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# Isolation and characterization of β-cyclodextrin sulfates by preparative gradient polyacrylamide gel electrophoresis, capillary electrophoresis and electrospray ionization - mass spectrometry

A β-cyclodextrin sulfate mixture has been fractionated using discontinuous gradient polyacrylamide gel electrophoresis. Semidry electrotransfer of the sample onto a positively charged nylon membrane and visualization of a portion of this membrane with Alcian blue stain showed multiple bands. The bands were cut from the remaining portion of the membrane and after washing with 8 M urea, the β-cyclodextrin sulfate fractions were eluted with 2 M sodium chloride and dialyzed. Analysis of each fraction using high resolution analytical gradient polyacrylamide gel electrophoresis as well as capillary electrophoresis, using indirect detection, showed some of the fractions to be pure while others were mixtures. Each β-cyclodextrin sulfate fraction was complexed with a basic synthetic peptide and analyzed by electrospray ionization mass spectrometry to define the mass of the components in each mixture and thereby to determine the purity of each sample.

## 1 Introduction

Interest in cyclodextrin sulfates as therapeutic agents and pharmaceutical excipients is a recently emerging area. The cyclodextrin family of carbohydrates is classified based on the number of 1 → 4 linked glucose residues present in the ring, where 6, 7 and 8 residues correspond to α-, β- and γ-cyclodextrin, respectively. β-Cyclodextrin sulfates (Fig. 1) have been shown to have some of the same biological properties as heparin, including the ability to inhibit angiogenesis (new vessel growth) [1], inhibit complement activation [2], modify cell growth and exhibit antiviral activity [3]. β-Cyclodextrin sulfates have also been used as excipients to carry water-insoluble drugs [4]. The utility of cyclodextrin sulfates for these pharmaceutical applications is dependent on the degree of sulfate substitution. A sulfate density of approximately 50% (*i.e.*, one half of the hydroxyl groups being substituted) results in biological activities similar to those of heparin [3]. Chemical sulfonation of cyclodextrin is typically achieved by using $SO_3$-complexes as sulfonating agents [5, 6]. These preparations vary in the number and position of sulfate substitution [7, 8]. Although conditions have been developed to reproducibly prepare cyclodextrin derivatives having varying degrees of sulfate substitution, the resulting cyclodextrin sulfates are still mixtures. Recently, our laboratory described a method for the analysis of cyclodextrin sulfates ion-paired with a basic peptide by electrospray ionization-mass spectrometry (ESI-MS). Using ESI-MS, it is possible to define the composition of these mixtures [8]. The current study describes a method to isolate β-cyclodextrin sulfate species useful in defining the role of sulfate density on biological activity.

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**Abbreviation:** MWCO, molecular weight cut-off

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## 2 Materials and methods

### 2.1 Materials

Sulfated β-cyclodextrin was obtained from American Maize Products (Hammond, IN) with a reported $M_r$ of 1539. The linear heparin oligosaccharides used as electrophoresis standards were prepared from bovine lung heparin using heparin lyase [9]. Spectrapore dialysis membranes of 500 and 1000 molecular weight out-off (MWCO) were purchased from Spectrum Medical (Los Angeles, CA). A Hoefer Scientific (San Francisco, CA) vertical slab gel apparatus was used for β-cyclodextrin separation. High resolution, analytical gradient polyacrylamide gel electrophoresis (PAGE) analysis of β-cyclodextrin was performed using a Bio-Rad (Richmond, CA) Protean II vertical slab gel. Both gel systems were equipped with a Bio-Rad Model 1420B power source, and water was cooled with a VWR (Niles, IL) model 1160 circulating water bath operating at 4°C. Semidry electrotransfer was performed using a Semi-Phor model TE70 unit from Hoefer Scientific. 3MM

