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Matrix-assisted ultraviolet laser-desorption ionization and electrospray-ionization time-of-flight mass spectrometry of sulfated neocarrabiose oligosaccharides

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Abstract

Several commercial sulfated neocarrabiose oligosaccharides were analyzed by matrix-assisted ultraviolet laser-desorption ionization time-of-flight mass spectrometry (UV-MALDI-TOF-MS). UV-MALDI-TOF-MS was carried out in the linear and reflectron modes and, as routine, in both the positive- and negative-ion modes. 2,5-Dihydroxybenzoic acid and *nor*-harmane were used as matrices. In the positive- and negative-ion modes, with both matrices, peaks corresponding to $(M + Na)^+$ and $(M - Na)^-$ ions, respectively, were obtained, with only some signals due to glycosidic linkage cleavages (prompt fragmentation). With 2,5-dihydroxybenzoic acid abundant matrix signals were observed; *nor*-harmane afforded very few matrix signals in both ion modes, but more desulfation (prompt fragmentation) of the compounds occurred. When the desorption/ionization process was highly efficient, the post-source decay (PSD) fragmentation patterns were also investigated; most of the fragments detected derived from glycosidic linkage cleavages. Electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) in the negative-ion mode confirmed, with the observation of the $(M - Na)^-$ and the multiply charged anions, the identity and the purity of the samples.

Keywords: Sulfated oligosaccharides; Sulfated neocarrabiose oligosaccharides; *kappa*-Carrageenan oligosaccharides; UV-MALDI-TOF-MS; PSD UV-MALDI-TOF-MS; ESI-TOF-MS

1. Introduction

Matrix-assisted ultraviolet laser-desorption ionization time-of-flight mass spectrometry (UV-MALDI-TOF-MS) was developed primarily for the analysis of proteins.[1–3] Today, its use has been extended to many other classes of macromolecules, and particularly to carbohydrates.[4]

Recent studies, with low-molecular-weight laminarans,[5] fructans,[6] dextrans[7] and maltooligosaccharides,[7] illustrated the power of positive-ion mode MALDI-MS for defining the degree of polymerization profiles of polysaccharides. However, very few MALDI-MS analyses of sulfated oligosaccharides[8–10] have been reported, and only one of sulfated polysaccharides[11] has been reported. The sulfated oligosaccharides with molecular weights below 2000 were detected in the negative-ion mode.[8–10]

Sulfated oligosacharides and polysaccharides are difficult to study by MALDI, partly because of the labile nature of the sulfate group.[4] Thus, results can be erratic, indicating that there are still several experimental factors that affect the molecular weight measurements, i.e., the sample preparation method, the selected matrix and the type of mass analyzer used.

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kappa-Carrageenan[12] is essentially a linear sulfated polysaccharide consisting of alternating 3-linked β-D-galactopyranosyl 4-sulfate and 4-linked 3,6-anhydro-α-D-galactopyranosyl units. In an attempt to investigate the feasibility of UV-MALDI-TOF-MS for the analysis of carrageenans and depolymerized carrageenans, several commercial sulfated neocarrabiose oligosaccharides[13] (Fig. 1) were tested in the positive- and negative-ion modes and in linear and reflectron modes using 2,5-dihydroxybenzoic acid and *nor*-harmane as matrices. PSD experiments were also carried out, and structures of the fragment ions were assigned.

In addition, analysis of these sulfated oligosaccharides was performed by electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) in the negative-ion mode. Some of them have been previously analyzed by electrospray-ionization triple quadrupole mass spectrometry (ESI-QqQ-MS) in the negative-ion mode;[14] however, our studies carried out under milder conditions (lower capillary-tip potential) and with a TOF analyzer, gave rise to different spectral patterns.

2. Results

Table 1 lists the sulfated neocarrabiose oligosaccharides (sodium salts) studied, their sulfate content, and molecular weight.

UV-MALDI-TOF-MS.—2,5-Dihydroxybenzoic acid (gentisic acid, DHB) and *nor*-harmane (9*H*-pyrido[3,4-*b*]indole), were employed as matrices and UV-MALDI-TOF-MS experiments were carried out in the linear, reflectron, and PSD modes, and as a routine, in both the positive- and negative-ion modes. Laser-desorption ionization time-of-flight mass spectra (LD-TOF-MS) of the two matrices were carried out previous to the analysis of each sample.

Prompt fragmentation. Positive-ion mode. Table 2 shows the m/z, the relative intensities (RI) and the assignment of the peaks observed in the linear mass spectra of the sulfated oligosaccharides **1**–**7** when DHB was used as matrix. Even though molecular ions $(M + Na)^+$ were observed for all the samples, matrix cluster ions [$(nDHB + H)^+$, among others] covered a broad mass range of the spectrum. Moreover, for compound **5** the small molecular ion peak [$(M + Na)^+$, m/z 1368.4], was almost overlapped by the matrix signal at m/z 1375.6. Fig. 2A shows the spectrum of compound **6**. Besides, an analyte cluster ion [$(2M + Na)^+$] was observed for compound **3** at m/z 1693.8.

In the linear mode, only compound **3** gave a signal that is due to the fragmentation of a glycosidic linkage and corresponds to a B_3-ion (m/z 574.7), following the systematic nomenclature of Domon and Costello.[15] In the reflectron mode, for compounds **1**, **2** and **6**, in addition to the molecular ions, *Y*-cleavages with peaks at m/z 305.3 (Y_1-ion), 611.7 (Y_3-ion) and 1121.2 (Y_5-ion), respectively, were observed.

