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Research Paper

Minor polysaccharidic constituents from the red seaweed *Hypnea musciformis*. Appearance of a novel branched uronic acid

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ABSTRACT

Two polysaccharide fractions isolated from *Hypnea musciformis* after room temperature- and hot water extraction, soluble after KCl precipitation of the more abundant carrageenans, were subfractionated by ion-exchange chromatography eluting with increasing concentrations of NaCl. The lowest NaCl concentration (0.2 M) eluted agarans. The DL-hybrids (or mixtures) eluted at intermediate concentrations of NaCl. The D/L-galactose ratio and the sulfate proportion increased with the NaCl concentration. Different types of substitution were present, mainly at C-3 with sulfate, Xyl and methylated Gal stubs, as well as low amounts of 3,6-AnGal. A novel constituent, identified as 3-*C*-carboxy-D-erythrose[1] in its β-furanosic form, was found linked to C-6 of β-Gal units. A search carried out in other species like *Iridaea undulosa* and *Kappaphycus alvarezii* also revealed the same constituent. Finally, the late-eluting fractions were mostly carrageenans, with a structure consistent with that of a κ/ι/ν-carrageenan hybrid.

1. Introduction

The known history of carrageenans started when Schmidt (1844) reported the isolation of the mucilage from the red seaweed *Chondrus crispus*. From that time up to the 1970s it was established that carrageenans have a structure based on a linear chain with a repeating pair of a 3-linked β-D-galactopyranosyl residue and a 4-linked α-D-galactopyranosyl residue. The last residue occurs frequently as a 3,6-anhydro-α-D-galactopyranosyl moiety. Carrageenans are substituted with large amounts of sulfate ester groups (Stortz & Cerezo, 2000; Stortz, 2005; Usov, 2011). They differ from agarans, found in other red seaweeds, that have the 4-linked residue belonging to the L-series. A landmark in carrageenan studies was the discovery that, in certain families, different sexual stages produce different carrageenans (Chen, McLachlan, Neish, & Shacklock, 1973; McCandless, Craigie, & Walter, 1973; Pickmere, Parsons, & Bailey, 1973). A new landmark was the finding in the early 1990s, of agarans or agaran/carrageenan hybrids in three carrageenophyte species (Craigie & Rivero-Carro, 1992). Those DL-hybrids, though commonly found within the Cryptonemiales and other non-carrageenophytic taxa, were found for the first time in carrageenan-producing seaweeds (Ciancia & Cerezo, 2010; Stortz, 2005; Usov, 2011). These hybrids were shortly afterwards found in gametophytes (Ciancia, Matulewicz, & Cerezo, 1993; Ciancia, Matulewicz, & Cerezo, 1997; Flores, Cerezo, & Stortz, 2002) and sporophytes (Stortz & Cerezo, 1993; Stortz, Cases, & Cerezo, 1997) of the carrageenophytes *Gigartina skottsbergii* and *Iridaea undulosa*, even within their fibrilar cell walls (Flores, Stortz, Rodríguez, & Cerezo, 1997; Flores, Stortz, & Cerezo, 2000). Some L-galactose was also found in the carrageenophytes *Rhodophyllis divaricata* and *Sarcodia montagneana* (Chopin, Kerin, & Mazerolle, 1999). The presence of agarans and hybrids was later confirmed in other carrageenophytic seaweeds like *Kappaphycus alvarezii* (Estevez, Ciancia, & Cerezo, 2000; Estevez, Ciancia, & Cerezo, 2004), *Gymnogongrus torulosus* (Estevez, Ciancia, & Cerezo, 2001; Estevez, Ciancia, & Cerezo, 2008), *Hypnea musciformis* (Cosenza, Navarro, Fissore, Rojas, & Stortz, 2014) and *Gymnogongrus tenuis* (Perez Recalde et al., 2016). It may be asserted that these agarans or hybrids appeared in every carrageenophyte where they were searched for, helped by the availability of new techniques that allow detection of small amounts of L-galactose and its derivatives (Cases, Cerezo & Stortz, 1995; Falshaw & Furneaux, 1995; Navarro & Stortz, 2003). At the same time, several agarophytes were found to produce carrageenans or DL-hybrids (Navarro & Stortz,

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[1] According to the IUPAC rules for carbohydrates (Mc Naught, 1996; rule 18.3), the name of the acyclic form should be 3-*C*-hydroxymethyl-L-threuronic acid, whereas for the cyclic form, the ring closure takes precedence over the carboxyl group and thus, 3-*C*-carboxy-D-erythrofuranose should be imposed. (2*R*,3*R*)-apiuronic acid (ApiA) is used as an alternative trivial name throughout the text.

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2003; Takano, Shiomoto, Kamei, Hara, & Hirase, 2003), although none of them were from the main agar-producing orders Gelidiales and Gracillariales (Stortz, 2005; Takano et al., 2003). The proportion of agaran structures within the carrageenophytes was usually low, and required KCl precipitation and/or alkaline treatment to concentrate the L-galactose-rich structures, although an important proportion of agaran diads was found in *G. torulosus* (Estevez et al., 2008; Usov, 2011).

Hypnea musciformis is a widely-distributed red seaweed known to produce large amounts of κ-carrageenan (Alves et al., 2012). In a previous work (Cosenza et al., 2014) we extracted the polysaccharides from *Hypnea musciformis* with room temperature- and hot water, and fractionated them with potassium chloride. All the fractions were characterized rheologically and the carrageenan fractions were characterized chemically. In order to acquire a better knowledge of the structure of agarans and DL-hybrids present in carrageenophytes, in the current work we subfractionated the 2 M KCl-soluble fractions (**R-2S** and **H-2S**), and to characterize chemically the structures of the isolated subfractions. The discovery of the presence of 3-*C*-carboxy-D-erythrofuranosyl stubs in significant amounts is an important outcome of this work.

2. Materials and methods

2.1. Material

Samples of *Hypnea musciformis* (Wulfen) Lamoroux Foslie were collected near Natal (Rio Grande do Norte), Brazil (5° 37′ 52″ S, 35° 13′ 03″ W). The seaweeds were sorted, air dried, cleaned manually and milled to a fine powder before extraction. All reagents were of analytical grade.

2.2. Extraction and fractionation

The extraction from the seaweed and the KCl fractionation procedures to give fractions **R-2S** and **H-2S** were described elsewhere (Cosenza et al., 2014). Those fractions were used as starting materials for further fractionation by ion exchange chromatography. For that purpose a mixture of 1.2 g of DEAE-Sephadex A-50 and 1 g of Sephadex G-100 (Stortz et al., 1997) were swollen together in 0.2 M NaCl for 3 h at 90 °C. A glass column of 45 × 2.5 cm was filled with the degassed gel and after thorough washing, solutions of the samples **R-2S** and **H-2S** containing 0.3–0.5 g of the fractions were laid on the top of the column. Elution was carried out with increasing concentrations of aqueous NaCl (up to 3 M). The fractions obtained from the column were followed by the phenol/sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), pooled, dialyzed (molecular weight cut-off 6000–8000) and freeze-dried.

2.3. General methods

Total carbohydrates were determined by the phenol-H_2SO_4 method (Dubois et al., 1956) using galactose as standard. The percentages of sulfate were measured by turbidimetry (Dodgson & Price, 1962) after hydrolysis with 1 M HCl, and also by ion chromatography, as the accuracy of the turbidimetric method decreases for samples with low sulfate content (≈ < 6% SO_3Na). Soluble proteins were determined by the procedure of Lowry, Rosebrough, Farr and Randall (1951).

