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Chemically bonded β-cyclodextrin stationary phase for liquid chromatographic separation of substituted aromatic compounds

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Abstract. The effects of the mobile phase composition and the relative position of substituents in aromatic compounds on the retention behaviour on the β-cyclodextrin bonded copolymer of hydroxylethyl methacrylate and ethylene dimethacrylate (HEMA gels) were investigated. The average β-cyclodextrin amount immobilized was determined by modified Hultmann's method. Increasing the proportion of water in the mobile phase resulted in an increase of the retention times and the relative retention of isomers. The elution order of solute isomers was independent of the methanol concentration in the mobile phase. D-, L-, and D,L-tryptophan were used to estimate the ability of the β-cyclodextrin bonded HEMA gel to separate optical isomers. It was found that the particle size, particle size distribution, and the length of the spacer influenced the efficiency of the column.

Key words: liquid chromatography, HEMA gel, β-cyclodextrin, aromatic compounds.

INTRODUCTION

Cyclodextrins, which are known to be cyclic oligosaccharides consisting of six or more α-(1,4)-linked D-glucopyranose units, form stable inclusion complexes with a variety of organic molecules both in the solid state and in aqueous solutions [1]. The stability of such inclusion is, in general, most closely related to the fitness of the size of guest molecules to that of the cavity of cyclodextrin units, although many other factors such as Van der Waals forces, dipole–dipole interaction, hydrogen bonding, and hydrophobic interaction also play a role in determining the ease of complex formation. The general scheme of complex formation can be expressed as follows:

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$$C + S \leftrightarrow C \cdot S,$$

where C is cyclodextrin, S is a guest molecule, and $C \cdot S$ is a cyclodextrin–guest molecule complex.

The complex formation constant can be found from

$$K_D = \frac{[C] \cdot [S]}{[C \cdot S]}.$$

If two different guest molecules S^1 and S^2 compete for cyclodextrin cavity simultaneously, the complex formation can be described as:

$$\begin{array}{ccc} & C + S^1 + S^2 & \\ K_{D1} \swarrow\nearrow & & \nwarrow\searrow K_{D3} \\ & K_{D2} & \\ C \cdot S^1 + S^2 & \leftrightarrow & C \cdot S^2 + S^1 \end{array}$$

Cyclodextrin acts like an enzyme, with active sites associated both with the substrate and the inhibitor. The dissociation constants from the scheme above reflect the stability of the complex. The dissociation constants can be determined using spectroscopy and kinetic methods [2].

Many attractive features of cyclodextrin, covalent or noncovalent catalysis, and enantiomeric catalysis arise from the specific interaction between cyclodextrin units and guest molecules [3]. Cyclodextrins and their derivatives have been successfully employed as chiral selectors for analytical separations in gas chromatography (GC) and liquid chromatography (LC). During the chromatographic separation process the underivatized or derivatized cyclodextrins as host molecules form intermediate diastereomeric complexes with chiral guest molecules; differences in the stability of these complexes determine the enantioselectivity of separations. With liquid chromatographic enantiomer separations the chiral selector may either be added to the (aqueous) mobile phase or may be contained in the stationary phase, preferably fixed to the surface either by SiO bonds or as substituents in polymers, which form an immobilized coating on such a surface. In GC and LC the interaction of the chiral substrate with the selector is usually realized on a coated solid surface. Because of the immobilization of the selector on the support surface and at the low separation temperatures in LC, high enantioselectivities are achieved. In GC the enantioselectivities are lower than in LC because the separations of larger and more polar molecules can only be executed at temperatures at which these compounds are volatile enough to be present at a reasonable vapour pressure in the gaseous mobile phase for fast mass transport through the column [4].

Substituted aromatic compounds were separated successfully on silicagel with chemically bonded β-cyclodextrin. The silica activation was done using [3-[(2-aminoethyl)amino]propyl] trimethoxysilane [3, 5, 6].

To overcome the pH limits for silicagel sorbents a wide range of polymer sorbents are used [7, 8]. The HEMA gels (copolymers of hydroxylethyl metha-

crylate and ethylene dimethacrylate) enable to apply alkaline eluent at pH above 7 and their chemical modification can be easily done using conventional methods. There is no need to dry the sorbent before chemical bonding and to use a dry box.