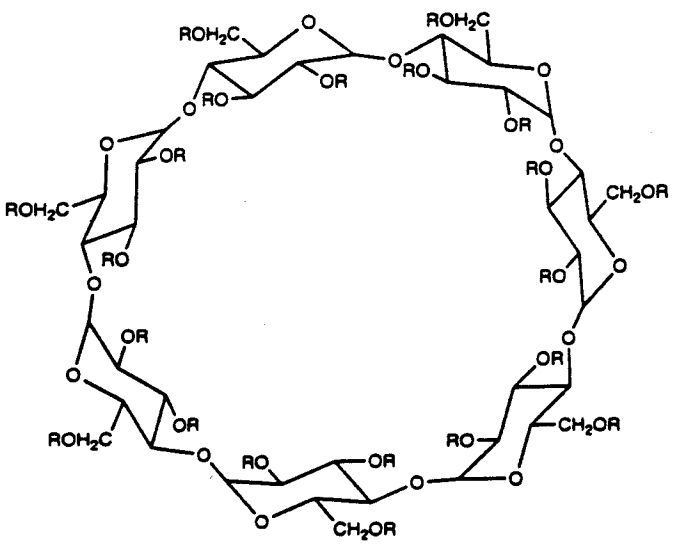


*Figure 1.* Structure of β-cyclodextrin. There are 21 potential sites for sulfate substitution. R = $SO_3$ or H.



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Chromatography paper was from Whatman (Maidstone, England). Nytran Plus, positively charged nylon transfer membrane, was from Schleicher & Schuell (Keene, NH). Acrylamide (ultrapure), tris(hydroxymethyl)aminomethane (Tris), Alcian blue dye, bromophenol blue dye and ammonium persulfate were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Glycine hydrochloride, disodium ethylenediaminetetraacetic acid (EDTA), Azure A dye, boric acid, sucrose, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and urea were purchased from Fisher Chemical Company (Fair Lawn, NJ). Capillary electrophoresis (CE) was performed using a Dionex Capillary Electrophoresis system with advanced computer interface, Model I, equipped with a high-voltage power supply capable of constant or gradient voltage control using a fused-silica capillary, 50 μm ID × 375 μm OD, 65 cm long, from Dionex Corporation (Sunnyvale, CA). UV spectroscopy was performed using a Shimadzu (Tokyo, Japan) model UV 160 spectrometer equipped with a thermostated cell. Freeze-drying was performed using a Virtis (Gardiner, NY) lyophilizer. All other reagents used were analytical grade.

### 2.2 Solutions

Resolving gel buffer (and lower chamber buffer): 0.1 M boric acid, 0.1 M Tris, 0.01 M disodium EDTA, pH 8.3. Stacking gel buffer: 0.1 M boric acid, 0.01 M disodium EDTA, 0.1 M Tris-HCl, pH 6.3. Upper chamber buffer: 1.24 M glycine, 0.2 M Tris. Acrylamide solution (12%): 11.52% w/v acrylamide containing 0.48% w/v *N,N'*-methylenebisacrylamide and 1% sucrose in resolving gel buffer. Acrylamide solution (19.5%): 17.9% w/v acrylamide containing 1.62% w/v *N,N'*-methylenebisacrylamide and 11.5% sucrose in resolving gel buffer. Acrylamide solution (22%): 20.02% w/v acrylamide containing 2% w/v *N,N'*-methylenebisacrylamide and 15% w/v sucrose in resolving gel buffer. Stacking gel solution: 4.75% w/v acrylamide and 0.25% w/v *N,N,'*-methylenebisacrylamide in stacking gel buffer. Semidry transfer buffer: 50 mM Tris, 60 mM glycine, 20% v/v methanol. Alcian blue staining solution: 0.5% Alcian blue in 1 % acetic acid.

### 2.3 Isolation of sulfated β-cyclodextrins by prepative gradient PAGE

Chemically sulfonated β-cyclodextrin (250 mg) was exhaustively dialyzed against 4 × 4 L distilled water using a 1000 MWCO membrane to remove excess salt. The sample was freeze-dried and diluted to 100 mg/mL with distilled water. Preparative gradient gels (18 × 32 cm separated by 1.5 mm spacers, 12 → 19.5% total acrylamide) were prepared by mixing 35 mL of each gel solution with 150 μL 10% w/v ammonium persulfate and 35 μL TEMED in a gradient former while stirring. After pouring the gels, a layer of water was added and the gel allowed to polymerize. The water was removed and stacking gel solution (7.5 mL, 5% total acrylamide) added using a comb to create a single large well. Gradient gels were assembled in the apparatus and 5–10 mg sample containing a trace of bromophenol blue tracking dye was applied. Electrophoresis was performed at 400 V for 16–18 h with circulating water cooling at 4 °C.

