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Chiroptical characterization of homopolymeric block fractions in alginates

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ABSTRACT

Homopolymannuronic and homopolyguluronic fractions were obtained by partial hydrolysis of the alkaline extracts from the brown seaweeds *Ascoseira mirabilis, Desmarestia menziessi, Desmarestia ligulata* and *Durvillaea* sp. collected in southern Chile. Full characterization of the fractions was achieved by FT-IR and NMR spectroscopy. Total hydrolysis with 90% formic acid of the homopolymeric fractions allowed the preparation of mannuronic and guluronic acids. Both monomers and homopolymeric fractions as neutral salts were studied by CD and ORD. Chiroptical spectra were similar in shape and sign to those previously published in the literature, and permitted to assign D configuration to mannuronic acid and L configuration to guluronic acid in alginic acids. Specific optical rotation values at the sodium D light for the homopolymannuronic (∼−100°) and homopolyguluronic (∼−110°) acid fractions were obtained. These high negative values are proposed for the assignment of the absolute configuration of monomers in homopolymeric fractions.

1. Introduction

Alginic acid, the major structural polysaccharide from brown seaweeds, is a copolymer of β-D-mannuronic (M) and α-L-guluronic acid (G). Both monomers are linked 1→4 forming heteropolymeric and homopolymeric blocks (Haug, Larsen, & Smidsrød, 1974; Painter, 1983). The M/G ratio and the distribution of the two uronic acids in blocks depend on the species, and within a particular species depend on the tissue type, habitat, season of harvest, etc. (Chandía, Matsuhiro, Mejías, & Moenne, 2004; Craigie, Morris, Rees, & Thom, 1984; Davis, Llanes, Volesky, & Mucci, 2003; García-Ríos, Ríos-Leal, Robledo, & Freile-Pelegrin, 2012; Larsen, Salem, Sallam, Mishrikey, & Beltagy, 2003; Leal, Matsuhiro, Rossi, & Carusso, 2008; Panikkar & Brasch, 1997; Venegas, Matsuhiro, & Edding, 1993). Salts of alginates present $[\alpha]_D$ around −130°, but the high negative values of specific rotation did not give information about their composition (Mahmood & Siddique, 2010; Roff & Scott, 1971).

Physical methods such as vibrational spectroscopy, nuclear magnetic resonance spectroscopy (NMR), and circular dichroism (CD) have been used for the characterization of alginate and its fractions (Campos-Vallette et al., 2010; Cárdenas-Jirón, Leal, Matsuhiro, & Osorio-Román, 2011; Chandía, Matsuhiro, & Vásquez, 2001; Grasdalen, Larsen, & Smidsrød, 1981; Grasdalen, 1983; Morris, Rees, & Thom, 1980; Panikkar & Brasch, 1997; Rees, 1972). CD was especially applied for the study of cation binding in alginates, it was known that gelation of alginate by addition of Ca^{2+} ion produced changes in CD spectra due to the coordination of n orbitals of carboxylate group of homopolyguluronic acid with the divalent cation (Grant, Morris, Rees, Smith, & Thom, 1973; Kohn, 1975; Morris, Rees, & Thom, 1973; Morris, Rees, Thom, & Welsh, 1977; Morris, Rees, & Young, 1982; Thom, Grant, Morris, & Rees, 1982).

Full characterization of the structure of natural polysaccharides requires the determination of the absolute configuration of the constituent monosaccharides. These determinations have early been done using optical rotational data; the method is simple but requires the total acid hydrolysis of polysaccharides, and

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the isolation and purification of the component monosaccharides. It is difficult to apply for the study of complex biological polysaccharides; however, Stroop, Bush, Marple, and Le Course (2002) used a polarimeter as a detector in anion exchange chromatography for the determination of absolute configuration of monosaccharides in bacterial polysaccharides. CD spectroscopy was also employed for the determination of absolute configuration of *O*-benzoylated-monosaccharides of bacterial polysaccharides (Kaluarachchi & Bush, 1989). Gas-liquid chromatography (g.l.c.) methods are also employed for the determination of absolute configuration of neutral sugars and hexosamines; for example, the absolute configuration can be determined by reaction of monosaccharides with S-1-amino-2-propanol and analysis by g.l.c. of the diastereomic acetylated derivatives (Cases, Stortz, & Cerezo, 1995). This reaction is complicated to perform on uronic acids, because it requires a previous esterification with methyl alcohol, followed by the reduction of the esters in order to obtain the corresponding neutral monosaccharides (Ponce, Pujol, Damonte, Flores, & Stortz, 2003). Considering all the above mentioned issues, it is assumed that chiroptical analysis will give enough information on the absolute configuration of the constituent uronic acids of homopolymeric block fractions of sodium alginates. Based on known CD spectra of alginates and uronic acid glycosides, and specific rotation of alginates, it can hypothesize that the optical rotatory dispersion (ORD) curves of sodium salts of L-guluronic, D-mannuronic and homopoly-L-guluronic, and homopoly-D-mannuronic block fractions are plain and negative from 600 to ~220 nm, whereas the Cotton effects appear. Thus, the value of the optical activity at a single wavelength within the plain curve region allows the assignment of the absolute configuration of the two constituent uronic acids.

In this context, homopolymeric fractions of sodium alginates extracted from brown seaweeds were fully characterized by IR and NMR spectrometries, and analyzed by chiroptical methods, and the possible applicability of specific optical rotation to determine the configuration of mannuronic acid and guluronic acid in homopolymeric fractions is discussed.

2. Experimental

2.1. Materials and methods

Ascoseira mirabilis Skottsberg and *Desmarestia menziessi* J. Agardh were collected in Hannah Point, South Shetland Islands, Antarctica (62°55′S, 60°37′W); *Desmarestia ligulata* (Stackhouse) Lamouroux was collected in Caleta Cocholgüe, Bío Bío Region (36°35′48″S, 72°58′40″W); *Durvillaea* sp. was collected in Magellan Strait (53°47′8″S, 70°58′37″W). Analytical grade solvents were purchased from Merck (Darmstadt, Germany); reagent grade chemicals were purchased from Sigma (St. Louis, MO, USA) and were used as received.

Absorbance was measured in a Genesys 5 Thermospectronic spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). FT-IR spectra in KBr pellets of seaweeds and polysaccharides were registered in the 4000–400 cm^{-1} region using a Bruker IFS 66*v* instrument (Bruker, Coventry, UK) according to Leal et al. (2008), samples were previously milled under liquid nitrogen and dried for 8 h *in vacuo* at 56 °C. ^{1}H NMR (400.13 MHz) and ^{13}C (100.62 MHz) spectra were recorded in D_2O, after isotopic exchange (3 × 0.75 mL) at 70 °C or 25 °C, on a Bruker Avance DRX 400 spectrometer (Bruker, Coventry, UK) using the sodium salt of 3-(trimethylsilyl)-1-propionic-2,2,3,3-d_4 acid (TMSP-d_4) as internal reference. The two-dimensional experiments were performed using a pulse field gradient incorporated into an NMR pulse sequence. The number of scans in each experiment was dependent on the sample concentration. The molecular weight of alginates and their fractions was determined by the reducing end method (Cáceres, Carlucci, Damonte, Matsuhiro, & Zúñiga, 2000). Preparation and characterization of homopolymannuronic acid and homopolyguluronic acid fractions from *Macrocystis pyrifera* were previously described (Matsuhiro, Martínez-Gómez, & Mansilla, 2015).