Regardless of the above-mentioned advantages there are few attempts to separate the substituted aromatic compounds on HEMA gels with chemically bonded β-cyclodextrin.

Both β- and α-cyclodextrin (β-CD and α-CD) can form inclusion complexes with derivatized and underivatized amino acids. α-CD is significantly smaller than β-CD and tends to complex many small molecules more tightly than β-CD. Thus, there exists a possibility that smaller racemate moleculs, which cannot be resolved on β-CD bonded phases, can be resolved on α-CD phases [9]. Armstrong et al. separated racematic tryptophan and analogues on an α-CD bonded phase column [10].

EXPERIMENTAL

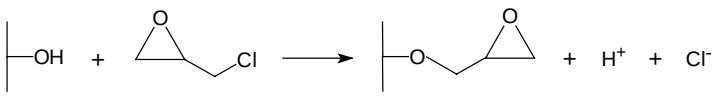
Materials and chromatography

HEMA gels were purchased from TESSEK (Czech Republic). Solvent (methanol) and reagents were of analytical grade and were purchased from Aldrich. All chromatographic studies were carried out with a system consisting of a Model INKROM IP-2 (Estonia) and a Model JASCO UV-975 variable-wavelength UV–VIS detector (Japan). Each stationary phase was packed by slurry method into a 150 × 3 mm i.d. TESSEK glass column. The mixtures of methanol–water (80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, and 0/100) were used as eluents at flow rates of 1.0 mL/min (sorbent I) and 0.5 mL/min (sorbent II). The retention times of ortho-, meta-, and para-toluidine; ortho-, meta-, and para-nitroaniline; ortho- and para-nitrophenol; D-, L-, and D,L-tryptophan were measured.

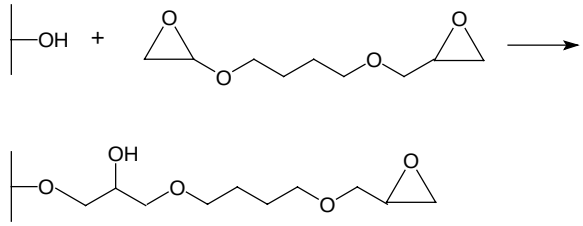
The concentration of sample solutes was generally 0.2 mM. The sample volume of 20 μL was injected. The detector wavelength was adjusted to 254 nm (tryptophan) or 280 nm (toluidine, nitroaniline, nitrophenol).

Preparation of the chemically bonded β-cyclodextrin stationary phase

HEMA gels were epoxy activated according to schemes 1 and 2. HEMA gels were washed with distilled water and suspended in 1 M NaOH solution. Epichlorhydrin and 1,4-butanediol diglycidyl ether were added to prepare sorbents I (particle size 25–40 μm) and II (particle size 10 μm), respectively. The mixture was stirred at room temperature for 10 h.

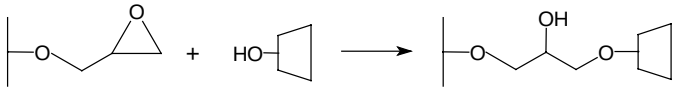


Scheme 1. Activation of HEMA gel with epichlorhydrin (sorbent I).

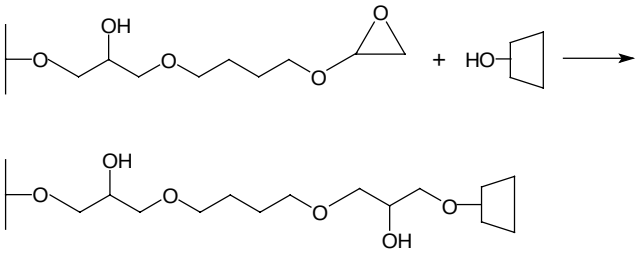


Scheme 2. Activation of HEMA gel with 1,4-butanediol diglycidyl ether (sorbent II).

β-Cyclodextrin was immobilized to the activated sorbent in alkaline media according to schemes 3 and 4. The activated sorbent was filtered and washed with water, and suspended in 0.2 M Na_2CO_3 solution. β-Cyclodextrin and 1 M NaCl solution were added. The mixture was stirred at room temperature for 12 h. The sorbents were filtered and washed thoroughly with water, 0.1 M acetate buffer (pH = 4.0), and methanol, and dried in vacuo at 50 °C for 10 h.



Scheme 3. Immobilization of β-cyclodextrin to sorbent I in alkaline media.



