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Comparison of charge state distribution in commercially available sulfated cyclodextrins used as chiral resolving agents in capillary electrophoresis

Roy Estrada III[a,*], Gyula Vigh[b]

[a] TM Chemicals LP, 2525 Independence Parkway South, Deer Park, TX 77536, USA
[b] Department of Chemistry, Texas A&M University, P.O. Box 30012, College Station, TX 77842-3012, USA

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ABSTRACT

The charge state distributions of randomly sulfated cyclodextrins from Sigma–Aldrich and Beckman-Coulter, as well as single isomer sulfated cyclodextrins from TM Chemicals LP were investigated using hydrophilic interaction liquid chromatography (HILIC). A cross-linked diol phase and an unbonded silica phase were used as HILIC stationary phases. Groups of sulfated cyclodextrins with different charge states were resolved from each other, while regioisomers in a charge group were partially separated. A ladder of sulfated cyclodextrins having a charge state distribution from 1 to 14 was prepared and was used to determine the charge state heterogeneity of the commercially available sulfated cyclodextrin samples. Wide charge state and regioisomer distributions are seen for the randomly sulfated cyclodextrins, while HILIC analysis of every single isomer sulfated cyclodextrin sample indicates the presence of a single species.

1. Introduction

Due to their versatility, sulfated cyclodextrins (CDs) are currently the most commonly used chiral resolving agents in capillary electrophoresis (CE) [1]. Their proven ability to separate neutral, zwitterionic, basic and even anionic analytes [2–6] makes them the preferred choice among several other types of chiral selectors in CE. Sulfated CDs can be obtained as single isomers or as randomly sulfated mixtures. At present, most of the commercially available sulfated CDs are mixtures of randomly sulfated species [7,8]. For randomly substituted CDs in general, only the average degree of substitution (DS) is known with certainty while the actual isomeric heterogeneity and/or charge distribution are not conclusively determined. This is unfortunate because it is not the DS, rather the actual distribution of the differently substituted cyclodextrin regioisomers that dictates the ensemble enantioselectivity of the mixture [9–11]. This is because different regioisomers of substituted CDs have different electrophoretic and complexation behaviors and, when used in a mixture, different regioisomers may have opposing effects on the overall separation of an enantiomeric pair. This underlines the importance of having the ability to characterize both the charge state distribution and the isomeric heterogeneity of sulfated CDs. Without a good analytical tool to characterize these random mixtures, it becomes difficult, if not impossible, to effectively control the variability of charge distribution between sulfation batches. This, in turn, can compromise the reproducibility of the separation of a pair of enantiomers.

Capillary electrophoresis (CE) has been successfully used to characterize sulfoalkyl ether and carboxymethyl ether derivatives of cyclodextrins [3,12–14]. Although CE can provide excellent separation efficiencies for ionic analytes, the increase in mobility brought about by the addition of a sulfate group onto a highly sulfated CD (e.g., DS > 11) can be easily hidden by electromigration dispersion and by the mobility altering effects of ionic strength, which become more pronounced for highly ionic analytes [15].

Hydrophilic interaction liquid chromatography (HILIC) has been proven to be very effective in separating analytes with high ionic charge [16–18] and so was used in this work to characterize sulfated CDs. The objective was to determine the extent of charge heterogeneity and the relative abundances of the differently charged species in both the single isomer and randomly sulfated CDs that are available commercially.

2. Experimental

2.1. Materials

The sodium salts of the single isomer sulfated CDs – hexakis(2,3-di-*O*-methyl-6-*O*-sulfo)-α-cyclodextrin (HxDMS), hepta(6-*O*-sulfo)-β-cyclodextrin (HS), heptakis(2,3-*O*-diacetyl-6-*O*-sulfo)-β-cyclodextrin (HDAS), heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β

* Corresponding author. Tel.: +1 281 930 2524; fax: +1 281 930 2724.
E-mail addresses: restrada@texasmolecular.com, roy3rd@gmail.com (R. Estrada III).