2.2. Extraction and purification of alginates

Milled seaweed sample (100 g) was extracted with 500 mL of *n*-hexane in a Soxhlet equipment during 8 h. The defatted seaweed was dried for 72 h at room temperature and soaked into 2000 mL of an ethanol:formaldehyde (80:20 v/v) mixture during 72 h. The seaweed was dried at room temperature and extracted with 1800 mL of 3% Na_2CO_3 aqueous solution at 95 °C for 4 h. The mixture was centrifuged at 3000*g* (Kubota KS-300P, Tokyo, Japan) and the supernatant was dialyzed against distilled water using Spectra/Por membrane (MWCO 3500) (Spectrum Laboratories, Rancho Dominguez, CA, USA), concentrated *in vacuo*, poured over three times of its volume in ethanol and centrifuged. The pellet was dissolved in hot water, and freeze-dried. The extract was purified, by sequential precipitation in HCl and $CaCl_2$ solutions as previously described (Venegas et al., 1993).

2.3. Total hydrolysis of alginate samples

Total acid hydrolysis of alginate was performed with 90% aqueous solution of formic acid according to Chandía et al. (2001). The hydrolyzate was neutralized with 0.1 M NaOH, freeze-dried and analyzed by high performance liquid chromatography (HPLC) with an ion exchange column of Whatman Partisil 10-Sax (250 × 4.6 mm) using a Waters 600 HPLC equipment and a UV Waters 2990 detector (Waters Co., Framingham, MA, USA) (Gacesa, Squire, & Winterburn, 1983).

2.4. Fractionation of alginate samples

Alginate sample was partially hydrolyzed with HCl according to Haug et al. (1974). Briefly, a sample of purified extract was heated with 0.3 M HCl for 0.5 h at 100 °C under nitrogen atmosphere, cooled and centrifuged. The supernatant was dialyzed against distilled water, concentrated and poured into five volumes of ethanol, affording fraction 1. The pellet was stirred with 0.3 M HCl for 2 h at 100 °C; neutralized and precipitated with 1 M HCl at pH 2.85. By centrifugation, a soluble fraction (fraction 2) and a precipitate (fraction 3) were obtained. Fractions were purified by dialysis and precipitated into ethanol.

2.5. Chiroptical methods

Circular dichroism (CD) and optical rotatory dispersion (ORD) spectra of sample in distilled water (0.8 mg/mL) at pH 7.0, were recorded on Jasco J-815 circular dichroism spectropolarimeter, equipped with an ORD and Peltier cell holders (JASCO, Hachioji, Tokyo, Japan), using a wavelength range of 190–250 nm and 190–600 nm for CD and ORD, respectively. All the measurements were performed under nitrogen atmosphere at 20.0 °C using a quartz cell with an optical pathway of 1 cm; sucrose (0.8 mg/mL) was used as standard. Molar ellipticity, [θ], was calculated according to the following equation:

$$[\theta] = \frac{100 \times \theta}{C \times L} \quad (1)$$

where θ is degree of ellipticity, L the optical pathway and C the concentration in g/dmol, respectively.

Table 1
Yield of alkaline extracts and fractions, M/G ratio of extracts and molecular weight of extracts and fractions.

Seaweed sample[a]	Sodium alginate yield (%)	M/G Ratio	Blocks yield (%)			Molecular weight (g/mol)			
			F1	F2	F3	Sodium alginate	F1	F2	F3
1	5.3	0.72	37.0	24.8	38.2	13,000	9500	11,400	11,900
2	9.2	0.93	31.1	38.3	30.6	87,000	18,000	25,900	28,700
3	30.3	2.78	15.7	70.8	13.5	278,100	16,100	29,500	19,900
4	15.3	0.62	6.5	22.6	70.9	48,500	15,000	18,300	18,600

[a] 1: *Ascoseira mirabilis*, 2: *Desmarestia menziessi*, 3: *Durvillaea* sp., 4: *Desmarestia ligulata*.

A Perkin-Elmer 241 polarimeter (PerkinElmer, Waltham, Massachusetts, USA) was used to measure specific optical rotation of aqueous solution (0.8 mg/mL) employing sodium D line (589.0 nm) at 22–25 °C. All specific optical rotation data are expressed as mean ± standard deviation. Each measurement was done in quadruplicate (n = 4) at $P < 0.05$.

3. Results and discussion

3.1. Extraction and characterization of alginates, block fractions and monomers

Fig. 1 presents the FT-IR spectra of milled and dried samples of seaweeds in the region 1600–700 cm^{-1}. The IR spectra of the seaweeds *Ascoseira mirabilis* (Fig. 1A) and *Desmarestia menziesii* (Fig. 1B) are not well resolved; however, the second-derivative spectra show clearly the signal assigned to mannuronic acid residue at 820 cm^{-1}. A marked difference between the spectra of *Desmarestia ligulata* and *Durvillaea* sp. is especially observed in the second derivative spectra. Fig. 1D depicts signals in *Desmarestia ligulata* spectrum assigned to β-mannuronic acid (817.8 cm^{-1}) and α-guluronic acid (791.5 cm^{-1}), whereas the spectrum of *Durvillaea* sp. presents (Fig. 1C) in the finger print region, the characteristic triad (937.1, 885.5 and 818.9 cm^{-1}) of mannuronic acid; no signal assigned to guluronic acid was observed (Chandía et al., 2001; Leal et al., 2008). These results indicate that the alginate from *Durvillaea* sp. is highly enriched in mannuronic acid. It can be mentioned that this is the first time that dried and milled samples of brown seaweeds are analyzed by IR spectroscopy; this methodology will be useful for the field study of alginophytes.

Seaweed samples were treated with *n*-hexane, followed by ethanol-formaldehyde mixture in order to eliminate fats and phenols, and extracted with 3% sodium carbonate aqueous solution. Alkaline extraction, followed by purification with HCl and $CaCl_2$ afforded white solids in 5–30% yield. Table 1 shows that the seaweeds collected in Antarctica contain the lowest yield of alkaline extract. M/G ratio was determined by total acid hydrolysis and HPLC analysis of the hydrolysis products, results depicted on Table 1 indicate that the composition of the alkaline extracts varies substantially. *Desmarestia menziessi* extract shows to contain similar proportions of both uronic acids, whereas the polysaccharide from *Durvillaea* sp. is highly enriched in mannuronic acid as expected from the IR spectra analysis of the seaweed.

It is noteworthy that the FT-IR spectra of the alkaline extracts (Fig. 2) are very similar to those of the corresponding seaweeds (Fig. 1). The FT-IR spectra of the alkaline extract from *Durvillaea* sp. (Fig. 2C) show in the anomeric or finger print region signals at 953.6 (δ C—C—H), 885 (δ C—O—C of β glycosidic linkage) and 824 (δ C—O—C, δ C—C—C, ring δ C—C—O) cm^{-1} which form the characteristic pattern of β-mannuronic acid residues (Cárdenas-Jirón et al., 2011). In the case of *Desmarestia ligulata*, the FT-IR spectra of both seaweed and alkaline extract (Fig. 2D) show besides the band at 823.3 cm^{-1} assigned to mannuronic acid, a second band in the anomeric region (~780 cm^{-1}) assigned to deformations vibrations of α-guluronic acid residues (Leal et al., 2008).

