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Separation and analysis of cyclodextrins by capillary electrophoresis with dynamic fluorescence labelling and detection

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Abstract

Mixtures of α-, β- and γ-cyclodextrins were complexed with 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) and separated by capillary electrophoresis. The enhanced fluorescence emission of 2,6-ANS when complexed with the cyclodextrins allowed isolated zones to be visualized. Detection limits for α-, β- and γ-cyclodextrin were determined to be 62, 2.4, and 24 μM, respectively. Enhanced resolution of cyclodextrin mixtures could be obtained by adjusting the concentration of ANS in the running buffer or by suppressing the electroosmotic flow. Components in 2,6-di-O-methyl-β-cyclodextrin with differing degrees of substitution were separated by this technique and compared with electrospray mass spectra of the same mixture.

1. Introduction

Cyclodextrins (CDs) are toroidally shaped polysaccharides formed from 6, 7 and 8 glucopyranose units for the α, β and γ species, respectively [1]. The interior of the torus is an electron rich, hydrophobic environment while the exterior contains many sites capable of hydrophilic interactions. CDs are used to increase the solubility and bioavailability of hydrophobic pharmaceuticals in aqueous solutions [2,3], and as selectivity reagents for the resolution of structural, positional and stereo isomers in the separation sciences [4–6].

CDs are difficult to analyze as they are uncharged and demonstrate no appreciable UV–Vis absorbance. Furthermore, the reactivity of these compounds is such that they are not easily labelled with a visualizing agent. Methods previously used for the analysis of CD mixtures include thin-layer chromatography [7], high-performance liquid chromatography (HPLC) [8–11] and capillary electrophoresis (CE) [12]. The chromatographic methods typically suffer from poor sensitivity, long analysis times and poor resolution. Electrophoretic separation methods would not normally be considered for separating CDs as the molecules are uncharged except at very high pH. However, Nardi et al. [12] were able to separate mixtures of native cyclodextrins by CE when the CDs were complexed with the benzoate ion. The benzoate anion also served as a means of visualizing the complex via indirect UV absorbance detection. Although this pro-

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cedure was able to fully resolve α-, β- and γ-CD, the resolution was not sufficient to separate more complex mixtures, and the quantity of analyte required for detection was relatively high.

To improve the sensitivity and separating power of the cyclodextrin analysis, improved methods for mobilizing and visualizing these molecules must be employed. One promising method for detecting CDs is to employ a fluorescent probe. CDs are known to dramatically alter the fluorescence quantum efficiency of many fluorophores which form an inclusion complex [13]. This phenomenon has been recently exploited for detection of CDs by HPLC [14]. Steady state fluorescence and anisotropy measurements have been used to determine the binding constant (K) for β-CD and several isomers of anilinonaphthalenesulfonates (ANS) under varying pH and temperature conditions [15]. Of the fluorophores studied, 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) consistently provided the highest binding constant, 1860 M^{-1} at pH 11.0, and so was judged best for the studies described here. In this work, we exploit the properties of host–guest complexes of CDs and 2,6-ANS to develop a more sensitive means of separating and observing mixtures of CDs by CE.

2. Experimental

2.1. Instrumentation

Fluorescence excitation and emission spectra were acquired using a multifrequency cross-correlation phase and modulation spectrofluorometer (Model K2, ISS, Champaign, IL, USA) with a 300-W Xe arc excitation source. The monochromators on this instrument were adjusted so that both excitation and emission signals had a spectral bandpass of 4 nm. All spectra were collected in the DC excitation and emission mode.

The CE apparatus was constructed from components. A regulated high-voltage DC power supply (Model EH50R02, Glassman High Voltage, White House Station, NJ, USA) operated at 30 kV was used to drive the electrophoretic separation. A platinum wire electrode approximately 6 cm in length was used to establish electrical contact between the high-voltage supply and a 1.5-ml inlet buffer reservoir. The inlet reservoir and high-voltage end of the capillary were enclosed in a Plexiglass box to minimize arcing and to protect the operator from accidental shock. All separations were carried out in a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D., 360 μm O.D., and approximately 1 m in length. The outlet buffer reservoir was grounded through a 1-MΩ resistor to allow the current passing through the capillary to be monitored with a voltmeter.

