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ORIGINAL PAPER

Use of 6-*O*-mono-substituted derivatives of β-cyclodextrin-bearing substituent with two permanent positive charges in capillary electrophoresis

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This study details the use of two permanently positively charged mono-substituted β-cyclodextrin derivatives, 6^{I}-deoxy-6^{I}-(*N*,*N*,*N*′,*N*′,*N*′-pentamethyl-ethylene-1,2-diammonio)-cyclomaltoheptose dichloride (PEMEDA-β-CD) and the newly synthesised 6^{I}-deoxy-6^{I}-(*N*,*N*,*N*′,*N*′,*N*′-pentamethyl-propylene-1,3-diammonio)-cyclomaltoheptose (PEMPDA-β-CD) as chiral selectors in capillary electrophoresis. Cyclodextrin (CD) derivatives were tested as additives in various buffers at various pH values with the optional addition of an organic modifier. Fourteen anionogenic analytes were tested, including native amino acids, *N*-blocked amino acids and profens, which were detected using a UV-VIS detector at optimal wavelengths of 214 nm, 254 nm or 280 nm. A borate buffer (15 mmol L^{-1}) at pH 9.5 without the addition of an organic modifier was chosen as a suitable background electrolyte. In addition, the effect of the concentration of the chiral selector on the separation and enantioseparation of selected analytes was monitored. The additions of cyclodextrin derivatives varied within the concentration range of 0.0–5.0 mmol L^{-1}. Both chiral selectors were suitable for the enantioseparation of *N*-Boc-D,L-tryptophan, which was already separated on the baseline at 0.5 mmol L^{-1} concentration of the chiral selector.
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Keywords: capillary electrophoresis, chiral separation, chiral selectors, mono-substituted cyclodextrin derivatives

Introduction

Capillary electrophoresis (CE) is a widely used technique for the chiral separation of optically active substances (Juvancz et al., 2008), due to its simplicity and the option of using a great many modifications, leading to lower consumption of solvents and samples. The analyses are rapid with high levels of efficiency and resolution. On the other hand, in comparison with other separation methods, CE affords lower selectivity and higher limits of detection and the capillaries can be overloaded (Tsioupi et al., 2013). In addition to the choice of a suitable solvent or buffer, the separation is significantly affected by the type of chiral selector (Fanali, 2000; Tsioupi et al., 2013). Over the years, many chiral selectors have been prepared, such as various antibiotics, oligo- and polysaccharides, chiral crown-ethers, cyclofructanes and others (Ward & Ward, 2012). However, cyclodextrins (CDs) and their derivatives are the most extensively used group (Tsioupi et al., 2013). Many of these substances are synthesised and used every year in enantioseparations. The advantages of CDs include their good solubility in water and stability and their ready commercial availability; also, they do not absorb in the UV region and have a variety of properties due to the possibility of forming derivatives (Juvancz et al., 2008). In relation to their

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many favourable properties and, especially, their ability to form inclusion complexes, they are used not only in many branches of analytical chemistry or in the sample pre-treatments (Szente & Szemán, 2013), but also in pharmacy, where they are used for solubilising drugs and to prolong their release time. They are also applied in the food and cosmetics industries (Hedges, 1998). CDs can also be successfully employed in the development of rapid chiral electrophoretic separations using microchips (Sánchez-Hernández et al., 2012; Skelley & Mathies, 2003). For example, Stockton et al. (2009) used two CD derivatives simultaneously for the analysis of polycyclic aromatic hydrocarbons on chips.

Most CD derivatives are commercially available in the form of randomly substituted derivatives. Accordingly, these are not individual chemicals but a mixture of positional isomers and homologues with various substituents (mono-, di-, tri- and more substituted derivatives), where each has a somewhat different separation ability. These products are characterised in terms of the degree of substitution, which corresponds to the number of substituents on the CD ring (Cucinotta et al., 2010; Fanali, 2000; Juvancz et al., 2008). The properties of these products cannot be precisely defined, hence they yield results with reduced reproducibility. Accordingly, attempts are made to synthesise mono-substituted derivatives of CD. They have only one hydroxyl group substituted at a defined position in their whole molecule. Their synthesis is more difficult and yields are usually in amounts measured in milligrams but it is possible to achieve greater reproducibility and selectivity and better understanding of the enantioseparation mechanism (Cucinotta et al., 2010; Del Valle, 2004). The low synthesis yields, in particular, are a further reason why CE is suitable for studying the properties of these CD derivatives, as it is easy to alter the analysis conditions and the consumption of stock solutions is minimal (Cucinotta et al., 2010).

CD derivatives can be divided into neutral, positively or negatively charged, amphoteric and polymerising derivatives (Tsioupi et al., 2013). The use of a great many functional groups for their modification has been described in a number of reviews (Cucinotta et al., 2010; de Boer et al., 2000; Del Valle, 2004; Fanali, 2000; Juvancz et al., 2008; Tsioupi et al., 2013). Modification of CDs also changes their properties and it is thereby possible to increase their solubility or improve their complexing ability. In a recent study, such readily soluble CD derivatives, which were also forming supramolecular aggregates in the solution, were described as a separation medium for CE (Benkovics et al., 2016). The solubility, especially, is a key factor in using the CD derivatives for CE, where ionisable groups or groups with permanent charge are preferred. Also the interaction between an ionisable analyte and a selector can be enhanced (Cucinotta et al., 2010). In addition, a selector thus modified has its own electrophoretic mobility in an electric field at a suitable pH (Fanali, 2000), hence it is frequently possible to separate even uncharged analytes (Galaverna et al., 2001). Terabe et al. (1985) and Terabe (1989) were the first to successfully employ charged CD derivatives, where they employed electrokinetic electrophoresis as a separation technique.