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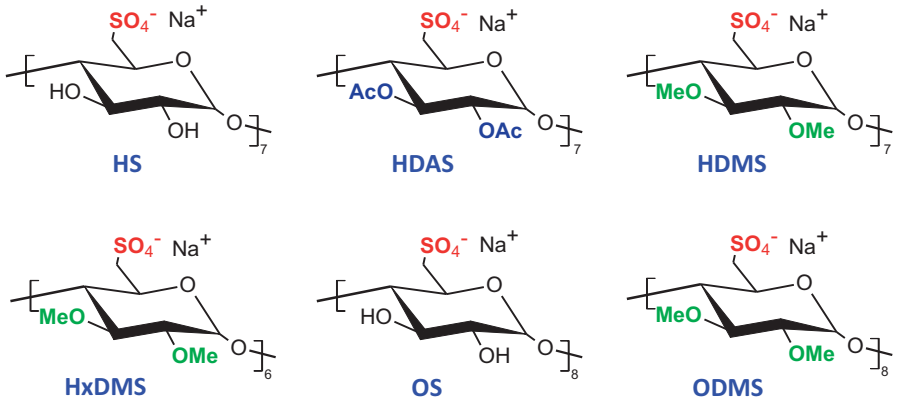

Fig. 1. Structures of the single isomer sulfated CDs.

-cyclodextrin (HDMS), octa(6-*O*-sulfo)-γ-cyclodextrin (OS) and octakis(2,3-di-*O*-methyl-6-*O*-sulfo)-γ-cyclodextrin (ODMS) – were obtained from TM Chemicals LP (Deer Park, TX, USA) [19]. Two different lots (Lot S902078 and Lot M001869) of highly sulfated-β-cyclodextrin (HS-β-CD) and highly sulfated-γ-cyclodextrin (HS-γ-CD) were purchased as 20% aqueous solutions from Beckman-Coulter (Brea, CA, USA). Sulfated β-cyclodextrin (SAS-β-CD) from Sigma–Aldrich (St. Louis, MO, USA) was also obtained. The sulfated CD charge ladder was prepared in-house as described in Section 2.3.

2.2. Chromatography

HPLC separations were obtained on a system consisting of a 507 autoinjector and 126 solvent delivery module (Beckman-Coulter) and a SEDEX Model 55 (SEDERE, Alfortville, France) evaporative light scattering detector (ELSD) which was connected to a 406 analog interface module (Beckman-Coulter). The Gold 8.1 software package (Beckman-Coulter) was used to control the HPLC system and to collect data. Analytical HILIC separations were done using either a Luna HILIC column (3 μm, 200 Å, 150 mm × 4.6 mm) or a Kinetex HILIC column (2.6 μm, 100 Å, 100 mm × 4.6 mm) from Phenomenex (Torrance, CA, USA). Semi-preparative HILIC was done using the same Luna HILIC column as above, but under nonlinear chromatographic conditions. Ammonium formate (Sigma–Aldrich) was used as a salt additive for the HILIC separations. HPLC-grade water was purchased from Aqua Solutions (Deer Park, TX, USA), while HPLC-grade acetonitrile was from VWR (South Plainfield, NJ, USA).

2.3. Preparation of the sulfated CD ladder

The sulfated CD ladder was prepared according to a modified procedure of Bernstein and others [20]. Native β-cyclodextrin (Cargille Corporation, Cedar Rapids, IA, USA), 10.2 g, was stirred in 160 mL dimethyl formamide (Sigma–Aldrich) with 40 mL of dimethyl isopropyl amine (Sigma–Aldrich) at 56 °C. Sulfur trioxide pyridine complex (Sigma–Aldrich) was added incrementally at a rate of 3 equivalents for every 30 min. After each incremental addition, a 10 mL aliquot was taken and quenched with 1 mL of isopropanol. A total of 33 equivalents of the sulfur trioxide pyridine complex were added. A composite sample was made from a select number of aliquots which was then added to 10 volumes of acetone to produce an off-white gum. The gum was dissolved in the run sample solvent and was analyzed by HILIC.

