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Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of oligosaccharides and oligosaccharide alditols obtained by hydrolysis of agaroses and carrageenans, two important types of red seaweed polysaccharides

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ABSTRACT

MALDI-TOF mass spectrometry analyses of several oligosaccharides (aldoses) and oligosaccharide alditols derived from agaroses, kappa- and iota-carrageenans using different matrices (2,5-dihydroxybenzoic acid, *nor*-harmane, ferulic acid, and the ionic liquid matrices 2,5-dihydroxybenzoic acid–*n*-butylamine and ferulic acid–*n*-butylamine) were conducted. These carbohydrates were selected as model compounds to study the MALDI prompt and post-source decay (PSD) fragmentation processes of both families of oligosaccharides. Sulfated alditols showed in the negative-ion mode the molecular ion as $[M-Na]^-$ together with the species yielded by their prompt fragmentation (mainly desulfation) while the sulfated oligosaccharides (aldoses) showed mainly glycosidic prompt fragmentation (glycosidic *C*-cleavages and desulfation). Non-sulfated aldoses and alditols, which could only be analyzed in positive-ion mode ($[M+Na]^+$), did not suffer any prompt fragmentation. The former yielded cross-ring fragmentation in the PSD mode. Best results were obtained by using 2,5-dihydroxybenzoic acid and/or *nor*-harmane as matrices for all the compounds studied.

1. Introduction

Galactans biosynthesized by red seaweeds (Rhodophyta) essentially consist of linear chains of alternating 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl units and, in many cases, the latter residue appears in the 3,6-anhydro form. These galactans are classified either as carrageenans if the 4-linked residue is in the D-configuration or agarans if this unit is in the L-configuration.[1] Most of these polysaccharides are found as anionic polymers with varying degrees of sulfation. Furthermore, some algal species produce these galactans with an almost idealized repeating unit such as kappa-carrageenan (3-linked β-D-galactopyranose 4-sulfate and 4-linked 3,6-anhydro-α-D-galactopyranose), iota-carrageenan (3-linked β-D-galactopyranose 4-sulfate and 4-linked 3,6-anhydro-α-D-galactopyranose 2-sulfate), agarose (3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose), 6′-sulfated agarose (3-linked β-D-galactopyranose 6-sulfate and 4-linked 3,6-anhydro-α-L-galactopyranose) and methylated agarose (3-linked 6-*O*-methyl-β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose).[2–9] The use of partial hydrolysis methods on repetitive agarans and carrageenans is an attractive way to obtain oligosaccharides with a specific sulfation pattern in relative high yields.[10–13] These oligosaccharides can be utilized as useful standards and model compounds for the study of complex native algal galactans,[14] and are also potential sources of different positional isomers and/or diastereoisomers of sulfated oligosaccharides which have proved to exhibit a variety of physiological activities.[14,15] The extent of these activities has been correlated with the degree of polymerization and the percentage and position of the sulfate groups.[14,15] Therefore, analytical techniques capable of resolving complex mixtures of isomeric oligosaccharides obtained from agarans and carrageenans were developed and structural elucidation of these oligosaccharides was mainly carried out by NMR spectroscopy.[13,14]

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Recently, MALDI mass spectrometry (MALDI MS) has become a major technique for the analysis of carbohydrates being their structural complexity a major problem for these studies.[16–18] Particularly, sulfated oligosaccharides are difficult to analyze by this technique, partly because of the labile nature of the sulfate group.[16–18] Results can be erratic, indicating that there are still several unknown structural and experimental factors that affect the measurements.

As part of a research project related with the study of MALDI MS analysis of carbohydrates,[19–24] we report here the behavior of selected model compounds, oligosaccharides (aldoses) and oligosaccharide alditols, using different matrices [2,5-dihydroxybenzoic acid (DHB), *nor*-harmane, ferulic acid (FA), and the ionic liquid matrices DHB–*n*-butylamine (DHB–Bu) and FA–Bu]. These compounds, obtained from agaroses, kappa- and iota-carrageenans, were chosen to study the MALDI and the fragmentation processes (prompt fragmentation and PSD) of both families of carbohydrates.

2. Results and discussion

2.1. Oligosaccharides

To investigate the effect of the sulfate group on the stability of the molecular ions yielded by MALDI, the mass spectra of the neutral oligosaccharides G→LA (**1**), G→LA→G (**2**) and G→LA→G→LA (**3**) were compared with those of monosulfated G4S→DA (**4**) and disulfated G4S→DA→G4S→DA (**5**) (nomenclature of Knutsen et al.).[3] Experiments were conducted in positive- and negative-ion linear and reflectron modes and when the intact molecular ion was detected, in PSD mode too. Table 1 lists the molecular weights and Figure 1 shows the molecular structures of the studied oligosaccharides and the main fragmentations (nomenclature of Domon and Costello is used).[25]

By using DHB as matrix, compound **1** showed in positive linear and reflectron modes the intact molecular ion as $[M+Na]^+$ (Table 2) together with minor signals corresponding to $[M+H]^+$ and the analyte cluster ion $[2M+Na]^+$. PSD analysis gave as main fragment the sodiated species at *m/z* 257.8 produced by a cross-ring $^{0,3}X_1$ cleavage of the galactose ring with shedding of the $C_3H_6O_3$ (Table 3). It was impossible to determine spectra with *nor*-harmane as matrix since the molecular ion peak $[M+Na]^+$ was overlapped by a matrix signal at *m/z* 338.8 with a shoulder at *m/z* 350.9.

Compound **2** showed in similar experiments the ion peak $[M+Na]^+$ together with a signal corresponding to the $[M+K]^+$ species, being the former the most intense ion. In the PSD mode not only the fragment produced by cross-ring $^{0,4}X_2$ and/or $^{0,4}X_0$ cleavage was observed at *m/z* 448.2, but also the signal at *m/z* 346.9 due to a C_2- and/or Y_2-fragmentation. Similar results were obtained for this compound in the positive-ion linear and reflectron modes when *nor*-harmane was used as matrix.

Compound **3** gave the molecular ion peak $[M+Na]^+$ at *m/z* 652.9 only by using DHB as matrix. In the PSD analysis from this precursor ion a quite efficient decomposition process yielded a signal at *m/z* 289.8, a protonated species formed after glycosidic B_2- and/or Z_2-fragmentation with further loss of H_2O (Table 3). The presence of the G→LA repeating unit in the structure of compound **3** would account for this behavior. No signals of this compound were observed in the MALDI mass spectrum by using *nor*-harmane as matrix.

Although analyses in the negative-ion mode were attempted by using DHB and *nor*-harmane as matrices, compounds **1**–**3** did not yield $[M-H]^-$ or any stable anion fragment.