Positively charged CDs, in contrast to negatively charged ones, are used less frequently and none of them dominate in the application. Cationic CDs can be strong electrolytes, where the substituents are quaternary ammonium salts, or weak electrolytes, when the side-chains contain primary, secondary or tertiary amino groups, which could be charged by protonisation via the pH of the environment. The side-chains may have different lengths, contain different numbers of nitrogen atoms or could be formed by heterocycles (Cucinotta et al., 2010).

Permanently positively charged CDs possess a great advantage that it is not unconditionally necessary to maintain an exact pH value of the background electrolyte, but rather to adjust their value so that the effective mobilities of the complexed and free analyte are the opposite. In this way, it is possible to obtain the best enantioselective separation and also the greatest stability of the complex formed. In addition, when mono-substituted CD derivatives are used, the charge and, subsequently, the electrostatic interactions between the analyte and CD are always localised at the same site; this contributes to the greater asymmetry of the medium (Cucinotta et al., 2010; Nzeadibe & Vigh, 2007). However, their use is frequently limited to their possible adsorption on the negatively charged wall of the capillary, which can complicate a separation (Tsioupi et al., 2013). This problem can be resolved by using a coated capillary as, for example, in Galaverna et al. (2001), who used diamino-β-CD.

Cationic CD derivatives were synthesised and successfully used for the CE enantioseparation of dipeptides and tripeptides (2-hydroxy-3-trimethylammoniumpropyl-β-CD (Süß et al., 2002)), various carboxylic and hydroxy acids (diamino-β-CDs (Galaverna et al., 2001)), dansyl amino and hydroxy acids or profens (pyrrolidinium-β-CD (Xiao et al., 2010), mono-6^{A}-(2-methoxyethyl-1-ammonium)-6^{A}-β-CD (Wang et al., 2013) and mono-6^{A}-propylammonium-β-CD (Tang et al., 2006)), N-acyldehydroamino acids (6^{I}-deoxy-6^{I}-methylamino-β-CD (Mikuš & Kaniansky, 2002)), hydroxyl acids (mono-6^{A}-(2-hydroxyethyl-1-ammonium)-6^{A}-β-CD (Zhou et al., 2013)) and many other model compounds (Cucinotta et al., 2010; Tsioupi et al., 2013). Some compounds exhibited better enantioseparation, when non-aqueous CE was used, for example ketoprofen with quaternary ammonium β-CD (Wang & Khaledi, 1998).

PEMEDA-β-CD (Fig. 1, $n = 2$) is a mono-substituted doubly positively charged derivative of

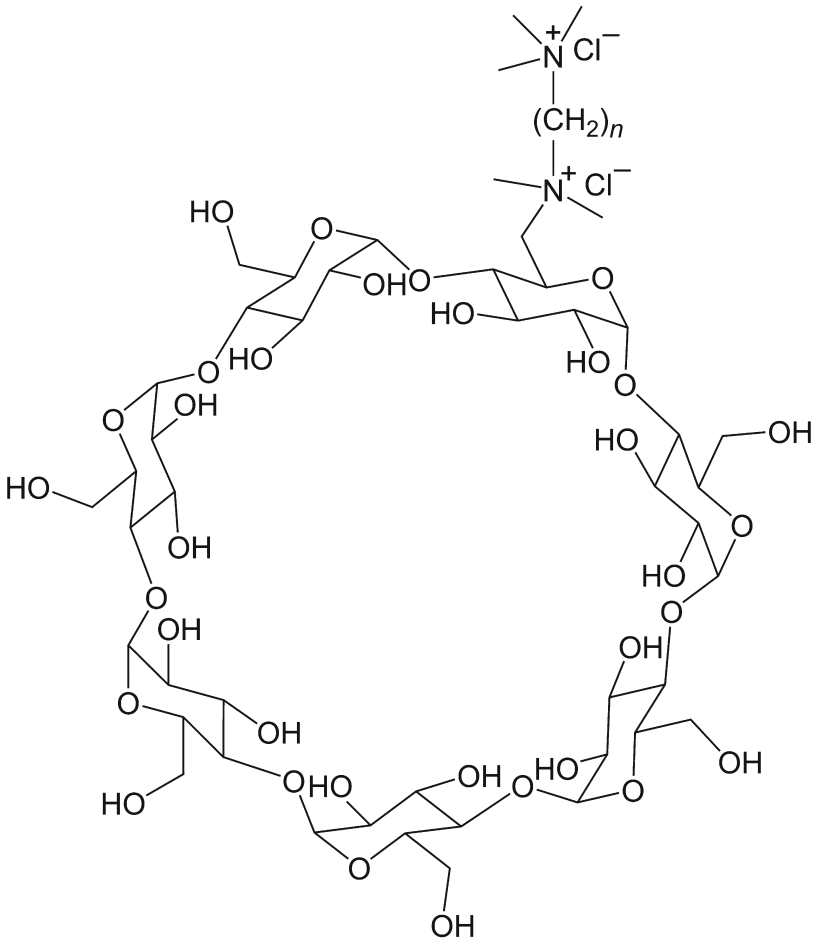


Fig. 1. Structural formula of PEMEDA-β-CD (n = 2) and PEMPDA-β-CD (n =3).

β-CD which has only one glucose unit at the C-6 position attached to the N,N,N',N',N'-pentamethyl-ethylene-1,2-diammonium group. This substituent contains two quaternary ammonium groups interlinked by two carbon atoms. Nzeadibe and Vigh (2007) tested the chiral properties of mono-substituted PEMEDA-β-CD as an additive in CE on a group of selected analytes; however, the synthesis of all homologues was described in detail by Popr et al. (2014).

PEMPDA-β-CD (Fig. 1, n = 3), bearing the N,N,N',N',N'-pentamethyl-propylene-1,3-diammonium group at the C-6 position, differs from PEMEDA-β-CD only in the length of the carbon chain (three carbon atoms) between two quaternary ammonium groups. Popr et al. (2014) was the first to describe the synthesis of a whole series of these substances (including α- and γ-CD derivatives and derivatives with different numbers of ammonium groups), which they managed to synthesise with high yields and without the need for further pre-purification by chromatographic methods. It follows from the available literature that none of these homologues has been used as an additive in CE.