Scheme 4. Immobilization of β-cyclodextrin to sorbent II in alkaline media.

Determination of immobilized β-cyclodextrin

The amount of β-cyclodextrin immobilized was evaluated by a modified Hultmann's method. β-Cyclodextrin (30 mg) and the prepared sorbent (100 mg) were hydrolysed in 2 M H_2SO_4 solution at 100 °C for 5 h. After cooling the 100 μL aliquots were taken to react with 2.5 mL of *o*-toluidine. The derivatives were obtained at 90–95 °C for 5 min. The mixture was cooled and the absorbance was measured on KFK-3 spectrocolorimeter. The resulting quinolidine derivative is a coloured substance with the absorbance maximum at 630 nm.

RESULTS AND DISCUSSION

In general it is not easy to evaluate the concentration of an immobilized substrate. It is quite interesting to compare the β-cyclodextrin sorbent prepared with that of Kawaguchi et al. [3], which was ethylenediamine monosubstituted β-cyclodextrin, abbreviated as β-CD-en. The average amount of β-cyclodextrin was 49.2 μmol/g for β-CD-en-silica gel and 32 μmol/g for β-CD-HEMA gel. When the resolution for ortho, meta, and para isomers increased, the amount of cyclodextrin immobilized increased.

The retention times (t_R) of substituted benzene derivatives on β-CD-HEMA gel were measured by changing the methanol–water ratio in the mobile phase from 100/0 to 20/80. The results are given in Table 1.

The retention times of toluidine isomers were measured using a methanol–water (50/50) mixture. The following capacity factors k' [$k' = (t_R - t_M)/t_M$] were obtained for sorbent I and sorbent II: *o*-toluidine – 0.88 and 1.09, *m*-toluidine – 0.91 and 1.20, and *p*-toluidine – 0.68 and 1.04. The elution order of toluidine isomers on β-CD-HEMA gels was different from that on β-CD-en-silica gel. Kawaguchi's group eluted the solutes with a methanol–water mixture (30/70) and obtained the following capacity factors k' for β-CD-en-silica gel: *o*-toluidine – 0.93, *m*-toluidine – 1.17, and *p*-toluidine – 1.30.

For measuring the retention times of nitroaniline isomers retention was high enough to increase the methanol content to 70%. The capacity factors k' obtained for sorbent I and II were listed: *o*-nitroaniline – 1.71 and 2.07, *m*-nitroaniline – 1.75 and 2.13, and *p*-nitroaniline – 1.79 and 2.28. Kawaguchi's group, using a methanol–water mixture (30/70), obtained the capacity factors k' for nitroaniline isomers as follows: *o*-nitroaniline – 2.20, *m*-nitroaniline – 2.76, and *p*-nitroaniline – 12.80. The elution order of isomers on β-CD-en-silica gel was the same as on β-CD-HEMA gels.

The retention of nitrophenol isomers was even stronger and a mixture of methanol and water (80/20) was used to get the following capacity factors k' for both sorbents: *o*-nitrophenol – 1.23 and 1.58, and *p*-nitrophenol – 2.52 and 2.40. The elution of nitrophenol by Kawaguchi's group was done applying a methanol–water mixture (30/70). The following capacity factors k' of isomers obtained were listed: *o*-nitrophenol – 1.67, *m*-nitrophenol – 3.92, and *p*-nitrophenol – 6.92 [3].

Table 1. Retention times (min) on sorbents I and II at different water–methanol ratios

Compound	Ortho		Meta		Para		Eluent
	I	II	I	II	I	II	
Toluidine	2.05	5.25	2.08	5.50	1.83	5.10	50/50
Nitroaniline	5.48	5.10	5.55	5.20	5.63	5.45	70/30
Nitrophenol	4.28	5.80	–	–	6.75	7.65	80/20

– No result was obtained.

The elution order of isomers for each solute was independent of the methanol content in the mobile phase.

The retention times of isomers are invariable on the unmodified stationary phase as shown in Table 2, indicating that the sample molecules interact primarily after all with cyclodextrin units bonded on the surface of HEMA gels.

The effect of the mobile phase composition on retention times of toluidine and nitroaniline isomers was investigated. The results obtained are given in Table 3.