Fluorescence excitation was performed with an argon ion laser (Innova 90-5, Coherent, Palo Alto, CA, USA) operating at 363.8 nm. To minimize fluctuations in the signal, the intensity of the laser beam was actively regulated with a laser stabilization accessory (Model 50SA, LiCONiX, Santa Clara, CA, USA). An excitation beam of approximately 14 mW was focused with a fused-silica lens (focal length 100 mm, Newport Research Corporation, Irvine, CA, USA) into a region of the separation capillary with the polyimide coating removed. The resulting fluorescence emission signal was collected and imaged on the entrance slit of a double monochromator (Model DH-10, Instruments SA, Edison, NJ, USA) with a microscope objective (15×, Oriel, Stratford, CT, USA). The spectrometer was adjusted to provide a spectral bandpass of 8 nm centered at 424 nm. Output from the photomultiplier tube (Model R1527-03, Hamamatsu, Bridgewater, NJ, USA) was amplified with a current-to-voltage amplifier (Model 428, Keithley Instruments, Cleveland, OH, USA). For these studies, the photomultiplier tube was biased at −700 V and the amplifier gain maintained at $1 \cdot 10^5$ V A^{-1} with a time constant of 300 ms. An Objective C program running on a NeXTCube computer (NeXT Computer, Redwood City, CA, USA) monitored the output from the current amplifier with a 16-bit analog-to-digital converter (Model XL-1900 mainframe with XL-ADC2 ADC, Elexor Associates, Morris Plains, NJ, USA) which digitized the signal at 7

Hz. The column efficiency, theoretical plates, retention time, peak areas, etc. were determined by a program which calculated statistical moments for these data [16].

Electrospray spectra were acquired with a quadrupole mass spectrometer configured with an electrospray interface (Model 201 mass filter with options ES and E2000, Vestec, Houston, TX, USA). The quadrupole mass analyzer was controlled and data recorded by a dedicated processor (Model 900 DSP, Teknivent, Maryland Heights, MO, USA) monitored by a 80486-based computer running commercial software (Vector/Two version 1.4, Teknivent).

Samples solutions were introduced into the electrospray interface by means of a syringe pump (Model 341B, Sage Instruments, Boston, MA, USA) at a flow-rate of 1.4 μl min^{-1}. The electrospray needle was held between 2 and 3 kV for all analyses. The electrospray voltage, distance between the needle and the first skimmer cone, and sample flow-rate were adjusted to achieve a stable spray current of approximately 0.2 μA. Instrument operating temperatures were maintained at the following values for all analyses; spray chamber 50°C, ion lenses 150°C, block 250°C. The repeller voltage was 20 V and the pressure in the analyzer $5 \cdot 10^{-6}$ Torr (1 Torr = $1.3 \cdot 10^{2}$ Pa). Calibration of the mass analyzer was performed while sequentially aspirating solutions of poly(ethylene glycol) with average molecular masses of 400, 900, and 1500 u.

2.2. Reagents and materials

Chemicals and reagents were purchased from the following sources and used without further purification: α-CD, Sigma (St. Louis, MO, USA); 2,6-ANS, Molecular Probes (Eugene, OR, USA); anhydrous disodium hydrogen phosphate, methanol and sodium hydroxide, Fisher Scientific (Pittsburgh, PA, USA); tetrabutylammonium bromide (TBAB), β-CD, γ-CD and heptakis (2,6-di-O-methyl)-β-CD (DM-β-CD), Aldrich (Milwaukee, WI, USA). The DM-β-CD was specified to contain 30% of the 2-6-di-O-methyl substituent by plasma desorption mass spectrometry with the remainder primarily higher and lower O-methyl homologues.

