**Disaccharide compositional analysis of chondroitin sulphate using WAX HILIC-MS with pre-column procainamide labelling; application to the placenta in pre-eclampsia**

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**Footnotes**

† Electronic supplementary information (ESI) available.

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**Abstract**

Chondroitin sulphate (CS) and dermatan sulphate are linear negatively charged heteropolysaccharides. These glycosaminoglycans are involved in cellular signalling via binding to growth factors. CS is expressed in a range of tissue and biological fluids and is highly expressed in the placenta. There is evidence that decorin; a CS proteoglycan is significantly decreased in pre-eclampsia and fetal growth restriction. It is considered that GAG chain composition may influence cellular processes that are altered in pre-eclampsia. The goal of the present study was to develop an LC-MS method with precolumn procainamide labelling for the disaccharide compositional analysis of CS. The method was used to investigate whether the disaccharide composition of placenta-extracted CS is altered in pre-eclampsia. The study revealed differential disaccharide compositions of placental chondroitin sulphate disaccharide between pre-eclampsia and other pregnancy samples. This suggests that the method may have diagnostic potential for pregnancy disorders. Furthermore, the findings suggest that CS sulphation might play a significant role in maternal labour.

## Introduction

Chondroitin sulphate and dermatan sulphate are growth factor- and cytokine binding-glycosaminoglycans (negatively charged linear heteropolysaccharides) thus involved in cellular signalling[1, 2]. CS is present on cell surfaces and in great amounts in the extracellular matrix as vital components of proteoglycans (PG) where they help to maintain structural integrity[1, 3]. They are also present in biological fluids including urine[4], blood[5], CSF (cerebrospinal fluid)[6] and are highly expressed in the placenta[7]. Placental CSPGs are in the intervillous spaces, fibrous tissue and on cell surfaces to a lesser extent[7]. Specifically, decorin, syndecan-1 (SDC-1), Integrin Beta-1 and biglycan have been identified in the placenta[8, 9].

The placenta is central to the development of pre-eclampsia -, a serious pregnancy complication affecting 3-5% of all pregnancies. In normal placental development, invading extravillous trophoblasts (EVT) transform the high resistance decidua and myometrial uterine spiral arteries to low resistance blood vessels for adequate placental perfusion[10]. This spiral artery remodelling is incomplete in pre-eclampsia resulting in placental hypoperfusion and poor nutrient supply to the developing foetus[11]. Growth factors, the local cellular environment, oxygen tension and cytokines all influence the EVT invasion process[11].

Decorin is present in the wall of placental foetal blood vessels and the decidua[12, 13]. It is involved in endothelial cell development, angiogenesis and has been shown to have anti-proliferative, anti-migratory and anti-invasive effects on extravillous trophoblasts[14]. There is evidence that decorin is significantly decreased in pre-eclampsia [15] and foetal growth restriction[13]. SDC-1, primarily a HSPG also carry CS chains; both chains cooperating in the binding to cytokines and fibroblast growth factor[16]. SDC-1 is greatly expressed on the apical side of syncitiotrophoblasts[9] and is vital for proper placental implantation and angiogenesis[17]. However, there are contrasting reports of increased[18], unaltered[15] and decreased[19, 20] levels of placental SDC-1 in pre-eclampsia. The full actions of specific growth factors are achieved via their binding to glycosaminoglycans (GAG), largely driven by interactions with the sulphate groups on the GAG chain. Therefore, it is likely that GAG chain composition independent of total amounts of GAGs may influence cellular processes which are altered in pre-eclampsia. In addition, hemodynamic modulation involving stiffness of maternal abdominal aorta, high resistance of small arteries and arterioles is also implicated in pre-eclampsia[21]. It is possible that this modulation may be influenced by GAGs involved in extracellular matrix alterations[22] such as those on CSPGs.

CS chains comprise 40 - 100 differentially sulphated disaccharide subunits of uronic acid (UA) β(1→3) linked to N-acetylgalactosamine (GalNAc) resulting in structural heterogeneity[23-25]. The position two of the UA, four, and six of the GalNAc can be sulphated. These yield non, mono, di, and tri-sulphated disaccharides with the latter generally minimally present[25]. The UA of CS is glucuronic acid (GlcA) which is replaced by iduronic acid (IdoA) at varying degrees in dermatan sulphate. Both chains are however usually present as co-polymeric structures[23].

For disaccharide compositional analysis, GAGs are generally liberated from de-fatted tissues using proteolytic enzymes such as papain[24] or actinase E[26], extracted using anion exchanger diethylaminoethyl (DEAE)[27] or quaternary ammonium columns[28, 29] and purified using alcohol precipitation[28], molecular weight cut-off filters[30] or desalting columns[27]. The extracted GAG are then often depolymerised into constituent disaccharides using specific GAG lyases[31] or alternatively, by hydrazinolysis followed by nitrous acid deaminative cleavage[32, 33]. These

disaccharides can then be analysed using high-performance liquid chromatography (HPLC) with mass spectrometry (MS) with or without derivatisation. Analysis of native GAG disaccharides avoids the extra sample derivatisation steps. However, anomeric separation is a significant complication[34, 35]. In addition, SAX-HPLC which is often used here is incompatible with MS due to the high salt composition of mobile phase. Derivatisation with hydrophobic fluorophores have significant advantages of improving RP LC separation[29], increasing MS sensitivity[36], offering fluorescent detection [37] and avoiding anomeric LC separation[34]. In the current study derivatisation of GAG disaccharides with procainamide offered several benefits. These advantages include the facilitation of HILIC separation, utilisation of solvents more compatible with MS and the detection of derivatives using ESI MS in the positive ion mode. We have previously shown that procainamide derivatisation also significantly increases GAG disaccharide MS sensitivity relative to AMAC derivatisation[38].

Mixed mode chromatography such as WAX-HILIC offers unique selectivity for the separation of polar and charged analytes because it offers multiple retention modes[39]. Here, hydrophilicity is combined with electrostatic interaction retention mechanisms. This is particularly useful for the separation of GAG disaccharides where more than one sulphation positional isomer is present in addition to differentially sulphated disaccharides. To the best of our knowledge, WAX-HILIC-MS analysis of procainamide-labelled CS disaccharide has not been previously reported.

The aim of this study was to develop a novel WAX-HILIC-MS method for the disaccharide compositional analysis of CS. Three HILIC columns with different chemistries were tested for the separation of eight procainamide-labelled disaccharides. Upon optimisation of separation, we applied this method to investigate if the disaccharide composition of placenta-extracted CS is altered in pre-eclampsia.

