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Short communication

Positive ion MALDI-TOF mass spectra are more suitable than negative ion spectra to characterize sulphated glycosaminoglycan oligosaccharides

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ABSTRACT

Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) is increasingly used in the field of carbohydrate characterization. The majority of oligosaccharides can be investigated with only a minimum extent of fragmentation. However, significant but unwanted sulphate loss occurs if the negative ion MALDI mass spectra of sulphated carbohydrates are recorded. We will show here by using differently sulphated oligosaccharides of the chondroitin sulphate (CS) type that this problem can be minimized by recording the positive ion spectra in the presence of the common MALDI matrix DHB (2,5-dihydroxybenzoic acid). Although the sensitivity of the positive ion spectra is reduced in comparison to the negative ion spectra, the reduction of the sulphate loss is a major advantage – particularly regarding the analysis of mixtures.

1. Introduction

Charged polysaccharides of the glycosaminoglycan (GAG) type such as hyaluronan (HA) or chondroitin sulphate (CS) are important constituents of the extracellular matrix (ECM) of connective tissues [1]. Besides their structural roles in the ECM, particularly HA is currently attracting considerable interest [2]: HA has many biomedical applications [3] and is used, for instance, as an additive to increase the viscosity of the joint fluids from patients suffering from rheumatic diseases. Since the discovery of the "sulphation code", particularly (over)sulphated polysaccharides (such as heparin) are also experiencing considerable scientific interest [4].

Mass spectrometry (MS) is unequivocally a powerful tool of GAG characterization and (normally in combination with other methods such as NMR) widely used to elucidate the structures of isolated GAG oligosaccharides [5]. Unfortunately, intact high molecular weight GAGs are not detectable by MS [6] (independent of the applied ionization technique) because of their high molecular weights and their sulphate residues, which represent strong electrolytes. The sulphate residues make sulphated GAG oligosaccharides rather refractive to MS characterization and reduce the achievable ion yields significantly [1].

Although electrospray ionization (ESI) MS is more often used [7], it is well known that oligosaccharides derived from non-sulphated and low-sulphated GAGs can be also conveniently characterized by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) MS [8]. MALDI MS offers several important advantages: for instance, MALDI devices can be handled very easily, a high sample throughput is possible, there are nearly exclusively singly charged ions and MALDI tolerates impurities to a higher extent than ESI MS. However, the sulphate loss that occurs under MALDI MS conditions is a considerable problem and complicates particularly mixture analysis. Although liquid crystalline matrices (sometimes in combination with the addition of CsCl) have been indicated to be helpful to minimize the sulphate loss, these approaches are so far not widely used [9].

Therefore, the aim of this study is the comparison of the positive and negative ion MALDI-TOF mass spectra of isolated oligosaccharides from HA and CS. 9-Aminoacridine (9-AA) [10] and 2,5-dihydroxybenzoic acid (DHB) [11] will be applied as matrices due to their different p*K* values of 9.99 and 2.76, respectively. This makes them differently suitable to record negative and positive ion mode spectra. Although 9-AA is quite a "new" matrix, a number of useful applications of this matrix have been recently described [12,13].

It will be shown that the positive ion MALDI mass spectra of strongly acidic oligosaccharides can be recorded with only slightly reduced sensitivities in comparison to the negative ion spectra. However, the most important advantage of the positive ion mass