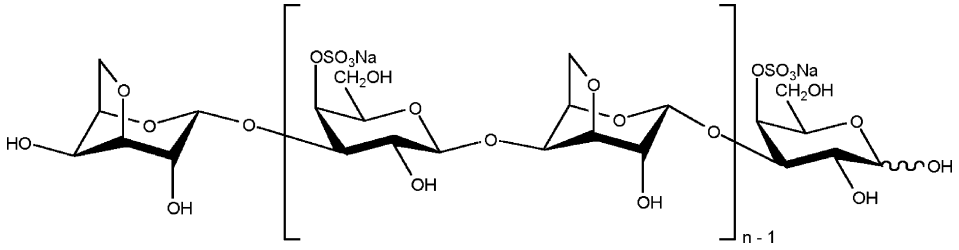

Fig. 1. Structure of the sulfated neocarrabiose oligosaccharides. For compound **2** there is no sulfate group at the 4^3-position. Compound **5** is also sulfated at the 2^4-position.

Table 1
List of the sulfated neocarrabiose oligosaccharides (sodium salts) analyzed in this study

Compound	Name	n[a]	Sulfate (as $NaSO_3$) %	Molecular weight[b]
1	Neocarrabiose 4^1-sulfate	1	23.9	426.3
2	Neocarratetraose 4^1-sulfate	2	13.9	732.6
3	Neocarratetraose $4^1,4^3$-disulfate	2	24.5	834.6
4	Neocarrahexaose $4^1,4^3,4^5$-trisulfate	3	24.6	1242.9
5	Neocarrahexaose $2^4,4^1,4^3,4^5$-tetrasulfate	3	30.3	1345.0
6	Neocarraoctaose $4^1,4^3,4^5,4^7$-tetrasulfate	4	24.7	1651.3
7	Neocarradodecaose $4^1,4^3,4^5,4^7,4^9,4^{11}$-hexasulfate	6	24.8	2467.9

[a] n corresponds to the number of repeating units in the oligosaccharide (see Fig. 1).
[b] Average molecular weights.

Table 2
Positive- and negative-ion mode UV-MALDI-TOF-MS of the sulfated model compounds carried out in the linear mode using DHB as matrix[a]

Compound	Positive ion-mode			Negative ion-mode		
	m/z	RI	Assignment	*m/z*	RI	Assignment
1	449.1	100	$(M+Na)^+$	578.8	42	$(M-DHB\text{-}H)^-$
				403.0	100	$(M-Na)^-$
2	754.8	100	$(M+Na)^+$	709.6	100	$(M-Na)^-$
3[b]	857.1	100	$(M+Na)^+$	811.8	100	$(M-Na)^-$
	754.9	17	$(M-NaSO_3+H+Na)^+$			
	574.7	29	B_3-ion[c]			
4	1266.1	100	$(M+Na)^+$	1219.2	100	$(M-Na)^-$
5	1368.4	100	$(M+Na)^+$	1321.6	100	$(M-Na)^-$
6	1674.2	100	$(M+Na)^+$	1628.2	100	$(M-Na)^-$
	1572.0	16	$(M-NaSO_3+H+Na)^+$			
7	2491.8	100	$(M+Na)^+$	2445.4	94	$(M-Na)^-$
	2389.2	22	$(M-NaSO_3+H+Na)^+$	2343.7	100	$(M-NaSO_3+H-Na)^-$

[a] Matrix signals and signals of compounds with RI lower than 10% have been excluded from the Table.
[b] In the positive-ion mode the peak at *m/z* 1693.8 (5%) was assigned to $(2M+Na)^+$.
[c] Cationized with Na^+.

The presence in the linear mode of a peak at *m/z* 754.9 for compound **3**, 1572.0 for compound **6,** and 2389.2 for compound **7**, is consistent with the loss of one sulfate group $(M-NaSO_3+H+Na)^+$ and indicates that desulfation took place in the ionization area of the mass spectrometer before applying the acceleration voltage (prompt fragmentation). Desulfation has been reported in the negative-ion ESI-MS of neocarrabiose oligosaccharides.[14]

Analysis of the sulfated oligosaccharides was also carried out in the positive-ion mode with *nor*-harmane as matrix. Even though fewer matrix cluster ions were observed, a low desorption/ionization efficiency was achieved. The lowest molecular weight oligosaccharide, compound **1**, afforded the most intense signals and gave in the linear mode, besides the molecular ion $(M+Na)^+$, *m/z* 449.6, analyte cluster ions corresponding to $(nM+Na)^+$ with $n=2$ and 3 at *m/z* 875.3 and 1301.2, respectively. For compounds **2**, **3** and **6** only weak signals due to molecular ions $(M+Na)^+$ were observed. No analyte signals were detected for compounds **4**, **5** and **7**.

Negative-ion mode. When DHB was used as matrix, only the molecular ion peaks $(M-Na)^-$ were found in the linear mode spectra of compounds **2**–**6** (Table 2). Similar to what was observed in the positive-ion mode, the molecular ion of compound **5** (*m/z* 1321.6) was small and appeared almost overlapped by the DHB signal at *m/z* 1328.5.

In the spectrum of compound **7** the signal at *m/z* 2343.7, due to desulfation, was also detected. For compound **1**, the peak at *m/z* 578.8 was assigned to $(M+DHB-H)^-$.

Even though no cleavage of glycosidic linkages was observed for any of the compounds in the linear mode, for compound **4** in the reflectron mode, peaks at *m/z* 954.6 and 937.8 (anionized by the loss of Na^+) consistent with C_5- and B_5-cleavages, respectively, were detected.

