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An automated mass spectrometry-based screening method for analysis of sulfated glycosaminoglycans

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ABSTRACT

Glycosaminoglycans (GAGs) are linear polysaccharides, consisting of repeated disaccharide units, attached to core proteins in all multicellular organisms. Chondroitin sulfate (CS) and dermatan sulfate (DS) constitute a subgroup of sulfated GAGs for which the degree of sulfation varies between species and tissues. One major goal in GAG characterization is to correlate structure to function. A common approach is to exhaustively degrade the GAG chains and thereafter determine the amount of component disaccharide units. In large-scale studies, there is a need for high-throughput screening methods since existing methods are either very time- or samples consuming. Here, we present a new strategy applying MALDI-TOF MS in positive ion mode for semi-qualitative and quantitative analysis of CS/DS derived disaccharide units. Only a few picomoles of sample are required per analysis and 10 samples can be analyzed in 25 min, which makes this approach an attractive alternative to many established assay methods. The total CS/DS concentration in 19 samples derived from *Caenorhabditis elegans* and mammalian tissues and cells was determined. The obtained results were well in accordance with concentrations determined by a standard liquid chromatography-based method, demonstrating the applicability of the method for samples from various biological matrices containing CS/DS of different sulfation degrees.

1. Introduction

Glycosaminoglycans (GAGs) are linear polysaccharides occurring in cells of all multicellular organisms, from invertebrates to mammals, in the form of proteoglycans (PGs) or as free chains [1,2]. Different PGs are expressed in a cell- and tissue specific manner and fulfill diverse biological functions [3]. The GAG family includes both unsulfated species, i.e., hyaluronan, and sulfated ones, including the glucosaminoglycans heparin and heparan sulfate (HS), and the galactosaminoglycans chondroitin sulfate (CS) and dermatan sulfate (DS). CS/DS consists of repeated disaccharide units formed by variably sulfated *N*-acetylgalactosamine (GalNAc) and uronic acid; glucuronic acid in CS and iduronic acid in DS. CS/DS bear many essential functions in an organism. For example, they influence tissue morphogenesis and organogenesis and support hemostasis during wound repair or coagulation processes by influencing cell proliferation, differentiation and migration [4,5]. As GAGs play important roles in developmental and physiological processes, they are also affected in pathology. The sulfation degree of CS has been found to vary with the development of osteoarthritis (OA) [6] and CS-based drugs have therefore been used for treatment of signs and symptoms of OA for nearly 40 years [7,8]. Several genetic disorders are caused by mutations in genes encoding glycosyltransferases and sulfotransferases, resulting in non-functioning biosynthesis of CS/DS side chains [9].

To better understand basic mechanisms of various biological processes, it is essential to correlate GAG structure to function. The analysis of GAGs remains a challenge, due to the huge heterogeneity of GAG populations. Chemical properties including size and charge also complicate the analyses. Most analytical approaches for structural characterization rely on exhaustive degradation of GAG chains into disaccharide units and subsequent determination of the type and amount of these components [10,11]. Digestion can be performed either enzymatically using bacterial polysaccharide enzymes [10] or chemically. A few assays, i.e. the Alcian Blue [12] and the Carbazole assays [13], based on dye and color reactions followed by absorbance measurements are today routinely used for quantification of total GAGs. These methods are straightforward to perform and can be executed in most laboratories. However, the amounts required for analyses are high, on the micro

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to nanomole level, and the accuracy is limited [14]. Also, limited or no information on the disaccharide composition is obtained. Chromatographic methods, on the other hand, are accurate and sensitive. By separating the component disaccharide units with respect to physico-chemical properties, both qualitative and quantitative information is readily obtained. High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are commonly used for GAG analysis in combination with either spectrophotometric detectors or mass spectrometry (MS) [10,11]. In case of MS, mainly on-line electrospray ionization (ESI) in combination with various mass analyzers has been applied [11,15,16]. One advantage of using MS-based strategies is their low detection limits. Sub-picomole levels of GAGs are readily determined using HPLC–ESI-MS methods, while several competitive techniques often require nanomoles of material [17]. Despite these advantages, chromatographic methods are very time-consuming.

