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Alginic acids in *Lessonia trabeculata*: characterization by formic acid hydrolysis and FT-IR spectroscopy

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Abstract

The ratio of mannuronic acid to guluronic acid (M/G) in alginic acids from blade, stipe and holdfast of *Lessonia trabeculata* collected in three locations of the Chilean coast was determined by total hydrolysis with formic acid. It was found that the reaction of alginic acids with 90% formic acid for 6 h at 100°C, followed by treatment with 1.5 N formic acid for 2 h at 100°C produced the total hydrolysis. The values of M/G ratio, determined by HPLC, were slightly lower than those obtained by the traditional hydrolysis method employing 80% sulphuric acid. No systematic tissue-to-tissue variations in the M/G ratio were observed.

FT-IR spectroscopy, especially in the second-derivative mode, was applied for characterizing alginates and partial hydrolysis fractions. The alginate fraction enriched in polymannuronic acid presented two bands at ~893 and ~822 cm^{-1} while the polyguluronic acid enriched fractions showed four characteristic absorption bands at around 947, 903, 813 and 780 cm^{-1}.

Keywords: Alginic acid; Hydrolysis; *Lessonia trabeculata*; Phaeophyta; FT-IR

1. Introduction

The brown seaweed *Lessonia trabeculata* (Villouta & Santelices, 1986) of the order Laminariales (Phaeophyta) dominates the shallow subtidal environments of northern Chile (Vásquez, 1992).

The major structural polysaccharide of brown seaweeds is alginic acid, a linear 1,4-linked copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G). The two uronic acids can be arranged in homopolymeric [poly(β-D-mannosyluronic acid) (MM) and poly(α-L-gulosyluronic acid) (GG)] or heteropolymeric (MG) blocks.

The alginic acid of *L. trabeculata* from central Chile was studied in this laboratory (Matsuhiro & Zambrano, 1989). Venegas, Matsuhiro, and Edding (1993) have found differences in the mannuronic to guluronic acid ratios and block compositions of alginic acid extracted from populations of *L. trabeculata* growing in exposed and sheltered habitats.

The characterization of the subtidal environments contaminated by iron mining through the quantification of Fe in seawater, in different tissues and alginic acid of *L. trabeculata*, have been recently reported (Vásquez, Vega, Matsuhiro, & Urzúa, 1999).

Related to our work on the chemical modifications of polysaccharides and their conjugation to proteins (Jerez, Matsuhiro, & Urzua, 1997; Jerez, Matsuhiro, Urzúa, & Zúñiga, 1999; Lillo & Matsuhiro, 1997) aqueous alkaline extracts from *L. trabeculata* from three different locations of northern Chile were examined in search for homopolyguluronic enriched alginic acids. This work presents the results of the studies on the total hydrolysis of alginates with formic acid and the characterization of alginates and the partially hydrolysed alginates by FT-IR spectroscopy.

2. Experimental

2.1. Materials and methods

L. trabeculata samples were collected in winter and spring in San Lorenzo (30°15′), Carrizal Bajo (28°05′) and Chapaco (28°05′). The extraction of sodium alginate from *L. trabeculata* was previously reported (Matsuhiro & Zambrano, 1989, 1990). Alginate samples were purified according to Venegas et al. (1993). D-Mannuronic acid, D-galacturonic acid and D-glucuronolactone from Sigma were used as standards. L-Guluronic acid was prepared by H_2SO_4

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hydrolysis of alginic acid from *L. trabeculata*. FT-IR spectra in KBr pellets were registered in the 4000–400 cm^{-1} region using a Bruker IFS 66 v instrument according to Cáceres, Faúndez, Matsuhiro, and Vásquez (1997). ^1H NMR spectra were recorded at 200 MHz and 75°C using methanol as internal reference. The samples were prepared according to Panikkar and Brasch (1997).

2.2. Total hydrolysis of alginate samples

2.2.1. With sulphuric acid

Sodium alginate (50 mg) was mixed with 0.5 ml of 80% H_2SO_4 at 0°C and the mixture was left for 18 h at room temperature. Then, it was cooled to 0°C and 6.5 ml of water was added. The resulting mixture was refluxed for 6 h and then treated according to Haug and Larsen (1962). An aliquot was analysed by high performance liquid chromatography (HPLC) with an anionic exchange column of Whatman Partisil 10-Sax (250 × 4.6 mm) using 0.02 M KH_2PO_4 aqueous solution as eluant (Gacesa, Squire, & Winterburn, 1983) on a Merck-Hitachi L-6000[a] HPLC apparatus equipped with a L-4000A UV detector.