This experimental work was carried out to test the use of PEMEDA-β-CD and PEMPDA-β-CD as chiral selectors in CE and to compare their enantioselective properties using a group of selected analytes under optimised conditions.

Experimental

Chemicals

PEMEDA-β-CD dichloride and PEMPDA-β-CD dichloride were obtained by the conversion of PEMEDA-β-CD diiodide or PEMPDA-β-CD diiodide using ion-exchange chromatography. PEMEDA-β-CD diiodide or PEMPDA-β-CD diiodide were synthesised using the published procedures, both with a yield of 98 %. Characterisation with determination of the exact positions of the substituents was performed using 2D NMR methods (Popr et al., 2014).

D,L-Fenoprofen (purity $\geq$ 97 %), D,L-suprofen (purity $\geq$ 98.0 %), D,L-ketoprofen (purity $\geq$ 98.0 %), N-Boc-L-tyrosine (purity $\geq$ 98.0 %), N-Boc-D-tryptophan (purity $\geq$ 98.0 %), N-Boc-L-tryptophan (purity $\geq$ 99.0 %), D,L-tryptophan (purity $\geq$ 99.0 %), D,L-phenylalanine (purity 99 %), N-Boc-D-phenylalanine (purity $\geq$ 99.0 %), N-Boc-L-phenylalanine (purity $\geq$ 99.0 %), N-acetyl-L-tryptophan (purity $\geq$ 99.0 %),

Table 1. Concentrations of chiral selectors in various buffers used in CE measurements

Chiral selector	PEMEDA-β-CD	PEMPDA-β-CD		
Buffer	Borate[a]	Borate[a]	Phosphate[b]	Phosphate[c]
c^d/(mmol L^{-1})	0.1 0.5 1.0 2.5 5.0 – –	0.1 0.5 1.0 1.5 2.5 3.0 5.0	1.0	1.0

a) pH = 9.5; *b*) pH = 8.5; *c*) pH = 6.5; *d*) concentration of chiral selector.

Table 2. Results obtained by TLC for non-electrolysed and electrolysed solutions of PEMEDA and PEMPDA-β-CD in the form of dichlorides after application of voltage of 15 kV

t^a/min	I^b_{PEMEDA}/μA	I^c_{PEMPDA}/μA	PEMEDA-ST[d]	PEMEDA-SA[e]		PEMPDA-ST[f]	PEMPDA-SA[g]	
			R^h_{F}	R^i_{F1}	R^j_{F2}	R^h_{F}	R^i_{F1}	R^j_{F2}
0	50.3	47.6	0.48	0.48	nd	0.38	0.38	nd
30	48.1	44.4	0.48	0.45	nd	0.38	0.38	nd
60	44.5	42.0	0.48	0.45	nd	0.38	0.35	nd
90	40.4	39.4	0.48	0.48	nd	0.38	0.35	0.65
120	36.0	35.6	0.45	0.45	nd	0.38	0.38	0.68
150	30.9	31.4	0.48	0.45	0.83	0.35	0.35	0.68
180	22.7	27.7	0.45	0.48	0.85	0.38	0.35	0.65

a) Time of voltage application; *b*) current passing through PEMEDA-β-CD solution; *c*) current passing through PEMPDA-β-CD solution; *d*) PEMEDA standard; *e*) PEMEDA sample; *f*) PEMPDA standard; *g*) PEMPDA sample; *h*) retardation factor of non-electrolysed solution of CD derivative; *i*) retardation factor of non-decomposed selector in electrolysed sample; *j*) retardation factor of degradation product; nd – not detected.

N-acetyl-D,L-tryptophan (purity ≥ 99.0 %), *N*-acetyl-D-phenylalanine (purity 99 %), *N*-acetyl-L-phenylalanine (purity 99 %), mandelic acid (purity 99 %), *R*-(−)-mandelic acid (purity 98 %), D,L-3-phenyllactic acid (purity 98 %), L-(−)-3-phenyllactic acid (purity 98 %), *N*-benzoyl-L-phenylalanine (purity ≥ 99.0 %), *N*-benzoyl-D-phenylalanine (purity ≥ 99.0 %), methanol (MeOH) for HPLC (purity ≥ 99.9 %) and acetonitrile for HPLC (purity ≥ 99.9 %) were purchased from Sigma–Aldrich (St. Louis, USA). D,L-Tyrosine (purity 99 %) and *N*-Boc-D-tyrosine (purity ≥ 98.0 %) were obtained from Fluka (Buchs, Switzerland). Disodium tetraborate (p.a. purity) and potassium dihydrogen phosphate (p.a. purity) were purchased from Lachema (Brno, Czech Republic). Sodium hydroxide (NaOH) (p.a. purity), 35 % hydrochloric acid (HCl) (p.a. purity), 96 % sulfuric acid (p.a. purity), ammonium acetate (p.a. purity), tris(hydroxymethyl)aminomethane (purity > 98 %) were obtained from Lach-Ner (Neratovice, Czech Republic). Potassium hydroxide (KOH) (p.a. purity) and acetic acid (99.8 %) (p.a. purity) were supplied by Penta (Chrudim, Czech Republic). Milli Q deionised water was obtained using an Millipore instrument (Milford, USA).

Preparation of standards and buffers

The buffers were prepared from an aqueous solution of disodium tetraborate decahydrate, potassium dihydrogen phosphate or tris(hydroxymethyl)aminomethane with the required concentrations and the pH of the solutions was then adjusted using 0.1 mol L^{-1} NaOH, 1 mol L^{-1} KOH or diluted 36 % HCl in a ratio of 1 : 1 with water.

