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Separation and analysis of cyclodextrins by capillary zone electrophoresis

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Abstract

α-, β-, and γ-Cyclodextrins were separated by capillary zone electrophoresis using indirect UV photometric detection. The cyclodextrins are separated and detected after complexation with benzoic acid or benzoic acid derivatives using a constant electroosmotic flow. Enhanced resolution of mixtures of cyclodextrins was obtained by adjusting the pH of the electrolyte and the concentration of benzoic acid. High pH conditions resulted in short analysis times with a low selectivity, whereas high benzoic acid concentrations resulted in long analysis times with a high selectivity. Detection limits were found at 50 μM for α-, β-, and γ-cyclodextrins, respectively. The method allows the separation and analysis of cyclodextrins in complex samples such as fermentation broth, urine, plasma, and pharmaceutical formulations with minimal sample pretreatment. © 1997 Elsevier Science Ltd. All rights reserved.

Keywords: Capillary zone electrophoresis; Cyclodextrins; Benzoic acid

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of 6–8 (1 → 4)-linked α-D-glucosyl residues denoted α-, β-, and γ-CD, respectively [1]. CDs are produced from starch by the action of extracellular enzymes, cyclodextrin glycosyltransferases (EC 2.4.1.19). The analysis of CDs produced by these enzymes is difficult since the enzymes concomitantly produce mixtures of cyclodextrins as well as different starch hydrolysis products.

Due to their ability to entrap various small molecules into their hydrophobic central cavity, CDs are able to modify the physicochemical properties of the guest molecules by increasing their solubility and stability, as well as by modifying their reactivity. CDs have found numerous applications in the agricultural, food, chemical, and pharmaceutical industries [1]. Furthermore, CDs have been shown to be valuable as selectivity reagents for the resolution of structural, positional, and stereoisomers in analytical chemistry [2].

A wide range of methods has been used for the analysis of CDs. These include absorbance and fluorescence spectroscopy [3], thin-layer chromatography [4,6], high-performance liquid chromatography (HPLC) [5–10], and electrophoresis [11–13].

The use of absorbance and fluorescence spectroscopy for the analysis of CDs has been reviewed by Mäkelä et al. [3]. These methods fail to discrimi-

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nate between the individual CDs and are highly sensitive to changes in the pH. Furthermore, three assays are necessary to measure the concentration of α-, β-, and γ-CD in one sample.

For the separation of CDs by HPLC, several stationary phases have been described, e.g. resins modified with specific absorbents [5] and reversed-phase media [6–9] used in combination with either refractive index detection [5–7], indirect photometric detection [8], or pulsed amperometric detection [9]. The chromatographic methods require extensive sample preparation when analysing complex samples such as fermentation broth containing CDs, oligosaccharides, residual starch, as well as proteins, salts, and other substances. Using reversed-phase HPLC with refractive index detection, the CDs eluted together with the linear oligosaccharides also present in the sample [10]. In order to remove the oligosaccharides, the samples had to be incubated with exo-acting amylolytic enzymes (e.g. β-amylase) for several hours prior to analysis. Furthermore, the chromatographic methods often suffer from poor sensitivity and resolution in combination with long separation times.

Separation and analysis of CDs by electrophoresis has been carried out in polyacrylamide gels [11] and, more recently, using capillary electrophoresis [12,13]. Electrophoretic separation of CDs is normally not possible since CDs are only charged at a very high pH. Nardi et al. [12] have demonstrated that CDs can be separated by formation of inclusion complexes with benzoic acid (BA) using capillary electrophoresis. The presence of BA also allows detection of the CDs by indirect UV absorbance measurement due to a reduction in the absorbance of the benzoate anion following complexation with the CDs [14]. Although a baseline separation between α-, β-, and γ-CD could be obtained using this method, only the detection of β-CD was possible in complex samples [12]. This technique has been successfully used for the separation of the charged CD derivatives, sulfoalkyl ether β-CDs [14]. Similarly, the fluorophore 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) has been used for the separation and detection of CDs in a capillary electrophoresis system [13]. The separation relied on the complex formation of CDs with the anionic 2,6-ANS. These complexes could be detected by fluorescence spectroscopy due to enhanced fluorescence of 2,6-ANS following complexation with CDs. This method had a good sensitivity for β-CD, allowing the separation of 2,6-di-*O*-methyl β-CDs in a heterogeneous sample, whereas α- and γ-CD were only barely detectable.

In this work we report an improved method for the detection of CDs based on the work of Nardi et al. [12]. It comprises a fast and sensitive method for the simultaneous analysis of α-, β-, and γ-CD in complex samples.

