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Charged cyclodextrin-mediated sample stacking in micellar capillary electrophoresis
A simple method for enhancing the detection sensitivity of hydrophobic compounds

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Abstract

The development of on-line sample stacking techniques for enhancing limits of detection of neutral analytes in micellar capillary electrophoresis (MCE) has recently gained much attention. Utilizing high-conductivity sample matrices to invoke sample stacking is promising, but requires the limited use of sample solubilizing agents such as alcohols in the sample matrix. In this study, we show how simple replacement of the sample solvent (methanol) with a solution of sulfated β-cyclodextrin (sβ-CD) allows a significant increase in the sensitivity of detection of model hydrophobic analytes. This increase in sensitivity is accompanied by significant peak sharpening. Sulfated CDs in the sample matrix allow for effective solubilization of hydrophobic analytes without the use of organic solvents such as methanol. The testing of various sample matrix sβ-CD concentrations for their effect on peak sharpening identified 3 to 5% as optimal for the estrogen standards. The use of a sβ-CD sample matrix allowed for hydrostatic injections (3.5 kPa) of 297 s, compared with 4 s when the analytes were dissolved in methanol. A mechanism explaining the sβ-CD-induced effect involves an analyte transfer mechanism where the sβ-CDs, despite providing anodic mobility to analytes in the sample zone, are able to transfer analytes to trailing separation buffer micelles for ''recycling'' back into the sample zone without compromising the stacking process. The overall improvement in sensitivity allows detection of estrogens in the parts-per-billion range and stands to improve the utility of MCE as a bioanalytical technique.

Keywords: Stacking; Micellar electrokinetic chromatography; Estrogens; Sulfated β-cyclodextrin; Cyclodextrins

1. Introduction

The development of sensitivity enhancement techniques such as field-amplified stacking [1] and on-line isotachophoresis [2] have had a profound effect on the applicability of capillary zone electrophoresis (CZE) for the analysis of low-concentration analytes in the research and clinical laboratory. Of these, sample stacking is the most commonly used mode for zone sharpening in CZE. In CZE, sample ions are stacked by exploiting conductivity differences between the sample matrix and separation buffer. The

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sample ions have an enhanced electrophoretic mobility if they are in a lower conductivity (lower ionic strength) matrix than the separation buffer. When voltage is applied to the system, sample ions in the sample plug accelerate toward the adjacent separation buffer zone. Upon reaching this boundary, the higher conductivity of the separation buffer induces a decrease in the velocity of the sample ions. Subsequently, the sample components are stacked into a smaller zone than the original sample plug. When the conductivity gradient dissipates, the stacked analytes undergo normal CZE separation [1].

The first report of stacking neutral compounds in micellar capillary electrophoresis (MCE), also known as micellar electrokinetic chromatography (MEKC), was by Liu et al. in 1994 [3]. Neutral analytes were dissolved in micellar solutions at a concentration above the critical micelle concentration (CMC) but much lower than the separation buffer. Complexation of the neutral analytes with the charged micelles resulted in charged analyte/micelle complexes that had higher mobility in the sample zone versus the separation buffer due to field amplification, analogous to that demonstrated in CZE [1]. When the sample plug was injected, field-amplified stacking resulted followed by the MCE separation. A variation of this stacking method used reversed polarity to stack analytes at the detector-end of the sample plug, and then move the sample matrix out of the injection end of the capillary via electroosmotic flow (EOF) [3]. The polarities were then switched to allow MCE separation. The advantage of this method is the increased injection volume, since the sample matrix is no longer in the capillary potentially disrupting a constant electric field. Later, Quirino and Terabe studied a different mechanism where analytes were solubilized only in water [4,5]. Micelles from the separation buffer entered and moved rapidly through the sample matrix due to field amplification. The analytes were complexed by micelles as they traversed the sample matrix, stacking analytes at the injection end of the capillary [4]. The same polarity-switching technique to move sample matrix from the capillary that Liu et al. described [3] was also employed with the water sample matrix [5].

Quirino and Terabe describe another neutral analyte stacking and sample matrix removal method without switching polarities during the analysis [6]. Low pH separation buffers were used to reduce EOF and reversed polarity was employed. While the EOF was of adequate magnitude to push the sample matrix from the injection end of the capillary, the analyte/micelle complex possessed sufficient electrophoretic mobility to overcome the EOF and migrate towards the detector. Stacking occurred due to a low-conductivity sample matrix via field-amplification of micelles as they entered the sample zone.

Two methods of sample stacking during injection have also been developed. Nielsen and Foley [7] induced zone sharpening of neutral analytes during electrokinetic injection. Utilizing a low-conductivity sample matrix consisting of positively-charged micelles, the sample could be stacked while electrokinetically injecting into a separation buffer of higher conductivity due to the field amplification effect. The separation could be carried out at normal polarity since positive and neutral surfactants were used in the sample matrix. Similarly, Quirino and Terabe injected neutral analyte/anionic micelle complexes into a water plug using reversed polarity [8]. The separation buffer pH was acidic to reduce the EOF below the electrophoretic mobility of the analyte/micelle complex. Using this method, the water plug would exit the injection end of the capillary via EOF, while the analytes would migrate toward the detector when complexed with a micelle.

These stacking methods provide lower detection limits, but are fundamentally limited by the field amplification method. Field amplification requires a lower conductivity sample matrix than the separation buffer and, consequently, the concentration of the charged micelles incorporated into the sample matrix as ''analyte carriers'' is limited. As the concentration of the charged micelle is decreased, the complexation of analytes and, therefore, the efficiency of stacking is decreased. Nielsen and Foley began to address this problem by forming mixed micelles of neutral and charged surfactants in the sample matrix [7]. Quirino and Terabe addressed this issue by dissolving the analytes in water and using the micelles that entered the sample matrix as analyte carriers, with the same limitations [5].

An alternative method for stacking utilizes high conductivity sample matrices to stack the micelles in the run buffer against the sample matrix. The ''micelle front'' formed by a ''reversed field amplified stacking'' efficiently collects the uncharged, hydro-

phobic analytes as it moves through the high conductivity sample matrix [9,10]. A similar approach employs a sample matrix and run buffer of equal conductivity, a lack of pseudostationary phase in the sample matrix, and lack of EOF to stack the analytes [11].

In this manuscript, a stacking mechanism is examined which utilizes a high conductivity sample matrix with a pseudostationary phase (sulfated cyclodextrins) in the presence of normal EOF. It is demonstrated that sulfated β-cyclodextrin (sβ-CD) as a sample matrix component provides a marked sample stacking effect and subsequent enhancement in sensitivity for detecting hydrophobic compounds, as well as being an excellent solubilizing agent. Three of the most common estrogens in urine from women, estrone, estradiol and estriol were utilized. Ji et al. accomplished the separation of these clinically-relevant estrogens via MCE, although detection limits were not sufficient to detect physiological concentrations [12]. In this manuscript, the mechanism through which cyclodextrin-mediated stacking occurs was explored via optimization of stacking and detectability of the estrogen standards as model hydrophobic compounds. As a result of MCE often being exploited for the analysis of neutral and/or hydrophobic analytes, it may be plausible to extend this simple method for enhancing analyte detectability to many analyte systems amenable to MCE.