The proportion of monosaccharides constituting the polysaccharides was determined using a reductive hydrolysis procedure (Jol, Neiss, Penninkhof, Rudolph, & De Ruiter, 1999; Stevenson & Furneaux, 1991). After acetylation (CF_3COOH:Ac_2O 1:1, 10 min, 50 °C), the alditol acetates were analyzed by gas-liquid chromatography (GLC) on an HP 5890A apparatus fitted with a capillary column SP 2330 (30 m x 0.25 mm i.d., thickness 0.20 μm), and equipped with a flame ionization detector operating at 240 °C. The injector and detector temperatures were 240 °C and the oven temperature started at 200 °C, rose 2 °C/min to 230 °C, and stayed at this temperature for 20 min. Nitrogen was used as the carrier gas at a head pressure of 15 psi. Aliquots were injected with a split ratio of 80:1. Cyclizable 6-sulfate was determined by 3,6-anhydrogalactose measurement (Navarro & Stortz, 2003) after alkaline treatment (NaOH 1 M for 5 h at 80 °C). The absolute configuration of the monosaccharides was determined after hydrolysis and reductive amination with (*S*)-1-amino-2-propanol and (*S*)-α-methylbenzylamine followed by acetylation (pyridine and Ac_2O) and GLC analysis (Cases et al., 1995). The configuration of the 3,6-anhydrogalactose was determined after mild hydrolysis and derivatization with (S)-α-methylbenzylamine, as described (Navarro & Stortz, 2003). Mild acid hydrolysis to remove furanosic stubs was carried out (0.02 M CF_3COOH, 2 h, 100 °C) as described elsewhere (Mariño, Marino, Lima, Baldoni, & de Lederkremer, 2005). When necessary, GLC-EI/MS analyses were carried out on a Shimadzu QP 5050 A GC/MS apparatus working at 70 eV using similar conditions to those described above, but using He as gas carrier and a split ratio of 60:1. The molar percentage of the different monosaccharides was calculated by considering that the FID responses are proportional to the molecular weight of the derivatives. Molecular weight determinations of the fractions were carried out by gel permeation chromatography, as depicted elsewhere (Cosenza et al., 2014).

2.4. Alkylation analysis

The triethylammonium salts of the fractions (5 mg, prepared by passage of the fractions through an Amberlite IR-120 column previously treated with $Et_3N{\cdot}HCl$ solution and rinsed with water until chloride ion was absent) were methylated as described by Ciucanu and Kerek (1984), using NaOH and CH_3I in dimethyl sulfoxide. The methylated products (isolated by dialysis and lyophilization) were hydrolyzed and derivatized as described above (Section 2.3), analyzed by GLC as described elsewhere (Cases, Stortz, & Cerezo, 1994; Stevenson & Furneaux, 1991), and characterized by GLC–MS. Desulfation-methylation was carried out as described by Navarro, Flores and Stortz (2007). Ethylation analysis, necessary for distinguishing the substitution patterns of the naturally methylated galactose units, was carried out as described by Cases et al. (1994).

2.5. Isolation and derivatization of (2R,3R)-apiuronic acid from Hypnea musciformis

The procedure reported by Spellman, McNeill, Darvill, Albersheim and Hendrick (1983) for the isolation of aceric acid was followed. Briefly, 100 mg of **H-2S** were hydrolyzed in 2 M CF_3COOH for 2 h at 120 °C. After removing the acid *in vacuo*, the hydrolyzate was redissolved in water, brought to pH = 9, and applied to a Bio-Rad AG1-X8 (acetate form) column (2 × 20 cm) pre-equilibrated with 0.2 M acetic acid. The column was eluted with 200 mL of 0.2 M acetic acid, 300 mL of 2 M acetic acid and 300 mL of 4 M acetic acid. The apiuronic acid was recovered from the last eluate with a purity of ≈ 90%.

For the generation of 2-*C*-hydroxymethyl-L-threonolactone, 10 mg of apiuronic acid dissolved in 1 M NH_3 were reduced overnight with 20 mg of $NaBH_4$. Borate was removed by the addition of Amberlite IR-120 resin (H^+ form) and successive washings with methanol (5 × 2 mL), and then the lactone was isolated by freeze-drying. Acetylation was carried out with Ac_2O/TFA 1:1 (v/v) for 30 min at 50 °C. GLC analysis of the acetylated lactone was performed on an Ultra-2 column (30 m × 0.32 mm i.d., thickness 0.17 μm) with a program temperature starting at 160 °C for 5 min, rising 1 °C/min to 220 °C, then 2 °C/min to 250 °C and then main-

taining this temperature for 20 min. N_2 was used as the carrier gas at a head pressure of 7 psi. Aliquots were injected with a split ratio of 70:1, and the injector and detector were kept at 250 °C. CI-MS of this product was carried out in positive mode with a Thermo DSQ II mass spectrometer with a quadrupole analyzer, using methane gas.

2.6. Determination of the configuration of (2R,3R)-apiuronic acid

For the determination of the configuration of C-2, reduction of the carboxylic group of apiuronic acid present in **H-2S** was carried out with the aid of the 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and $NaBH_4$, as described elsewhere (Taylor & Conrad, 1972). The reaction, carried out twice, gave ca. 50% of reduction. The presence of apiose was confirmed by GLC–MS after reductive hydrolysis and acetylation. The configuration of the apiose (and thus, that of C-2 of the apiuronic acid) was determined by reductive amination (Cases et al., 1995) using α-picoline borane as the reducing agent (Cosenza, Navarro, & Stortz, 2011). (*R*)-1-amino-2-propanol was used as the chiral amine, and acetylation was performed for 9 h with $NaOAc/Ac_2O$. A D-apiose-enriched extract from commercial parsley, obtained as described in Supplementary data, was used as the standard.

The configuration of C-3 was inferred from NOESY determinations, and also by using the method DP4+ (Grinblat, Zanardi, & Sarotti, 2015), modified slightly: the geometry of the most stable conformers of the lactone (Section 2.5, aldehyde-reduced apiuronic acid), as previously established by MM3, was determined by M06-2X/6–31 + G(d,p) optimizations with the polarizable continuum method (PCM) in water; the GIAO NMR single point calculations were carried out by using mPW1PW91/6–31 + G(d,p), and their Boltzmann-averaged populations from free energy calculations at the same level, with PCM in water. The last Boltzmann-averaged ensemble was used to calculate the theoretical $^3J_{H1,H2}$ and $^3J_{H1',H2}$ for each epimer, using the Altona equation as established in PC Model 9.1 (Serena Software). All the DFT calculations were carried out using Gaussian 09W.

2.7. Nuclear magnetic resonance

The NMR spectra were obtained on a Bruker Avance II 500 spectrometer at 500.13 (^{1}H) and 125.77 (^{13}C) MHz equipped with a 5-mm probe, at room temperature, using ca. 20 mg polysaccharide in 0.7 mL of D_2O. Acetone was added as internal standard (referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm in ^{13}C, 2.22 ppm in ^{1}H). The spectra of (2*R*,3*R*)-apiuronic acid and its reduced derivative were carried out with ca. 8 mg dissolved in 0.7 mL of D_2O. Methanol was used as internal standard, calibrating the methyl group to 49.5 ppm in ^{13}C and 3.34 ppm in ^{1}H. For the acetylated derivative of reduced apiuronic acid, the sample was dissolved in $CDCl_3$, and calibrated considering the signals of residual chloroform as 77.16 ppm in ^{13}C and 7.26 ppm in ^{1}H. All 2D NMR experiments (HSQC, DEPT-HSQC, HMBC, COSY and NOESY) were performed using standard pulse sequences.