The alkaline extracts were partially acid hydrolyzed affording three fractions; according to Haug et al. (1974) fraction (F1) obtained in the first hydrolysis step was enriched in heteropolymeric (MG) blocks, the soluble fraction at pH 2.85 (F2) was mainly composed of polymannuronic acid, and the insoluble fraction at pH 2.85 (F3) was enriched in polyguluronic acid. The alkaline extracts from *A. mirabilis* and *Desmarestia menziessi* showed to contain similar proportions of the three fractions, whereas *Durvillaea* sp. was enriched in polymannuronic acid, and *Desmarestia ligulata* is mainly composed of polyguluronic acid (Table 1). It can be mentioned that the alkaline extract from *Desmarestia ligulata* collected in Magellan region was studied in this laboratory (Leal et al., 2008), it presented higher M/G ratio (0.77) and contained lower proportion of fraction insoluble at pH 2.85 (56.4%) in relation to the sample collected in Bío Bío region. In order to have fully characterized pure homopolymannuronic acid and homopolyguluronic acid blocks for chiroptical studies, fractions obtained by partial acid hydrolysis of alkaline extracts were analyzed by FT-IR and NMR. As an illustration the normal and second derivative FT-IR spectra of fraction F1 of the alkaline extract from *Desmarestia menziessi*, fraction F2 from *Durvillaea* sp., and fraction F3 from *Desmarestia ligulata* are shown in Fig. 3. Cárdenas-Jirón et al. (2011) calculated IR absorptions of the alternating tetrasaccharide of 1 → 4 linked β-D-mannopyranuronate and α-L-gulopyranuronate (MG) residues, values were very similar to those found in the FT-IR spectrum of F1 fraction of the alkaline extract from the hybrid *Lessonia-Macrocystis* seaweed and allowed to confirm the presence of a heteropolymeric fraction (Leal et al., 2012). Absorptions at 1028.0, 991.8, 964.2, 888.3, and 824.4 cm^{-1} in Fig. 3A were assigned to MG residues, according to published data and sustained the heteropolymeric nature of fraction F1. On the other hand, FT-IR spectra of fraction F2 (Fig. 3B) present signals in the finger print region at 955.5, 879.5 and 822.7 cm^{-1} forming the characteristic triplet assigned to homopolymannuronic block fraction (Chandía et al., 2001, 2004; Leal et al., 2008; Matsuhiro, Leal, & Mansilla, 2012); furthermore, values are in accordance with those calculated for MM tetrasaccharide (Cárdenas-Jirón et al., 2011). It is noteworthy that the FT-IR spectra of fraction F3 show the presence of two bands in the finger print region at 810.9 and 777.7 cm^{-1} ascribed to deformations vibrations of α-guluronic acid (Chandía et al., 2001; Leal et al., 2008), and the absence of a band around 820 cm^{-1} assigned to vibrations of mannuronic acid residues; altogether these results indicate the presence of a homopolyguluronic acid fraction. At this point, it should be emphasized that generally in the literature, the band at 808 cm^{-1} has been incorrectly attributed to the presence of mannuronic acid residues in alginates following the proposed assignment by Mackie (1971).

Structures of heteropolymeric and homopolymeric fractions deduced by FT-IR spectroscopy were confirmed by NMR spectroscopy. Chemical shifts of the ^1H NMR spectrum of F2 obtained by partial hydrolysis of the alkaline extract from *Durvillaea* sp. were assigned with the aid of COSY experiment (figures not shown), and are presented on Table 2. Values are in agreement with those previously published for fractions 2 of the alkaline extracts from

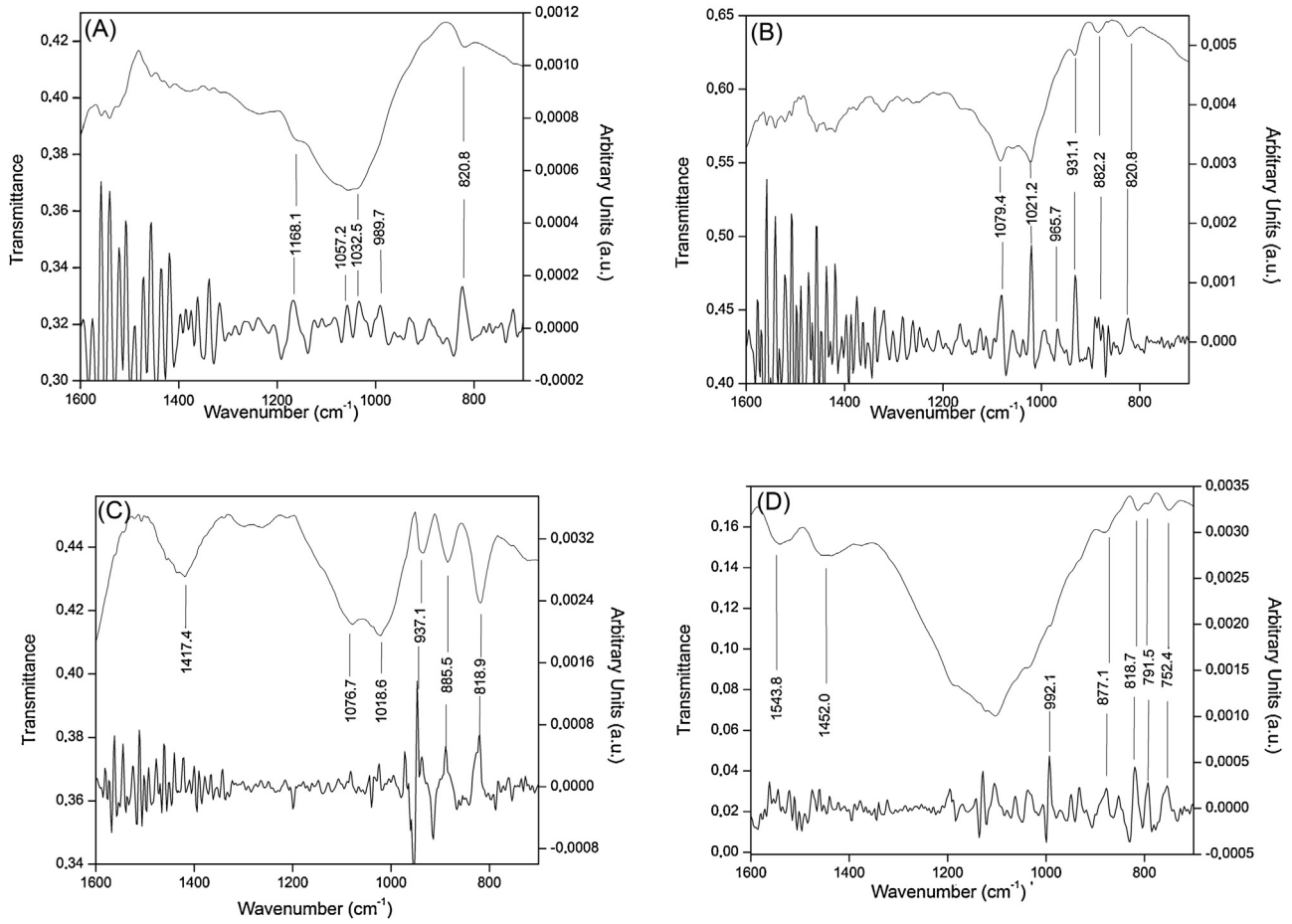

Fig. 1. FT-IR and second derivative spectra of milled and dried samples of brown seaweeds: (A) *Ascoseira mirabilis*, (B) *Desmarestia menziesii*, (C) *Durvillaea* sp., and (D) *Desmarestia ligulata*.

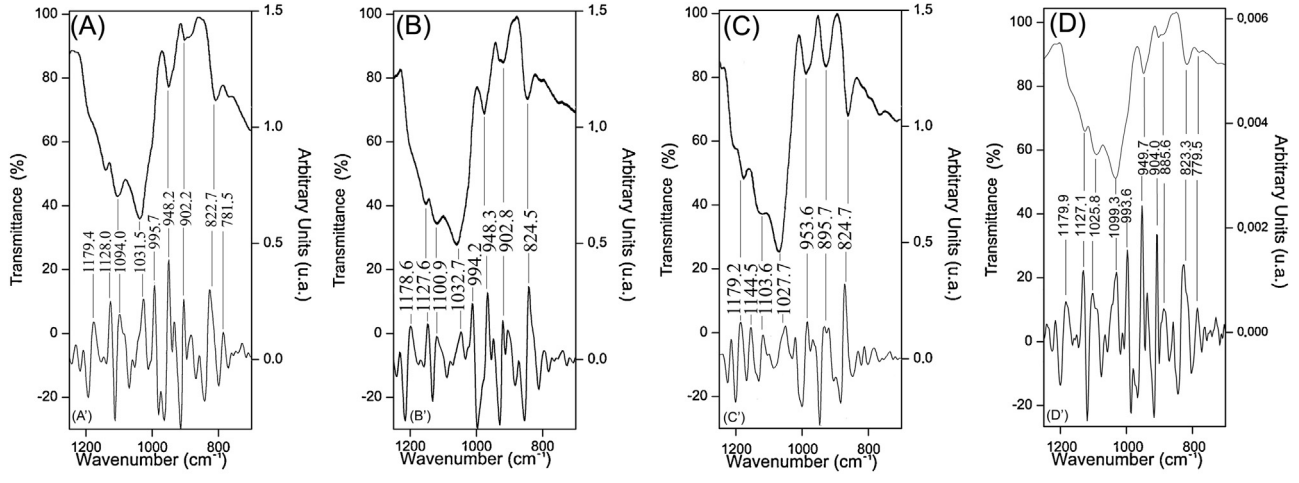

Fig. 2. Normal FT-IR and second derivative spectra of alkaline extract from: (A) *Ascoseira mirabilis*, (B) *Desmarestia menziessi*, (C) *Durvillaea* sp., and (D) *Desmarestia ligulata*.

