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METHYLATED CYCLODEXTRIN-BONDED STATIONARY PHASES FOR LIQUID CHROMATOGRAPHY

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SUMMARY

Ethylenediamine-monosubstituted per-O-methyl-α- and -β-cyclodextrin were coupled to succinamidopropyl silica. The unreacted, terminal carboxyl groups of the resulting methylated cyclodextrin stationary phases do not interact significantly with several disubstituted benzene derivatives having various functional groups. However, the secondary amino –NH– group in the spacer arm linking the cyclodextrin units to silica gel interacts strongly with solutes having small pK_a values, such as aminobenzoic and nitrobenzoic acids. Methylated α-cyclodextrin stationary phases with non-nitrogen-containing spacer arms were prepared by reaction of bare silica gel with two organotrichlorosilanes incorporating methylated α-cyclodextrin. These new stationary phases do not exhibit the strong interaction with the benzoic acids.

INTRODUCTION

Recently, cyclodextrins (CDs) have been immobilized on silica gel beads via several different spacer arms[1–4], and the unmodified CD stationary phases obtained have been shown to be useful in the liquid chromatographic separation of structural isomers[1–3], enantiomers[4,7–10] and diastereomers[5].

We previously compared the retention behaviour of disubstituted benzene derivatives on several β-CD stationary phases obtained by reaction of diethylenetriamine-monosubstituted β-CD with succinamidopropyl, glutaraldehyde-activated and epoxy-substituted silica derivatives[3]. It was found that the unmodified β-CD stationary phase prepared from succinamidopropyl silica (Su-Silica) is superior to the others, because the unreacted, terminal carboxyl groups of Su-Silica do not interact significantly with solutes having various functional groups. This spacer arm, however, contains both amido and secondary amino –NH– groups and it is probable that the –NH– groups affect the solute retention in some cases. On the unmodified CD stationary phases obtained by coupling ethylenediamine-monosubstituted CDs to Su-Silica, we had to use mixtures of methanol and phosphate buffer instead of methanol and water in order to elute amino-, chloro-, methyl- and nitrobenzoic acids, and their retention was strongly dependent upon the ionic strength of the buffer[11,12]. Com-

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pared with these solutes which are readily ionizable to anionic forms, the retention of cresol, nitroaniline and dinitrobenzene was scarcely affected by a change in ionic strength. On the other hand, the benzoic acids could readily be eluted with water or methanol–water after acylation of these unmodified CD stationary phases with acid anhydrides. This phenomenon strongly suggests the influence of the –NH– groups in the spacer arm on the retention of the acids. Feitsma *et al.*[10] and Fujimura *et al.*[6] recently referred to the anomalous retention behaviour of aromatic carboxylic acids on their unmodified β-CD stationary phases prepared by treating amino-bonded silicas with monotosylated β-CD. It was proposed that the secondary amino groups in their spacer arms and the unreacted, terminal amino moieties participate in the retention.

Acylation of the unmodified CD stationary phases effected remarkable changes in solute retention, as mentioned above. In this treatment, it is possible to acylate both the –OH groups of the CD units and the –NH– groups in the spacer arm, *i.e.*, $-(CH_2)_3NHCO(CH_2)_2CONH(CH_2)_2NH$–CD. Therefore, in order to investigate the effect of the spacer arm on the retention, we used the methylated CD stationary phases reported previously[13]. These stationary phases, having $-OCH_3$ instead of –OH groups in their CD units, were acetylated with acetic anhydride in pyridine, together with the stationary phases without CD units. The retention behaviour of several solutes was studied on these stationary phases before and after acetylation. Further, an attempt was made to obtain methylated CD stationary phases having the same spacer arm described above, but by a simplified procedure. We also tried to prepare methylated α-CD stationary phases with non-nitrogen-containing spacer arms.

EXPERIMENTAL

Materials and chromatography

Silica gel (super micro bead B-5, mean particle diameter 5.9 μm and specific surface area 411 m^2/g) and α-CD were gifts from Fuji-Davison (Kasugai, Japan) and Wako (Osaka, Japan), respectively. All other reagents of analytical reagent grade were obtained from Wako or Tokyo Kasei (Tokyo, Japan).

All chromatographic studies were carried out with an LC-6A pump (Shimadzu, Kyoto, Japan), a special dampner (Gasukuro Kogyo, Tokyo, Japan), a KHP-UI-130A injector (Kyowa Seimitsu, Tokyo, Japan) and a 440 UV detector operating at 254 nm (Waters, Milford, MA, U.S.A.).

Each stationary phase was packed into a stainless-steel column (10 cm × 0.4 cm I.D.) by a balanced density slurry method. The flow-rate of the eluent (water, methanol–water or methanol–0.15 *M* phosphate buffer) was 1.0 ml/min. The concentration of the sample solutes was 0.2 m*M*, and a volume of less than 20 μl was injected.