2.8. Determination of apiuronic acid in polysaccharides from other seaweeds

Alkali-treated 2 M KCl-soluble fractions from *Kappaphycus alvarezii* were obtained as reported by Estevez et al. (2004). The KCl-soluble fraction from tetrasporic *Iridaea undulosa* was the T_3 fraction used by Stortz and Cerezo (1993). Carrageenan C from *I. undulosa* used by Stortz and Cerezo (1993) was alkali-treated and precipitated with 2 M KCl in order to obtain the 2 M KCl-soluble fraction. The apiuronic acid content was determined by GLC after reductive hydrolysis and acetylation, as a relative area with respect to the remaining sugars.

3. Results and discussion

3.1. Fractionation of *R-2S* and *H-2S* and analysis of the subfractions

R-2S and **H-2S** are the fractions soluble in 2 M KCl from the room temperature- and hot water extraction of the seaweed *Hypnea musciformis* (Cosenza et al., 2014). They have previously shown to be composed by a variety of monosaccharides, including L-galactose, and showed poor gelling abilities or no gelling ability at all (Cosenza et al., 2014). Both fractions were submitted to anion-exchange chromatography, using a mixture of an anion exchanger and gel permeation media (Stortz et al., 1997). After testing at an analytical scale for the optimal ratio between both media, a preparative separation was carried out with a 1.2:1 ratio, using steps of increasing concentrations of NaCl for elution. Fig. 1 shows the profiles of the elution: only one peak per NaCl concentration was obtained. Starting from **R-2S**, seven fractions were isolated (Fig. 1a), named considering the NaCl concentration at which they eluted as **R-0.2**, **R-0.4**, **R-0.6**, **R-0.8**, **R-1.0**, **R-1.5**, and **R-2.0**. The total recovery of these subfractions was 51.6%. Their yields and composition are shown in Table 1. Starting from **H-2S**, the equivalent seven fractions were isolated, with a total recovery of 49.8%. Their analysis is shown in Table 2. **R-0.6** was the main fraction isolated from **R-2S**, closely followed by **R-0.4** and **R-0.8**. However, all fractions were obtained with yields over 4% (Table 1). On the other hand, **H-0.8** was the main fraction isolated from **H-2S**. **H-0.6** was also important, but some other fractions were obtained in very low yields (Table 2). A rise in the proportion of sulfate groups and the molecular weight was observed for both fractionations as the concentration of NaCl increased. The first eluting fractions had very low molecular weights (MW < 16 kDa); for the **R** subfractions, moderate MWs were obtained, whereas for **H**, subfractions had MWs in the order of 300 kDa. In general, **H** fractions showed higher molecular weights than **R** fractions.

The analysis of the sugar composition allows to establish different groups within the fractions. For **R-2S**, galactose and its derivatives are always the main component of the subfractions. According to the D/L- ratio, four subgroups can be established: **R-0.2** has a ratio close to 1, suggesting that the galactan moiety has the structure of an agaran, **R-0.4** to **R-1.0** (four subfractions) show a proportion of units of the L-series that indicates a predominance of agaran diads within the DL-hybrids or mixtures; **R-1.5** shows a predominance of carrageenan diads, and **R-2.0**, almost devoid of L-galactose, is a carrageenan containing a large proportion of 3,6-anhydrogalactose and precursor units (Table 1). The first fractions, rich in agaran structures, are those containing larger proportions of xylose and methylated galactose units, typical of agarans. Glucose, probably from contaminating Floridean starch, also appears increased in these fractions. These first fractions show some 3,6-anhydro-L-galactose, but they are devoid of precursor 6-sulfated units.

The fractions derived from **H-2S** showed a similar behavior. However, the proportion of glucose is predominant in the first two fractions, as expected considering the higher solubility of starch in the boiling water used to extract carrageenan **H**. The proportion of protein is also significant in these fractions, but it decreases towards the end of the profile. Sulfate proportion tends to increase with the elution. An exception occurs for the first fraction, **H-0.2**, which shows a quite high sulfate proportion, already reported previously for a similar separation system (Stortz et al., 1997). The galactan moiety of **H-0.2** looks like an agaran (D-/L-ratio close to 1), whereas