It was found that the content of water in the mobile phase increased retention times and the relative retention of isomers increased. This improves separation of isomers, but the time of separation will increase as well. These trends are illustrated in figs 1 and 2.

D-, L-, and D,L-tryptophan retention was investigated using distilled water and mixtures of methanol and water (40/60, 20/80). The retention times obtained for sorbent II are given in Table 4. Unfortunately, the difference between the retention times was too small to resolve the diastereomers.

Chromatographic data were listed: capacity factor $k' = (6.02 - 2.56)/2.56 = 1.35$, selectivity $\alpha = 1.04$, and resolution $R_s = 0.67$.

Armstrong et al. [10] using a mixture of water–buffer (pH = 5.1, 1% TEAA) as the mobile phase, obtained the following chromatographic data for D,L-tryptophan on α-CD bonded phase: capacity factor $k' = 2.70$ (capacity factor k' is given only for L-tryptophan), selectivity $\alpha = 1.20$, and resolution $R_s = 1.90$.

Table 2. Retention times (min) on the unmodified stationary phase of sorbent II (eluent 70/30)

Compound	Ortho	Meta	Para
Toluidine	3.0	3.0	3.0
Nitroaniline	4.5	4.5	4.5
Nitrophenol	5.5	–	5.7

– No result was obtained.

Table 3. Retention times (min) of toluidine and nitroaniline isomers at different methanol contents in the mobile phase

Eluent	Toluidine			Nitroaniline		
	Ortho	Meta	Para	Ortho	Meta	Para
70/30	–	–	–	5.10	5.20	5.45
60/40	–	–	–	7.43	7.71	8.40
50/50	5.25	5.50	5.10	–	–	–
40/60	7.52	8.00	7.21	19.86	21.00	23.85
20/80	14.72	15.45	14.28	–	–	–

– No result was obtained.

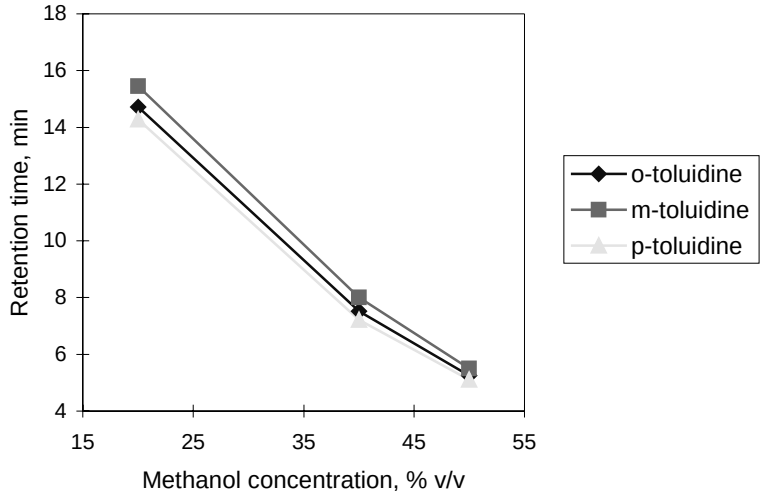


Fig. 1. Effect of the methanol concentration in the mobile phase on the retention times of toluidine isomers.

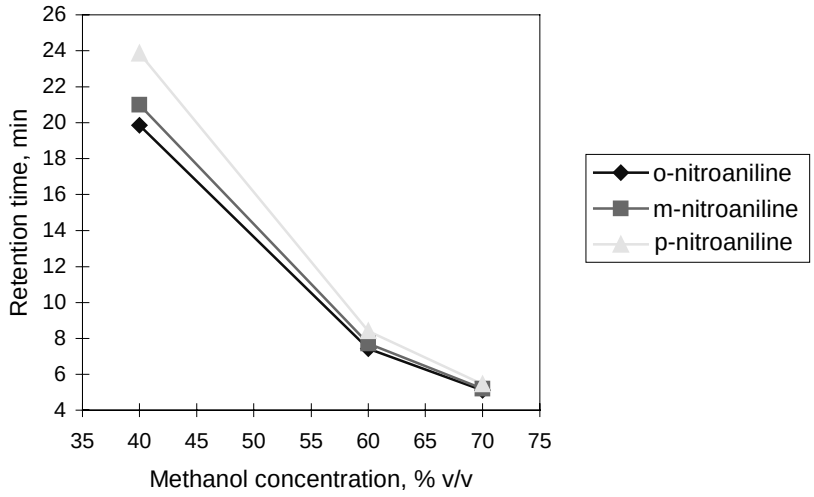


Fig. 2. Effect of the methanol concentration in the mobile phase on the retention times of nitroaniline isomers.