### 2.4 Electrotransfer of sulfated β-cyclodextrins to positively charged nylon membranes

Before assembling the transfer unit as described previously [9], the gel and three nylon membranes were soaked in semidry transfer buffer for 15 min. The transfer stack, from anode to cathode, included three layers of filter paper, three layers of Nytran Plus nylon membrane, gel, and three layers of filter paper. Transfers were performed at 200 mA constant current for 4 h. Thin vertical strips (approximately 2 mm) were cut at each side and through the middle of the membranes, stained with Alcian blue for 2 min and destained with water. The stained strips were aligned with the remaining membranes and the unstained, horizontal cyclodextrin sulfate bands in the membranes were cut based on the staining pattern. The nylon strips were each placed into separate vials containing 10 mL of 8 M urea and mixed end-over-end for 24 h at room temperature to remove acrylamide contaminants from the membrane. The urea solution was discarded and the strips washed by end-over-end for 1 h with 5 mL of water. Cyclodextrin sulfate was eluted from the membrane by end-over-end shaking with 5 mL of 2 M sodium chloride for 24 h. Each sample was desalted by exhaustive dialysis against 12 × 4 L water using 500 MWCO membranes and the retentate was freeze-dried.

### 2.5 Analytical gradient PAGE of sulfated β-cyclodextrins

Analytical gradient gels (16 × 20 cm separated by 1 mm spacers, 12 → 22% total acrylamide) were prepared as

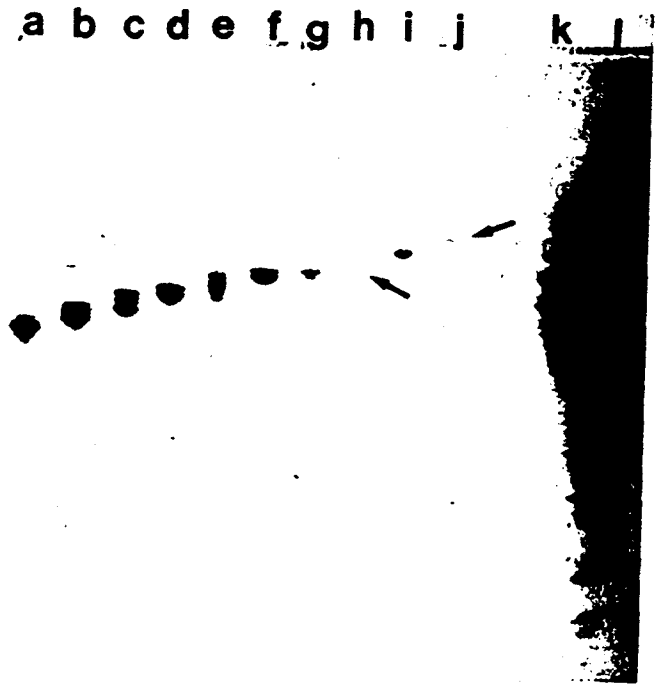


*Figure 2.* Analytical discontinuous gradient PAGE of cyclodextrin sulfates recovered from positively charged nylon membranes. The direction of migration is from top to bottom. Lanes (a)–(j) fractionated cyclodextrin sulfates; (k) cyclodextrin sulfate mixture; (l) bovine lung heparin, partially digested using heparin lyase I, with the degree of polymerization (dp) shown on the right. The indicated dp corresponds to the common fully sulfated heparin sequences ΔUAp2S-α-(1→4)-[D-GlcNpS6S-α-(1→4)-L-IdoAp2S]$_n$-α-(1→4)-D-GlcNpS6S, where $n$ = 0, dp = 2; $n$ = 1, dp = 4; *etc.* Arrows point to faint bands that can be seen in lanes (h) and (j).