The sulfated compounds **4** and **5** (Table 1, Fig. 1) showed a quite different behavior by MALDI MS analysis. Even though in positive-ion mode several matrices were assayed, that is, DHB, *nor*-harmane, FA, and the ionic liquid matrices DHB–Bu and FA–Bu, no signal could be obtained. Results were different in the negative-ion mode. When DHB was used as matrix, the intact molecular ion $[M-Na]^-$ was only detected for compound **4** at *m/z* 403.1; with *nor*-harmane, together with this signal, peaks at *m/z* 373.1 due to the loss of HCHO from the molecular ion and at *m/z* 258.7 due to a C_1-cleavage, were found with low relative intensity. PSD analysis of compound **4** using DHB as matrix showed as only fragment the ion at *m/z* 241.8 due to a [C_1-ion$-H_2O$] and/or B_1-cleavage of the glycosidic bond. This peak together with a weak signal at *m/z* 259.1 attributed to C_1-cleavage were observed when *nor*-harmane was the MALDI matrix (Table 3). The electrospray-ionization (ESI) mass spectrum in negative-ion mode of compound **4** gave signals at *m/z* 421.2 $[M+H_2O-Na]^-$, 403.2 $[M-Na]^-$, 259.1 and 241.2 (both yielded by prompt fragmentation).

Finally, disulfated compound **5** did not show the intact molecular ion as $[M+Na]^+$ and/or $[M-Na]^-$ by using DHB and *nor*-harmane as matrix (Table 2). In order to overcome this problem, FA and the ionic liquid matrices DHB–Bu and FA–Bu were assayed. In spite of the effort, only signals at *m/z* 710.2, 565.7, 403.2, 258.6 and 241.0 were detected with DHB as matrix. These signals were also obtained, with higher relative intensity, with *nor*-harmane as matrix (Fig. 2, Table 2). The signal at *m/z* 403.4 can be assigned to the $[(G4S→DA)-Na]^-$ fragment obtained by C_2- and/or Y_2-fragmentation of the central glycosidic bond. Fragments observed at *m/z* 709.5 and 565.0 would be the final species produced after desulfation of the species at *m/z* 811.6 ($[M-Na]^-$) and the fragment produced by a C_3-type fragmentation respectively. Fragments at *m/z* 372.9 and 258.5 also derive from *C*-fragmentations, but formation of the former would include shedding of HCHO; the signal at *m/z* 240.7could be due to a [C_1-ion$-H_2O$] and/or B_1-cleavage of the glycosidic bond (Table 2). The presence of these fragments and those detected by ESI MS of compound **5** (see Section 4) confirms that the molecular ions produced by both MALDI and ESI are quite unstable and they suffer prompt fragmentation in the ionization chamber. In agreement with the behavior observed in MALDI experiments, ESI analysis only yielded ions in negative-ion mode. But, on the contrary, by ESI process the stable intact molecular ion was detected as the double charged species $[M-2Na]^{2-}$ at *m/z* 394.0. As it is known double charged ions are not detected in general by MALDI MS analysis. Together with this double charged anion, an important signal at *m/z* 403.0 and a small signal at *m/z* 241.1 were observed. Although MALDI and ESI are soft ionization methods the minor vibration energy remaining in the gas molecular ion of **5** is enough to induce its prompt decomposition.

The results obtained in this section of the present study support our previous suggestion that prompt fragmentation observed in the MALDI MS analysis of a mixture of kappa-carrageenan oligosaccharides was mainly due to glycosidic *C*-cleavages.[20]

Table 1
List of the oligosaccharides and oligosaccharide alditols analyzed in this study

Compound	Structure[a]	Molecular weight
Oligosaccharide aldoses		
1	G→LA	324.3
2	G→LA→G	486.4
3	G→LA→G→LA	630.5
4	G4S→DA	426.3[b]
5	G4S→DA→G4S→DA	834.6[c]
Oligosaccharide alditols		
6	G→LAOH	326.3
7	G6M→LAOH	340.3
8	G6S→LAOH	428.3
9	G4S→DA2SOH	530.4[c]
10	G4S→DA2S→G4S→DA2SOH	1040.7[d]

[a] Nomenclature of Knutsen et al.[3]
[b] Monosodiated.
[c] Disodiated.
[d] Tetrasodiated.