2.3. Procedures

At the start of each day, the capillary was sequentially rinsed with 1 *M* sodium hydroxide, de-ionized water and the running buffer. Buffer solutions were prepared from a 100 m*M* solution of sodium phosphate adjusted to pH 12 with 1 *M* sodium hydroxide. This solution was diluted with deionized water to produce a 30 m*M* buffer. Benzoic acid buffers were prepared from sodium benzoate adjusted with 5 *M* hydrochloric acid to pH 4.0. Just prior to analysis, 2,6-ANS was added to these buffers to produce the stated concentration of fluorophore.

In the initial investigation of 2,6-ANS as a mobile phase additive for CE, it was observed that the inlet buffer reservoir became visibly discolored after a limited exposure to electrophoretic conditions (typically 5 analyses). The fluorescence background signal monitored at the detector increased proportionally with this discoloration and was dramatically reduced when the buffer in the inlet reservoir was replaced with a fresh solution of 2,6-ANS. This reaction only occurred when voltage was applied to the inlet buffer reservoir, so it was inferred that the 2,6-ANS molecule was being electrochemically altered. A reaction consistent with these observations and known chemical properties of the molecule would be the oxidation of 2,6-ANS to form an imine. To minimize the generation of interfering species, the electrode area available for reaction was reduced by covering all but the end of the electrode with a PTFE sleeve. The production of the interfering species was greatly inhibited and more than 20 separations could be performed without significant hindrance from the electrochemical reaction product.

3. Results and discussion

3.1. Anilinonaphthalenesulfonate fluorescence measurements

Fig. 1 shows the steady-state excitation and emission spectra for 2,6-ANS ($6.7 \cdot 10^{-6}$ *M*) in distilled water, and in solutions saturated with α-CD (0.15 *M*), β-CD (0.016 *M*), and γ-CD