Compounds **1**–**7** were also analyzed using *nor*-harmane as matrix (Table 3). This matrix produced more intense signals but more desulfation (succesive loss of 102.0 Da from the molecular ion peak) than DHB, and for compounds **5**–**7**, the most intense peak of the spectrum did not correspond to the molecular ion $(M-Na)^-$, but to desulfated anions. Moreover, the number of sulfate groups lost is $(x-1)$, where x is the number of sulfate groups of the analyte. Fig. 3A shows the spectrum of compound **6**.

As observed in the positive-ion mode with this matrix, analyte cluster ions were also detected in the negative-ion mode spectrum of compound **1** at *m/z* 829.2 $(2M-Na)^-$ and *m/z* 1255.6 $(3M-Na)^-$, respectively.

In the spectrum of compound **5**, peaks at *m/z* 1260.3 and 1158.5 were found and attributed to $(M+H_2O-NaSO_3)^-$ and $(M+H_2O-2NaSO_3+H)^-$, respectively.[16] Similar type of fragments were obtained for

compounds **6** (m/z 1463.8) and **7** (m/z 2077.6). Retention of water can be explained taking into account its interaction with the negative charge site of the sulfated analytes.[17]

Cleavage of glycosidic linkages was only observed in the reflectron mode for compound **4** (m/z 955.3, C_5-ion) and in the linear mode for compound **7** (m/z 1483.6, Y_7-ion).

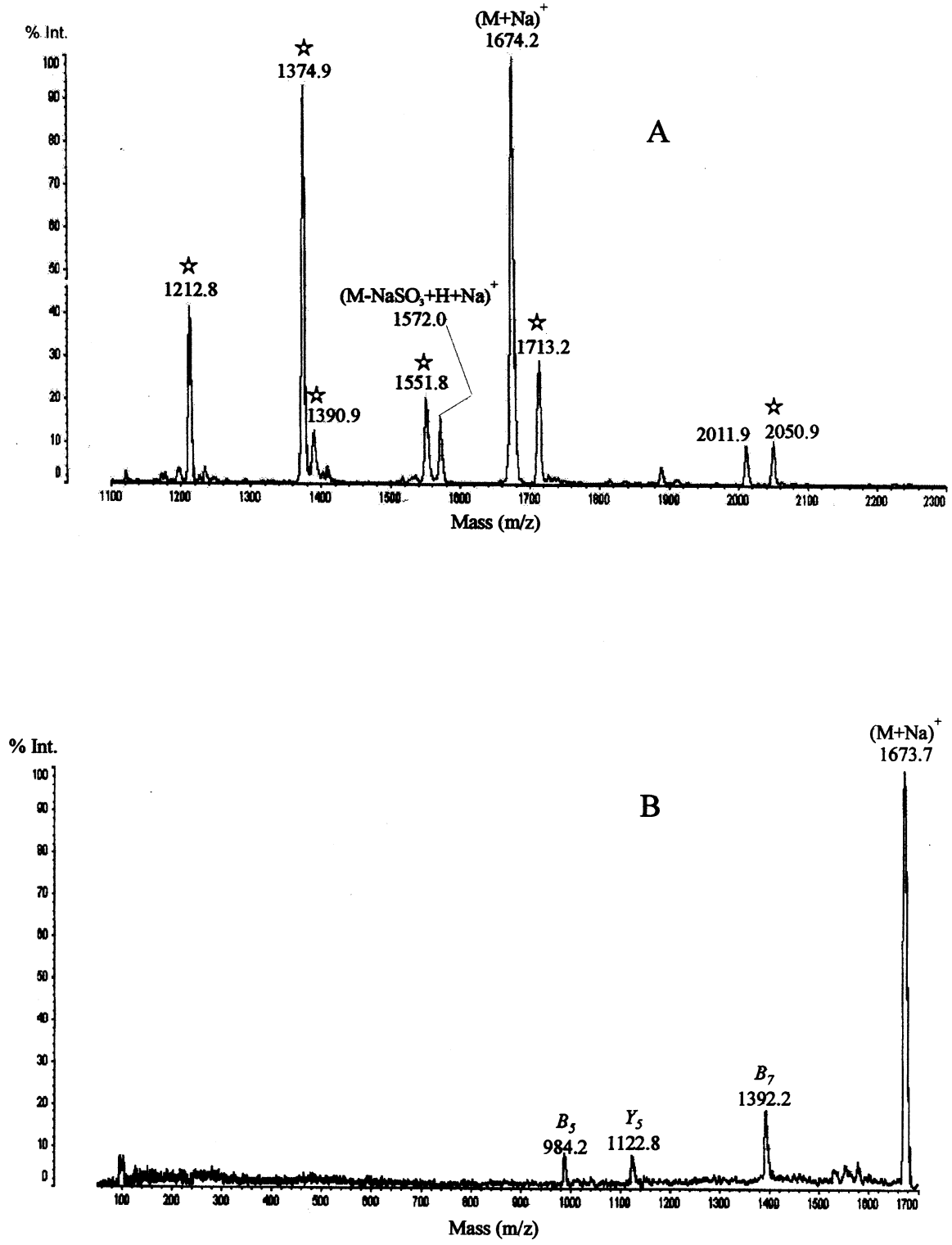

Fig. 2. Positive-ion mode UV-MALDI-TOF (A) and PSD UV-MALDI-TOF (B) mass spectra of compound **6** obtained using DHB as matrix. Peaks with an asterisk in spectrum (A) correspond to the matrix.

