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Capillary electrophoretic separation of α-, β-, γ- and δ-cyclodextrins using a dual electrolyte system

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Abstract

Capillary electrophoresis was employed for the separation α-, β-, γ- and δ-cyclodextrins (CDs). A separation of α-, β-, γ- and δ-CDs could be obtained by the use of a range of aromatic anions as background electrolytes. Using a dual electrolyte system consisting of 3.5 m*M* methyl orange and 40 m*M* 4-*tert*.-butylbenzoic acid, α-, β-, γ- and also δ-CDs could be separated with a 6–13-times higher sensitivity compared to previously published methods. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Buffer composition; Cyclodextrins; Anions, aromatic; Oligosaccharides

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides produced from starch by the enzyme cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) and occur in mixtures of cyclic and linear oligosaccharides. They are composed of a number of (1→4)-linked α-D-glucose units of which CDs with 6, 7 and 8 glucose units (α-, β- and γ-CDs), are predominantly produced [1,2]. The existence of larger CDs with 9, 10, 11, and 12 (1→4)-linked α-D-glucose units, denoted δ-, ε-, ζ- and η-CDs, respectively, was first described by French and co-workers [3,4]. Due to low yields and difficulties in their purification, they have only recently been purified and characterised [5–7].

Analysis of CDs has been performed using various techniques of which thin-layer chromatography [8] and high-performance liquid chromatography [9–13] are often employed. However, these techniques are hampered by interference with linear oligosaccharides and tedious sample preparations. Capillary electrophoresis (CE) excels in separation and analysis of α-, β- and γ-CDs and their derivatives, even in complex samples [14–17]. This is due to the high selectivity of CE for inclusion complexes formed between the solute containing CDs and the electrolyte. However, for the analysis of δ-CD and larger CDs, which only are produced in minute amounts, the sensitivity of the known methods is too low. In the CE method, the neutral CDs are carried towards the cathode and the detector by the electroosmotic flow (EOF) with a given mobility. The EOF is caused by cations attracted to the negatively charged silanol groups on the inside of the fused-silica capillary. These cations move towards the cathode dragging the solute in the capillary in the same direction. The aromatic anions in the background electrolyte (BGE) move towards the anode against the EOF and away from the detector, with a mobility

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specific for the anion used. An equilibrium is formed between free CDs being dragged towards the cathode by the EOF and CD–aromatic anion complexes migrating toward the anode, with a reduced mobility compared to the mobility of the free anion. This results in separation of the CDs from other neutral species which do not form complexes with the aromatic anions.

Previously, we have reported that CE allows the analysis of α-, β- and γ-CDs in complex samples [17]. In this paper, we present a method which allows the concomitant separation of α-, β-, γ- and δ-CDs in complex samples using a dual electrolyte system. The sensitivity of this method for α-, β- and γ-CDs is 6–13-fold higher compared to previously published methods [17].

2. Experimental

2.1. Chemicals

2-Methylbenzoic acid (2-MBA), 3-methylbenzoic acid (3-MBA), 4-methylbenzoic acid (4-MBA), 4-ethylbenzoic acid (4-EBA), 2,4-dimethylbenzoic acid (2,4-DMBA), 2,5-dimethylbenzoic acid (2,5-DMBA), 3,5-dimethylbenzoic acid (3,5-DMBA), 3,5-dimethoxybenzoic acid (3,5-DMOBA), 4-*tert*.-butylbenzoic acid (4-*t*-BBA) and salicylic acid (SA) were obtained from Aldrich, Steinheim, Germany. Benzoic acid (BA), ibuprofen (4-isobutyl-α-methylphenylacetic acid, sodium-salt) (IB), methyl orange {4-[*p*-(dimethylamino)-phenylazo]benzenesulphonic acid, sodium salt} (MO) and maltoheptaose were obtained from Sigma, St. Louis, MO, USA. 3-Phenylpropionic acid (3-PPA), 4-biphenylacetic acid (4-BPAA) and soluble starch were obtained from E. Merck, Darmstadt, Germany. Oxoid yeast extract was obtained from Unipath, Basingstoke, UK. Bacto tryptone was obtained from Difco Labs., Detroit, MI, USA. Brown rice from Neue Allgemeine Reisgesellschaft, Hamburg, Germany was used.

Pharmaceutical grade α-, β- and γ-CDs were gifts from Wacker Chemie. δ-CD was provided by Dr. H. Ueda, Hoshi University, Tokyo, Japan. All chemicals were of analytical grade, unless stated otherwise.