2. Results and discussion

The electrophoretic separation of CDs was optimised using electrolyte combinations of BA (50, 100, and 200 mM), tris(hydroxymethyl)aminomethane (Tris) (100, 200, and 500 mM) at various pH (7, 8, 9, 10). Fig. 1 shows the selectivity expressed as the relative retention ratio (α) between the water peak, which is equal to the migration time of the electroosmotic flow through the capillary, and the fastest eluting CD, which is γ-CD when using BA as background electrolyte (BGE). It is evident that the highest selectivity is obtained at a low pH and a high BA concentration. The reason for the differences in selectivity at different pH values could be found in the speed of the electroosmotic flow. The elution time of the water peak with 100 mM BA and 100 mM Tris was 7.5 min at pH 7.0 compared to 3.8 min at pH 10.0. A low pH results in longer separation times and also in a higher selectivity.

A BA concentration of 200 mM gave the best separation of γ-CD from the water peak. The increase in BA concentration could have shifted the

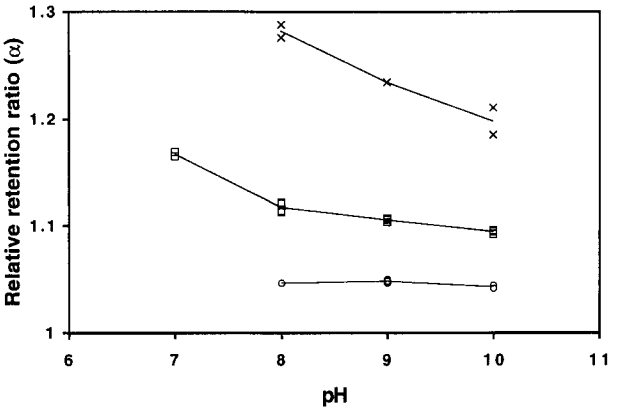

Fig. 1. Relative retention ratio ($\alpha = RT_{\gamma\text{-CD}} / RT_{water}$) between the water peak and γ-cyclodextrin with different benzoic acid concentrations as a function of the pH. Experiments were performed on a 57 cm (50 cm to detector) 50 μm i.d. fused silica capillary. A 2 mM γ-CD solution was loaded for 5 s by pressure (0.5 psi) and separation was performed at 30 kV constant voltage. ×: 200 mM benzoic acid; □: 100 mM benzoic acid; ○: 50 mM benzoic acid. RT = retention time.

Table 1
Separation of α-, β-, and γ-CD from the water peak in the presence of benzoic acid and various benzoic acid derivatives. BGE consisted of 100 mM benzoic acid or benzoic acid derivative in 100 mM Tris, pH 7. Separations were performed on a 47 cm (40 cm to the detector) 50 μm i.d. fused silica capillary. Samples were loaded by pressure (0.5 psi) for 5 s. Separation was carried out at 60 μA constant current

Electrolyte [a]	Relative retention ratio (α)		
	α-CD	β-CD	γ-CD
Benzoic acid	1.25	1.46	1.15
2,5-Dimethyl benzoic acid	1.55	1.64	1.38
2,4-Dimethyl benzoic acid	1.49	1.60	1.49
3,5-Dimethyl benzoic acid	1.58	1.44	1.58
3,5-Dimethoxy benzoic acid	1.49	1.57	1.49

[a] 100 mM in 100 mM Tris, pH 7.

equilibrium between free CD and complexed CD towards the complexed form. However, an increased baseline noise was observed when using 200 mM BA, which made the determination of the peak area difficult.

The effect of Tris concentration on the separation was also investigated. No effect was observed between 100 and 200 mM Tris at any combination of the pH values or BA concentrations tested. At 500 mM Tris, the CDs and the water peak could not be detected. From these results, a buffer system was chosen, which consisted of 100 mM BA and 100 mM Tris adjusted to pH 7.

BA derivatives were investigated for their ability to improve the separation of CDs (Table 1). Only BA and 2,5-dimethyl benzoic acid (DMBA) were able to separate the CDs (Fig. 2), while 2,4-DMBA, 3,5-DMBA, and 3,5-dimethoxy benzoic acid (DMOBA) did not separate α- and γ-CD. 2,5-DMBA gave the best separation of the CDs from the water peak. The order of effective mobility of the $CDBA^-$ complex was $\mu_{\gamma CDBA^-} < \mu_{\alpha CDBA^-} < \mu_{\beta CDBA^-}$ for BA and 2,5-DMBA. For 2,4-DMBA and 3,5-DMOBA, equal mobilities for γ- and α-CD complexes were observed when analysing 3.33 mM of each cyclodextrin. The presence of 3,5-DMBA in the electrolyte resulted in reverse mobilities for the CDs, with β-CD having the lowest mobility and α- and γ-CD the highest. The difference in mobilities of the CDBA complexes is due to different association constants between the CDs and the BAs. The addition of BGE to the samples resulted in sharper and higher peaks, which were most pronounced for γ-CD.

