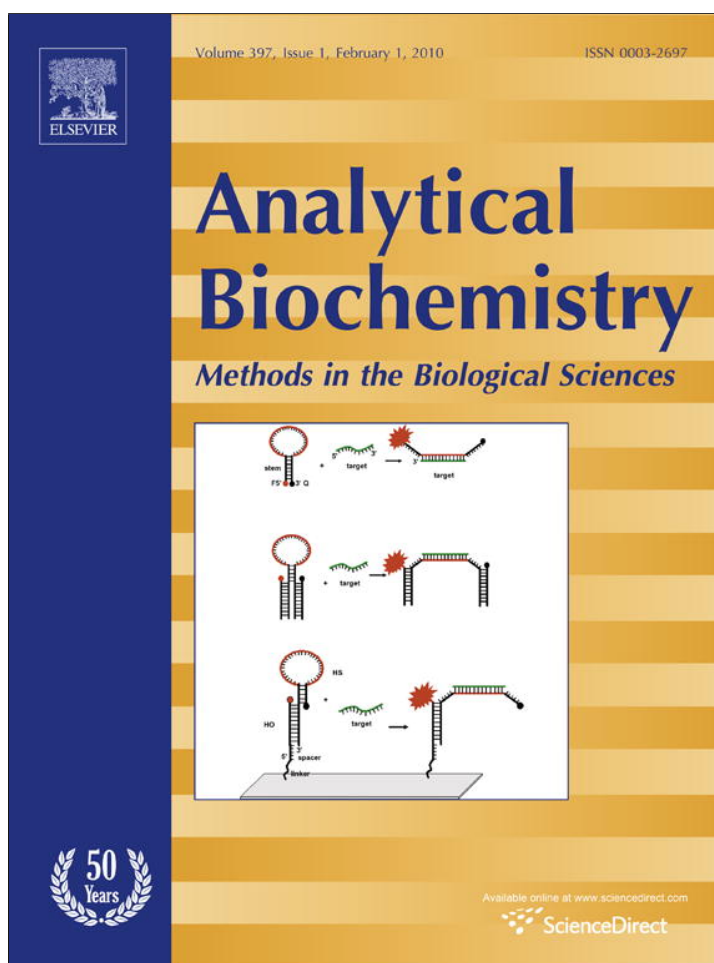


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Ultrapformance liquid chromatography with electrospray ionization ion trap mass spectrometry for chondroitin disaccharide analysis

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ABSTRACT

Chondroitin sulfate (CS) has an important role in cell division, in the central nervous system, and in joint-related pathologies such as osteoarthritis. Due to the complex chemical structure and biological importance of CS, simple, sensitive, high resolution, and robust analytical methods are needed for the analysis of CS disaccharides and oligosaccharides. An ion-pairing, reversed-phase, ultrapformance liquid chromatography (IPRP-UPLC) separation, coupled to electrospray ionization mass spectrometry with an ion trap mass analyzer, was applied for the analyses of CS-derived disaccharides. UPLC separation technology uses small particle diameter, short column length, and elevated column temperature to obtain high resolution and sensitivity. Hexylamine (15 mM) was selected as the optimal ion-pairing reagent.

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Proteoglycans, major components of extracellular matrices and cell surfaces, consist of core proteins to which glycosaminoglycans (GAGs)¹ are covalently linked. GAGs are linear polyanionic polysaccharides composed of repeating disaccharide units that can be divided into four main classes: hyaluronan; chondroitin sulfate (CS) and dermatan sulfate (DS); heparin and heparan sulfate; and keratan sulfate. CS and DS differ from the other three classes in that they contain disaccharides with galactosamine and are known as galactosaminoglycans [1].

CS and DS consist of repeating disaccharide units of residues of uronic acid (β -D-glucuronic in CS and α -L-iduronic in DS) and N-acetyl- β -D-galactosamine (GalNAc) in alternating (1–4) and (1–3) linkages (Fig. 1). The GalNAc residues of most of the disaccharide units are substituted with 4- or 6-O-sulfo groups in CS types A and B (DS) or CS type C, respectively [2]. CS GAGs are polydisperse

with molecular weight distribution depending on the tissue source and having average molecular weights that vary from 10 to 140 kDa [3].

