Elsevier Editorial System(tm) for Analytical Biochemistry: Methods in the Biological Sciences

Manuscript Draft

Manuscript Number:

Title: Quantitative analysis of glycosaminoglycans, chondroitin/dermatan sulfates, hyaluronic acid, heparan sulfate and keratan sulfate by LC-ESI-MS/MS

Article Type: Full Length Article

Section/Category: Carbohydrates

Keywords: glycosaminoglycan (GAG), high-sulfated disaccharide, electrospray ionization (ESI), tandem mass spectrometry (MS/MS), selected reaction monitoring (SRM)

Corresponding Author: Mrs. Harumi Osago,

Corresponding Author's Institution: shimane Univ.

First Author: Harumi Osago

Order of Authors: Harumi Osago; Tomoko Shibata; Nobumasa Hara; Suguru Kuwata; Michihaya Kono; Mikako Tsuchiya

Abstract: For simultaneous quantitative analysis of glycosaminoglycans (GAGs), we developed a method using LC-electrospray ionization-tandem MS (LC-ESI-MS/MS) with a selected reaction monitoring (SRM) mode. For the first time, we demonstrated the simultaneous quantification of a total of 17 variously sulfated disaccharides of 4 GAG classes (8 chondroitin/dermatan sulfates, 1 hyaluronic acid, 6 heparan sulfate and 2 keratan sulfate) with a sensitivity of less than 0.5 pmol within 18 min using this method. By one-shot analysis of GAGs with our MS/MS method, we showed the differences in the composition of GAG classes and the sulfation patterns between the porcine articular cartilage and yellow ligament. In addition to the internal disaccharides described above, some saccharides derived from the nonreducing terminal were also simultaneously detected. The simultaneous quantification of both internal and nonreducing terminal saccharides could be useful to estimate the chain length of GAGs. This method would help establish comprehensive GAGomic analysis of biological tissues.

Suggested Reviewers: Shunji Tomatsu
stomatsu@nemours.org

April. 14, 2014

Editor
Analytical Biochemistry

Dear editor:
I am sending the text and graphic PDF file for a manuscript entitled "Quantitative analysis of glycosaminoglycans, chondroitin/dermatan sulfates, hyaluronic acid, heparan sulfate and keratan sulfate, by LC-ESI-MS/MS" by Osago *et al*. This manuscript has not been published before and it is not under consideration for publication, in any form or language. Its publication has been approved by all listed authors and explicitly by the responsible authorities in the laboratories where the work was carried out. If accepted the above manuscript in the Analytical Biochemistry, it will not be published elsewhere in the same form, in either the same or another language, without the consent of the Editors and Publisher of the Journal.

Your kind consideration of our paper would be greatly appreciated.

Sincerely yours,

Harumi Osago
Department of Biochemistry
Shimane University Faculty of Medicine
89-1 Izumo 693-8501 Japan
Tel. 81-853-20-2120
Fax 81-853-20-2120
E-mail biochem1@med.shimane-u.ac.jp

Quantitative analysis of glycosaminoglycans, chondroitin/dermatan sulfates, hyaluronic acid, heparan sulfate and keratan sulfate by LC-ESI-MS/MS

Harumi Osago [a]*, Tomoko Shibata [b], Nobumasa Hara [a], Suguru Kuwata [c], Michihaya Kono [c], Yuji Uchio [c], and Mikako Tsuchiya [a]

[a] Department of Biochemistry

[b] Center for Integrated Research in Science

[c] Department of Orthopaedic Surgery

Shimane University Faculty of Medicine, Izumo, 693-8501, Japan

Quantitative GAG analysis by LC-ESI-MS/MS

* Corresponding author:

Fax: 81-853-20-2120

E-mail address: biochem1@med.shimane-u.ac.jp

Abstract

For simultaneous quantitative analysis of glycosaminoglycans (GAGs), we developed a method using LC-electrospray ionization-tandem MS (LC-ESI-MS/MS) with a selected reaction monitoring (SRM) mode. For the first time, we demonstrated the simultaneous quantification of a total of 17 variously sulfated disaccharides of 4 GAG classes (8 chondroitin/dermatan sulfates, 1 hyaluronic acid, 6 heparan sulfate and 2 keratan sulfate) with a sensitivity of less than 0.5 pmol within 18 min using this method. By one-shot analysis of GAGs with our MS/MS method, we showed the differences in the composition of GAG classes and the sulfation patterns between the porcine articular cartilage and yellow ligament. In addition to the internal disaccharides described above, some saccharides derived from the nonreducing terminal were also simultaneously detected. The simultaneous quantification of both internal and nonreducing terminal saccharides could be useful to estimate the chain length of GAGs. This method would help establish comprehensive GAGomic analysis of biological tissues.

Keywords: glycosaminoglycan (GAG), high-sulfated disaccharide, electrospray ionization (ESI), tandem mass spectrometry (MS/MS), selected reaction monitoring (SRM)

Abbreviations

GAG: glycosaminoglycan, **CS:** chondroitin sulfate, **DS:** dermatan sulfate, **HA:** hyaluronic acid, **HS:** heparan sulfate, **KS:** keratan sulfate, **UA:** uronic acid, **GlcA:** glucuronic acid, **DMMB:** 1,9-dimethylmethylene blue, **MS/MS:** tandem mass spectrometry, **Q1:** first chamber, **Q3:** third chamber, **SRM:** selected reaction monitoring, **CE:** collision energy

1. Introduction

The glycosaminoglycans (GAGs) are classified into 4 classes, namely, chondroitin/dermatan sulfates (CS/DS), hyaluronic acid (HA), heparan sulfate (HS) and keratan sulfate (KS), based on the repeating disaccharide unit and the glycoside linkage pattern (Table 1) [1]. Except HA, all GAGs are modified with several sulfate groups at multiple positions and covalently bind to core proteins as proteoglycans. All GAGs are constitutively localized to the extracellular matrix of many connective tissues such as cartilage. Their negative charge provides swelling pressure for visco-elasticity in the tissues. Age- or disease-related changes in composition of GAG classes, sulfation pattern [2, 3, 4, 5] and chain length [6, 7] have been reported. For example, CS and HS are recently reported to have opposite effects on axonal growth in the spinal cord [8]. Furthermore, multiple classes of GAGs are suggested to play each role in cancer metastasis [9]. Though the high-sulfated disaccharides are minor components of GAG chains, they have also been reported to be involved in neurite outgrowth promoting activity [10] and altered in several diseases [11, 12]. Thus, a fast, accurate, quantitative and comprehensive method that provides the information of the class, sulfation pattern and chain length of GAGs, is required for basic and clinical science.

So far the quantitative composition analysis of GAG disaccharides has been done by UV [13, 14], fluorescence [14, 15] and MS [16]. However, the closely related, but subtly different structure of GAGs has complicated their detailed and extensive analysis. Among the "omics" fields such as proteomics, lipidomics and glycomics, MS has been used to identify and precisely quantify thousands of target molecules from complex biological samples. The high-throughput molecular profiling approaches have now become indispensable tools for not only molecular and cellular biology but also clinical medicine. Since the selected reaction monitoring (SRM) mode on a triple quadrupole MS is a highly sensitive and selective quantification method and has been previously used for targeted quantitative "omics" [17], we have tried to introduce our approach to the GAG research field for "GAGomics". In this paper, we describe the specific and quantitative one-shot analysis of variously sulfated saccharides of 4 GAG classes for the first time. With this method, we showed the differences in the composition of the GAG classes and sulfation patterns between the porcine articular cartilage and yellow ligament and the estimation of the chain length of cartilage CS.

2. Materials and Methods

2.1. Materials

Standard unsaturated disaccharides of GAGs listed in Table 1, purified GAG chains: CS type A (CSA), CS type B (CSB), CS type C (CSC) and KS, and GAG degrading enzymes: chondroitinase ABC, keratanase II and hyaluronidase SD were purchased from Seikagaku Biobusiness Corporation (Tokyo, Japan). GalNAc-4S and -6S were from Dextra Laboratories, Ltd. (Reading, UK). The preparation of two standard disaccharides of KS is described later. Thermolysin from *Bacillus thermoproteolyticus rokko* (cat. no. T7902) and aggrecan were from Sigma-Aldrich. The porcine tissues were obtained from a local slaughterhouse. Acetonitrile (LC/MS grade) and all other reagents were from Wako Pure Chemical Industries (Osaka, Japan). A porous graphitized carbon column Hypercarb (particle size 3 μm, column size 2.1 mm × 150 mm) was purchased from Thermo Fisher Scientific Inc. (MA, USA).

2.2. Measurement of total GAG content

Total GAG content was quantified with 1,9-dimethylmethylene blue (DMMB) using the Blyscan GAG assay kit (Biocolor Ltd.) [18].