D-Mannuronic and L-guluronic acids were separated by ion-exchange chromatography on a DEAE Sephadex A-25 column (100 × 1.3 cm). Elution was carried out with water and solutions of increasing concentrations of acetic acid (0.5, 1.0, 1.5, 2.0 and 2.5 M) (Haug & Larsen, 1962).

2.2.2. With formic acid

Samples of sodium alginate (6.0 mg) and 5.0 ml of 90% formic acid in a sealed tube were heated for 2, 4 or 6 h at 100°C in an oven. Each of the resulting solutions was diluted with 25 ml of distilled water and hydrolysed for 2 h at 100°C. After evaporation of the solution in vacuo, the excess of acid was removed by repeated evaporations with distilled water. The residue was dissolved in 1 ml of distilled water and 10 μl of triethylamine was added. After 10 min the M/G ratio was determined by HPLC according to Gacesa et al. (1983). All hydrolyses were conducted in duplicate.

An aliquot of the hydrolysates (3 ml; 5 mg/ml) was neutralized with 0.5 N NaOH and chromatographed on a Sepharose 6 B column (100 × 1.5 cm). Elution was carried out with 0.2 M NaCl and monitored with phenol–sulphuric acid and carbazole–sulphuric acid reagents (Chaplin, 1986). The column was calibrated with 2 ml (5 mg/ml) solutions of Blue dextran 2000 and with D-glucose. Fractions of 3 ml were collected. Solutions of D-galacturonic acid (2 ml; 4 mg/ml) and unhydrolysed alginate (3 ml; 5 mg/ml) were chromatographed under the same conditions.

In parallel experiments samples were hydrolysed for 2 h in sealed tubes at 100°C in an oven and re-hydrolysed after dilution, for 4 or 6 h at 100°C.

2.3. Partial hydrolysis

A 1% aqueous solution of sodium alginate was made up to 0.3 M in HCl by addition of 3.0 M HCl and heated for 0.5 h at 100°C under nitrogen. The mixture was cooled and centrifuged. The solid was suspended in 0.3 M HCl and heated for 2 h at 100°C under nitrogen. The precipitate was collected by centrifugation, suspended in water, and redissolved by neutralization to a final concentration of 1%. The solution was adjusted to pH 2.85 by addition of 1 M HCl. The precipitate was collected by centrifugation and solubilized by neutralization (Haug, Larsen, & Smidsrod, 1974; Panikkar & Brasch 1997).

Each of the supernatant of the centrifugations was poured into five volumes of ethanol; the precipitate was separated by filtration and washed with ethanol. The resulting solid was dissolved in minimum volume of water at 50°C, cooled and poured into five volumes of ethanol. The process was repeated once more.

3. Results and discussion

3.1. Total hydrolysis

Alginic acid is very resistant to hydrolysis by mineral acids, and complete liberation of the uronic acids without decomposition is difficult to achieve. Destruction, particularly of L-guluronic acid units can occur. The traditional hydrolysis method employing 80% H_2SO_4 (Adams, 1965; Haug & Larsen, 1962) is frequently used for the complete release of mannuronic and guluronic acids from alginic acid. According to Anzai, Uchida, and Nishide (1990) a shorter hydrolysis period increased the recovery of monouronates but may not influence the M/G ratio.