## Experimental

### Materials

Chondroitin sulphate (sodium salt from bovine cartilage), hyaluronic acid (sodium salt from rooster comb), chondroitinase ABC (from *Proteus vulgaris*), actinase (from *Streptomyces griseus*), procainamide hydrochloride, sodium cyanoborohydride, CHAPS, molecular weight cut off filters (3 kDa and 10 kDa, Millipore), 0.22 µm Millex-GP filters (Millipore), anion exchange spin columns (Q Mini H, Sartorius) and Hank's balanced salt solution were purchased from Sigma-Aldrich (Poole, U.K.). Chondroitin sulphate unsaturated disaccharides ($0S_{CS}$, $2S_{CS}$, $4S_{CS}$, $6S_{CS}$, $2S6S_{CS}$, $2S4S_{CS}$, $4S6S_{CS}$, $TriS_{CS}$) and disaccharide internal standard (ΔUA,2S – GlcNCOEt-6S) were purchased from Iduron (Manchester, U.K.). LC-MS grade dimethyl sulphoxide (DMSO), acetic acid, Tris-HCL, calcium chloride, sodium chloride, sodium acetate, urea, chloroform, methanol, UPLC water, HPLC grade acetonitrile (ACN) and ammonium formate were purchased from Fisher Scientific (Loughborough, U.K.). Lyophilisation was carried out using a Scan Vac CoolSafe 55-4 freeze dryer (Labogene, Lynge, Denmark).

### Procainamide labelling of CS disaccharides

Procainamide labelling of disaccharides was carried out as previously reported[38] with a slight modification. Samples containing CS disaccharides (with or without the disaccharide internal standard) were lyophilised and re-constituted in 20 µL of 0.4 M procainamide prepared in a mixture of DMSO and acetic acid (7:3, v/v). They were subsequently incubated at room temperature for 15 min and 20 µL of 1 M sodium cyanoborohydride was added. The mixture was vortexed and then incubated at 45 ºC for 4 h. This was subsequently diluted in 60 µL of 80 % ACN and an aliquot (2 µL) was injected onto the UPLC column.

### HILIC-UPLC-UV-MS/FLR Conditions

A Waters ACQUITY H-Class UPLC system was used coupled to an SQD2 or fluorescence (FLR) detector with an in-line diode array detector (DAD). To achieve optimum separation of procainamide-labelled CS disaccharides, an ACQUITY Glycan BEH Amide (1.7 µm; Waters, Ireland, U.K.), ACE HILIC-B (3 µm; HiChrom, Reading, UK), and GlycanPac AXH-1 (1.7 µm; Thermo Fisher Scientific, Loughborough, UK) HILIC based 2.1 x 150 mm columns were tested. The columns were maintained at 55 $^0$C and a flow rate of 0.35 mL/min was used. Solvent A, B and C were ammonium formate (pH 4.4), ACN and water respectively. For the detection of procainamide labelled disaccharides, a wavelength of 299 nm was set on the DAD and an excitation and emission wavelength of 330 and 380 nm respectively was used on the FLR.

The MS conditions previously optimised[38] and used for the detection of procainamide-labelled disaccharides in the positive ESI mode were as follows; the capillary voltage was set at 3.1 kV and the probe was positioned 4.83 and 7 mm horizontally and vertically respectively. The source and desolvation temperature were set at 110 ºC and 250 $^0$C respectively. The cone and source gas flow were set at 1 and 800 L/hr respectively. A cone voltage of 50 V was used

for the TIC and subsequently optimised for each procainamide-labelled disaccharide and used in the SIR chromatogram. The cone voltage was varied between 20 – 75 V to achieve this. The needle draw rate for the sample injector was set at 140 µL/min and samples were maintained at 10 ºC in the autosampler. The instrument was operated, and data collected and analysed using MassLynx® (v 4.1).

### Quantitative analysis

A mixture of eight procainamide labelled CS disaccharides and internal standard was analysed using optimal ULPC-MS conditions over a concentration range of 0.005 – 20 µg/mL. This was used to determine the linearity ($r^2$) of the method. Five replicate injections were made, and data obtained was used to calculate the intra-assay coefficient of variation (CV) at three different concentrations (0.078, 1.25 and 10 µg/mL) and the method detection limit (MDL). The MDL was defined as the concentration with a signal distinguishable from the noise at a 99 % confidence interval.

### Ethics Considerations

Informed consent was obtained from each volunteer and ethical approval was granted by the Barnet, Enfield & Haringey NHS and Hammersmith and Queen Charlotte's & Chelsea Hospitals NHS research ethics committee for the collection of placenta samples. Natural Science Ethics Committee at Middlesex University, London granted ethical approval for the current study (project number 1989).

### Tissue preparation and clinical groups

Placenta tissues were collected and processed within 1 h. Samples were collected near the maternal surface adjacent to the maternal decidua. Tissues were washed in Hank's balanced salt solution to remove contaminants and red blood cells. The amnion and decidua tissue were removed, and the resulting villous trophoblast-enriched tissue was fixed in 80 % ethanol until use. Trophoblast enrichment ensures that placenta tissue samples used in the current study are largely free of maternal tissue and amniotic membrane contamination.

Twenty-seven placenta samples were collected from gestational aged-matched pregnancies and used in this study. They were grouped as follows; term delivery from uncomplicated pregnancy prior to labour onset (Term non-labour; TNL, n =10), uncomplicated pregnancy at term after labour onset (Term labour; TL, n = 7), pre-term delivery upon spontaneous pre-term labour (Pre-term labour; PTL, n = 5) and pre-term delivery from women with pre-eclampsia prior to labour onset (PE, n = 5). The onset of regular uterine contractions with cervical dilation before the 37$^{th}$ week of gestation was designated pre-term labour[40].

**Women with pregnancies complicated by pre-eclampsia tend to deliver pre-term, and often via caesarean section. Therefore, in this study we have utilised other pregnancy groups – preterm labour and uncomplicated pregnancy prior to labour onset (Term non-labour, i.e., term pregnancy**

**delivered via a caesarean section prior to labour onset) to enable us understand whether chondroitin sulfation is also influenced by gestational age or labour[19].**

### Sample preparation

Placenta tissue GAGs were extracted using a method adapted from[29,41]. Tissues were cut into tiny pieces and de-fatted using three sequential chloroform: methanol (v/v) solutions; 2:1, 1:1 and 1:2 each for 10 h. The solvent was removed, and the tissues were allowed to air dry for 20 min at room temperature. Tissues were then re-suspended in 750 µL of water and proteolysed for 18 h at 55 ºC using 250 µL of actinase E (20 mg/mL in 0.1 M Tris/ 0.1M $CaCl_2$). The resulting solution was filtered using 0.22 µm syringe filter to remove particulates and peptides were removed using a 10 kDa molecular weight cut-off filter. The retentate was collected, lyophilised and re-suspended in 8 M urea and 2 % CHAPS (w/v). This was then loaded onto an anion exchange column, washed with 0.2 M NaCl, and GAGs were eluted using 16 % NaCl. The extracted GAGs were desalted using a 3 kDa molecular weight cut-off filter and stored at -20 ºC prior to use.