Today, there is an increasing interest to perform systemic analyses. When screening a large number of samples for GAG content, fast and reliable analytical methods are required. Matrix-assisted laser desorption time of flight (MALDI-TOF) MS is an attractive alternative to ESI-MS since it is more tolerant towards salts and therefore does not require extensive desalting before analyzing the samples. This makes MALDI-TOF MS-based methods rather quick. However, it is sometimes questioned as being applicable for quantitative analysis due to the high shot-to-shot variability. Even though MALDI-TOF MS has been applied in studies of GAGs for other purposes [11], only a few reports regarding the quantification of GAGs have been presented [17–20]. To the best of the authors' knowledge, the quantification of sulfated GAGs by using a standard calibration is mentioned only in two articles [17,19]. Both these investigations used MALDI operating in negative mode to estimate the total CS/DS content in samples of mammalian origin.

Here, we present a novel MALDI-TOF MS-based screening method, operating in positive mode, for semi-qualitative analysis and quantitative estimation of the CS/DS content in various samples. In contrast to previous MALDI-based studies [17,19], the correlation between signal-to-noise and concentration is investigated not only for monosulfated but also for non- and disulfated species. Technically, the spot-to-spot variation was successfully compensated for both by use of an internal standard (IS) and by adding spectra obtained by laser shots on 50 points on each sample spot. The applicability for samples of diverse sources, including mammalian as well as *Caenorhabditis elegans*, was evaluated and the results were compared to those obtained using a standard HPLC-method.

2. Materials and methods

2.1. Disaccharide standards

Eight standards, representing all possible combinations of sulfated and non-sulfated disaccharide units resulting after eliminase reaction by chondroitinase ABC on CS/DS were commercially obtained from Iduron Ltd. (Manchester, UK). As seen in Fig. 1, the units were *O*-sulfated in either position C2 of the 4,5-unsaturated hexuronate ring (HexA), or position C4 or C6 in the GalNAc unit. Explicitly, the obtained standards were the non-sulfated unit ΔHexA-GalNAc (0S), three mono-*O*-sulfated ΔHexA-GalNAc units; ΔHexA2S-GalNAcS (2S), ΔHexA-GalNAc4S (4S) and ΔHexA-GalNAc6S (6S), three disulfated units; ΔHexA2S-GalNAc4S (24S), ΔHexA2S-GalNAc6S (26S), ΔHexA-GalNAc4,6S (46S) and the trisulfated ΔHexA2S-GalNAc4,6S (246S) unit. The standards were dissolved in ultrapure water, frozen and used as stock solutions for all further investigations.

2.2. Sample preparation

GAGs were isolated from *C. elegans*, Chinese hamster ovary (CHO) 677 cells [21], porcine lung, mouse lung, heart and brain tissue according to the procedure described by Ledin et al. [10]. Briefly, 10–50 mg of dry tissues or cell pellet were proteolytically degraded, nucleic acids were digested and the polyanionic carbohydrates were isolated by anion exchange chromatography. Mammalian GAG samples were eluted from anion exchange columns by step-wise elution with 2 M NaCl and desalted on PD-MiniTrap™ G-25 columns (GE Healthcare Biosciences). Because of the vast excess of non-sulfated chondroitin over sulfated GAG structures in *C. elegans* samples, the protocol was altered to a two-step procedure: Samples were first adjusted to 0.25 M NaCl and bound to anion exchange columns. Non-bound material was collected, adjusted to 0.1 M NaCl and bound to a second anion exchange column. All samples were eluted with 1.5 M NaCl and desalted as the mammalian samples. The samples were redissolved in a final 100 μL of 40 mM Tris/acetate buffer, pH 8.0, for enzymatic cleavage. The polysaccharides were exhaustively digested to CS/DS disaccharides using 50 mU of the bacterial lyase chondroitinase ABC (Seikagaku Corp., Tokyo, Japan) and incubated 3 h (mammalian samples) or overnight (*C. elegans*, to ensure that all C0S is cleaved) at 37 °C. The incubation was