CS plays an important role in fundamental biological processes, including cell division and in the central nervous system [4–6]. CS/DS hybrid chains are crucial to neuronal cell migration and maturation during brain development [5]. CS represents a low-potency effective treatment for the symptoms of osteoarthritis, the most common musculoskeletal disease, impacting 10% of the world's population [7–10]. CS also has other potential therapeutic effects as an antiinflammatory, an antioxidant, and a reducer of allergic response as well as in the treatment of urinary pathologies [11].

GAGs are anionic biopolymers owing to the presence of sulfo and carboxyl groups. Due to the biological significance of CS, rapid and sensitive analytical methods are required for their characterization at the level of disaccharide composition. Therefore, a number of researchers have applied various analytical methods to understand the role of CS in biological systems and for the diagnosis of CS-related diseases [1,3,12,13]. Ion-pairing reversed-phase liquid chromatography (IPRP-LC) is one of the most powerful techniques for the analysis of GAG-derived oligosaccharides [14–20]. In this technique, ion pair reagents, typically amines with hydrophobic alkyl chains, are added to the mobile phases and separation is performed on an RP-LC column. Common ion-pairing reagents used for GAG analysis include tri-*n*-butylamine (TrBA) [15,16,18,19], *n*-pentylamine (PTA) [14], *n*-hexylamine (HXA) [14], tetrabutylammonium (Bu₄N⁺) [15,17], and tetrapropylammonium (Pr₄N⁺) [15]. The use of ion-pairing reagents in the separation

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¹ Abbreviations used: GAG, glycosaminoglycans; CS, chondroitin sulfate; DS, dermatan sulfate; GalNAc, N-acetyl- β -D-galactosamine; IPRP-LC, ion-pairing reversed-phase liquid chromatography; TrBA, tri-*n*-butylamine; PTA, *n*-pentylamine; BEH, bridged ethyl hybrid; HXA, *n*-hexylamine; Bu₄N⁺, tetrabutylammonium; Pr₄N⁺, tetrapropylammonium; ESI-MS, electrospray ionization ion trap mass spectrometry; UPLC, ultrapformance liquid chromatography; HPLC, high-performance liquid chromatography; UV, ultraviolet; MALDI, matrix-assisted laser desorption/ionization; HXA, *n*-hexylamine; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Δ Di-OS, Δ UA-GalNAc; Δ Di-4S, Δ UA-GalNAc4S; Δ Di-6S, Δ UA-GalNAc6S; Δ Di-UA2S, Δ UA2S-GalNAc; Δ Di-diS_B, Δ UA2S-GalNAc4S; Δ Di-diS_D, Δ UA2S-GalNAc6S; Δ Di-diS_E, Δ UA-GalNAc4S6S; Δ Di-TriS, Δ UA2S-GalNAc4S6S; EIC, extracted ion chromatogram; LOD, limit of detection.

step also results in the removal of alkali or alkaline earth metal cations from samples, minimizing cation adduction that often complicates the interpretations of electrospray ionization ion trap mass spectrometry (ESI–MS). Unfortunately, some nonvolatile ion-pairing reagents can contaminate of the interface of MS. Higher concentrations of ion-pairing reagents, beneficial for optimized separation, can also reduce the sensitivity of ESI–MS detection. Although ion-pairing chromatography offers an effective way to separate GAG-derived oligosaccharides, the separation times can be relatively long. The recent introduction of ultraperformance liquid chromatography (UPLC) offers an approach for overcoming these long analysis times. In UPLC, it is possible to use shorter columns (150 mm) packed with supports having small particle diameters (1.7 μm) and to run these at higher flow rates to increase speed, efficiency, and resolution in comparison with other traditional high-performance liquid chromatography (HPLC) methods [21–23].