Table 3
Negative-ion mode UV-MALDI-TOF-MS of the sulfated model compounds carried out in the linear mode using *nor*-harmane as matrix[a]

Compound	*m/z*	RI	Assignment
1[b]	829.2	27	$(2M-Na)^-$
	403.4	100	$(M-Na)^-$
2	709.3	100	$(M-Na)^-$
3	811.7	100	$(M-Na)^-$
	709.8	35	$(M-NaSO_3+H-Na)^-$
4	1219.9	100	$(M-Na)^-$
	1118.2	74	$(M-NaSO_3+H-Na)^-$
5	1322.3	27	$(M-Na)^-$
	1260.3	21	$(M+H_2O-NaSO_3)^-$
	1220.4	50	$(M-NaSO_3+H-Na)^-$
	1158.5	42	$(M+H_2O-2NaSO_3+H)^-$
	1118.6	98	$(M-2NaSO_3+2H-Na)^-$
	1016.7	100	$(M-3NaSO_3+3H-Na)^-$
6	1627.8	88	$(M-Na)^-$
	1539.8	12	
	1525.8	40	$(M-NaSO_3+H-Na)^-$
	1463.8	28	$(M+H_2O-2NaSO_3+H)^-$
	1423.8	48	$(M-2NaSO_3+2H-Na)^-$
	1321.8	100	$(M-3NaSO_3+3H-Na)^-$
7	2444.9	33	$(M-Na)^-$
	2342.1	28	$(M-NaSO_3+H-Na)^-$
	2077.6	29	$(M+H_2O-4NaSO_3+3H)^-$
	2037.1	31	$(M-4NaSO_3+4H-Na)^-$
	1934.6	100	$(M-5NaSO_3+5H-Na)^-$
	1483.6	29	Y_7-ion[c]
	1447.9	48	

[a] Matrix signals and signals of compounds with RI lower than 10% have been excluded from the Table. Peaks in boldface type were not assigned.
[b] The peak at *m/z* 1255.6 (3%) was assigned to $(3M-Na)^-$.
[c] Anionized by loss of Na^+.

PSD mode. Post-source decay (PSD) fragmentation is the option used for obtaining information about molecular structure from UV-MALDI-TOF-MS, and it has been already applied to neutral oligosaccharides and glycoconjugates.[4] In the present study, PSD analysis was undertaken to investigate fragmentation/desulfation, as well as to obtain information concerning the use of this technique for the determination of the sequence of units in an unknown oligosaccharide.

Positive-ion mode. It has been reported[18,19] that PSD spectra of the $(M+Na)^+$ ions of neutral oligosaccharides tend to be dominated by glycosidic and secondary cleavage reactions with only weak contribution from cross-ring products. The PSD spectra of the sulfated oligosaccharides using both matrices showed major ions as the result of *Y*- and/or *B*-cleavages (Table 4). For compound **1,** besides the molecular ion, only the peak corresponding to an Y_1-ion was detected with both matrices; for compound **3** the B_3-ion was observed. On the other hand, compound **4** gave with DHB peaks at *m/z* 1121.9 and 984.3, due to Y_5- and B_5-cleavages, respectively. Fig. 2B shows the PSD spectrum of compound **6** obtained using DHB as matrix.These results indicate, as expected, a preferential cleavage of the 3,6-anhydrogalactosidic linkages.

Negative-ion mode. Compound **1** gave, with DHB (Table 4) peaks at *m/z* 260.5 (Y_1-cleavage) and 103.9. This type of fragmentation has been previously observed in the collision-induced dissociation (CID) ESI-QqQ mass spectrum of the same oligosaccharide;[14] the latter peak corresponds to $NaSO_3^-$. The PSD spectrum of compound **4** produced with the same matrix the peaks at *m/z* 956.5 (C_5-cleavage) and 938.0 (B_5-cleavage), both of which were previously observed in the reflectron mode and are due to prompt fragmentation.

With *nor*-harmane as matrix, more fragments were detected. In the mass spectrum of compound **1**, besides the signal at *m/z* 259.0 (Y_1-ion), peaks at *m/z* 241.2 and 97.0 were observed. The peak at *m/z* 241.2 is derived from a Z_1-cleavage of the glycosidic bond; the signal at *m/z* 97.0 corresponds to HSO_4^-. For compound **2**, fragments at *m/z* 564.9 and 258.5, due to Y_3- and Y_1-cleavages, respectively, were formed; compound **3** gave fragments at *m/z* 666.9 and 528.8 ascribed to Y_3- and B_3-cleavages. Fig. 3B shows the PSD spectrum of compound **6**, and the fragmentation pattern is indicated in Fig. 4. The signal at *m/z* 1527.8 was assigned to desulfation; no sulfate elimination was observed for the other oligosaccharides. For compound **7**, besides the ions corresponding to glycosidic linkage cleavages of the *Y*- and *B*-types, a doubly charged ion $(M-2Na)^{2-}$[18] at *m/z* 1209.8 (calcd *m/z* 1210.9) was found.

ESI-TOF-MS.—The samples were analyzed by ESI-TOF-MS in the positive- and negative-ion modes; no signals were detected in the former mode, but good spectra were obtained in the latter.