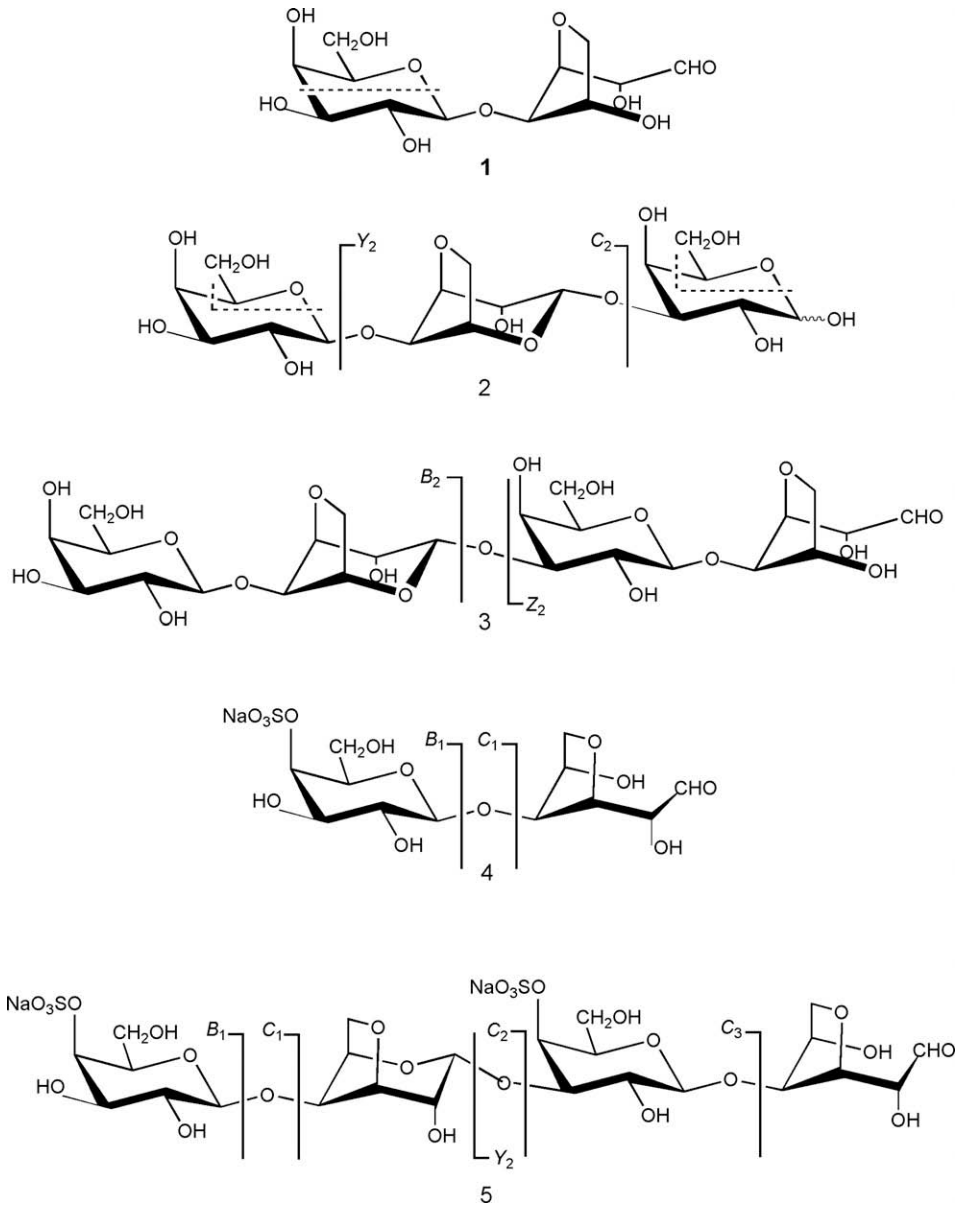

Figure 1. Molecular structure and main fragmentations of oligosaccharides **1**–**5**.

2.2. Oligosaccharide alditols

The behavior of oligosaccharide alditols in MALDI MS analysis was carefully studied using as model compounds the family listed in Table 1, constituted by G→LAOH (**6**), G6M→LAOH (**7**), G6S→LAOH (**8**), G4S→DA2SOH (**9**), and G4S→DA2S→G4S→DA2-SOH (**10**) (Fig. 3).

Compound **6** gave in the positive-ion mode, with DHB and *nor*-harmane as matrices, only the peak corresponding to the sodiated molecular ion $[M+Na]^+$ with *m/z* 349.5. Spectra obtained with the DHB showed in general S/N ratio higher than those with *nor*-harmane (Table 4). Compound **6** showed to be quite stable because no significant fragmentation was observed in PSD experiments. The analysis of the methylated model compound **7** yielded quite similar results to those obtained in the former case (Table 4).

The behavior changed drastically when a sulfate group was present in the alditol structure. Thus, the monosulfated derivative **8** could be analyzed in both positive- and negative-ion modes (Table 4). By using DHB as matrix, the molecular ion was observed in positive-ion mode as $[M+Na]^+$ (*m/z* 451.4) and in negative-ion mode as [M−Na] (*m/z* 405.2); the signal at *m/z* 286.4, in positive-ion mode, was attributed to a *C*- and/or *B*-fragmentation. PSD analysis with DHB in negative-ion mode gave a fragment at *m/z* 242.3 due to a *C*- and/or *B*-glycosidic linkage cleavage (Table 3). The analysis carried out with *nor*-harmane as matrix, showed similar results to those described above for DHB. In all cases the spectra were obtained with quite high S/N ratio.

In spite of the fact that the monosulfated alditol **8** did not show desulfated ions, even in PSD experiments, the disulfated alditol **9** (MW 530.4, Table 1) gave, by using *nor*-harmane as matrix, the molecular ion peak $[M-Na]^-$ at *m/z* 507.8, together with signals at *m/z* 405.5, 248.7 and 226.5 (Table 4). The first signal could be rationalized as due to $[M-NaSO_3+H-Na]^-$, while the other two could be produced by a *Z*-cleavage (Fig. 3). Desulfated fragments were also observed by PSD analysis (Table 3) at *m/z* 405.4 and 387.1 (dehydration of the fragment of *m/z* 405.4). The spectra obtained by using DHB as matrix in the negative-ion mode, showed in linear and reflectron modes the above-mentioned signals at *m/z* 507.6 and 405.6

Table 2
Molecular and daughter ions observed in the MALDI-TOF mass spectra of oligosaccharides **1**–**5**

Compound	Matrix	Ion-mode	*m/z* (RI)[a]	Assignment[b]
1	DHB	Positive	347.7 (100%)	$[M+Na]^+$
			325.5 (12%)	$[M+H]^+$
2	DHB	Positive	524.5 (55%)	$[M+K]^+$
			508.0 (100%)	$[M+Na]^+$
3	DHB	Positive	652.9 (100%)	$[M+Na]^+$
4	*Nor*-harmane	Negative	403.1 (100%)	$[M-Na]^-$
			373.1 (24%)	$[M-HCHO-Na]^-$
			258.7 (13%)	$[C_1\text{-ion}-Na]^-$
	DHB	Negative	403.1 (100%)	$[M-Na]^-$
5	*Nor*-harmane	Negative	709.5 (3%)	$[M-NaSO_3+H-Na]^-$
			565.0 (18%)	$[C_3\text{-ion}-NaSO_3+H-Na]^-$
			403.4 (31%)	$[C_2\text{-ion and/or } Y_2\text{-ion}-Na]^-$
			372.9 (13%)	$[C_2\text{-ion}-HCHO-Na]^-$
			258.5 (100%)	$[C_1\text{-ion}-Na]^-$
			240.7 (37%)	$[C_1\text{-ion}-Na-H_2O]^-$ and/or $[B_1\text{-ion}-Na]^-$
	DHB	Negative	710.2 (3%)	$[M-NaSO_3+H-Na]^-$
			565.7 (7%)	$[C_3\text{-ion}-NaSO_3+H-Na]^-$
			403.2 (22%)	$[C_2\text{-ion}-Na]^-$
			258.6 (42%)	$[C_1\text{-ion}-Na]^-$
			241.0 (100%)	$[C_1\text{-ion}-Na-H_2O]^-$ and/or $[B_1\text{-ion}-Na]^-$

[a] RI = relative intensity.
[b] The different cleavages of the glycosidic bond are indicated according to the nomenclature of Domon and Costello.[25]

Table 3
Signals observed in the PSD spectra of compounds **1**–**4**, **8,** and **9**