three Chilean seaweeds (Leal et al., 2008). The ^{13}C NMR spectrum of this fraction presented six signals (figure not shown) which were assigned to homopolymannuronic acid with the aid of 2D NMR spectra and literature data (Grasdalen 1983; Grasdalen et al., 1981; Leal et al., 2008; Matsuhiro, Torres, & Guerrero, 2007; Panikkar & Brasch, 1997). Fig. 4A depicts the $^{13}C/^{1}H$ HMBC NMR spectrum of F2 fraction whereas C4′/H1 and C1/H4′ correlations are indicative of the presence of 1 → 4 linkage in homopolymannuronic acid block. ^{13}C NMR chemical shift values (Table 2) are shifted ~2.00 ppm downfield in relation to those previously published; this discrepancy can be explained considering that methanol was previously used as internal reference (Leal et al., 2008). Fig. 4B displays $^{13}C/^{1}H$ HSQC NMR spectrum of F3 fraction obtained by partial hydrolysis of sodium alginate from *Desmarestia ligulata*, it shows in the ordinate axis five signals (carboxylate carbon signal appeared at 178.34 ppm) which well correlate with ^{1}H signals in the

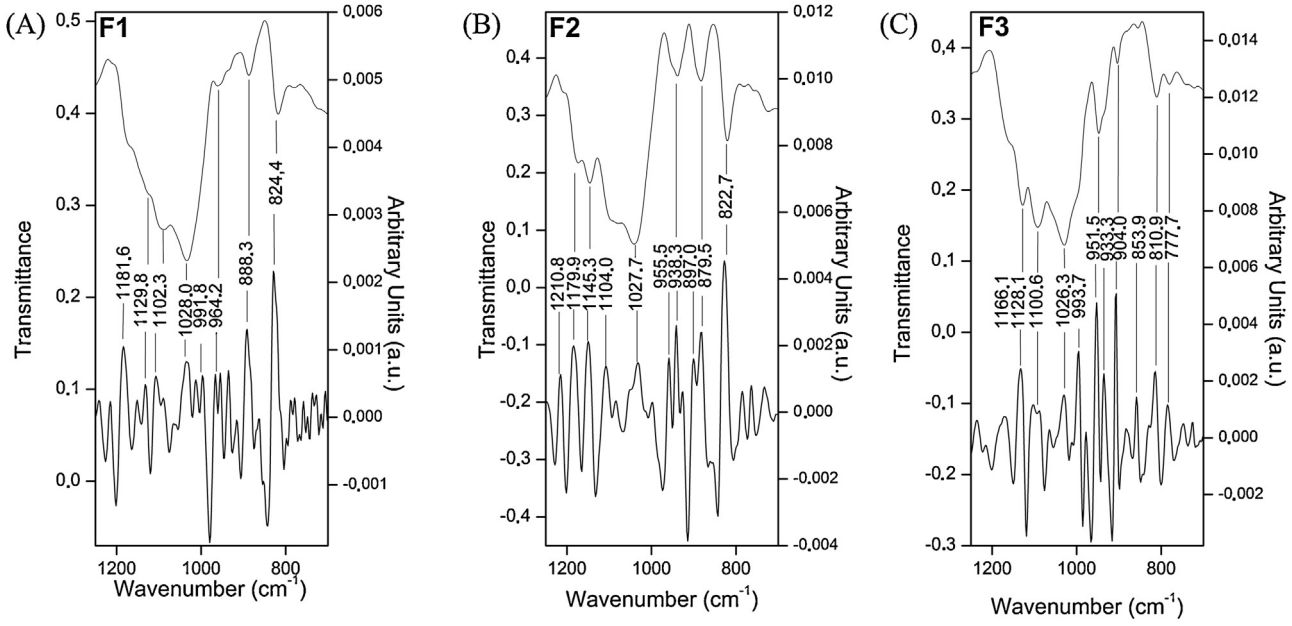

Fig. 3. Normal FT-IR and second derivative spectra: (A) F1 fraction obtained by partial acid hydrolysis of the alkaline extract from *Desmarestia menziesii*, (B) F2 fraction obtained by partial acid hydrolysis of the alkaline extract from *Durvillaea* sp., and (C) F3 fraction obtained by partial acid hydrolysis of the alkaline extract from *Desmarestia ligulata*.

Table 2
Chemical shifts assignments in the ^{1}H and ^{13}C NMR spectra of sodium salts of homopolymannuronic acid (MMM), homopolyguluronic acid (GGG), and its constituent monomers.[a]

	δ (ppm)					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	C6
MMM	4.65/102.93	4.04/72.72	3.77/74.05	3.88/80.69	3.74/78.70	178.08
GGG	5.04/103.69	3.92/67.78	4.00/71.83	4.11/82.95	4.46/69.94	178.34
α-Mannuronate	5.23/96.51	3.91/73.32	3.87/72.98	3.85/71.88	4.11/75.77	179.96
β-Mannuronate	4.93/96.78	3.94/74.13	3.68/75.51	3.72/71.44	3.81/79.28	179.22
β-Guluronate	4.93/96.41	3.68/71.52	4.11/73.96	4.62/70.28	4.38/77.40	179.97

[a] Spectra were registered in D_2O solution, using the sodium salt of 3-(trimethylsilyl)-1-propionic-2,2,3,3-d_4 acid (TMSP-d_4) as internal reference, at 70 °C for homopolymeric blocks, and at 25 °C for the monomers.

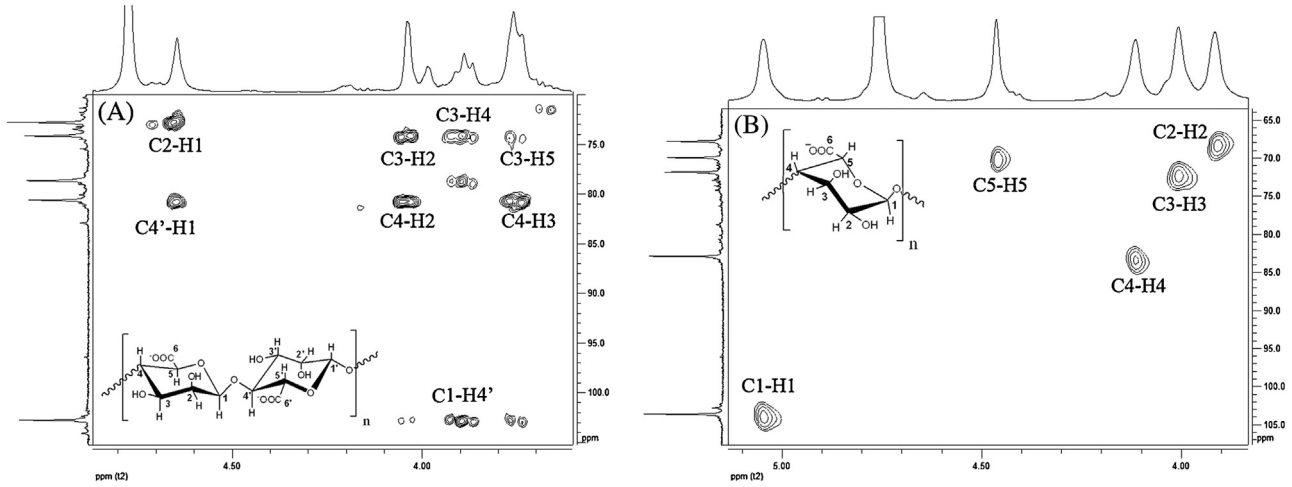

Fig. 4. (A) $^{13}C/^{1}H$ HMBC NMR spectrum of F2 fraction obtained by partial hydrolysis of sodium alginate from *Durvillaea* sp., (B) $^{13}C/^{1}H$ HSQC NMR spectrum of F3 fraction obtained by partial hydrolysis of sodium alginate from *Desmarestia ligulata*. Spectra were recorded in D_2O at 70 °C.