Prior to use, all the buffers were filtered through Nylon 66 Membranes 0.2 μm × 47 mm commercial filters from Supelco Analytical (Bellefonte, USA) to prevent plugging of the capillary.

The solutions of CD derivatives prepared by dissolving the appropriate amount of the selector in the background electrolyte to obtain the required concentrations are summarised in Table 1. Solutions with a chiral selector had to be prepared afresh each week and frequently changed during measurement because of the instability of the chiral selector (see below).

Stock solutions of standards of the tested analytes with a concentration of 0.5 mg mL^{-1} were prepared. Fenoprofen, suprofen and ketoprofen were dissolved in 100 % MeOH and the other standards in a 1 : 1 mixture of pure MeOH and the appropriate buffer.

Table 3. Separation parameters obtained for selected analytes in 15 mmol L^{-1} borate buffer (pH 9.5) with various concentrations of added PEMPDA-β-CD

Analyte	$c_{\text{PEMPDA-}\beta\text{-CD}}$/(mmol L^{-1})												
	0	1				2.5				5			
	μ_{ef}^{a}	μ_{EOF}^{b}	μ_{ef1}^{c}	μ_{ef2}^{d}	α^{e}	μ_{EOF}^{b}	μ_{ef1}^{c}	μ_{ef2}^{d}	α^{e}	μ_{EOF}^{b}	μ_{ef1}^{c}	μ_{ef2}^{d}	α^{e}
D,L-fenoprofen[f]	−1.85	1.65	−0.53	−0.53	1.00	1.32	−0.07	−0.07	1.00	1.18	0.44	0.44	1.00
D,L-suprofen[g]	−2.02	1.63	−0.57	−0.57	1.00	1.18	−0.06	−0.06	1.00	1.25	0.24	0.24	1.00
D,L-ketoprofen[g]	−2.00	1.83	−1.26	−1.28	1.06	1.23	−0.33	−0.34	1.08	1.18	−0.04	K	–
D,L-tyrosine[f]	−1.56	1.62	−0.94	−0.94	1.00	1.35	−0.93	−0.94	1.04	1.11	−0.71	−0.73	1.08
N-Boc-D-tyrosine[f]	−1.96	1.62	−1.17	–	–	1.29	−0.49	–	–	1.04	−0.21	–	–
N-Boc-L-tyrosine[f]	−1.78	1.74	–	−1.24	–	1.33	–	−0.65	–	0.98	–	−0.20	–
N-Boc-D,L-tyrosine[f,h]	−1.63	1.56	−1.12	−1.15	1.11	1.38	−0.67	−0.71	1.13	0.95	−0.22	−0.23	1.10
D,L-tryptophan[f]	−1.15	1.46	−0.97	−0.97	1.00	1.31	−0.97	−0.97	1.00	1.12	−0.54	−0.54	1.00
N-Boc-D-tryptophan[f]	−1.84	1.60	−0.86	–	–	1.29	−0.31	–	–	1.01	−0.04	–	–
N-Boc-L-tryptophan[f]	−1.86	1.80	–	−1.10	–	1.31	–	−0.44	–	1.00	–	−0.10	–
N-Boc-D,L-tryptophan[f,h]	−1.75	1.65	−1.00	−1.17	1.59	1.38	−0.30	−0.46	1.85	1.02	−0.03	−0.15	5.44
D,L-phenylalanine[f]	−1.63	1.96	−1.11	−1.11	1.00	1.42	nd	nd	–	1.13	nd	nd	–
N-Boc-D-phenylalanine[f]	−2.04	1.67	−1.20	–	–	1.39	−0.60	–	–	1.02	−0.21	–	–
N-Boc-L-phenylalanine[f]	−2.06	1.60	–	−1.23	–	1.35	–	−0.62	–	1.25	–	−0.15	–
N-Boc-D,L-phenylalanine[f,h]	−2.07	1.84	−1.33	−1.38	1.16	1.38	−0.57	−0.64	1.24	1.22	−0.13	−0.19	1.55

a) Electrophoretic mobility of analyte; *b*) magnitude of EOF; *c*) electrophoretic mobility of faster enantiomer; *d*) electrophoretic mobility of slower enantiomer; *e*) enantioselectivity; *f*) measured at wavelength of 214 nm; *g*) measured at wavelength of 254 nm; *h*) ratio of D- and L-form of 1 : 2; nd – not detected; K – coelution with a marker of EOF; all μ values are given in 10^{-4} cm^2 V^{-1} s^{-1}.

For analysis, it was necessary to further dilute the samples with the buffer to a final concentration of 0.25 mg mL^{-1}.

CE measurements

The electrophoretic measurements were performed on a PrinCE 250 autosampler (PrinCE Technologies, Emmen, the Netherlands) using a UV-VIS spectrophotometric detector Spectra 100 (Thermo Separation Products, Waltham, USA) using an unmodified fused silica capillary with an internal diameter of 75 μm and an external diameter of 375 μm (CACO, Slovakia), with an overall length of 66.5 cm and an effective length of 51.5 cm. The samples were injected at a pressure of 10 kPa for 2.5 s. A voltage of 15 kV was applied to the capillary, which was thermostated at 20 °C. The UV absorption spectra were recorded at wavelengths of 214 nm, 254 nm or 280 nm depending on the analyte measured. The MeOH in which the samples were dissolved was used as a marker of electroosmotic flow (EOF). Prior to the initial use, the capillary was activated by rinsing with 1 mol L^{-1} NaOH solution for 1 h and then for 10 min with deionised water. Every day, at the beginning of measurement, the capillary was conditioned by rinsing with deionised water for 2 min, then with a 1 mol L^{-1} NaOH solution for 4 min, and then again with deionised water for 2 min and finally with the background electrolyte for 6 min. Between the individual measurements the capillary was rinsed for 2 min with the solution of the background electrolyte, as appropriate with the addition of the chiral selector. After the final measurement, the capillary was stored overnight without the background electrolyte after previous rinsing: 2 min with deionised water, 3 min with 1 mol L^{-1} NaOH solution, 6 min with deionised water and 6 min with air. All conditional pre-treatments were carried out at a pressure of 100 kPa.