A modification of the formic acid method used in the hydrolysis of fucans containing uronic acids (Mian & Percival, 1973; Nishino, Yokoyama, Dobashi, Fujihara, & Nagumo, 1989) was assayed for sodium alginate extracted from blades of *L. trabeculata* collected in San Lorenzo (sample 1) and from stipes of *L. trabeculata* collected in

Table 1
M/G values of alginates obtained in different hydrolysis conditions (samples of sodium alginate from blades of *L. trabeculata* collected in spring in: (1) San Lorenzo; (2) Carrizal Bajo)

Hydrolysis conditions		Sample	M/G	Sample	M/G
Sulphuric acid 80% H_2SO_4 (20°C, 18 h), 2 N H_2SO_4 (100°C, 6 h)		1	0.51	2	0.54
Formic acid[a]					
First step	Second step				
2 h	2 h	1	0.09	2	0.28
2 h	4 h	1	0.12	2	0.41
2 h	6 h	1	0.19	2	0.37
4 h	2 h	1	0.40	2	0.46
6 h	2 h	1	0.43	2	0.47

[a] First step: 90% HCOOH (100°C). Second step: 1.5 N HCOOH (100°C).

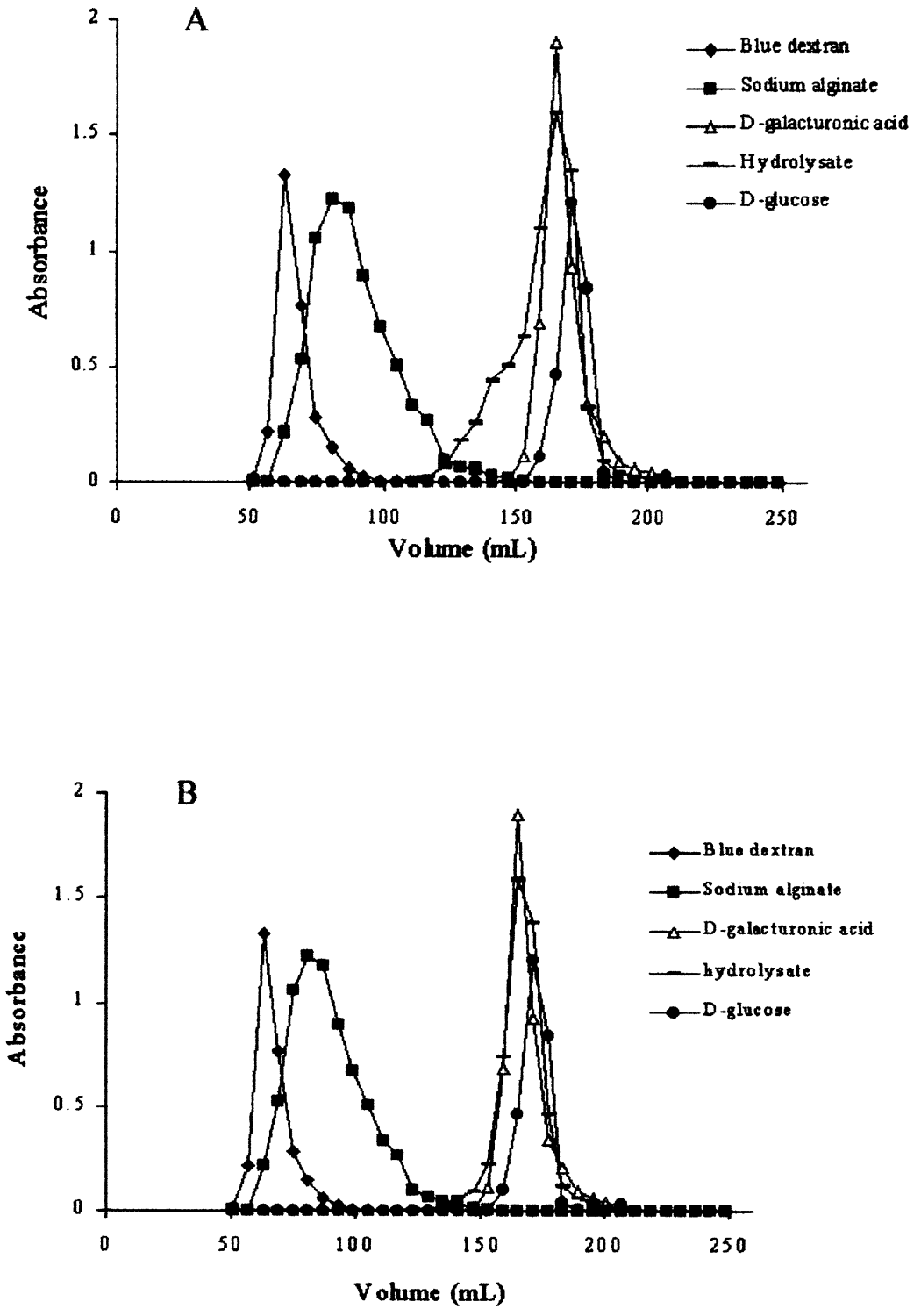

Fig. 1. Elution pattern of alginic acid hydrolysates on Sepharose 6B column. Conditions of hydrolysis at 100°C: (A) 90% HCOOH for 2 h, 1.5 N HCOOH for 2 h, (B) 90% HCOOH for 6 h, 1.5 N HCOOH for 2 h. Glucose was determined with the phenol–sulphuric acid reagent. Samples of alginate, hydrolysates and galacturonic acid were determined with the carbazol reagent.

Carrizal Bajo (sample 2). The mannuronic acid to guluronic acid ratio (M/G) in the hydrolysate was determined by HPLC according to Gacesa et al. (1983). In Table 1 the M/G values obtained in different conditions are presented.

Analysis by gel permeation chromatography (GPC) of the hydrolysate obtained by heating sample 1 at 100°C with 90% formic acid for 2 h, and then for 2 h with 1.5 N formic acid, indicates that a small amount of partially degraded

Table 2
Yield and M/G ratio of alginic acid from *L. trabeculata* (samples of *L. trabeculata* collected in winter in: (3) San Lorenzo; (4) Chapaco; (5) Carrizal Bajo)

Sample	B (Blade)		S (Stipe)		H (Holdfast)	
	Yield (%)	M/G	Yield (%)	M/G	Yield (%)	M/G
3	29.8	0.43	27.4	0.50	19.8	0.34
4	13.1	1.21	13.3	1.73	12.9	1.17
5	15.8	0.88	13.5	0.64	9.1	0.62