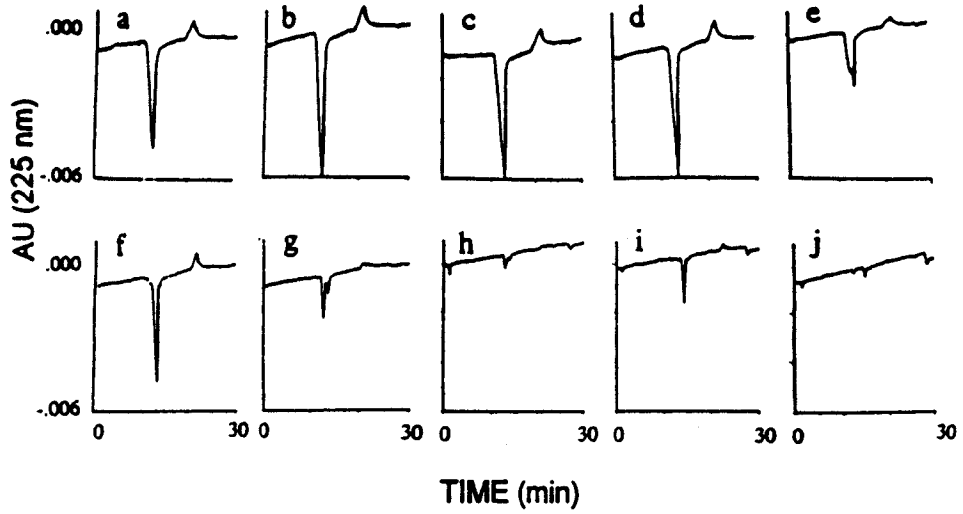


*Figure 3.* Reverse polarity capillary electrophoresis analysis of β-cyclodextrin sulfates. See Section 2.6 for conditions used. The labeled panels correspond to the fractionated β-cyclodextrin sulfates shown in Fig. 2.

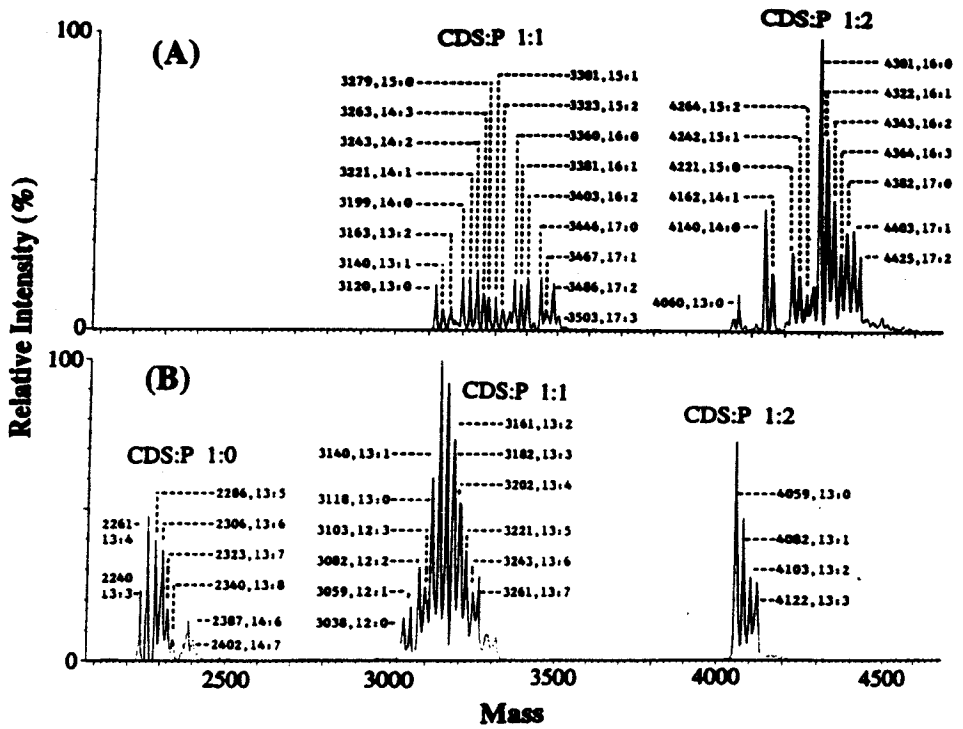


*Figure 4.* ESI-MS analysis of selected β-cyclodextrin sulfate fractions. (A) Transformed ESI mass spectrum of a β-cyclodextrin sulfate mixture (Figs. 2i and 3i) that exhibits six cyclodextrin sulfate components containing from 13 to 17 sulfate groups. An average of 15.6 sulfate groups per cyclodextrin molecule was calculated from this spectrum. (B) Transformed ESI mass spectrum of a relatively pure β-cyclodextrin sulfate fraction (Figs. 2b and 3b) that exhibits three cyclodextrin sulfate components with a major component containing 13 sulfates and two minor components containing 12 and 14 sulfate groups each. An average of 13.0 sulfate groups per cyclodextrin molecule was calculated from this spectrum. The labels above the clusters of peaks indicate the molar ratio of cyclodextrin sulfate (CDS) to basic peptide (P) in the ion pair. Each peak is labeled with its transformed molecular weight and the molar ratio of the number of sulfate groups (#S) to the number of sodium ions (#Na) present in each β-cyclodextrin sulfate component.