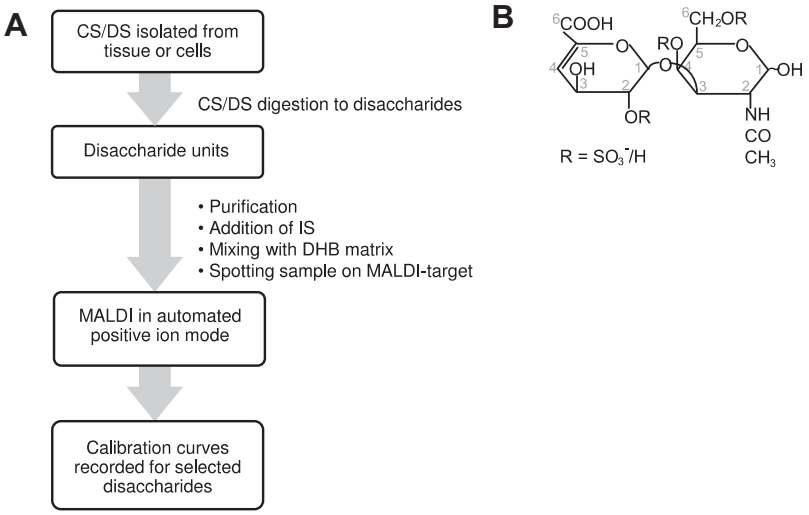

Fig. 1. (A) Schematic drawing of the experimental procedure. (B) Structure of the disaccharide unit resulting after enzymatic digestion of chondroitin and dermatan sulfate by chondroitinase ABC. The disaccharide units can be sulfated at any of the three indicated positions, or combinations thereof.

terminated by boiling samples for 5 min at 96 °C and the samples were finally diluted 10-fold with H_2O.

2.3. Reversed phase-ion pairing (RPIP)-HPLC

RPIP-HPLC was performed on a Luna 5u C18 reversed phase column (4.6 × 150 mm, Phenomenex) run at 1.1 mL min^{-1} in solution A (8.5% acetonitrile, 1.2 mM *tetra-N*-butylammonium hydrogen sulfate) and disaccharide units were eluted by a NaCl-gradient in solution A over 35 min exactly as described in Ledin et al. [10]. Re-equilibration of the column was performed for 15 min in solution A before next sample application. Samples of 10–90 μL were injected by an auto-robot and detection achieved by post-column addition of 2-cyanoacetamide (0.25%) in NaOH (0.5%) at a flow rate of 0.35 mL min^{-1} as described elsewhere [22]. Signals were quantified against known amounts of standard disaccharides, analyzed in parallel.

2.4. MALDI-TOF mass spectrometry

For MALDI-TOF MS analysis, a series of standards, ranging from 2 to 200 ng μL^{-1}, were prepared in 4 mM Tris/Ac buffer, pH 8.0. To construct the calibration curves, maltopentaose was used as an internal standard. A stock solution of internal standard at a concentration of 50 ng μL^{-1} was prepared. Disaccharide standard solutions and samples were mixed with the internal standard at a ratio of 2:1 (v: v). The samples were then mixed with the matrix 2,5-dihydroxybenzoic acid (DHB) and loaded on an MTP384 ground steel target (Bruker Daltonics, Bremen, Germany) using the dried droplet technique.

Mass spectra were obtained using an Ultraflex II MALDI-TOF MS (Bruker Daltonics). An automated method was designed and applied. For each sample, the mass range from m/z = 140 to 1200 was analyzed in positive polarity mode. The laser power was set to 50%, while the frequency was kept at 100.0 Hz. Each single mass spectrum was obtained as the sum of 100 shots at a preselected point. For each target spot, 50 mass spectra were automatically recorded and summed. The mass spectra were processed in FlexAnalysis 3.0 (Bruker Daltonics) using an automated macro. The macro was programmed to extract mass-to-charge (m/z), signal-to-noise (S/N), intensity and the area of several characteristic peaks, i.e. those at m/z = 402, 504, 605 and 707 and 851 (IS), from each spectrum. The information from each mass spectrum in a series was written to the same output file, which was thereafter imported to Microsoft Excel.