2.3. Extraction of GAGs from the porcine articular cartilage and yellow ligament

The porcine articular cartilage and yellow ligament (6 mg) were completely solubilized with thermolysin (12 U) at 70 °C overnight in 200 mM ammonium acetate (pH 8.0) containing 5 mM $CaCl_2$ in a total volume of 600 µl, and centrifuged at 20,000 g for 15 min. The supernatant was added to 9 times the volume of chilled ethanol, kept overnight at −20 °C and centrifuged at 20,000 g for 15 min. The precipitates from the cartilage and ligament were reconstituted with 60 and 30 µl of distilled water, respectively.

2.4. Digestion and alkaline-reduction of GAGs

For preparation of the standard KS disaccharides, KS (20 µg) was incubated with keratanase II (5 mU) for 3 h at 37 °C in 50 mM Tris-HCl (pH 7.5) containing 0.05% BSA in a total volume of 50 µl. CS chains (CSA, CSB and CSC, 46 µg), aggrecan (50 µg), cartilage GAGs (15 µg) or ligament GAGs (15 µg) were incubated with chondroitinase ABC (10 mU) for 12 h at 37 °C in 50 mM ammonium acetate (pH 8.0) containing 0.05% BSA in a total volume of 120 µl. For complete digestion, the cartilage GAGs (5 µg) were incubated with a mixture of chondroitinase ABC (5 mU), keratanase II (5 mU) and hyaluronidase SD (2.5 mU) for 12 h at 37 °C in 0.05% BSA, 1 mM $CaCl_2$, 50 mM sodium acetate (pH 6.0) and 30 mM Tris-HCl (pH 8.0) in a total volume of 60 µl. For LC-MS/MS analysis, an aliquot of the

digests was heated for 5 min at 100 °C, added to an equal volume of 10 mM ammonium bicarbonate, (pH 10.0) (solvent A) and filtered.

For the analysis of the linkage region [19, 20], the chondroitinase ABC digests of aggrecan (50 µg) or cartilage GAGs (15 µg) were alkaline-reduced with 0.1 M $NaBH_4$/0.05 M NaOH overnight at 50 °C in a total volume of 150 µl, added to 2 µl of 99.5% acetic acid and 148 µl of solvent A, filtered, and applied to LC-MS/MS.

2.5. LC-MS/MS

MS was performed with a triple quadrupole mass spectrometer equipped with an electrospray ion source (Sciex API 3000; AB SCIEX) in the negative ionization mode. The separation was performed on a Hypercarb column using the Shimadzu HPLC system (LC-10AD pumps, SIL-HTC autosampler, and DGU-14AM degasser) at a flow rate of 0.15 ml/min. The column temperature was maintained at 40 °C. The mobile phases consisted of solvent A and 100% acetonitrile (solvent B). The samples without or with the alkaline-reduction were developed with gradient I (the percentage of solvent B was linearly changed as follows: 0 min, 5%; 9 min, 15%; 10.5 min, 15%; 11 min, 70%; 11.5 min, 70%; 12 min, 5%; and 25 min, 5%) and gradient II (the percentage of solvent B was linearly changed

as follows: 0 min, 5%; 7 min, 10%; 7.5 min, 20%; 12.5 min, 20%; 13 min, 50%; 18 min, 50%; 18.5 min, 70%; 19 min, 70%; 19.5 min, 5%; and 35 min, 5%), respectively. The turbo gas temperature was set at 400 °C and the ion spray voltage was adjusted at −4500 V. The flow rates of the nebulizer, curtain, and collision gases were 8, 8, and 6 (instrument units), respectively. Nitrogen was used as a collision gas as well as a nebulizer. The entrance potential (EP) was −10 V, and the other parameters are provided in Table 2. The mass spectrometer was operated at low resolution for mass filters of first and third (Q1 and Q3, respectively) chambers in the SRM mode. A channel was defined by a combination of a transition of Q1 and Q3 mass filters with other parameters indicated in Table 2. Data acquisition and analysis were performed using Analyst 1.4 software. Standard calibration curves were generated with 3 independent experiments using 1/X-weighted linear regressions and were used to determine the concentrations of the unknown samples. The nomenclature for disaccharide fragmentation by Domon and Costello was used [21].

3. Results

3.1. Simultaneous analysis of 17 disaccharides of CS/DS, HA, HS and KS

Based on the precursor ion and the specific product ion detected with MS and

MS/MS analyses of the disaccharide standards of CS/DS, HA, HS and KS (Table 1), the channels of the SRM mode were set (Table 2).

3.1.1. Setting of the channels for disaccharides

Non-sulfated disaccharides of CS/DS, HA and HS

For ΔHS-0S, we set channel A (378.4 → 175.0) based on a previous report [22]. The MS and MS/MS analyses of ΔCS-0S and ΔHA showed the same patterns as ΔHS-0S (Fig. 1a). All these 3 disaccharides were detected with this channel and were distinguished by different retention times under our LC condition (Fig. 1b).

Mono-sulfated disaccharides of CS/DS and HS

By the MS analysis of 4 mono-sulfated disaccharides, ΔCS-2S, ΔCS-4S, ΔCS-6S and ΔHS-6S, $[M-H]^-$ was produced at approximately *m*/*z* 458.2 (data not shown). The MS/MS analysis showed the same patterns of the product ions as obtained previously with fast atom bombardment (FAB)-MS/MS [23]. By the release of the sulfate group, all the disaccharides produced the product ion at approximately *m*/*z* 97.0, corresponding to $[HSO_4]^-$(Fig. 2a). Only ΔCS-2S, having sulfated uronic acid (UA), produced the product ion

at *m*/*z* 237.0 (B_1 ion). For ΔCS-4S and ΔCS-6S, the most abundant product ions were detected at approximately *m*/*z* 300.0 (Y_1 ion) and *m*/*z* 282.0 (Z_1 ion), respectively, consistent with the results of a previous report [24]. ΔHS-6S did not produce specific product ions. We set channels B-1 (458.2 → 97.0), B-2 (458.2 → 300.0), B-3 (458.2 → 282.0) and B-4 (458.2 → 237.0). The transitions of the former 3 channels had been reported previously [22, 24]. We compared the specificity and sensitivity of the channel B series for the mono-sulfated disaccharides (Fig. 2b). ΔCS-2S, ΔCS-4S and ΔCS-6S were specifically detected with channels B-4, B-2 and B-3, respectively. ΔHS-6S, which was separately eluted from the other 3 disaccharides, was identified with channel B-1 (Table 3). For ΔHS-NS, we set channel E (416.0 → 138.0) based on a previous report [25].

High-sulfated disaccharides of CS/DS

By the MS analysis of 3 di-sulfated disaccharides, ΔCS-2S4S, ΔCS-2S6S and ΔCS-4S6S, signals of approximately *m*/*z* 268.8, 538.3 and 458.2 ($[M-2H]^{2-}$, $[M-H]^-$ and $[M-SO_3-H]^-$, respectively) were produced (data not shown). From the tri-sulfated disaccharide ΔCS-tS, signals at *m*/*z* 308.7, 268.8 and 458.2 ($[M-2H]^{2-}$, $[M-SO_3-2H]^{2-}$ and $[M-2SO_3-H]^-$, respectively) were produced (data not shown). By the MS/MS analysis of the

$[M-2H]^{2-}$ of all the 4 high-sulfated disaccharides, the product ions of approximately *m*/*z* 97.0 ($[HSO_4]^-$) and *m*/*z* 458.0 ($[M-SO_3-H]^-$ and $[M-2SO_3-H]^-$ for di-sulfated and tri-sulfated disaccharides, respectively) were produced (Fig. 3a). The disaccharides having a sulfate group at the C4 position of GalNAc (ΔCS-2S4S, ΔCS-4S6S and ΔCS-tS) produced the Y_1 ion of approximately *m*/*z* 300.0. The disaccharides having a sulfated uronic acid (ΔCS-2S4S, ΔCS-2S6S and ΔCS-tS) produced the B_1 ion at *m*/*z* 237.0.

For detection of the 3 di-sulfated disaccharides ΔCS-2S4S, ΔCS-2S6S and ΔCS-4S6S, we set channels 268.8 → 97.0 (channel C-1), 538.3 → 300.0 (channel C-2), 268.8 → 300.0 and 538.3 → 97.0. Channel C-2 specifically detected ΔCS-2S4S and ΔCS-4S6S, whereas channel C-1 detected all the 3 di-sulfated disaccharides. The transitions 268.8 → 300.0 and 538.3 → 97.0 did not sensitively detect them (data not shown). These 3 disaccharides could be detected with the channel C series (Q1: $[M-2H]^{2-}$ or $[M-H]^-$) but less sensitively than those detected with the channel B series (Q1: $[M-SO_3-H]^-$) (Fig. 4). Only ΔCS-4S6S was separately eluted from mono-sulfated disaccharides (Fig. 5). From these results, for the quantitative analysis of ΔCS-2S4S, ΔCS-2S6S and ΔCS-4S6S, we selected channels C-2, C-1 and B-2, respectively (Table 3).