3. Results and discussion

3.1. Single isomer CDs

The structures of the single isomer sulfated CDs that were analyzed in this work are shown in Fig. 1. These are α-CDs (HxDMS), β-CDs (HS, HDAS and HDMS) and γ-CDs (OS and ODMS) that are fully sulfated on the 6-carbon positions and contain either hydroxy, methoxy or acetoxy groups in the 2- and 3-carbon positions. The structures and purity of these CDs have been unambiguously determined by multiple methods in [2,4,5,21–23]. These single isomer sulfated CDs have isomeric purities above 97% according to indirect UV-detection CE analyses under optimized electroosmotic flow conditions [2,4,5,21–23].

HILIC separations of each of the CDs (Fig. 2) show that they have indeed very good isomeric purities. Area percentages for the main components, excluding that of the sodium ion peak, range from 97.7% for HS to 99.9% for ODMS. The minor components eluting before and after the main components are most likely under- and over-sulfated species, respectively. The narrow, well-defined peaks indicate the absence of other regioisomers, in agreement with high resolution NMR data available from the manufacturer (data not shown).

3.2. Randomly sulfated CDs

The structures of the randomly sulfated CDs from Beckman-Coulter and Sigma–Aldrich are illustrated in Fig. 3. Chen et al. [7] reported the characterization of the degree of sulfation and heterogeneity of randomly sulfated CDs using a number of analytical techniques including ESI-MS, elemental analysis and indirect UV-detection CE. Due to the possibility of the cleavage of sulfate esters in the gas phase [24], the authors were judicious not to use the ESI-MS data to determine the charge distributions. Nevertheless, ESI-MS provided an indication that the maximum degree of sulfation in the sample was 14. Indirect UV-detection CE on the other hand showed a single peak for each of the highly sulfated CDs, making it impossible to discern the presence and the actual relative concentration distribution of the differently sulfated CD species.

Since HILIC has been shown to effectively separate complex mixtures of analytes with high ionic charge, such as multiphosphorylated peptides [16] and heparin [17,18], it was used in this study to characterize the randomly sulfated CD samples. We anticipated that a difference of even one sulfate group will result in an adequate shift in the partitioning behavior in HILIC to facilitate a separation. Fig. 4 shows the chromatograms of the HILIC