Samples containing placenta-extracted GAGs were lyophilised and re-suspended in a buffer solution comprising 50 mM Tris and 60 mM sodium acetate, pH 8.0. CS was then enzymatically depolymerised by incubation with 10 mIU chondroitinase ABC for 24 h at 37 ºC. CS and HA standard (20 µg) were also depolymerised similarly in triplicate for quantitative analysis. The reaction mixture was heated in a boiling water bath for 10 min to inactivate the enzymes and centrifuged at 12,000 g for 10 min. The supernatant containing CS disaccharides was collected and 0.5 µg of disaccharide internal standard (ΔUA,2S - GlcNCOEt,6S) was added. The sample was then lyophilised, disaccharides were labelled with procainamide and an aliquot (2 µL) was injected onto the UPLC column.

### Data Analysis

The HPLC-peak areas were used to determine the concentrations of CS disaccharides from standard curve for each analyte and normalised with respect to the disaccharide internal standard (ΔUA,2S – GlcNCOEt-6S). Individual CS disaccharide concentrations were converted into a percentage of total CS disaccharides in a particular biological sample. ANOVA was used to determine significant differences between groups. A generalised linear model (GLM) analysis was used to determine the independent influence of pre-eclampsia (PE), labour (TL, TNL) and pre-term delivery (PTL, PE) on placental GAG disaccharide composition. All statistical analysis was done using Minitab® (v 17).

## Results & discussion

### LC separation of procainamide-labelled CS disaccharides

As previously described[38], procainamide disaccharide labelling by reductive amination was utilised for chondroitin sulphate disaccharides. Three different HILIC based column chemistries were then tested for the separation of these procainamide-labelled CS disaccharides. Due to the structural similarities of these disaccharides and thus resulting difficulty in LC separation, isocratic elution conditions were initially employed. Solvent A, B, and C were ammonium formate (150 mM, pH 4.4), ACN and UPLC water respectively. With all three columns, excess unreacted procainamide was poorly retained and eluted before the derivatised disaccharides (Figure 1). This was expected as procainamide is less hydrophilic relative to procainamide-labelled CS disaccharides.

Procainamide-labelled CS disaccharides could not be separated using the ACE HILIC-B column and only the unsulphated disaccharide was eluted using a solvent composition of A:B:C of 20:80:0%, v/v(Figure 1a). The other procainamide-labelled CS disaccharides could not be eluted even by increasing composition of the aqueous solvent in the mobile phase up to 50 % A. Reducing the concentration of solvent A to 50 mM did not improve elution either. strong adsorption of acidic analytes on HILIC columns is possible, due to their high affinity for aminopropyl-silica[42]. The ACE HILIC-B column is a pH-responsive positively charged proprietary material, bonded to silica, that can be adjusted within a suggested operational pH span of 2.0 to 7.0. This may explain the seemingly irreversible retention of highly negatively charged sulphated CS disaccharides. The Glycan BEH Amide column was tested with different isocratic conditions ranging from 10 – 20 % A (0 % C). There was excellent resolution between procainamide-labelled CS disaccharides except for $6S_{CS}$ and $2S6S_{CS}$ which co-eluted (Figure 1b). The use of 100 mM, 50 mM and 10 mM ammonium formate (pH 4.4) did not significantly improve resolution between these two CS disaccharides. Interestingly, the more hydrophilic mono-sulphated $2S_{CS}$ eluted before the unsulphated $0S_{CS}$; thus, the order of elution did not strictly follow an increasing degree of sulphation as expected. This phenomenon was also observed for procainamide-labelled heparan sulphate disaccharides on the Glycan BEH Amide column but at a decreased buffer concentration[38].

Using the GlycanPac AXH-1 column, the previously co-eluting disaccharide critical pair ($6S_{CS}$ and $2S6S_{CS}$ on the Glycan BEH Amide column) was completely resolved using a mobile phase composition composed of A, B and C of 11:85:4 %, v/v (Figure 1c). Furthermore, all procainamide-labelled CS disaccharides except for the sulphational positional di-sulphated isomer 2S6SCS and 2S4SCS were completely separated to baseline. However, it was possible to integrate the HPLC-peak area for both these disaccharides (Figure 1c). Consequently, this column was chosen for further analyses. A reduction in the concentration of Solvent A (ammonium formate, pH 4.4) to 50 mM did not further improve resolution but resulted in co-elution of $2S4S_{CS}$ and $2S6S_{CS}$ and an overall decrease in the retention of all procainamide-labelled CS disaccharides (Supplementary Figure S-1). Therefore, the final LC conditions used are as follows; Solvent A/B/C (11/85/4 %, v/v from 0 – 23 min, transitioning to 11/60/29 from 23 - 26 min. The composition was maintained until 35 min after which the composition returned to the initial conditions at 38 min. Following each injection, the column was re-equilibrated for 22 min. The gradient step in the elution conditions was made after the separation of the critical pair, to shorten the method run time.