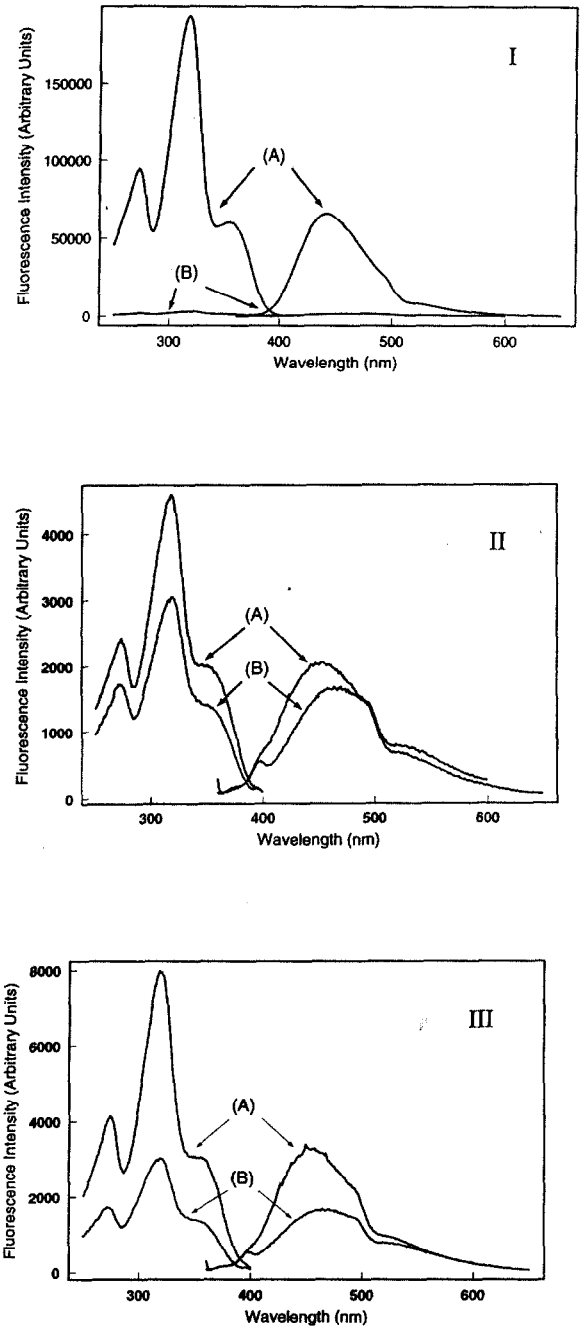

Fig. 1. Fluorescence excitation and emission spectra for $6.7 \cdot 10^{-6}$ *M* 2,6-ANS. A is the spectrum in a solution saturated with CD, B is the spectrum in distilled water. Figs. I, II and III correspond to β-CD (0.016 *M*), α-CD (0.15 *M*), and γ-CD (0.18 *M*) respectively. All excitation spectra were monitored at 460 nm and emission spectra were excited at 351 nm.

(0.18 *M*). From these spectra, it is clear that the uncomplexed 2,6-ANS demonstrates a fluorescence signal which is enhanced in the presence of the CDs. For all CDs studied, when an inclusion complex is formed, the fluorescence excitation spectrum remains virtually unchanged but a significant shift to shorter wavelengths is observed in the emission signal. In addition to the shift in the wavelength of maximum fluorescence emission, the CD–2,6-ANS host–guest complex produces a large change in fluorescence emission intensity. This enhancement is as much as a factor of 55 at 422 nm for β-CD, but α- and γ-CD also significantly enhance the fluorescence signal of 2,6-ANS when complexed. Of the CDs investigated, (α-, β-, γ-, hydroxypropyl-β-, methyl-β-, and DM-β-CD), the derivatized β-CDs were found to provide the largest enhancement of fluorescence emission. The fluorescence emission signal for 2,6-ANS complexed with DM-β-CD increased by a factor of 245 at 426 nm. The maximum excitation and emission wavelengths as well as fluorescence enhancement factors are summarized in Table 1. For the work described here, all fluorescence emission measurements were made at 424 nm which was found to give close to maximum enhancement for all the CDs studied.

3.2. Separation of native cyclodextrins

Not only does the 2,6-ANS molecule allow the CDs to be visualized, it also imparts a charge to this otherwise neutral molecule to allow separation in an electric field in much the same way that benzoate was previously used [12]. Fig. 2 shows the molecular species and relative mobilities of these species in the capillary. The uncomplexed CD has no net charge under all but strongly basic conditions [17] and so migrates at the velocity of the electroosmotic flow. The 2,6-ANS molecule and the complex are negatively charged. Since the size of the complex is greater than that of 2,6-ANS, the magnitude of the electrophoretic mobility $|\mu|$ will be lower, as indicated by the relative size of the vectors in Fig. 2. The electroosmotic flow, with mobility

Table 1
Fluorescence signal properties for cyclodextrin–2,6-ANS complexes

	λ^{ex}_{max} (nm)	λ^{em}_{max} (nm)	Fluorescence emission enhancement
Uncomplexed 2,6-ANS	318	464	–
α-CD–2,6-ANS	318	452	1.8 (416 nm)
β-CD–2,6-ANS	317	441	55 (422 nm)
γ-CD–2,6-ANS	318	454	2.3 (426 nm)
Heptakis (2,6-di-O-methyl)-β-CD–2,6-ANS	317	437	245 (426 nm)

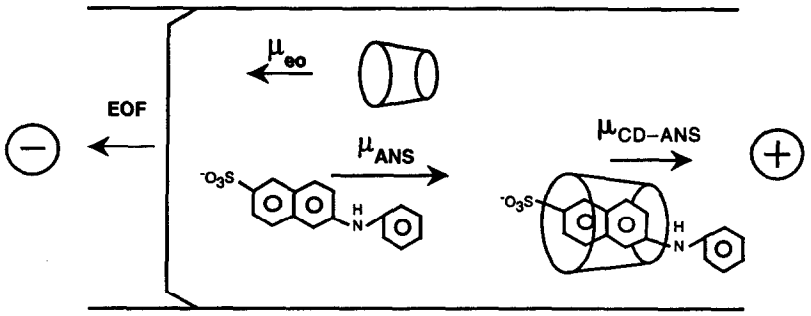

Fig. 2. Schematic diagram showing mobilities of 2,6-ANS, CD and CD–2,6-ANS complex in a bare fused-silica capillary.