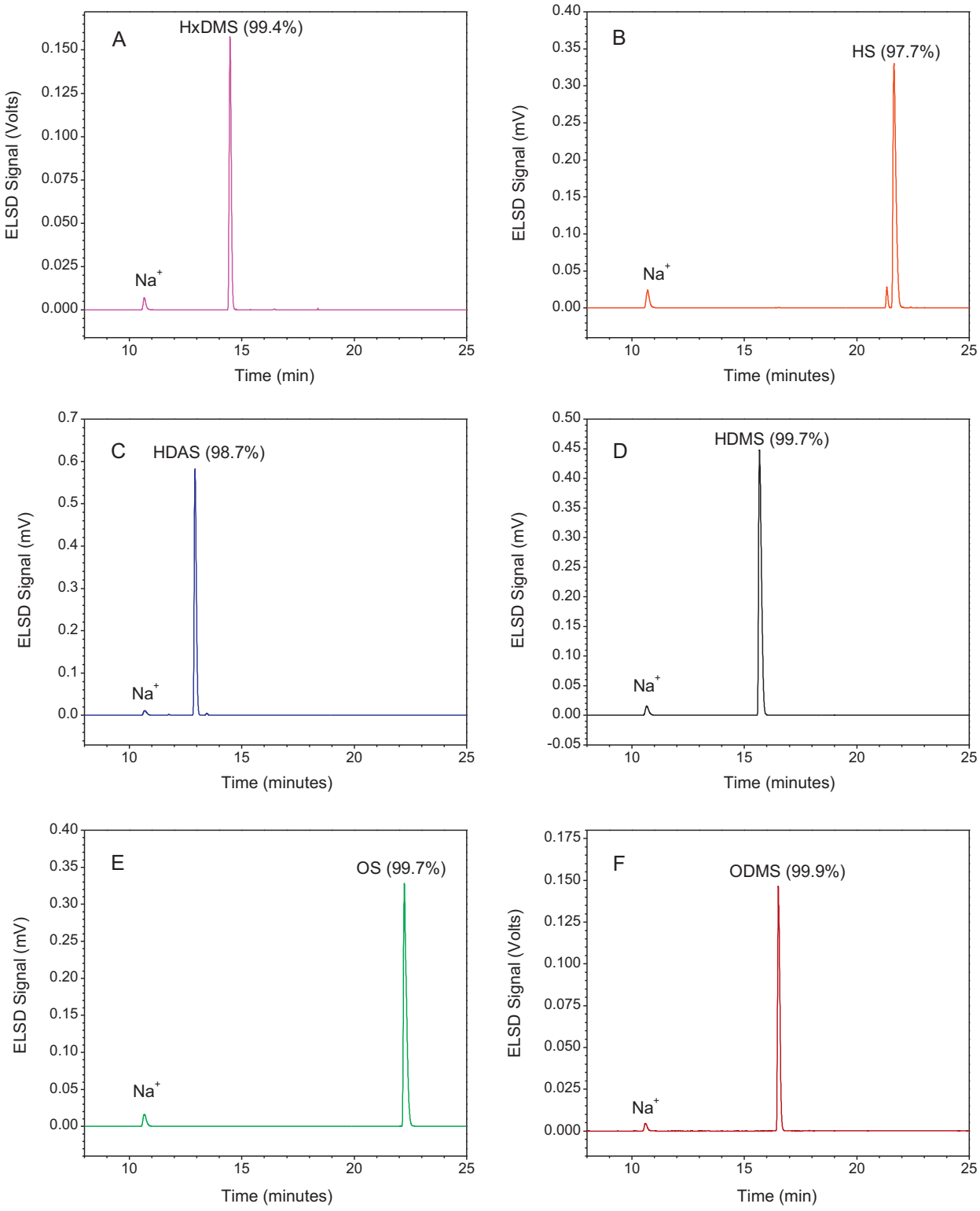

Fig. 2. HILIC analysis of the single isomer sulfated CDs: (A) HxDMS, (B) HS, (C) HDAS, (D) HDMS, (E) OS and (F) ODMS. HILIC was done using a Luna HILIC column with a binary gradient of 96–56% B in 36 min at 1 mL/min (A: 5 mM ammonium formate in water; B: 5 mM ammonium formate in 95%, v/v, acetonitrile: 5%, v/v, water).

separations of the highly sulfated CDs from Beckman-Coulter and Sigma–Aldrich. HILIC was able to resolve the complex random mixtures into discrete bands. The peak distributions resemble Gaussian profiles, which is to be expected of random sulfation reactions.

When comparing the peak widths of the randomly sulfated CDs with those of the single isomer CDs shown in Section 3.1, it becomes obvious that each peak in the random mixtures is composed of multiple, closely related components. It appears that each of these peaks is composed of a group of positional isomers that have the