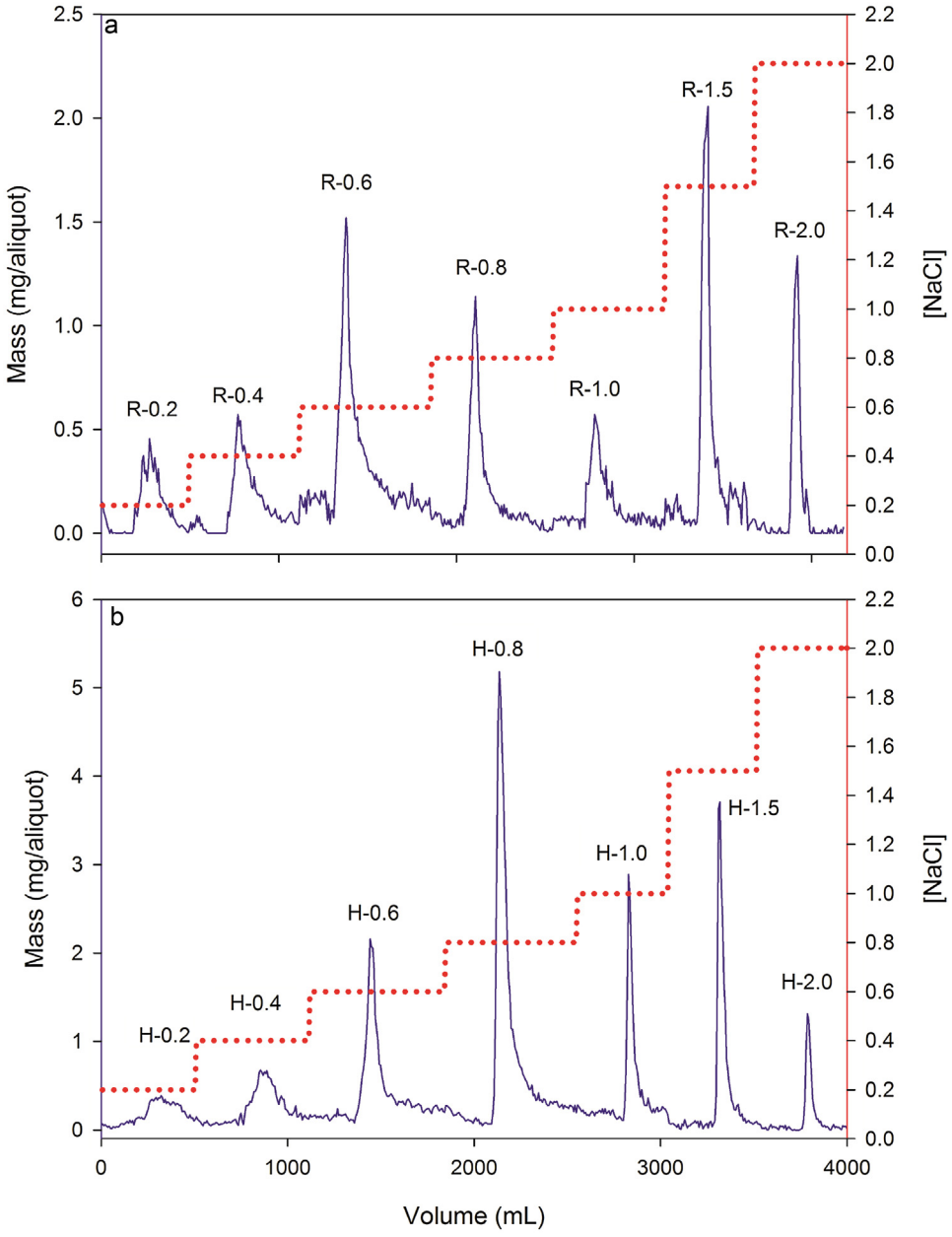


Fig. 1. Elution profiles of ion-exchange chromatography on DEAE-Sephadex A-50/Sephadex G-100 of fractions **R-2S** (a) and **H-2S** (b), with the acronyms of the isolated fractions.

H-2.0 is, as **R-2.0**, a carrageenan almost devoid of L-galactose and rich in 3,6-anhydrogalactose. The remaining five fractions are DL-hybrids or mixtures, most of them enriched in agaran diads (with the exception of **H-1.0**, which contains equal amounts of agaran and carrageenan diads, Table 2). Once again, methylated galactoses and xylose appear in significant amounts in the agaran-rich fractions. It is worth noting that 3-*O*-methyl-L-galactose is absent in these fractions, although it appeared in the subfractions of **R-2S**. Its enantiomer, almost absent in the room temperature fractions, is more abundant in the subfractions of **H-2S**. The agarans in **H-2S** showed less 3,6-An-L-Gal and more precursor units that those in **R-2S**. Besides, **H-2S** and at least five of its subfractions show a novel constituent which was identified as 3-*C*-carboxy-D-erythrose (2*R*,3*R*-apiuronic acid, see Section 3.2), in proportions ranging from 3 to 21% of the monosaccharide content. Its concentration is higher in fractions rich in DL-hybrids.

3.2. *Identification of 3-C-carboxy-D-erythrose (2R,3R-apiuronic acid)*

Some subfractions isolated from the **H-2S** fraction, most notably **H-0.8** and **H-1.0** presented an isolated ^{13}C resonance at ca. 109 ppm in their HSQC spectra (see Section 3.3), consistent with the presence of a sugar acting as a furanosic substituent. As mentioned above (Section 3.1), the same fractions showed, after reductive hydrolysis and acetylation, an unexpected component that appeared slightly after xylitol acetate in the chromatographic conditions used on the SP-2330 column (Section 2.2). On the other hand, GLC carried out on a less polar column (e.g., Ultra-2), resulted in complete resolution from xylitol acetate, with the novel peak appearing at about half its retention time. This fact suggested a different chemical nature (distinct polarity) for the derivative of the novel constituent. The electron impact mass spectrum (EI-MS, Supplementary data) of the reduced peracetylated sugar showed a pattern

Table 1
Composition and yields of the **R-2S** and of its subfractions isolated after ion-exchange chromatography.

	Fraction							
	R-2S	**R-0.2**	**R-0.4**	**R-0.6**	**R-0.8**	**R-1.0**	**R-1.5**	**R-2.0**
Yield (%)[a]	18.9	4.5	8.6	13.5	9.5	4.8	6.3	4.4
Protein (%)	17.9	14.2	20.8	9.7	ND[b]	ND	ND	ND
Ratio D/L-Gal[c]	1: 0.45	1: 0.96	1: 0.47	1: 0.64	1: 0.59	1: 0.56	1: 0.25	1: 0.05
Molar ratio Gal[c]:S	1: 1.13	1: 0.63	1: 0.59	1: 0.74	1: 1.06	1: 1.50	1: 1.46	1: 1.42
Molar ratio Gal:Gal 6-sulfate[d]	1: 0	1: 0	1: 0	1: 0	1: 0.07	1: 0.04	1:0.09	1: 0.26
MW[e] (kDa)	W[f]	9	14	35	65	55	93	151
Monosaccharides (mol/100 mol)								
D-Gal	39	16	24	26	30	42	52	62
L-Gal	16	18	17	21	21	25	17	5
3,6 An-D-Gal	6	–	6	5	3	6	16	29
3,6 An-L-Gal	3	4	2	2	1	1	–	–
D-Xyl	12	11	12	13	13	5	4	–
D-Glc	14	32	23	20	21	13	4	–
D-Man	2	5	3	2	1	2	3	1
L-Ara	1	2	1	1	1	1	2	2
L-Rha	1	1	1	1	–	1	1	1
L-Fuc	2	–	1	1	–	3	1	–
2-*O*-Me-D-Gal	2	5	5	3	3	–	–	–
3-*O*-Me-D-Gal	2	–	–	–	1	–	–	–
3-*O*-Me-L-Gal	Tr.	4	5	4	4	–	–	–
6-*O*-Me-Gal[g]	Tr.	2	–	1	1	1	–	–

[a] Yields for **R-2S** is given in g/100 g **R**. Yields for the subfractions given in g/100 g **R-2S**.
[b] ND = not determined.
[c] "Gal" includes the proportion of galactose, 3,6-anhydrogalactose and methylated galactoses.
[d] Gal 6-sulfate was determined by 3,6-AnGal measurement after alkaline treatment (Navarro & Stortz, 2003).
[e] Molecular weight, as determined for the maximum value of the GPC peak.
[f] Very wide peak, with more than one maximum.
[g] Configuration not determined.

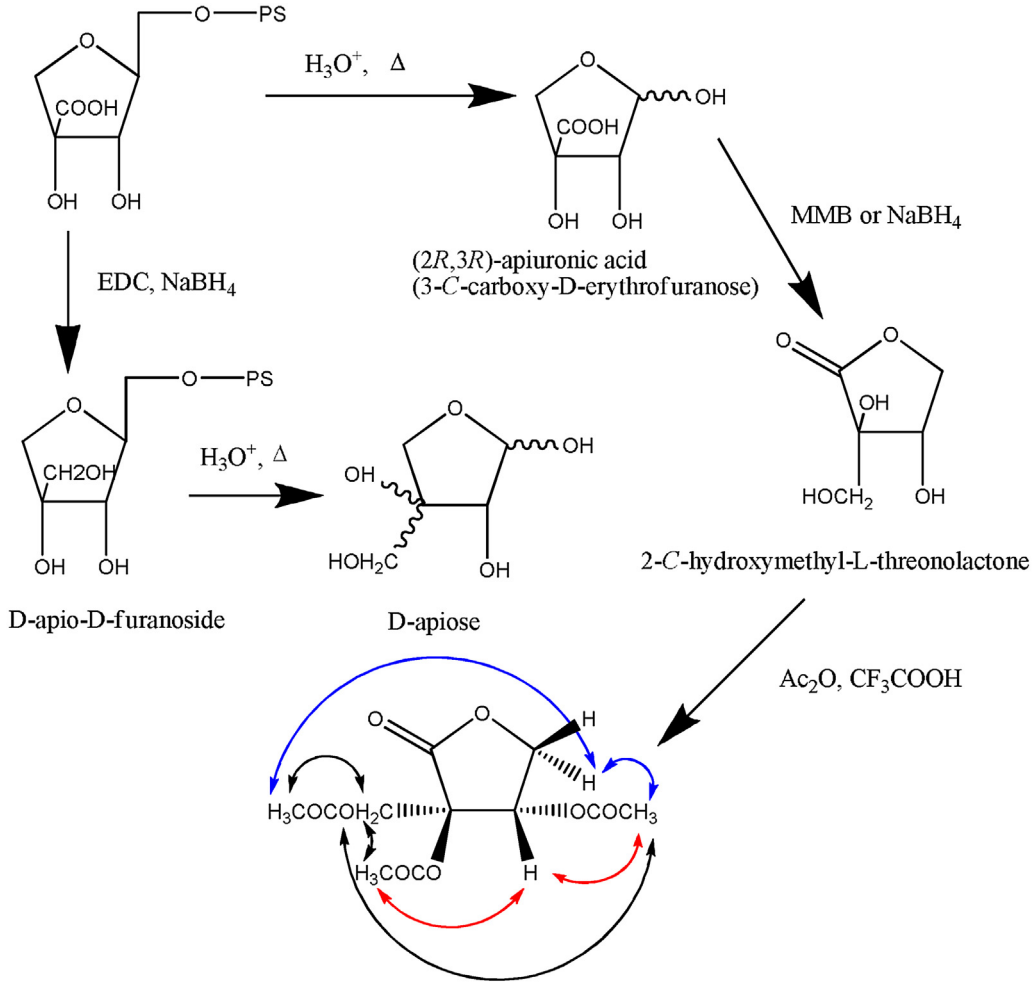
Table 2
Composition and yields of the **H-2S** and of its subfractions isolated after ion-exchange chromatography.