2. Materials and methods

2.1. Materials

Sodium tetraborate, sodium hydroxide, hexamethonium bromide, 17β-estradiol (E1), estrone (E2) and estriol (E3) were obtained from Sigma (St. Louis, MO, USA). Sodium phosphate, dibasic, and HPLC-grade acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, USA). Cholic acid, sodium salt hydrate, was obtained from Aldrich (Milwaukee, WI, USA).

Unless otherwise specified in the text, the sβ-CDs used possessed 7 to 11 degrees of substitution (DS) and were obtained from Aldrich. Highly sulfated α-, β- and γ-CDs, with 11, 12 and 13 DS, respectively, were a gift from Beckman Instruments (Fullerton, CA, USA). Tertiary amine β-CD (DS 3.5), carboxymethyl β-CD (DS 14), and amphoteric β-CD (tertiary amine, DS 2; carboxy methyl, DS 2) were a gift from Supelco (Bellefonte, PA, USA).

2.2. Buffer and sample preparation

Separation buffers were prepared with Milli-Q water (Millipore, Bedford, MA, USA), and were filtered through a 0.2-μm filter (Gelman) before use. Borate/phosphate buffers were not adjusted for pH (range 8.7–8.9). All pH measurements were performed before the addition of any organic solvents. Estrogen standards were prepared in methanol at 100 or 150 μg/ml concentrations of each estrogen. The sβ-CD sample buffers were prepared in water at concentrations of 1–10% (w/v) corresponding to approximately 5 to 50 m*M*. All other s-CD buffers were prepared in water at stated concentrations.

2.3. Instrumentation

MCE separations were carried out with a Beckman P/ACE System 2100 interfaced with an IBM Value Point 486 computer utilizing System Gold software (V. 8.1, Beckman Instruments) for control of the instrument and data collection. Migration times for individual peaks as well as peak area and height were obtained through the System Gold version 8.0 software.

2.4. Capillary electrophoresis separation conditions

Polyimide-coated, bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) used in these experiments were 47 cm (40 cm to the detector)×50 μm I.D.×375 μm O.D. Polarity was normal (the inlet was the anode), the capillary temperature was maintained at 20°C and detection was by UV absorbance at 200 nm. The capillary was conditioned daily by rinsing with a minimum of 20 column volumes each with 0.1 *M* NaOH, water and separation buffer (in order). For a typical analysis the following method was used: a five capillary volume rinse with separation buffer (1 min at 140 kPa, 20

p.s.i.), 2- to 297-s sample injection (3.5 kPa, 0.5 p.s.i.), 2–5 sample matrix injection, separation at 30 kV, followed by a 10 capillary volume rinse with water (140 kPa, 20 p.s.i.) (1 p.s.i.=6894.76 Pa). The rinses with separation buffer were carried out with non-electrolyzed separation buffer, and the electrophoresis buffer was replaced within 10 runs.

2.5. Injection volume determination

The fill rate of the capillary was determined by first flushing the capillary with separation buffer then injecting the sβ-CD/estrogen sample matrix at low pressure. The time of absorbance change was noted and used to calculate the fill rate. A similar fill rate was determined for separation buffer injected at low pressure into a capillary filled with sample matrix (i.e., the viscosities of the two solutions were similar). The fill rate was used to calculate the approximate volume of sample injected during different time frames.

3. Results and discussion

3.1. sβ-CD as a sample matrix

MCE is a mode of CE that is useful for the separation of mixtures of uncharged and hydrophobic compounds [13]. The presence of charged surfactant in the buffer functions not only to maintain the solubility of neutral hydrophobic analytes, but also allows for their separation due to differential partitioning of the analytes into micelles during the electrophoretic process. A result of the fact that not all analytes are easily solubilized in a micelle-containing separation buffer, the use of organic-based sample matrices (e.g., methanol) are often required, despite the fact that they are associated with limitations. Most importantly, injection volumes are restricted to relatively small sample plugs, therefore limiting the detection sensitivity of the analysis.

3.1.1. Limits of detection (LODs) for estrogens in methanol

The LOD was determined for estrogens in methanol by first determining the maximum hydrostatic

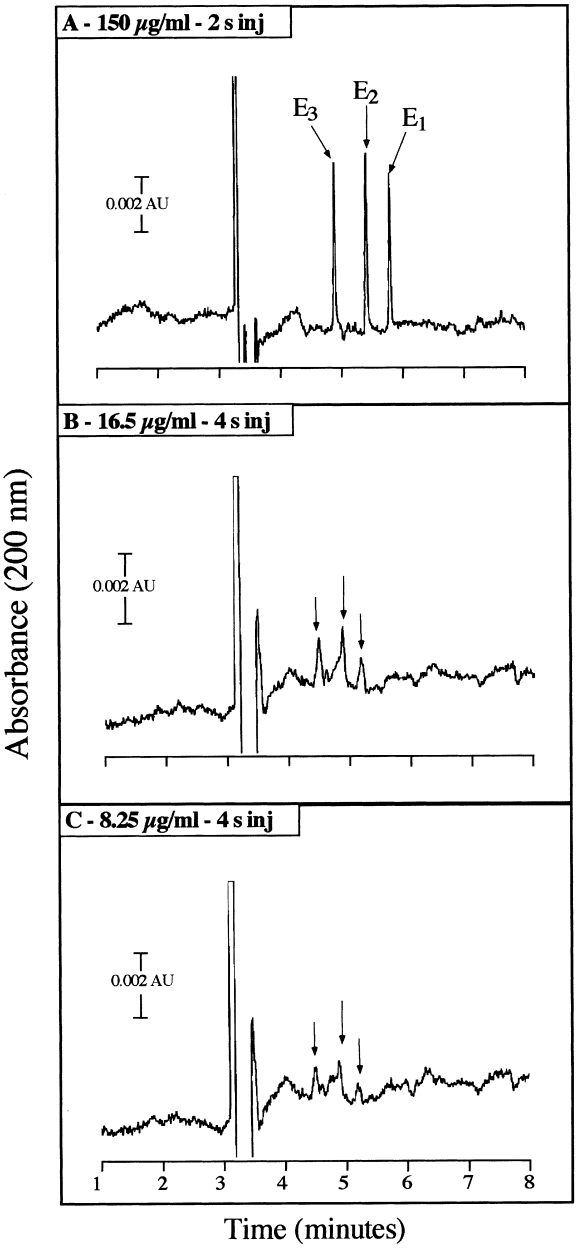

Fig. 1. Maximizing injection of estrogen standards in a methanol matrix for MCE separation. Separation was carried out in 5 m*M* borate–5 m*M* phosphate–80 m*M* sodium cholate buffer–20% acetonitrile, pH 8.9, 30 kV. (A) 2-s hydrodynamic injection of 150 μg/ml estrogens. 4-s hydrodynamic injection of estrogens at concentrations of (B) 16.5 μg/ml and (C) 8.25 μg/ml. Estrogen standards were in 100% methanol. E_1; β-estradiol, E_2; estrone and E_3; estriol.[1]

