments (Table 1). A downfield signal in the ^{1}H NMR spectrum

Table 1. Polysaccharide sources, NMR assignments, and ESIMS ions of the oligosaccharide alditols **KA**, **KB**, **TA**, **TB**, **GA**, **WA**, and **WB**

		Oligosaccharide													
		KA		**KB**		**TA**		**TB**		**GA**		**WA**		**WB**	
Sources															
Seaweed		*K. alvarezii*				*G. skottsbergii*				*G. domingensis*		*A. spicifera*			
Processing[a]		A. T.				A. T.				—		A. T.			
Hydrolyzed galactan		κ-Carrageenan				θ-Carrageenan				Agarose 6-OSO_3^-		Pyruvylated agarose 2-OSO_3^-			
Oligosaccharide yield (%)		9.5		6.8		12.8		3.0		11.7		5.5		2.0	
^{1}H and ^{13}C NMR assignments[b]		^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Gal*p* (ext.)	1	4.51	103.0	4.61	102.2	4.54	103.0	4.59	102.4	4.57	102.5	4.72	100.5	4.58	102.0
	2	3.53	70.8	3.52	70.7	3.55	70.7	3.50	70.6	3.54	70.5	4.25	78.8	3.64	70.2
	3	3.80	71.7	3.80	71.7	3.68	72.6	3.68	72.5	3.68	72.4	3.28	71.9	3.72	71.6
	4	4.67	76.3	4.70	76.4	3.94	68.5	3.94	68.5	3.98	68.4	4.00	68.6	4.17	70.8
	5	3.82	74.4	3.83	74.4	3.75	75.2	3.74	75.2	3.97	72.8	3.73	75.1	3.62	66.2
	6	3.80	60.7	3.81	60.8	3.79	61.0	3.80	60.9	4.21	67.4	3.79	60.8	4.06	64.8
	6′	3.80		3.81		3.79		3.77		4.21		3.79		3.94	
3,6-An-Gal*p*	1			5.11	94.5			5.25	93.9						
	2			4.15	69.3			4.64	74.8						
	3			4.52	78.7			4.74	77.5						
	4			4.62	77.8			4.67	77.7						
	5			4.64	76.2			4.71	76.4						
	6			4.21	69.1			4.10	69.5						
	6′			3.95				4.24							
Gal*p* (int.)	1			4.58	102.7			4.57	102.8						
	2			4.06	69.0			3.68	69.1						
	3			3.99	78.1			3.87	81.2						
	4			4.84	73.4			4.13	66.2						
	5			3.83	74.4			4.64	74.9						
	6			3.81	60.8			3.80	60.8						
	6′			3.81				3.77							
3,6-An-GalOH	1	3.67	62.7	3.69	62.8	3.89	60.5	3.89	60.5	3.73	62.9	3.67	62.9	3.71	62.9
	1′	3.67		3.69		3.89		3.89		3.66		3.67		3.71	
	2	3.82	71.2	3.89	71.2	4.57	77.9	4.56	77.8	3.97	70.9	3.93	71.0	3.96	71.0
	3	3.98	83.2	3.93	83.2	4.13	81.6	4.14	81.5	3.93	83.7	3.93	83.5	3.96	83.5
	4	4.16	87.0	4.22	87.0	4.34	86.3	4.36	86.3	4.31	86.0	4.30	86.2	4.31	85.6
	5	4.47	75.5	4.50	75.6	4.50	75.7	4.50	75.8	4.40	75.3	4.44	75.2	4.40	75.2
	6	3.98	72.6	4.09	72.7	4.03	72.6	4.03	72.6	3.97	72.8	4.01	73.0	4.01	72.9
	6′	3.82		3.93		3.85		3.66		3.86		3.86		3.87	
ESIMS ions															
		m/z		*m/z*		*m/z*		*m/z*		*m/z*		*m/z*		*m/z*	
Positive-ion mode		451		859		451		859		451		451		441	
		$[M+Na]^+$		$[M+Na]^+$		$[M+Na]^+$		$[M+Na]^+$		$[M+Na]^+$		$[M+Na]^+$		$[M+Na]^+$	
Negative-ion mode		405		813		405		813		405		405		395	
		$[M-Na]^-$		$[M-Na]^-$		$[M-Na]^-$		$[M-Na]^-$		$[M-Na]^-$		$[M-Na]^-$		$[M-Na]^-$	
				395				395							
				$[M-2Na]^{2-}$				$[M-2Na]^{2-}$							

[a] A. T., alkaline treatment.

[b] NMR experiments were performed at 25 °C for **KA**, **TA**, **TB**, **GA**, and **WB**, 40 °C for **WA** and 50 °C for **KB**.

(4.67 ppm) and its corresponding ^{13}C NMR signal (76.3 ppm) were assigned to H-4/C-4 resonances of the β-D-Gal*p* unit. These values are in accordance with α-sulfation at C-4, as expected considering the structure of the parent polysaccharide, indicating that the **KA** was β-D-Gal*p* 4-OSO_3-(1→4)-3,6-An-D-GalOH (carrabiitol 4^2-sulfate—Fig. 2A—Table 1). ESIMS analyses in the negative- and positive-ion modes showed molecular ions with *m*/*z* 405 $[M-Na]^-$ and 451 $[M+Na]^+$, respectively, corresponding to results expected for a monosulfated disaccharide alditol (M_n 428, sodium salt).