Software Clarity (DataApex, Prague, Czech Republic), Origin 8 (OriginLab, Northampton, USA) and Microsoft Excel 2003 (Microsoft, Redmond, USA) were used to evaluate the data.

Detection of decomposition products of CD derivatives

The solutions of PEMPDA-β-cyclodextrin and PEMEDA-β-cyclodextrin were prepared with a concentration of 2.5 mmol L^{-1} in 15 mmol L^{-1} borate buffer at pH 9.5 with a volume of 200 μL, to which a voltage was applied (test solutions), and the identical solutions to which a voltage was not applied; these were intended as stock solutions for comparison. In each case, a voltage of 15 kV was applied to the test solutions for 4 h and every 30 min, 5 μL samples were taken from the input vial and used for thin-layer chromatography (TLC). The TLC separation system consisted of silica gel plates (TLC Silica gel 60 F_{254}, Merck, Darmstadt, Germany) with a length of 4 cm and a mixture of MeOH, acetic acid and 1 % aqueous solution of ammonium acetate ($\varphi_r = 10 : 1 : 9$) as the developing system. The test sample of the individual

Table 4. Separation parameters obtained for selected analytes in 15 mmol L^{-1} borate buffer (pH 9.5) with various concentrations of added PEMEDA-β-CD

Analyte	$c_{\text{PEMEDA-}\beta\text{-CD}}$/(mmol L^{-1})												
	0	1				2.5				5			
	μ_{ef}^{a}	μ_{EOF}^{b}	μ_{ef1}^{c}	μ_{ef2}^{d}	α^{e}	μ_{EOF}^{b}	μ_{ef1}^{c}	μ_{ef2}^{d}	α^{e}	μ_{EOF}^{b}	μ_{ef1}^{c}	μ_{ef2}^{d}	α^{e}
D,L-fenoprofen[f]	−1.85	1.65	−0.40	−0.40	1.00	1.34	−0.06	−0.06	1.00	1.11	0.35	0.35	1.00
D,L-suprofen[g]	−2.02	1.57	−0.65	−0.65	1.00	1.53	0.05	0.05	1.00	1.10	0.26	0.26	1.00
D,L-ketoprofen[g]	−2.00	1.70	−0.83	−0.86	1.06	1.19	−0.31	−0.33	1.07	1.09	−0.03	K	–
D,L-tyrosine[f]	−1.56	1.63	−1.32	−1.32	1.00	1.38	−0.94	−0.94	1.00	1.04	−0.64	−0.64	1.00
N-Boc-D-tyrosine[f]	−1.96	1.67	−1.17	–	–	1.46	−0.71	–	–	1.10	−0.37	–	–
N-Boc-L-tyrosine[f]	−1.78	1.82	–	−1.33	–	1.56	–	−0.83	–	1.06	–	−0.39	–
N-Boc-D,L-tyrosine[f,h]	−1.63	1.64	−1.16	−1.19	1.09	1.30	−0.67	−0.70	1.10	1.03	−0.27	−0.30	1.16
D,L-tryptophan[f]	−1.15	1.71	−1.13	−1.13	1.00	1.34	−0.98	−0.98	1.00	1.12	nd	nd	–
N-Boc-D-tryptophan[f]	−1.84	1.82	−1.11	–	–	1.45	−0.50	–	–	1.12	−0.11	–	–
N-Boc-L-tryptophan[f]	−1.86	1.77	–	−1.21	–	1.42	–	−0.62	–	1.06	–	−0.19	–
N-Boc-D,L-tryptophan[f,h]	−1.75	1.69	−1.08	−1.26	1.65	1.56	−0.56	−0.77	1.74	1.12	−0.10	−0.24	2.69
D,L-phenylalanine[f]	−1.63	1.75	−1.21	−1.21	1.00	1.46	nd	nd	–	1.13	nd	nd	–
N-Boc-D-phenylalanine[f]	−2.04	1.78	−1.29	–	–	1.42	−0.75	–	–	1.14	−0.31	–	–
N-Boc-L-phenylalanine[f]	−2.06	1.67	–	−1.31	–	1.51	–	−0.80	–	1.14	–	−0.32	–
N-Boc-D,L-phenylalanine[f,h]	−2.07	1.54	−1.18	−1.24	1.23	1.35	−0.78	−0.85	1.22	1.09	−0.23	−0.28	1.28

a) Electrophoretic mobility of analyte; *b*) magnitude of EOF; *c*) electrophoretic mobility of faster enantiomer; *d*) electrophoretic mobility of slower enantiomer; *e*) enantioselectivity; *f*) measured at wavelength of 214 nm; *g*) measured at wavelength of 254 nm; *h*) ratio of D- and L-form of 1 : 2; nd – not detected; K – coelution with a marker of EOF; all μ values are given in 10^{-4} cm^2 V^{-1} s^{-1}.

CD derivatives to which the voltage was applied together with a reference sample was separated on the thin layer. After drying the layer, the chromatographic spots were detected using carbonisation detection performed by immersing the plates in 50 % sulfuric acid with subsequent heating.

Results and discussion

Selection of background electrolyte

With a view to promoting dissociation of the carboxyl groups present in the analyte molecules, the selection of the separation buffer and its pH values was oriented towards the alkaline region. The properties of the buffer were tested on three representatives from individual groups of selected analytes (D,L-ketoprofen as profen, D,L-tyrosine as native amino acid and *N*-Boc-D-tyrosine as amino acid blocked with *tert*-butoxycarbonyl on the amino functional group).