The most widely used detection method coupled to LC for GAG-derived oligosaccharides has been ultraviolet (UV) absorption at 232 nm. Although UV is simple to use, it has low selectivity because common impurities often absorb light at the same wavelength as analyte. Fluorescence detection affords enhanced sensitivity but requires either pre- or postcolumn derivatization [24]. MS offers a powerful alternative for the detection and structural characterization of GAG-derived oligosaccharides. Moreover, unlike UV and fluorescence, MS offers standard-free analysis by providing information on analyte identity. Although both matrix-assisted laser desorption/ionization (MALDI) and ESI detectors have been used in such analyses [25–27], the transfer of LC effluent to a target plate with matrix solution results in longer analysis time [28], making ESI detectors much easier to interface with LC. For higher sensitivity, nano-ESI can be employed [29]. Simultaneous detection by UV absorbance and MS offers the advantages of both detection methods and has been used in the current study [30].

We report a new method for the direct analysis of CS-derived disaccharides. This method provides a rapid, efficient, and highly

sensitive analysis of these disaccharides. IPRP–UPLC is coupled with ESI ion trap MS (ESI–MS). This method combines a chromatographic separation using reversed-phase bridged ethyl hybrid (BEH) C18 columns packed with 1.7- μm particles with both MS and UV detection. The UPLC separation offers improved resolution, efficiency, and greater sensitivity for analyzing CS-derived disaccharides. *n*-Hexylamine (HXA) is used as an ion-pairing reagent, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) is used as an organic modifier in the mobile phase.

Materials and methods

Materials

Aliphatic amine, HXA, and organic modifier HFIP were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were of the highest purity available. Unsaturated disaccharide standards of CS/DS ($\Delta\text{Di-0S}$ [$\Delta\text{UA-GalNAc}$], $\Delta\text{Di-4S}$ [$\Delta\text{UA-GalNAc4S}$], $\Delta\text{Di-6S}$ [$\Delta\text{UA-GalNAc6S}$], $\Delta\text{Di-UA2S}$ [$\Delta\text{UA2S-GalNAc}$], $\Delta\text{Di-diS}_B$ [$\Delta\text{UA2S-GalNAc4S}$], $\Delta\text{Di-diS}_D$ [$\Delta\text{UA2S-GalNAc6S}$], $\Delta\text{Di-diS}_E$ [$\Delta\text{UA-GalNAc4S6S}$], and $\Delta\text{Di-TriS}$ [$\Delta\text{UA2S-GalNAc4S6S}$], where ΔUA is 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid, GalNAc is 2-acetamido-2-deoxy-D-galactose, and S is sulfo) were obtained from Seikagaku (Japan).

Methods

LC separation

The separation was performed on an Acquity UPLC BEH C18 column (2.1 \times 150 mm, 1.7 μm , Waters, Milford, MA, USA) using solution A for 10 min, followed by a linear gradient from 10 to 40 min of 0 to 50% solution B. The column temperature was maintained at 45 $^\circ\text{C}$. The flow rate was 100 $\mu\text{l}/\text{min}$. Solutions A and B for UPLC were 0 and 75% acetonitrile, respectively, containing the same concentration of 15 mM HXA as an ion-pairing reagent and 100 mM HFIP as an organic modifier. Buffers were filtered using 0.2- μm membrane filters (Millipore, Billerica, MA, USA). Detection was