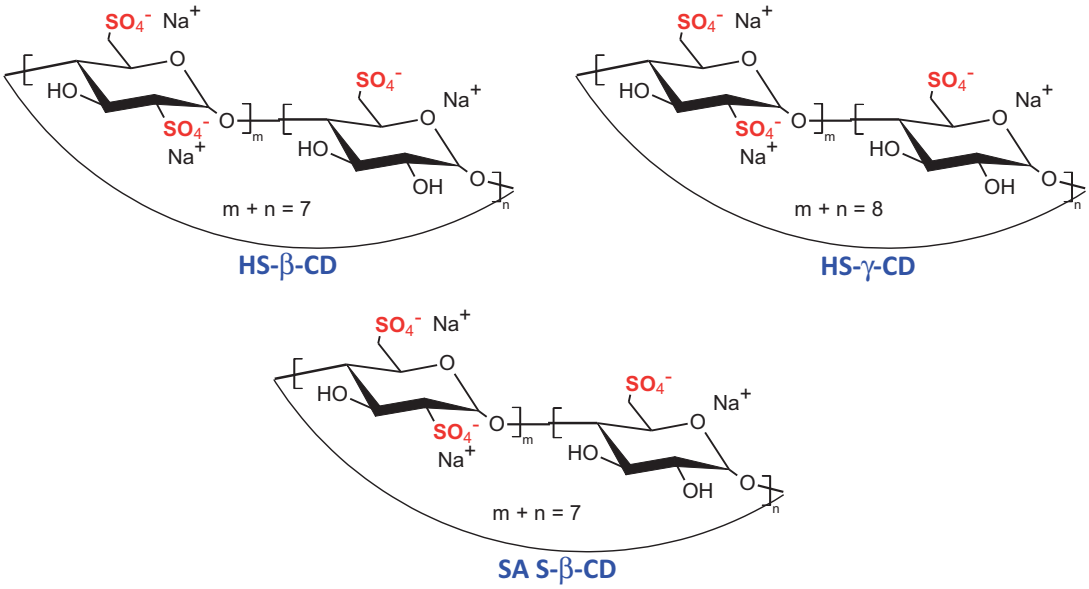

Fig. 3. Structures of the randomly sulfated cyclodextrins from Beckman-Coulter (HS-β-CD and HS-γ-CD) and Sigma–Aldrich (SAS-β-CD).

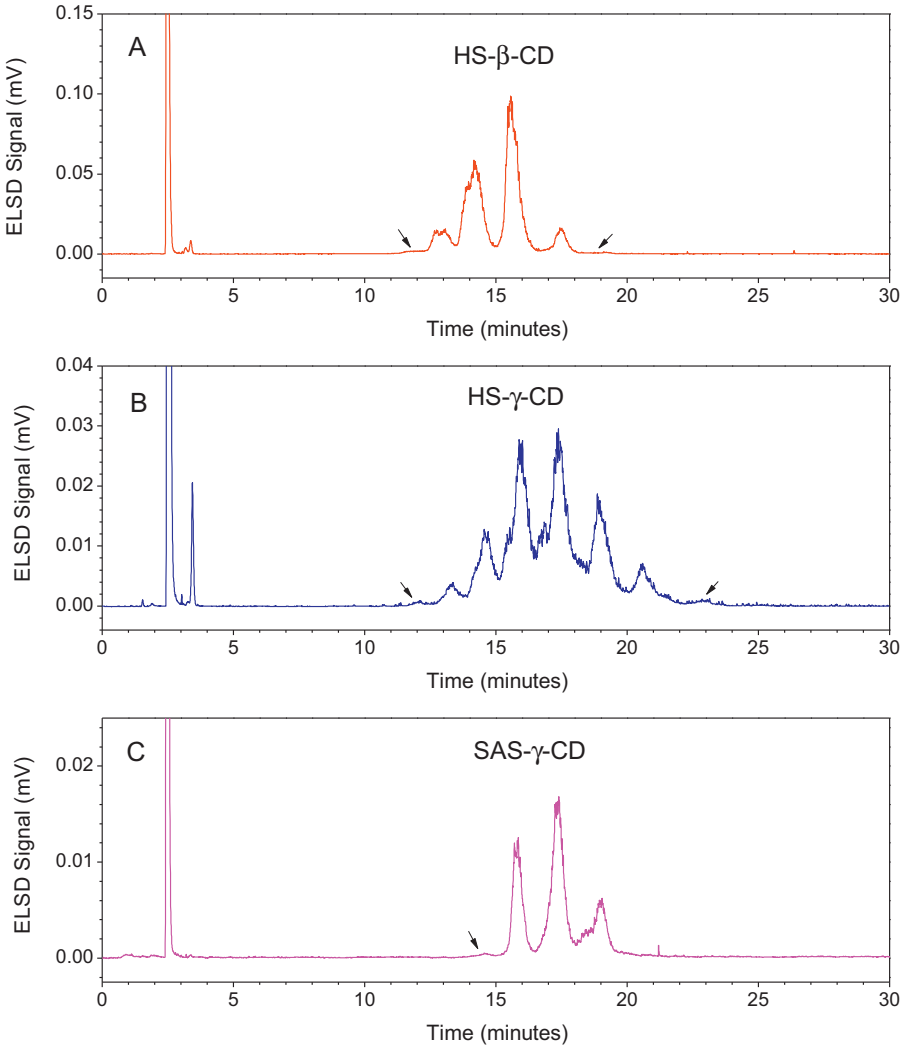

Fig. 4. HILIC analysis of randomly sulfated CDs: (A) HS-β-CD, (B) HS-γ-CD and (C) SAS-β-CD. Isocratic HPLC was carried out using a Luna HILIC column at 1.5 mL/min using 30 mM ammonium formate in 72%, v/v, acetonitrile: 28%, v/v, water as eluent. The arrows point to small yet detectable peak groups (charge groups).