Preparation of methylated CD stationary phases

Careful treatment of α-CD (10 g) or β-CD (7.5 g) with trityl chloride (10 g) in pyridine gave mono(6-O-trityl)-α-CD or -β-CD as a main product, respectively. Ethylenediamine-monosubstituted per-O-methyl-α-CD (Me-α-en) or -β-CD (Me-β-en) was prepared by methylation of all the remaining hydroxyl groups of each mono-

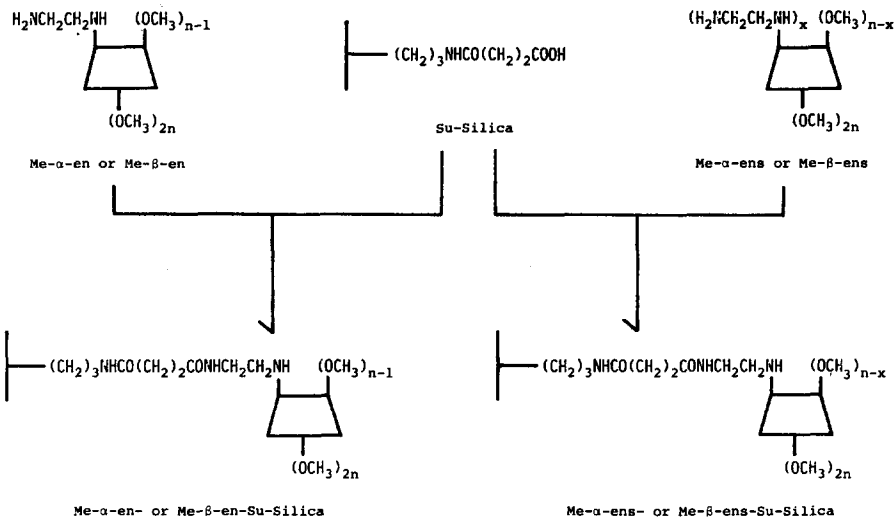

Fig. 1. Immobilization of ethylenediamine-substituted per-O-methyl-CDs on Su-Silica. α-CD; $n = 6$; β-CD; $n = 7$; $x = 1$–3 for Me-α-ens and Me-β-ens.

trityl-CD, removal of the trityl group and tosylation of the free hydroxyl group produced, followed by displacement of the sulphonate with ethylenediamine.

Me-α-en or Me-β-en was coupled to Su-Silica as described previously (Fig. 1), and the stationary phase obtained is denoted by Me-α-en-Su-Silica or Me-β-en-Su-Silica, respectively[13].

A mixture of mono-, di- and tri-(6-O-trityl)-α- or -β-CD was obtained by reaction of trityl chloride (10 g) with α-CD (4.5 g) or β-CD (5 g) for 10 h at room temperature in a smaller amount of pyridine compared with the above-mentioned monotritylation. By treating each mixture in much the same way as described above, ethylenediamine-substituted per-O-methyl-α-CD (Me-α-ens) or -β-CD (Me-β-ens) was derived and gave the corresponding methylated CD stationary phase (Me-α-ens-Su-Silica or Me-β-ens-Su-Silica, respectively).

The amounts of ethylenediamine-substituted per-O-methyl-CDs immobilized were evaluated by elemental analysis: 46.5 μmol/g for Me-α-en-Su-Silica, 58.2 μmol/g for Me-β-en-Su-Silica, 48.5 μmol/g for Me-α-ens-Su-Silica and 35.2 μmol/g for Me-β-ens-Su-Silica.

Preparation of stationary phases without CD units

N-Ethylethylenediamine or *n*-propylamine was immobilized instead of the CD derivatives on Su-Silica; the resulting stationary phases are denoted by Et-en-Su-Silica or Pr-Su-Silica, respectively. The amount of N-ethylethylenediamine immobilized was 199 μmol/g and that of n-propylamine, 341 μmol/g.

Acetylation of stationary phases

Each stationary phase (1.5 g) was suspended in pyridine (20 ml) and acetic anhydride (6 ml) was added. After stirring for 6 h at 45°C, the acetylated stationary phase was filtered off, thoroughly washed successively with methanol, water and

methanol and dried *in vacuo*. The acetylated stationary phases are denoted by prefixing the names of the parent stationary phases by Ac-.

RESULTS AND DISCUSSION

Effect of unreacted carboxyl groups on retention

The amount of carboxyl groups on Su-Silica utilized in this work is 993 μmol/g, and the proportion of the carboxyl groups used for the immobilization of ethylenediamine-monosubstituted CD (Me-α-en or Me-β-en) is only about 1/20. It is therefore assumed that the unreacted carboxyl groups will affect the retention of solutes on the methylated CD stationary phases. Table I gives the retention times of the *ortho*, *meta* and *para* isomers of eight disubstituted benzene derivatives having various functional groups on Su-Silica in methanol–water (20:80). These solutes are apparently rapidly eluted, and the contribution of the unreacted carboxyl groups on Su-Silica to the retention is considered to be small.