Compound	Matrix	Ion-mode	*m/z* (RI)[a]	Origin[b]
1	DHB	Positive	347.7 (100%)	$[M+Na]^+$
			257.8 (6%)	$^{0,3}X_1$
2	DHB	Positive	508.0 (100%)	$[M+Na]^+$
			448.2 (5%)	$^{0,4}X_2$ and/or $^{0,4}X_0$
			346.9 (7%)	C_2 and/or Y_2
3	DHB	Positive	652.9 (11%)	$[M+Na]^+$
			289.8 (100%)	$(B_2$ and/or $Z_2-H_2O)$[c]
4	*Nor*-harmane	Negative	403.1 (100%)	$[M-Na]^-$
			259.1 (6%)	C_1
			242.3 (17%)	$[C_1-H_2O]^-$ and/or B_1
	DHB	Negative	403.1 (100%)	$[M-Na]^-$
			241.8 (56%)	$[C_1-H_2O]^-$ and/or B_1
8	DHB	Negative	405.2 (100%)	$[M-Na]^-$
			242.3 (40%)	$[C_1-H_2O]^-$ and/or B_1
9	DHB	Negative	507.6 (100%)	$[M-Na]^-$
			405.2 (21%)	$[M-NaSO_3+H-Na]^-$
			387.6 (21%)	$[M-NaSO_3+H-Na-H_2O]^-$
	Nor-harmane	Negative	507.8 (100%)	$[M-Na]^-$
			405.4 (16%)	$[M-NaSO_3+H-Na]^-$
			387.1 (14%)	$[M-NaSO_3+H-Na-H_2O]^-$

[a] RI = relative intensity.
[b] The different glycosidic and cross-ring cleavages are indicated according to the nomenclature of Domon and Costello.[25]
[c] Protonated fragment.

together with a peak at *m/z* 387.9 due to dehydration of the latter (Table 4). Compound **9** was also analyzed by using FA and the ion liquid matrices FA–Bu and DHB–Bu. FA only yielded, the molecular ion $[M-Na]^-$ with low intensity; on the other hand, FA–Bu gave in linear and reflectron modes signals at *m/z* 507.7, 405.5 and 387.3 which were assigned as indicated in Table 4 for DHB. Finally by using DHB–Bu, together with the signal at *m/z* 507.9, a large number of high relative intensity signals of matrix clusters were observed. Sulfated alditol **9** was also detected in positive-ion mode as $[M+Na]^+$ by using DHB and *nor*-harmane as matrices.

Finally, compound **10** (Table 1, Fig. 3) was analyzed by using different matrices (Table 4). Although DHB and *nor*-harmane yielded similar spectra in negative-ion mode, best results were obtained when the latter was used as matrix, because minor amounts of matrix clusters were formed. *Nor*-harmane yielded signals at *m/z* 1018.6, 812.6, 710.0, 691.5, 504.7 and 402.9 assigned to $[M-Na]^-$, $[M-2NaSO_3+2H-Na]^-$, $[M-3NaSO_3+3H-Na]^-$, $[M-3NaSO_3+3H-Na-H_2O]^-$, $[C_2\text{-ion}-Na]^-$ and $[C_2\text{-ion}-NaSO_3+H-Na]^-$, respectively (Fig. 4). It is interesting to point out that as it was previously observed in the MALDI MS analysis of sulfated neocarrabiose oligosaccharides,[19] the loss of sulfate moieties from a molecular ion with *x* sulfate groups, not necessarily produces the complete family of $[M-nNaSO_3+nH-Na]^-$ ions with $n = 1, 2, 3, (x-1)$. In the present example, although the corresponding ions with $n = 2$ and $n = 3$ were observed, the species $[M-NaSO_3+H-Na]$ was not detected. PSD analysis was not successful because of the low intensity of the $[M-Na]^-$ signal.

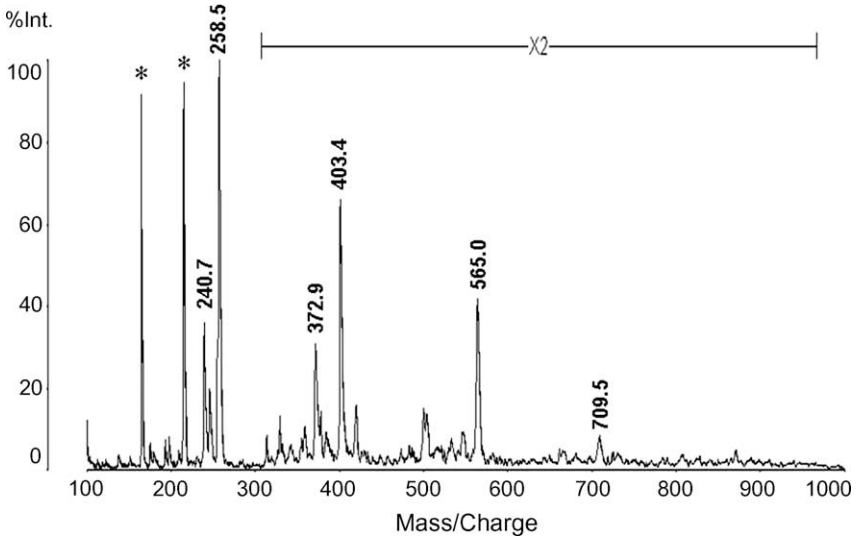

Figure 2. Negative-ion mode MALDI-TOF mass spectrum of compound **5** obtained using *nor*-harmane as matrix. Peaks with an asterisk correspond to the matrix.