[1]Note abbreviations, literature varies with respect to nomenclature.

injection allowable without peak broadening, loss of resolution or current failure. Fig. 1A shows electrophoresis of a 2-s injection (3.4 kPa positive pressure) of the standards in methanol at 150 μg/ml in a capillary equilibrated with a separation buffer consisting of 5 m*M* phosphate, 5 m*M* borate, 80 m*M* sodium cholate and 20% acetonitrile at pH 8.9. Ji et al. had shown these conditions to be optimal for separation of these estrogens [12]. Lower concentrations of the estrogens were tested and the detectability of the estrogens at 33 μg/ml using a 2-s injection plug is poor at best ($S/N=10$, data not shown). The injection time could be increased to a functional maximum of 4 s (calculated sample plug 1.8 mm, 35 nl) at which point the detectability was slightly improved but the peak shape degraded with increasing injection time. Replicate analyses with 5-s methanol injections commonly failed, most likely due to the methanol plug serving as an insulator between the separation buffer and the inlet vial. As a result, a 4-s injection was deemed to be the maximum.

The LOD was determined by electrophoresing samples from serial two-fold dilutions of the 33 μg/ml stock solution. Fig. 1B and C shows the electropherograms resulting from 4-s injections of the standards when dissolved in methanol at 16.5 μg/ml (panel B) and 8.25 μg/ml (panel C). It is clear from these data that the ability to quantitatively detect any of the estrogens at a concentration below 8.25 μg/ml is tenuous at best. This is problematic in a system where estrogenic compounds are present at low ng/ml or even high pg/ml concentrations [12]. The signal-to-noise (S/N) ratio with a 4-s injection at concentrations of 16.5 and 8.25 μg/ml was 8/1 and 5/1, respectively. Hence, the LOD was concluded to roughly be in the 8–16 μg/ml range.

3.1.2. Optimal sβ-CD concentration

Preliminary data had shown that sβ-CD was a good sample matrix component for solubilization of steroids as well as effecting stacking in MCE [9]. Concentrations of 1 to 10% (w/v) sβ-CD in water were examined to determine optimal concentration in the sample matrix. The results are shown in Fig. 2. Calculated plate heights decrease as sβ-CD is increased from 1 to 3%, but then increase above 6% sβ-CD. Consequently, 3% (14.6 m*M*) sβ-CD was

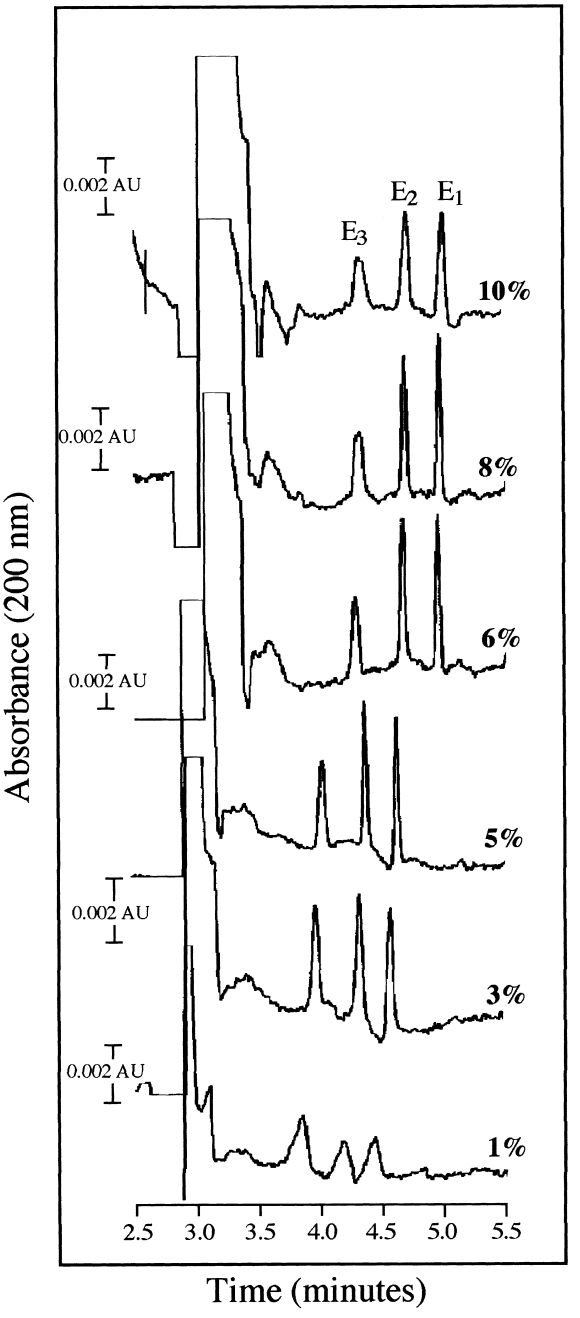

Fig. 2. Optimal sβ-CD concentration for stacking. Standard injection of estrogens in 1%, 3%, 5%, 6%, 8% and 10% sβ-CD. 64-s hydrodynamic injection. Separation buffer: 5 m*M* borate–5 m*M* phosphate–80 m*M* sodium cholate–20% acetonitrile, pH 8.9.

used as the standard concentration for the following experiments.

3.1.3. LODs for estrogens in sβ-CD

A maximum hydrostatic injection was determined for the estrogens dissolved in 3% sβ-CD at a concentration of 8.25 μg/ml by increasing the injection time from 4 s to 8, 16, 32 and 64 s in consecutive runs. Fig. 3 illustrates the dramatic improvement afforded by the sβ-CD sample matrix injection (Fig. 3A) showing that a much better signal-to-noise ratio is obtained with the same 4-s injection relative to that in the methanol matrix (see Fig. 1C). Injections of 32 s and 64 s were accomplished with little loss of resolution as shown in Fig. 3B and C. As the injection time is increased from 4 to 64 s, peak area (PA) increased linearly for all three estrogens with an r^2 value for E_1, E_2 and E_3 of 0.997, 0.996 and 0.997, respectively (data not shown). Not surprisingly, the response of peak height was nonlinear, showing signs of plateauing with injections of 32 s or greater. This is evident in Fig. 3 where, unlike estrogens in methanol, good peak shape is maintained with larger injections but some broadening is obvious with an injection time of 64 s. Using the peak areas for E_1 in Figs. 1C and 3C, a 23-fold enhancement in sensitivity has been achieved