carbohydrate region. Table 2 presents the ^{1}H and ^{13}C NMR chemical shifts of fraction 3, assignments are in very good agreement with those previously published for the sodium salt of homopolyguluronic acid from *Macrocystis pyrifera* (Matsuhiro et al., 2015).

The obtained homopolymannuronate and homopolyguluronate fractions were fully hydrolyzed with formic acid; HPLC analysis indicated the sole presence of mannuronic acid and guluronic acid, respectively. Fig. 5 presents the $^{13}C/^{1}H$ HSQC NMR spectra of the sodium salts of mannuronic acid and guluronic acids. Correlations

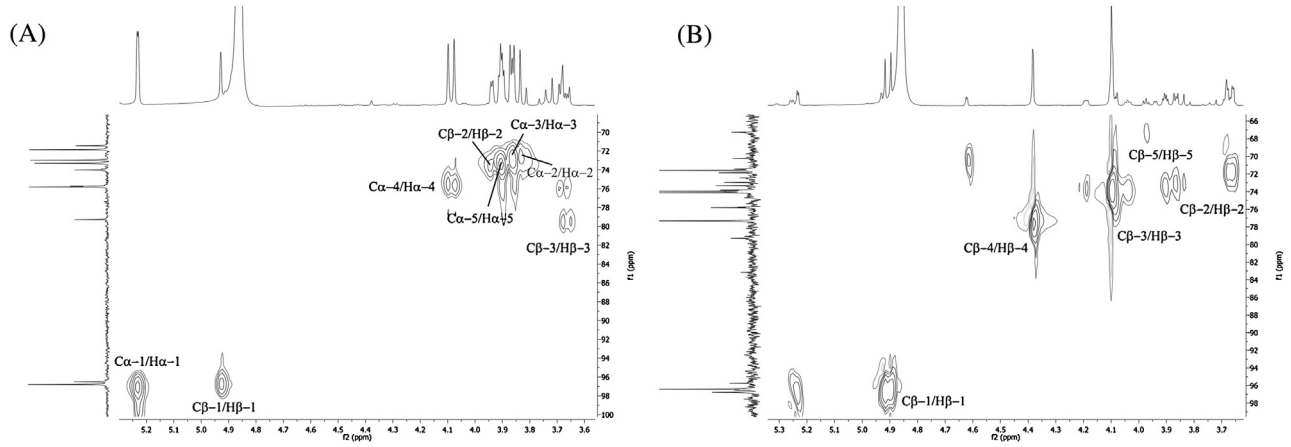

Fig. 5. $^{13}C/^{1}H$ HSQC NMR spectra: (A) sodium mannuronate, and (B) sodium guluronate obtained by total acid hydrolysis of homopolymannuronate fraction from *Durvillaea* sp., and homopolyguluronate fraction from *Desmarestia ligulata*, respectively. Spectra were recorded in D_2O at 25 °C.

shown in Fig. 5A indicate equilibrium between α and β sodium mannopyranuronate anomers. From ^{1}H NMR spectrum (figure not shown) α/β ratio of 1.7 was found; Heyraud, Gey, Leonard, Rochas, and Girond (1996) in a NMR spectroscopy study of oligomannuronates obtained α/β ratio of 2.2 for the reducing end of a trimer. 2D NMR spectra analysis allowed the assignments of the signals of both anomers (Table 2). Agrawal (1992) reported the ^{1}H and ^{13}C NMR chemical shifts for the anomers of the sodium salt of mannuronic acid from spectra taken in D_2O using acetone as internal reference; ^{1}H NMR chemicals shifts presented on Table 2 are in good agreement with those reported by Agrawal, while ^{13}C chemicals shift are shifted ~2.00 ppm downfield.

On the other hand, the $^{13}C/^{1}H$ HSQC NMR spectrum of sodium gulopyranuronate (Fig. 5B) shows a large predominance of the β anomer; this prevalence has been previously observed in di- and trisaccharides of guluronic acid. For example, Brown and Preston (1991) studied by ^{1}H NMR the unsaturated (at the non-reducing end) of trisaccharide of guluronic acid obtained by enzymatic hydrolysis of sodium alginate, they assigned the signal at 4.89 ppm with 0.80 integration value to H-1 to the β-reducing end. Similarly, Heyraud et al. (1996) found the major presence of β anomer at the reducing end of saturated and unsaturated trimers of gulopyranuronate by NMR spectroscopy. Liu, Jiang, Liao, and Guan (2002) prepared unsaturated di- and trisaccharides by enzymatic hydrolysis of homopolyguluronate, they reported ^{1}H chemicals shifts of H-1 (4.85, and 4.87 ppm, respectively). Furthermore, Grasdalen, Mo, Bjørgum and Siddiqui (1990) investigated the mutarotation of the sodium salt of α-L-guluronate by NMR in D_2O; the authors reported a large predominance of the β-anomer at equilibrium. Herein, the full assignment of the ^{1}H and ^{13}C chemical shifts of the sodium salt of β-gulopyranuronate by $^{13}C/^{1}H$ HSQC and $^{1}H/^{1}H$ COSY (figure not shown) NMR spectra is presented (Table 2).

3.2. CD and ORD spectra of monomers and homopolymeric fractions

Both monomers and homopolymeric fractions were analyzed as sodium salts by CD and ORD in distilled water. Fig. 6A depicts the CD spectrum of sodium mannopyranuronate in the 250–190 nm region, it displays negative first and positive second Cotton effects at 214.8 nm and 199.2 nm, respectively. The spectrum corresponds, as established by NMR, to the equilibrium mixture of α- and β-mannopyranuronate, with predominance of the α-anomer. The CD spectrum shows marked similarity in sign and shape to that published by Morris, Rees, Sanderson, and Thom (1975) for the sodium salt of methyl β-D-mannopyranuronoside in $^{4}C_1$ conformation. According to these authors, the stereochemistry of carbons C-4 and C-5 close to the carboxylate group played an overriding role on the energies of electronic transitions whereas, the configuration of distant carbons had little influence. They found that D-uronic acid presented positive $n \rightarrow \pi^*$ transition band around 200 nm, and a second negative band at longer wavelength when the OH on C-4 was in equatorial position. Fig. 6B displays the CD spectrum of the sodium gulopyranuronate, it presents a single negative band at 206.6 nm, and is similar to that of methyl α-L-guluronoside published by Morris et al. (1975), that showed a negative band around 210 nm. According to the literature, methyl glycosides of pyranuronic acids with an axial hydroxyl group on C-4, such as galactopyranuronic, gulopyranuronic, and idopyranuronic acids, presented a single band around 210 nm, assigned to the normal $n \rightarrow \pi^*$ transition of the carboxyl group (Closson & Haug, 1964; Morris et al., 1975; Cziner, Stevens, Morris, & Rees, 1986). In this case, also it seems that the configuration of the anomeric carbon has little effect on the electronic transitions of carboxylate group.