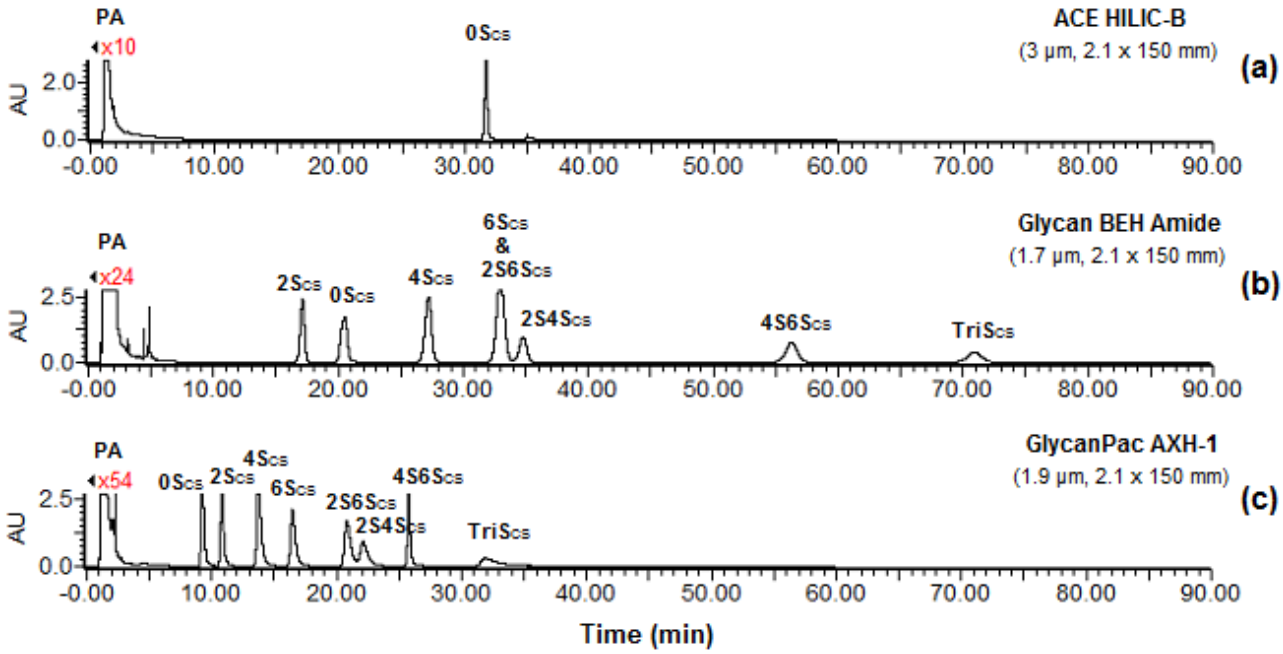



**Figure 1:** LC separation of procainamide-labelled CS disaccharides using HILIC columns (a) ACE HILIC-B (b) Glycan BEH Amide and (c) GlycanPac AXH-1. Mobile phase A, B and C was ammonium formate (pH 4.4), acetonitrile and water respectively. Detection was Diode array, and a wavelength of 299 nm was used for extraction of chromatograms. PA (procainamide peak).

Of the three HILIC based columns tested for procainamide-labelled CS disaccharides, optimal separation was achieved using the GlycanPac AXH-1 column. Separation of procainamide-labelled disaccharides was achieved within 40 min on this column. Elution was in the order of increasing sulphation and hydrophilicity demonstrating the effectiveness of the HILIC and WAX separation mechanisms on the GlycanPac AXH-1 column; the unsulphated disaccharide ($0S_{CS}$) eluted first, followed by the mono- ($2S_{CS}$, $4S_{CS}$, $6S_{CS}$), di- ($2S6S_{CS}$, $2S4S_{CS}$, $4S6S_{CS}$), and tri-sulphated disaccharide ($TriS_{CS}$). These disaccharides were detected using fluorescent, UV and mass spectrometric detection (Figure 2).

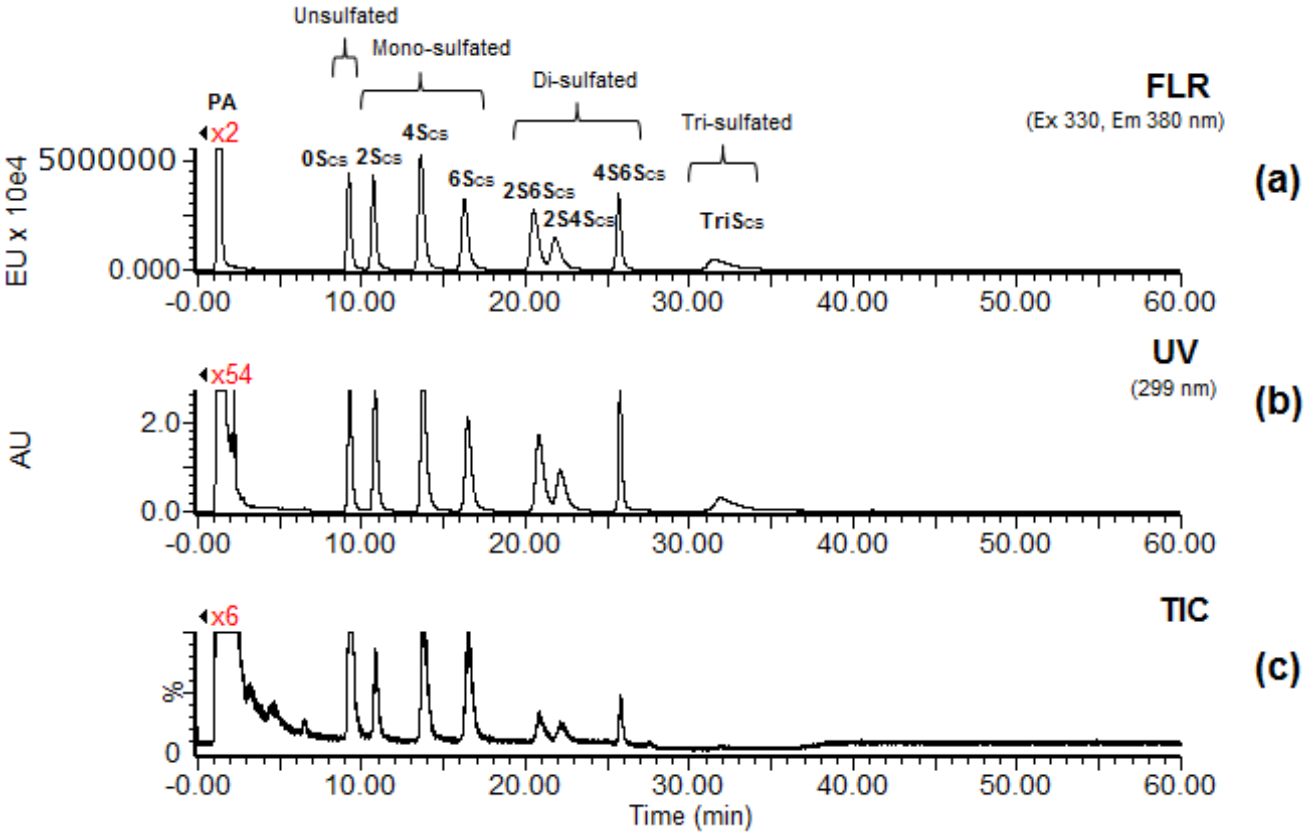


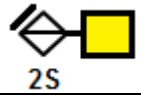
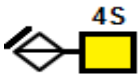
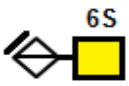
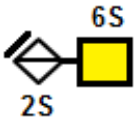
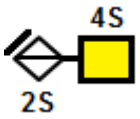
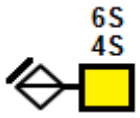
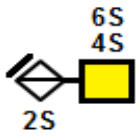
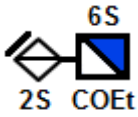

**Figure 2:** Separation of procainamide-labelled CS disaccharides on GlycanPac AXH-1 (a) Fluorescent (b) UV and (c) TIC. EU (emission units), PA (procainamide peak).