μ_{eo}, opposes the movement of 2,6-ANS and the CD–ANS inclusion complex.

Fig. 3 shows a representative separation of a mixture of α-, β-, and γ-CD at pH 12. The peak corresponding to neutral species appears at 6.6 min while those of the CDs appeared shortly thereafter. To account for these data we consider the equilibrium,

$$CD + ANS \leftrightarrows CD\text{–}ANS \quad (1)$$

The equilibrium constant for this reaction, K, is given by:

$$K = \frac{[CD\text{–}ANS]}{[CD][ANS]} \quad (2)$$

The similarity in size between the CD cavity and the fluorophore causes 2,6-ANS to form inclusion complexes with β-CD preferentially, but α- and γ-CD also form complexes to a lesser extent. In previous work, the equilibrium constants for

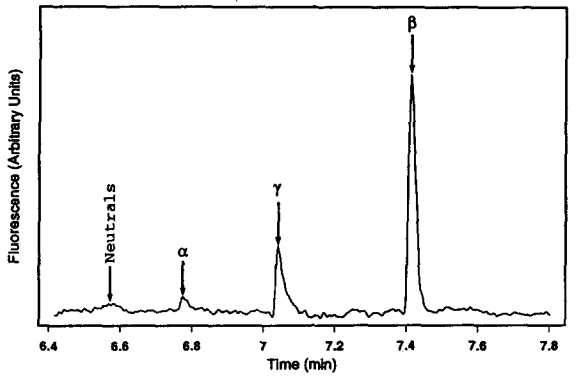

Fig. 3. CE separation of α-, β-, and γ-CD. Analysis buffer: 40 m*M* phosphate, pH 11.76, 1 m*M* 2,6-ANS. The analysis was carried out in a capillary of dimensions 50 μm I.D., 360 μm O.D., and 1 m in length in a field of 300 V cm^{-1}. The sample was introduced into the capillary by electrokinetic injection; 5 kV for 2 s from a sample containing 1.44 mg ml^{-1} α-CD, 0.017 mg ml^{-1} β-CD and 0.24 mg ml^{-1} γ-CD. Detection was by fluorescence excited at 363 nm and monitored at 424 nm.

most β-CD–ANS complexes have been found to show no significant sensitivity to solution pH [15]. From Fig. 3 it can be seen that $|\mu|$ for α-CD–2,6-ANS < γ-CD–2,6-ANS < β-CD–2,6-ANS. These results are consistent with those seen in Fig. 1, where the magnitude of the fluorescence enhancement per unit concentration of CD is observed to be α-CD–2,6-ANS < γ-CD–2,6-ANS < β-CD–2,6-ANS. These observed differences in mobility and fluorescence enhancement must in part be due to differences in the binding constants for CD–2,6-ANS. The mixture of CDs may therefore be separated by differences in the fraction of molecules which are complexed with 2,6-ANS. For β-CD–2,6-ANS, $K = 2 \cdot 10^3$ [15] and therefore in 1 m*M* 2,6-ANS Eq. 2 shows that a fraction 0.67 of the β-CD is complexed if buffer constituents do not interfere with the binding. Although binding constants for α- and γ-CD have not been measured, they are evidently less than that for β-CD and so the fractions complexed to 2,6-ANS will be less than for β-CD. Increasing the 2,6-ANS concentration in the running buffer improves the separation of the native CDs, but also increases the background fluorescence signal, making detection more difficult. In general, the optimum separation/detection conditions were when the minimum amount of 2,6-ANS necessary to achieve the desired separation was added to the analysis buffer.