	Fraction							
	H-2S	**H-0.2**	**H-0.4**	**H-0.6**	**H-0.8**	**H-1.0**	**H-1.5**	**H-2.0**
Yield (%)[a]	15.9	1.7	5.5	10.6	18.3	5.8	6.1	1.7
Protein (%)	13.9	16.1	21.6	14.0	<2	<2	<2	ND[b]
Ratio D/L-Gal[c]	1: 0.40	1: 0.97	1: 0.83	1: 0.79	1: 0.60	1: 0.33	1: 0.71	1: 0.05
Molar ratio Gal[c]:S	1: 0.56	1: 1.16	1: 0.34	1: 0.34	1: 0.79	1: 1.08	1: 0.97	1: 1.92
Molar ratio Gal:Gal 6-sulfate[d]	1: 0.12	ND	1: 0	1: 0.11	1: 0.16	1: 0.05	1:0.18	ND
MW[e] (kDa)	W[f]	6	16	68	225	230	272	315
Monosaccharides (mol/100 mol)								
D-Gal	41	9	12	22	33	47	48	60
L-Gal	18	7	12	25	20	16	34	4
3,6 An-D-Gal	4	–	–	–	–	1	–	27
3,6 An-L-Gal	1	5	4	–	–	–	–	–
D-Xyl	10	9	9	10	13	10	5	1
D-Glc	13	66	51	22	15	1	2	4
D-Man	1	–	2	–	–	1	–	3
L-Rha	Tr.	1	–	–	–	1	1	1
L-Fuc	1	–	–	–	–	2	–	–
2-*O*-Me-D-Gal	2	1	3	5	–	–	–	–
2-*O*-Me-L-Gal	Tr.	–	–	1	1	–	–	–
3-*O*-Me-D-Gal	1	2	4	6	2	–	–	–
ApiA[g]	8	–	3	9	16	21	10	–

[a] Yields for **H-2S** is given in g/100 g **H**. Yields for the subfractions given in g/100 g **H-2S**.
[b] ND = not determined.
[c] "Gal" includes the proportion of galactose, 3,6-anhydrogalactose and methylated galactoses.
[d] Gal 6-sulfate was determined by 3,6-AnGal measurement after alkaline treatment (Navarro & Stortz, 2003).
[e] Molecular weight, as determined for the maximum value of the GPC peak.
[f] Very wide peak, with more than one maximum.
[g] (2*R*,3*R*)-apiuronic acid, determined as the aldehyde-reduced product.

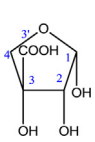
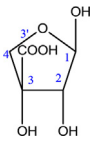
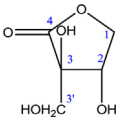
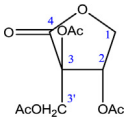
different from those of regular sugar alditol acetates. The molecular weight of the derivative, determined by CI-MS was 274, compatible with an acetylated aldopentonolactone (which would derive, prior to reduction, from an aldopenturonic acid) and the EI-MS of the derivative had some similarities with those of the acetylated aldopentonolactones. However, none of the four different aldopentonolactone acetates matched the retention times of the novel peak (data not shown).

A clue to the identity of the novel peak came from the work of Spellman et al. (1983), who identified 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid) as a component of plant pectins. The EI-MS spectrum of their derivative looked very similar to ours, with a shift



Scheme 1. Reactions carried out with the novel constituent from *H. musciformis* and key NOE interactions for the acetylated lactone.

Table 3
^1H and ^{13}C NMR data for (2*R*,3*R*)-apiuronic acid and some derivatives, at 500 and 125 MHz, respectively (values in parenthesis are $J_{H,H}$, in Hz)[a].

								
	Chemical shift (ppm)							
	^1H[b]	^{13}C	^1H[b]	^{13}C	^1H[b]	^{13}C	^1H[c]	^{13}C
H1/C1	5.35 (4.9)	96.4	5.19 (4.6)	102.3	4.17 (5.9, 9.1) 4.61 (6.8, 9.1)	72.1	4.08 (6.4, 9.6) 4.84 (8.5, 9.7)	68.8
H2/C2	4.35 (4.8)	75.7	4.23 (4.6)	80.4	4.56 (6.8, 5.9)	73.5	5.83 (6.4, 8.6)	71.7
H3/C3		79.9		81.7		77.6		78.0
H3′/C3′		178.9		177.8	3.82 (11.9) 3.95 (11.9)	61.3	4.20 (11.3) 4.71 (11.1)	61.5
H4/C4	4.20 (9.9) 4.01 (9.9)	76.4	4.36 (9.9) 3.87 (9.9)	75.7		178.7		170.4
Ac-O2							2.11	20.0, 169.7
Ac-O3							2.16	20.1, 169.3
Ac-O3′							2.09	20.3, 170.1

[a] Numbering system is indicated in the figures.
[b] In D_2O.
[c] In $CDCl_3$.

of −14 mass units in some of the main fragments. This suggested the presence of an analog of aceric acid lacking the methyl group, i.e., a 3-*C*-carboxytetrose which can only be closed as a furanosic ring. After applying the same protocol proposed by Spellman et al. (1983) for the isolation of aceric acid, the novel acid (Scheme 1) was obtained from **H-2S** with 90% purity (by GLC). 1D and 2D NMR spectroscopy confirmed the structure, disregarding configurational issues. Complete assignment of α- and β-anomers is shown in

Table 3. Additionally, reduction of the polysaccharide **H-2S** with a soluble carbodiimide and further hydrolysis gave apiose (3-*C*-(hydroxymethyl)-tetrose, Scheme 1), thus confirming the nature of the derivative as an apiuronic acid.