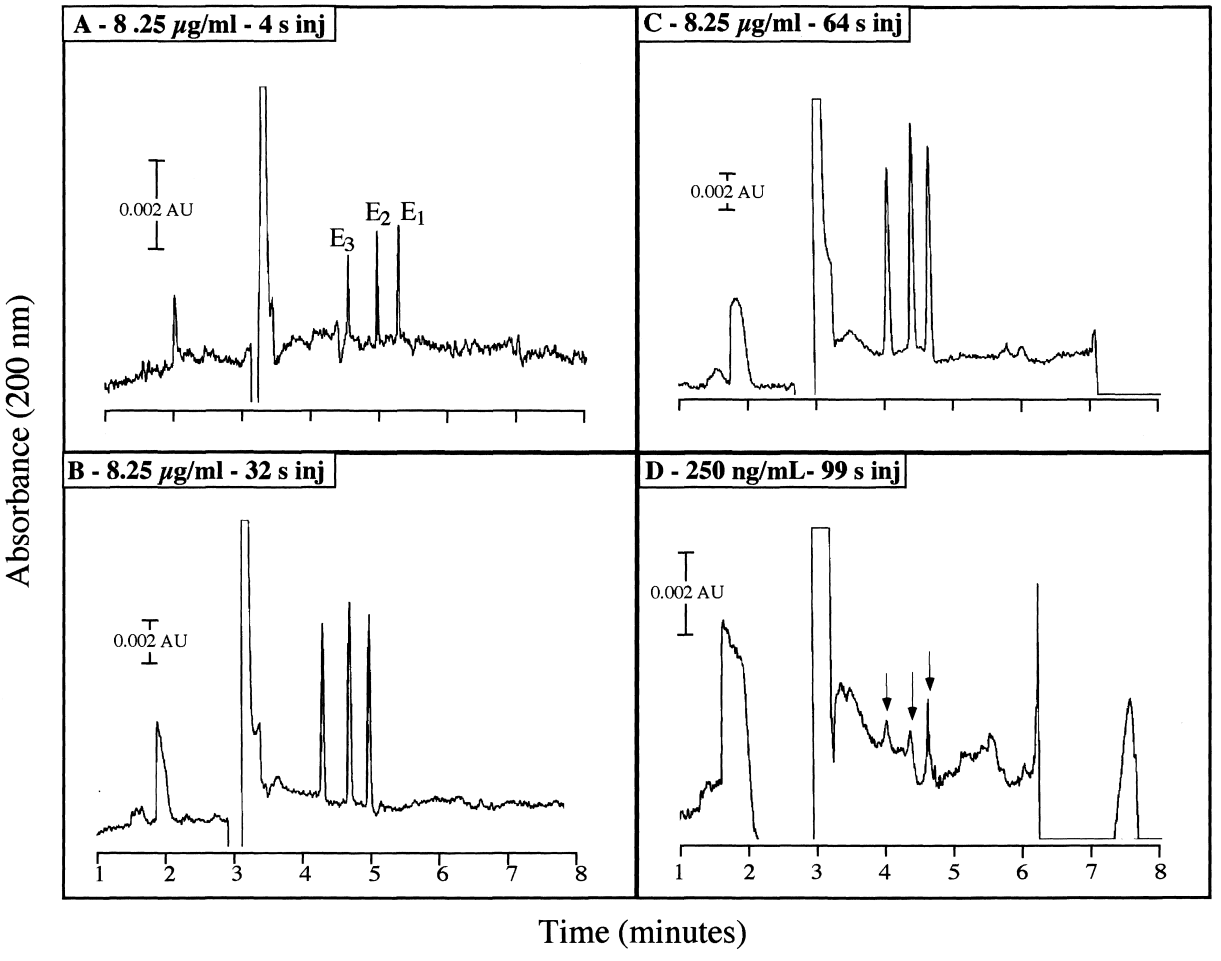

Fig. 3. Improved injection capability with estrogen standards solubilized in sulfated β-cyclodextrin. Separation of 8.25 μg/ml estrogens solubilized in 3% sβ-CD. Hydrodynamic injection of (A) 4 s, (B) 32 s and (C) 64 s. (D) 99-s injection of 250 ng/ml estrogens in 3% sβ-CD. Separation buffer: 5 m*M* borate–5 m*M* phosphate–80 m*M* sodium cholate–20% acetonitrile, pH 8.9.

(0.03301±0.00874 vs. 0.74303±0.03873 for duplicate injections).

With a 64-s injection, the LOD for estrogens dissolved in 3% sβ-CD was determined by electrophoresing samples from serial two-fold dilutions of an 8.25 μg/ml stock solution. Detection is easily accomplished with the estrogen standards at a concentration of 531.5 ng/ml. Discernable and quantifiable peaks could be observed as low as 250 ng/ml when the injection time was extended to 99 s (Fig. 3D). Under these conditions, an average *S/N* ratio of 11/1 was obtained indicating a *S/N* ratio of 5/1 could be obtained with concentrations as low as 118 ng/ml. A 99-s injection extends the detection limit roughly 70-fold over that attainable with methanol as a solvent. Injections of up to 297 s could be achieved still maintaining baseline resolution. Fig. 4 illustrates 99-, 198- and 297-s injections. These injections correspond to filling 12, 24 and 36% of the capillary volume with sample plugs of 111, 222 and 333 nl, respectively. As noted before with high-salt sample matrices, the less hydrophobic analytes experience the greatest reduction in plate number at long injections [10].

It was reasoned that some loss of resolution in long injections was due to the decreased effective