2.2. Capillary electrophoresis

CE was performed on a Beckman P/ACE 5010 system equipped with 200, 214, 254, 280 and 340 nm filters and a P/ACE UV absorbance detector (Beckmann Instruments, Fullerton, CA, USA). Electrophoresis and electropherogram analysis was performed using System Gold version 8.10 software (Beckmann Instruments). Fused-silica capillaries of 50 μm I.D. were obtained from Composite Metal, CA, USA. The capillaries were conditioned daily with a high-pressure purge (138 kPa) of 1 *M* HCl followed by 1 *M* NaOH, both for 5 min. Prior to analysis, the capillaries were rinsed with 1 *M* NaOH for 0.5 min using a high-pressure purge (138 kPa), followed by a high-pressure purge (138 kPa) of water for 0.5 min. The capillary was then filled with BGE using a high-pressure purge (138 kPa) for 2 min. Sample loading was performed by applying pressure (3.4 kPa) to the anionic side of the capillary for 5 s. Prior to separation, a small amount of BGE was loaded onto the capillaries by pressure (3.4 kPa) for 10 s in order to prevent escape of sample at the beginning of the separation. The separation was carried out at 30 kV constant voltage. Three BGE resevoirs were applied, one to fill the capillary and two to perform the separation. Capillary temperature was maintained at 25°C. BGE solutions were degassed and filtered through a 0.22-μm syringe filter prior to use and were stable for approximately 20 analyses. Capillary length was 77 cm (70 cm to the detector).

Stock solutions of 10 m*M* MO and/or 200–250 m*M* aromatic anion (see Section 2.1) in 2 m*M* disodiumphosphate, pH 7.0 or 8.0 was used to prepare the different BGE solutions. Two m*M* phosphate buffer, pH 7.0 or 8.0 was added to a final volume of 10 ml.

2.3. Sample preparation

Standard CDs and maltoheptaose were dissolved in double distilled water and used without further sample preparation. A crude CGTase preparation was produced by growing *Paenibacillus* sp. F8 in a medium consisting of 2% (w/v) brown rice, 0.5% (w/v) yeast extract, 0.5% (w/v) tryptone, 0.2% (w/v) K_2HPO_4, 0.2% (w/v) Na_2HPO_4, 0.02% (w/v)

$MgSO_4 \cdot 7H_2O$, 0.02% (w/v) $CaCl_2 \cdot 7H_2O$ and 0.1% (w/v) $(NH_4)_2SO_4$ for 24 h at 37°C in a shaking incubator (New Brunswick Scientific, Edison, NJ, USA) at 200 rpm. CGTase enzyme was concentrated by ammonium sulphate precipitation (70% saturation). CDs were produced by incubating 5% (w/v) soluble starch in 50 m*M* phosphate buffer, pH 7.0, 2 m*M* $CaCl_2$ with a concentrated CGTase preparation for 10 h at 50°C. The enzyme reaction was stopped by addition of three volumes of 4°C cold methanol and cooling at 4°C for 30 min. After insoluble material was removed by centrifugation, the CD containing supernatant was decanted and dried in a heating block at 100°C. The dried sample was dissolved in double distilled water prior to analysis.

3. Results and discussion

3.1. Single electrolyte BGEs

Several aromatic anions were tested for their ability to separate CDs as well as other starch hydrolysis products and for their capability to form inclusion complexes. Using mixtures containing 2 m*M* of α-, β-, γ- and δ-CDs, satisfactory separations were obtained using 75 m*M* of 4-MBA, 4-EBA, BA, 4-*t*-BBA, 2-MBA, 3-MBA, 3,5-DMBA, 2,5-DMBA, 3,5-DMOBA, 2,4-DMBA, 4-BPAA or IB in 2 m*M* phosphate buffer, pH 7.0. With SA and 3-PPA, the CD species could not be completely separated. Analysis of starch hydrolysates obtained by incubation of soluble starch with CGTase from *Paenibacillus* sp. F8 showed that 4-MBA, SA, BA, 4-*t*-BBA, 2-MBA, 3-MBA, 3-PPA, 2,5-DMBA, 2,4-DMBA, 4-BPAA and IB were unable to separate δ-CD from non-cyclic maltooligosaccharides. These non-cyclic starch hydrolysis products migrated as a broad peak close to the water peak indicating heterogeneity and low complex forming ability. Maltoheptaose eluted in the beginning of the broad starch peak. These compounds could however be effectively removed by precipitation with methanol [18]. Fig. 1 shows electropherograms of CDs and maltoheptaose, using 75 m*M* of 2,4-DMBA, 2,5-DMBA and 3,5-DMBA, respectively in 2 m*M* phosphate buffer, pH 7.0, as BGE.