alginate fractions are present (Fig. 1A). On the other hand, results of the GPC analyses of the hydrolyste from sample 1 obtained after 6 h of heating (Fig. 1B) in the first step, followed by 2 h in the second step, shows that the complete release of monouronic acids was achieved.

The M/G determination for sodium alginate samples from *L. trabeculata* collected in three different locations was conducted with the formic acid hydrolysis method using a 6-h period of heating in the first step and 2 h in the second step. The results are presented in Table 2. Higher values of M/G were obtained from the sample collected in Chapaco, a Fe contaminated area (Vásquez et al., 1999). The M/G values previously found for the alginate of *L. trabeculata* collected in summer in Las Cruces (33°29′S, 71°38W) varied between 1.08 and 1.94 (Matsuhiro & Zambrano, 1989). The large variation of the M/G values obtained in alginates from *L. trabeculata* may be attributed to environmental adaptation (Craigie, Morris, Rees, & Thom, 1984).

In the alginic acid samples analysed in this work, no systematic tissue-to-tissue variation in the M/G values was found. According to some authors, alginic acids from blades and stipes might show higher M/G values than those extracted from holdfast. In holdfast, the high content of polyguluronate would provide mechanical strength for its adhesion (Cheshire & Hallam, 1985; Stockton, Evans, Morris, Powell, & Rees, 1980).

3.2. Partial hydrolysis

Three alginate samples were partially hydrolysed with HCl. According to the literature (Haug et al., 1974), the fraction (F_1) obtained in the first hydrolysis step with 0.3 M HCl was mainly composed of heteropolymeric blocks, the soluble fraction (F_2) at pH 2.85 was enriched in polymannuronic acid, and the insoluble fraction (F_3) at pH 2.85 was enriched in polyguluronic acid. In the case of alginate with a lower M/G value (0.43) a very good yield (65%) of the insoluble fraction (F_3) was obtained. For samples 1 and 2 the yield of the insoluble fractions were 11 and 39%, respectively.