For **KB**, three ^{13}C NMR signals were found in the anomeric region corresponding to C-1 of the terminal β-D-Gal*p* (102.2 ppm), internal β-D-Gal*p* (102.7 ppm), and 3,6-An-α-D-Gal*p* (94.5 ppm) units. These data and the signal at 62.8 ppm (C-1 of 3,6-An-D-GalOH unit) indicated that **KB** was a tetrasaccharide alditol. With

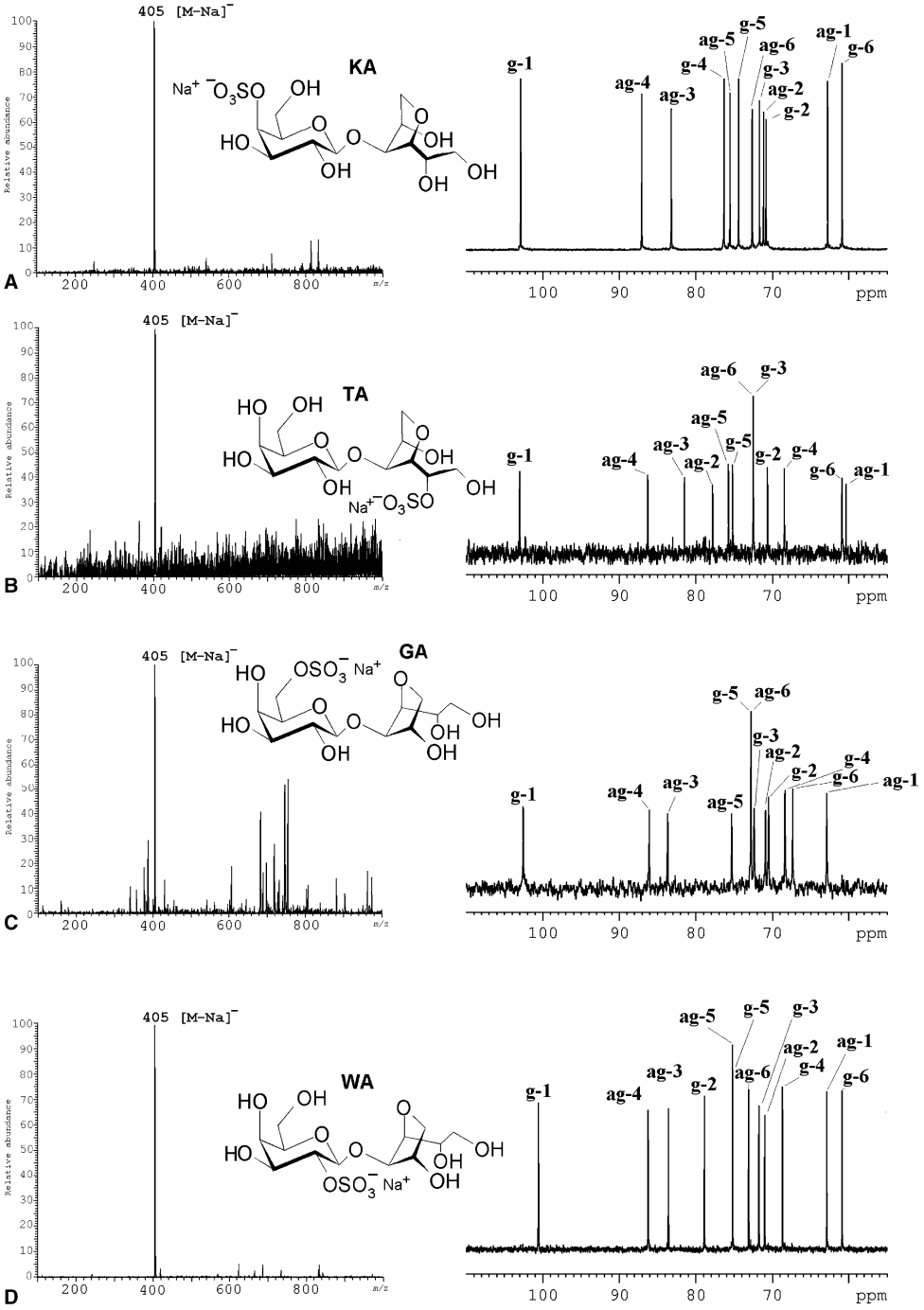


Figure 2. Negative-ion mode ESIMS and ^{13}C NMR spectra of the sulfated disaccharide alditols **KA** (A), **TA** (B), **GA** (C), and **WA** (D). **g** and **ag** correspond to Gal*p* and 3,6-AnGalOH, respectively.