LC separation of native CS disaccharides using WAX-HILIC has been previously reported[43]. However, only four disaccharides were separated and LC co-elution of sulphation positional disaccharide isomers ($4S_{CS}$, $6S_{CS}$) in a single peak was observed which required a mass spectrometer to distinguish them. In comparison, the current study demonstrates the separation of all eight commercially available CS disaccharides. Although a longer run time was required, good resolution (1.3) is achieved between $4S_{CS}$ and $6S_{CS}$ which are typically the major disaccharides in biological samples. To the best of our knowledge, this is the first method demonstrating the procainamide labelling and separation of all eight unsaturated CS disaccharides using a mixed mode WAX HILIC. The ability for fluorescent detection is an advantage of pre-column procainamide labelling which allows CS disaccharide analysis in laboratories which have a fluorescent detector without the need for a relatively more expensive mass spectrometer.

## Optimisation of MS conditions

Labelling of CS disaccharides with procainamide resulted in a mass increase of 219 Da (Table 1), which is consistent with previous studies of procainamide-labelled HS disaccharides[38] and N-linked glycans[44]. Procainamide-labelled CS disaccharides standards and the internal disaccharide standard were then used to optimise MS conditions for their detection in the positive electrospray ionisation mode. Multiple ions were observed in the MS spectrum for these disaccharides corresponding to $[M + H]^+$, $[M –SO_3 + H]^+$, $[M –2SO_3 + H]^+$ and $[M –3SO_3 + H]^+$ species (Table 1, Supplementary Figure S-2). The sulphonic acid group of GAG disaccharides are heat-labile, and their MS in-source fragmentation (- 80 *m/z*) is well known[31, 38]. The $[M + H]^+$ ion was the most intense for the procainamide-labelled unsulphated CS disaccharide (ΔUA – GalNAc), $[M –SO_3 + H]^+$ for the mono- and di-, while $[M –2SO_3 + H]^+$ was the most intense ion for the tri-sulphated disaccharide (Supplementary Figure S-2). The MS signals of these ions were further optimised by varying their cone voltages. The optimum cone voltage which yielded the most intense MS signals for the chosen disaccharide ion (Table 1, Supplementary Figure S-3) was used to set up the selected ion recording method (SIR) (Figure 3). In the SIR chromatogram, the procainamide-labelled internal disaccharide standard co-eluted with $2S4S_{CS}$. However, this posed no issue since the MS ions utilised for detection had different *m/z* values. The optimised SIR method was used for quantitative analysis of disaccharides in biological samples.

**Table 1:** Disaccharide code, symbol, theoretical molecular weight (MW), observed MS ions, assignment and optimum MS cone voltage for procainamide-labelled disaccharides

| Disaccharide code | Symbol | Theoretical MW (native disaccharide) | Observed ions (procainamide labelled, *m/z*) | Assignment | Optimum MS cone voltage (V) |
|---|---|---|---|---|---|
| **0S$_{CS}$** (ΔUA – GalNAc) |  | 379.0 | 599.4 | **[M + H]$^+$** | 60 |
| **2S$_{CS}$** (ΔUA,2S – GalNAc) |   | 459.0 | 679.4<br>**599.4** | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$** | 70 |
| **4S$_{CS}$** (ΔUA – GalNAc,4S) |   | 459.0 | 679.4<br>**599.4** | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$** | 70 |
| **6S$_{CS}$** (ΔUA – GalNAc,6S) |   | 459.0 | 679.4<br>**599.4** | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$** | 75 |
| **2S6S$_{CS}$** (ΔUA,2S – GalNAc,6S) |   | 539.0 | 759.4<br>**679.4**<br>599.4 | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$**<br>[M –2SO$_3$ + H]$^+$ | 55 |
| **2S4S$_{CS}$** (ΔUA,2S – GalNAc,4S) |   | 539.0 | 759.4<br>**679.4**<br>599.4 | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$**<br>[M –2SO$_3$ + H]$^+$ | 55 |
| **4S6S$_{CS}$** (ΔUA – GalNAc,4S,6S) |   | 539.0 | 759.4<br>**679.4**<br>599.4 | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$**<br>[M –2SO$_3$ + H]$^+$ | 55 |
| **TriS$_{CS}$** (ΔUA,2S – GalNAc,4S,6S) |   | 619.0 | 839.6<br>**759.4**<br>679.4<br>599.5 | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$**<br>[M –2SO$_3$ + H]$^+$<br>[M –3SO$_3$ + H]$^+$ | 55 |
| Internal standard (ΔUA,2S – GlcNCOEt, 6S) |   | 553.0 | 773.4<br>**693.4**<br>613.5 | [M + H]$^+$<br>**[M –SO$_3$ + H]$^+$**<br>[M –2SO$_3$ + H]$^+$ | 55 |

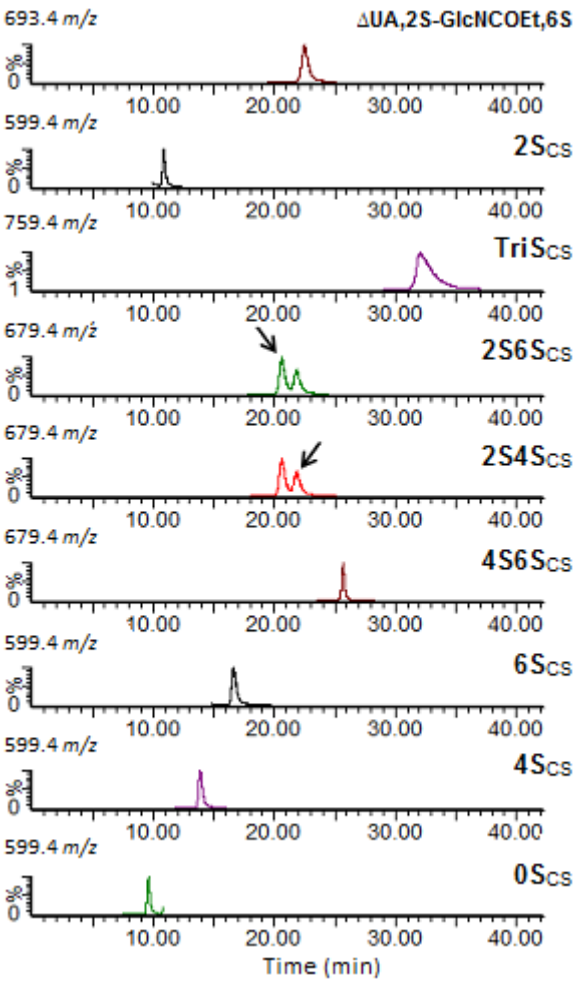



**Figure 3:** SIR chromatogram of procainamide-labelled CS disaccharides and internal disaccharide standard (20 µg/mL each) using optimum MS conditions. The monitored MS ion for each disaccharide is also shown.