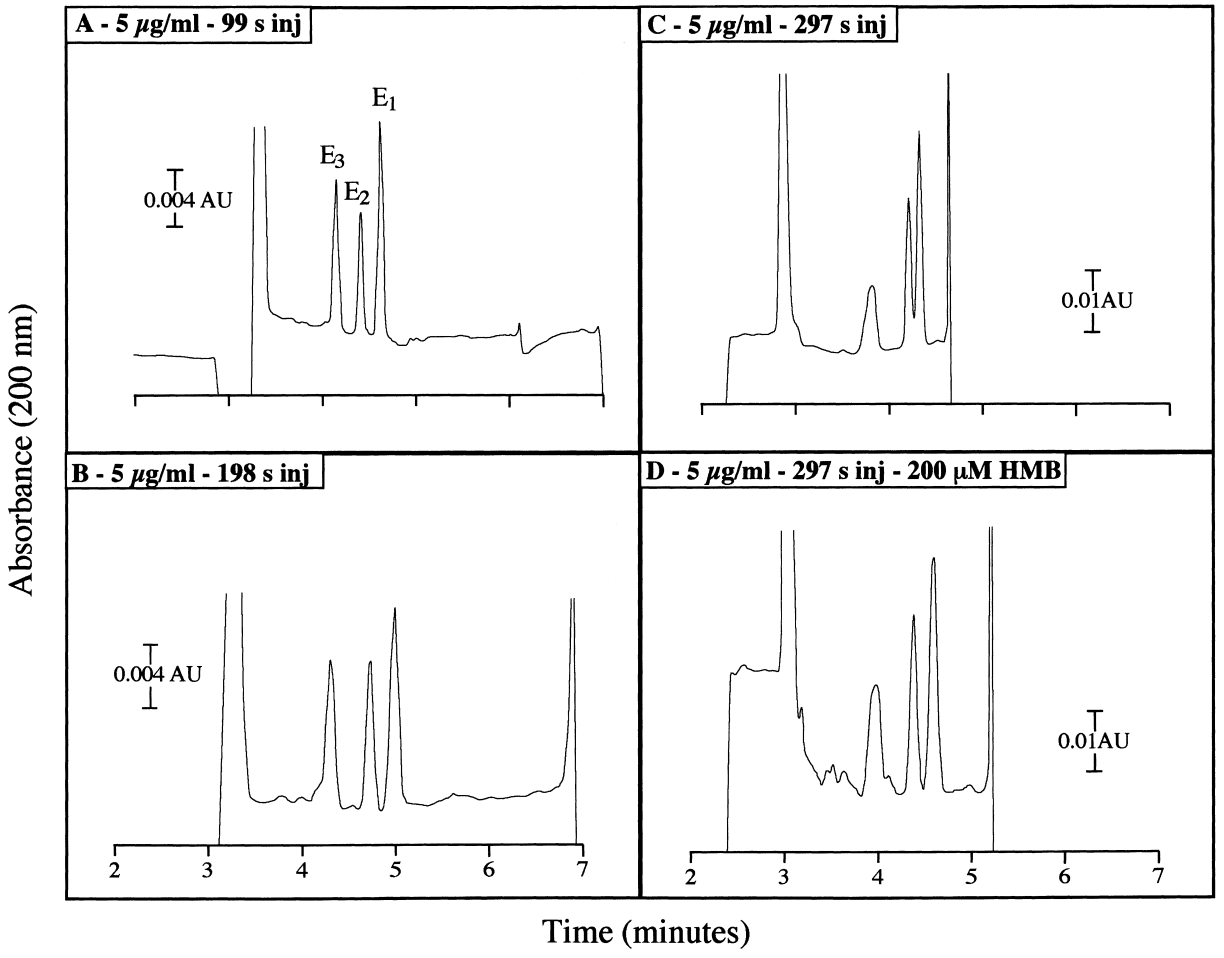

Fig. 4. Improved injection capability with estrogen standards solubilized in sulfated β-cyclodextrin. Standard run of estrogens at 5 μg/ml in a 3% sβ-CD sample matrix. Hydrodynamic injection of (A) 99 s, (B) 198 s and (C) 297 s. (D) Standard separation buffer with 200 μ*M* HMB added. 297-s injection of 5 μg/ml estrogens. Standard separation buffer: 5 m*M* borate–5 m*M* phosphate–80 m*M* sodium cholate–20% acetonitrile, pH 8.9.

capillary length for separation with long sample injections. Consequently, the EOF was reduced in order to increase separation time in the capillary with a 297 s injection (36% capillary volume). Hexamethonium bromide (HMB), a *bis*-quaternary ammonium alkane, was added to the buffer at concentrations of 100, 200 and 250 μ*M* to reduce the EOF [14]. The reduction in EOF alters the migration time of the estrogens by 10 to 15 s, but the additional separation time allows baseline resolution of the three components (Fig. 4D).

3.2. Mechanism for sulfated β-cyclodextrin-mediated stacking

The mechanism in operation with the sβ-CD-mediated stacking is not based on standard field amplification, as the sample matrix has a higher conductivity than the separation buffer. In this sense, it is similar to methods which rely on high-conductivity sample matrices to concentrate separation buffer micelles before they enter the sample zone [10]. However, the presence of pseudostationary phase in the sample matrix should have deleterious effects on stacking with mechanisms that operate without field-amplification in the sample zone. Thus, the inclusion of either organic modifiers [10] or a pseudostationary phase [11] in the sample matrix should have a destructive effect on stacking of hydrophobic analytes in these MCE modes.

We hypothesize that stacking occurs with the sβ-CD in the sample matrix via two concurrent mechanisms (Fig. 5). Complexation between the sβ-CD and the estrogens imparts a component of mobility for the estrogens towards the injector end of the capillary. The sβ-CDs, some with bound estrogens, contact the micelles in the trailing separation buffer (Fig. 5B) where cholate effectively displaces steroids

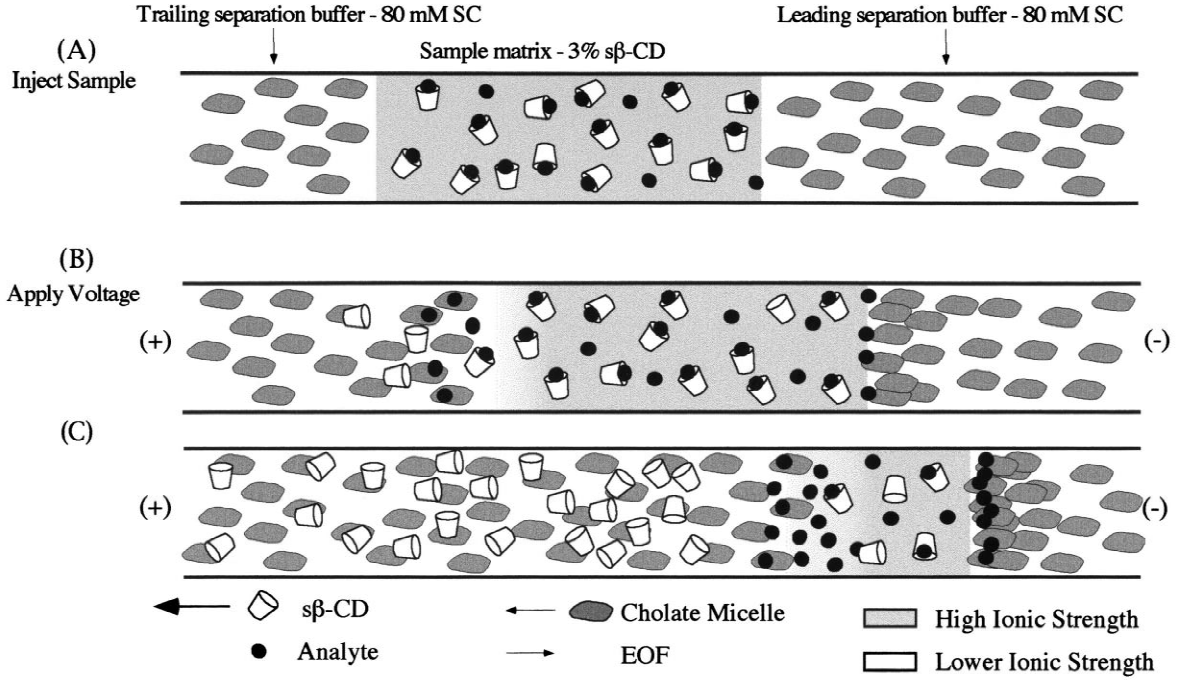

Fig. 5. Proposed mechanism for stacking of hydrophobic compounds solubilized in sulfated β-cyclodextrin. Panel A illustrates the composition of the capillary inlet after injection and before voltage is applied, where the estrogens are encapsulated by the sβ-CD. (Panel B) When voltage is applied, the cholate and sβ-CD/estrogen complexes migrate towards the inlet. As sβ-CD/estrogen complexes reach the injection end separation buffer interface, the estrogens transfer to the cholate micelles due to their preferential complexation with the cholate. Simultaneously, the high conductivity sample matrix induces a stacking effect on the micelles in the leading run buffer. (Panel C) As analyte molecules partition in and out of the micelles in the trailing buffer they are displaced towards the sample matrix. As the EOF moves the entire system toward the detector, uncomplexed analytes become complexed with the micelles in the leading micelle front effectively stacking the analytes while permitting a solubilizing reagent in the sample matrix.

from the CDs [15]. In this case, displacement of the estrogens by the trailing cholate would leave the sβ-CD carriers free from analytes at this interface. Partitioning in and out of the micelle phase will impart the estrogens with a larger cathodic mobility than the cholate. This is due to the net mobility of chocolate being a sum of its EOF and anodic electrophoretic mobility, while the estrogen's net mobility is a sum of EOF and any electrophoretic mobility imparted by discontinuous complexation with cholate. The net effect will displace, in the direction of the sample matrix, the estrogens from the trailing micelle zone (Fig. 5C).