Calibration curves were obtained by plotting the S/N of the disaccharide normalized to the S/N of the internal standard against the amount spotted on target, and subsequent linear regression. For the non-sulfated unit (0S), the calibration curve of the peak at m/z = 402 was recorded (Calibration curve A). In case of monosulfated disaccharide units, the calibration curves for both sulfated (m/z = 504) and desulfated signals (m/z = 402) were recorded. These are referred to as Calibration curve B and C, respectively, in the following description. For *C. elegans* samples, the concentration was calculated from the 0S calibration curves, Calibration curve A. For mammalian samples, the linear equation of the 4S trendline (Calibration curve B) was used to calculate the concentration of monosulfated disaccharide unit. Then, the concentration of 0S disaccharide was determined using Calibration curve A after having subtracted the contribution from the desulfated 4S, calculated from Calibration curve C. For the majority of mammalian samples, the contribution from the nonsulfated peak was below detection limit and the concentration was solely determined from Calibration curve B. Each biological sample was analyzed twice and the precision of the method was calculated as the square root of the average $RSD^{\wedge 2}$ obtained for all samples.

3. Results and discussion

3.1. Method performance and optimization

A schematic drawing of the suggested automated MALDI-TOF MS method for determination of disaccharide unit concentration is shown in Fig. 1. To investigate the performance of our method, variably sulfated disaccharide standards were analyzed in positive ion mode by MALDI-TOF MS. Disaccharides sulfated at the three possible sites (Fig. 1B) and combinations thereof were successfully ionized and detected. Characteristic mass spectra were obtained for non-, mono-, di- and trisulfated CS/DS disaccharides and a list of characteristic peaks for each of the standards is compiled in Table 1. For all analyzed units, sodium adduct peaks were observed with the dominating peak containing one sodium ion per sulfate and carboxy-group. Also, peaks containing an additional sodium ion were observed. Mass spectra of some of the disaccharide units recorded in positive ion mode using the DHB matrix have been presented in literature [17,23,24] and the detected masses obtained here agree well with those. The characteristic mass spectra of all sulfated disaccharides contained not only the peaks of the standards under study, but also signals assigned to the corresponding desulfated species (Suppl. Fig. 1). This was expected, since desulfation during the ionization process is a recognized phenomenon [24].

In order to evaluate the possibility to design a quantitative method, the correlation between peak signal-to-noise and disaccharide amount at the picomole level was investigated. The drawback of using MALDI in quantitative studies is the high shot-to-shot variability. It was, however, noticed that after careful optimization, including manual selection of spots on the MALDI-target and averaging several mass spectra per disaccharide standard sample, linear calibration curves could be obtained (data not shown). However, one or two standards were usually considered as outliers and therefore excluded from the linear regression. Yet, as the concept of this project was to develop a rapid and reliable screening method to be executed in automated mode, the performance was not acceptable. To compensate for experimental variations, an internal standard was added to the samples. In many analytical methods, an isotopically labeled variant of the compound under study is selected as IS to guarantee similar behavior upon ionization and detection. This strategy was not applicable in the present investigation, since several analytes of various sulfation degrees were to be quantified in the same sample. Instead, a stable, non-sulfated sugar whose mass did not interfere with any of the disaccharide units, maltopentaose, was evaluated as IS. To further improve the performance, an automated method was designed, summing up the signal of 50 spectra per spot and disaccharide unit amount.

Calibration curves for the non-sulfated (0S) and the monosulfated (2S, 4S and 6S) disaccharides were generated using this fully automated method. For all units, linear correlation in the range of around 3–40 pmol (corresponding to ~1.5–20 ng) was observed when normalizing the disaccharide signal to the IS (Fig. 2). For the

Table 1
Characteristic peaks detected for CS/DS-derived disaccharides in positive ion mode.

m/z	Identity of the peak	Sulfation level	Number of Na^+
402.1	0S	Non-sulfated	1
424.1	0S	Non-sulfated	2
504.0	2S, 4S or 6S	Monosulfated	2
526.0	2S, 4S or 6S	Monosulfated	3
606.0	24S, 46S or 26S	Disulfated	3
628.0	24S, 46S or 26S	Disulfated	4
707.9	246S	Trisulfated	4
729.9	246S	Trisulfated	5

monosulfated disaccharides, calibration curves of the corresponding desulfated species were also recorded. R^2-values between 0.98 and 0.99 were obtained in all cases. The degree of desulfation upon ionization was estimated by comparing the calibration curves of the $m/z = 504$ and 402 signal for each monosulfated disaccharide unit. It was concluded that sulfation in position 2 is most stable, followed by modification in position 4 and 6. It was also observed that under identical MALDI conditions, the signal intensity for the 6S disaccharide unit was 5–6 times lower than for corresponding amounts of 2S and 4S, indicating that the 6S-standard was more difficult to ionize. The detection limit was, consequently, also highest for 6S. The standard of lowest concentration, corresponding to 1.3 pmol was readily detected for 2S and 4S, while this signal could not be observed for 6S.