the complete ^{1}H and ^{13}C NMR assignments (Table 1), it was possible to confirm the expected sulfation at C-4 for both terminal (76.4 ppm) and internal (73.4 ppm) β-D-Gal*p* units. The structure proposed for **KB**, β-D-Gal*p* 4-OSO_3-(1→4)-3,6-An-α-D-Gal*p*-(1→3)-β-D-Gal*p* 4-OSO_3-(1→4)-3,6-An-D-GalOH (carratetraitol $4^4,4^2$-disulfate—Fig. 3A—Table 1), was in agreement with the molecular ions with *m*/*z* 813 $[M-Na]^-$, 395 $[M-2Na]^{2-}$ (negative-ion mode) and *m*/*z* 859 $[M+Na]^+$ (positive-ion mode) in the ESIMS analyses. These MS results are in accordance with the expected average mass for a disulfated tetrasaccharide alditol (M_n 836, disodium salt).

The partial reductive hydrolysis of θ-carrageenan was carried out following the same procedure used for κ-carrageenan, namely that established by Usov and Elashvili.[10] ^{13}C NMR analysis indicated that the commonly used partial hydrolysis time (8 h) was not sufficient to generate oligosaccharides (data not shown), so that the hydrolysis time was increased to 12 h. The hydrolyzate was then submitted to fractionation and purification by anion-exchange and gel-filtration chromatography giving rise to two main oligosaccharidic fractions, **TA** and **TB** (Fig. 1B).

Twelve ^{13}C NMR signals were present in the **TA** spectrum, indicating a disaccharide alditol (Table 1). The signal corresponding to C-2 of 3,6-An-D-GalOH 2-sulfate (77.9 ppm) appeared at 6.7 ppm downfield when compared with the previously described assignment for 3,6-An-L-GalOH,[10,12,13] confirming the expected sulfated position. The signal attributable to C-2 of the β-D-Gal*p* unit was identified at 70.7 ppm, indicating that this carbon is non-substituted. However, being aware of the θ-carrageenan structure, sulfation of the β-units was also expected. These data suggested a specific C-2 sulfate hydrolysis from the β-D-Gal*p* units during the