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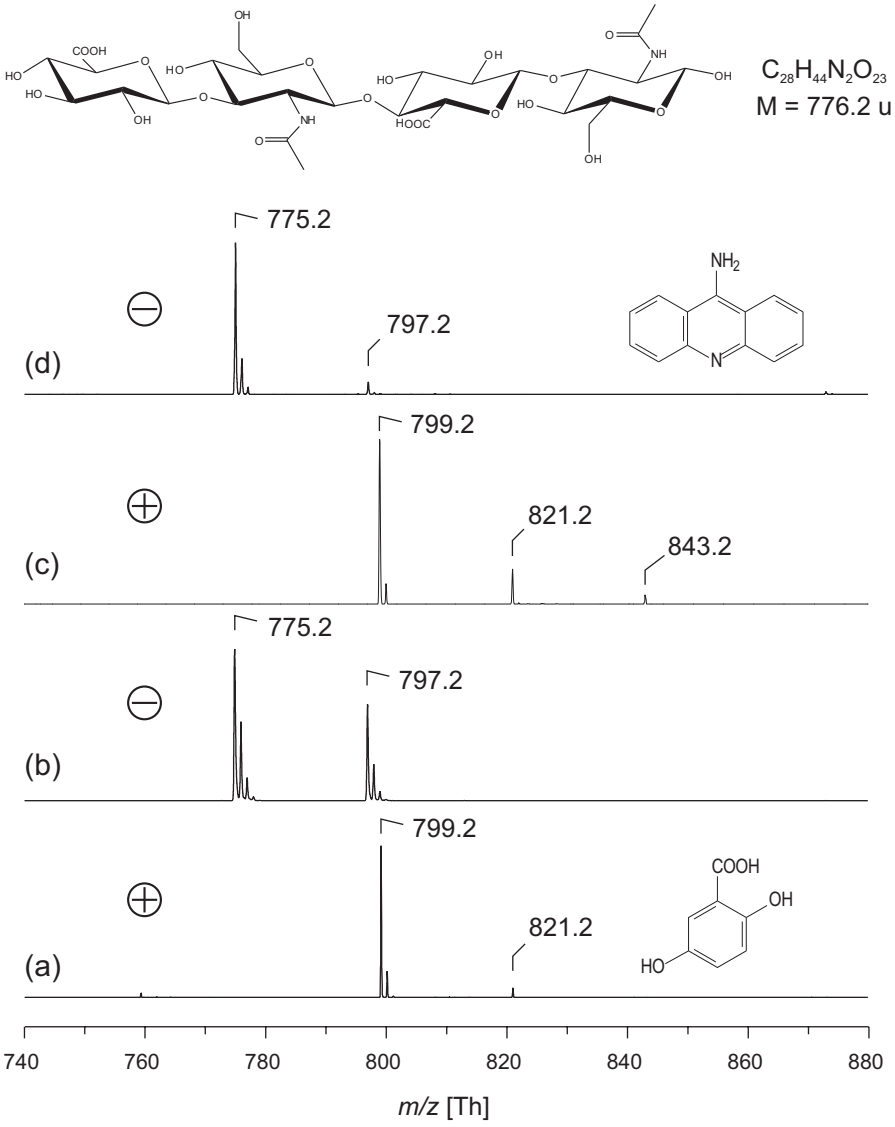

Fig. 1. MALDI-TOF mass spectra of an isolated HA tetrasaccharide. 5 μl of a 1 mg/ml HA-4 solution was mixed with 5 μl of either DHB or 9-AA. 1 μl of the mixture was applied on the target. Spectra (a) and (b) were recorded in the presence of DHB while (c) and (d) were recorded with 9-AA as matrix. The structure of HA-4 and its monoisotopic mass is given at the top of the figure, while the structures of both matrices are given in the corresponding traces. Please note that there are only slight differences between the positive (a) and (c) and the negative ion spectra (b) and (d) regarding the achievable spectral qualities.

spectra is the significantly reduced loss of the sulphate residues. We conclude that the application of ionic liquid matrices is not an absolute necessity to monitor sulphated GAGs [9] but common DHB is also a powerful matrix that helps to overcome many problems.

2. Experimental

2.1. Chemicals

All chemicals, solvents, enzymes and MALDI matrices were obtained in highest commercially available purity from Sigma–Aldrich (Deisenhofen, Germany). They were used without further purification. The HA tetrasaccharide was from Hyalose (Oklahoma City, USA) while the CS tetrasaccharide was obtained by hyaluronidase digestion of the native CS polysaccharide (from bovine trachea) and subsequent purification by TLC [14]. Shortly, 500 μl of a 20 mg/ml CS solution was digested by 250 μl 10 mg/ml testicular hyaluronidase. As the enzyme generates a mixture of different oligosaccharides, preparative normal-phase (silica gel 60) thin-layer chromatography (TLC) using butanol:formic acid:water (3:4:1, v/v/v) was used to isolate the tetrasaccharide. This compound was scraped from the silica gel and re-eluted with water. Its concentration was determined using a modified carbazol method [15]. The purities of all compounds were checked by means of high resolution NMR spectroscopy prior to use. An oversulphated chondroitin disaccharide (Δdi-trisulphate CS) was additionally purchased from DEXTRA (Reading, UK) and used as supplied.