To demonstrate the quantitative capabilities of this technique, solutions containing pure α-, β- and γ-CD were separated in a buffer containing 1.0 m*M* 2,6-ANS. Both peak height and peak area measurements provided a linear signal response over an extended range of concentration. A linear signal response was observed for β-CD over a concentration range from $5.2 \cdot 10^{-6}$ to $1.6 \cdot 10^{-3}$ *M*. The signals from α- and γ-CD increased linearly with concentration from $2.1 \cdot 10^{-4}$ to $6.3 \cdot 10^{-2}$ *M* and $1.8 \cdot 10^{-5}$ to $6.1 \cdot 10^{-3}$ *M*, respectively. From these data, the detection limits (signal-to-noise of 3) are estimated to be 62, 2.4, and 24 m*M* for α-, β- and γ-CD, respectively. The limiting noise source in these measurements was fluctuations in the fluorescence background signal from the uncomplexed 2,6-ANS. The upper limits of detection were established either by the solubility of the CD or the non-linear increase in the concentration of the CD–2,6-ANS complex once a significant fraction of the CD was complexed.

3.3. Separation of derivatized cyclodextrins

To demonstrate the potential resolving power of this technique for the analysis of complex mixtures of CDs, a separation of DM-β-CD was performed. Derivatized CDs are usually a mixture of CDs with various degrees of substitution. These derivatized molecules are being investigated by the pharmaceutical industry as possible delivery complexes for very hydrophobic drugs [2] and so monitoring their chemical composition is a critical part of any toxicological study.

Initial attempts to separate DM-β-CD under the conditions used to separate α-, β- and γ-CD provided incomplete resolution of the molecules with different degrees of substitution. From these data and previous reports [12], it was clear that the dominant mobilization mechanism for the inclusion complexes was the electroosmotic flow in the capillary. This flow would not separate the different DM-β-CD species and overwhelmed the electrophoretic processes so that little separation was obtained. To increase the ratio of electrophoretic to electroosmotic mobility, two changes were made to the separation procedure. First, the separation was performed in a benzoate buffer (30 m*M*) which contained a small amount (1 m*M*) of 2,6-ANS. A previous investigation of the complexation of benzoic acid with the CDs suggests that electrophoretic mobility can be enhanced by increasing the concentration of complexing reagent [12]. The presence of this additional charged inclusion species increases the proportion of time the mobility of the CD is influenced by the electric field, without increasing the fluorescence background signal. Addition of benzoate to the buffer did enhance the separation but also interfered with the inclusion of 2,6-ANS, causing detection limits to suffer. This effect can be modeled as a competition between the benzoate anion and the 2,6-ANS for the CD cavity. Under

conditions where [benzoate] >> [2,6-ANS], as used here, binding of the 2,6-ANS will be given by,

$$K_{obs} = \frac{K_{ANS}}{1 + K_B[B]} \quad (3)$$

where K_{obs} is the observed binding constant, K_{ANS} the binding constant for 2,6-ANS, and K_B and [B] the binding constant and concentration of the competitor (the benzoate anion). Drawing from the analogy of chiral separation by CE, the concentration of the competitor can be optimized to provide maximum mobility difference while still providing adequate sensitivity [18]. Modeling of these interactions has found that the maximum mobility difference is found when the product of the binding constant and the concentration (K_{obs}[CD]) is unity. From previous work [12] it can be estimated that the binding constant K for the β-CD–benzoate complex is 50 M^{-1}. As previously mentioned, K_{ANS} for 2,6-ANS–β-CD is $2 \cdot 10^3$ M^{-1}, and the derivatised β-CDs have been inferred from fluorescence studies to have even stronger binding. By applying Eq. 3, K_{obs} in 30 mM benzoate is reduced by a factor of 2.5 in comparison with K_{ANS}, bringing K_{obs}[CD] closer to unity. This explains the action of the benzoate ion on improving the separation, as well as increasing the time the CD–2,6-ANS is influenced by the electric field.

The second method for improving the separation is to alter the analysis buffer to suppress the electroosmotic flow. Reduced electroosmosis increases the time required for the analysis but enhances resolution [19].