The second mechanism occurs simultaneously, where the sβ-CD is acting as a high-conductivity, high-mobility sample matrix component. Under these conditions, the lower-mobility cholate will stack against the detector end of the sample matrix (Fig. 5B). EOF will carry analytes through this stacked micelle front, which in turn efficiently concentrates the analytes into a narrow zone [10]. The key point here is that the high-anodic mobility sβ-CD does not interfere or antagonize analyte/micelle front interaction. This is because the sβ-CD component of the sample matrix migrates toward the injector end of the capillary, not the detector end, where the micelle front has formed. It has already been demonstrated that inclusion of organic modifier in the sample matrix interferes with analyte/micelle front interaction, because the net mobility of a neutral organic modifier in the sample matrix is toward the micelle front, with EOF [10]. Hence, a pseudostationary phase with the same charge, but higher mobility, than the separation buffer, can be included in this type of stacking mechanism without deleterious effects. This should be a great advantage for analyzing compounds which are difficult to solubilize in aqueous media. Data relevant to the postulated ''analyte transfer mechanism'' are discussed below.

3.3. Mechanism verification

3.3.1. Requirement of negatively charged β-cyclodextrin

If the mechanism postulated above is correct, the observed stacking effect should be considerably less effective if the sample matrix cyclodextrin component either did not enhance the ionic strength, or had a mobility that was not anodic. This was tested with two different types of cyclodextrins. Uncharged β-CD, with no intrinsic electrophoretic mobility and no ionic component, tertiary amine β-CD, which has cathodic mobility, carboxymethylamine β-CD, which is zwitterionic, and carboxymethyl β-CD, which is another form with anodic mobility, were utilized. The estrogen standards were dissolved in the following sample matrices for comparison: (1) separation buffer (5 m*M* phosphate–5 m*M* borate–80 m*M* sodium cholate–20% acetonitrile, pH 8.9), (2) 3% amphoteric β-CD (tertiary amine, DS 2; carboxy methyl, DS 2), (3) 3% tertiary amine β-CD (DS 3.5), (4) 3% β-CD and (5) 3% carboxymethyl β-CD (DS 14), and electrophoresed under standard conditions with the cholate separation buffer. As sample matrices, the 3% amphoteric β-CD and 3% tertiary amino β-CD were ineffective, appearing to have adsorbed to the capillary surface. The estrogens were not observed and subsequent runs using the standard run procedure with sβ-CD as the sample matrix showed decreased resolution and increased migration times for the analytes (data not shown). Use of the separation buffer alone as a sample matrix was also ineffective for detection of the estrogens (Fig. 6A), as indicated by the broad peaks with poor S/N ratios. Detectable peaks were observed with the β-CD, indicating that zone sharpening was occurring (Fig. 6B). Quirino and Terabe described this method of stacking where the micelles from the separation buffer are accelerated in the sample buffer due to field amplification [4]. As the micelles move across the sample matrix, analytes are collected and stacked at the injection end of the sample plug. However, the sharpening effect is greatly diminished without the high-conductivity matrix needed to cause micelle stacking outside the sample zone. Fig. 6C shows the separation obtained with 3% carboxymethyl β-CD as the sample matrix. Stacking has occurred, although not as effectively as with sβ-CD (see Fig. 4C). According to the proposed mechanism, the highly charged carboxymethyl β-CD (DS 14) should stack the analytes as efficiently as the sβ-CD. The decreased effectiveness of stacking with this CD is unclear but may be a result of the higher charge precluding density complexation with the negatively-charged cholate, or a larger variation in the degree of carboxylation.

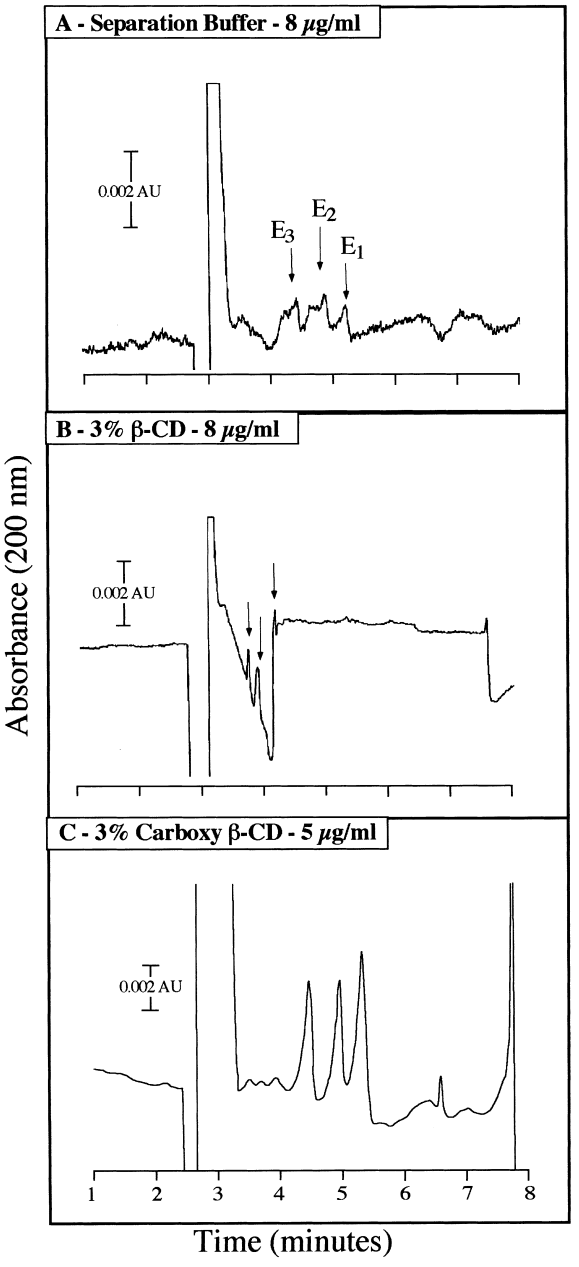

Fig. 6. Separation of estrogens in various sample matrices. 64-s injection of 8 μg/ml estrogens using a sample matrix of (A) standard separation buffer and (B) 3% β-CD. (C) 99-s injection of 5 μg/ml estrogens in carboxymethyl β-CD. Standard separation buffer: 5 m*M* borate–5 m*M* phosphate–80 m*M* sodium cholate–20% acetonitrile, pH 8.9.