2.2. MALDI-TOF MS measurements

All MALDI-TOF mass spectra were recorded on a Bruker Autoflex™ mass spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [16,17].

The system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage was 20 kV. 100 single laser shots were averaged for each mass spectrum. Positive and negative ion spectra were acquired. The laser fluence was kept about five percent above threshold to obtain an optimum signal to noise (S/N) ratio. In order to enhance the spectral resolution all spectra were

acquired in the reflector mode using delayed extraction conditions. 0.5 M (77 mg/ml) DHB was prepared in methanol [18] and 9-AA (10 mg/ml) in 60:40 (v/v) isopropanol/acetonitrile [19]. The tetrasaccharide solutions (about 1 mg/ml) were mixed 1:1 (v/v) with the individual matrices prior to deposition onto the MALDI targets. 1 μl of the matrix/sample mixture was applied onto the target. Spectra were processed with the program Flex Analysis™ provided by Bruker Daltonics.

3. Results and discussion

HA is the only GAG without sulphate residues. As the carboxylate residue represents only a weak electrolyte with a p*K* value of about 3.21 [3], the characterization of HA oligosaccharides by means of MALDI MS is possible without any problems. Therefore, the purified HA tetrasaccharide was initially investigated in order to check the efficiencies of both matrices, 9-AA and DHB, because 9-AA has not yet been used in the GAG field but only to analyze lipids [20] as well as small acidic molecules [21]. Besides its basic properties, the very low background [21] of 9-AA is a significant advantage of this matrix. The obtained MALDI mass spectra are shown in Fig. 1. Spectra (a) and (b) were recorded in the presence of DHB while 9-AA was used in traces (c) and (d). The expected masses of HA-4 can be detected in the positive (a) and (c) as well as the negative ion spectra (b) and (d). The mass of the free acid of HA-4 is 776.2 ($C_{28}H_{44}N_2O_{23}$). Therefore, the peak at *m*/*z* 775.2 in the negative ion spectra (b) and (d) corresponds to the singly charged ion subsequent to deprotonation, while the peak at *m*/*z* 797.2 is caused by H^+/Na^+ exchange. It is not surprising that the intensity of the Na^+ adduct is higher in the case of DHB (b) because this matrix has a higher polarity and was prepared in a more polar solvent (containing more salt as impurity) than 9-AA (d). It is surprising that this behaviour changes if the positive ion spectra are considered: using 9-AA (c) the ions with higher sodium contents (*m*/*z* 821.2 and 843.2) are more abundant relative to the pure Na^+ adduct at *m*/*z* 799.2 in comparison to DHB (a). It is not the aim of this paper to clarify this aspect in detail but it seems reasonable to assume that the enhanced basicity of the 9-AA leads to more pronounced H^+ abstraction accompanied by addition of Na^+. It is obvious that 9-AA is the matrix of choice in the negative ion mode (d) because here the analyte ionizes as a single species. This results in the best sensitivity and is illustrated by the data shown in Table 1 where the sample amount (in order to achieve an arbitrarily defined

Table 1
MALDI MS detection limits of HA-4 in the presence of either 9-AA or DHB as matrices. A signal-to-noise ratio of 10 was defined as the detection limit. Selected peaks from the positive (+) and negative (−) ion mass spectra were selected. n.a. indicates that no detection was possible under these conditions. For details see text.

Detection limit	9-AA	DHB
m/*z* 775.2 (−)	130 pg	500 pg
m/*z* 797.2 (−)	500 pg	800 pg
m/*z* 799.2 (+)	5 ng	500 pg
m/*z* 821.2 (+)	100 ng	50 ng
m/*z* 843.2 (+)	170 ng	n.a.