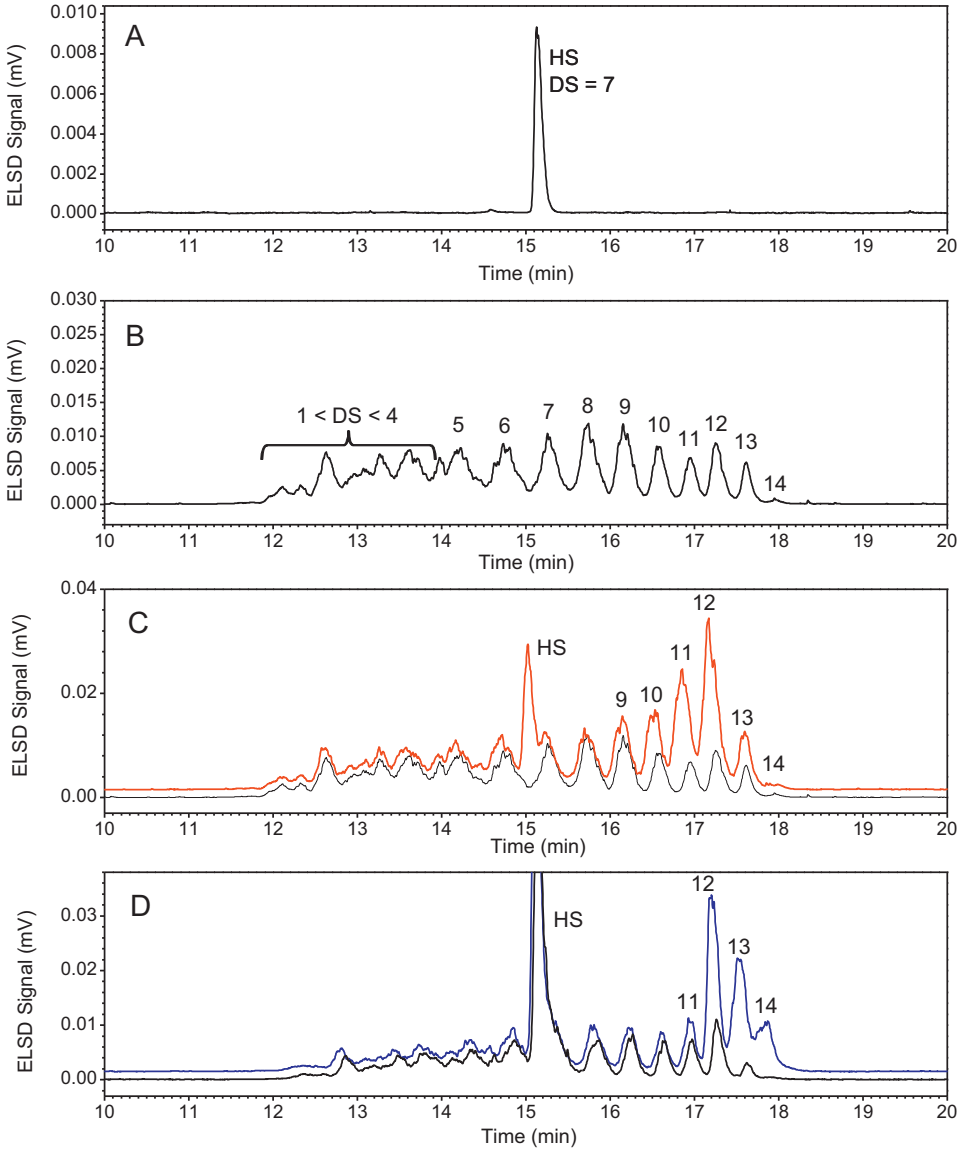

Fig. 5. Determination of the charge state distribution of the randomly sulfated CDs by comparing the retention position of their peaks in HILIC with those of HS and the sulfated CD ladder. (A) HS standard, (B) sulfated CD ladder, (C) sulfated CD ladder spiked with HS and HS-β-CD and (D) sulfated CD ladder spiked with HS and SAS-β-CD. In panels C and D the lower traces are the chromatograms of the CD charge ladder, the top traces are those of the spiked analytes. The HILIC separation was done using a Luna HILIC column with a binary gradient from 90% to 75% B in 16 min at 1.5 mL/min (A: 5 mM ammonium formate in water; B: 5 mM ammonium formate in 94%, v/v, acetonitrile: 6%, v/v, water).

same degree of sulfation. Thus, the HILIC separation unmistakably revealed a wide heterogeneity in the degree of sulfation for HS-β-CD, HS-γ-CD and SAS-β-CD containing as many as 6, 8 and 4 peak groups (charge groups), respectively.

3.3. *Characterization of the charge distribution of the randomly sulfated β-CDs*

In order to determine the actual charge distribution of the randomly sulfated β-CDs, we prepared a sulfated CD charge ladder by randomly sulfating native β-CD with an incremental addition of the sulfating agent. An aliquot of the reaction mixture was taken after the addition of each increment and portions of selected aliquots were mixed at specific ratios so that the peak heights in the HILIC analysis were of the same order of magnitude. The charge number of each peak was then determined using HS as a DS standard. HS was used because it has been well characterized and proven to have a DS of 7 [4]. Fig. 5A andB compares