## Quantitative analysis

For the quantitative analysis of procainamide-labelled CS disaccharides and internal disaccharide standard, the following method validation parameters were determined using the HILIC-UPLC-MS method with SIR chromatogram and shown in Table 2. Excellent linearity ($r^2$) was obtained (0.998 – 1) over a concentration range of 0.005 – 20 µg/mL. Differential ionisation efficiency was observed for these disaccharides (Supplementary Figure S-3) reflecting the different method detection limits (MDL). The procainamide labelled disaccharides $0S_{CS}$ and $4S_{CS}$ showed the greatest ionisation efficiency and consequently the lowest MDL (0.005 µg/mL). The tri-sulphated disaccharide ($TriS_{CS}$) showed the least ionisation efficiency and thus the highest MDL (0.108 µg/mL). A similar pattern of decreasing ionisation efficiency with increasing sulphation was previously observed for procainamide labelled-HS disaccharides[38] and is expected considering that these disaccharides were detected using ESI operated in the positive ionisation mode. As an LC injection volume of only 2 µL was used in

the current study, the on-column sensitivity is in the pico – nano-molar range. A previous study using the same WAX HILIC column chemistry reported a higher method sensitivity[43]. However, their column was repacked in-house to achieve a smaller internal diameter (250 µm) allowing for microscale chromatography. Furthermore, a more sensitive QTOF MS was used which is more expensive than the single quadrupole MS used in the current study. Nevertheless, our method allows for the detection of CS disaccharides from as little as 2.5 µg of starting material. All calculated MDLs had an observed signal to noise ratio of at least 3:1. The use of procainamide-labelled internal disaccharide standard (ΔUA,2S – GlcNCOEt, 6S) allowed for correction of ion suppression/enhancement which is a significant advantage of the SIR WAX HILIC UPLC-MS method developed in the current study. The intra-assay precision determined by the coefficient of variation (CV) at three different disaccharide concentrations were as follows: 1.59 – 8.62 % at 0.078 µg/mL, 1.07 – 2.98 % at 1.25 µg/mL and 0.88 – 2.79 % at 10 µg/mL. Overall, the CV ranged from 0.88 – 8.62 % which is well within acceptable ranges of < 15 %.

**Table 2:** Method parameters; linearity ($r^2$), method detection limit (MDL) and precision measured as coefficient of variation (% CV)

| Disaccharide | $r^2$ | Concentration range (µg/mL) | MDL (µg/mL) [nM] | %CV (10 µg/mL) | %CV (1.25 µg/mL) | %CV (0.078 µg/mL) |
|---|---|---|---|---|---|---|
| $0S_{CS}$ | 0.998 | 0.005 - 20 | 0.005 [13.2] | 1.62 | 1.60 | 1.59 |
| $2S_{CS}$ | 0.999 | 0.005 - 20 | 0.006 [13.1] | 2.79 | 2.45 | 1.92 |
| $4S_{CS}$ | 0.999 | 0.005 - 20 | 0.005 [10.9] | 0.88 | 1.27 | 1.83 |
| $6S_{CS}$ | 0.999 | 0.005 - 20 | 0.007 [15.3] | 1.11 | 1.38 | 2.49 |
| $2S6S_{CS}$ | 1 | 0.078 - 20 | 0.023 [42.7] | 1.40 | 1.07 | 7.88 |
| $2S4S_{CS}$ | 0.999 | 0.019 - 20 | 0.022 [40.8] | 1.56 | 1.76 | 7.55 |
| $4S6S_{CS}$ | 1 | 0.078 - 20 | 0.025 [46.4] | 1.86 | 2.98 | 8.62 |
| $TriS_{CS}$ | 1 | 0.156 - 20 | 0.108 [17.4] | 1.49 | 2.30 | - |
| ΔUA,2S – GlcNCOEt, 6S | 1 | 0.019 - 20 | 0.007 [12.7] | 1.30 | 2.57 | 2.46 |

**CS disaccharide analysis of trophoblast-enriched placenta tissue**

Prior to the disaccharide compositional analysis of CS extracted from trophoblast-enriched placenta tissue, the suitability of the developed and optimised HILIC-UPLC-MS method was first tested on commercially sourced bovine cartilage CS. This revealed a total of six CS constituent disaccharides: $0S_{CS}$, $4S_{CS}$, $6S_{CS}$, $4S6S_{CS}$, $2S4S_{CS}$ and $2S6S_{CS}$ (Figure 4).