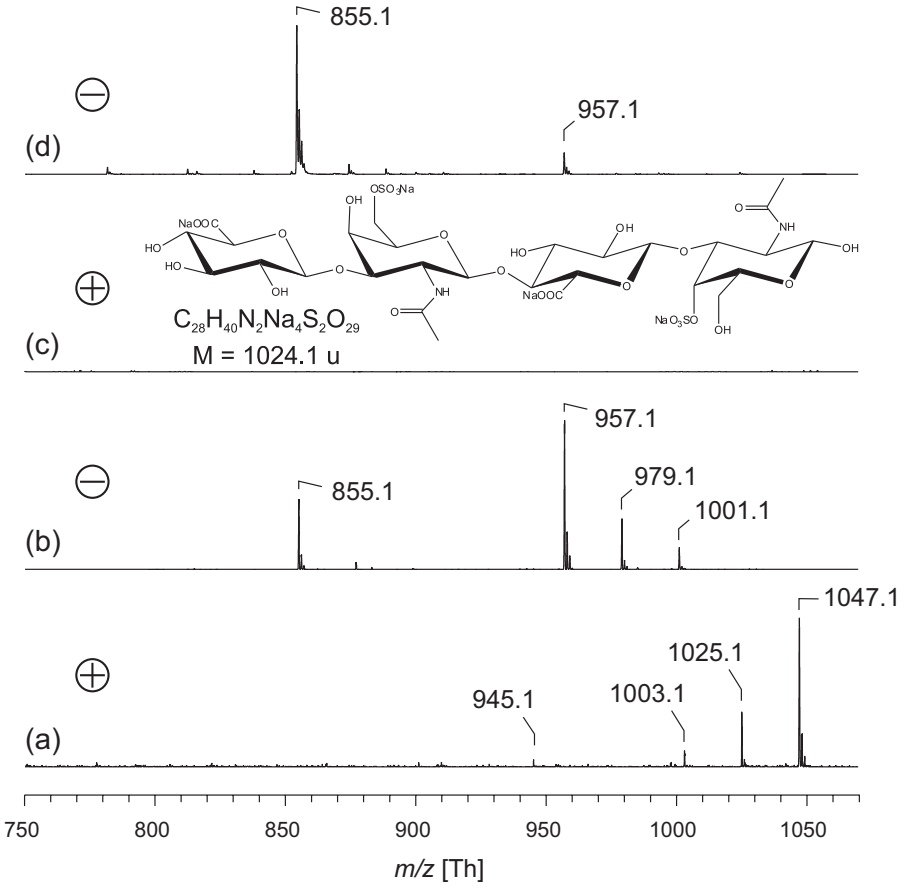

Fig. 2. MALDI-TOF mass spectra of an isolated CS tetrasaccharide. The structure of CS-4 and its monoisotopic mass is given at the top of the figure. The position of the sulphate residues (likely in 4- or 6-position on the N-acetylgalactosamine) is not relevant to this paper. 5 μl of the CS solution (1 mg/ml) was mixed with 5 μl of one of both matrices. 1 μl of this mixture was subsequently applied onto the target. Spectra (a) and (b) were recorded in the presence of DHB while (c) and (d) were recorded with 9-AA as matrix. Please note that 9-AA allows only recording the negative ion spectrum (d) while no peak could be observed in the positive ion spectrum (c). Loss of sulphate is also observed in the presence of DHB in the negative ion mode (b) but only to a very small extent in the positive ion spectrum (a).