One means of suppressing electroosmotic flow in a fused-silica capillary is by the addition of organic solvents to the analysis buffer [20]. Similarly, the addition of small quantities of ammonium salts to the buffer can suppress or even reverse electroosmotic flow [21]. Both of these techniques were used to enhance the separation of DM-β-CD. Representative separations of DM-β-CD are shown in Fig. 4. In Fig. 4A, the separation is performed under conditions similar to those used for the separation in Fig. 3, where the elution time for the molecules

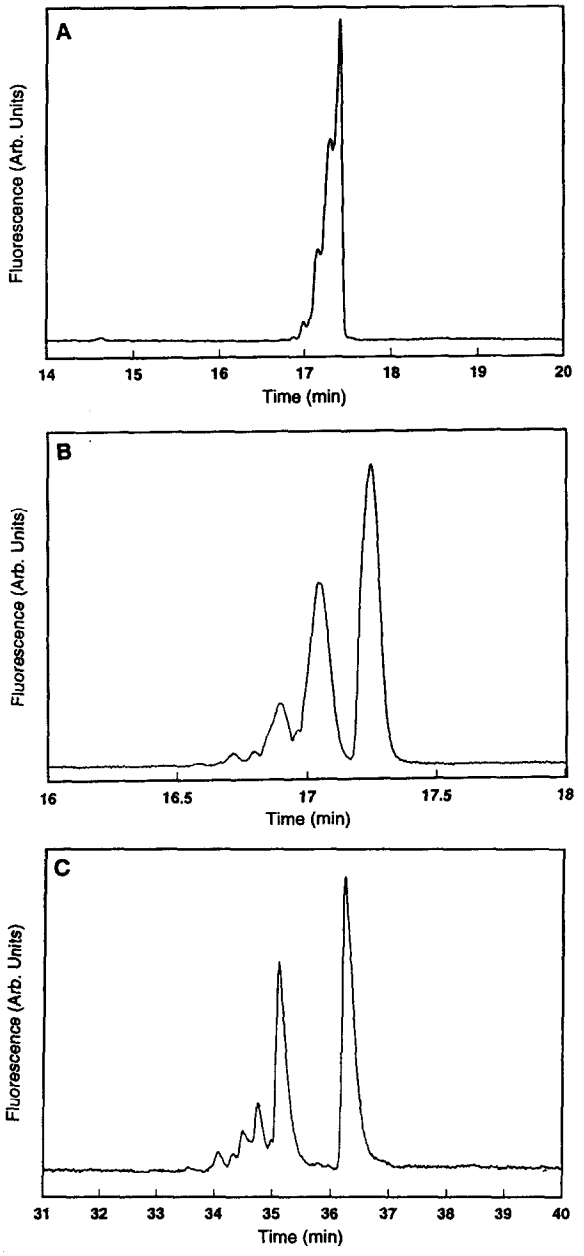

Fig. 4. CE separation of DM-β-CD. Analysis buffers: (A) 1 mM 2,6-ANS in 40 mM phosphate buffer (pH 11.76), (B) 1 mM 2,6-ANS in 30 mM benzoate (pH 8.0) with 20% methanol, (C) 40 μM TBAB and 1 mM 2,6-ANS in a pH 4.0 benzoate buffer (30 mM). The analysis was carried out in a capillary of dimensions 50 μm I.D., 360 O.D., and 1 m in length in a field of 300 V cm^{-1}. The sample was introduced into the capillary by electrokinetic injection; 5 kV for 2 s. Detection was by fluorescence excitation at 363 nm and monitored at 424 nm.

is 6.6 min. Although the electropherogram clearly indicates that the sample is heterogeneous, individual components are not resolved. In Fig. 4B, methanol is added to the running buffer to suppress electroosmosis and sodium benzoate added to enhance electrophoretic migration. A corresponding increase in resolution is observed. For this electropherogram, the time corresponding to the elution of neutral molecules is 14.8 min. Fig. 4C shows that addition of TBAB to the buffer further suppresses electroosmosis (elution time for neutral molecules is 26.9 min) and an additional increase in resolution is observed.