ΔCS-tS was detected not only with channel D (Q1: $[M-2H]^{2-}$) but also more

sensitively with the channel B (Q1: $[M-2SO_3-H]^-$) and C series (Q1: $[M-SO_3-H]^-$) (Fig. 3b). For ΔCS-tS, the most sensitive channel C-1 was selected (Table 3).

The high-sulfated disaccharides could be detected with multiple channels set for cognate disaccharides and more sensitively with the channels for the less sulfated ones. This phenomenon was observed with KS disaccharides [26], and can be explained by the in-source fragmentation-induced conversion of high-sulfated disaccharides to less-sulfated ones with the release of the labile sulfate group.

High-sulfated disaccharides of HS

The MS and MS/MS analyses of ΔHS-6SNS showed the same patterns as FAB-MS/MS [27]. The MS/MS analysis of the $[M-H]^-$ of ΔHS-2SNS and ΔHS-tS showed the product ion of approximately *m*/*z* 416.0 ($[M-SO_3-H]^-$ and $[M-2SO_3-H]^-$ for ΔHS-2SNS and ΔHS-tS, respectively) (Fig. 4a). ΔHS-2SNS and ΔHS-6SNS were detected with channel F (496.2 → 416.0). ΔHS-tS was detected not only with channel G (576.1 → 416.0) but also more sensitively with channel F (Fig. 4b). From these results, for ΔHS-2SNS, ΔHS-6SNS and ΔHS-tS, channel F was selected (Table 3).

Identification of the total 17 disaccharides of CS/DS, HA, HS and KS

For KS-6S and KS-6S6S, we set channel H (462.1 → 97.0) based on a previous report [26]. By the combination of LC and the MS/MS analysis using the SRM mode consisting of the 10 channels shown in Fig. 5, a total of 17 variously sulfated disaccharides (8 CS/DS, 1 HA, 6 HS, and 2 KS) were simultaneously and quantitatively identified within 18 min.

3.1.2. Quantification of standard disaccharides

Fifteen disaccharides of CS/DS/HA/HS classes can be quantified by using specific calibration curves constructed with increasing the amount of the standard disaccharides. The calibration curves for all disaccharide standard showed a linear response from 0.5 to 125 pmol with correlation coefficients generally greater than 0.99 (Table 3). Since authentic standard materials for KS-6S and KS-6S6S are not commercially available, they were quantified only in a relative quantification manner.

The digests of the 3 different CS chains by chondroitinase ABC, in which no GAG signals were detected with the DMMB assay, were analyzed by this method. Table 4 shows that our results of the disaccharide compositions of the CS chains are similar to those of

previous reports based on UV methods [13, 14].

3.1.3. Quantification of GAG disaccharides from the porcine articular cartilage and yellow ligament

The GAGs extracted from the solubilized porcine articular cartilage were digested with a combination of chondroitinase ABC, keratanase II and hyaluronidase SD, until no GAG signals were detected in the digest with the DMMB assay. The digest was analyzed by our MS/MS method (Fig. 6) and the result of the quantification is shown in Table 5. Seven disaccharides from the 3 GAG classes (4 CS/DS, 1 HA and 2 KS) were simultaneously quantified. In the CS class, non-, mono-, di- and tri-sulfated disaccharides were relatively 14, 85, 1 and 0%, respectively. These findings are consistent with those of a previous report based on fluorescence and MS detection showing that most CS of the porcine nose cartilage exists in the mono-sulfated form [28]. In the same report, HA was not detected. With our method, the amounts of CS and HA were 75.7 and 0.6 nmol/mg wet weight of the articular cartilage, respectively, and the ratio of HA to CS was estimated to be approximately 0.008. By a relative quantification with our standard KS disaccharides, the amount of KS class was estimated to be approximately 5 nmol/mg wet weight of the cartilage (data not shown). These

levels are similar to those in previous reports on the human [29] and rabbit cartilage [30]. The total disaccharides was estimated to be approximately 37 μg/mg wet weight of the cartilage, similar to the GAG content (33.3 μg/mg) quantified by the DMMB assay. These values are similar to those in a previous report on the human cartilage [31]. From these results, our quantitative composition analysis of GAG disaccharides seems to be able to obtain acceptable data.

In the chondroitinase ABC digest of the yellow ligament GAGs, 6 disaccharides of 2 GAG classes (5 CS/DS and 1 HA) were simultaneously quantified (Table 5). In the CS class, non-, mono-, di- and tri-sulfated disaccharides were relatively 11, 76, 13 and 0%, respectively. The amounts of CS and HA were 2.1 and 0.1 nmol/mg wet weight of the ligament, respectively, and the ratio of HA to CS was estimated to be approximately 0.05. This is a first report of the detailed composition analysis of GAG disaccharides of the yellow ligament.

3.2. Analysis of nonreducing and reducing terminals of CS

In the CS digest, some monosaccharides (GalNAc-4S, GalNAc-6S and GalNAc-4S6S) and disaccharides (GlcA-GalNAc-4S (CS-4S) and GlcA-GalNAc-6S (CS-6S)) derived from the nonreducing terminals had been detected [6]. For GalNAc-4S and

GalNAc-6S, channel J (300.3 → 97.0) (Table 2) was set based on the MS/MS analysis. Under our LC conditions, they eluted at 9.7 and 9.9 min, respectively, and were not chromatographically separated (data not shown). The calibration curve of each monosaccharide is considerably similar and linear within 1–50 pmol. For the other monosaccharide GalNAc-4S6S and the disaccharides CS-4S and CS-6S, channels K (380.3 → 97.0) and L (476.0 → 97.0) were set, respectively, based on their $[M-H]^-$ and the estimated product ion at *m*/*z* 97 ($[HSO_4]^-$). With these channels, some peaks corresponding to GalNAc-4S and/or GalNAc-6S and CS-4S and/or CS-6S were also detected from the digests of CS chains and cartilage GAGs. A peak corresponding to GalNAc-4S6S was also detected from the digests of CS chains (data not shown).

In the chondroitinase ABC digested and β-eliminated cartilage proteoglycan aggrecan, variously sulfated ΔUA(β1-3)GalNAc(β1-4)GlcA(β1-3)Gal(β1-3)Gal(β1-4)xylitol (ΔHexaSch-0S-ol) derived from the linkage region have been detected [20]. To detect ΔHexaSch-0S-ol and its mono- and di-sulfated forms (ΔHexaSch-mS-ol and ΔHexaSch-dS-ol, respectively), we set channels M (506.0 → 175.0), N (546.0 → 97.0) and O (586.3 → 97.0), respectively, based on previous reports [32, 33] (Table 2). These channels detected some peaks from the reduced digests of aggrecan and cartilage GAGs, but not from the

non-reduced digest of aggrecan (data not shown). These saccharides of nonreducing and reducing terminals of CS from proteoglycan were first recognized with the SRM mode.

4. Discussion

In this study, we have developed a simple method for the simultaneous analysis of 17 internal disaccharides from 4 GAG classes and 5 nonreducing terminal saccharides from CS using LC-ESI-MS/MS with the SRM mode. This quantitative composition analysis was confirmed to obtain reliable data comparable to those from previous reports based on UV method [13, 14]. By using this method, we first quantified 7 and 6 internal disaccharides from the porcine articular cartilage and yellow ligament GAGs, respectively. Moreover 2 nonreducing saccharides of CS/DS were also simultaneously detected from these 2 tissues. From these results, we were able to obtain information on the classes, the sulfation patterns and the chain length of GAGs.

With our method, 2 isomeric disaccharides (ΔCS-2S and ΔCS-6S) were distinguished by the specific channels of SRM, and 11 disaccharides were just distinguished by combination of the specific channels and LC separation. Only 4 disaccharides (ΔCS-tS, ΔHS-NS, ΔHS-tS and KS-6S6S) were distinguishable at the MS level. As the result, single

injection analysis distinguished 17 multiple sulfated disaccharides (8 CS/DS, 1 HA, 6 HS, and 2 KS) within 18 min and can be quantified with a sensitivity of less than 0.5 pmol. From the GAG digests of the porcine articular cartilage and yellow ligament, 4 CS/DS, 1 HA and 2 KS disaccharides, and 5 CS/DS and 1 HA disaccharides were quantified, respectively. The results show that the ratio of the disaccharides of HA to CS/DS in the yellow ligament (0.05), even with incomplete digestion of HA, is higher than that of the cartilage GAGs (0.008) (Table 5). The characteristic GAG compositions of the animal tissues may reflect their different functions physiologically. Thus, this composition analysis of GAGs would accelerate the elucidation of the function of each GAG class.