After several attempts to evaluate disulfated disaccharides, the conclusion was that there is poor correlation between the concentration and S/N ratio of the peaks. Since desulfation occurs more extensively for the disulfated units than for monosulfated ones (Suppl. Fig. 1), the signals from the characteristic peaks are less predictable. However, predominately non- and monosulfated species are found in mammalian species [25] and hence these calibration curves would be most important when screening samples in the laboratory. Qualitative information on the presence of di- and trisulfated disaccharides would furthermore also be obtained by examining the mass spectra. To investigate the repeatability of the method, calibration curves for the two most abundant CS/DS disaccharides in mammalian tissues, 0S and 4S, were recorded at several occasions during a period of 10 months. Linear correlation in the expected range of 1.3–40 pmol with correlation coefficients, R^2, between 0.98 and 0.99 was observed in all cases, indicating that the method is robust and applicable despite smaller variations in instrumental performance.

MALDI-TOF MS-based quantification of CS/DS disaccharides has previously been presented by a few scientific groups [17,19]. All previous approaches dealt with determination of the total amount of disaccharides and no separate calibration curves were presented for different types of CS/DS disaccharide units. The current study is the first one to use MALDI-TOF MS in positive mode for quantitative estimation of sulfated GAGs. For a screening method, this could potentially be an advantage, since the instruments in many bioanalytical laboratories are routinely tuned and optimized in positive mode, e.g., for protein and peptide analyses. Also, a very common and inexpensive matrix, DHB, was applied in the experiments. The linear range, on the pmol-level, and sensitivity of our MALDI-based method is well comparable to or even somewhat better than what has been reported for negative mode MALDI assays [17,19]. As compared to colorimetric methods for determination of the total CS/DS concentration, our method is by far more sensitive. A detection limit of the Carbazole assay of around 1 nmol after optimization [14] has been reported, which is 1000 times higher than for the MALDI-based strategy.

3.2. Determination of concentrations in authentic samples

Since the goal of the investigation was to develop a generally applicable method for CS/DS derived from various tissues, a collection of authentic samples from different origins was analyzed. There were two major categories of samples: disaccharides from *C. elegans* and disaccharides from mammalian sources. For the *C. elegans* samples, only one disaccharide peak, corresponding to 0S, was observed in the mass spectra (see Fig. 3A). The finding that *C. elegans* contained predominantly the non-sulfated unit agrees well with literature [3]. Mammalian tissues are known to contain mainly monosulfated, but also non-sulfated and disulfated species