The identification of the species separated was attempted by comparing the separation with the electrospray ionization mass spectrum of DM-β-CD shown in Fig. 5. Three major components can be seen in both figures, with the intensity of the signals in the mass spectrum reversed from the trend in the electropherogram. In the mass spectrum, the charge is imparted to the homologues principally via a sodium adduct ion. The slight shoulder on the high mass side of the peaks is consistent with the formation of a potassium adduct. The three major peaks in the spectrum are assigned to be the β-CD (i) fully methylated on all the 2 and 6 positions of all 7 glucose subunits, (ii) the addition of an extra methyl group on species (i), (iii) the addition of 2 extra methyl groups on species (i). It is interesting to note that for species (i) and (ii) there are no geometric isomers, but for species (iii) there are 3 geometric isomers that have the same statistical weight. The mass spectrometer is *not* able to distinguish the geometric isomers of species (iii). The binding of the species (i), (ii) and (iii) with 2,6-ANS will differ, due to the possibility of steric interference of the extra methyl groups. A lower binding constant of a species in the electropherogram would lead to a shorter migration time. Therefore it is possible that the major peak at 36.4 min in Fig 4C is due to species (i), and the peak at 35.2 min to species (ii). The preceding peaks may all be due to species (iii), being a partial separation of the geometric isomers, to which the binding constant and hence the electrophoresis will be sensitive. This explanation is consistent with 30% of the mixture being the fully substituted 2,6-di-O-methylated-β-CD, species (i). Since in both the electropherogram and the mass spectrum, peak intensity is not necessarily a quantitative indication of the relative abundance of each chemical species, further work needs to be done to substantiate these arguments. The use of on-line CE–mass spectrometry [22] or fraction collection followed by mass spectrometry [23] could be used to conclusively determine whether the CE peaks were of single mass components. Even without this information, this CE separation could be used as a finger print method for the comparison of batch-to-batch and supplier-to-supplier variations in the derivatized CDs.

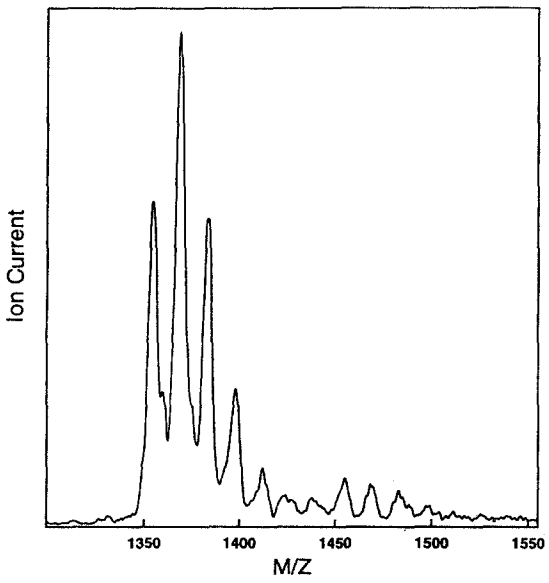

Fig. 5. Electrospray mass spectrum of DM-β-CD (1.2 mg ml^{-1}). Spray current: 0.2 μA. Solvent: 3% aqueous acetic acid–methanol (50:50). Approximately 1.6 μg of the DM-β-CD mixture was consumed for this analysis.

4. Conclusions

This work demonstrates the feasibility of analyzing complex mixtures of cyclodextrins by CE. Dynamic fluorescence labelling provides a simple, sensitive and selective means of monitoring the CD. The increase in fluorescence

associated with 2,6-ANS binding to β-CD allowed sensitive detection of β-CD, while α- and γ-CD demonstrated less of an enhancement and correspondingly higher detection limits. Although the instrument used in these studies employed a laser excitation source, equivalent sensitivity should be possible with less expensive arc-lamp fluorescence detectors as the detection limits are determined by the background fluorescence signal and not by shot noise or the detection electronics. Suppression of the electroosmotic flow increased the time required for the analysis but provided enhanced separation capabilities. Addition of organic modifiers and an ammonium salt allowed components of DM-β-CD with differing degrees of substitution to be separated. Further suppression of the electroosmotic flow with coated capillaries [24,25] or the application of a radial electric field [26,27] should further enhance the separation capability of this technique.

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