the HILIC analyses of HS and the sulfated CD ladder. The degrees of sulfation of the peaks were assigned based on their position relative to the HS standard. Therefore, the peak that coincides with HS has a DS of 7, the one after that has a DS of 8 and so on. The DS calibration shows that the highest ionic charge in the sulfated CD ladder is 14.

The charge distribution of the highly sulfated β-CD samples from Beckman-Coulter and Sigma–Aldrich were then determined by comparing the retention times of their peaks with those in the sulfated CD ladder. In Fig. 5C and D the lower traces are chromatograms of the CD charge ladder and the top traces are those of the spiked analytes (HS-β-CD and SAS-β-CD, respectively). The chromatogram overlay in Fig. 5C shows that the HS-β-CD consists of a mixture of 9–14-sulfated species with the 12-sulfated peak as the largest. This is congruent with the findings of elemental analysis of HS-β-CD in [7] which established its average degree of sulfation to be 12. On the other hand, the sulfated β-CD

Table 1
Comparison of peak area percentages of the major components in HS-β-CDs (Beckman-Coulter) from two different lots.

	Degree of substitution					
	9	10	11	12	13	14
Lot #1						
Mean % peak area ($n = 6$)	1.29	8.99	34.07	47.11	7.83	0.72
RSD, %	11.8	1.5	1.2	1.4	2.1	5.7
Lot #2						
Mean % peak area ($n = 6$)	1.02	7.76	32.59	48.57	9.17	0.89
RSD, %	8.2	1.3	0.9	1.0	2.7	12.9
Lot 1 to Lot 2% difference	23.3	14.6	4.4	−3.0	−15.8	−21.0

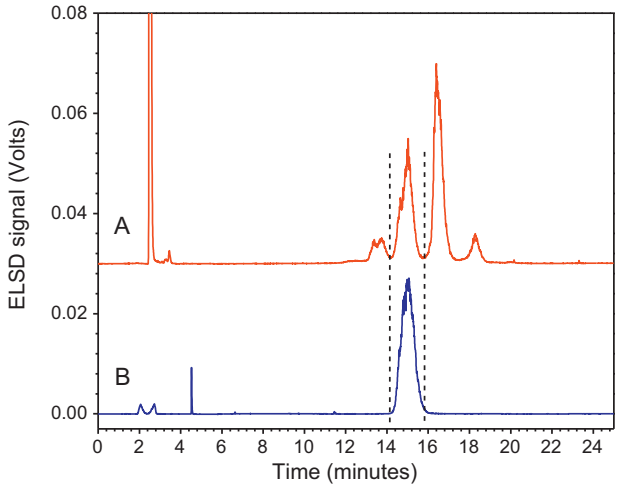

Fig. 6. Comparison of the HILIC analysis of HS-β-CD before (A) and after (B) fractionation of the DS = 11 peak. HPLC conditions are the same as in Fig. 4.