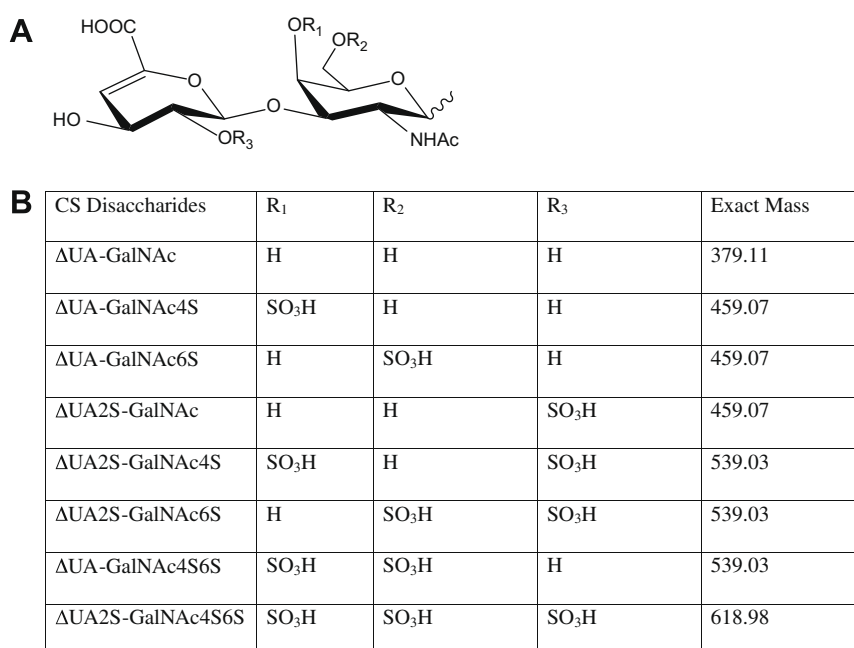


Fig. 1. The chemical structure of the commercially available CS-derived disaccharides. (A) General structure for the CS-derived disaccharide. (B) Pattern of sulfation and mass-to-charge ratio (m/z) for eight different CS disaccharides.

performed using both a UV absorbance detector at 232 nm and an ion trap mass detector.

Mass spectrometry

The LC–MS analysis was performed on an LC–MS system (LC/MSD trap MS, Agilent Technologies, Santa Clara, CA, USA). The column effluent entered the source of the ESI–MS for continuous detection by MS. The electrospray interface was set in positive ionization mode with the skimmer potential 40.0 V, capillary exit 40.0 V, and a source of temperature of 350 °C to obtain maximum abundance of the ions in a full-scan spectra (350–2000 Da, 10 full scans/s). Nitrogen was used as a drying gas (8 L/min) and a nebulizing gas (40 psi). The data were collected by UV detection and extracted ion chromatogram (EIC), respectively.

Results and discussion

Separation optimization requires the resolution of eight CS-derived disaccharides (Fig. 1), which depends on three main parameters: separation efficiency (N), separation selectivity (α); and capacity factor (k').

$$R_s = 1/4(\alpha - 1/\alpha)N^{1/2}(k'/1 + k')$$

Separation selectivity can be optimized by changing the composition of the mobile phase and/or stationary phase. Separation efficiency can be optimized by changing column length, solvent velocities, packing uniformity, particle diameter, column temperature, and flow rate. Capacity factor depends on the solvent strength [31].

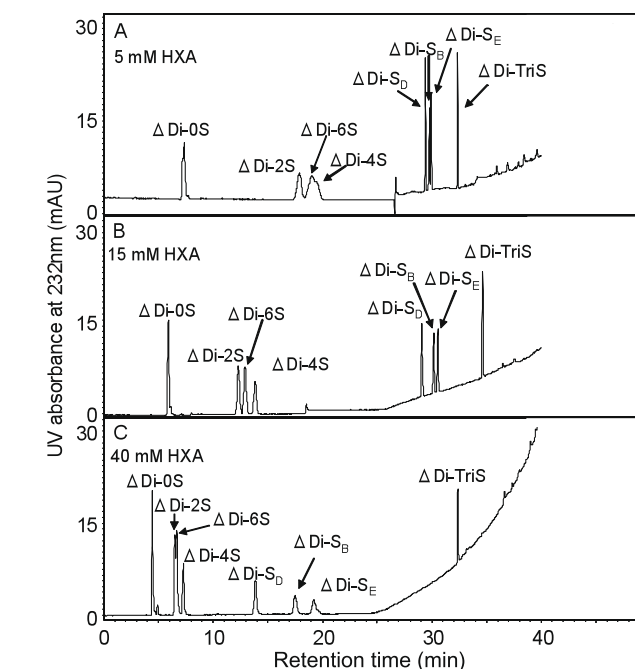


Fig. 2. Influence of various concentrations of the ion-pairing reagent (HXA) on the IPRP–UPLC separation of eight different CS disaccharide standards. The separation was performed on an Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm, Waters) using solution A for 10 min, followed by a linear gradient from 10 to 40 min of 0 to 50% solution B. Solutions A and B for UPLC were 0 and 75% acetonitrile, respectively, containing HXA (5, 15, and 40 mM in panels A, B, and C, respectively) as an ion-pairing reagent and a fixed amount of HFIP (100 mM in each panel) as a buffering acid. The detection wavelength was 232 nm, the flow rate was 100 μl/min, and the injection volume was 5 μl.