ESI-QqQ-MS in the negative-ion mode of compounds **1**, **3**, **4**, **6**, and **7** have already been reported.[14] Our spectra, which were determined under milder conditions (lower capillary-tip potential) and with a TOF analyzer, allowed the detection of signals corresponding to $(M-Na)^-$ for all the compounds (see Experimental). For compounds **1** and **2**, this peak was the most intense signal of the spectrum; for oligosaccharides with a higher number of sulfate groups, small/trace signals were obtained for $(M-Na)^-$, and intense peaks corresponding to multiply charged anions were observed.

Compound **5,** the oligosaccharide with higher sulfate content (Table 1), is the only one in which a signal at *m/z* 391.0 could be attributed to a desulfated anion $(M-NaSO_3+H\text{-}3Na)^{3-}$. Fig. 5 shows the spectrum of compound **5**.

3. Discussion

Important peaks corresponding to matrix cluster ions were observed in the positive- and negative-ion mode UV-MALDI-TOF mass spectra of compounds **1**–**7**, when DHB was used as matrix. Moreover, for compound **5** these signals interfered with the molecular-ion detection. With this matrix a high desorption/ionization efficiency was achieved in the positive-ion mode. A particularly good spectrum was obtained for compound **2**, the oligosaccharide with the lowest sulfate content investigated. This result is in agreement with the fact that, for neutral oligosaccharides, very good spectra were obtained with this matrix.[4] In the negative-ion mode all the compounds gave only the molecular ion $(M-Na)^-$, with the exception of compound **7**, for which loss of one sulfate group was observed (m/z 2343.7). Only a few fragments, but of considerable RI, were detected in the reflectron mode (compound **4**, m/z 954.6 and 937.8; compound **7**, m/z 2343.3).

A low desorption/ionization efficiency was observed for these compounds in the positive-ion ion mode when *nor*-harmane was used as matrix. Good spectra were obtained in the negative-ion mode, but extensive desulfation occurred. A few matrix cluster ions were detected in both ion modes.

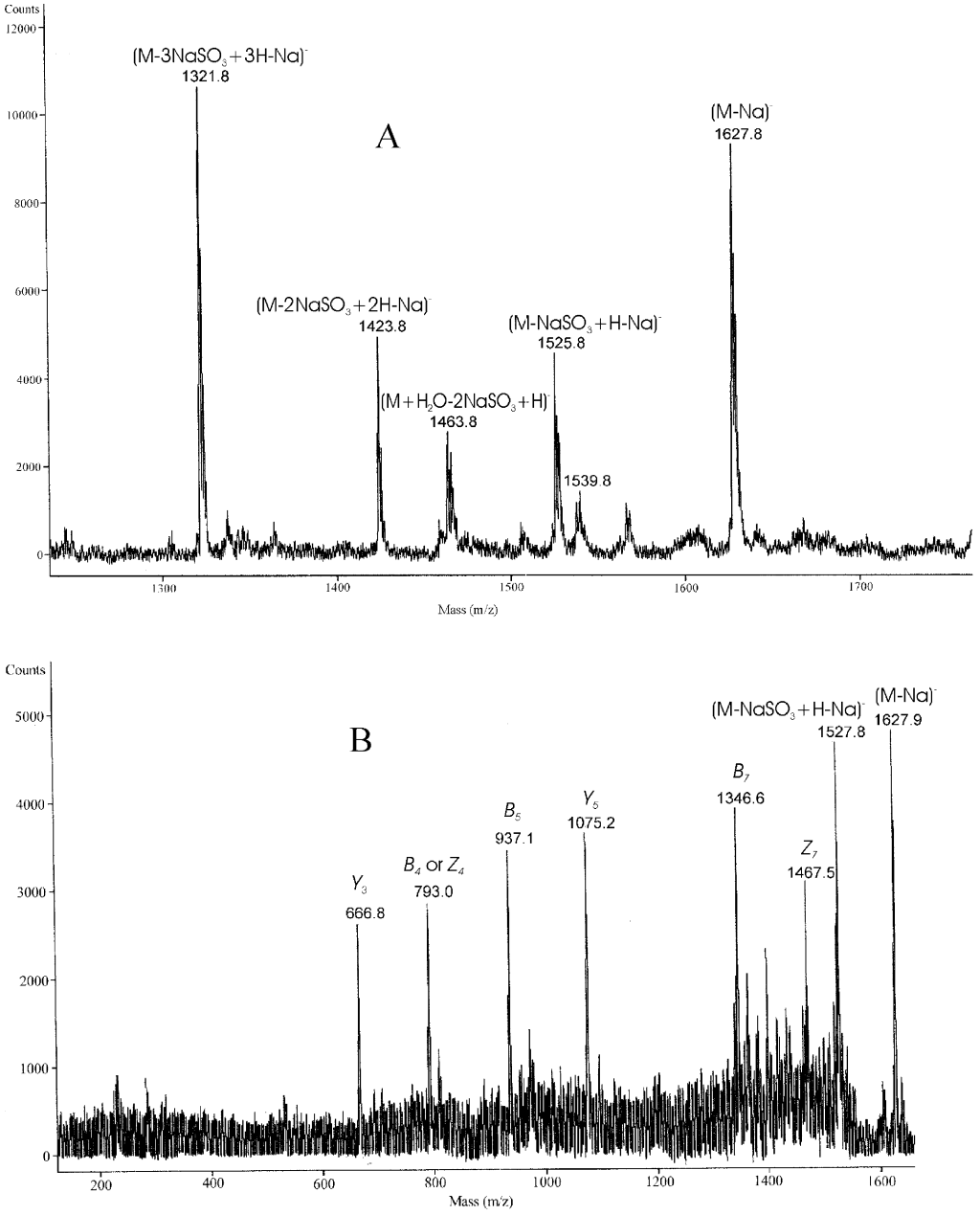

Fig. 3. Negative-ion mode UV-MALDI-TOF (A) and PSD UV-MALDI-TOF (B) mass spectra of compound **6** obtained using *nor*-harmane as matrix.