For the first time, in a SRM mode we distinguished 8 high-sulfated disaccharides over 3 classes (ΔCS-2S4S, ΔCS-2S6S, ΔCS-4S6S, ΔCS-tS, ΔHS-2SNS, ΔHS-6SNS, ΔHS-tS and KS-6S6S) at the same time. We found a higher level of di-sulfated CS/DS disaccharides in the ligament (13%) than in the cartilage (1%) (Table 5). Galactosaminoglycan uronyl-2-sulfotransferase, responsible for the synthesis of high-sulfated CS/DS, has been shown to transfer a sulfate to the C2 position of iduronyl residues in DS rather than glucuronyl residues in CS [34]. Thus, high levels of di-sulfated disaccharides would reflect the presence of DS as shown in the DS-rich CSB chain (Table 4) and the porcine intestinal

mucosa [28]. DS-rich proteoglycans such as decorin or biglycan are known to be abundant in the ligaments. In fact, the human yellow ligament has been reported to have plenty of these DS-rich proteoglycans with di-sulfated disaccharides [35]. Our data clearly demonstrated the differences between the cartilage and ligament not only in the composition of the GAG classes but also in the sulfation patterns of CS/DS.

In the nonreducing terminal of CS, the presence of some monosaccharides and disaccharides was shown, and GalNAc-4S was reported to be a main component in the human cartilage [6]. The chain length of CS was calculated from the ratio of internal disaccharides to nonreducing termini, and its alteration has been shown during the development [6] or in disorders such as osteoarthritis [7]. By quantification of cartilage GAGs, the chain length of CS was calculated from the ratio of internal disaccharides (75.7 nmol/mg wet weight cartilage, Table 5) to GalNAc-4S and/or GalNAc-6S (5.4 nmol/mg wet weight cartilage, data not shown), and the value obtained was approximately 14, which was similar to that of previous reports on the human cartilage [6, 7]. We can discriminate the nonreducing terminal saccharides from the cognate internal disaccharides based on the difference in masses. Thus, even one-shot analysis without any derivatization steps enables to estimate the chain length. Our method could facilitate the evaluation of the alteration of the

length of CS chains and its application could be expanded to evaluate the length of other GAG chains of animal tissues.

In this paper, we described a simple and rapid method suitable for simultaneously quantification analysis of complex GAG mixtures derived from biological samples. Using this method, we successfully demonstrated characteristic GAG features of the connective tissues in terms of the class, sulfation pattern and chain length. The difference in these GAG features should be closely associated with their biological functions. Thus, this novel method would help establish the basic system for GAGomic analysis and lead to an understanding of the structure-function relationships of GAGs and the significance of their alteration in the disorders of complex biological systems.

5. Acknowledgements

We would like to thank Dr. Yoko Ohashi (The University of Electro-Communications, Japan) and Dr. Toshikazu Minamisawa (Seikagaku Corporation) for their helpful suggestions. This work was supported by JSPS KAKENHI Grant Numbers 23930010, 25930009 and 25462371.

References

[1] J. Zaia, On-line separations combined with MS for analysis of glycosaminoglycans, Mass Spectrom. Rev. 28 (2009) 254–272.

[2] M.T. Bayliss, D. Osborne, S. Woodhouse, C. Davidson, Sulfation of chondroitin sulfate in human articular cartilage. The effect of age, topographical position, and zone of cartilage on tissue composition, J. Biol. Chem. 274 (1999) 15892–15900.

[3] M. Sharif, D.J. Osborne, K. Meadows, S.M. Woodhouse, E.M. Colvin, L. Shepstone, P.A. Dieppe, The relevance of chondroitin and keratan sulphate markers in normal and arthritic synovial fluid, Br. J. Rheumatol. 35 (1996) 951–957.

[4] M. Warda, F. Zhang, M. Radwan, Z. Zhang, N. Kim, Y.N. Kim, R.J. Linhardt, J. Han, Is human placenta proteoglycan remodeling involved in pre-eclampsia?, Glycoconj. J. 25 (2008) 441–450.

[5] S. Tomatsu, T. Fujii, M. Fukushi, T. Oguma, T. Shimada, M. Maeda, K. Kida, Y. Shibata, H. Futatsumori, A.M. Montano, R.W. Mason, S. Yamaguchi, Y. Suzuki, T. Orii, Newborn screening and diagnosis of mucopolysaccharidoses, Mol. Genet. Metab. 110 (2013) 42–53.

[6] A.H. Plaas, S. Wong-Palms, P.J. Roughley, R.J. Midura, V.C. Hascall, Chemical and

immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan, J. Biol. Chem. 272 (1997) 20603–20610.

[7] A.H. Plaas, L.A. West, S. Wong-Palms, F.R. Nelson, Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate, J. Biol. Chem. 273 (1998) 12642–12649.

[8] K. Takeuchi, N. Yoshioka, S. Higa Onaga, Y. Watanabe, S. Miyata, Y. Wada, C. Kudo, M. Okada, K. Ohko, K. Oda, T. Sato, M. Yokoyama, N. Matsushita, M. Nakamura, H. Okano, K. Sakimura, H. Kawano, H. Kitagawa, M. Igarashi, Chondroitin sulphate *N*-acetylgalactosaminyl-transferase-1 inhibits recovery from neural injury, Nat. Commun. 4 (2013) 2740.

[9] A. Weyers, B. Yang, D.S. Yoon, J.H. Park, F.M. Zhang, K.B. Lee, R.J. Linhardt, A Structural Analysis of Glycosaminoglycans from Lethal and Nonlethal Breast Cancer Tissues: Toward a Novel Class of Theragnostics for Personalized Medicine in Oncology?, OMICS. 16 (2012) 79–89.

[10] M. Hikino, T. Mikami, A. Faissner, A. Vilela-Silva, M.S.G. Pavao, K. Sugahara, Oversulfated dermatan sulfate exhibits neurite outgrowth-promoting activity toward embryonic mouse hippocampal neurons: implications of dermatan sulfate in

neuritogenesis in the brain, J. Biol. Chem. 278 (2003) 43744–43754.

[11] S. Mizumoto, J. Takahashi, K. Sugahara, Receptor for advanced glycation end products (RAGE) functions as receptor for specific sulfated glycosaminoglycans, and anti-RAGE antibody or sulfated glycosaminoglycans delivered in vivo inhibit pulmonary metastasis of tumor cells, J. Biol. Chem. 287 (2012) 18985–18994.

[12] P. Martinez, G. Vergoten, F. Colomb, M. Bobowski, A. Steenackers, M. Carpentier, F. Allain, P. Delannoy, S. Julien, Over-sulfated glycosaminoglycans are alternative selectin ligands: insights into molecular interactions and possible role in breast cancer metastasis, Clin. Exp. Metastasis 30 (2013) 919–931.

[13] K. Yoshida, S. Miyauchi, H. Kikuchi, A. Tawada, K. Tokuyasu, Analysis of unsaturated disaccharides from glycosaminoglycuronan by high-performance liquid chromatography, Anal. Biochem. 177 (1989) 327–332.

[14] A. Kinoshita, K. Sugahara, Microanalysis of glycosaminoglycan-derived oligosaccharides labeled with a fluorophore 2-aminobenzamide by high-performance liquid chromatography: application to disaccharide composition analysis and exosequencing of oligosaccharides, Anal. Biochem. 269 (1999) 367–378.

[15] Y. Takegawa, K. Araki, N. Fujitani, J. Furukawa, H. Sugiyama, H. Sakai, Y. Shinohara,

Simultaneous analysis of heparan sulfate, chondroitin/dermatan sulfates, and hyaluronan disaccharides by glycoblotting-assisted sample preparation followed by single-step zwitter-ionic-hydrophilic interaction chromatography, Anal. Chem. 83 (2011) 9443–9449.

[16] B. Yang, Y.D. Chang, A.M. Weyers, E. Sterner, R.J. Linhardt, Disaccharide analysis of glycosaminoglycan mixtures by ultra-high-performance liquid chromatography-mass spectrometry, J. Chromatogr. A 1225 (2012) 91–98.

[17] S. Ohtsuki, Y. Uchida, Y. Kubo, T. Terasaki, Quantitative targeted absolute proteomics-based ADME research as a new path to drug discovery and development: methodology, advantages, strategy, and prospects, J. Pharm. Sci. 100 (2011) 3547–3559.

[18] R.W. Farndale, D.J. Buttle, A.J. Barrett, Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue, Biochim. Biophys. Acta 883 (1986) 173–177.

[19] H.C. Robinson, J.J. Hopwood, The alkaline cleavage and borohydride reduction of cartilage proteoglycan, Biochem. J. 133 (1973) 457–470.

[20] K. Sugahara, I. Yamashina, P. De Waard, H. Van Halbeek, J.F. Vliegenthart, Structural studies on sulfated glycopeptides from the carbohydrate-protein linkage region of

chondroitin 4-sulfate proteoglycans of swarm rat chondrosarcoma. Demonstration of the structure Gal(4-O-sulfate)beta 1-3Gal beta 1-4XYL beta 1-O-Ser, J. Biol. Chem. 263 (1988) 10168–10174.