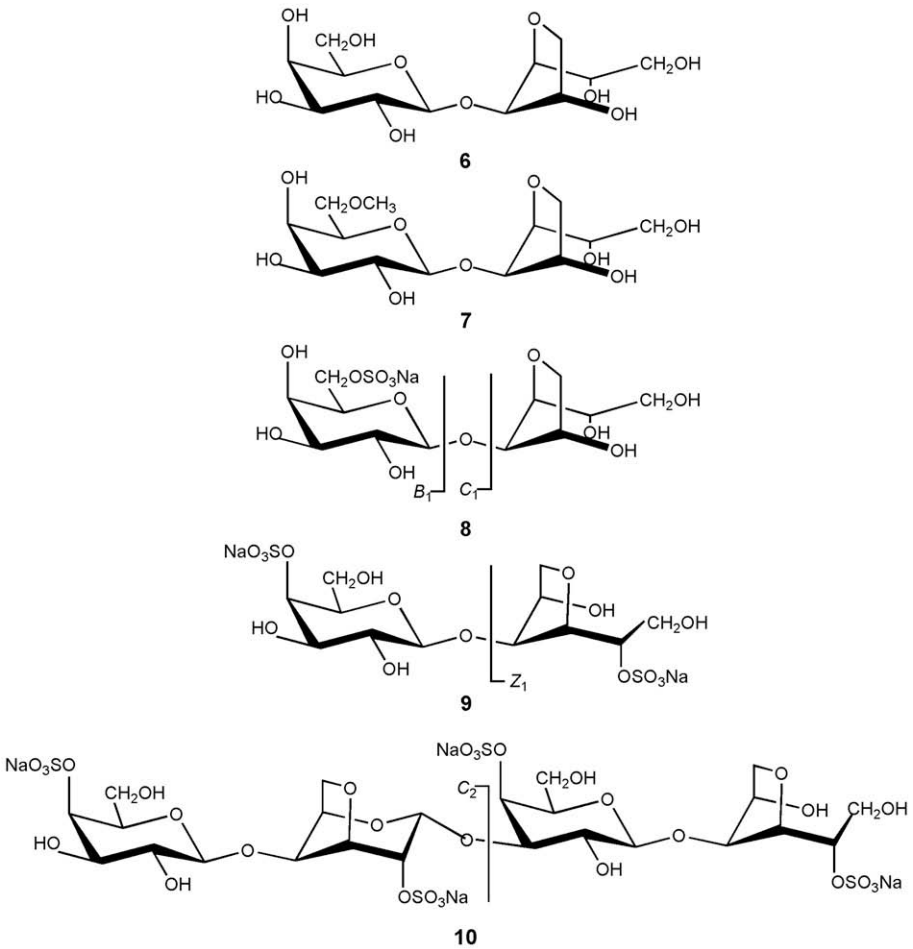

Figure 3. Molecular structure and cleavage of the glycosidic bonds in oligosaccharide alditols **6**–**10**.

An additional and interesting conclusion is obtained by the comparison of the relative intensities of the $[M-Na]^-$ and $[M-n\mathrm{Na}SO_3+n\mathrm{H}-Na]^-$ signals for compounds **9** and **10**. Compound **9**, with $x = 2$, shows an $[M-Na]^-$ to $[M-NaSO_3Na+H-Na]^-$ intensity ratio of 1.6:1, while for compound **10**, with $x = 4$, the ratios of $[M-Na]^-$ to $[M-n\mathrm{Na}SO_3Na+H-Na]$ for $n = 2$ and 3 are 1.2:1 and 0.2:1, respectively. Thus, the stability of the molecular ion is minor when higher is the number of sulfate groups in the molecular structure of a sulfated oligosaccharide alditol. Thus, both matrices DHB and *nor*-harmane seem to yield molecular ions with enough vibrational

Table 4
Molecular and daughter ions observed in the MALDI-TOF mass spectra of oligosaccharide alditols **6**–**10**

Compound	Matrix	Ion-mode	*m/z* (RI)[a]	Assignment[b]
6	DHB	Positive	349.5 (100%)	$[M+Na]^+$
7	DHB	Positive	363.6 (100%)	$[M+Na]^+$
8	DHB	Positive	451.4 (63%)	$[M+Na]^+$
			286.4 (100%)	$[C_1\text{-ion}-H_2O+Na]^+$ and/or $[B_1\text{-ion}+Na]^+$
		Negative	405.2 (100%)	$[M-Na]^-$
9	DHB	Positive	553.2 (100%)	$[M+Na]^+$
		Negative	507.6 (43%)	$[M-Na]^-$
			405.6 (60%)	$[M-NaSO_3+H-Na]^-$
			387.9 (100%)	$[M-NaSO_3+H-Na-H_2O]^-$
	Nor-harmane	Positive	553.7 (100%)	$[M+Na]^+$
		Negative	507.8 (52%)	$[M-Na]^-$
			405.5 (33%)	$[M-NaSO_3+H-Na]^-$
			248.7 (100%)	$[Z_1\text{-ion}-H]^-$
			226.5 (98%)	$[Z_1\text{-ion}-Na]^-$
10	*Nor*-harmane	Negative	1018.6 (20%)	$[M-Na]^-$
			812.6 (17%)	$[M-2NaSO_3+2H-Na]^-$
			710.0 (100%)	$[M-3NaSO_3+3H-Na]^-$
			691.5 (15%)	$[M-3NaSO_3+3H-Na-H_2O]^-$
			504.7 (3%)	$[C_2\text{-ion}-Na]^-$
			402.9 (37%)	$[C_2\text{-ion}-NaSO_3+H-Na]^-$

[a] RI = relative intensity.
[b] The different cleavages of the glycosidic bond are indicated according to the nomenclature of Domon and Costello.[25]

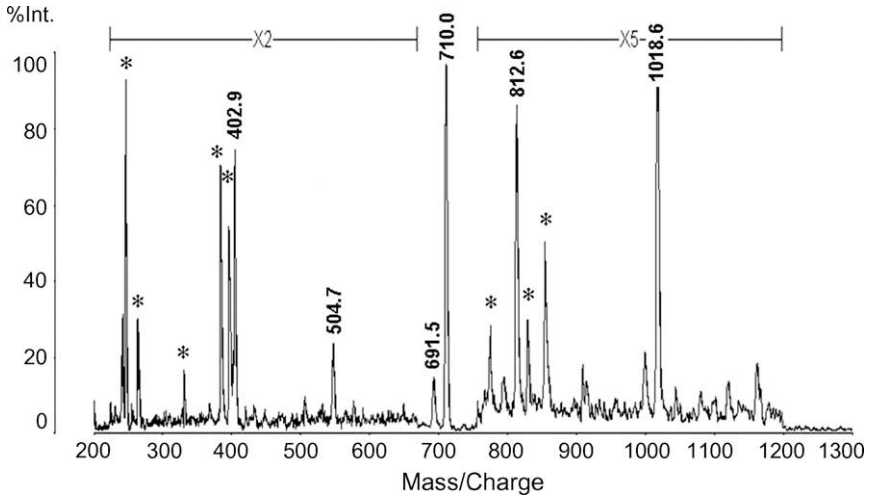

Figure 4. Negative-ion mode MALDI-TOF mass spectrum of compound **10** obtained using *nor*-harmane as matrix. Peaks with an asterisk correspond to the matrix.

energy to suffer prompt fragmentation, mainly desulfation. Similar results were obtained with the other MALDI matrices attempted (FA, DHB–Bu and FA–Bu).

3. Conclusions

The results previously discussed showed that non-sulfated oligosaccharides **1–3** and non-sulfated oligosaccharide alditols **6** and **7**, which could only be analyzed in positive-ion mode ($[M+Na]^+$), did not suffer any prompt fragmentation. On the other hand, sulfated compounds **4** and **8–10** gave in the negative-ion mode the molecular ion as $[M-Na]^-$ and compounds **4**, **9** and **10** also showed prompt fragmentation (Tables 2 and 4). Compound **5**, the G4S→DA→G4S→DA studied, had a quite peculiar behavior because the intact molecular ion as $[M-Na]^-$ or $[M+Na]^+$ was not detected in the corresponding ion mode, although experiments were conducted with different matrices in order to overcome this problem. The species yielded by prompt fragmentation in the negative-ion mode, indicated that glycosidic *C*-cleavages and desulfation were the main processes. The presence of the aldehyde as terminal functional group in this sulfated oligosaccharide diminishes its thermal stability and the glycosidic *C*-cleavages efficiently decompose the molecular ion.