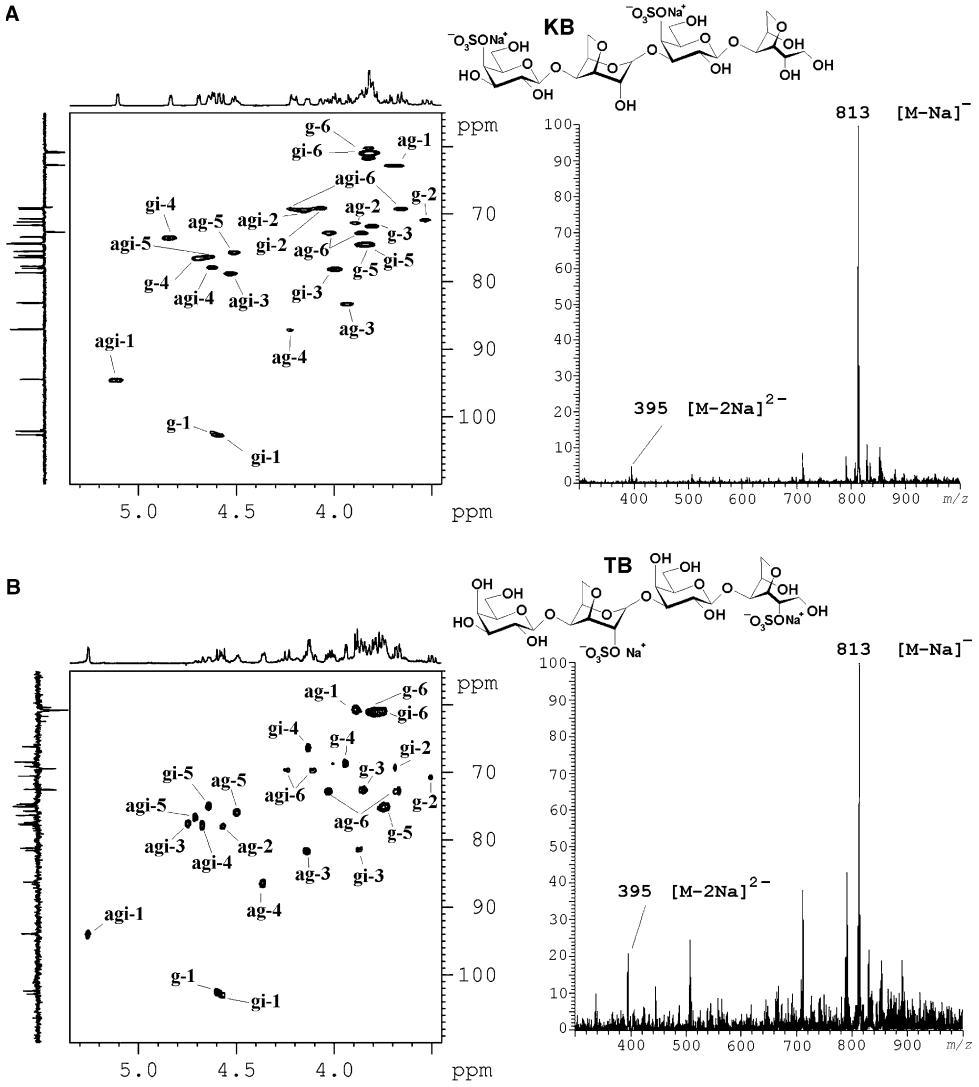


Figure 3. 2D NMR HMQC and negative-ion mode ESIMS spectra of the disulfated tetrasaccharide alditols KB (A) and TB (B). **g**, **agi**, **gi**, and **ag** correspond to external Gal*p*, 3,6-AnGal*p*, internal Gal*p*, and 3,6-AnGalOH, respectively.

hydrolytic process. ESIMS analyses on **TA** showed identical results to the ones obtained for **KA**, confirming the presence of only one sulfate group attached to the disaccharide alditol. These results demonstrated that the **TA** structure is β-D-Gal*p*-(1→4)-3,6-An-D-GalOH 2-OSO_3 (carrabiitol 2^1-sulfate—Fig. 2B—Table 1).

As well as for **KB**, **TB** showed NMR data compatible to a tetrasaccharide alditol. Having its complete ^1H and ^{13}C NMR assignments, we observed that **TB** showed non-substituted C-2 for both terminal (70.6 ppm) and internal (69.1 ppm) β-D-Gal*p* units. Sulfation at C-2 of the 3,6-An-D-GalOH was characterized by the resonance at 77.8 ppm. The signal corresponding to sulfated C-2 of the 3,6-An-α-D-Gal*p* unit (74.8 ppm) appeared less displaced in comparison to the galactitol unit, due to the β-effect caused by the glycosidic linkage. Mass spectrometric analyses showed molecular ions with *m/z* 813 $[M-Na]^-$ and 395 $[M-2Na]^{2-}$ (negative-ion mode) and *m/z* 859 $[M+Na]^+$ (positive-ion mode). These results indicated that **TB** is a specifically sulfated tetrasaccharide alditol, β-D-Gal*p*-(1→4)-3,6-An-α-D-Gal*p* 2-OSO_3-(1→3)-β-D-Gal*p*-(1→4)-3,6-An-D-GalOH 2-OSO_3 (carratetraitol $2^1,2^3$-disulfate, M_n 836, disodium salt—Fig. 3B—Table 1).

Standard partial reductive hydrolysis conditions were utilized for agarose 6-sulfate, and the hydrolyzate was processed following the same steps used for κ- and θ-carrageenan. However, only a disaccharide structure was found in NaCl continuous gradient (**GA**—Fig. 1C). Its ^{13}C NMR DEPT analysis showed an inverted signal at 67.4 ppm corresponding to C-6 of the β-D-Gal*p* units. This 6.5 ppm displacement, in comparison to a non-substituted disaccharide alditol,[10,12,13] indicates the expected C-6 sulfation. The structure of **GA**, β-D-Gal*p* 6-OSO_3-(1→4)-3,6-An-L-GalOH (agarobiitol 6^2-sulfate—Fig. 2C—Table 1) was confirmed by ESIMS analyses in the negative- and positive ion-modes, which gave the molecular ions with *m/z* 405 $[M-Na]^-$ and *m/z* 451 $[M+Na]^+$, respectively (M_n 428, sodium salt). Usov and Elashvili[12] characterized agarobiitol 6-sulfate from the partial reductive hydrolyzate of *Laurencia* sp. water-soluble galactan; our data appears to be in good agreement with the cited reference.

For the purposes of this work, we also made use of two acidic disaccharide alditols previously produced from the alkali-treated *A. spicifera* agaran: β-D-Gal*p* 2-OSO_3-(1→4)-3,6-An-L-GalOH (agarobiitol 2^2-sulfate—**WA**—Fig. 2D—Table 1)[13,44] and 4,6-*O*-(1′-carboxyethylidene)-β-D-Gal*p*-(1→4)-3,6-An-L-GalOH ($4^2,6^2$-*O*-(1-carboxyethylidene)-agarobiitol—**WB**—Table 1).[13]

3.3. Capillary electrophoresis analysis

The first capillary electrophoresis conditions used for our experiments were the ones described by Carney and Osborne[19] for the separation of disaccharides derived from chondroitin (40 mM phosphate, 40 mM SDS, 10 mM tetraborate, pH 9.0, at 15 kV and 40 °C). These disaccharides, which were produced by lyase depolymerization, showed an intense absorbance at 232 nm (UV detection) due to the presence of conjugated double bonds, providing an analysis of high sensitivity. However, red seaweed-derivatized oligosaccharide alditols do not have any chromophoric group; thus, low sensitivity could be a limitation when using a UV detection system. In an effort to improve the sensitivity of our capillary electrophoresis analyses, we used an indirect UV detection scheme by addition of *p*-toluenesulfinic acid (TSA) to the buffer.