Although the BGE solutions presented in Fig. 1 gave good separations of α-, β-, γ- and δ-CDs, their detection limit was rather high. Especially for δ-CD, which occurs only in small amounts compared to α-, β- and γ-CDs. The detection limit could be improved by lowering the voltage or the BGE concentration, thereby reducing noise caused by Joule heating (results not shown). However, lowering the voltage can lead to unacceptable long analysis times, where-

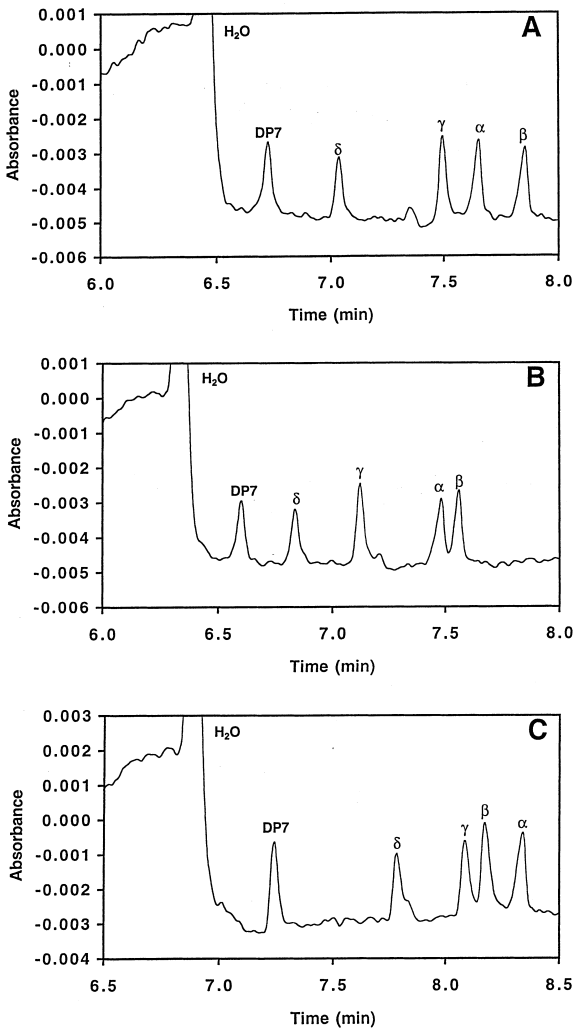

Fig. 1. Electropherograms of α-, β-, γ- and δ-CDs and maltoheptaose separated using 75 m*M* of (A) 2,4-DMBA, (B) 2,5-DMBA and (C) 3,5-DMBA in 2 m*M* phosphate buffer, pH 7.0 as BGE. H_2O indicates the water peak caused by the flow of the EOF, DP7 indicates maltoheptaose and the greek letters the corresponding CDs. Absorbance was measured at 254 nm using indirect detection.

as lowering of the BGE concentration can result in poorer separations.

3.2. Methyl orange

To improve the sensitivity of the CE system for the separation and analysis of CDs, an acid–base indicator known to change its absorbance upon inclusion with CDs was added to the BGE. Phenolphthalein, bromocresol green and MO have been used in more or less specific detection methods for the quantification of CDs based on absorption spectrometry [19]. Phenolphthalein and bromocresol green have a high specificity towards β- and γ-CDs only, making them unsuitable for the detection of all CD species. MO on the other hand gives relative equal sensitivity towards α-, β- and γ-CDs at neutral pH (relative sensitivity α:β:γ=214:100:86) [19]. The largest absorbance difference between the free MO and MO complexed with CDs was found at 515, 505 and 480 nm for α-, β- and γ-CDs, respectively [20]. MO increased the absorbance of the CDs considerably. Using MO in 2 m*M* phosphate buffer, pH 8.0 at concentrations from 1 to 10 m*M* revealed that although the different MO–CD complexes displayed a high effective mobility (μ_{eff}), MO alone was not able to separate the CDs. However, the high mobility observed even at low concentrations of MO indicated that MO has a higher inclusion complex formation constant compared to the benzoic acid derivatives and/or a higher mobility of the anion–CD complex.