It is important to note that this behavior is completely different to that observed in the MALDI-TOF linear and reflectron spectra of sulfated neocarrabiose oligosaccharides using DHB and *nor*-harmane as matrices where desulfation but no glycosidic *C*-cleavages was detected as prompt fragmentation.[19]

Furthermore, monosulfated compounds **4** and **8** gave, in the negative-ion PSD experiments (Table 3), cleavages of the glycosidic bonds similarly to what was observed for the sulfated neocarrabiose oligosaccharides.[19] On the other hand, for compound **9** desulfation was observed. PSD spectra of non-sulfated oligosaccharides **1** and **2** showed, in the positive-ion mode cross-ring fragmentation; for compound **2** a glycosidic cleavage was also detected.

The experiments conducted by using several compounds as MALDI matrices allowed finding suitable conditions for effective analysis of the alditols and oligosaccharides studied. The results obtained can be summarized as follows:

(a) Alditols with more than one sulfate group suffered desulfation. Glycosidic cleavages were also observed, yielding the more abundant ions in the case of compound **9** when *nor*-harmane was used as matrix.
(b) Sulfated oligosaccharides with more than one sulfate group gave, as it was expected,[19] desulfation, but glycosidic fragmentation was more important.
(c) Non-sulfated oligosaccharides and non-sulfated alditols did not show any prompt fragmentation.
(d) Non-sulfated oligosaccharides yielded cross-ring fragmentation in the PSD mode.

4. Experimental

4.1. Materials

Agarose was obtained from Vetec Quimica (Rio de Janeiro, RJ, Brazil). Kappa-carrageenan, iota-carrageenan, 6′-sulfated and methylated agarose were extracted from *Kappaphycus alvarezii*,[26,27] *Eucheuma denticulatum*[28], and *Gracilaria domingensis*,[14,29] respectively, as it was previously reported.

4.2. Preparation of oligosaccharides and oligosaccharide alditols

Commercial agarose (150 mg) was first dissolved in hot (~90 °C) water (13.5 mL) and then 1 M TFA solution (1.5 mL) was added in one portion. The resulting mixture was heated at 65 °C for 2 h, cooled to room temperature, diluted with water (15 mL), and then concentrated under vacuum. The residue was co-evaporated with toluene three times to give a sirup. This material was dissolved in water (2 mL), applied on a BioGel P-2 column (1.5 × 70 cm) and eluted with water. Oligosaccharide detection was performed by the phenol–H_2SO_4 acid method[30] and TLC. TLC was carried out on Silica Gel 60 (2:2:1 BuOH–AcOH–H_2O) with detection by charring 0.5% orcinol in concd H_2SO_4–EtOH 1:20. The fractions obtained were concentrated and freeze-dried to give agarobiose **1** and agarotetraose **3**. Oligosaccharide G→LA→G (**2**) was prepared as previously described by Yang et al.[31] with modifications[32]: a 1% mixture of commercial agarose in concentrated HCl was kept at room temperature for 30 min. The reaction was then neutralized by the addition of solid $NaHCO_3$, the resulting mixture was freeze-dried and the remaining solid was submitted to three successive extractions with DMSO. The combined extracts were treated with three volumes of $CHCl_3$ to precipitate the oligosaccharides, which were separated by centrifugation. The pellet was then applied on a BioGel P-2 column and eluted with water; the eluate was monitored by the phenol–H_2SO_4 method which evidenced a rich sugar fraction that gave compound **2**.

Kappa-carrageenan was submitted to partial acid hydrolysis as described for commercial agarose. The hydrolysate was dissolved in water, applied on a Sephadex G-25 column (1.5 × 100 cm), and eluted with water. The fractions obtained were concentrated and freeze-dried to give kappa-carrabiose **4** and kappa-carratetraose **5**.[26]

Agaran sulfate isolated from *Acanthophora spicifera* was submitted to partial reductive hydrolysis as previously described.[10] The hydrolyzate was dissolved in water, applied to a DEAE–Sephadex A-25 (Cl^-) column (5 × 22 cm) and eluted with water and NaCl aqueous solutions. The aqueous eluate was concentrated and desalted by gel filtration chromatography on a BioGel P-2 column (1.5 × 100 cm) using water as eluant. The fraction obtained was concentrated and freeze-dried to give agarobiitol **6**.[13]

6′-Sulfated and methylated agarose, obtained from *G. domingensis*,[29] was submitted to partial reductive hydrolysis as previously described.[10] The hydrolysate was dissolved in water and applied on a Sephadex G-25 column (1.5 × 100 cm) and eluted with water. The main fraction was concentrated under vacuum, dissolved in water, applied on a BioGel P-2 column (1.5 × 100 cm), and eluted with water. The second subfraction obtained was concentrated and freeze-dried to give 6^2-*O*-methyl agarobiitol **7**.[33]

6′-Sulfated and methylated agarose, obtained from *G. domingensis*,[29] was submitted to partial reductive hydrolysis as previously described.[10] The hydrolysate was dissolved in water, applied on a DEAE–Sephadex A-25 (Cl^-) column (2.5 × 12 cm), eluted with water and a continuous NaCl gradient (0–0.15 M). The main fraction eluted with NaCl was concentrated and desalted by aqueous elution on a BioGel P-2 column (1.5 × 100 cm) to give agarobiitol 6^2-sulfate **8**.[14]

Iota-carrageenan, obtained from *E. denticulatum*,[28] was submitted to partial reductive hydrolysis as previously described.[10] The hydrolysate was dissolved in water and applied to a DEAE–Sepharose CL-6B ($CO_3{}^{2-}$) column (3.0 × 15 cm). Successive elutions were performed with water and ammonium carbonate solutions. Carrabiitol 2^1,4^2-disulfate **9** and carratetraitol 2^1,4^2,2^3,4^4-tetrasulfate **10** were eluted with 0.10 M and 0.25 M ammonium carbonate solution respectively.[33]

TLC, NMR spectroscopy, and ESI MS were used to control the purity of the compounds and their characterization. The ^{1}H NMR spectra of compounds **1**, **3–10** and the ^{13}C NMR spectra of all the compounds are available as electronic supplementary data.