[21] B. Domon, C.E. Costello, A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates, Glycoconjugate J. 5 (1988) 397–409.

[22] T. Oguma, S. Tomatsu, A.M. Montano, O. Okazaki, Analytical method for the determination of disaccharides derived from keratan, heparan, and dermatan sulfates in human serum and plasma by high-performance liquid chromatography/turbo ionspray ionization tandem mass spectrometry, Anal. Biochem. 368 (2007) 79–86.

[23] D.J. Lamb, H.M. Wang, L.M. Mallis, R.J. Linhardt, Negative ion fast-atom bombardment tandem mass spectrometry to determine sulfate and linkage position in glycosaminoglycan-derived disaccharides, J. Am. Soc. Mass Spectrom. 3 (1992) 797–803.

[24] J. Zaia, C.E. Costello, Compositional analysis of glycosaminoglycans by electrospray mass spectrometry, Anal. Chem. 73 (2001) 233–239.

[25] T. Oguma, H. Toyoda, T. Toida, T. Imanari, Analytical method of heparan sulfates using high-performance liquid chromatography turbo-ionspray ionization tandem mass

spectrometry, J. Chromatogr. B Biomed. Sci. Appl. 754 (2001) 153–159.

[26] T. Oguma, H. Toyoda, T. Toida, T. Imanari, Analytical method for keratan sulfates by high-performance liquid chromatography/turbo-ionspray tandem mass spectrometry, Anal. Biochem. 290 (2001) 68–73.

[27] T. Ii, M. Kubota, S. Okuda, T. Hirano, M. Ohashi, Negative-ion fast atom bombardment tandem mass spectrometry for characterization of sulfated unsaturated disaccharides from heparin and heparan sulfate, Glycoconj. J. 12 (1995) 162–172.

[28] N.Volpi, High-performance liquid chromatography and on-line mass spectrometry detection for the analysis of chondroitin sulfates/hyaluronan disaccharides derivatized with 2-aminoacridone, Anal. Biochem. 397 (2010) 12–23.

[29] G. Rizkalla, A. Reiner, E. Bogoch, A.R. Poole, Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease, J. Clin. Inves. 90 (1992) 2268–2277.

[30] A. Asari, S. Miyauchi, K. Miyazaki, A. Hamai, K. Horie, T. Takahashi, T. Sekiguchi, A. Machida, K. Kohno, Y. Uchiyama, Intra- and extracellular localization of hyaluronic acid and proteoglycan constituents (chondroitin sulfate, keratan sulfate, and protein core) in articular cartilage of rabbit tibia, J. Histochem. Cytochem. 40 (1992) 1693–1704.

[31] A.M. Hitchcock, K.E. Yates, S. Shortkroff, C.E. Costello, J. Zaia, Optimized extraction of glycosaminoglycans from normal and osteoarthritic cartilage for glycomics profiling, Glycobiology 17 (2007) 25–35.

[32] R.P. Estrella, J.M. Whitelock, N.H. Packer, N.G. Karlsson, Graphitized carbon LC-MS characterization of the chondroitin sulfate oligosaccharides of aggrecan, Anal. Chem., 79 (2007) 3597–3606.

[33] H. Lu, L.M. McDowell, D.R. Studelska, L. Zhang, Glycosaminoglycans in Human and Bovine Serum: Detection of Twenty-Four Heparan Sulfate and Chondroitin Sulfate Motifs Including a Novel Sialic Acid-modified Chondroitin Sulfate Linkage Hexasaccharide, Glycobiol. Insights 2010 (2010) 13–28.

[34] M. Kobayashi, G. Sugumaran, J. Liu, N.W. Shworak, J.E. Silbert, R.D. Rosenberg, Molecular cloning and characterization of a human uronyl 2-sulfotransferase that sulfates iduronyl and glucuronyl residues in dermatan/chondroitin sulfate, J. Biol. Chem. 274 (1999) 10474–10480.

[35] M. Yukawa, K. Takagaki, T. Itabashi, K. Ueyama, S. Harata, M. Endo, Structural varieties of small proteoglycans in human spinal ligament, Connect. Tissue Res. 42 (2001) 209–222.

Legends to figures

Figure 1

Identification of non-sulfated disaccharides of CS/DS, HA and HS classes

(a) Product ion spectra with fragmentation scheme of ΔCS-0S and ΔHA. (b) Chromatogram with channel A showing the separation of ΔCS-0S, ΔHA and ΔHS-0S (100 pmol).

Figure 2

Identification of mono-sulfated disaccharides of CS/DS and HS classes

(a) Product ion spectra with fragmentation scheme of ΔCS-2S, ΔCS-4S, ΔCS-6S and ΔHS-6S. (b) Chromatogram with the indicated channels showing the separation of the disaccharides (100 pmol).

Figure 3

Identification of di- and tri-sulfated disaccharides of CS/DS class

(a) Product ion spectra with fragmentation scheme of ΔCS-2S4S, ΔCS-2S6S, ΔCS-4S6S and ΔCS-tS. (b) Chromatogram with the indicated channels showing the separation of the disaccharides (100 pmol). The intensity magnification of the chromatogram with channel B-4

is 3 times that of the other channels.

Figure 4

Identification of di-and tri-sulfated disaccharides of HS class

(a) Product ion spectra with fragmentation scheme of ΔHS-2SNS and ΔHS-tS. (b) Chromatogram with the indicated channels showing the separation of ΔHS-2SNS, ΔHS-6SNS, and ΔHS-tS (100 pmol).

Figure 5

Separation of a mixture of 17 disaccharides derived from 4 classes

(a) The simultaneous measurement of 15 disaccharides of CS/DS, HA and HS classes (100 pmol) and 2 disaccharides of KS class (approximately 2 nmol) by the SRM analysis of MS/MS combined with LC separation. (b) The extracted ion chromatograms with indicated channels.

Figure 6

Profile of GAG disaccharides of the cartilage

GAGs derived from the porcine cartilage were simultaneously digested with chondroitinase ABC, keratanase II and hyaluronidase SD. Internal disaccharides in the digest were quantitatively detected with the indicated channels. The intensity magnification of the chromatograms with channels C-2 and H are 1000 and 100 times those of the other channels, respectively.

Table 1 **Investigated compounds**

Table 2 **Parameters for SRM analysis of GAGs**

Table 3 **Linearity and sensitivity of SRM analysis with specific channels**

Table 4 **Disaccharides composition analysis of CS chains**

Table 5 **Quantification profile of GAG disaccharides in the articular cartilage and yellow ligament**