Early ORD studies of a series of aldopyranoses and their methyl glycopyranosides by Listowsky, Avigad, & Englard (1965) showed that these compounds presented plain ORD curves between 600 and 185 nm, no Cotton effect appeared above 200 nm, indicating that the small proportions of aldehyde form did not contribute to the optical rotatory dispersion; the authors established the direction of rotatory contribution of each chiral carbon in pyranose ring. They informed that β-glycopyranosides of D-sugars presented more levorotatory curves than α-anomers and predicted a negative rotational contribution of the equatorial substituted anomeric carbon. Later, the same group analyzed the ORD properties of uronic acid and acetamido sugars by comparison with ORD curves of methyl glycopyranosides. They informed that α-methyl glycosides of D-glucuronic, D-mannuronic acid, and D-galacturonic acid showed positive Cotton effect around 205 nm, whereas propyl glycoside of β-D-glucuronic acid presented a negative Cotton effect (Listowsky, Avigad, & Englard, 1968). Herein, ORD spectra in the 600–200 nm region of the sodium salts of mannuronic acid (Fig. 6A′) and guluronic acid (Fig. 6B′), obtained by total hydrolysis of the corresponding homopolymeric block, are shown. To the best of our knowledge, the ORD spectra of these compounds have not been previously described. The sodium salts of α- and β-mannopyranuronates present plain negative curve from 600 to 230 nm, a negative Cotton effect at 223 nm due to the carboxylate chromophore, and a sharp change in rotation toward the positive direction. The presence of a negative dispersion curve may be explained by the negative contribution of axial hydroxyl group on

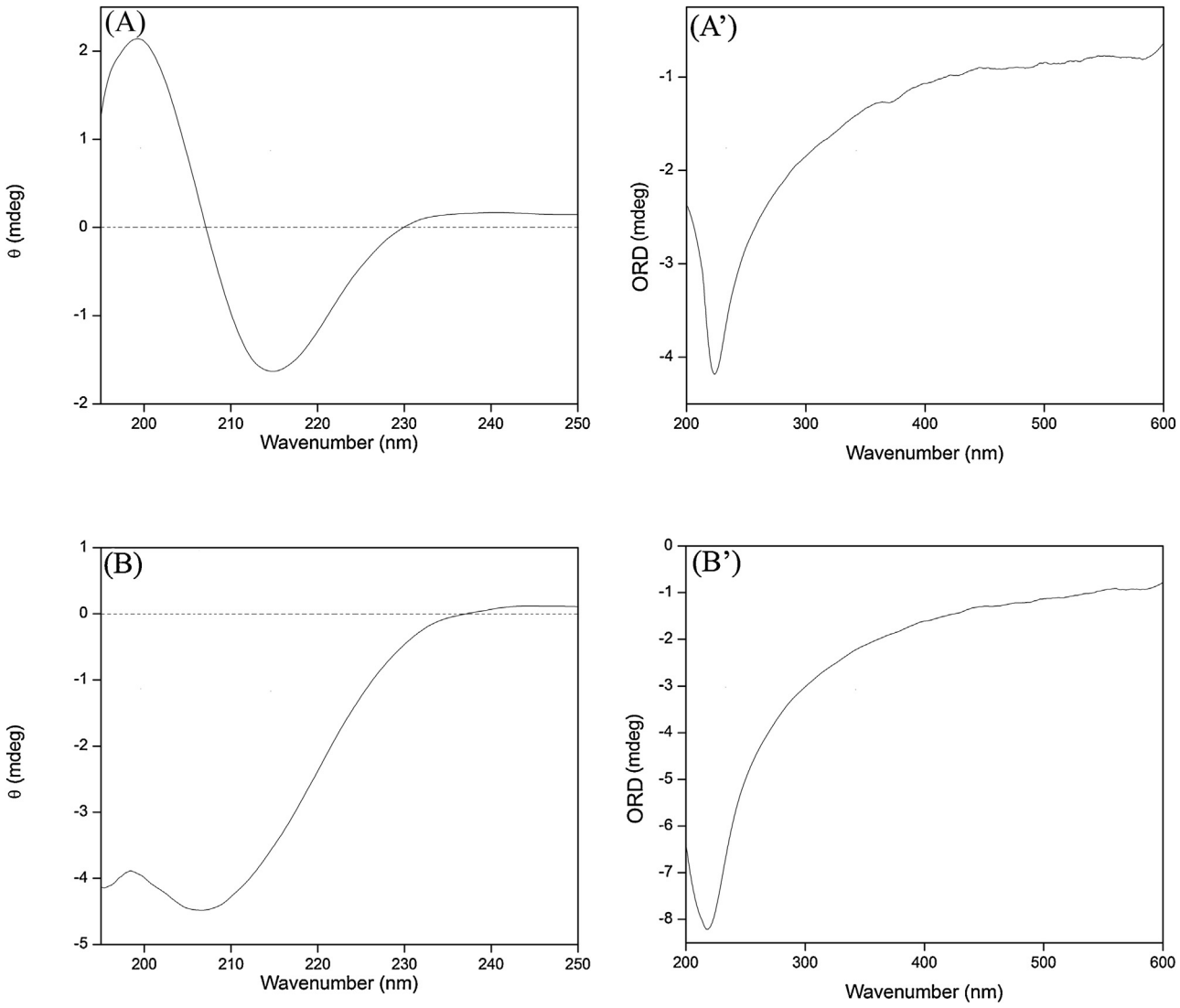

Fig. 6. CD (A and B) and ORD (A′ and B′) spectra of sodium salts of mannuronic acid and guluronic acid, respectively. Uronic acid were obtained by total acid hydrolysis of the corresponding homopolymeric fractions.

C-2 of mannopyranuronate in $^4C_1$ conformation and the low contribution of the mixture of α-β anomeric hydroxyl groups (Listowsky et al., 1965). Pace, Tanford, and Davidson (1964) found that the negative ORD curve of L-fucose at equilibrium is almost the mirror image of the positive curve of D-galactose; for the former, the influence of negative contributions of axial hydroxyl group on C-4 and of equatorial hydroxyl group on C-2 in $^1C_4$ conformation were important contributions to the sign of the dispersion curve. L-Gulopyranuronate in $^1C_4$ conformation presents the same configuration on C-2 and C-4 as L-fucose, and an axial hydroxyl group on C-3; however, the magnitude of its positive contribution may be smaller than those of C-2 and C-4 (Listowsky et al., 1965). Fig. 6B′ depicts a plain negative curve from 600 to 220 nm, a negative Cotton effect at 217.9 nm and a sharp change in rotation probably due to a positive Cotton effect in the near UV.

Fig. 7 depicts the CD and ORD spectra of sodium salt of homopolymannuronic blocks obtained by partial acid hydrolysis of the alkaline extracts from *Desmarestia ligulata, Durvillaea* sp. and *Macrocystis pyrifera*. It is noteworthy, in the case of the F2 block fractions from the first two seaweeds, that the CD and ORD spectra (Fig. 7A and B) are very similar in sign and shape, but more intense than those of the monomer. The CD spectrum presents a negative peak around 214 nm and a positive peak at 199 nm, and is also very similar to that published by Morris et al. (1975) for polymannuronate fraction from commercial alginate. In the case of CD spectrum from homopolymannuronate fraction from *Macrocystis pyrifera* (Fig. 7C) peak/trough depth indicated that the fraction is contaminated by polyguluronate, as previously shown by NMR spectroscopy (Matsuhiro et al., 2015). As expected, the ORD curves of sodium salts of homopolymannuronic acid block fractions present plain negative curves from 600 nm down to ~230 nm, similar in shape to the corresponding monomer; the Cotton effect is shown at 222–223 nm and a sharp change toward positive values. On the other hand, CD and ORD spectra of homopolyguluronate (Fig. 8), obtained by partial acid hydrolysis of the alkaline extract from *Ascoseira mirabilis, Desmarestia menziessi*, and *Macrocystis pyrifera* show a marked similarity with the CD and ORD spectra of the monomer in sign and shape, but more intense in size; CD spectra are also similar to that published by Morris et al. (1975) for polyguluronate of commercial alginate. In the case of homopolyguluronic also, the ORD curves are plain and negative from 600 to ~220 nm, the negative Cotton effect appears at 217–218 nm, followed by a sharp change toward positive values. Tables 3 and 4 present the [θ] values obtained from CD and ORD spectra for the