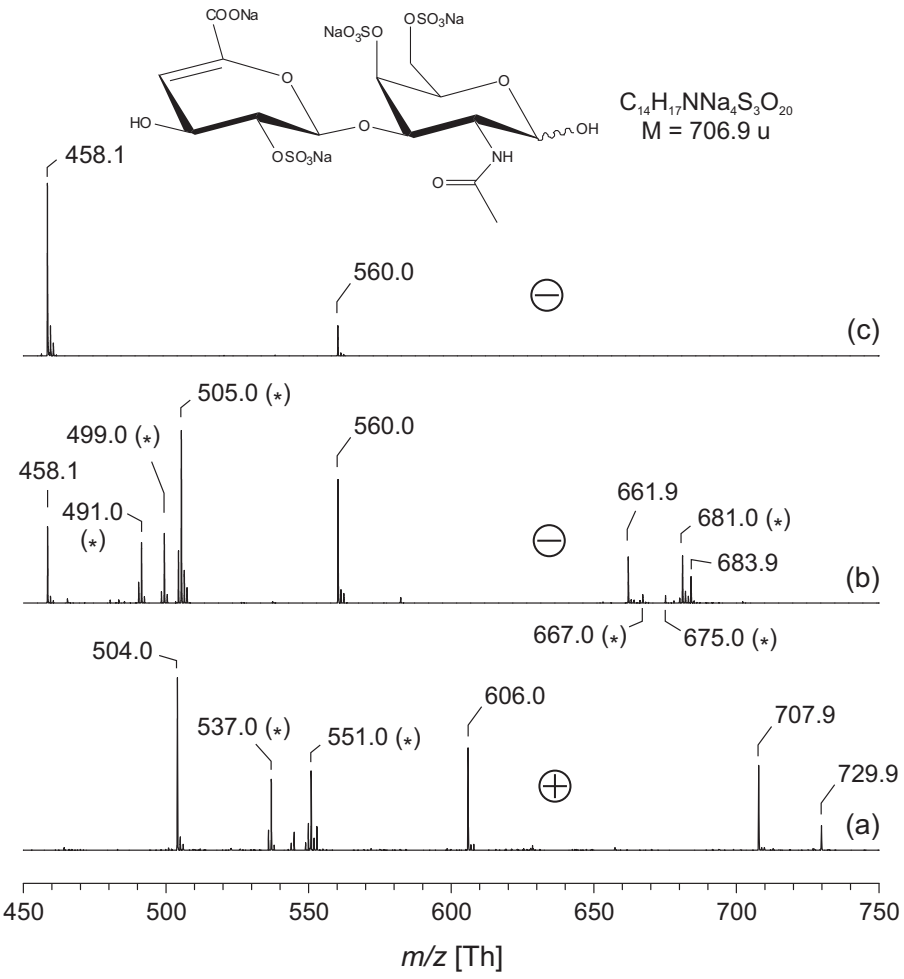

Fig. 3. MALDI-TOF mass spectra of commercially available trisulphated CS disaccharide. Its structure and its monoisotopic mass is given at the top of the figure. The position of the sulphate residues is not relevant to this paper. 5 μl of this CS solution (1 mg/ml) was mixed with 5 μl of one of both matrices. 1 μl of this mixture was subsequently applied onto the target. Spectra (a) and (b) were recorded in the presence of DHB while (c) was recorded with the matrix 9-AA. The polarities of the measurements are indicated in the individual traces. Please note the high abundance of DHB-derived cluster ions that are marked by asterisks. All peaks are labelled by their *m*/*z* ratios. For details see text.

signal-to-noise (S/N) ratio of 10) is plotted against the peaks assigned in Fig. 1.

It is not surprising that the negative ion spectra can be recorded with a higher sensitivity when 9-AA is used, while DHB (due to its acidic character) is the matrix of choice for positive ion detection.

In comparison to HA, the MALDI MS characterization of sulphated GAGs is much more challenging. A common CS tetrasaccharide (CS-4) bears in total 2 sulphate residues, one on each N-acetylgalactosamine unit either in 4- or 6-position. This chemical modification in comparison to HA is accompanied by significant but unwanted sulphate loss [11]. The MALDI-TOF mass spectra of CS-4 are shown in Fig. 2. There are obviously significant differences between the negative (b) and (d) and positive ion (a) and (c) spectra. The monoisotopic mass of the sodium salt of CS-4 is 1024.1 ($C_{28}H_{40}N_2Na_4S_2O_{29}$). Therefore, one would expect positive ions at either *m*/*z* 1025.1 (H^+ adduct) or 1047.1 (Na^+ adduct). These peaks are only detectable in the presence of DHB (a) but not in the presence of 9-AA (c). This is not very surprising due to the large differences of the p*K* values of both matrices. However, it is astonishing to which significant extent the negative ion mass spectra differ from the expected masses. As the mass of neutral CS is 1024.1, one would expect *m*/*z* 1001.1 in the negative ion spectrum [M−Na^+]. The corresponding peak is detected exclusively in the presence of DHB (b) but not at all in the presence of 9-AA (d). In the latter case there is only one intense signal at *m*/*z* 855.1 that corresponds to the loss of one sulphate residue [M−4Na^++3H^+−SO_3]. This peak is also detectable in the presence of DHB (b) but with much lower intensity. In contrast, in this case (b), the detection of the expected mass of the intact CS-4 ion containing two sulphate residues is possible and results in the peaks at *m*/*z* 1001.1 [M−Na^+], 979.1 [M−2Na^++H^+] and 957.1 [M−3Na^++2H^+]. It is not surprising that the DHB provides more intense H^+ adducts than 9-AA because DHB is an acidic compound [18]. However, it is surprising that 9-AA is obviously a poor matrix for CS-4 detection because in the positive ion spectrum (c) nothing at all is detectable while in the negative ion spectrum (d) the sulphate loss predominates and the intact tetrasaccharide with two sulphate residues is not detectable. It is also surprising that the positive ion mass spectrum in the presence of DHB (a) gives the by far best results regarding the detection of the intact CS-4 ion. The opposite would have been expected due to the strong negative charge of the CS-4.