Table 4. Retention times (min) of D-, L-, and D,L-tryptophan on sorbent II

Eluent	D-tryptophan	L-tryptophan	D,L-tryptophan
40/60	4.16	4.05	4.10
20/80	4.52	4.27	4.35
0/100	6.29	6.02	6.15

Comparison of our data with those of Armstrong's group shows that α-CD gives better selectivity than β-CD for separating D,L-tryptophan into diastereomers.

Column efficiency (number of theoretical plates) was calculated from the following equation:

$$N = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2,$$

where $W_{0.5}$ is peak width at half height.

The number of theoretical plates found for sorbent I is very low:

$$N = 5.54\left(\frac{2.10}{0.75}\right)^2 = 47.$$

The number of theoretical plates calculated for sorbent II was sufficiently higher:

$$N = 5.54\left(\frac{5.20}{0.38}\right)^2 = 1037.$$

The selectivity of the separation of para- and ortho-nitroaniline is expressed as:

$$\alpha = \frac{t'_{\mathrm{B}}}{t'_{\mathrm{A}}}.$$

The value of selectivity α was found for sorbent I ($\alpha = 5.58/5.43 = 1.03$) and for sorbent II ($\alpha = 5.40/5.05 = 1.07$) using eluent (70/30).

Resolution can be expressed as follows:

$$R_{\mathrm{s}} = \frac{2(t_{\mathrm{B}} - t_{\mathrm{A}})}{W_{\mathrm{B}} + W_{\mathrm{A}}}.$$

For the above pairs of isomers this expression gave the following data: for sorbent I: $R_{\mathrm{s}} = 2(2.1)/8.4 = 0.5$; for sorbent II: $R_{\mathrm{s}} = 2(3.0)/6.5 = 0.92$.

CONCLUSIONS

The β-cyclodextrin bonded copolymer of hydroxylethyl methacrylate and ethylene dimethacrylate (HEMA gels) was prepared. The average β-cyclodextrin amount immobilized was found by a modified Hultmann's method. By increasing the proportion of water in the mobile phase the retention times and the relative retention of isomers was increased. The elution order of solute isomers

was found to be independent of the methanol content in the mobile phase. D-, L-, and D,L-tryptophan were used to estimate the ability of the β-cyclodextrin bonded HEMA gel to separate the optical isomers. The sorbent particle size, the fraction particle size distribution, and the length of the spacer were found to influence the efficiency of the prepared columns.

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Asendatud aromaatsete ühendite vedelikkromatograafiline lahutamine β-tsüklodekstriinsel statsionaarsel faasil

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On uuritud mobiilse faasi koostise ja asendajate asukoha mõju aromaatsete ühendite retentsiooniaegadele β-tsüklodekstriiniga modifitseeritud hüdroksü-

etüül- ja etüleendimetakrülaadi kopolümeeril (HEMA-geelil), mis on epoksüaktiveeritud epikloorhüdriini või 1,4-butaandiooldiglütsidüüleetriga. Immobiliseeritud β-tsüklodekstriini kogus statsionaarses faasis on määratud modifitseeritud Hultmanni meetodil. Vee sisalduse kasv mobiilses faasis (metanooli ja vee segu) põhjustab nii testainete kui ka isomeeride retentsiooniaegade erinevuse suurenemise, võimaldades seega isomeere paremini lahutada, kuid pikendades samas eksperimendi läbiviimise aega. Eksperimendi tulemusena on ilmnenud, et testainete isomeeride elueerimise järjekord ei sõltu mobiilse faasi koostisest. β-tsüklodekstriiniga modifitseeritud HEMA-geeli optiliste isomeeride lahutamisvõime hindamisel on testainetena kasutatud D-, L- ja D,L-trüptofaani. Saadud retentsiooniaegade põhjal on kindlaks tehtud, et nimetatud statsionaarse faasiga ei saa ratseemilist segu komponentideks lahutada. On leitud, et kolonni efektiivsust mõjutab olulisel määral sorbendi tera suurus, fraktsiooni monodisperssus ja kasutatud kovalentse silla pikkus.