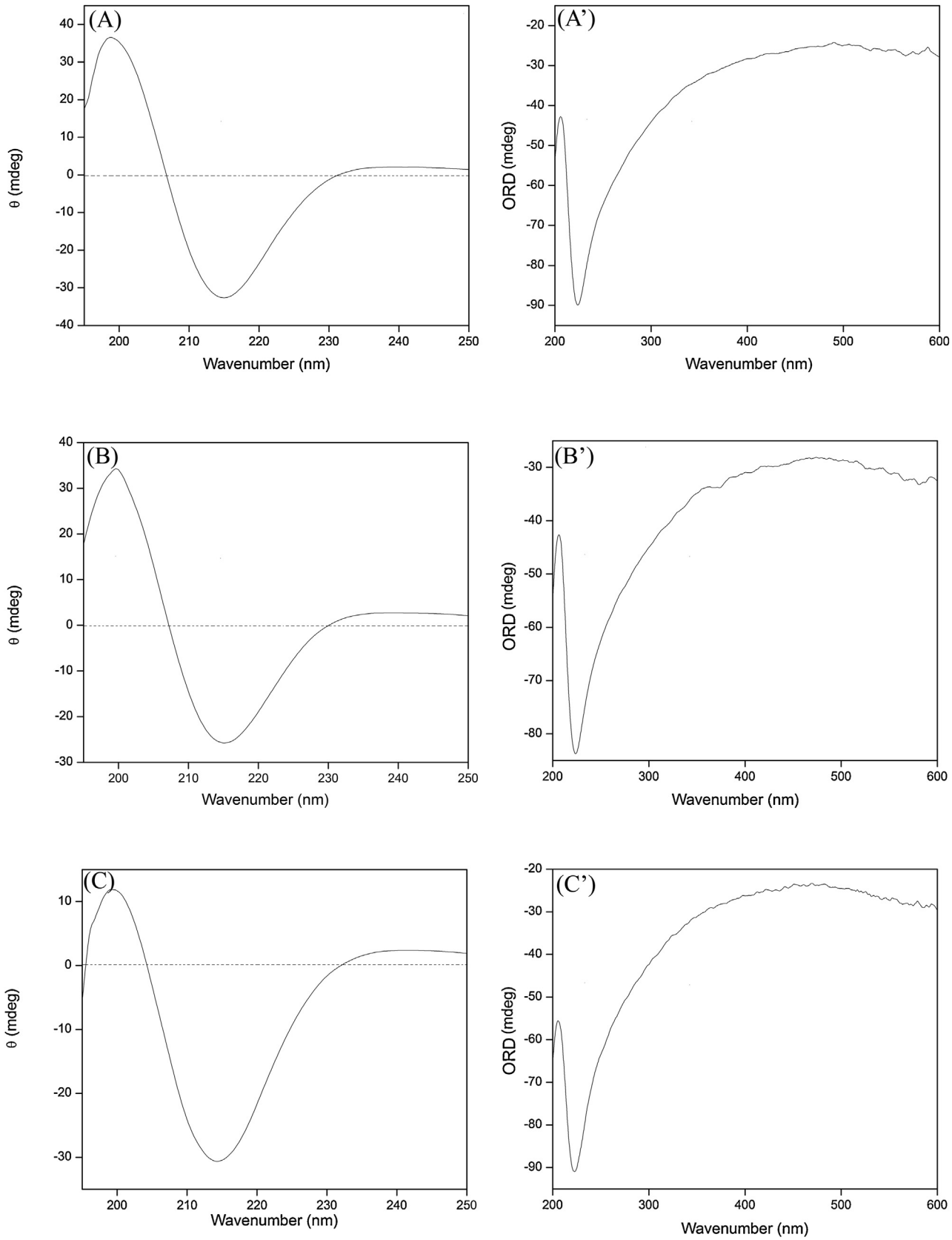

Fig. 7. (A), (B), and (C) CD spectra; (A′), (B′), and (C′) ORD spectra of homopolymannuronate (fractions F2) obtained by partial acid hydrolysis of the alkaline extract from *Desmarestia ligulata*, *Durvillaea* sp., and *Macrocystis pyrifera*, respectively.

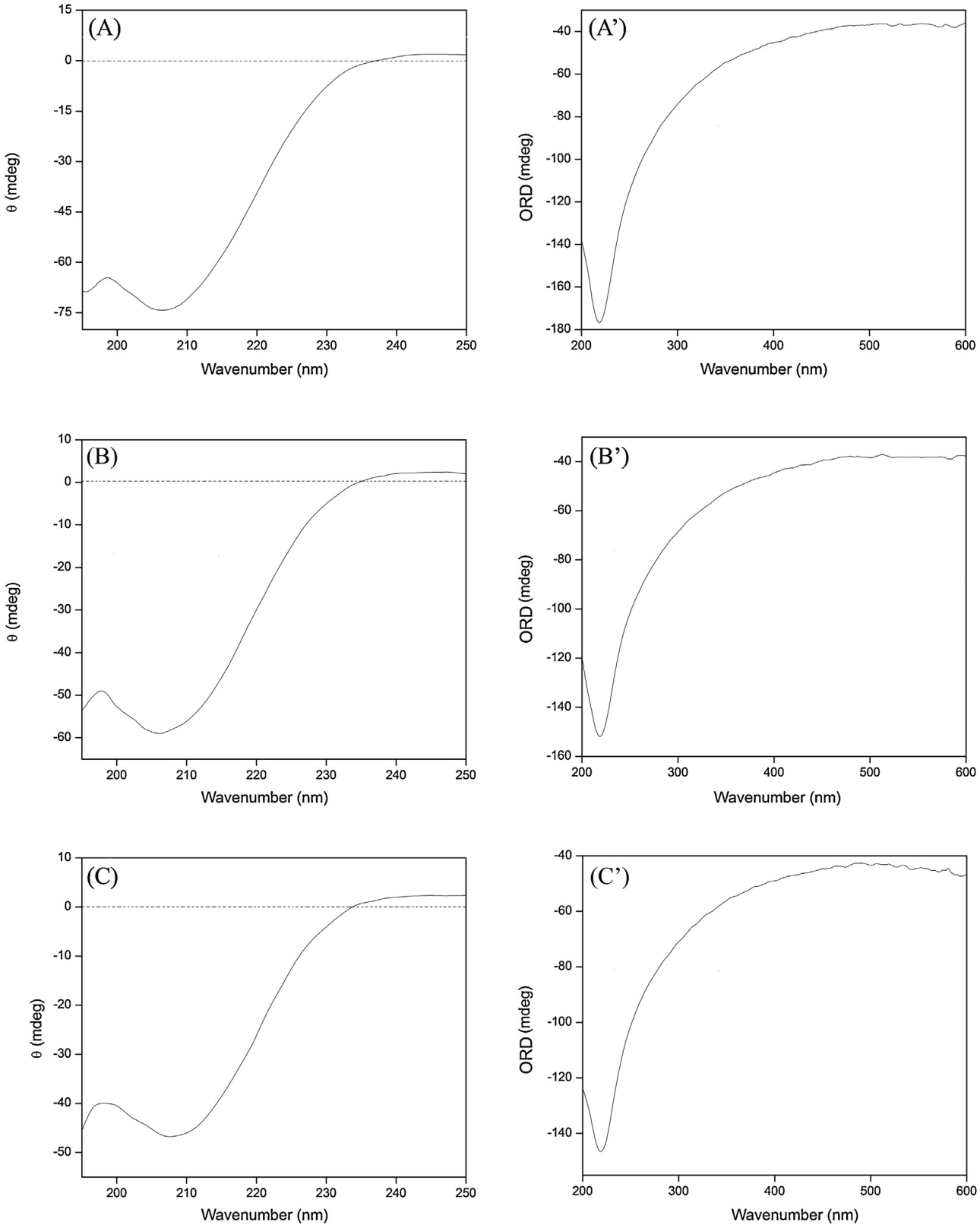

Fig. 8. (A) CD, and (A′) ORD spectra of F3 fraction obtained by partial hydrolysis of alkaline extract from *Desmarestia ligulata*; (B) CD and (B′) ORD spectra of fraction F3 obtained by partial hydrolysis of alkaline extract from *Desmarestia menziessi*; (C) CD and (C′) ORD spectra of fraction F3 obtained by partial acid hydrolysis of alkaline extract from *Macrocystis pyrifera.*