3.3.2. Cholate concentration importance in flanking the sample plug

According to the proposed mechanism described above, if dissociation of the bound estrogen from the sβ-CD at the sβ-CD/sodium cholate (SC) interface was occurring, one would expect the stacking effect to be dependent on the SC concentration in the ''chaser plug'' (a plug injected behind the sample matrix). Lowering the concentration of SC in the chaser plug would reduce the relative affinity of the analyte for the micelle buffer, and should degrade the stacking effect. This was tested experimentally by following a 99-s hydrostatic pressure injection of the estrogen standards (5 μg/ml) in the sβ-CD sample matrix, with a 64-s injection of a buffer with varied SC concentration. The chaser plug consisted of standard separation buffer (5 m*M* phosphate–5 m*M* borate–20% acetonitrile) containing 80, 60, 40, 20 and 0 m*M* cholate. Each of the solutions containing less that 80 m*M* SC were supplemented with NaCl to maintain ionic strength, e.g., 40 m*M* NaCl was added to 40 m*M* cholate. Fig. 7 shows the degradation of peak shape with decreasing cholate concentration in the chaser plug, supporting the analyte transfer hypothesis. According to this postulate, a lower concentration of micelles at the inlet end of the sample matrix/separation buffer interface would cause the analytes to be transferred over a longer range than if a high concentration of micelles was immediately at the interface.

3.3.3. CD complexation with estrogens

The effect of CD complexation with the estrogens in the sample matrix during stacking was investigated using highly sulfated α-, β- and γ-CDs (sα-CD, DS 11, sβ-CD, DS 12, sγ-CD, DS 13). Relative affinities of the CDs for the estrogens were assessed by injecting estrogen standards (150 μg/ml in methanol, 2 s, into separation buffers containing the different s-CDs. Time of appearance of the estrogens after the EOF marker indicated the degree of complexation with the s-CDs. The s-CD form that complexes most efficiently with the estrogens will elicit the longest retention times.

To assess the relative affinities of the s-CD forms for the estrogens, a separation buffer containing 5 m*M* borate, 5 m*M* phosphate and 14.6 m*M* of either sα-CD, sβ-CD or sγ-CD, pH 9 was used. Fig. 8

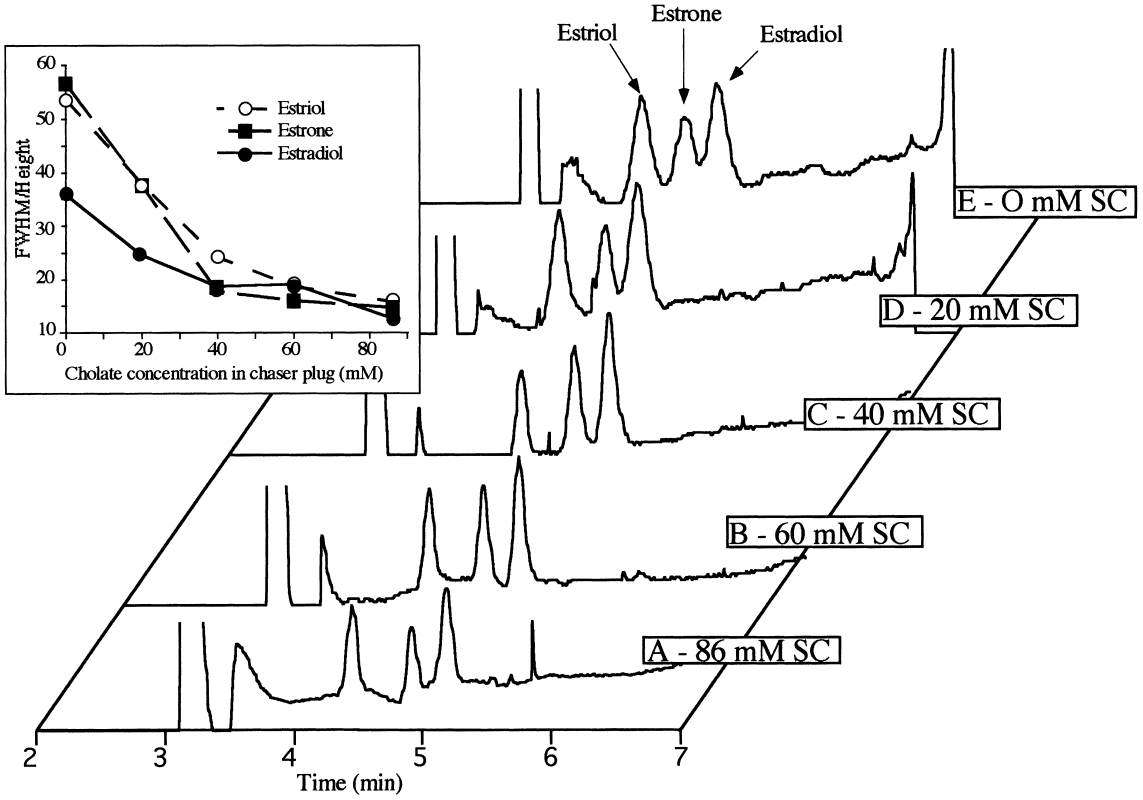

Fig. 7. Effect of cholate concentration in chaser plug on stacking. 99-s hydrostatic injections of 5 μg/ml estrogens, dissolved in 3% sβ-CD, with a post-sample injection of 64 s. Post-sample injections, chaser plugs, consisted of separation buffer varied in cholate concentration and matched to the original separation buffer ionic strength with sodium chloride. Cholate concentration equaled (A) 80 m*M*, (B) 60 m*M*, (C) 40 m*M*, (D) 20 m*M* and (E) 0 m*M*. Inset illustrates the ratio of the full width at half maximum (FWHM) height to the height for the three peaks at varying cholate chaser plug concentrations.

shows that sγ-CD possesses the highest affinity for the estrogens, as illustrated by the absence of the estrogen peaks even after 6 h of separation time (Fig. 8C, 60 min of separation time shown). Based on the migration times of the estrogens, it is seen that sβ-CD (Fig. 8B) complexes less than sγ-CD, while the estrogens are complexed least with sα-CD (Fig. 8A). This observation is consistent with the idea that steroid compounds complex most-strongly with the γ form of CD through increased hydrophobic interaction of the analyte with the larger CD [16]. The relative affinities for the sulfated forms may be diminished by the decreased hydrophobic surface available due to the sulfate groups around the opening and base of the s-CDs.