Establishing the configuration of the two stereogenic centers required further experiments. The configuration of C-2 was determined from the two peaks appearing after an authentic sample of D-apiose (obtained from parsley) reacted with racemic 1-amino-2-propanol and the single peak appearing with the *R*-enantiomer (Cases et al., 1995). Reaction of apiose from EDC-reduced **H-2S** with the *R*-enantiomer of 1-amino-2-propanol led to just one peak, overlapping with that corresponding to the D-apiose, indicating that C-2 has the *R* configuration.

In order to determine the relative configuration at C-3, we decided to proceed with the product of aldehyde reduction of the apiuronic acid (2-*C*-hydroxymethyl-L-threonolactone, Scheme 1), as mutarotation complicates the analysis of the original uronic acid. 1D and 2D NMR spectra allowed the complete assignment for the lactone and its acetylated derivative (Table 3). Although the NOESY spectra was confusing for the lactone, it gave unequivocal correlations for the acetylated lactone (Scheme 1), leading to the conclusion that the hydroxyl groups on C-2 and C-3 are *trans*, thus indicating that C-3 has also the *R* configuration. This is consistent with both hydroxyl groups being *cis* in the original apiuronic acid, as indeed occurs in natural apiofuranosides (Hulyalicarj, Jones, & Perry, 1965). The relative configuration at C-3 was also confirmed by molecular modeling of all the possible conformers for both C-3 epimers of the lactone. Each Boltzmann-averaged ensemble gave characteristic theoretical coupling constants ($^3J_{H1,H2}$ and $^3J_{H1',H2}$) and chemical shifts. It has been shown that accurate geometric and energy calculations of five-member lactones in combination with experimental NMR data are useful in the assessment of configurational features (Viturro, Maier, Stortz, & de la Fuente, 2001). The experimental *J* values (6.8 and 5.9 Hz, Table 3) have a better match with those of the *trans*-lactone (expected *J*s 6.2 and 5.3 Hz) than with those of the *cis*-lactone (expected *J*s 4.5 and 2.0 Hz). The comparison of chemical shifts was carried out with the aid of the DP4+ method (Grinblat et al., 2015). The calculations gave a perfect match with the *trans*-lactone in comparison with the *cis*-lactone. The three independent methods consistently indicate the 3*R* configuration for the lactone, thus the original substituent of the polysaccharides is (2*R*,3*R*) apiuronic acid or 3-*C*-carboxy-D-erythrofuranose.

The appearance of apiuronic acid in red seaweed galactans (and in any other polysaccharide) is, to the best of our knowledge, unprecedented. In 1965, Mendicino and Picken mentioned the presence of D-apiose in seaweeds, although a careful appraisal of their publication indicated its presence only in aquatic plants, besides its known abundance in parsley. However, Estevez et al. (2004) reported a signal at 109.6 ppm in the ^{13}C NMR spectrum of some fractions soluble in 2 M KCl from *Kappaphycus alvarezii*. This may be regarded as an indication of its presence in this seaweed too. Thus, we searched for the novel component in fractions from other carrageenophytes. The tetrasporic fraction from *Iridaea undulosa* soluble in 2 M KCl (T_3, Stortz & Cerezo, 1993; Stortz et al., 1997) exhibited 0.4% of the novel component. We have also isolated the 2 M KCl-soluble fractions of alkali-treated room-temperature- and hot water extracts from *Kappaphycus alvarezii* (Estevez et al., 2000, 2004), and the room-temperature extract from cystocarpic *Iridaea undulosa* (Flores et al., 2002; Stortz & Cerezo, 1993). They also showed the peaks corresponding to the novel component, in proportions of 1.1, 1.7, and 0.7%, respectively. Therefore, we can conclude that its presence is not specific of the current species, although its proportion appears much higher in *H. musciformis* (ca. 8% in **H-2S** vs. 1.7% in the equivalent fraction from *K. alvarezii*). It should be mentioned that this component appears in the current seaweed only in the hot water extract, and concentrated only in the fractions that are neither pure agarans nor pure carrageenans, but only in the carrageenan-agaran hybrids.

3.3. Structural determination of the subfractions

In order to determine the main structural features of the isolated subfractions, they were submitted to methylation analysis, desulfation-methylation in some cases, furanose hydrolysis, and NMR spectroscopy. All of the subfractions were methylated using the method of Ciucanu and Kerek (1984). Results are shown in Table 4.

A general pattern of great complexity is observed. Methylation proceeded smoothly in most cases, but for **H-1.0** the large amounts of non-methylated galactose (Table 4) indicate undermethylation. Attempts to remethylate this fraction or trying harsher methods failed to decrease the proportion of non-methylated galactose. Within this complexity, several issues may be asserted:

a) The first fractions in each group (**R-0.2** to **R-0.8** and **H-0.2** to **H-0.8**) carry important amounts of glucans, apparently bonded $1\rightarrow3$ and $1\rightarrow4$, with branching on O-6. The usual structure assigned to Floridean starch is equivalent to that of regular starch, *i.e.*, 4-linkages and branches on O-6 (Usov, 2011). However, there were reports suggesting the presence of 3-linkages in the Floridean starch from *Dilsea edulis* (Peat, Turvey & Evans, 1959). *Hypnea musciformis* appears to be particularly rich in those bonds: most fractions have larger amounts of $1\rightarrow3$ glucose bonds than $1\rightarrow4$ bonds (Table 4).
b) Fractions **R-2.0** and **H-2.0** show the characteristics of almost pure carrageenans of the κ-family. Methylation analysis seems to underestimate the amount of 2,3- and 3- methylated units, representative of μ/ν-precursors, but they should be present according to the proportion of 6-sulfate determined, at least in **R-2.0** (Table 1). The predominance of 2,6-di-*O*-methylgalactose and the high proportions of 3,6-anhydrogalactose both non-methylated and 2-*O*-methylated are indicative of the presence of a carrageenan hybrid with predominance of ι-diads, but also rich in κ-diads and μ/ν-precursors.
c) Disregarding the glucan contamination, **R-0.2** and **H-0.2** are agaran structures. In **R-0.2**, the β-D-galactose units are mainly unsubstituted (2,4,6-Gal) or substituted in O-2 and O-4 (6-Gal, 12% shown to be entirely of the D-series), whereas the α-L-galactose units are mainly unsubstituted or substituted in O-3 (about 40% each), or as 3,6-anhydrogalactose or its 2-sulfate (20%). The substituents may be terminal xylose, galactose or 3-*O*-methyl-L-galactose units, or sulfate. The fraction **H-0.2** is qualitatively similar, but β-Gal units are less substituted, and 3,6-AnGal moieties appear more often.
d) The remaining fractions correspond to DL-hybrids or carrageenan-agaran mixtures. The proportion of trimethylated sugars (non substituted galactoses) decreases as the elution progresses, as expected considering that the latter fractions have larger proportions of sulfate groups. Substitution occurs mostly on O-4 or O-6 of β-D-Gal units and on O-3 of α-L-Gal units. Less substitution is found on O-2 of β-D-Gal units, although it occurs on most fractions (Table 4).
e) Xylose appears mostly as single stub side chains. However, some dimethylated units have been found (Table 4). This can be ascribed to undermethylation, as literature (Usov, 2011) reports single stubs in other red seaweeds. However, the presence of double or multiple branching cannot be discarded. Some galactose also appears as side chains, mainly on the first-eluting fractions. The 3-*O*-methyl-L-galactose appearing in the first eluting subfraction of the **R-2S** fraction corresponds probably to

Table 4
Methylation analysis of the subfractions obtained from **R-2S** and **H-2S** (mols/100 mols of galactoses).