In our optimization studies, the mobile phase, stationary phase, column length, particle diameter, mobile phase flow rate, and column temperature were examined. By varying these parameters, separation and selectivity were optimized for CS-derived disaccharide analysis.

Effect of ion-pairing reagent on retention time and separation efficiency

Mobile phase composition (the concentration of ion-pairing reagent [HXA] and organic modifier [HFIP]), gradient profile, and buffer pH were evaluated for the separation of the eight CS disaccharides. The HFIP plays a role as buffering acid to protonate the HXA because the ion-pairing reagent does not carry a positive charge. HFIP converts HXA into the ammonium ion by decreasing the pH of the mobile phases. The concentration of HXA on capacity factor and the separation efficiency were evaluated at 5, 15, and 40 mM. The retention time decreased with increasing HXA concentration. Whereas peaks eluted at shorter retention times using 40 mM HXA, ΔDi-2S and ΔDi-6S could not be separated. At 5 mM HXA, ΔDi-6S and ΔDi-4S could not be separated. However, 15 mM HXA provided excellent separation efficiency with good peak shapes for the CS-derived disaccharides (Fig. 2).

The mechanism of solute retention in IPRP–LC is governed mainly by the hydrophobic interactions between the analyte and the stationary phase. The basis of the separation in IPRP–LC is still controversial, and two models—partition and adsorption—have been proposed as the separation process in the column chromatography [32]. In the partition model, the separation process can be explained by the formation of ion pairs between the positively charged ion-pairing reagents and the negatively charged CS disaccharides. In the adsorption model, positively charged ion-pairing reagents adsorb onto the stationary phase in the C18 column through their hydrophobic chain. This adsorption of ion-pairing reagents creates a pseudo-ion-exchange support, resulting in interaction between negatively charged CS disaccharides and already

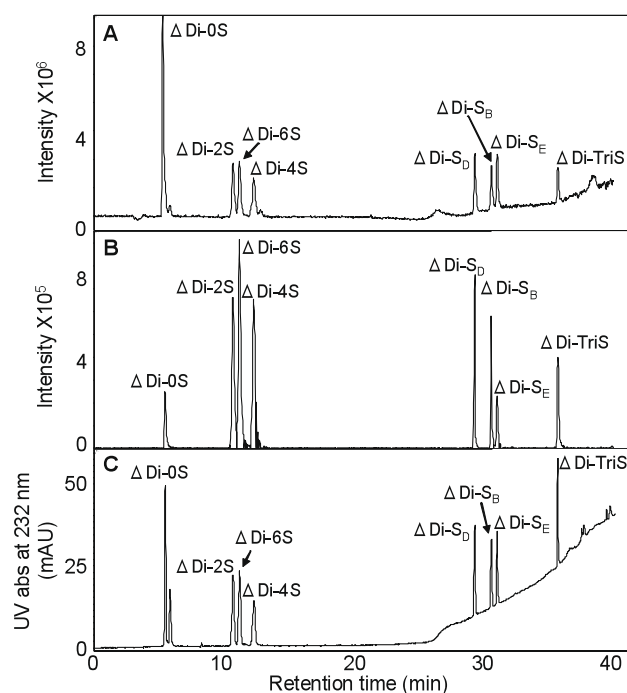


Fig. 3. IPRP–UPLC chromatograms of eight CS-derived disaccharide standards with mass and UV detection. Experimental conditions are the same as described in Fig. 2. Detection relied on total ion chromatogram (A), EIC (B), and UV (C).

absorbed positively charged ion-pairing agents. Both cases predict our experimental results, indicating that the retention time increases with increasing ion-pairing reagent concentration.