Figure1

Click here to download Figure: Fig1.pdf

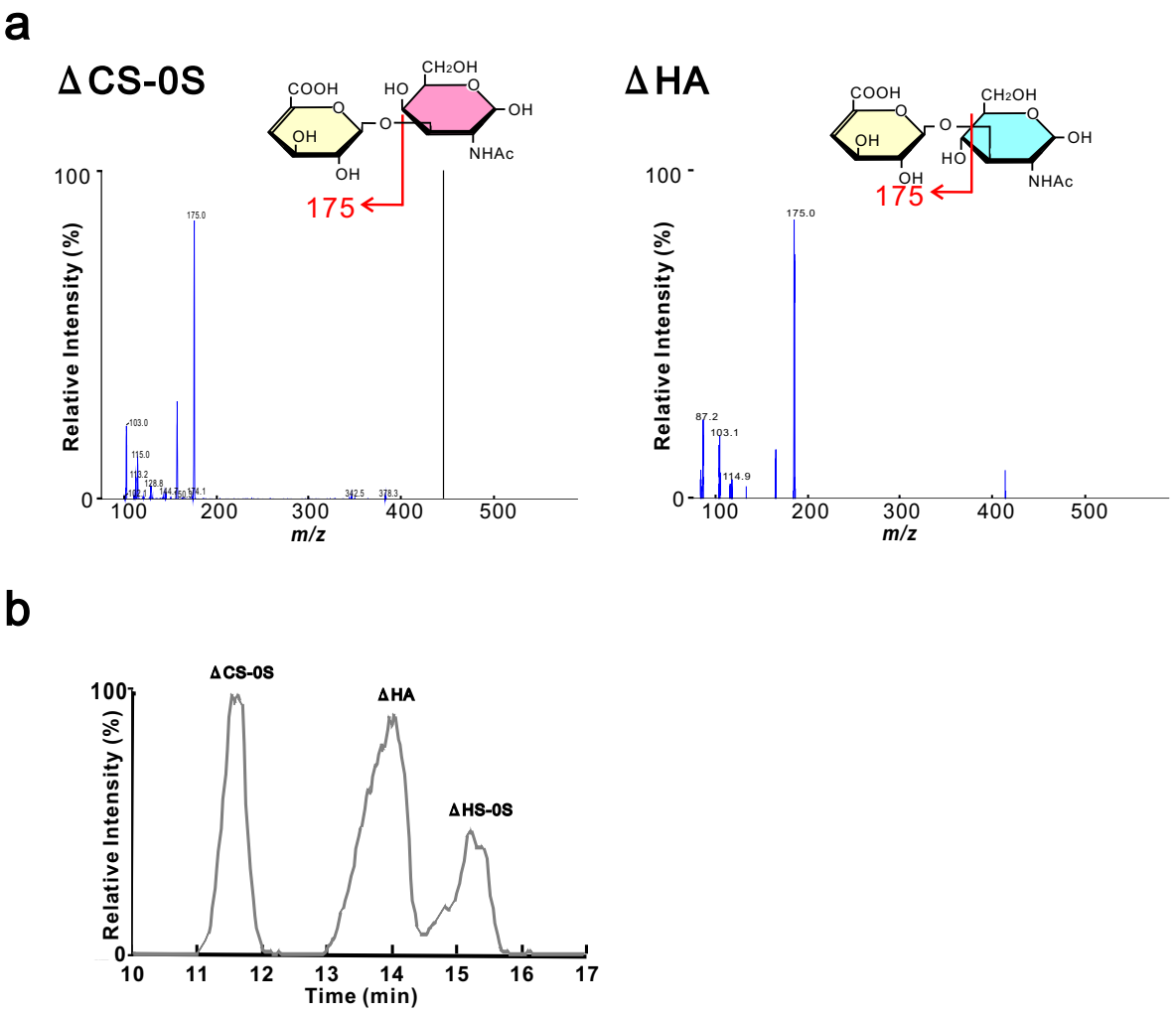


Figure2

Click here to download Figure: Fig2.pdf

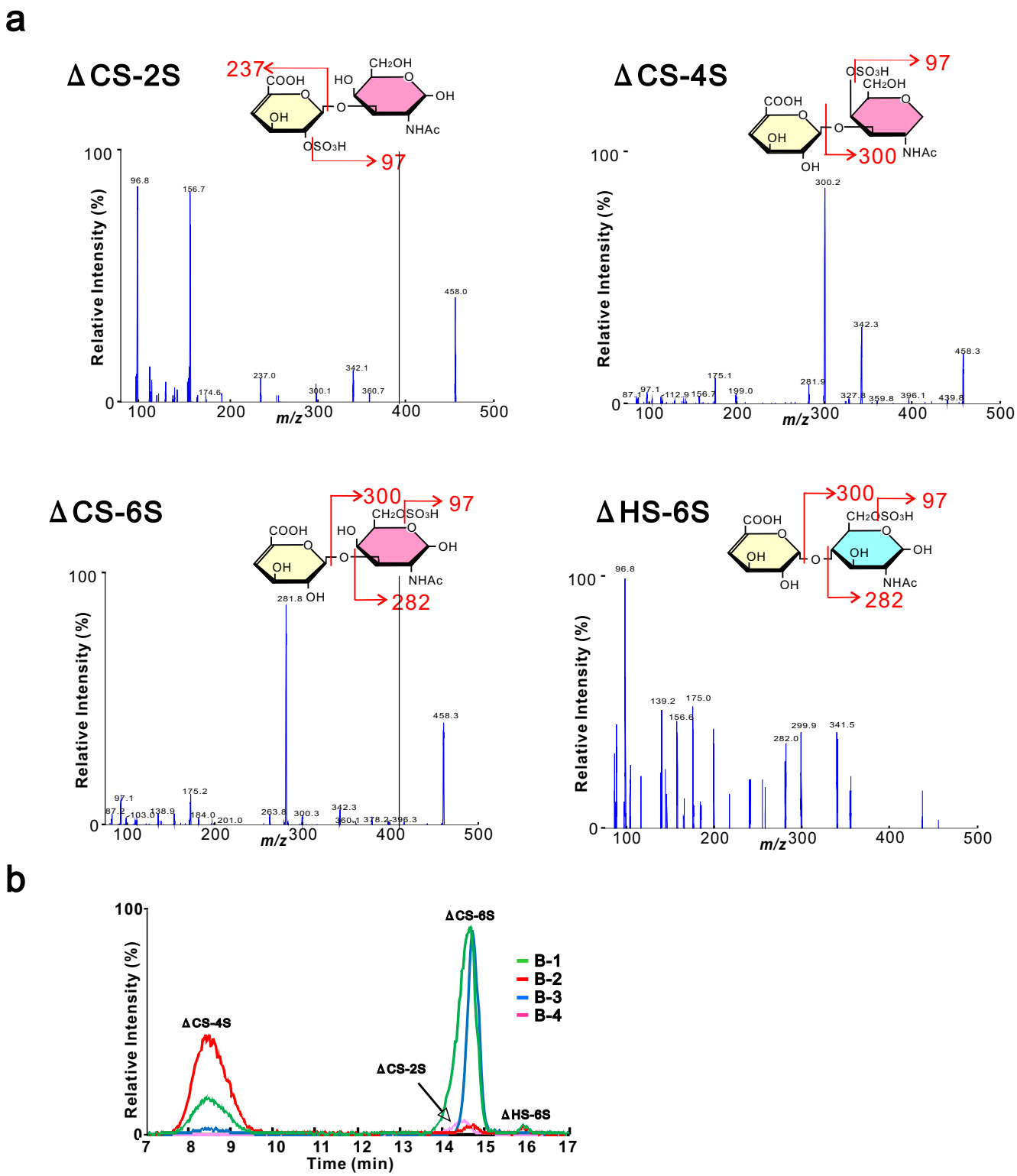


Figure3

Click here to download Figure: Fig3.pdf

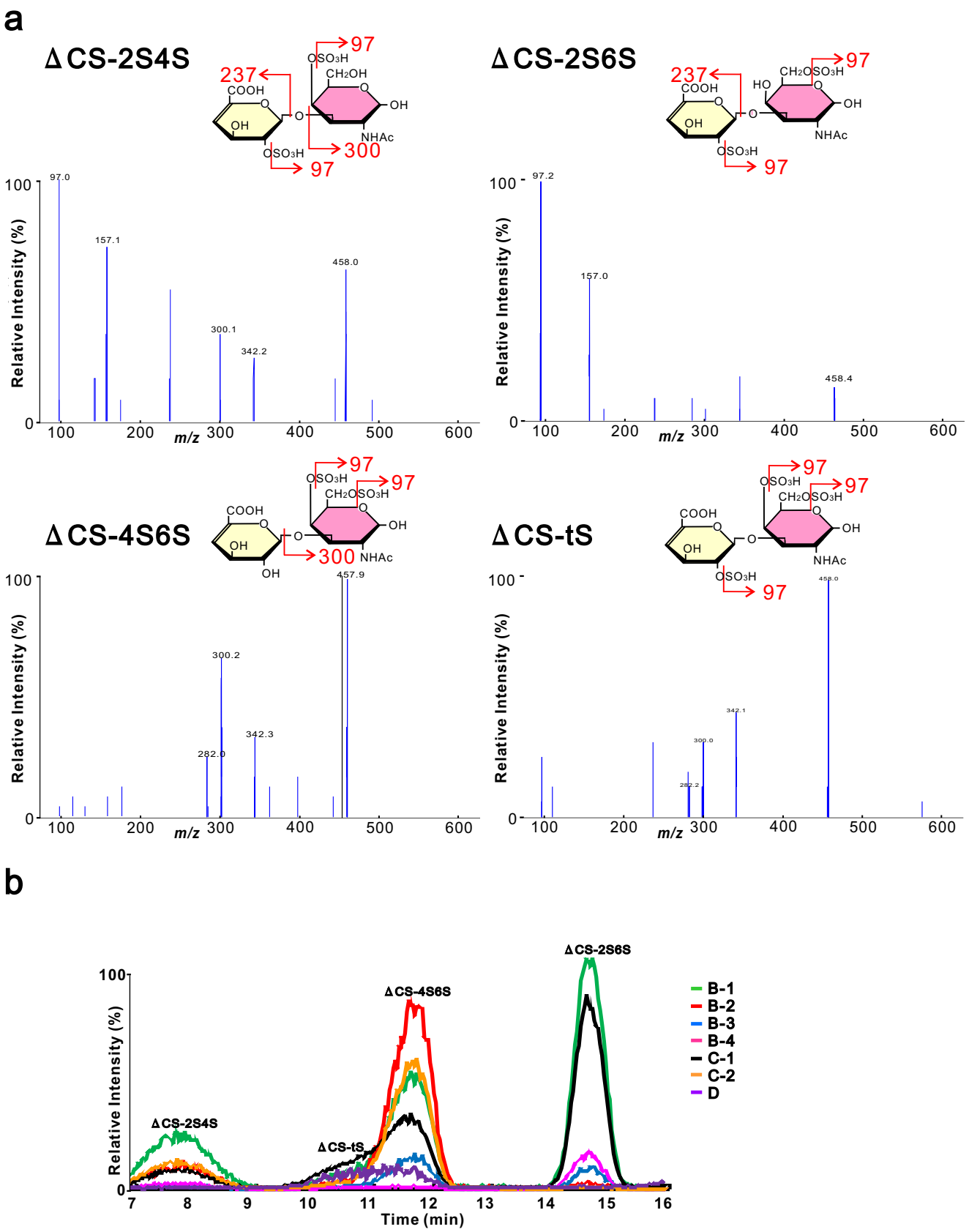


Figure4

Click here to download Figure: Fig4.pdf

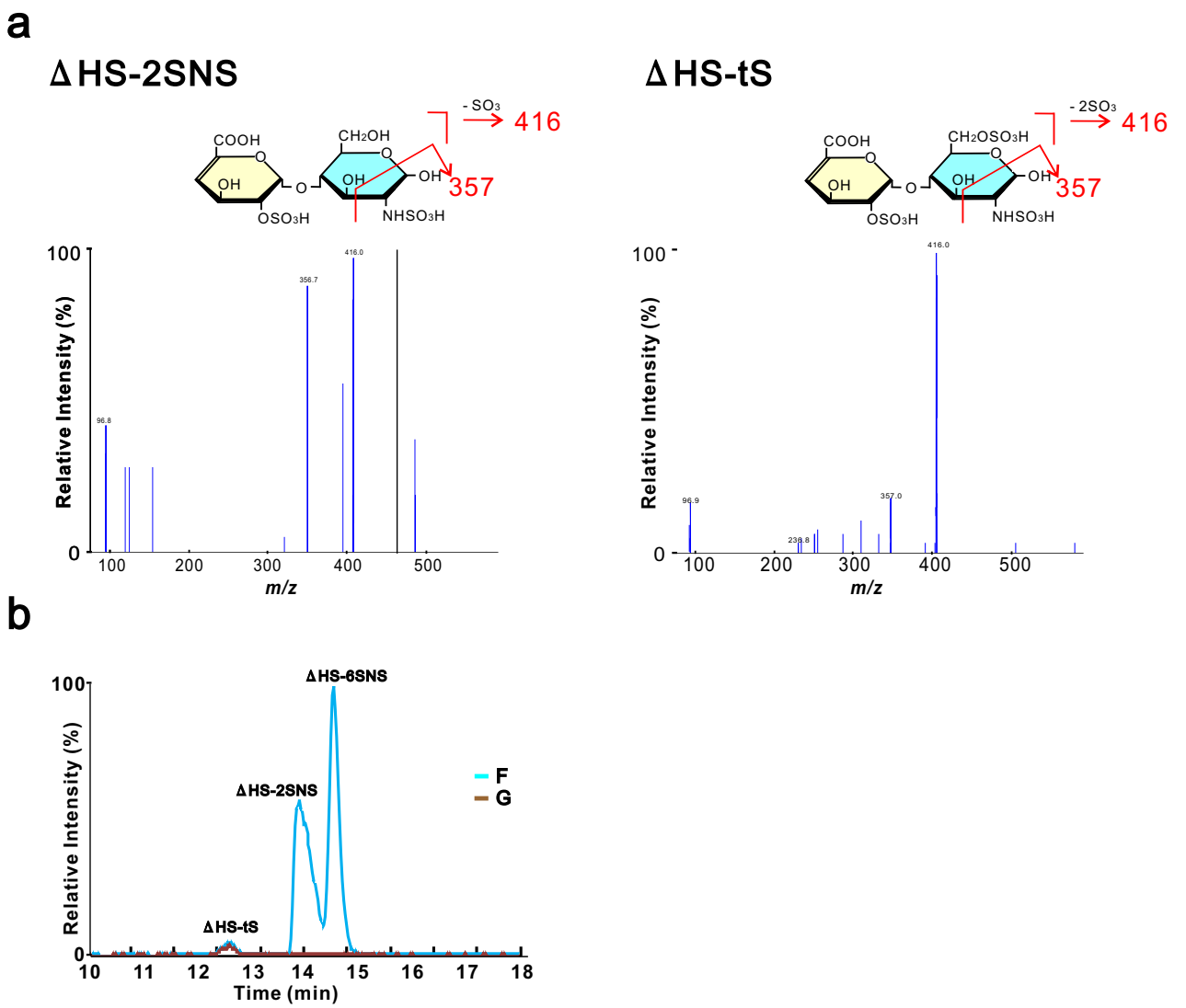


Figure5

Click here to download Figure: Fig5.pdf

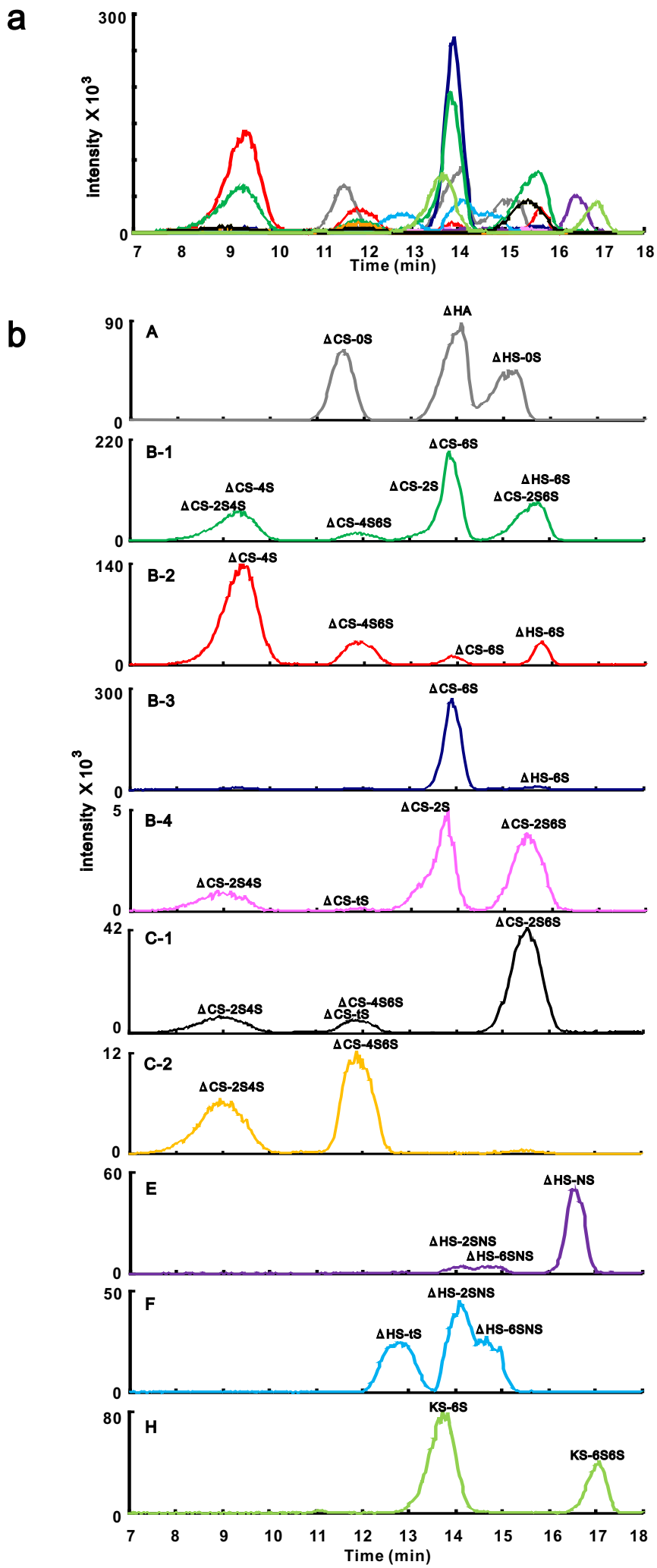


Figure6

Click here to download Figure: Fig6.pdf

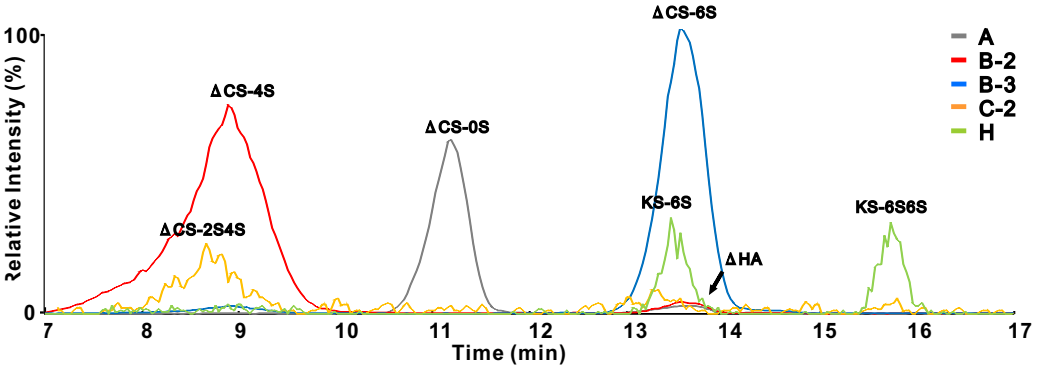


Table1

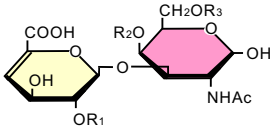
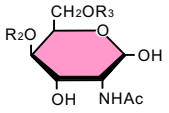
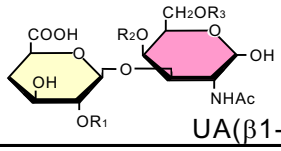
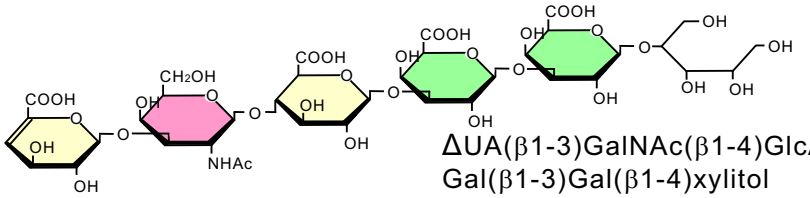
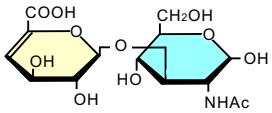
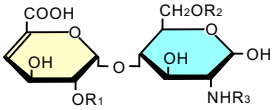
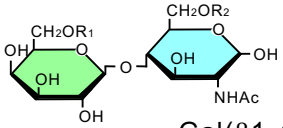
Class	Abbreviation	Structure	R_1	R_2	R_3	M.W.
CS/DS	ΔCS-0S	 ΔUA(β1-3)GalNAc	H	H	H	379.3
	ΔCS-2S		SO_3H	H	H	459.4
	ΔCS-4S		H	SO_3H	H	459.4
	ΔCS-6S		H	H	SO_3H	459.4
	ΔCS-2S4S		SO_3H	SO_3H	H	539.4
	ΔCS-2S6S		SO_3H	H	SO_3H	539.4
	ΔCS-4S6S		H	SO_3H	SO_3H	539.4
	ΔCS-tS		SO_3H	SO_3H	SO_3H	619.5
	GalNAc-4S	 GalNAc	-	SO_3H	H	301.3
	GalNAc-6S		-	H	SO_3H	301.3
	GalNAc-4S6S		-	SO_3H	SO_3H	381.3
	CS-4S	 UA(β1-3)GalNAc	H	SO_3H	H	477.4
	CS-6S		H	H	SO_3H	
	ΔHexaSch-0S-ol	 ΔUA(β1-3)GalNAc(β1-4)GlcA(β1-3) Gal(β1-3)Gal(β1-4)xylitol				1013.9
HA	ΔHA	 ΔUA(β1-3)GlcNAc	-	-	-	379.3
HS	ΔHS-0S	 ΔUA(α1-4)GlcN	H	H	Ac	379.3