Table 4
Positive- and negative-ion mode PSD experiments of the sulfated model compounds carried out using DHB and *nor*-harmane as matrices[a,b,c]

Compound	Matrix	Gate (m/z)	Fragments (m/z, type)
Positive-ion mode			
1	DHB	424–474	449.7, 305.9 (Y_1)
2	DHB	715–796	755.2, **636.9**, 611.2 (Y_3), 474.2 (B_3)
3	DHB	809–909	858.4, 576.5 (B_3)
4	DHB	1200–1338	1265.4, 1121.9 (Y_5), 984.3 (B_5)
6	DHB	1585–1769	1673.7, 1392.2 (B_7), 1122.8 (Y_5), 984.2 (B_5)
1	*nor*-Harmane	449.0	449.3, 305.3 (Y_1)
2	*nor*-Harmane	755.0	755.0, 574.5 (Z_3 -H_2O), 472.7 (B_3), 304.5 (Y_1)
3	*nor*-Harmane	857.0	857.6, 575.3 (B_3)
6	*nor*-Harmane	1673.8	1673.9, 1391.6 (B_7), 1121.2 (Y_5), 983.2 (B_5), 838.8 (B_4 or Z_4), 714.5 (Y_3), 576.4 (B_3)
Negative-ion mode			
1	DHB	382–425	403.8, 260.5 (Y_1), 103.9 ($NaSO_3^-$)
3	DHB	762–862	812.3
4	DHB	1153–1289	1220.1, 956.5 (C_5), 938.0 (B_5)
1	*nor*-Harmane	403.0	403.3, **357.4**, **355.4**, 259.0 (Y_1), 241.2 (Z_1), 97.0 (HSO_4^-)
2	*nor*-Harmane	709.0	709.0, 564.9 (Y_3), **478.6**, 258.5 (Y_1)
3	*nor*-Harmane	810.9	811.0, 666.9 (Y_3), 528.8 (B_3)
4	*nor*-Harmane	1219.0	1219.0
6	*nor*-Harmane	1627.9	1627.9, 1527.8 $(M-NaSO_3+H-Na)^-$, 1467.5 (Z_7), 1346.6 (B_7), 1075.2 (Y_5), 937.1 (B_5), 793.0 (B_4 or Z_4), 666.8 (Y_3)
7	*nor*-Harmane	2444.8	2444.8, **2385.1**, **2314.5**, 1891.7 (Y_9), 1755.2 (B_9), 1483.3 (Y_7), 1344.9 (B_7), 1209.8 $(M-2Na)^{2-}$, 1074.7 (Y_5)

[a] PSD experiments were carried out when signals of the molecular ions were intense enough, in the reflectron mode.
[b] Peaks in boldface type were not assigned.
[c] Positive fragments are cationized with Na^+. Negative fragments, designated according to Domon and Costello,[15] are anionized by loss of Na^+.

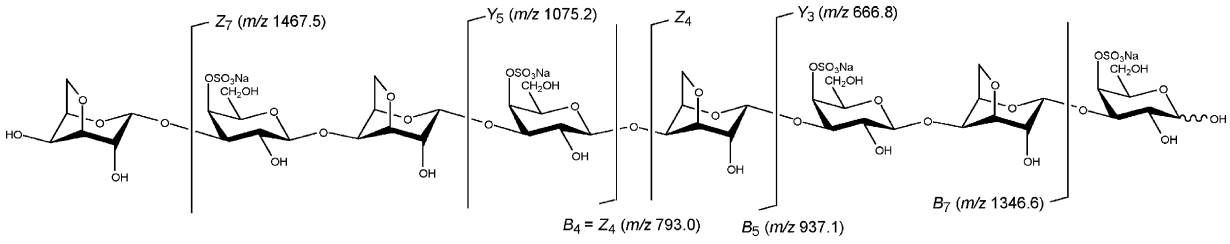

Fig. 4. PSD fragmentation pattern of compound **6** obtained in the negative ion-mode using *nor*-harmane as matrix.

In the negative-ion mode and with *nor*-harmane, spectral analysis was consistent with a maximum loss of $(x-1)$ sulfate groups, where x is the number of sulfates in the analyte, suggesting that the non-ionized sulfate groups are more easily lost than the stabilized sulfate anion (see later) or that the monocharged anion, with only one sulfate group, is the most stable in the UV-MALDI process.

In the PSD spectra, signals corresponding to glycosidic linkage cleavages were observed. As expected, fragmentation mainly occurred in the more labile 3,6-anhydrogalactosidic linkages. No sulfate elimination was detected (except for compound **6** in the negative-ion mode and with *nor*-harmane). This result, together with the sulfate elimination observed in the linear and reflectron modes, is consistent with the fact that the loss of sulfate takes place in the ionization area of the mass spectrometer before applying the acceleration voltage (prompt fragmentation).

ESI-TOF-MS in the negative-ion mode confirmed, with the observation of the $(M-Na)^-$ (small/trace for the higher molecular weight oligosaccharides) and the

multiply charged anions, the identity and purity of the compounds. Desulfation was only detected for compound **5** and was attributed to the loss of the sulfate group at the 2-position of the 3,6-anhydrogalactose residue (see later). These results differ from those previously reported[14] where desulfation at the 4-position of the galactose units was observed for the different oligosaccharides, probably due to the higher vibrational energy acquired in the volatilization/ionization process.