Different buffers with or without addition of the CD derivative were tested: 25 mmol L^{-1} TRIS-chloride buffer, pH 9.5, 25 mmol L^{-1} phosphate buffer, pH 8.5 or 6.5 and 25 mmol L^{-1} or 15 mmol L^{-1} borate buffer, pH 9.5. Of all the buffers tested, only borate buffers appeared to be suitable. When 25 mmol L^{-1} of borate buffer was used, an increase in the voltage or increase in the concentration of the chiral selector led to exceeding the current value of 100 μA, even when an organic modifier was added to the background electrolyte solution. Hence, 15 mmol L^{-1} of borate buffer at pH 9.5 without addition of an organic modifier was chosen as a suitable electrolyte. Tests were also performed with the addition of MeOH and acetonitrile to the background electrolyte with the addition of a selector of 10 vol. %. However, it was concluded that this addition did not contribute to improving the separation but only to its prolongation because of the retarded EOF.

Verification of stability of chiral selectors

Measurement under optimal conditions was also carried out using PEMPDA-β-CD in the form of the diiodide. This chiral selector is probably decomposed almost immediately after applying the voltage to the electrodes, as there was a rapid decrease in the current value and the analysis could not be performed. Accordingly, TLC was used to monitor the stability of the solution of chiral selectors in the form of dichlorides after application of the voltage. A decrease in the absorbance was recorded with a simultaneous decrease in the current over time. The current values measured over three hours and the retardation factor calculated for the detected spots are summarised in Table 2.

From measurement of PEMPDA-β-CD in the form of the dichloride, it was apparent that the CD derivative is partially decomposed after 1.5 h. Initially, units of percent of the original amount decomposed; however, up to half the selector is decomposed after 2 h or more. The assumed degradation product was less polar and its retardation factor was higher than that of the non-decomposed selector. Conflicting with the expectation that PEMEDA-β-CD would be less resistant to decomposition than PEMPDA-β-CD because of the shorter distance between the positively charged

nitrogen atoms in the substituent, it was found from the measurement of PEMEDA-β-CD in the form of the dichloride that this selector was partially decomposed after more than two hours of measurement. After 150 min, approximately 10 % of the selector was decomposed and, after 3 h, approximately a third of the selector was decomposed. Once again, the migration of the degradation product on the thin layer was more rapid because of its lower polarity. Both selectors are probably degraded because of the elevated temperature and high pH of the solution in which the selector is dissolved. Because the quaternary ammonium salt is characterised by lower stability in such a medium, Hofmann elimination occurs, with the formation of an olefin and an amine (Cope & Mehta, 1963). Popr et al. (2015) came to similar conclusions when they tested the stability of mono-substituted tetraalkylammonium-CD derivatives in the form of diiodides at elevated temperatures and in a NaOH solution. The degradation products were further separated and identified. Subsequently, ^{1}H NMR at 50 °C in the presence of NaOH was used to determine the decomposition half-life of the two derivatives. The decomposition half-life was 7.9 h for PEMEDA-β-CD in the form of the diiodide and 20.1 h for PEMPDA-β-CD in the form of the diiodide. While PEMEDA-β-CD decomposed more rapidly, steady-state decomposition occurred sooner, while PEMPDA-β-CD decomposed more slowly and without reaching a steady state. Hence it may be concluded that not only the temperature and pH of the solution but also whether or not a current is passing through the solution and which kind of salt is present in the solution have a substantial influence on the decomposition of the chiral selectors.

Effect of concentration of chiral selector on electroosmotic mobility

The magnitude of the EOF was monitored using MeOH as a marker, because it was always present as a solvent in the sample solutions. The addition of a chiral selector to the background electrolyte had a substantial effect on the magnitude of the EOF even at very low selector concentrations. The concentrations of the individual chiral selectors were gradually increased from zero up to 5.0 mmol L^{-1}. As the concentration increased, the EOF became slower, as is shown for PEMPDA-β-CD in Fig. 2. The dependence was almost identical for the second selector, hence no difference was observed between the individual chiral selectors. The effect on the EOF could be caused by adsorption of the chiral selector on the wall of the silica capillary because of the two positive charges in the structure of the selector. It was not possible to determine the electrophoretic mobility of the CD derivatives alone because the selector did not contain any chromophore suitable for UV detection. Because of

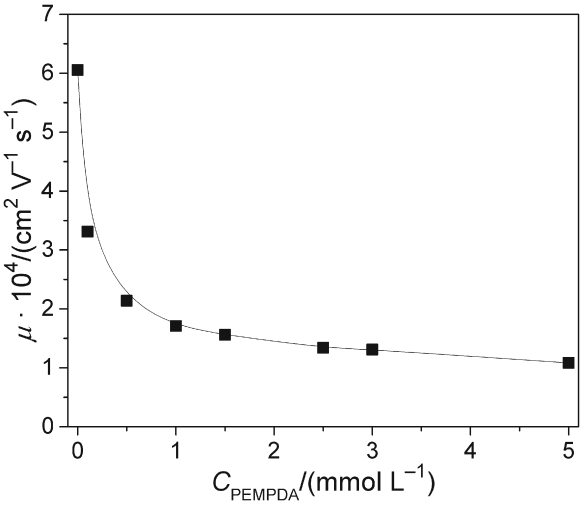


Fig. 2. Effect of PEMPDA-β-CD concentration on magnitude of EOF.

the permanent positive charge of the molecule, it can be anticipated that the actual mobility of PEMPDA- and PEMEDA-β-CD alone will be positive and independent of the pH value of the medium.

PEMPDA-β-CD as chiral selector in CE

The mobilities of all the analytes after the addition of PEMPDA-β-CD in the form of the dichloride to the background electrolyte of concentrations of 1.0 mmol L^{-1}, 2.5 mmol L^{-1} and 5.0 mmol L^{-1} were measured under optimal conditions (15 mmol L^{-1} borate buffer at pH 9.5 without the addition of organic modifier). The selected analytes contain a suitable chromophore as well as an ionisable functional group, preferably carboxyl. Because the chiral selector has permanently positively charged groups, the carboxyl group made it possible to promote the potential for electrostatic interaction between the selector and the analyte at a suitable pH. In order to further promote these interactions, one of the selected groups was amino acids, which have an amino group blocked by various functional groups.