The resulting buffer (40 mM phosphate, 40 mM SDS, 10 mM tetraborate, 10 mM TSA, pH 9.0) was used in order to separate the positional isomers **KA** and **TA**. Even though the indirect UV detection system provided sufficient sensitivity for this experiment, the isomers could not be resolved, giving rise to only one peak (data not shown). We first attempted to obtain a better resolution by increasing the pH of the buffer to 10.0, with the object of a more effective complexation of the borate ions and the free hydroxyl groups of the oligosaccharide alditols.[27,46–51] The analysis at pH 10.0 showed a slight increase in the resolution of the isomers, which was evident by the appearance of a shoulder in the main peak, suggesting the presence of two partially overlapped peaks (Fig. 4A).

With the encouragement of a borate complex-based separation, we increased borate concentration by 10 mM. The resulting buffer (40 mM phosphate, 40 mM SDS, 20 mM tetraborate, 10 mM TSA, pH 10.0)

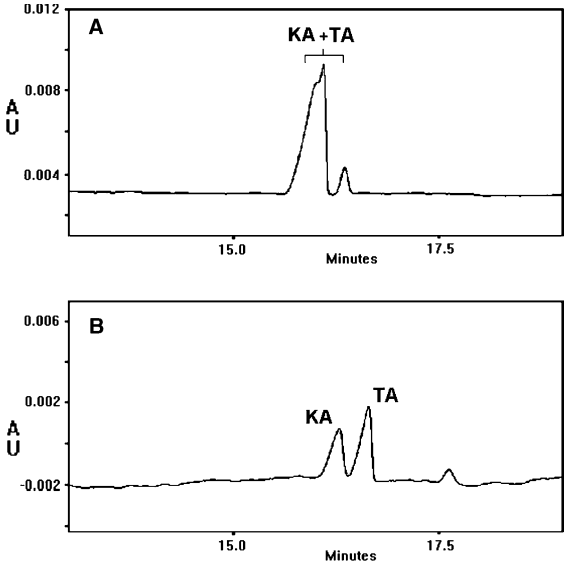


Figure 4. Effect of borate addition on the peak resolution of the sulfated disaccharides KA and TA. Electrophoresis was carried out at 15 kV, 40 °C in 40 mM phosphate/40 mM SDS/10 mM TSA, pH 10.0 buffer system in the presence of 10 mM (A) and 20 mM borate (B).

allowed a complete separation of the two isomers (Fig. 4B). Examination of each individual isomer showed that **KA** migrates faster than **TA** toward the cathode (outlet) under these conditions. Another experiment using a pH 11.0 buffer was attempted, but the resulting electropherogram had excessive baseline instability and improvement of resolution was not achieved. We also tested various voltages and temperatures to optimize the analysis time. The fastest analyses were performed using 15 kV, although the baseline instability was high for all voltages above 10 kV. The best results were achieved using 45 °C and 10 kV.

Having established the ideal buffer composition, temperature, and voltage conditions for the separation of **KA** and **TA**, the other oligosaccharides were submitted to CE analyses. Figure 5 shows the electropherogram obtained for a mixture of the seven oligosaccharides now described. The isomeric tetrasaccharides **KB** and **TB** demonstrated the same order of elution as their disaccharide analogues, namely that the κ-carrageenan derived oligosaccharide (**KB**, 21.2 min) migrates faster toward the cathode than the θ-carrageenan derivative (**TB**, 21.6 min), with resolution between peaks ($R_{KB/TB}$) of 1.0 (Table 2, Fig. 5). The sulfated disaccharides **GA** (19.8 min) and **WA** (20.7 min) appeared with higher retention times than **KA** (18.6 min) and **TA** (19.0 min), with the agaran-derivative pair being better resolved ($R_{GA/WA} = 2.7$) than the carrageenan-derivative one ($R_{KA/TA} = 1.2$). In the pyruvylated disaccharide (**WB**) analysis, it was possible to visualize a main peak surrounded by smaller peaks (minor impurities), all of them with higher migration rates than those found for the sulfated oligosaccharides.

4. Discussion

Several red seaweed galactans show complex patterns of substitution due to the presence of *O*-sulfate, *O*-methyl and/or *O*-glycosyl groups at different positions, and pyruvic acid acetals at O-4,6 of the β-D-Gal*p* units.[1,52–55] Moreover, a new member of the red seaweed galactan family, the D,L-hybrid, is under discussion.[14–17]

The use of partial hydrolysis, followed by characterization of the resulting oligosaccharides, is one of the most reliable methods to determine the structural details of complex algal galactans, such as their substitution patterns and unit sequence. The main obstacle in this kind of study is the relatively low yield of the numerous oligosaccharides, due to the great number of possible combinations of substitution positioning and α-unit forms (3,6-AnGal*p* or Gal*p*). In the case of D,L-hybrid galactans, the number of possible oligosaccharides is still higher, and conventional preparative chromatographic methods are not capable of resolving the resulting complex diasteroisomeric mixture.