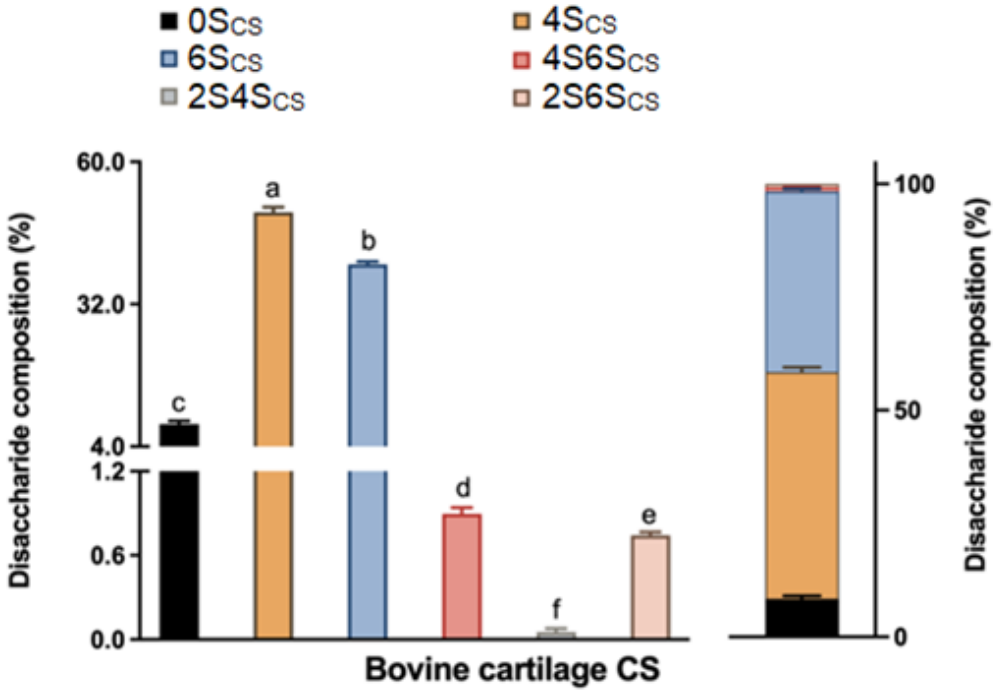



**Figure 4:** Percentage disaccharide composition of bovine cartilage CS. Results are expressed as mean ± standard deviation. Means that do not share a letter are significantly different (p values < 0.001, ANOVA).

The mono-sulphated $4S_{CS}$ (~ 50%), was the most abundant disaccharide followed by $6S_{CS}$, (~ 40 %) and then the non-sulphated disaccharide ($0S_{CS}$, ~ 8.4 %). The three di-sulphated disaccharides ($4S6S_{CS}$, $2S4S_{CS}$ and $2S6S_{CS}$) were each less than 1 % (Figure 4). The mono-sulphated $2S_{CS}$ and the tri-sulphated $TriS_{CS}$ were not detected in commercially available bovine cartilage CS. These results are in broad agreement with previous studies including one which analysed a reference standard approved by the European Pharmacopeia Commission. Review of these studies of enzymatically depolymerised bovine cartilage CS show that $2S_{CS}$ and $TriS_{CS}$ were also not reported while the non-sulphated $0S_{CS}$ ranged from about 3 – 8 %, the di-sulphated disaccharides from non-detection to 2 %, and a $4S_{CS}/6S_{CS}$ ratio of 1.5 – 2.0[45-47]. The $4S_{CS}/6S_{CS}$ ratio is about 1.3 in the current study. The similarities between the results obtained in the current study and those in published literature demonstrate that the developed HILIC-UPLC-MS method can be reliably used for CS disaccharide compositional analysis.

Interestingly, an additional LC-peak eluting close the non-sulphated CS disaccharide was observed in the chromatogram of bovine cartilage CS which was suspected to be the non-sulphated disaccharide of hyaluronic acid ($0S_{HA}$) (Supplementary Figure S-4). This was subsequently confirmed using chondroitinase ABC-treated HA standard. As chondroitinase

ABC or AC II can also depolymerise HA[48, 49], the ability of the developed HILIC-UPLC-MS method to differentiate between $0S_{CS}$ and $0S_{HA}$ is an important advantage of the method as HA is often present in biological samples. In addition, HA is a known contaminant of commercially available CS standard and CS-based pharmaceutical formulations[48, 50].

To investigate whether CS sulphation is altered in pre-eclampsia, disaccharide compositional analysis of CS extracted from the placenta of women following term delivery from uncomplicated pregnancy prior to labour onset (Term non-labour; TNL), uncomplicated pregnancy at term after labour onset (Term labour; TL), pre-term delivery upon spontaneous pre-term labour (Pre-term labour; PTL) and pre-term delivery from women with pre-eclampsia (PE) were analysed. This revealed a total of six CS disaccharides ($0S_{CS}$, $4S_{CS}$, $6S_{CS}$, $4S6S_{CS}$, $2S4S_{CS}$ and $2S6S_{CS}$) in the placenta of all four clinical groups (Figure 5a). The mono-sulphated $2S_{CS}$ and the tri-sulphated $TriS_{CS}$ were also not detected in the placenta. Only one other study[22] has characterised the disaccharide composition of placental CS in pre-eclampsia. However, they detected only five and three CS disaccharides in the control and pre-eclampsia group respectively. The very small sample size (n = six; three controls, three pre-eclampsia) and analytical method used in their study may have contributed to this. The di-sulphated CS disaccharides; $2S4S_{CS}$ and $2S6S_{CS}$ which could not be resolved from each other using their method and $4S6S_{CS}$ were not detected in their study. Their lack of detection of these di-sulphated disaccharides may be due to method sensitivity as these disaccharides were each less than 1.5 % of total CS disaccharides in the current study.

Overall, $6S_{CS}$ was the most abundant disaccharide in the placenta in all clinical groups, followed by $0S_{CS}$ and then by $4S_{CS}$ (Figure 5a). CSPGs account for more than 74 % of total PGs in the placenta; 2 and 72 % of cell-associated and fibrous tissue PGs respectively and their chains contain less than 10 % and about 60 % non-sulphated disaccharide respectively[7] . In the current study, the non-sulphated $0S_{CS}$ was about 35 % of total CS disaccharides which likely reflects CS present both in the fibrous tissue and on cells of the placenta. This is similar to a previous study where placental cotyledon-CS comprised of about 30 % $0S_{CS}$[26]. Compared to term labour, sulphated CS disaccharides; $4S_{CS}$, $2S4S_{CS}$ and $2S6S_{CS}$ but not $6S_{CS}$ were decreased in pre-eclampsia but did not achieve independent statistical significance. Decreased mRNA expression of the enzymes responsible for the 4-O, 6-O and 2-O sulphation of CS; C4ST-1, C6ST and UA2ST respectively, has been previously reported in trophoblast-enriched pre-eclamptic placental tissues[22]. However, extrapolation of this finding should be treated with caution as various isoforms of these enzymes are known except for UA2ST.