The fractions were analysed by FT-IR in the solid state. This spectroscopic technique, especially in the second-derivative mode, has been used to differentiate between agar and carrageenan-type polysaccharides and to distinguish agar-producing from carrageenan-producing seaweeds (Matsuhiro, 1996; Matsuhiro & Rivas, 1993). In Table 3, the FT-IR frequencies and the second-derivative frequencies of alginates in the anomeric or fingerprint region are presented. According to Mackie (1971), alginates showed in the IR spectra two characteristics bands at 808 and 787 cm^{-1}, assigned to mannuronic and guluronic acids, respectively. The FT-IR spectra and the second-derivative spectra gave more information than the classical IR. Irrespective of the M/G ratio of the alginate, all the F_3 fractions give the band assigned to guluronic acid at around 781 cm^{-1} and three additional bands. The FT-IR spectrum and its second-derivative spectrum of fraction F_3 obtained by partial hydrolysis of the alginate from stipe of *L. trabeculata* collected in Carrizal Bajo is shown in Fig. 2. The band at around 948 cm^{-1} was assigned to α 1 → 4 linkage, and the band at 903 cm^{-1} was attributed to the α-L-gulopyranuronic asymmetric ring vibration (Mathlouthi & Koenig, 1986). It is noteworthy that the third band, at around 814 cm^{-1}, which is present in the spectra of all the F_3 fractions enriched in polyguluronate, is absent in the polymannuronic enriched F_2

Table 3
FT-IR frequencies (cm^{-1}) of sodium alginate samples and of the fractions obtained by partial hydrolysis in the 1000–700 cm^{-1} region
F_1: soluble fraction, F_2: subfraction soluble at pH 2.85 of insoluble fraction, F_3: subfraction insoluble at pH 2.85 of insoluble fraction, b: broad band. Frequencies in parenthesis were obtained from the second-derivative spectra

Sample	Frequencies (cm^{-1})							
4S (M/G 1.73)		(947.7)		(904.3)	880.0			
F_1	964.6				889.6	(819.7), b816.7		
F_2	964.8				(893.6)	(821.2), b818.3		
F_3		947.2		903.2		(822)	813.3	781.6
5S (M/G 0.64)		947.7		(903.8)	880.0		812.1	
F_1	967.5	(942.1)			897.6	(818.1), b816.8		
F_2	963.9				893.9	(822.3), b818.8		
F_3		947.7		903.7		(823.0)	812.9	781.1
3B (M/G 0.43)		947.3		902.2	(890.0)	(822), 815.7		780.9
F_1	963.2		912.1		889.0	(819.8)	814.4	
F_2		946.9			(893.1), 887.5	(821.4), 818.7		
F_3		948.7		904.1		(822.2)	812.1	782.0