Table 3 lists all the analytes that were detected in at least one of the selected concentrations of the chiral selector, even when separation of the individual enantiomers did not occur.

Mandelic acid, *N*-acetyltryptophan, *N*-acetylphenylalanine, *N*-benzoylphenylalanine, and phenyllactic acid were not detected at all in solutions with the addition of a chiral selector (measured at a wavelength of 214 nm with the exception of *N*-acetyltryptophan detected at 280 nm). In this case, there was probably minimal or no interaction with the CD derivative because of the substantial retardation of the EOF; because of their presence in the anionic forms at the selected pH, their migration time was so long that no

response was recorded even after 2 h. The peaks could be registered only when the effect of the electric field was augmented by a pressure of 2 kPa to increase the speed of the electrolyte. The analysis then proceeded more rapidly but enantioseparation did not occur.

D,L-Fenoprofen and D,L-suprofen interacted with the selector and an increase in the selector concentration to 5.0 mmol L^{-1} led to a very good interaction with the selector, as manifest in the positive electrophoretic mobility of fenoprofen and suprofen which, however, was not enantioselective. D,L-Ketoprofen interacted with the selector in a different way, which meant that its enantiomers began to separate slightly. However, as the concentration increased, the peaks only became narrower and the enantioselectivity did not improve. In addition, at the highest concentration of the selector, i.e. 5.0 mmol L^{-1}, the zone of ketoprofen co-eluted with the zone corresponding to the marker for the EOF, i.e. MeOH, and it was not possible to precisely define the top of the peak(s). Standards were not available for the individual enantiomers. Only the racemate was available, hence it was not possible to determine which peak corresponded to the D- and which to the L-form.

Out of the unblocked amino acids, the best results were obtained for D,L-tyrosine. The separation of the enantiomers occurred with an increasing concentration of cyclodextrin derivative. D,L-Tryptophan interacted with the selector but the enantiomers were not separated and the weakest interaction without separation occurred for D,L-phenylalanine. Standards for the individual enantiomers were not available for any of the unblocked amino acids hence, for D,L-tyrosine, being the only one to exhibit at least partial separation, it was not possible to determine which peak corresponded to the D-form and which to the L-form.

The best results were obtained for amino acids blocked by *tert*-butoxycarbonyl on the amino functional group. Consequently, its mixtures of enantiomers in a ratio of 1 : 2 (D : L) were also measured in solutions with other concentrations of chiral selectors.

For phenylalanine blocked with *tert*-butoxycarbonyl, interaction with the chiral selector occurred at low concentrations and was apparent in the partial separation of the individual enantiomers. The enantioseparation did not improve significantly with increasing selector concentration. There was only a decrease in the analysis time. The ratio of the individual peaks indicated that the D-form of *N*-Boc-phenylalanine interacted more with PEMPDA-β-CD.

In the presence of PEMPDA-β-CD, *N*-Boc-D,L-tyrosine is only partly separated and its interaction with the chiral selector was the weakest of the thus-blocked selected amino acids. As with *N*-Boc-D,L-phenylalanine, increasing the concentration of the selector did not lead to any substantial improvement in the enantioseparation. Maximum enantioselectivity was achieved at a selector concentration of

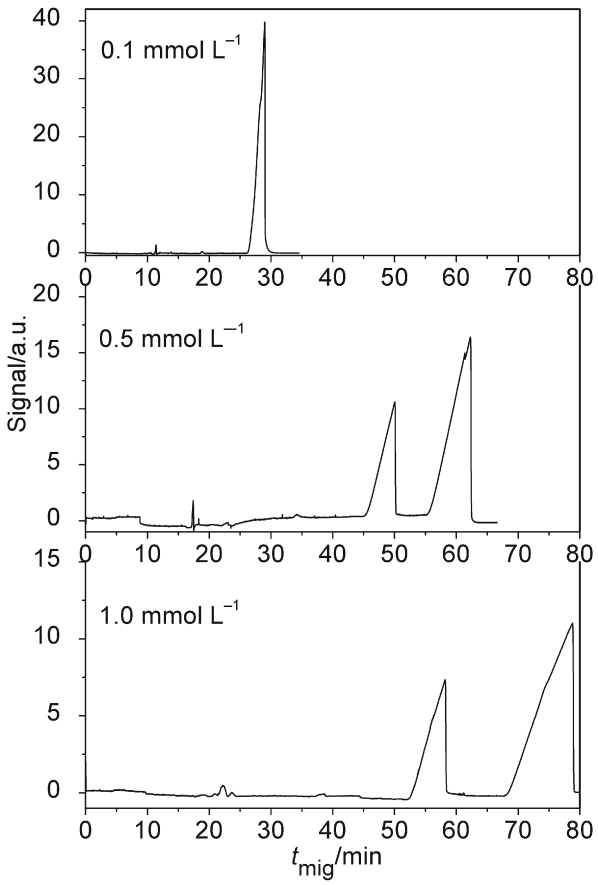


Fig. 3. Electropherograms of enantioseparation of *N*-Boc-D,L-tryptophan in ratio of D- and L-forms of 1 : 2 measured at various concentrations of PEMPDA-β-CD added to 15 mmol L^{-1} borate buffer at pH 9.5; applied voltage: 15 kV; UV detection at 214 nm.

2.5 mmol L^{-1}. It was determined from the ratio of the peaks that, once again, the D-form of *N*-Boc-tyrosine interacted with PEMPDA-β-CD more than the L-form.