The use of three different clinical control groups (term non-labour, term labour and pre-term labour; PTL) allowed us to demonstrate the levels of CS disaccharides in pre-eclampsia independent of the effects of gestational age and labour which is a significant strength of the current study. Using a GLM analysis, decreased levels of $4S6S_{CS}$ was independently associated with pre-eclampsia ($p = 0.003$), pre-term delivery ($p < 0.001$) and with labour ($p < 0.001$) (Figure 5a). In addition, increased levels of other 4-O sulphated disaccharides were independently associated with labour; $4S_{CS}$ ($p < 0.001$) and $2S4S_{CS}$ ($p = 0.001$). Furthermore, decreased levels of the di-sulphated $2S6S_C$ were independently associated with pre-term delivery ($p = 0.018$) while decreased levels of the non-sulphated $0S_{CS}$ was independently associated with labour ($p = 0.001$) (Figure 5a). It is difficult to discuss the clinical significance of some of these results. However, highly sulphated CS comprising majorly $4S6S_{CS}$ has been shown to bind growth factors such as HB-EGF, FGF-10 and FGF-2 [51] therefore, can mediate growth factor signalling. This disaccharide is decreased in pre-eclampsia in the current study

but may play a limited role in placental growth factor binding as it comprises only about 1% of placental CS. When the disaccharides were grouped based on sulphation, increased levels of the mono- ($p = 0.002$), di- ($p = 0.001$) and total sulphated CS disaccharides ($p = 0.001$) were independently associated with labour (Figure 5b). The consequent decrease in the levels of the non-sulphated CS disaccharide was also independently associated with labour ($p = 0.001$). This is an interesting finding which suggests that placental CS sulphation may be involved in in preparing the placenta for labour. However, further studies are required to confirm this.

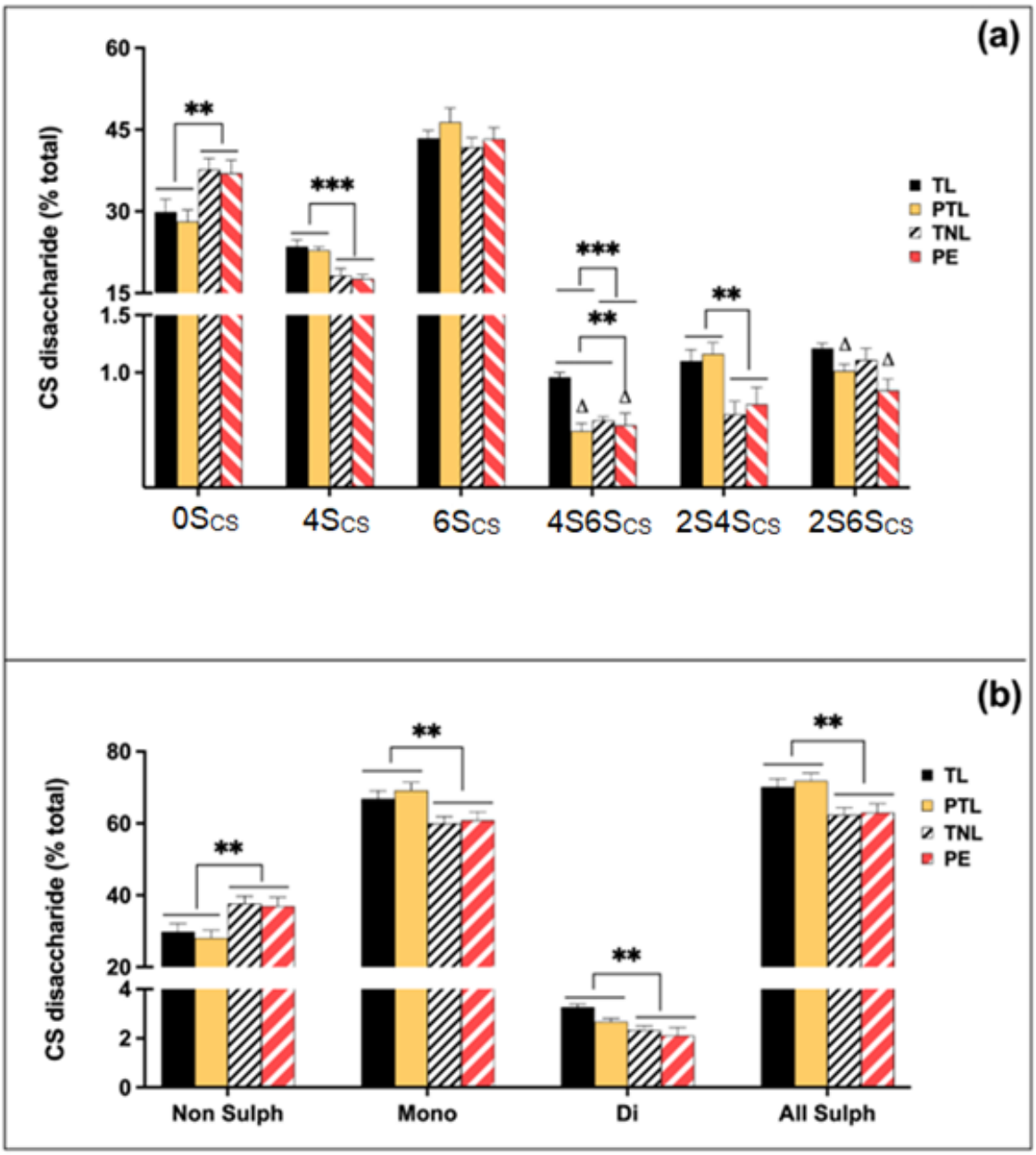



**Figure 5:** The disaccharide composition of placenta tissue extracted-CS from different clinical groups (TL, PTL, TNL and PE) **(a):** the individual disaccharides, **(b):** disaccharides grouped based on sulphation. Results are reported as mean ± SEM. ** = $p \leq 0.01$ and *** = $p \leq 0.001$ (GLM). Δ indicates disaccharides significantly decreased in association with pre-term delivery ($p < 0.05$, GLM).

## Conclusion

In this study, we have developed a WAX HILIC-UPLC-MS method with procainamide labelling which allows for the separation and detection of all eight commercially available CS disaccharides. The method also allows hyaluronic acid non-sulphated disaccharide to be resolved from its isomer in CS thus enabling the detection of hyaluronic acid which is a common contaminant of CS samples. Furthermore, procainamide labelling enables fluorescent detection, therefore a mass spectrometry is not necessary for the implementation of this method. Using the developed method, disaccharide compositional analysis of bovine cartilage CS was performed with results similar to previous studies, confirming the reliability of the method. The method was then used to determine the disaccharide composition of placenta-extracted CS in pre-eclampsia with results showing that the disaccharide composition of CS in the placenta in pre-eclampsia and other pregnancy complications are different. The results also suggests that CS sulphation may be important in maternal labour.

## Author Contributions

Imeobong U Antia: Investigation, formal analysis, conceptualisation, methodology, visualisation, writing – original draft and writing – review & editing.

Frank A Hills: Conceptualisation, sample collection, methodology, supervision, writing – review & editing

Ajit J Shah: Conceptualisation, methodology, supervision, writing – review & editing

## Conflicts of Interest

There are no conflicts to declare

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