	R-0.2	R-0.4	R-0.6	R-0.8	R-1.0	R-1.5	R-2.0	H-0.2	H-0.4	H-0.6	H-0.8	H-1.0	H-1.5	H-2.0
2,3,4,6-Glc[a]	4	–	2	–	–	–	–	–	–	–	–	–	–	–
2,3,6-Glc	4	2	–	–	–	–	–	14	23	6	4	4	–	1
2,4,6-Glc	7	12	14	6	4	–	–	45	–	–	–	–	–	–
2,3-Glc	1	8	8	8	–	–	–	12	6	6	7	–	–	–
2,4-Glc	–	7	–	–	–	–	–	15	27	6	3	–	–	–
Glc	3	4	1	3	1	–	1	–	4	3	2	5	1	–
2,3,4,6-Gal	10	7	9	5	4	3	–	4	2	6	–	2	2	–
2,3,4-Gal	–	2	3	–	–	–	–	–	–	1	9	–	1	–
2,3,6-Gal	16	13	9	7	4	2	–	13	16	9	5	6	6	2
2,4,6-Gal	20	17	9	10	11	12	4	30	18	10	7	–	9	5
3,4,6-Gal	4	3	6	3	2	2	1	–	5	6	2	–	1	–
2,3-Gal	–	2	3	2	2	2	2	–	–	–	–	7	1	2
2,4-Gal	3	6	10	12	8	4	1	1	8	16	45	23	10	2
2,6-D-Gal	3	11	8	13	18	29	47	4	9	3	1	6	9	49
2,6-L-Gal	19	17	17	19	26	13	–	19	21	19	1	18	38	–
4,6-Gal	–	2	6	5	5	2	–	–	–	9	11	4	2	–
2-Gal	1	4	4	4	5	6	5	3	3	4	2	4	4	4
3 + 4–Gal	–	–	2	3	3	2	4	–	–	3	15	3	5	2
6-Gal	12	–	5	6	5	5	1	–	–	–	–	–	5	2
Gal	1	–	–	2	2	1	1	–	–	4	–	21	2	–
3,6-An−2-Gal[b]	7	12	8	8	5	9	13	21	12	7	2	6	3	14
3,6-AnGal	4	4	1	1	–	8	21	4	6	3	–	–	2	18
2,3,4-Xyl	8	10	9	7	4	2	1	9	7	14	10	4	2	–
2,3 + 3,4-Xyl	2	5	5	3	2	–	–	3	–	4	2	–	–	–
2,4-Xyl	1	–	–	–	–	–	–	–	4	3	3	5	–	–
Xyl	2	–	–	–	–	–	–	5	–	3	–	5	1	–
2,3-ApiA[c]	–	–	–	–	–	–	–	–	4	29	55	24	8	–
ApiA	–	–	–	–	–	–	–	–	–	–	–	4	2	–

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methylglucose, etc.
[b] 3,6-An-2-Gal = 3,6-anhydro-2-*O*-methylgalactose.
[c] 2,3-Api = 2,3-di-*O*-methylapiuronic acid, see text.

terminal units: ethylation analysis of **R-0.4** showed that this sugar appeared as its 2,4,6-tri-*O*-ethyl-3-*O*-methyl derivative.

f) Some fractions, particularly **H-0.8**, show notably larger amounts of 3-linked (β) units than 4-linked ones (α). As those fractions still carry 4-linked glucose units in spite of the high ionic strength necessary to elute this fraction, a possible explanation can be that some glucose units are part of the galactan structure replacing α-galactose units. This has been already suggested in a previous work by Stortz et al. (1997) for similar polysaccharides from the tetrasporic stage of *Iridaea undulosa*.

g) Methylation analysis of the fractions (Table 4) showed a derivative of apiuronic acid methylated at O-2 and O-3. The derivative had an EI-MS compatible with the structure proposed for the novel sugar, and indicated that it performs as a non-reducing terminal substituent.

h) Fraction **H-0.8**, rich in apiuronic acid was submitted to partial hydrolysis in order to cleave furanosic bonds. The product, obtained after dialysis, was almost free of apiuronic acid, but contained about the same proportions of the remaining sugars, reinforcing the suggestion that apiuronic acid acts as a single stub side chain. Methylation analysis of the partially hydrolyzed product showed a large decrease in the proportion of 2,4-di-*O*-methylgalactose with a concomitant increase in the proportion of 2,4,6-tri-*O*-methylgalactose, without major changes in the remaining sugars. This indicates that apiuronic acid is linked to O-6 of β-galactose units.

On some selected fractions, desulfation followed by methylation was carried out. Desulfation proceeded with sulfate losses ranging from 40 to 85%, whereas recovery yields were 54 to 100%. Table 5 shows the results of the methylation analysis of the desulfated fractions. Desulfation followed by methylation may help to distinguish substitution by sulfate groups and by other moieties, mainly glycosyl side stubs. Fractions **R-0.6** and **H-0.6** were only moderately affected by desulfation as they carried originally low

Table 5
Methylation analysis of some subfractions after desulfation (mols/100 mols of galactoses)[a].

	R-0.6d	R-1.5d	H-0.6d	H-0.8d	H-1.0d	H-1.5d
2,3,4,6-Gal[b]	11	5	3	9	2	4
2,3,4-Gal	–	–	3	3	–	1
2,3,6-Gal	17	15	15	9	21	36
2,4,6-Gal	20	40	7	24	30	30
3,4,6-Gal	6	–	5	–	–	–
2,3-Gal	–	–	–	–	1	2
2,4-Gal	7	10	19	30	29	13
2,6-D-Gal	4	6	4	2	6	4
2,6-L-Gal	16	4	29	5	2	2
4,6-Gal	3	–	3	4	–	–
2-Gal	3	4	–	–	3	2
3 + 4–Gal	1	–	–	10	1	–
6-Gal	–	–	–	–	2	–
Gal	1	–	–	4	–	–
3,6-An−2-Gal[c]	9	15	7	–	2	5
3,6-AnGal	2	–	5	–	–	1

[a] The glucose, xylose and apiuronic acid components are not shown in the Table. A **d** is added to the fraction acronym to indicate that it has been desulfated.
[b] 2,3,4,6-Gal = 2,3,4,6-tetra-*O*-methylgalactose, etc.
[c] 3,6-An-2-Gal = 3,6-anhydro-2-*O*-methylgalactose.

proportions of sulfate. However, it may be inferred that sulfates located on O-2, O-4 and O-6 of β-galactose units are the most common (Tables 4 and 5). Branching is important in these fractions. On the other hand, the desulfation-methylation of the late-eluting fractions **R-1.5** and **H-1.5** shows a backbone rich in both trimethylated galactoses (and/or 3,6-anhydrogalactose), suggesting lower degrees of branching. 2,6-Di-*O*-methyl- and 6-*O*-methyl-galactose appear as the most diminished after desulfation, suggesting that O-4 of β-units and O-3 of α-units are the main sulfation locations, followed by O-2 of β-units or double-sulfated units (O-2 and O-4, or O-2 and O-6). For fraction **H-1.0** comparison is complicated, as the original fraction was undermethylated (Table 4). However, sulfation on O-6 of β-units and on O-3 of α-units are the most likely

Table 6
Tentative assignments for the anomeric signals in the NMR (HSQC) spectra of the subfractions of **R-2S** and **H-2S**[a].