With a wide spectrum of affinities exhibited by the s-CDs for the estrogens, the effects of s-CDs in sample matrices (not in the separation buffer) were observed to assess the importance of complexation of estrogens during stacking. Fig. 8D–F display the separations employing sample matrices of 14.6 m*M* sα-CD, sβ-CD and sγ-CD in conjunction with the standard cholate separation buffer. As shown, the separations with sα-CD and sβ-CD in the sample matrices provide similar separations. This contrasts the results with the sγ-CD sample matrix where the estrogens have been stacked, but insufficient separation has occurred (Fig. 8F). The estrogen separation is presumably impeded by the strong complexation with the sγ-CD (Fig. 8C), and the es-

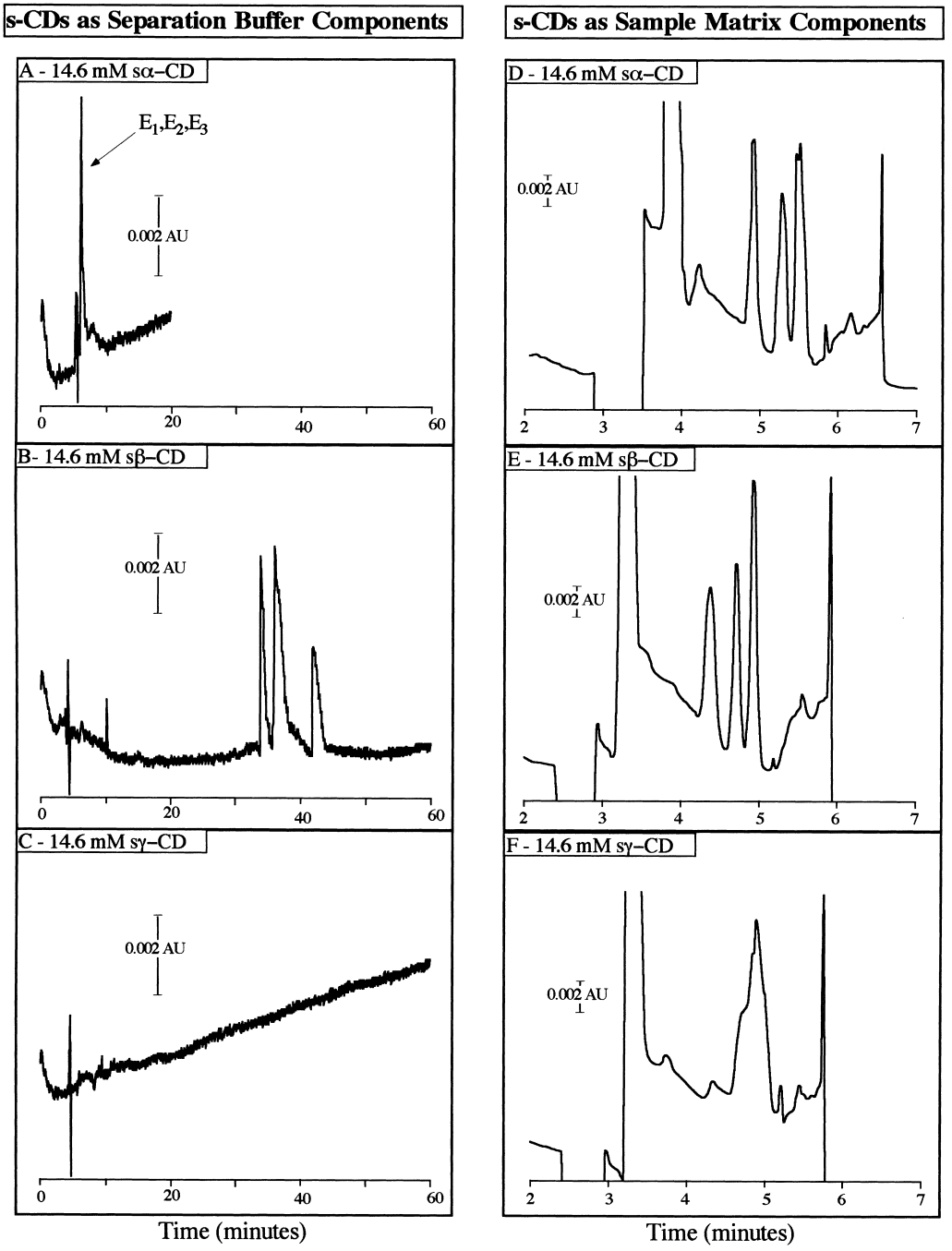

Fig. 8. Complexation of the estrogens with sα-CD, sβ-CD and sγ-CD. 2-s injection of 100 μg/ml estrogens in methanol, separated in a buffer containing 5 m*M* borate, 5 m*M* phosphate and (A) 14.6 m*M* (~3%) sα-CD, (B) 14.6 m*M* sβ-CD and (C) 14.6 m*M* sγ-CD. 198-s injection of 5 μg/ml estrogens in sample matrices of (D) 14.6 m*M* sα-CD, (E) 14.6 m*M* sβ-CD and (F) 14.6 m*M* sγ-CD using a 5 m*M* borate, 5 m*M* phosphate, 20% acetonitrile and 80 m*M* sodium cholate buffer, pH 8.9.

trogens cannot effectively partition from the sample matrix to the separation buffer. As a result, adequate separation does not occur. This important observation stresses the need for a stacking mechanism to be active during the stacking process but inert during the separation. In the case with sγ-CD as a sample matrix component, it is likely that the stacking due to high-conductivity occurs, but the analyte transfer is interrupted by the high affinity of the sγ-CD for the estrogen analytes. In the case of the sβ-CD, it is clear that moderate complexation and, hence, increased solubilization of hydrophobic analytes, in concert with efficient analyte transfer at the injector side of the sample matrix, provides for effective stacking and separation. For the sα-CD, only slight complexation was observed (Fig. 8A), although occurs (Fig. 8D).

Each of the three s-CD sample matrices provides approximately the same high-conductivity, but each exhibits a different affinity for the estrogen analytes. As such, the plate numbers with the s-CD sample matrices (Fig. 8D–E, average of 18 437 for sα-CD, 13 745 for sβ-CD, not obtainable for sγ-CD) indicate that the difference in stacking effectiveness for the three s-CDs is in the efficiency of analyte transfer from the sample matrix to the separation buffer at the conclusion of stacking. However, analyte transfer can be enhanced by altering the composition of the separation buffer, e.g., by adding organic modifiers to the separation buffer, without compromising the stacking mechanism [10].

4. Conclusions

Sulfated β-CD sample matrices not only provide an ideal solubilizing matrix for estrogens but increase sensitivity for CE detection. The sβ-CD allows for extremely long injections (up to 36% of the capillary) while still providing baseline resolution of the estrogen analytes. Data supporting a proposed analyte transfer mechanism has been shown where the sβ-CDs, despite providing anodic mobility to analytes in the sample zone, are able to transfer analytes to trailing separation buffer micelles for ''recycling'' back into the sample zone. Thus, analytes are not lost nor is stacking compromised. The analytes are eventually stacked by EOF pushing the analytes into micelles stacked on the detector-side of the sample matrix due to the high conductivity of the sβ-CD. The data in this report show a high anodic mobility pseudostationary phase can be included in the sample matrix during high-salt stacking with a lower-anodic mobility micellar phase in the separation buffer. The importance of this is that solubilizing agents other than typical organic modifiers, which are deleterious to stacking in this mode, can be included in the high-conductivity matrices.

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