In order to specify the characteristics of (a) each matrix and (b) each detection mode in more detail, the detection limits achievable under the different conditions were also compared. A signal-to-noise (S/N) ratio of 10 was arbitrarily defined as the detection limit. A serial dilution of the CS-4 stock solution between 50 pg and 500 ng was performed and the matrix moiety was kept constant. The required sample amounts are summarized in Table 2.

Table 2
Detection limits of CS-4 in the presence of 9-AA and DHB as MALDI matrices. A signal-to-noise ratio of 10 was defined as the detection limit. Selected peaks from the positive (+) and negative (−) ion mass spectra were selected. n.a. indicates that no detection was possible under these conditions. For details see text.

Detection limit	9-AA	DHB
m/z 855.1 (−)	62.5 pg	2500 pg
m/z 957.1 (−)	125 pg	500 pg
m/z 1047.1 (+)	n.a.	2500 pg
m/z 945.1 (+)	n.a.	300 ng

From the data given in Table 2 it is obvious that there are major differences between both matrices: the detection limits in the negative ion mode using 9-AA are significantly lower in comparison to DHB. This can be easily explained by the basicity of the 9-AA. Under positive ion conditions, however, intact CS-4 is exclusively detectable in the presence of DHB – most probably due to the higher acidity of this matrix compound that seems to reduce sulphate loss [19]. It has been reported that the addition of cesium ions does also reduce the sulphate loss [22]. Although we have also tried to repeat the measurements in the presence of Cs^+, only minimal improvements could be achieved (data not shown). Therefore, this aspect will not be further discussed in the present work.

Of course, the investigation of even higher sulphated saccharides than common CS-4 would be also of high interest. However, finding adequate samples is not easy: on the one hand, oligosaccharides of the heparin type are available [11] but these species contain in addition to the O-sulphate residues also N-SO_3 residues that might complicate the investigations. On the other hand, oversulphated CS can be hardly depolymerized by enzymes into defined products [1]. In order to overcome these problems, a commercially available (Dextra, No. C3207) CS disaccharide with three sulphate residues (Fig. 3) has been investigated. The same tendencies as already observed in Fig. 2 are obvious. The chemical formula of this compound is $C_{14}H_{17}NNa_4S_3O_{20}$ and corresponds to a molecular weight of 706.9 amu. In the positive ion MALDI spectrum recorded in the presence of DHB (Fig. 3a), the expected peaks at *m/z* 707.9 [M+H^+] and 729.9 [M+Na^+] are detectable although there is also a significant loss of one (*m/z* 606.0) and two sulphate residues (*m/z* 504.0). Since this loss is much more pronounced in comparison to the singly sulphated CS tetrasaccharide (Fig. 2a), the number of the sulphate residues is the obvious decisive factor and indicates that the number of sulphate residues per disaccharide unit determines the probability of their losses. Considering the negative ion spectrum in the presence of DHB (Fig. 3b) the same compounds but with slightly changed intensities can be detected: the intact molecule is detected at *m/z* 661.9 [M−Na^+] and 683.9 [M−2Na^++H^+], subsequent to the loss of one sulphate at *m/z* 560.0 and 582.0 and, finally, after two sulphate losses at *m/z* 458.1. Although this spectrum exhibits also intense DHB cluster ions (marked by asterisks) [18], DHB (Fig. 3b) is obviously a much more suitable matrix than 9-AA. Using this latter matrix, no matrix-derived ions are observed but the intact molecule with all sulphate residues is not detectable at all (Fig. 3c). Thus, 9-AA is surely no suitable matrix if sulphated carbohydrates are to be detected.

4. Conclusions

The aim of this study was (a) to investigate to which extent and under which conditions the loss of sulphate [22] from GAG oligosaccharides may be suppressed and (b) to investigate the suitability of DHB and 9-AA as MALDI matrices for the analysis of sulphated oligosaccharides. We conclude that the positive ion mass spectra in the presence of DHB are the method of choice to detect the intact oligosaccharides with only a moderate extent of sulphate loss. This is particularly important if mixtures of compounds with different sulphation patterns have to be characterized.

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