The resolution of eight different CS-derived disaccharides by IPRP-UPLC with ESI-MS detection has not been reported previously. Elution order depends on the number and position of sulfo groups that interact with HXA ion-pairing reagent, promoting hydrophobic interaction of the analyte with the stationary phase. CS trisulfated disaccharide elutes last from the column because it has the highest number of sulfo groups among eight CS disaccharides (Fig. 3). In contrast, nonsulfated CS disaccharide elutes first because it has no sulfo groups and, hence, has weak ion-pairing interaction through its carboxyl group and HXA. An increased number of sulfo groups increases retention time; thus, the unsulfated disaccharide is followed by the three monosulfated disaccharides, the three disulfated disaccharides, and finally the trisulfated disaccharide. The sulfo group position is a secondary factor that impacts the elution ordering. For example, among the monosulfated disaccharides, the isomer with the 4-*O*-sulfo group in the galactosamine residue (Δ Di-4S) elutes last, with the isomer with the 6-*O*-sulfo group in the galactosamine residue (Δ Di-6S) eluting earlier and the isomer with the 2-*O*-sulfo group in the uronic acid residue (Δ Di-2S) eluting earliest. This result suggests that the strength of interaction with the ion-pairing reagent clearly depends on sulfation position.

The 1.7- μ m particle size BEH UPLC column differs from a traditional 5- μ m octadecyl-silica (C18) column. The BEH column is mechanically stronger and can be operated over an extended pH range (pH 1–12), whereas the C18 silica-based column begins to dissolve at pH values above 8.0, particularly at elevated temperatures. The bridging of the ethyl groups in the silica matrix makes BEH columns much more resistant to high pH and elevated temperatures and, thus, is able to retain their separation characteristics for a longer time [23]. Thus, mobile phases at pH values in the range of 8.2 to 9.6 that cannot be used with silica-based C18 col-

umns can be routinely used with BEH columns. Also, the higher pH avoids the presence of α - and β -anomers associated with a single disaccharide [33].

IPRP-UPLC coupled to ESI-MS for analysis of chondroitin sulfate disaccharides in positive ion mode

The ESI-MS analysis of CS disaccharides was performed in both positive ion and negative ion modes. Surprisingly, the analysis in the positive ion mode provides the best results. The number of HXAH⁺ moieties adducted varied based on the charge of each CS disaccharide. The nonsulfated disaccharide showed a molecular ion of m/z 481.2, corresponding to the addition of one HXAH⁺ and an H⁺ (Fig. 4A). Monosulfated disaccharides (m/z 662.3) had two HXAH⁺ and an H⁺ (Fig. 4B–D). Minor peaks at m/z 583.2, corresponding to the sodiated species [M–H+HXNA+Na]⁺, were also observed in these spectra. Disulfated disaccharides with three HXAH⁺ and an H⁺ were observed at m/z 843.3 (Fig. 4E–G). These spectra also showed minor molecular ions corresponding to [M–2H+2HXNA+Na]⁺ and [M–2H+2HXNA+H]⁺ at m/z 764.2 and 742.2, respectively. In addition, fragment ion peaks observed at m/z 685.0 and 662.2 corresponded to desulfonation and were assigned as [M–S–H+2HXNA]⁺ and [M–S–2H+2HXNA+Na]⁺, respectively. The MS spectrum of trisulfated disaccharide showed a major peak corresponding to the molecular ion with four HXAH⁺ and an H⁺ (Fig. 4H). Minor peaks assigned as [M–3H+3HXNA+Na]⁺, [M–3H+2HXNA+2Na]⁺, [M–3H+HXNA+3Na]⁺, and [M–3H+4Na]⁺ were also observed at m/z 945.3, 866.2, 787.0, and 707.8, respectively.