The high efficiency of the ionization processes of these compounds in negative UV-MALDI-TOF-MS and in negative ESI-TOF-MS confirms the previous suggestion that sulfate groups stabilize the corresponding anionic forms.[9] The sulfate anion could be stabilized through a hydrogen bond-like interaction with the primary hydroxyl of the β-galactose unit. This is consistent with the elimination of the sulfate on the 2-position of the 3,6-anhydrogalactose residue (compound **5**) by the proposed intramolecular rearrangement reaction followed by expulsion of SO_3,[14] considering that it has a lower possibility of stabilization (see Fig. 1).

In conclusion, UV-MALDI-TOF-MS has proved to be a useful tool for the analysis of sulfated oligosaccharides. DHB was an effective matrix, but it should be kept in mind the presence of abundant matrix ions, in both ion modes. *Nor*-harmane gave extensive desulfation (prompt fragmentation) in the negative-ion mode. PSD analysis provided data about the sequence of monosaccharide residues. Negative ESI-TOF-MS is an important technique and it has been used complementary to UV-MALDI-TOF-MS, in order to establish if the analyte was pure or if it was a mixture of oligosaccharides with different degrees of sulfation; thus, prompt fragmentation could be studied.

4. Experimental

UV-MALDI-TOF-MS.—Matrix chemicals. The β-carboline (9*H*-pyrido[3,4-*b*]indole) *nor*-harmane, 2,5-dihydroxybenzoic acid (DHB, gentisic acid) and 3,5-dimethoxy-4-hydroxycinnamic acid (SA, sinapinic acid; used for protein calibrants) were obtained from Aldrich Chemical Co.

Analytes. The seven sulfated neocarrabiose oligosaccharides: neocarrabiose 4^1-sulfate (MW 426.3); neocarratetraose 4^1-sulfate (MW 732.6); neocarratetraose $4^1,4^3$-disulfate (MW 834.6); neocarrahexaose $4^1,4^3,4^5$-trisulfate (MW 1242.9); neocarrahexaose $2^4,4^1,4^3,4^5$-tetrasulfate (MW 1345.0); neocarraoctaose $4^1,4^3,4^5,4^7$–tetrasulfate (MW 1651.3); neocarradodecaose $4^1,4^3,4^5,4^7,4^9,4^{11}$-hexasulfate (MW 2467.9), were obtained from Sigma Chemical Co.

Calibrating chemicals. Caffeine (MW 149.19); tetrabutylammonium bromide (MW 322.37); 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); neurotensin (N6383, MW 1672.96) and bovine insulin (I5500, MW 5733.5) were purchased from Sigma-Aldrich.

Solvents. MeOH, EtOH and MeCN (Sigma-Aldrich, HPLC grade). Water of very low conductivity (Milli Q grade; 56–59 nS/cm with PURIC-S, ORUGANO Co., Ltd., Tokyo, Japan) was used.

Instruments. Measurements were performed with: (a) a Shimadzu Kratos, Kompact MALDI 4 (Pulsed Extraction) laser-desorption time-of-flight mass spectrometer (Shimadzu, Kyoto, Japan) and (b) an Applied Biosystems Voyager DE-STR laser-desorption time-of-flight mass spectrometer. Both spectrometers were equipped with pulsed nitrogen laser ($\lambda_{em} = 337$ nm;

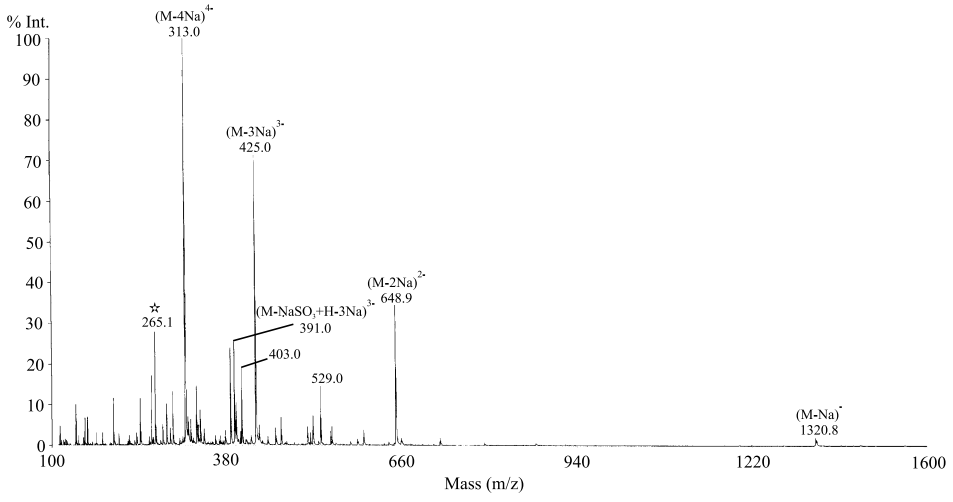

Fig. 5. Negative-ion mode ESI-TOF mass spectrum of compound **5**. The peak with an asterisk corresponds to the solvent.

pulse width = 3 ns), tunable PDE and PSD modes, as described elsewhere.[9,20–22] Data shown in Tables 2–4 were obtained with the former instrument, when DHB was used as matrix, and with the latter when *nor*-harmane was the matrix.

The samples were 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.