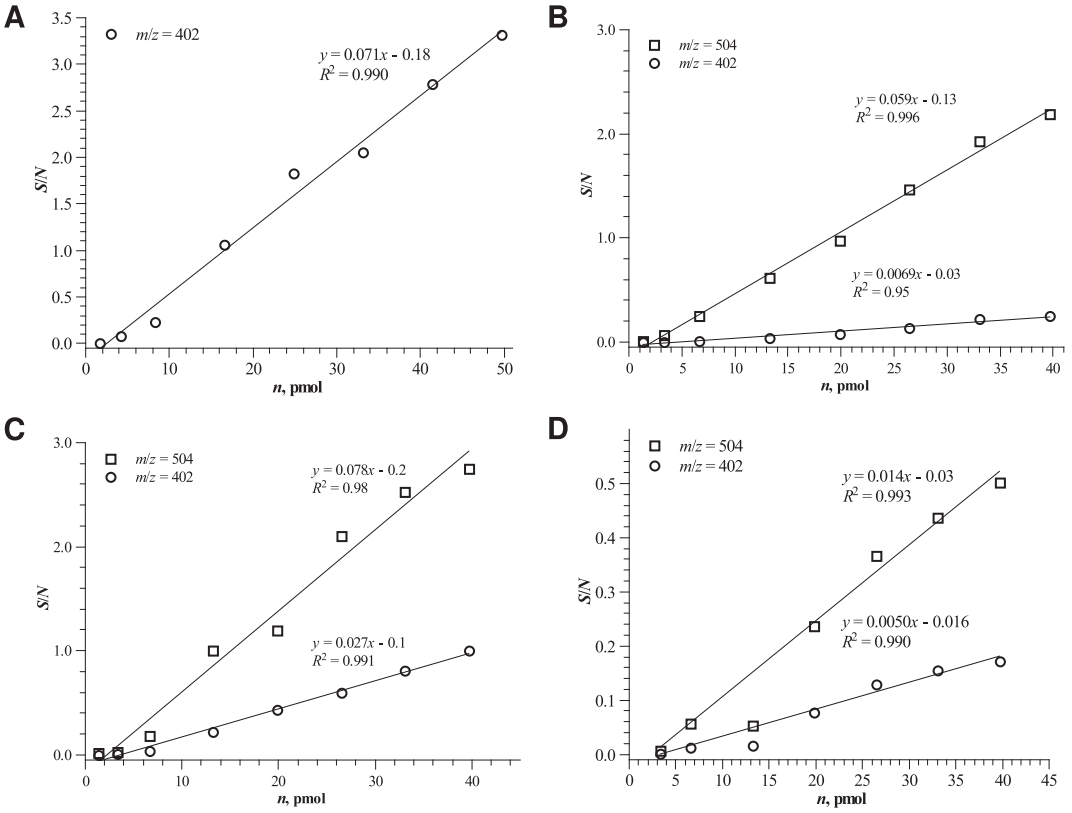

Fig. 2. Calibration curves for the CS/DS disaccharide standards in 4 mM Tris/Ac buffer. S/N values are normalized against the internal standard (maltopentaose) signal: (A) 0S standard, (B) 2S standard, (C) 4S standard, (D) 6S standard. Linear correlation between the normalized S/N and the amount spotted on target was observed for all units in the range of around 3–40 pmol. The standard of the lowest amount, 1.3 pmol, was detected for all but the 6S standard. For monosulfated species, also the peak corresponding to a desulfated disaccharide unit showed a linear response.

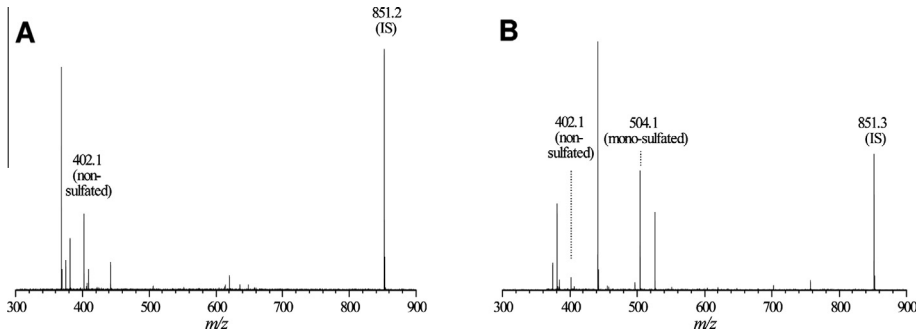

Fig. 3. Characteristic mass spectra of CS/DS disaccharides derived from native samples: (A) *C. elegans*, where only the non-sulfated unit was detected. (B) Mammalian tissue, here represented by mouse brain. Both the non- and monosulfated units are clearly visible.

Table 2
Determination of disaccharide concentrations in different samples, using the calibration curves for 0S and 4S. Two independent measurements were performed by MALDI-TOF MS for each sample and the results are shown in the columns called Run 1 and Run 2. The concentrations as determined by a reference RPIP-HPLC method are indicated for comparison.