3.3. Dual electrolyte systems

Different combinations of aromatic anions and MO were tested for their ability to separate the CDs, while maintaining their absorbance signal provided by the addition of MO. The addition of the aromatic anions significantly improved the separation of the CDs. However, for most aromatic anions in the BGE, α/β-CD co-migrated, whereas γ/δ-CD were separated from each other (results not shown). Five aromatic anions which alone provided satisfactory separation of α- and β-CDs were therefore tested for their ability to improve the separation of α- and β-CDs, while maintaining the separation of γ- and δ-CDs. Except for BGE containing 3,5-DMOBA, the BGE solutions displayed μ_{eff} in the order of μ_{eff}(δ-CD)>μ_{eff}(γ-CD)>μ_{eff}(β-CD)>μ_{eff}(α-CD). The ratio between μ_{eff} of the CD–BGE complexes for α/β-CD, respectively γ/δ-CD, is listed in Table 1A. IB was not able to separate α- and β-CDs, whereas the other aromatic anions gave an improved separation. Except for SA, the separation of γ- and δ-CDs was improved by the addition of aromatic anions. 4-*t*-BBA, 2,4-DMBA and 3,5-DMOBA all improved the separation of both α/β-CD and γ/δ-CD. The inclusion complex formation constants for α-, β-, γ- and δ-CDs estimated using CE [16] were 51, 382, 74, 47 M^{-1}, respectively, for 4-*t*-BBA; 45, 42, 8, 3 M^{-1}, respectively, for 2,4-DMBA, and 47, 63, 10, 8 M^{-1}, respectively, for 3,5-DMOBA. These results indicate that 4-*t*-BBA, as the strongest inclusion complex former, represented the best combination with the apparently strong inclusion complex former MO.

3.4. Optimisation of the MO/4-t-BBA BGE

The effect of the MO concentration on the separation of the four CDs is shown in Table 1B. Fifty m*M* of 4-*t*-BBA alone provided an excellent separation of α- and β-CDs (high μ_{eff} ratio), whereas γ- and δ-CDs co-migrated. Addition of 1 to 3 m*M* MO led to a decrease in the μ_{eff} ratio for α- and β-CDs, although a separation was still obtained. Addition of 4 and 5 m*M* MO, respectively, did not result in a further change in the μ_{eff} ratio of α- and β-CDs. γ- and δ-CDs co-migrated up to a concentrations of 3 m*M* MO. A separation of γ- and δ-CDs was obtained in BGE containing 4 and 5 m*M* MO, respectively. Five m*M* MO without addition of 4-*t*-BBA was unable to separate any of the CDs.

The effect of the concentration of 4-*t*-BBA on the separation of the CDs was investigated with a constant MO concentration of 3 m*M* (Table 1C). The results show that the μ_{eff} ratio increased slightly for both α/β-CD and γ/δ-CD with an increased 4-*t*-BBA content of the BGE.

The results shown in Table 1 (A, B and C) were obtained using fresh BGE solutions. Upon standing at room temperature, some of the BGE solutions formed precipitates. To overcome this problem, a series of BGE solutions containing combinations of 30 to 70 m*M* 4-*t*-BBA and 3 to 5 m*M* MO were prepared. The combination of 70 m*M* 4-*t*-BBA and 5

Table 1
Optimisation of CE separation of α-, β-, γ- and δ-CDs using combinations of aromatic anions and MO in 2 m*M* phosphate buffers, pH 8.0

MO (m*M*)	Aromatic anion	Concentration (m*M*)	μ_{eff} (β-CD)/μ_{eff} (α-CD)	μ_{eff} (δ-CD)/μ_{eff} (γ-CD)
(A) Effect of co-electrolyte on separation				
3	4-*t*-BBA	75	1.12	1.05
3	IB	75	1.01	1.13
3	SA	75	1.09	1.01
3	2,4-DMBA	75	1.14	1.04
3	3,5-DMOBA	75	1.09	0.92
(B) Effect of MO concentration on separation				
0	4-*t*-BBA	50	1.45	1.00
1	4-*t*-BBA	50	1.16	0.99
2	4-*t*-BBA	50	1.12	1.00
3	4-*t*-BBA	50	1.10	1.01
4	4-*t*-BBA	50	1.09	1.06
5	4-*t*-BBA	50	1.10	1.08
5	4-*t*-BBA	0	1.02	1.00
(C) Effect of 4-t-BBA concentration on separation				
3	4-*t*-BBA	25	1.07	0.99
3	4-*t*-BBA	50	1.10	1.01
3	4-*t*-BBA	60	1.11	1.02
3	4-*t*-BBA	70	1.12	1.05
3	4-*t*-BBA	80	1.13	1.06
3	4-*t*-BBA	90	1.13	1.06
3	4-*t*-BBA	100	1.14	1.07
(D) Optimisation of separation				
3	4-*t*-BBA	30	1.07	1.03
3.5	4-*t*-BBA	30	1.06	1.07
4	4-*t*-BBA	30	1.05	1.10
4.5	4-*t*-BBA	30	1.05	1.12
3	4-*t*-BBA	40	1.09	1.05
3.5	4-*t*-BBA	40	1.08	1.10