Cyclodextrin concentrations as low as 50 μM were detectable using a 5-s pressure injection of the sample (Fig. 3). Longer injection times resulted in distorted peaks.

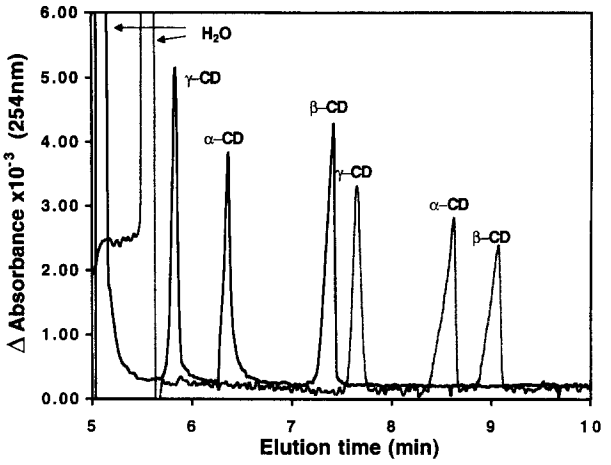

Fig. 2. Electropherograms of a mixture containing 3.33 mM of α-, β-, and γ-CD, using benzoic acid and 2,5-dimethyl benzoic acid as BGE. Separation was performed on a 47 cm (40 cm to the detector) 50 μm i.d. fused silica capillary. Samples were loaded by pressure (0.5 psi) for 5 s. Separation was carried out at 60 μA constant current. Black line: with benzoic acid; Grey line: with 2,5-dimethyl benzoic acid. Absorbance was measured at 254 nm.

An electropherogram of CDs contained in a fermentation broth from *Bacillus circulans* NRRL B-380 showed that they could be easily detected and separated in complex samples with either BA or 2,5-DMBA in the electrolyte (Fig. 4).

In the method described, the neutral CDs are carried towards the cathode and the detector by the

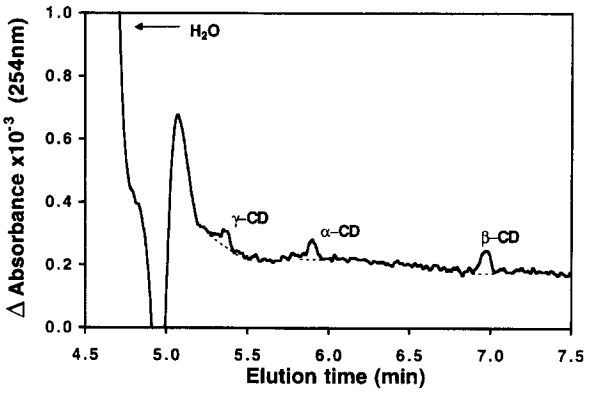

Fig. 3. Electropherogram of a mixture containing 50 μM of α-, β-, and γ-CD, using benzoic acid as BGE (100 mM benzoic acid, 100 mM Tris, pH 7). Separation was performed on a 47 cm (40 cm to the detector) 50 μm i.d. fused silica capillary. Samples were loaded by pressure (0.5 psi) for 5 s. Separation was carried out at 30 kV constant voltage. Absorbance was measured at 254 nm.

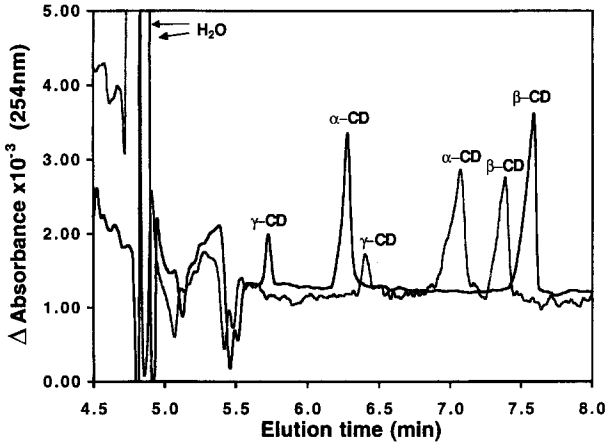

Fig. 4. Electropherograms of α-, β-, and γ-CD from a fermentation broth of *Bacillus circulans* NRRL B-380 with either 100 mM benzoic acid or 100 mM 2,5-dimethyl benzoic acid in 100 mM Tris, pH 7. Separation was performed on a 47 cm (40 cm to the detector) 50 μm i.d. fused silica capillary. Samples were loaded by pressure (0.5 psi) for 5 s. Separation was carried out at 30 kV constant voltage. Black line: with benzoic acid; Grey line: with 2,5-dimethyl benzoic acid. Absorbance was measured at 254 nm.