Sample	Sample origin	$c_{\text{MALDI-TOF MS}}$ (ng μL^{-1})			$c_{\text{RPIP HPLC}}$ (ng μL^{-1})
		Run 1	Run 2	Average	
1	*C. elegans*	7.6	13	10	12
2	*C. elegans*	10	8.9	9.6	9.3
3	*C. elegans*	9.4	10	9.7	11
4	*C. elegans*	11	18	15	12
5	*C. elegans*	7.3	6.6	7.0	14
6	*C. elegans*	5.2	4.2	4.7	13
7	*C. elegans*	4.6	3.2	3.9	5.2
8	CHO 667 cells	11	7.0	8.8	9.1
9	CHO 667 cells	6.2	7.3	6.7	7.8
10	CHO 667 cells	7.8	8.8	8.3	7.1
11	Mouse lung	4.4	3.2	3.8	4.1
12	Mouse lung	5.4	3.9	4.6	6.6
13	Mouse lung	6.9	6.3	6.6	10
14	Mouse heart	4.5	3.1	3.8	4.2
15	Mouse heart	4.1	3.7	3.9	3.6
16	Mouse brain	22	30	26	43
17	Mouse brain	20	30	25	24
18	Mouse lung	3.5	4.8	4.2	17
19	Mouse lung	19	19	19	14

[25]. For all mammalian samples presented in Table 2, two characteristic peaks at m/z = 402 and 504, were observed (see Fig. 3B). As expected, the monosulfated peak was the dominating signal for all mammalian samples. The signal derived from the disulfated unit was generally not observed. This was also anticipated, since the disulfated unit constitutes less than 10% of the total CS/DS population and since extensive desulfation occurs for this peak (Suppl. Fig 1).

The amount of CS/DS in the seven *C. elegans* samples (see Table 2) was determined from the 0S calibration curve. For the mammalian tissue samples, determination of the total disaccharide amount was performed by combining the information from calibration curves for the non- and monosulfated disaccharide units, as described in Section 2.4. This should be regarded as a simplified calculation, since the signal intensity of the non-sulfated species is not independent of the signal derived from the monosulfated disaccharide unit, due to ion suppression effects. However, the contribution from 0S is minor and the strategy was demonstrated to be well applicable for high-throughput screening experiments. A total of 19 samples isolated from different sources were analyzed and compared with values determined by RPIP-HPLC (Table 2). Two separate analyses of each sample were performed using the MALDI-TOF MS method in order to monitor the variation between runs.

From the results in Table 2, it can be concluded that the two methods generated comparable results. In general, the concentrations do not differ by more than a few ng μL^{-1}. Accurate values were obtained for *C. elegans* samples as well as for cells and tissue extracts of mammalian origin. The results obtained using the MALDI-TOF MS based method were in general somewhat lower than those obtained using the RPIP-HPLC-method, i.e. 14 out of 19 of the average values and 25 of the 38 values from separate runs in Table 2 were underestimated. The tendency was in particular observed for the mainly non-sulfated samples from *C. elegans*, even though only one calibration curve was required for determination of the amount of disaccharide units. One reason for this might be that the non-sulfated disaccharide unit elutes very early from the reverse phase column used in the RPIP-HPLC method, where unrelated contaminants can interfere and affect accuracy. A slight underestimation of the total CS/DS concentration for mammalian samples can be explained by the fact that mainly the 4S calibration curve was used to determine the concentrations, while both non- and disulfated units are present in the samples at lower levels. Only three samples, i.e. Sample 5, 6 and 18 deviated by more than 40% from the HPLC-results. Samples 5 and 6 were prepared in the same batch, and it is therefore likely that impurities in the samples influenced the results of either or both methods. For sample 18, we have no good explanation. The precision of the method was calculated to be 20%. Considering the purpose of our newly developed MALDI-TOF MS method for screening a large number of samples prior to further analysis, the accuracy and precision are sufficient and comparable to available techniques. Alternative strategies for screening samples are the more sample-consuming colorimetric methods. In a comparative study, results from the Carbazole assay was demonstrated to deviate from HPLC-based analyses by as much as 71% when determining the total amount of CS from mammalian samples [14]. The advantage of the described strategy is its simplicity and speed. Ten samples can be analyzed in less than 25 min, including generation of the required calibration curves, while analysis of the same number of samples by RPIP-HPLC takes approximately 10 h. Both semi-qualitative, i.e. what is the dominating sulfation degree, and quantitative information is obtained using the MALDI-TOF MS-based strategy. However, for more thorough investigations, HPLC-based methods in combination either with spectrophotometric detectors or ESI-MS are strongly suggested. The MALDI-based approach should be regarded as a complement and not as an alternative to these strategies.

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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.bbrc.2014.06.011.

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