described above by mixing 17.5 mL of each gel solution with 75 µL 10% w/v ammonium persulfate and 17 µL TEMED in a gradient former while stirring. Electrophoresis was performed at 400 V for 5 h with circulating water cooling and the bands were visualized by Alcian blue staining.

### 2.6 Analysis of oligosaccharides by capillary electrophoresis

The purity of each oligosaccharide was confirmed by the presence of a single major symmetrical peak on analysis using CE. The sample was separated and analyzed using a clean fused-silica capillary (50 µm ID, 375 µm OD, 65 cm long) [10]. The samples (approximately 1 mg/mL) were prepared in deionized, distilled water and loaded (8 nL) with gravity injection (hydrostatic pressure at a 45 mm head height for 15 s). Reverse-polarity analyses were carried out for 40 min at 18 kV using 25 mM benzoic acid-Tris base for indirect UV detection at 225 nm [10].

### 2.7 Electrospray mass spectrometry of sulfated β-cyclodextrins

Cyclodextrin sulfates were analyzed as ion-paired complexes with a basic peptide (RKRARKE, $M_r$ 943.1, obtained from Sigma Chemical, St. Louis, MO) by ESI-MS in the negative ion mode using a Micromass Quattro triple-quadrupole mass spectrometer as previously described [8]. Briefly, cyclodextrin sulfate (approximately 100 pmole) was mixed with excess peptide (mole ratio of ~ 1:5) in 1:1 water/acetonitrile. The ion-paired complexes showed no losses of $SO_3$ from the sulfate groups when analyzed using a capillary voltage of –2.7 kV and a nozzle skimmer voltage of –12 V.

## 3 Results and discussion

As part of our continuing study of the structure-activity-relationship (SAR) of polysulfated carbohydrates used in pharmaceutical applications, our laboratory has begun to examine commercially prepared β-cyclodextrin sulfates. Analysis by ESI-MS of cyclodextrin sulfates ion-paired with basic peptides has clearly demonstrated that they are heterogeneous mixtures containing varying levels of sulfation [8]. To further understand the SAR of the β-cyclodextrin sulfates, we next undertook their fractionation into charge and size uniform species. Initial attempts to fractionate β-cyclodextrin sulfate using strong anion exchange HPLC and weak anion exchange low pressure chromatography failed, apparently due to the irreversible interaction of β-cyclodextrin sulfate with the stationary phase. Even the use of up to 6 M sodium chloride failed to elute the sample from the columns studied. These attempts included a method previously shown to successfully fractionate 4-sulfobutyl ether derivatives of β-cyclodextrin [11]. Presumably, the higher charge of the β-cyclodextrin sulfates used in this study resulted in their irreversible adsorption to these anion exchange matrices

The β-cyclodextrin sulfate sample was next subjected to preparative discontinuous gradient polyacrylamide gel electrophoresis (PAGE). Electrotransfer to positive nylon membranes and elution with 2 M sodium chloride had already been applied in our laboratory to prepare homogeneous heparin oligosaccharides [9]. Initial attempts using an identical procedure to recover the bound β-cyclodextrin sulfates from the positively charged nylon membrane resulted in recoveries higher than theoretical, based on the mass of the recovered material. This suggested that soluble acrylamide oligomers were being recovered from the gel together with the desired β-cyclodextrin sulfate fractions. β-Cyclodextrin sulfate is believed to bind to the positively charged nylon transfer membrane primarily through ion-pairing interactions while the neutral oligomers of acrylamide primarily bind through hydrogen bonding interactions. Thus, the membrane was initially washed with 8 M urea to remove contaminating acrylamide oligomers, rinsed with water, and then the β-cyclodextrin sulfate fractions were released with 2 M sodium chloride, exhaustively dialyzed and freeze-dried. The mass of the recovered fractions was close to that expected based on the amount of sample initially loaded into the gel.