Fortunately, some red seaweed species produce repetitive galactans, and in many cases, it is possible to generate a repetitive structure by alkaline treatment from a relatively complex polysaccharide (e.g., *A. spicifera* aga-

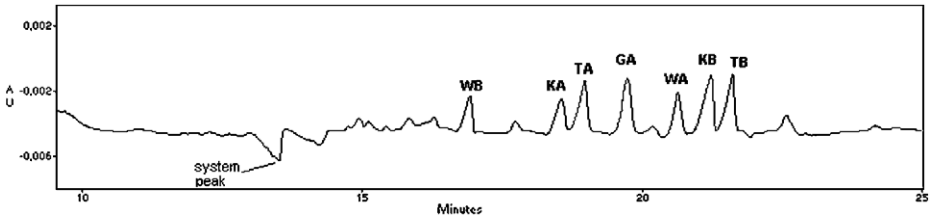


Figure 5. Electropherogram of all our oligosaccharide alditols. Electrophoresis was carried out at 10 kV, 45 °C in 40 mM phosphate/40 mM SDS/20 mM borate/10 mM TSA, pH 10.0.

Table 2. Retention times and resolution between electrophoresis peaks[a] of the oligosaccharides **WB**, **KA**, **TA**, **GA**, **WA**, **KB**, and **TB**

	Oligosaccharide alditol						
	WB	**KA**	**TA**	**GA**	**WA**	**KB**	**TB**
Retention time (min)	16.9	18.6	19.0	19.8	20.7	21.2	21.6
Resolution between peaks	$R_{WB/KA} = 5.3$ $R_{WB/TA} = 6.2$ $R_{WB/GA} = 9.2$ $R_{WB/WA} = 12.2$ $R_{WB/KB} = 12.9$ $R_{WB/TB} = 14.1$	$R_{KA/TA} = 1.2$ $R_{KA/GA} = 3.6$ $R_{KA/WA} = 6.3$ $R_{KA/KB} = 7.4$ $R_{KA/TB} = 8.5$	$R_{TA/GA} = 2.1$ $R_{TA/WA} = 4.6$ $R_{TA/KB} = 5.8$ $R_{TA/TB} = 6.8$	$R_{GA/WA} = 2.7$ $R_{GA/KB} = 4.1$ $R_{GA/TB} = 5.2$	$R_{WA/KB} = 1.6$ $R_{WA/TB} = 2.7$	$R_{KB/TB}$=1.0	

For electrophoresis conditions, see Figure 5.

[a] $RX/Y = 2(X - Y)/X_w + Y_w$, where X and Y are the retention times in min, and X_w and Y_w are the widths of the corresponding peaks.

ran).[13,44] We now make use of repetitive agarans and carrageenans, obtained from some selected Rhodophyta species, to produce oligosaccharides, which could be found in a complex galactan hydrolyzate. All four galactans now hydrolyzed were composed of repetitive diads of [→3)-β-D-Gal*p*-(1→4)-3,6-An-α-Gal*p*] with specific sulfation positioning for each one. The use of partial reductive hydrolysis, which specifically cleaves the 3,6-anhydrogalactosidic bonds, should produce only disaccharide alditols with its sulfate groups corresponding to the sulfation of the parent galactan. However, some unexpected oligosaccharides were obtained in terms of their degree of polymerization and sulfate content.

Partial reductive hydrolysis of agarose 6-sulfate generated, as expected, only a disaccharide alditol while, κ- and θ-carrageenan produced di- and tetrasaccharides. Both carrageenans have sulfated carbons that are one bond distant from carbons directly involved in the 3,6-anhydrogalactosyl linkage. In contrast, the sulfate groups in agarose 6-sulfate are three bonds distant from the target linkage. These data suggest that the presence of a sulfate group close to the 3,6-anhydrogalactosidic linkage may prevent hydrolysis of this linkage to some extent.

The first report describing partial reductive hydrolysis of alkali-treated crude extracts from *A. spicifera*, demonstrated that only disaccharide alditols were produced.[13] More recently,[44] a disulfated tetrasaccharide alditol from an alkali-treated, purified fraction, extracted from the same seaweed was also isolated. However, we noted that tetrasaccharides and higher oligosaccharides are found only as minor products (<0.1%) for the aforementioned alkali-treated galactan (unpublished results).

According to the produced and isolated oligosaccharide alditols, the sulfate groups of the κ-carrageenan and agarose 6-sulfate were substantially retained during the partial reductive hydrolysis. In contrast, the oligosaccharides produced from θ-carrageenan appeared to be completely desulfated at C-2 of the β-D-Gal*p* units, while hydrolysis of sulfate groups at the same position in the α-units was not detected. The longer hydrolysis time applied to the θ-carrageenan could have, in part, caused this specific hydrolysis. Sulfate hydrolysis, at the same position and unit, was also noted after partial reductive hydrolysis of *A. spicifera* agaran[13] and autohydrolysis of λ- and θ-carrageenans,[56] suggesting an important difference in the stability of the sulfate groups, depending on their position and the unit to which they are attached.