	ΔHS-6S		H	SO_3H	Ac	459.4
	ΔHS-NS		H	H	SO_3H	417.3
	ΔHS-2SNS		SO_3H	H	SO_3H	497.4
	ΔHS-6SNS		H	SO_3H	SO_3H	497.4
	ΔHS-tS		SO_3H	SO_3H	SO_3H	577.5
KS	KS-6S	 Gal(β1-4)GlcNAc	H	SO_3H	-	463.4
	KS-6S6S		SO_3H	SO_3H	-	543.4

Table2

Channel		Target		Transition *(m/z)*		Parameter			
		Saccharide	M.W.	Q1	Q3	DP	FP	CE	CXP
A		ΔCS-0S, ΔHA, ΔHS-0S	379.3	378.4	175.0	-30	-150	-18	-9
B	B-1	ΔCS-2S, ΔCS-4S, ΔCS-6S, ΔHS-6S	459.4	458.2	97.0	-60	-300	-60	-5
	B-2				300.0	-50	-300	-30	-5
	B-3				282.0	-70	-330	-30	-5
	B-4				237.0	-40	-270	-30	-5
C	C-1	ΔCS-2S4S, ΔCS-2S6S, ΔCS-4S6S	539.5	268.8	97.0	-40	-200	-50	-5
	C-2			538.3	300.0	-40	-350	-40	-5
D		ΔCS-tS	619.5	308.7	97.0	-45	-250	-40	-5
E		ΔHS-NS	417.3	416.0	138.0	-60	-240	-30	-3
F		ΔHS-2SNS, ΔHS-6SNS	497.4	496.2	416.0	-50	-330	-25	-5
G		ΔHS-tS	577.5	576.1	416.0	-40	-300	-40	-8
H		KS-6S	463.5	462.1	97.0	-70	-340	-80	-5
I		KS-6S6S	543.6	542.0	97.0	-80	-340	-80	-5
J		GalNAc-4S, GalNAc-6S	301.3	300.3	97.0	-36	-170	-40	-3
K		GalNAc-4S6S	381.3	380.3	97.0	-30	-300	-50	-5
L		CS-4S, CS-6S	477.4	476.0	97.0	-50	-300	-80	-5
M		ΔHexaSch-0S-ol	1013.9	506.0	175.0	-60	-300	-30	-5
N		ΔHexaSch-mS-ol	1093.3	546.0	97.0	-60	-300	-80	-5
O		ΔHexaSch-dS-ol	1174.0	586.3	97.0	-60	-300	-80	-5

DP: declustering potential, **FP:** focusing potential、**CE:** collision energy
CXP: collision cell exit potential, **mS:** mono-sulfate, **dS:** di-sulfate

Table3

Disaccharide	Channel	Range (pmol)	LOD (pmol)	cc