High resolution analytical discontinuous gradient PAGE analysis of the recovered β-cyclodextrin sulfate fractions was next used to assess their purity (Fig. 2). The migration of β-cyclodextrin sulfate mixture (lane k), consisting of seven glucose units, is highly retarded compared to a linear sulfated heparin oligosaccharide (lane l) of a similar degree of polymerization. This retardation is most likely due to the rigid cyclic structure of the β-cyclodextrin sulfate resulting in a larger relative hydrodynamic volume. Alternatively, unlike the cyclic oligosaccharides, linear sulfated oligosaccharides can undergo reptilate motion through the porous matrix of the gel. The original β-cyclodextrin sulfate mixture contained more than 12 components, all having seven glucose units and differing only in degree and position of sulfate groups [8]. All the major β-cyclodextrin sulfate components of the original sample (lane k) are visible in the fractionated material (lanes a–j). While some of the lanes appear to contain a single species (lanes i and j), most appear to contain from two to six components.

CE was next used to analyze each β-cyclodextrin sulfate fraction (Fig. 3). An indirect detection method was used relying on the absorbance of benzoic acid buffer at 225 nm. A single major (negative) peak was observed for several samples (a, b, c, d, f and i), suggesting a high degree of purity. It is important to note that while (i) and (j) showed a single band on high resolution gradient PAGE fractions, (c), (f) and (h) did not. These results suggest that CE might be incapable of separating isomeric β-cyclodextrin sulfates having identical numbers of sulfate groups. The fractions were next analyzed using ESI-MS as ion-paired complexes with a basic peptide. ESI-MS data (Fig. 4) showed only

**Table 1.** Distribution (%) of sulfate groups in β-cyclodextrin sulfate fractions[a)]

| No. sulfates (# S) | Fraction[b)] a | b | c | d | e | f | g | h | i | j |
|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 0.9 | –[c)] | – | – | – | – | – | – | – | – |
| 11 | 5.6 | – | – | – | – | – | – | – | – | – |
| 12 | 44.6 | 22.2 | – | – | – | – | – | – | – | – |
| 13 | 45.0 | 77.0 | 24.4 | 2.3 | 1.5 | – | 2.3 | 1.7 | 4.1 | 4.8 |
| 14 | 2.0 | 0.8 | 49.3 | 60.9 | 50.5 | 9.5 | 8.3 | 14.6 | 9.5 | 15.1 |
| 15 | 1.5 | – | 25.2 | 32.8 | 35.6 | 90.5 | 54.4 | 39.4 | 14.0 | 15.7 |
| 16 | 0.5 | – | 1.1 | 3.6 | 10.7 | – | 35.1 | 32.2 | 72.4 | 42.8 |
| 17 | – | – | – | 0.5 | 1.7 | – | – | 12.1 | – | 21.6 |
| 18 | – | – | – | – | – | – | – | – | – | 0.1 |
| Weighted average # sulfates[d)] | 12.5 | 13.0 | 14.0 | 14.4 | 14.6 | 14.9 | 15.2 | 15.4 | 15.6 | 15.6 |
| Sulfate density (%)[e)] | 59.4 | 61.7 | 66.8 | 68.5 | 69.6 | 71.0 | 72.5 | 73.2 | 74.1 | 74.3 |

a) Computed from the abundance of the corresponding sulfate-containing ions appearing in the transformed ESI mass spectra assuming equal response for each of the cyclodextrin sulfate components
b) Fractions (a)–(j) correspond to the component in lanes (a)–(j) in Figs. 2 and 3, respectively
c) Not detected
d) Weighted average # sulfates = [Σ(%) (#S)] / [Σ (%)]
e) Sulfate density = weighted average # sulfates × 100 / maximum # sulfates (21)

fraction (f) to be > 90% pure and (b) to be nearly pure (77%), having 15 and 13 sulfate groups, respectively. All the other fractions contained two or more clusters of molecular ions (Table 1).

## 4 Concluding remarks

In conclusion, preparative gradient PAGE offers a useful approach for the fractionation of β-cyclodextrin sulfates. The application of three analytical techniques, analytical gradient PAGE, CE and ESI-MS show that while some of the β-cyclodextrin sulfate fractions appear to be uniform in charge and mass, these fractions still contain mixtures of regio-isomers. New, higher resolution approaches will be required to prepare homogeneous β-cyclodextrin sulfates for biological evaluation.

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