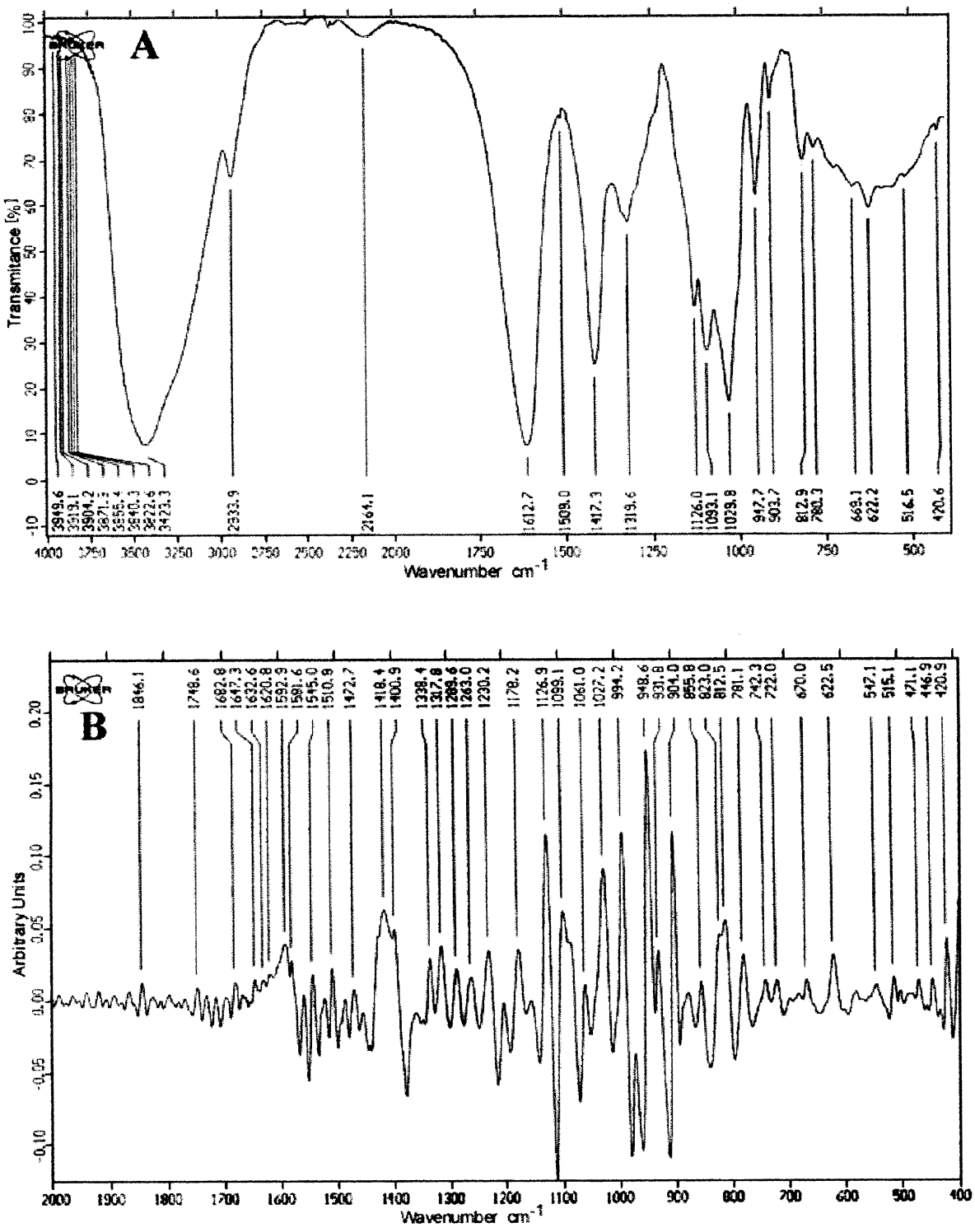

Fig. 2. FT-IR spectra of the fraction insoluble at pH 2.85 obtained by partial hydrolysis of alginate from stipes of *L. trabeculata* collected in Carrizal Bajo: (A) normal spectrum; (B) second-derivative spectrum.

fractions. On the other hand, the latter fractions showed in the normal spectra a broad band centred at 818 cm^{-1}, which in the second-derivative spectra is well resolved at 822 cm^{-1}, and was assigned to mannopyranuronic acid residues. Moreover, the F_2 fractions present the characteristic β-anomeric CH deformation band due to β-mannuronic residues around 898–884 cm^{-1}. The second-derivative spectra of F_3 fractions present a signal at 822.2 cm^{-1}, which