All the compounds were measured in the linear and reflectron modes, in both positive- and negative-ion modes. In all the cases that the signals of molecular ions were enough intense in the reflectron mode, the PSD experiments were also performed in positive and/or negative-ion mode.

Probe supports. The stainless steel polished surface twenty-sample-slides were purchased from Shimadzu Co., Japan (P/N 670-19109-01). 199-Welled gold sample plates (P/N V700401) were used in the Applied Biosystems Voyager DE-STR mass spectrometer

Sample preparation. 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 MeCN–H_2O. Analyte solutions were freshly prepared by dissolving the carbohydrates (1 mg) in 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, typically 0.5 μL of the matrix solution was placed on the sample probe tip, and the solvent was removed by blowing air at room temperature. Subsequently, 0.5 μL of the analyte solution was placed on the same probe tip covering the matrix and partially dissolving it, and the solvent was removed by blowing air. Then, two additional portions (0.5 μL) of the matrix solution were deposited on the same sample probe tip, producing a partial dissolution of the previously deposited thin-film matrix and analyte layers. The matrix to analyte ratio was 3:1 (v/v) and the matrix and analyte solution loading sequence was: (i) matrix, (ii) analyte, (iii) matrix, and (iv) matrix. *Method B*, the analyte stock solution was mixed with the matrix solution in a 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 partially redissolve, and the solvent was removed by blowing air.

The resulting crystalline layers were found to be relatively homogeneous in both cases. When DHB was used as matrix, signals of higher intensity and more narrow peaks were obtained with Method B. *nor*-Harmane as matrix showed signals of similar quality when either Method A or Method B was used. Thus, the results shown in the present paper are those obtained for each analyte under the optimum experimental conditions.

Spectrum calibration. Spectra were calibrated by use of external calibration reagents.[9,11,20–22] In the PSD mode: the whole fragmentation patterns of angiotensin I and β-cyclomaltoheptaose (β-CD) were used as described elsewhere.[22] The Kratos Kompact calibration program and the Voyager DE-STR calibration program were respectively used.

LD mass spectra of DHB and *nor*-harmane were determined in the positive- and negative-ion mode (m/z range 300–2500 Da) in order to determine the background.

ESI-TOF-MS.—All compounds were analyzed in positive- and negative-ion mode, although only in the latter mode signals were obtained. The negative-ion ESI-TOF mass spectra were acquired by directly infusing 0.5–50 μL of a solution (50–100 μM) of the sulfated carbohydrate in 9:1 MeOH–H_2O into the ESI ion source of a Mariner Applied Biosystems ESI-TOF mass spectrometer at room temperature, using 9:1 MeOH–H_2O as the solvent stream. Spectra were recorded in a m/z range between 100 and 4000 Da. The spray tip potential was + 2796.68 V, the nozzle potential was + 245.12 V and the skimmer voltage was + 11.01 V. The nozzle temperature was 140 °C. A Harvard PHD 2000 syringe infusion pump at a flow rate of 5 μL min^{-1} was used for the introduction of the carbohydrate solution. The N_2 flow rate was 0.40 L min^{-1}, the analyzer temperature was kept at 29.0 °C and pressure at 0.55 MPa. The mass calibration was achieved by using a 1 μM solution of the selected calibrant chemical: caffeine; tetrabutylammonium bromide; β-estradiol-3-sulfate-17-glucuronide dipotassium salt; cyclomaltooctoase and angiotensin I (z = 1, 1296.685 Da; z = 2, 648.342 Da), in 9:1 MeOH–H_2O.

*ESI-TOF mass spectra of compounds **1**–**7**.* **1**: m/z (RI) 501.5 (3%),425.0 [4%, $(M-H)^-$], 403.0 [100%, $(M-Na)^-$].

2: m/z (RI) 731.1 [6%, $(M-H)^-$], 709.2 [100%, $(M-Na)^-$].

3: m/z (RI) 811.1 [8%, $(M-Na)^-$], 465.0 (2%), 394.1 [100%, $(M-2Na)^{2-}$].

4: m/z (RI) 1242.4 [1%, $(M-H)^-$], 1219.1 [3%, $(M-Na)^-$], 823.4 (5%), 668.9 (3%), 598.0 [42%, $(M-2Na)^{2-}$], 530.0 [4%, B_3-ion], 466.0 (13%), 454.0 (11%), 391.0 [100%, $(M-3Na)^{3-}$].

5: m/z (RI) 1320.8 [3%, $(M-Na)^-$], 648.9 [37%, $(M-2Na)^{2-}$], 529.0 (15%), 425.0 [73%, $(M-3Na)^{3-}$], 403.0 (19%), 391.0 [28%, $(M-NaSO_3+H-3Na)^{3-}$], 313.0 [100%, $(M-4Na)^{4-}$].

6: m/z (RI) 1627.9 [tr, $(M-Na)^-$], 872.9 (3%), 813.0 [2%, $(M-H-Na)^{2-}$], 802.0 [18%, $(M-2Na)^{2-}$], 654.0 (2%), 573.0 (3%), 527.0 [54%, $(M-3Na)^{3-}$], 484.0 (9%), 389.5 [100%, $(M-4Na)^{4-}$].

7: m/z (RI) 2444.7 [2%, $(M - Na)^-$], 1621.8 (4%), 1209.9 [21%, $(M - 2Na)^{2-}$], 799.6 [23%, $(M - 3Na)^{3-}$], 593.5 [23%, $(M - 4Na)^{4-}$], 470.2 [22%, $(M - 5Na)^{5-}$], 388.0 [100%, $(M - 6Na)^{6-}$].

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