4.3. Electrospray-ionization mass spectrometry

The ESI MS equipment used was a Micromass Quatro LC–MS/MS triple quadrupole mass spectrometer. Compounds **1–8** were diluted in 7:3 CH_3CN–H_2O at 1 mg mL^{-1} and experiments were conducted as it has been described elsewhere.[14,26] Compounds **9** and **10** (0.125 mg mL^{-1}) were injected in 75:25 MeOH–20 mM *n*-pentylammonium formate solution.[33]

Compound **1**: positive-ion mode, *m/z* 671.4 (14%; $[2M+Na]^+$), 381.3 (8%), 365.0 (56%; $[M+H_2O+Na]^+$), and 347.1 (100%; $[M+Na]^+$).[26] Compound **2**: positive-ion mode, *m/z* 524.9 (85%; $[M+K]^+$) and 508.9 (100%; $[M+Na]^+$).[32] Compound **3**: positive-ion mode, *m/z* 671.6 (57%; $[M+H_2O+Na]^+$) and 653.4 (100%; $[M+Na]^+$).[26] Compound **4**: negative-ion mode, *m/z* 421.2 (45%; $[M+H_2O-Na]^+$), 403.2 (100%; $[M-Na]^+$), 259.1 (40%; $[C_1$-ion$-Na]^-$), and 241.2 (8%; $[B_1$-ion$-Na]^-$).[26] Compound **5**: negative-ion mode, *m/z* 421,0 (18%; $[C_2$-ion$+H_2O-Na]^-$), 403.0 (90%; $[C_2$-ion$-Na]^-$), 394.0 (100%; $[M-2Na]^{2-}$), and 241.1 (9%; $[B_1$-ion$-Na]^-$).[26]

Compound **6:** positive-ion mode, *m/z* 349.3 (100%; $[M+Na])^+$.[13] Compound **7**: positive-ion mode, *m/z* 363.4 (100%; $[M+Na])^+$.[33] Compound **8**: negative-ion mode, *m/z* 405.1 (100%; $[M-Na])^-$.[14]

Compound **9**: negative-ion mode, *m/z* 572.5 (11%; $[M-2Na+C_5H_{11}NH_3]^-$), 485.3 (12%; $[M-2Na+H]^-$), and 242.1 (100%; $[M-2Na]^{2-}$).[33] Compound **10**: negative-ion mode, *m/z* 1212.1 (11%; $[M-4Na+3C_5H_{11}NH_3]^-$), 1125.5 (29%; $[M-4Na+H+2C_5H_{11}NH_3]^-$), 1037.5 (22%; $[M-4Na+2H+C_5H_{11}NH_3]^-$), 951.3 (12%; $[M-4Na+3H]^-$), 562.4 (86%; $[M-4Na+2C_5H_{11}NH_3]^{2-}$), 518.8 (100%; $[M-4Na+H+C_5H_{11}NH_3]^{2-}$), 475.4 (53%; $[M-4Na+2H]^{2-}$), 345.7 (16%; $[M-4Na+C_5H_{11}NH_3]^{3-}$), and 316.4 (47%; $[M-4Na+H]^{3-}$).[33]

4.4. MALDI-TOF MS

4.4.1. Matrix chemicals

The β-carboline (9*H*-pyrido[3,4-*b*]indole) *nor*-harmane, 2,5-dihydroxybenzoic acid (DHB, gentisic acid), 3-methoxy-4-hydroxycinnamic acid (ferulic acid, FA), *n*-butylamine, and 3,5-dimethoxy-

4-hydroxycinnamic acid (sinapinic acid, SA; used for protein calibrants) were obtained from Aldrich Chemical Co.

4.4.2. Calibrant chemicals

Caffeine (MW 149.19); β-estradiol-3-sulfate-17-glucuronide dipotassium salt (MW 604.75), α-cyclodextrin (cyclomaltohexaose, MW 972.9), β-cyclodextrin (cyclomaltoheptaose, MW 1135.0), γ-cyclodextrin (cyclomaltooctaose, MW 1297.1); angiotensin I (MW 1296.49), and neurotensin (N6383, MW 1672.96) were purchased from Sigma–Aldrich.

4.4.3. Solvents

MeOH and CH_3CN (Sigma–Aldrich HPLC grade) were used as purchased without further purification. Water of very low conductivity (Milli Q grade; 56–59 nS/cm with PURIC-S, ORUGANO Co., Ltd, Tokyo, Japan) was used.

4.4.4. Instrument

Measurements were performed using a Shimadzu Kratos, Kompact MALDI 4 (Pulsed Extraction) laser desorption time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan), equipped with a pulsed nitrogen laser (λ_{em} = 337 nm; pulse width = 3 ns), tunable pulsed-delayed extraction (PDE) and PSD (MS/MS device) modes, as described elsewhere.[22–24]

Experiments were performed using firstly the full range setting for laser firing position in order to select the optimal position for data collection, and secondly fixing the laser firing position in the sample sweet spots. The sample was irradiated just above the threshold laser power for obtaining molecular ions and with higher laser power for studying cluster formation. Thus, the irradiation used for producing a mass spectrum was analyte dependent. Usually 50 spectra were accumulated. The sample was measured in the linear and reflectron modes, in both positive- and negative-ion modes.

4.4.5. Probe supports

Stainless steel polished surface twenty-sample-slides were purchased from Shimadzu Co., Japan (P/N 670–19109-01). Polished surface slides were used in order to get better images for morphological analysis in a stereoscopic microscope (NIKON Optiphot, Tokyo, Japan; magnification 400×) and a high-resolution digital microscope (Keyence VH-6300, Osaka, Japan; magnification 800×).

4.4.6. Sample preparation

The ionic DHB–Bu and FA–Bu liquid matrices were prepared as a 1:1 molar mixture of the carboxylic acid and *n*-butylamine in MeOH which, after evaporation under vacuum, yielded a viscous honey-like ionic liquid matrix. The DHB–Bu ionic liquid matrix has been previously described for the analysis of carbohydrates.[34,35]

Matrix stock solutions were made by dissolving 2 mg of the selected compound in 0.2 mL of 1:1 MeOH–H_2O or in 0.2 mL of 2:3 CH_3CN–H_2O. Analyte solutions were freshly prepared by dissolving the carbohydrates (1 mg) in pure water (0.5 mL). Best results were afforded when the matrix solutions were prepared in MeOH–H_2O.