sample from Sigma–Aldrich turns out to have a charge distribution of 11–14 (Fig. 5D overlay). Interestingly, SAS-β-CD has a higher average degree of sulfation than HS-β-CD from Beckman-Coulter. This finding contradicts the information in the Sigma–Aldrich website which states that the DS is from 7 to 11 [25].

3.4. Lot-to-lot variation of the charge distribution of HS-β-CD

HS-β-CD from two different lots were characterized and compared. Table 1 summarizes the results of the analyses. Method reproducibility determined from 6 parallel analysis of one lot is good (less than 3% RSD) for the area percentages for the different peaks except for the outlying ones, which do not have as good a signal-to-noise ratio as the others. Using this HILIC method, it was possible to discern a significant difference in the relative abundances of the main components between the two lots. Table 1 shows that HS-β-CD from Lot #1 is enriched in the 9-, 10- and 11-sulfated species while the other lot contains more of the 12-, 13- and 14-sulfated CDs.

3.5. Separation of regioisomers

In order to confirm the presence of multiple components within a peak in the HILIC chromatogram of the randomly sulfated CDs above, a band of HS-β-CD corresponding to a DS of 11 was isolated using semi-preparative HILIC and then separated in another column offering a different separation selectivity. Fig. 6 shows that the band that was preparatively isolated was clean and free of components from the neighboring bands. This fraction, which was obtained using the Luna HILIC column, was then analyzed

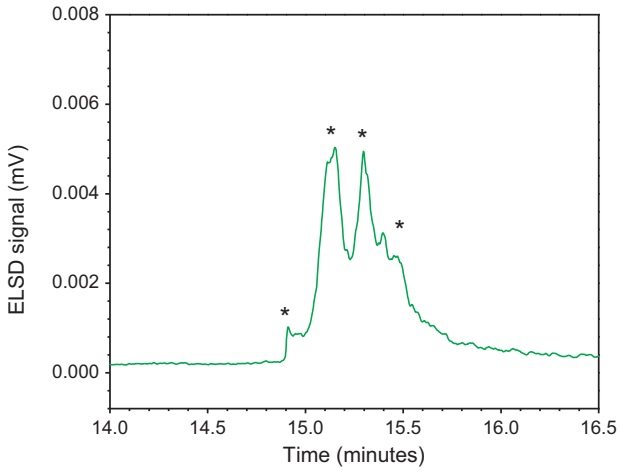

Fig. 7. HILIC analysis of the DS = 11 peak using a Kinetex HILIC column with a binary gradient of 96–81% B in 24 min at 1.5 mL/min (A: 5 mM ammonium formate in water; B: 5 mM ammonium formate in 94%, v/v, acetonitrile: 6%, v/v, water). The symbol * is used to mark possible regioisomers.

using a Kinetex HILIC column. We expected the latter column to bring additional selectivity due to the presence of surface silanol groups. HILIC analysis with the Kinetex column (Fig. 7) shows multiple components in the DS 11 band that are partially resolved from each other indicating that within each band in the HILIC separation there are multiple regioisomers of the same ionic charge.

4. Conclusions

The charge state distribution of three randomly sulfated cyclodextrins from Sigma–Aldrich and Beckman-Coulter, as well as six single isomer sulfated cyclodextrins from TM Chemicals LP were investigated using HILIC. Groups of sulfated CDs with different charge states were resolved even for the highly sulfated species and isomers in each charge group were partially separated. A ladder of sulfated CDs having a charge state distribution from 1 to 14 was synthesized and was used to determine the charge state heterogeneity of the samples tested. The charge state and isomer distributions were wide for the randomly sulfated CDs, while the single isomer sulfated CDs yielded a single charge group that contained a single species.

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