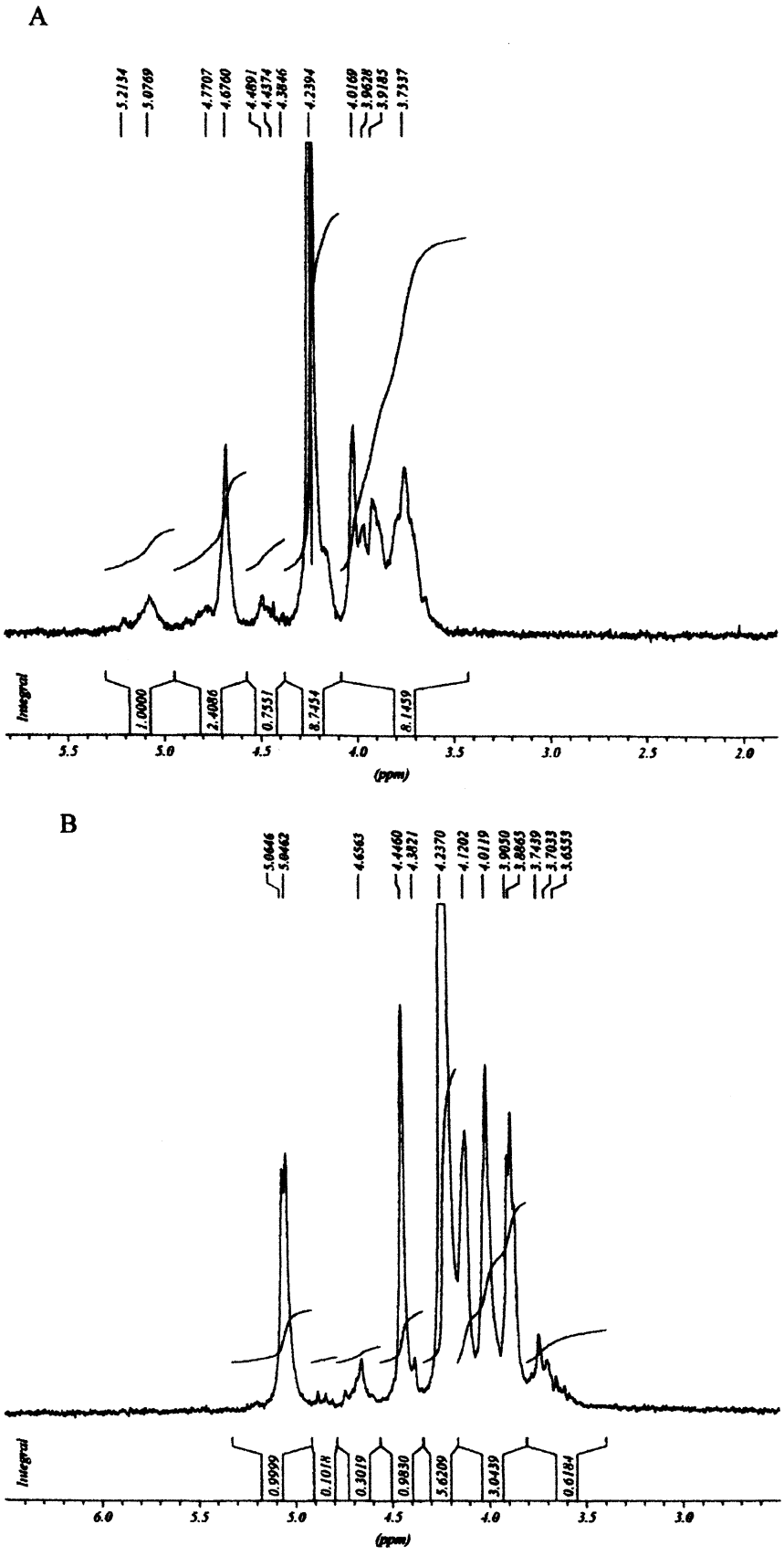

Fig. 3. ^{1}H NMR spectra of fractions obtained by partial hydrolysis of alginate from blades of *L. trabeculata* collected in San Lorenzo: (A) fraction soluble at pH 2.85; (B) fraction insoluble at pH 2.85.

is indicative of the presence of small amounts of mannuronic acid in the homopolyguluronic enriched fraction.

These results were in accordance with those obtained from the ^{1}H NMR spectra (Fig. 3). The spectrum of F_3 (Fig. 3B) from alginate sample 3 showed a doublet centred at 5.05 ppm assigned to H-1 and a signal at 4.45 ppm assigned to H-5 of L-guluronic acid in homopolymeric blocks. The smaller relative broad peak at ~4.7 ppm assigned to the anomeric proton of mannuronic acid residues and H-5 of L-guluronic acid residues adjacent to mannuronic acid (Grasdalen, 1983) indicate that fraction F_3 is a polyguluronic enriched fraction not purified by some mannuronic acid residues. The spectrum of fraction F_2 (Fig. 3A) corresponds to a polymannuronic enriched fraction with the characteristic anomeric proton signal centred at 4.70 ppm, the broad bands at 5.07 and 4.48 ppm are indicative of the presence of some guluronic acid residues.

4. Conclusions

Complete hydrolysis of alginic acids can be achieved with 90% formic acid. The method is clean and faster than the traditional 80% sulphuric acid hydrolysis. FT-IR spectroscopy, especially in the second-derivative mode, provides a good alternative method for the characterization of alginic acid and its heteropolymeric and homopolymeric blocks in the solid state. From the FT-IR spectroscopic analysis it can be concluded that although the M/G ratio of the alginic acid samples of *L. trabeculata* collected in the three localities varied considerably the different samples produced similar homopolymeric block systems.

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