To prepare the analyte-matrix deposit two methods were used: Method A (thin-film layer method) and Method B (mixture method).[19] Best results were obtained using *nor*-harmane as matrix followed by the adoption of Method B. In this method, the analyte stock solution was mixed with the matrix solution in 1:4 v/v ratio. A 0.5 μL aliquot of this analyte-matrix solution was deposited onto the stainless steel probe tip and dried with a stream of forced room temperature air. Then, an additional portion of 0.5 μL was applied to the dried solid layer on the probe, causing it to redissolve partially, and the solvent was removed by blowing air.

4.4.7. Spectrum calibration

Spectra in the linear and reflectron modes were calibrated by the use of external calibration reagents: (a) commercial proteins (angiotensin I and neurotensin) with SA as matrix in positive- and negative-ion modes, and (b) caffeine, β-estradiol-3-sulfate-17-glucuronide dipotassium salt, α-, β- and γ-cyclodextrins with *nor*-harmane as matrix in positive- and in negative-ion modes. The Kratos Kompact calibration program was used.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.10.009.

References

1. Painter, T. J. Algal Polysaccharides. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: New York, 1983; Vol. 2, pp 195–285.
2. Zablackis, E.; Vreeland, V.; Doboszewski, B.; Laetsch, W. M. *J. Phycol.* **1991**, *27*, 241–248.
3. Knutsen, S. H.; Mysladodski, D. E.; Larsen, B.; Usov, A. I. *Bot. Mar.* **1994**, *37*, 163–169.
4. Hurtado-Ponce, A. Q. *Bot. Mar.* **1995**, *38*, 215–219.
5. Ohno, M.; Nang, H. Q.; Hirase, S. *J. Appl. Phycol.* **1996**, *8*, 431–437.
6. Lechat, H.; Amat, M.; Mazoyer, J.; Gallant, D. J.; Buleon, A.; Lahaye, M. *J. Appl. Phycol.* **1997**, *9*, 565–572.
7. Estevez, J. M.; Ciancia, M.; Cerezo, A. S. *Carbohydr. Res.* **2000**, *325*, 287–299.
8. Aguilan, J. T.; Broom, J. E.; Hemmingson, J. A.; Dayrit, F. M.; Montaño, M. N. E.; Dancel, M. C. A.; Niñonuevo, M. R.; Furneaux, R. H. *Bot. Mar.* **2003**, *46*, 179–192.
9. Estevez, J. M.; Ciancia, M.; Cerezo, A. S. *Carbohydr. Res.* **2004**, *339*, 2575–2592.
10. Usov, A. I.; Elashvili, M. *Bot. Mar.* **1991**, *34*, 553–560.
11. Falshaw, R.; Furneaux, R. H. *Carbohydr. Res.* **1995**, *269*, 183–189.
12. Usov, A. I.; Elashvili, M. *Russ. J. Bioorg. Chem.* **1997**, *23*, 502–511.
13. Gonçalves, A. G.; Ducatti, D. R. B.; Duarte, M. E. R.; Noseda, M. D. *Carbohydr. Res.* **2002**, *337*, 2443–2453.
14. Gonçalves, A. G.; Ducatti, D. R. B.; Paranha, R. G.; Duarte, M. E. R.; Noseda, M. D. *Carbohydr. Res.* **2005**, *340*, 2123–2134.
15. Kazlowski, B.; Pan, C. L.; Ko, Y. T. *Carbohydr. Res.* **2008**, *343*, 2443–2450.
16. Harvey, D. J. *Mass Spectrom. Rev.* **1999**, *18*, 349–451.
17. Harvey, D. J. *Mass Spectrom. Rev.* **2006**, *25*, 595–662.
18. Harvey, D. J. *Mass Spectrom. Rev.* **2008**, *27*, 125–201.
19. Fukuyama, Y.; Ciancia, M.; Nonami, H.; Cerezo, A. S.; Erra-Balsells, R.; Matulewicz, M. C. *Carbohydr. Res.* **2002**, *337*, 1553–1562.
20. Ciancia, M.; Sato, Y.; Nonami, H.; Cerezo, A. S.; Erra-Balsells, R.; Matulewicz, M. C. *ARKIVOC* **2005**, *xii*, 319–331.
21. Fukuyama, Y.; Kolender, A. A.; Nishioka, M.; Nonami, H.; Matulewicz, M. C.; Erra-Balsells, R.; Cerezo, A. S. *Rapid. Commun. Mass Spectrom.* **2005**, *19*, 349–358.
22. Nonami, H.; Fukui, S.; Erra-Balsells, R. *J. Mass Spectrom.* **1997**, *32*, 287–296.
23. Erra-Balsells, R.; Nonami, H. *Environ. Control Biol.* **2002**, *40*, 55–73.
24. Erra-Balsells, R.; Nonami, H. *Environ. Control Biol.* **2008**, *46*, 65–90.
25. Domon, B.; Costello, C. E. *Glycoconjugate J.* **1988**, *5*, 397–409.
26. Ducatti, D. R. B. Hidrólise Ácida de Galactanas Para Produção de Oligossacarídeos e *C*-Glicosídeos *Building Blocks*: Semi-síntese de *N*-Alquil e

C-Dihidropiridina Glicosídeos. Ph.D Thesis, Universidade Federal do Paraná: Brazil, 2009, p 135.
27. Ducatti, D. R. B.; Massi, A.; Noseda, M. D.; Duarte, M. E. R.; Dondoni, A. *Org. Biomol. Chem.* **2009**, *7*, 578–588.
28. Viana, A. G.; Noseda, M. D.; Duarte, M. E. R.; Cerezo, A. S. *Carbohydr. Polym.* **2004**, *58*, 455–460.
29. Guimaraes, M.; Ascencio, S. D.; Viana, A. G.; Plastino, E. M.; Duarte, M. E. R.; Noseda, M. D. *Bot. Mar.* **2007**, *50*, 314–317.
30. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
31. Yang, B. Y.; Brand, J.; Montgomery, R. *Carbohydr. Res.* **2001**, *331*, 59–67.
32. Gonçalves, A. G. Modificações Químicas em Oligossacarídeos Derivados de Polissacarídeos de Algas Marinhas. Ph.D Thesis, Universidade Federal do Paraná: Brazil, 2005, p 162.
33. Ducatti, D. R. B. Oligossacarídeos Obtidos por Hidrólise Redutiva Parcial de Polissacarídeos de Algas Vermelhas: Estudos de Ressonância Magnética Nuclear. MSc. Thesis, Universidade Federal do Paraná: Brazil, 2005, p 113.
34. Mank, M.; Stahl, B.; Boehm, G. *Anal. Chem.* **2004**, *76*, 2938–2950.
35. Laremore, T. N.; Murugesan, S.; Park, T.-J.; Avci, F. Y.; Zagorevski, D. V.; Linhardt, R. J. *Anal. Chem.* **2006**, *78*, 1774–1779.