m*M* or 4.5 m*M* MO and the combination of 60 m*M* 4-*t*-BBA and 5 m*M* MO immediately resulted in the formation of precipitates. The other BGE mixtures were stored at 20°C and the formation of precipitates was checked after one and seven day(s) (Table 2). Six BGE solutions containing 30 m*M* 4-*t*-BBA in combination with 3, 3.5, 4 and 4.5 m*M* MO, and 40 m*M* 4-*t*-BBA in combination with 3 and 3.5 m*M* MO, did not form precipitates upon standing at 20°C for seven days. These BGE solutions were analysed for their ability to separate the CDs (Table 1D). An increased concentration of MO lowered the μ_{eff} ratio of α/β-CD and improved the effective mobility ratio of γ/δ-CD, as shown in Table 1B. Furthermore, an increase in 4-*t*-BBA concentrations led to an increased effective mobility ratio, as shown in Table 1C. A combination of 40 m*M* 4-*t*-BBA and 3.5 m*M* MO resulted in the best separation of all the four CDs. Fig. 2 shows the separation of standard mixtures of α-, β-, γ- and δ-CDs and maltoheptaose and

Table 2
Stability of MO-4-*t*-BBA buffer solutions

	4-*t*-BBA (m*M*)				
MO (m*M*)	30	40	50	60	70
5	7 days	1 day	1 day	[a]	[a]
4.5	N.O.	1 day	1 day	1 day	[a]
4	N.O.	7 days	1 day	1 day	1 day
3.5	N.O.	N.O.	1 day	1 day	1 day
3	N.O.	N.O.	7 days	7 days	1 day

N.O.: No precipitate observed after seven days.
[a] Precipitated immediately.

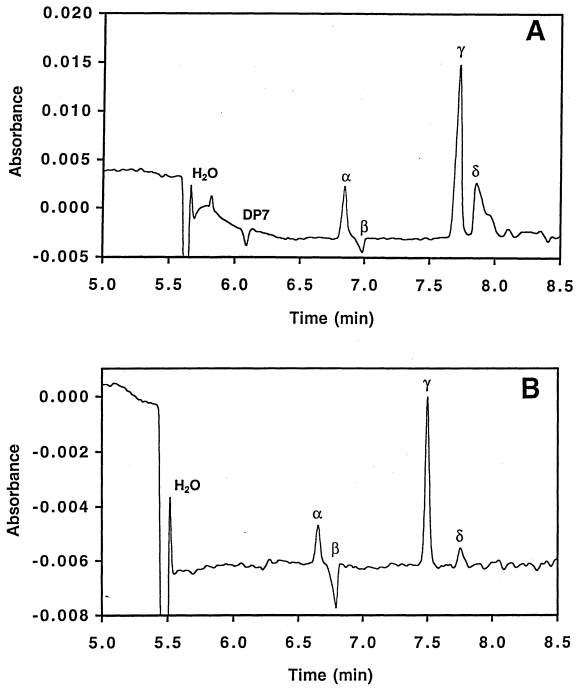

Fig. 2. Capillary electrophoretic separation of CDs using 3.5 m*M* MO and 40 m*M* 4-*t*-BBA in 2 m*M* phosphate buffer, pH 8.0. (A) Mixture of standards, 5 m*M* maltoheptaose, 2 m*M* β-CD and 1 m*M* of α-, γ- and δ-CDs, respectively. (B) Products from an incubation of soluble starch with CGTase from *Paenibacillus* sp. F8. H_2O indicates the water peak caused by the flow of the EOF, DP7 indicates maltoheptaose and the greek letters the corresponding CDs. Absorbance was measured at 280 nm using direct detection.

the separation of the products obtained by incubating the CGTase preparation from *Paenibacillus* sp. F8 with soluble starch.