Table 3
Molar ellipticity [θ] values for sodium salts of mannuronic (ManA) and guluronic (GulA) acids obtained by total hydrolysis of homopolymannuronic acid (fraction F2) from *Durvillaea* sp. and homopolyguluronic acid (fraction F3) from *Desmarestia ligulata*.

	Circular dichroism		Optical rotatory dispersion	
Monosaccharide[a]	Wavenumber (nm)	Molar ellipticity [θ] (deg cm^2/dmol)	Wavenumber (nm)	Molar ellipticity [θ] (deg cm^2/dmol)
ManA-Na	199.2 214.8	+461.9 −351.5	223.7	−894.3
GulA-Na	206.6	−871.3	217.9	−1774.8

[a] ManA-Na: sodium salt of mannuronic acid, GulA-Na: sodium salt of guluronic acid.

Table 4
Molar ellipticity [θ] values for sodium salts of homopolymannuronic acid (fraction F2 soluble at pH 2.85) and homopolyguluronic acid (fraction F3 insoluble at pH 2.85) obtained by partial hydrolysis of alkaline extracts from brown seaweeds.

		Circular dichroism		Optical rotatory dispersion	
Alkaline extract[a]	Block	Wavenumber (nm)	Molar ellipticity [θ] (deg cm^2/dmol)	Wavenumber (nm)	Molar ellipticity [θ] (deg cm^2/dmol)
1	F2	–	–	–	–
	F3	206.5	−1455.0	217.0	−3633.6
2	F2	–	–	–	–
	F3	207.1	−1311.1	218.2	−3034.3
3	F2	199.1 214.9	+851.9 −642.9	223.2	−1902.6
	F3	–	–	–	–
4	F2	199.5 214.8	+916.9 −806.2	223.2	−2057.9
	F3	206.8	−1837.2	218.0	−4237.0
5	F2	199.4 214.6	+300.1 −753.1	222.8	−2084.3
	F3	208.4	−1153.9	218.4	−3492.7

[a] From 1: *Ascoseira mirabilis*, 2: *Desmarestia menziessi*, 3: *Durvillaea* sp., 4: *Desmarestia ligulata*, 5: *Macrocystis pyrifera*.

Table 5
Specific optical rotation at sodium D line (598 nm) of alkaline extracts, block fractions and sodium salts of mannuronic acid (ManA-Na) and guluronic acid (GulA-Na) in H_2O.

Seaweed sample[a]	Optical rotation $[\alpha]_D$ (°)					
	Alkaline extract		Fraction F2	Fraction F3	ManA-Na	GulA-Na
1	−107.41 ± 7.21	−109.20 ± 5.27	−110.36 ± 8.22	−112.38 ± 1.28		−18.97 ± 2.90
2		−105.05 ± 3.37	−107.40 ± 7.52	−113.30 ± 1.77		
3		−108.24 ± 5.59	−91.00 ± 2.00	−98.76 ± 7.89		
4	−98.83 ± 3.01	−104.45 ± 4.12	−92.76 ± 1.79	−112.03 ± 2.69	−10.36 ± 2.31	−16.36 ± 2.39
5		−105.36 ± 8.12	−97.55 ± 3.33	−111.84 ± 2.13	−9.55 ± 1.59	−17.53 ± 4.24

[a] 1: *Ascoseira mirabilis*, 2: *Desmarestia menziessi*, 3: *Durvillaea* sp., 4: *Desmarestia ligulata*, 5: *Macrocystis pyrifera*.

sodium salts of monomeric and homopolymeric acids, respectively. A significant increase in intensity of the signals for the homopolymeric fraction is shown, but no relation with molecular weight (Table 1) was found. We previously reported the MW of fraction F2 (32,000) and fraction F3 (31,500) obtained by partial hydrolysis of the alkaline extract from *Macrocystis pyrifera* (Martínez-Gómez, Encinas, Matsuhiro, & Pavez, 2015), although these two fractions presented the highest MW, the effect of chain length on CD values is not noticeable. Altogether, results obtained by CD and ORD spectra indicate that mannuronic acid obtained by total hydrolysis of homopolymannuronate (F2) fractions presents D configuration, and guluronic acid obtained by total hydrolysis of homopolyguluronate (F3) fractions presents L configuration.

3.3. Specific optical rotation

Table 5 presents the specific optical rotation at the sodium D line of sodium salts of homopolymeric fractions and the constituent uronic acids. Alkaline extracts were viscous and only in two cases, optical rotation could be registered. Optical activity at sodium D line is highly negative Specific optical rotation of monomers were taken immediately after dissolution, samples were in equilibrium since no change was observed in a 24 h period. One of the first reports on the optical activity of mannuronic acid was published by Schoeffel and Link as early as 1933 (Schoeffel & Link, 1933). They found values of $[\alpha]_D = -14.35°$ for anhydrous β-D-mannuronic acid, and of $[\alpha]_D = -6.05°$ for α-D-mannuronic acid.$1/2H_2O$; in the case of their lactones values of $[\alpha]_D = +87.8–+89.3°$ were informed. On the other hand, Haug and Larsen (1970) informed $[\alpha]_D = +37°$ in water for L-guluronic acid from algae; this positive value is a bit far from that found in this work and probably it corresponded to a partial lactonization of the sample. Values of optical rotations for homopolymeric fractions are much higher than the values found for the monomers; these high negative values may be considered as the increase in rotation values although a clear additive effect of chain length is not observed. High negative values were also informed for fucoidans, polysaccharides mainly composed of sulfated L-fucopyranosil residues isolated from brown seaweeds (Bilan et al., 2004; Mulloy, Ribeiro, Alves, Vieiras, & Mourão, 1994). According to the group of Usov, the high $[\alpha]_D = \sim -134°$ of fucoidans can be used to establish that the constituent monosaccharide possess L configuration.

On the basis of CD and ORD spectra determined in this work, measurements of optical activity at the sodium line of homopolymannuronates and homopolyguluronate fractions permit the assignment of D configuration for mannuronic acid and L

configuration for guluronic acid. These facts are important because total hydrolysis of homopolymeric fractions, isolation of the constituent monomers, and the determination of the specific rotation are obviated. It is well known that the monomers are unstable, especially guluronic acid.

Altogether, the results obtained through chiroptical techniques in this work corroborate the proposed hypothesis establishing the application of the optical activity at sodium D line as a reliable method for the determination of the absolute configuration of mannuronic and guluronic acids in homopolymeric fractions. Within chiroptical methods, polarimetry is a simple method using equipment available in any chemistry laboratory.

4. Conclusion

FT-IR spectra of dried brown seaweeds give preliminary information about the composition of the alginic acid.

CD and ORD chiroptical methods give information about the nature, chirality and composition of homopolymeric fractions. However, it was found in this work that polarimetry is a simple and accessible method for optical activity determination.

High negative values of specific optical rotation at sodium D line of the sodium salts of homopolymannuronic and homopolyguluronic acids block fractions allow establishing D absolute configuration for mannuronic acid and L absolute configuration for guluronic acid.

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