The selective cleavage of the sulfate groups during the θ-carrageenan hydrolysis provided us with a monosulfated disaccharide (**TA**) and a disulfated tetrasaccharide (**TB**), which form positional isomer pairs with the κ-carrageenan derivative oligosaccharides (**KA** and **KB**, respectively). Another isomeric pair was obtained with the disaccharides **GA** and **WA**. Having produced and characterized these closely related sulfated oligosaccharide alditols with variations of their charge-to-mass ratio, stereochemistry of the 3,6-AnGal units, and sulfate positioning, we searched for an appropriate analytical technique capable of separating them. We chose CE because this technique is suitable for charged compounds and can provide separations with high resolution and low sample consumption.[18]

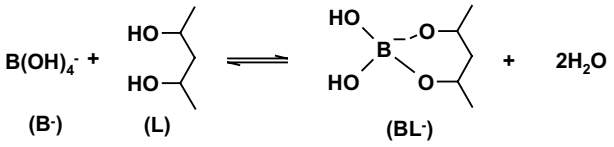
The first obstacle in our CE experiments was the low sensitivity of the direct UV detection due to the absence of chromophoric groups in our analytes. Despite the possibility of detecting carbohydrate–borate complexes directly by UV at a low wavelength,[27,57] we opted for indirect UV detection. In this methodology, an absorbing co-ion with the same charge as the analyte is added to the buffer. Detection is accomplished by displacement of the co-ion, leading to a decrease in the background absorbance.[58]

TSA (10 mM) was used as a detectable co-ion in our indirect UV detection scheme. This compound has an appropriate molar absorptivity, giving enough sensitivity (limit of detection around 1 mM) for our analyses. However, we did not achieve a good limit of detection, probably due to the multiple electrolyte buffer used, which caused a competitive displacement of the absorbing co-ion and, consequently, a decrease in the sensitivity.[58] On the other hand, TSA has a mobility similar to those of the oligosaccharide alditols under our CE conditions (absorbing co-ion system peak indicated in Fig. 5), providing our electropherograms with good peak shapes.

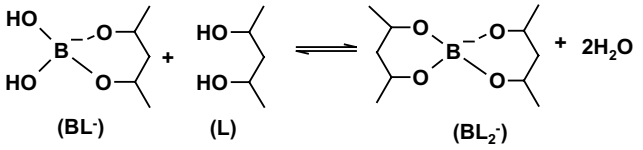
Capillary electrophoresis experiments were performed using an alkaline buffer with borate anions, and these conditions were essential for the success of the resolution of the acidic oligosaccharide alditols. This strategy was defined based mainly on differences between the availability of the hydroxyl groups for borate complexation,[27,46–51] which were shown by the prepared oligosaccharides. In this context, boron is a unique element in which a planar oxoacid $B(OH)_3$ (boric acid) is converted into tetrahedral oxoanion $B(OH)_4^-$ (tetrahydroxyborate) in basic solutions.[59–61] The following equilibrium (1) shows the formation of the $B(OH)_4^-$ anion from $B(OH)_3$ in alkaline media:

$$B(OH)_3 + OH^- \rightleftharpoons B(OH)_4^- \quad (1)$$

The tetrahydroxyborate anion is well known for its ability to form negatively charged complexes with vicinal cis and alditol hydroxyl groups in carbohydrates, mainly at high pH. The equilibria 2 and 3 show the formation of 1:1 monocomplex (BL^-) and a 1:2 dicomplex (BL_2^-):



(2)



(3)

This complexation induces changes in the charge-to-mass ratios of the ligands, and this property has been utilized since the earlier 1950s to determine the configuration of carbohydrates.[37,46,62] Buffers with high pH containing borate salts have been extensively used in CE for the separation of sugars, not only by inducing the formation of charged and mobile complexes from uncharged carbohydrates, but also by increasing the selectivity of the separation of charged ones.[27–40] Since **2** and **3** are dynamic, all carbohydrates with favorable configuration will be associated with tetrahydroxyborate to some extent.[27]

By observing the effects of pH increase and borate concentration (Fig. 4), as well as the migration of the disaccharides **KA**, **TA**, **GA** and **WA** (Fig. 5), we propose a mechanism of separation based on the increment of their negative charges provided by borate complexation. This mechanism is based on the fact that the negative charged analyte mobility, in high pH buffers, is the result of the electroendosmotic flow (EOF) toward the cathode and the electrophoretic migration of the analyte toward the anode. EOF is more significant than the electrophoretic migration under these conditions, and consequently, the resulting analyte mobility is toward the cathode.[18] In our experiments, the CE separation was then accomplished by differences in the slowing of the electrophoretic migration of the oligosaccharides against the EOF. This slowing was provided by their own negative charges and their ability to form complexes with tetrahydroxyborate anions.[31,39] The magnitude of the negative charge of this kind of complex is determined by their positions in the equilibrium, and therefore, by their stability. Given a constant amount of carbohydrates, the complex concentration increases with rising borate concentration according to the law of mass action as well as with rising pH. Therefore, the electrophoretic mobility is increased toward the anode.[27,63]