electroosmotic flow (EOF), with the mobility μ_{EOF} (Fig. 5). 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 benzoate anions in the BGE move towards the anode against the EOF and away from the detector, with the mobility μ_{BA^-}. An equilibrium is formed between free CDs being dragged towards the cathode by the EOF and CDBA complexes migrating toward the anode, with the mobility μ_{CDBA^-}. This results in the CDs being separated from other neutral species which do not form complexes with BAs.

This method allows the fast analysis of α-, β-, and γ-CD mixtures and analysis of cyclodextrin glycosyltransferase activity in complex samples using a capillary electrophoresis system and can also be easily automated.

3. Experimental

Materials.—Benzoic acid and Tris [tris(hydroxymethyl)aminomethane] were obtained from Sigma, St. Louis, MO, USA. Benzoic acid derivatives were obtained from Aldrich, Steinheim, Germany. Cyclodextrins, soluble starch, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and $(NH_4)_2SO_4$ were obtained form E. Merck, Darmstadt, Germany. Oxoid Yeast Extract was obtained from Unipath Ltd., Basingstoke, Hampshire, UK. Bacto Tryptone was obtained from DIFCO Lab., Detroit, MI, USA. All chemicals were analytical grade.

Capillary electrophoresis.—Capillary electrophoresis was performed on a Beckman P/ACE 5010 system equipped with a P/ACE UV Absorbance Detector. Capillaries were 50 μm i.d. fused silica capillaries obtained from Composite Metal, USA. The background electrolyte (BGE) consisted of mixtures of benzoic acids and Tris in various concentrations and pH; BA (50, 100, and 200 mM), Tris (100, 200, and 500 mM), pH (7, 8, 9, and 10). pH was adjusted with NaOH or HCl. Prior to analysis, the capillary was rinsed with a high-pressure purge (20 psi) of 1 M NaOH for 1.5 min. The capillary was filled with BGE using a high-pressure purge (20 psi) for 1.5 min. Sample loading was performed by applying pressure (0.5 psi) to the anionic side of the capillary. Separation was carried out at 30 kV constant voltage or 60 μA constant current. Capillary temperature was maintained at 25 °C. BGE solutions were only stable for approximately 20 analyses due to migration of BAs from the cathode to the anode, causing a decrease in the pH of the anode reservoir.

Fermentation broth was obtained by growing *Bacillus circulans* NRRL B-380 in 250 mL medium containing 2% (w/v) soluble starch, 0.5% (w/v) yeast extract, 0.5% (w/v) tryptone, 0.1% (w/v) KH_2PO_4, 0.035% (w/v) $MgSO_4 \cdot 7H_2O$, 0.02% (w/v) $CaCl_2 \cdot 2H_2O$, and 0.1% (w/v) $(NH_4)_2SO_4$ adjusted to pH 7 with NaOH in a 1-L conical flask. The medium was incubated for 24 h at 37 °C under

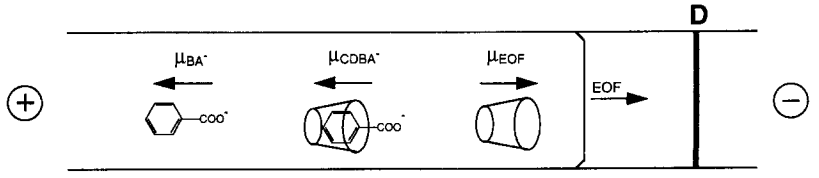

Fig. 5. Schematic diagram showing mobilities (μ) of benzoic acid (μ_{BA^-}), cyclodextrins (μ_{EOF}), and the cyclodextrin–benzoic acid complex (μ_{CDBA^-}), in a fused silica capillary in an electrical field. EOF: electroosmotic flow; D: detector.

continuous shaking (200 rpm). The supernatant was directly analysed by CE after centrifugation of the fermentation broth for 5 min at 16,000 *g*.

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