The separations described are based on the complex formation between the aromatic anions and the CDs. Two factors play a role in this process: the inclusion complex formation constant (the stability of the complex) and the mobility of the complex (μ_{max}). The μ_{eff} of the complex reaches μ_{max} when the inclusion complex formation constants are high. In the case of MO, the inclusion complex formation constants with CDs are likely to be high, so that μ_{eff} reaches μ_{max}. By addition of a second molecule capable of inclusion complex formation and with different μ_{max} and inclusion complex formation constant, the μ_{max} of MO will not be reached due to competition from the second electrolyte. Therefore, μ_{eff} will not reach μ_{max} and the differences in inclusion complex formation constants, and the proportions between MO and the CDs as well as between 4-*t*-BBA and the CDs will determine the effective mobilities of the CD complexes.

3.5. Sensitivity

Measurement of the absorbance at 280 nm resulted in the highest signal compared to 254 and 340 nm. The relative sensitivity at 254, 280, 340 nm was 14:100:29, respectively for δ-CD in 3.5 m*M* MO and 40 m*M* 4-*t*-BBA in 2 m*M* phosphate buffer, pH 8.0. α- and γ-CDs displayed similar sensitivities and β-CD showed the highest absorbance at 254 nm. The detector response for β-, γ- and δ-CDs was greatly influenced by the MO concentration at 280 nm (Fig. 3). While α-CD was unaffected, the detector response to γ- and δ-CDs increased with increasing MO concentration up to 3 and 4 m*M*, respectively. The signal from β-CD gave a negative absorbance which increased with increasing MO concentration. The variation observed in the signals for the four CDs, may be attributed to differences in their binding and in the resulting displacement of the two electrolytes in the sample zones.

Standard curves of α-, β-, γ- and δ-CDs were obtained using the optimal BGE solution (3.5 m*M* MO and 40 m*M* 4-*t*-BBA) in the range 0.05 to 10 m*M*, 0.25 to 10 m*M*, 0.05 to 10 m*M* and 0.1 to 2.5 m*M*, respectively. The following relationships were found by linear regression analysis: α-CD: $y=$

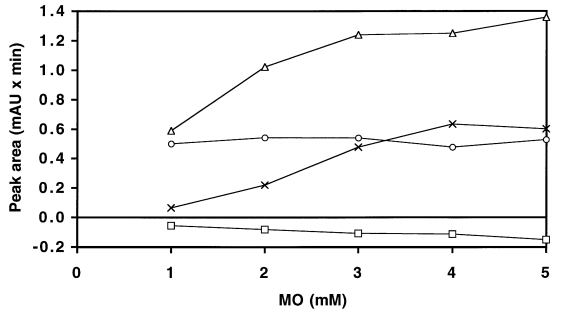

Fig. 3. Influence of MO concentration on the peak area of α-, β-, γ- and δ-CDs separated by CE. 4-*t*-BBA concentration was held constant at 50 m*M*. Absorbance was measured at 280 nm using direct detection. Circle: α-CD; square: β-CD; triangle: γ-CD; cross: δ-CD.

0.2456x+0.0204, R^2=0.989; β-CD: y=−0.0458x+0.003, R^2=0.9979; γ-CD: y=0.5847x+0.0144, R^2=0.9963; δ-CD: y=0.3168x+0.0252, R^2=0.9968, where y=peak area (mAU/min) and x=concentration of CD in sample (mM). Peak areas below 0.01 mAU/min could not be distinguished. Using a 5 s pressure purge (3.4 kPa), estimated detection limits of 0.05, 0.25, 0.025 and 0.05 mM for α-, β-, γ- and δ-CDs, respectively, could be obtained. Higher absorbance differences and thereby improved signals could possibly be obtained by using a diode array detector capable of detection in the visible light range, instead of a fixed-wavelength detector with a limited number of available filters, which was used in this study.

4. Conclusions

The separation and sensitive analysis of α-, β-, γ- and δ-CDs has been obtained using a dual electrolyte system consisting of MO and 4-*t*-BBA. MO has a large influence on the separation and detection of CDs. However, MO alone could not give satisfactory separations. The addition of aromatic anions such as 4-*t*-BBA to the BGE improved the separation considerably. This method allowed the analysis of α-, β-, γ- and δ-CDs in complex samples containing CGTase synthesis products.

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