ΔCS-0S	A	0.5 - 500	0.061	0.997
ΔCS-2S	B-4	2.0 - 250	0.500	0.995
ΔCS-4S	B-2	0.5 - 500	0.122	0.992
ΔCS-6S	B-3	0.5 - 500	0.122	0.997
ΔCS-2S4S	C-2	0.5 - 200	0.031	0.996
ΔCS-2S6S	C-1	0.5 - 250	0.031	0.988
ΔCS-4S6S	B-2	0.5 - 250	0.031	0.987
ΔCS-tS	C-1	1.0 - 500	0.122	0.990
ΔHA	A	0.5 - 250	0.031	0.990
ΔHS-0S	A	0.5 - 250	0.031	0.993
ΔHS-6S	B-1	0.5 - 125	0.031	0.986
ΔHS-NS	E	0.5 - 250	0.031	0.996
ΔHS-2SNS	F	0.5 - 125	0.031	0.995
ΔHS-6SNS	F	0.5 - 250	0.122	0.993
ΔHS-tS	F	1.0 - 500	0.244	0.988

cc: correlation coefficient LOD: limit of detection

Table4

Disaccharide composition (%)								
	CSA			CSB		CSC		
ΔCS-0S	1.2	1.6	1.2	0.4	0.7	1.6	1.7	1.0
ΔCS-2S	0.0	0.0	n.d.	0.0	n.d.	0.0	0.0	n.d.
ΔCS-4S	74.3	76.2	72.4	88.4	90.0	17.9	15.4	15.7
ΔCS-6S	23.1	19.3	24.9	2.7	1.9	73.1	72.9	73.8
ΔCS-2S4S	0.1	0.0	0.0	7.3	6.5	0.3	0.0	0.0
ΔCS-2S6S	0.7	2.7	0.9	1.0	0.6	5.0	9.3	8.8
ΔCS-4S6S	0.6	0.3	0.6	0.3	0.0	2.1	0.6	0.7
ΔCS-tS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ref		[13]	[14]		[13]		[13]	[14]

n.d. : not determined
ref : reference number

Table5

Disaccharide	Cartilage		Ligament	
	nmol/ mg wet weight			
ΔCS-0S	10.55	(14)	0.23	(11)
ΔCS-2S	0.00	(0)	0.00	(0)
ΔCS-4S	45.75	(60)	1.30	(62)
ΔCS-6S	18.68	(25)	0.30	(14)
ΔCS-2S4S	0.76	(1)	0.20	(10)
ΔCS-2S6S	0.00	(0)	0.07	(3)
ΔCS-4S6S	0.00	(0)	0.00	(0)
ΔCS- tS	0.00	(0)	0.00	(0)
Total	75.74	(100)	2.10	(100)
ΔHA	0.60		0.10	

Numbers in parentheses indicate the percentage of each disaccharide in the total of ΔCS .