Effect of spacer arm on retention

Tables II and III give the retention times of the eight solutes with methanol–water (20:80) on the methylated CD stationary phases before and after acetylation. Comparison of these results with those in Table I indicates that the coupling of Me-α-en and Me-β-en to Su-Silica has brought about increased retention of the solutes. This apparently suggests a positive contribution of the methylated CD units to the retention. In spite of a smaller amount of the methylated CD units in Me-α-en-Su-Silica, it retained the solutes more strongly than did Me-β-en-Su-Silica. This fact implies that the disubstituted benzene derivatives can fit the methylated α-CD cavity better than the methylated β-CD one. On Me-α-en-Su-Silica, *p*-iodoaniline could not be eluted within 30 min with methanol–water (20:80) and gave a retention time of 22.15 min with methanol–water (30:70). Being retained much more strongly, *p*-nitrophenol was eluted in 10.95 min with methanol–water (40:60). Moreover, neither aminobenzoic nor nitrobenzoic acid could be eluted within 60 min with any eluent of methanol–water (0:100–50:50), on Me-α-en-Su-Silica and Me-β-en-Su-Silica.

However, the treatment of these stationary phases with acetic anhydride in

TABLE I

RETENTION TIMES (min) OF DISUBSTITUTED BENZENE DERIVATIVES ON Su-SILICA

Eluent: methanol–water (20:80). t_0 = 1.10 min.

Solute	*o-*	*m-*	*p-*
Cresol	1.80	1.90	1.90
Iodoaniline	2.45	2.55	2.55
Toluidine	1.90	2.20	2.80
Nitroaniline	2.30	1.80	2.05
Nitrophenol	1.95	1.95	2.00
Dinitrobenzene	2.40	1.75	1.65
Aminobenzoic acid	1.10	1.45	1.50
Nitrobenzoic acid	1.60	1.70	1.50

TABLE II

RETENTION TIMES (min) ON METHYLATED α-CYCLODEXTRIN STATIONARY PHASES BEFORE AND AFTER ACETYLATION

Eluent: methanol–water (20:80).

Solute	*Me-α-en-Su-Silica**			*Ac-Me-α-en-Su-Silica***		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	3.05	3.65	4.55	4.25	5.05	5.85
Iodoaniline	7.50	20.15	–***	9.85	18.05	29.75
Toluidine	2.35	2.45	2.60	4.25	5.80	8.70
Nitroaniline	7.60	5.65	22.90	8.05	5.85	14.45
Nitrophenol	8.15	10.30	–***	6.10	9.05	11.15
Dinitrobenzene	4.80	2.95	2.60	7.15	4.30	3.95
Aminobenzoic acid	–***	–***	–***	2.55	1.60	2.75
Nitrobenzoic acid	–***	–***	–***	1.40	1.90	2.05

* t_0 = 1.00 min.
** t_0 = 1.20 min.
*** Not eluted.

pyridine resulted in ready elution of the benzoic acids, as shown in Tables II and III. This remarkable change in the retention is also true for the unmodified CD stationary phases obtained by coupling ethylenediamine-monosubstituted CDs to Su-Silica, as described in the Introduction. In the case of the unmodified CD stationary phases, the –OH groups of the CD moieties are also acetylated. Therefore, in order to explain convincingly the effect of the spacer arm on the retention, we acetylated the methylated CD stationary phases whose CD moieties were not modified. As is seen in Fig.

TABLE III

RETENTION TIMES (min) ON METHYLATED β-CYCLODEXTRIN STATIONARY PHASES BEFORE AND AFTER ACETYLATION

Eluent: methanol–water (20:80).

Solute	*Me-β-en-Su-Silica**			*Ac-Me-β-en-Su-Silica***		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	3.85	4.00	4.15	5.45	5.65	5.65
Iodoaniline	5.45	10.05	9.70	8.75	13.65	13.00
Toluidine	2.25	2.35	2.25	4.00	5.05	6.60
Nitroaniline	5.05	4.70	7.20	7.80	6.55	9.25
Nitrophenol	5.90	6.40	11.55	5.80	8.05	9.05
Dinitrobenzene	5.70	2.65	2.60	9.50	4.25	4.15
Aminobenzoic acid	–***	–***	–***	2.55	1.65	1.60
Nitrobenzoic acid	–***	–***	–***	1.35	1.75	1.65

* t_0 = 1.00 min.
** t_0 = 1.10 min.
*** Not eluted.