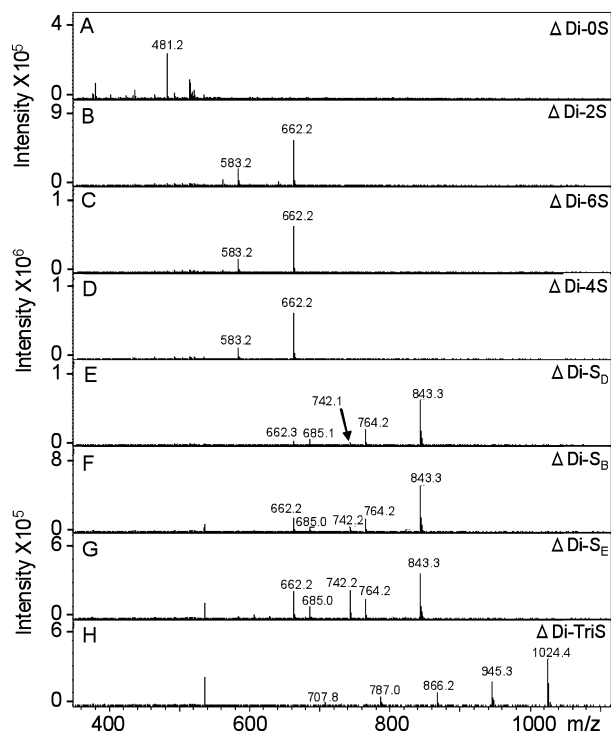


Fig. 4. Mass spectra of CS disaccharides: (A) Δ UA-GalNAc; (B) Δ UA2S-GalNAc; (C) Δ UA-GalNAc6S; (D) Δ UA-GalNAc4S; (E) Δ UA2S-GalNAc6S; (F) Δ UA2S-GalNAc4S; (G) Δ UA-GalNAc4S6S; (H) Δ UA2S-GalNAc4S6S.

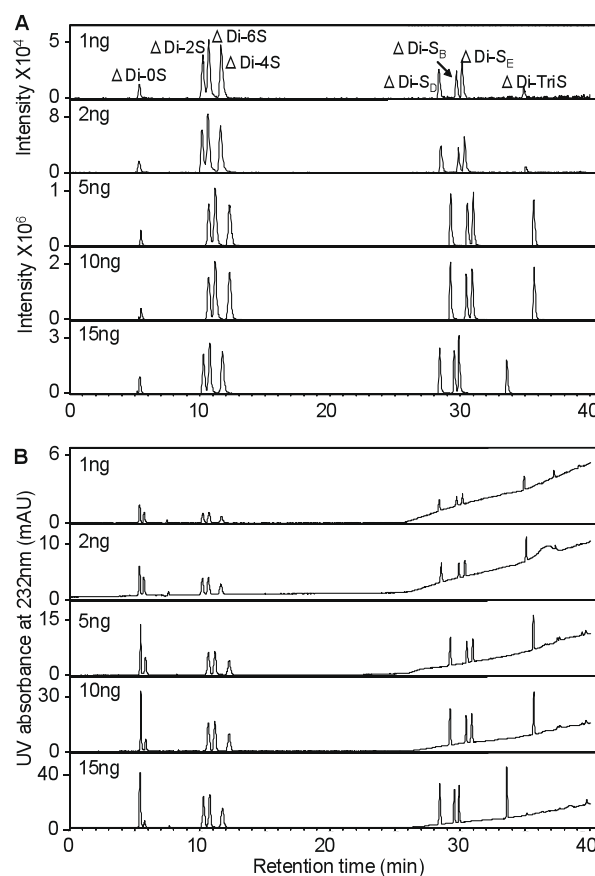


Fig. 5. Sensitivity analysis of eight different CS disaccharide standards by mass (A) and UV detector (B) corresponding to injections of 1, 2, 5, 10, and 15 ng of each of the standard disaccharides. Experimental conditions are the same as described in Fig. 2.

CS disaccharides were prepared in sample at amounts of 1, 2, 5, 10, and 15 ng and were analyzed by IPRP–UPLC–ESI–MS in the positive ion mode to test the sensitivity and linearity of this method (Fig. 5). The EICs and UV chromatograms of different amounts of disaccharide standards showed that less than 1 ng per disaccharide could easily be analyzed. The limit of detection (LOD) for disaccharides by EIC and UV detection were 150 and 300 pg, respectively.

Conclusions

This article has described an efficient separation of CS-derived disaccharides relying on UPLC. This method provides excellent separation with a low LOD. Furthermore, there is no need for sample preparation or further purification required for fluorescence detection. An online combination of IPRP–UPLC coupled to UV and ESI–MS offers a simple, efficient, and robust method for CS disaccharide analysis.

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