$^{13}C/^{1}H$ (ppm)	In fractions	Assignm.[a]	Linked to[a]	Reference
109.5/5.06	**H-0.4 to −1.5**	ApiA	C-6 of G	This work
105.4/4.68	**R-1.5, R-2.0**	G4S	D	[c,d]
104.4/4.70	**R-0.4, R-0.6, H-0.4 to −0.8**	G(6R)	L	[e,f]
104.4/4.55	**H-0.8**	G(6R)	L	[e,f]
104.4/4.51	**R-0.6, R-0.8, R-1.5**	G(6R)	L	[e,f]
104.4/4.48	**R-0.2 to −1.0, H-0.2 to −1.5**	β – Xyl	C-6 of G	[e,f]
103.9/4.51	**H-0.6**	NA[b]		
103.5/4.70	**R-0.2 to R-0.6**	G2(,4)S	L	[g]
103.5/4.52	**R-0.2 to R-0.8**	G2(,4)S	L	[g]
103.4/4.79	**H-0.2, H-0.4, H-0.8**	G2(,4)S	L	[g]
103.3/4.59	**R-0.6 to −1.0, H-0.4 to −0.8**	G2(,4)S	L	[g]
103.2/4.54	**H-0.2**	G2(,4)S	L	[g]
102.9/4.64	**R-0.4 to 2.0, H-0.2, H 0.8, H-1.5, H-2.0**	G(6R)	LA	[h]
		G4S	DA	[c,d]
102.7/5.20	**H-0.8**	NA		
101.9/5.30	**H-1.5**	L	G (6R)	[h]
101.6/5.30	**R-0.6 to −1**	L	G (6R)	[h]
101.4/4.95	**R-0.2**	NA		
101.3/5.18	**R-0.6**	L3S	G(6R)	[i]
101.3/5.08	**R-0.2 to −0.8, H-0.4, H-0.8**	L3Y	G(6R)	[f]
99.6/5.35	**R-0.2, R-0.4, H-0.2, H-0.4**	α-Glc	C-3/C-4 of Glc	[e,f]
99.3/4.97	**H-0.2**	LA	G	[h]
98.8/5.15	**R-0.2, R-0.6, H-0.2, H-0.4**	LA	G6R	[f]
98.6/4.99	**R-0.2 to −0.6, H-0.2, H-0.4**	LA	G	[h]
95.1/5.11	**R-0.4 to −2.0, H-0.4, H-1.5, H-2.0**	DA	G4S	[c,d]
92.6/5.29	**R-1.5, R-2.0, H-1.5, H-2.0**	DA2S	G4S	[c,d]

[a] Using the nomenclature for galactans of Knutsen, Myslabodsky, Larsen & Usov (1994).
[b] NA = not assigned.
[c] van de Velde, Knutsen, Usov, Rollema, & Cerezo (2002).
[d] Stortz, Bacon, Cherniak, & Cerezo (1994).
[e] Usov, Bilan, & Shashkov (1997).
[f] Navarro and Stortz (2008).
[g] Miller, Falshaw, Furneaux, & Hemmingson (1997).
[h] Lahaye, Yaphe, Viet, & Rochas (1989).
[i] Kolender and Matulewicz (2002).

to occur. In fraction **H-0.8**, the desulfation analysis points to O-6 and/or O-2 of β-units as the most probable sulfate locations.

NMR spectroscopy is an useful tool for structural determination of polysaccharides. However, the spectra of complex polysaccharides present many signals that are usually difficult to assign. Furthermore, most of the samples were not completely soluble at the concentrations needed to measure NMR spectra. Thus, many of the spectra had poor signal-to-noise ratios, and some small signals could not be observed. The region of the non-anomeric carbons did not yield any useful information, but some interesting results could be obtained from the anomeric region of the HSQC spectra. Table 6 shows some of the signals found and their potential assignments.

Table 6 shows results compatible with those obtained by chemical analysis. Early eluting fractions are richer in agaran diads, whereas late eluting fractions are richer in carrageenan diads. Some different peaks are assignable to similar units, probably present in a slightly different environment (influenced by the preceding and following units). Only one signal attributable to C-1/H-1 of glucose was found in spite of the finding of 1 → 3 and 1 → 4 linkages. This can be explained on the basis that both the chemical shifts of C-1′ and H-1′ are almost identical in nigerose (3-linked disaccharide) and maltose (4-linked disaccharide) (Goffin et al., 2009). Only signals for xylosyl units linked to O-6 of β-Gal units are found. Those linked to O-3 of α-L-Gal units, that should appear at ca. 101.7/4.66 ppm (Usov & Elashvili, 1991) are not observed, but a nearby signal (101.4/4.95 ppm) present in **R-0.2** (an agaran sample, Table 6) could arise from this substituent. The ^{1}H signal at 5.06 ppm and the ^{13}C signal at 109.5 ppm may be attributed to H-1/C-1 of apiuronic acid as the HSQC spectrum of a partially hydrolyzed **H-0.8** showed a clear decrease of the signal area. In addition, the ^{13}C NMR signal for the anomeric carbon of EDC-reduced **H-2S** also appears around 109 ppm, indicating a *trans*-relationship between O-1 and O-2 (Ishii & Yanagisawa, 1998). For the actual configuration of the sugar (see Section 3.2), this corresponds to the β-anomer (Scheme 1).

Perez Recalde et al. (2016) have summarized the different structural units found in the agarans encountered so far in carrageenophytes. Following the nomenclature of Knutsen et al. (1994), the agarans and hybrids from *Hypnea musciformis* contain, in the different fractions, a large diversity of structural units, as follows: G, G2S, G4S, G6S, G6Y, G2,4S, G2 M, G2,6S, L, L3S, L3Y, L3,6S, LA, and LA2S, where Y is a glycosyl substituent (xylose, galactose, 3-*O*-methyl-L-galactose or (2*R*,3*R*)-apiuronic acid). However, structural units appear scattered through the different subfractions. *Kappaphycus alvarezii*, another source of κ-carrageenan, has also shown a large diversity of units (Estevez et al., 2000, 2004).

4. Conclusion

So far, every time that a carrageenophyte was studied in detail, after separating the bulk of gelling carrageenans, a mixture of non-gelling polysaccharides was isolated. This mixture has always presented pure agarans, pure carrageenans, a glucan, and a group of different DL-hybrids ranging from those richer in agaran structures to those richer in carrageenan structures. As the mixed tetrasaccharide containing an α-D- and an α-L-Gal has never been isolated, there is a possibility that these so called hybrids do not exist (Ciancia & Cerezo, 2010), and that they are actually non-separable mixtures. *Hypnea musciformis* is not an exception. Besides the large amounts of κ-carrageenan produced by this seaweed (Cosenza et al., 2014), the 2 M KCl soluble fractions gather all the components of this mixture, with a great variety of sulfation locations, side stubs and structures. The most important feature, however, is the presence of 3-*C*-carboxy-β-D-erythrofuranosyl

((2*R*,3*R*)-apiofuranosyluronic acid) side stubs attached to O-6 of β-D-galactose units in some fractions, and its positive identification in products from other seaweeds previously studied where, probably due to its low proportion and to its chromatographic overlapping, its presence was neglected. The existence of this side stub just in the so called hybrids opens new questions about its biosynthesis and about its role. Could they be markers of hybrid regions? Its discovery opens the door for further studies on the subject.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.09.071.

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