The scheme presented in Figure 6 correlates the increase of the retention times shown by the monosulfated disaccharide alditols, according to the availability of their free vicinal cis and alditol hydroxyl groups. Specifically, we observed that **WA** had the highest retardation among the sulfated disaccharides, due to the presence of three sites for $B(OH)_4^-$ complexation, allowing the formation of a 4/6 or 3/4 complexes in the Gal*p* unit,[64] and 1/2 complex in the 3,6-AnGalOH unit. The disaccharide **GA** has two potential sites: 3/4 (Gal*p* unit), and 1/2 (3,6-AnGalOH unit). **TA** also has two potential sites for complexation (4/6 or 3/4 in the Gal*p* unit), although OH-4 would be involved for both possible complexes. For **KA**, only one site is available at 1/2 hydroxyl vicinal groups of the alditol unit, therefore, it demonstrates the lowest retardation in its migration, when compared to the other monosulfated disaccharide alditols. The higher retention time shown by the tetrasaccharides **KB** and **TB** is consistent with their higher charge-to-mass ratio in comparison with the disaccharides, and their order of elution follows the same mechanism proposed for the separation of the analogous disaccharides **KA** and **TA**. The lowest retention time is shown by the pyruvylated disaccharide **WB**, which can be explained by the milder acidity of the carboxyl in comparison with the sulfate groups.

van den Berg et al.[64] studied the formation of borate complexes of various mono- and disaccharides in

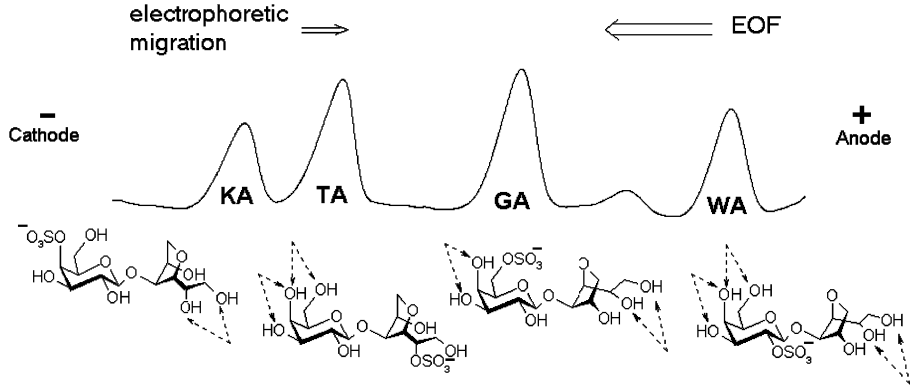


Figure 6. Correlation between the increase of the retention times of the monosulfated disaccharide alditols with the availability of their free vicinal cis and alditol hydroxyl groups; the arrows show the favorable hydroxyl groups for borate complexation.

aqueous solution (pH 7.0) by ^{11}B and ^{13}C NMR. The association constants ($K_{loc} = [BL^-]/[B^-][L]$) were determined for some galactopyranosides, and the values for 3/4 and 4/6 BL^- complexes were ~13 and ~5, respectively. The 3/4 BL_2^- complexes were also detected with an association constant of ~10. These data suggest that the 3/4 complexes have a more important participation than the 4/6 in the formation of the borate–galactopyranoside complexes. However, in our experiments, the 4/6 complex appears to be the determining factor, principally in the separation of **GA** and **WA**. This could be explained by changes in the equilibrium due to the Coulombic repulsion[27,47,48,51] between borate and the O-6 sulfate group in **GA** (3/4 complex destabilization).

It has been reported that alditols form borate complexes, that are much more stable than the corresponding pyranosyl sugars.[65] Mainly from the close retention times shown by **KA** and **TA** ($R_{KA/TA} = 1.2$), and the good resolution between **TA** and **GA** ($R_{TA/GA} = 2.1$), we conclude that complexation at positions 1/2 of the 3,6-AnGalOH unit had some participation in our CE separations. However, we can speculate that the proposed complex is less stable than a non-cyclized alditol–borate complex due to the interference of the 3,6-anhydro ring. Furthermore, according to the literature, diols in terminal positions are not as stable as internal *threo*-diols in alditols.[37,47]

Other factors can influence the borate-based separation of the oligosaccharide alditols now described, namely: (a) electrolytes, other than borate, which are components of our buffer can influence the separation; (b) aqueous borate solutions contain not only tetrahydroxyborate ions but also more highly condensed polyanions such as $[B_3O_3(OH)_5]^{2-}$ and $[B_4O_5(OH)_4]^{2-}$ (tetraborate); (c) trans-1,2-diols and hydroxyl groups linked to alternate carbons can be involved in the complexation.[47,48,51,59] However, we recognize that the scheme presented in Figure 6 is an over-simplified way of explaining our CE results.

In conclusion, partial reductive hydrolysis was used to prepare oligosaccharide alditols from repetitive agarans and carrageenans. The characterized positional isomers of sulfated oligosaccharide alditols were then completely resolved by CE in a borate buffer. In addition, we correlated the relative migration of the oligosaccharides with their possible borate complexes.

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