The best results were obtained for *N*-Boc-D,L-tryptophan, where the individual enantiomers were already separated on the baseline at 0.5 mmol L^{-1} concentration of PEMPDA-β-CD. It was found that the addition of only 0.1 mmol L^{-1} PEMPDA-β-CD substantially affected the magnitude of the EOF and the shape of the peak indicated that the D- and L-forms were beginning to separate. An increase in the selector concentration led to a better interaction of the individual enantiomers with the selector and also the negative mobility decreased. It was found from the ratio of the areas of the individual peaks that *N*-Boc-D-tryptophan interacted more strongly with the chiral selector (see Fig. 3). However, an increase in the concentration to 5 mmol L^{-1} meant that the separated *N*-Boc-D-tryptophan entered the zone of the solvent (MeOH), rendering it more difficult to evaluate the separation.

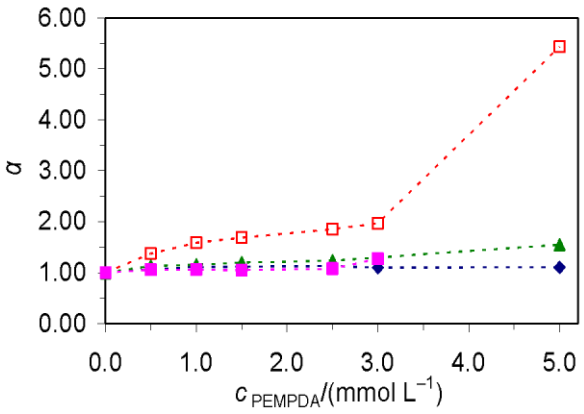


Fig. 4. Effect of concentration of PEMPDA-β-CD on enantioselectivity of *N*-Boc-D,L-tryptophan (□), *N*-Boc-D,L-phenylalanine (▲), *N*-Boc-D,L-tyrosine (◆) and D,L-ketoprofen (■).

It can be concluded that PEMPDA-β-CD in the form of the dichloride would be useful as a new synthetic chiral selector, especially in the enantioseparation of *N*-Boc-D,L-tryptophan in CE while using borate buffer at high pH values, because enantioseparation did not occur at lower pH values. Overall, it may be concluded that *tert*-butoxycarbonyl is useful as a protective group for amino groups in the interaction of these substances with the selector, as shown in Fig. 4. Hence it is probable that electrostatic interactions have a significant effect on the separation. From the values of the electrophoretic mobilities of the individual enantiomers and also of the mixtures, it is apparent that the D-enantiomer of amino acids blocked with this group affords a stronger interaction with the selector than the L-enantiomer. However, because of the substantial effect on the electroosmotic flow, probably caused by the interaction of the selector with the capillary wall, and the need to continually prepare fresh buffer and chiral selector solutions, the application of PEMPDA-β-CD as chiral selector in CE appears to be limited.

To date, the literature contains only one study of the properties of this CD derivative. Popr et al. (2015) tested the inclusion properties and measured the stability constant of inclusion complexes for PEMPDA-β-CD in the form of the diiodide and compared the values measured with the parameters measured under the same conditions for native β-CD. They carried out the measurement for three aromatic molecules containing various functional groups in a phosphate buffer at three different pH values and used the isothermal titration calorimetry technique. It was found that the highest stability constant value could be obtained for the interaction of PEMPDA-β-CD with salicylic acid in a buffer at pH 2.5 and that its value was somewhat higher than for native CD. In addition, a CD derivative was successfully immobilised on negatively charged plates and they noted that salicylic acid most readily occupied the selector cavities. Hence, they also found electrostatic interactions to be important in the separation.

PEMEDA-β-CD as chiral selector in CE

The same group of analytes as for PEMPDA-β-CD were also measured under optimised conditions with the second chiral selector. Table 4 presents the electrophoretic mobilities of all the analytes, for which they were measured at three different concentrations, even where enantioseparation did not occur.

Minimal differences were observed between the individual selectors. The greatest difference was found in the analysis of D,L-tyrosine, which did not even begin to partially separate with an increasing concentration of PEMEDA-β-CD.

Only partial separation of the individual enantiomers occurred, as with the previous selector, for D,L-ketoprofen, *N*-Boc-D,L-tyrosine and *N*-Boc-D,L-phenylalanine. The best results were again obtained in the separation of *N*-Boc-D,L-tryptophan for which, as with the previous selector, the individual enantiomers already began to separate at a concentration of 0.5 mmol L^{-1} PEMEDA-β-CD.

However, in general, the interactions were somewhat weaker, following from the negative electrophoretic mobility values, especially at higher concentrations of the chiral selector.

The properties of PEMEDA-β-CD were previously recognised, as noted above. Nzeadibe and Vigh (2007) first tested a chiral selector in CE for separation of the enantiomers of a wide range of substances, i.e. various weak acids and bases, selected neutral substances, etc. However, such good enantioselectivity as was achieved for 1.0 mmol L^{-1} PEMEDA-β-CD in the enantioseparation of *N*-Boc-D,L-tryptophan was not obtained for any of the analytes, even at higher selector concentrations or other pH values.

Conclusions

CD derivatives are a constantly developing group of substances with various properties and they can be successfully used in chiral separations. To obtain better reproducibility and selectivity, mono-substituted CD derivatives are preferable; two of these which are, in addition, permanently positively charged, PEMEDA-β-CD and PEMPDA-β-CD, were tested and their properties were compared under optimised conditions. Both selectors were found to have a substantial effect on the magnitude of the EOF and to exhibit good enantioselective properties in the separation of *N*-Boc-D,L-tryptophan under optimised conditions (15 mmol L^{-1} borate buffer at pH 9.5 without the addition of an organic modifier), where the individual enantiomers were already baseline-separated

at a chiral selector concentration of 0.5 mmol L^{-1}. Only minor differences were observed between the selectors, specifically in the separation of D,L-tyrosine, for which partial enantioseparation occurred only when PEMPDA-β-CD was used.

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