1, the spacer arm contains three –NH– groups, *i.e.*, two amido and one secondary amino groups. Acetylation with acetic anhydride is readily performed for primary and secondary amino groups but not for amido, –NH–, ones. Consequently, the remarkable change in the retention of the acids is ascribed to acetylation of the secondary amino –NH– group.

If this inference is reasonable, a similar remarkable change in the retention is expected for a stationary phase containing a secondary amino group but not CD units. N-Ethylethylenediamine was coupled to Su-Silica to produce Et-en-Su-Silica [–$(CH_2)_3NHCO(CH_2)_2NHC_2H_5$], and the retention behaviours of the solutes both on Et-en-Su-Silica and its acetylated stationary phase (Ac-Et-en-Su-Silica) were studied (Table IV). The expected large decrease in the retention after acetylation is apparent for aminobenzoic and nitrobenzoic acids, and for *o*- and *p*-nitrophenol. These solutes have smaller pK_a values, compared with the others. Rough calculations show that the percentage of the anionic form at pH 6.8, for instance, is 98.8% for *p*-aminobenzoic acid (pK_a = 4.89), 99.96% for *p*-nitrobenzoic acid (pK_a = 3.43) and 28.9% for *p*-nitrophenol (pK_a = 7.19). On the other hand, only 2.4% of *m*-nitrophenol (pK_a = 8.4) is in the anionic form at the same pH, and the retention of *m*-nitrophenol is not significantly influenced by acetylation of Et-en-Su-Silica. These results strongly suggest a strong interaction of the anionic forms of the solutes with the secondary amino group in the spacer arm. A similar large decrease in the retention of aminobenzoic and nitrobenzoic acids or of *o*- and *p*-nitrophenol was also observed by using methanol–phosphate buffer instead of methanol–water as eluents. This fact is most likely caused by the masking the secondary amino group, thus removing or reducing the strong interaction mentioned above.

It is of interest to examine the retention behaviour of aminobenzoic and nitrobenzoic acids both on Pr-Su-Silica and the stationary phase obtained by treating it with acetic anhydride, because these two stationary phase contain amido –NH– but no amino –NH–. No appreciable change in the retention was observed after the

TABLE IV

RETENTION TIMES (min) ON Et-en-Su-SILICA AND Ac-Et-en-Su-SILICA

Eluent: methanol–water (20:80).

Solute	*Et-en-Su-Silica**			*Ac-Et-en-Su-Silica***		
	o-	*m-*	*p-*	*o-*	*m-*	*p-*
Cresol	2.95	3.05	3.10	4.75	4.90	4.95
Iodoaniline	4.00	4.20	4.10	7.65	8.85	8.50
Toluidine	2.05	2.10	2.00	3.15	3.20	3.15
Nitroaniline	4.05	2.65	3.35	6.80	4.30	5.45
Nitrophenol	22.95	5.25	27.50	5.90	6.05	6.10
Dinitrobenzene	4.35	2.45	2.25	8.15	4.25	3.90
Aminobenzoic acid	–***	–***	–***	6.20	4.50	4.35
Nitrobenzoic acid	–***	–***	–***	6.25	11.05	10.95

* t_0 = 0.85 min.
** t_0 = 1.15 min.
*** Not eluted.

treatment with acetic anhydride. This suggests that the amido –NH– has not been acetylated. N-Propylacetamide (1 g) was treated with acetic anhydride (20 ml) in pyridine (30 ml) for 6 h at 45°C. The isolated compound gave an identical infrared spectrum to that of the starting N-propylacetamide. No acetylated product could be obtained. This model reaction also indicates that the amido –NH– in the spacer arm is not acetylated.

Methylated CD stationary phases derived from mixtures of tritylated CDs

α- or β-CD was treated with an excess of trityl chloride in pyridine to give a mixture of mono-, di- and tri-(6-O-trityl)-α- or -β-CD. Methylation of all the remaining hydroxyl groups of the derivatives in each mixture and then removal of the trityl groups produced a mixture of the corresponding hydroxy-per-O-methyl derivatives. Hitherto, the methylated CD stationary phases had been prepared by reaction of Su-Silica with ethylenediamine-monosubstituted per-O-methyl-CDs derived from monohydroxy-per-O-methyl-CDs. Their isolation from the mixtures by column chromatography is quite time-consuming and troublesome. If the mixtures can be used for preparing methylated CD stationary phases without the column isolation step, it is of great advantage. Therefore, the mixture of the hydroxy-per-O-methyl derivatives of α- or β-CD was treated as described in the Experimental. The resulting mixture of ethylenediamine-substituted per-O-methyl-α-CD (Me-α-ens) or -β-CD (Me-β-ens) was coupled to Su-Silica. The methylated CD stationary phases obtined (Me-α-ens-Su-Silica and Me-β-ens-Su-Silica) were compared with those from ethyl-

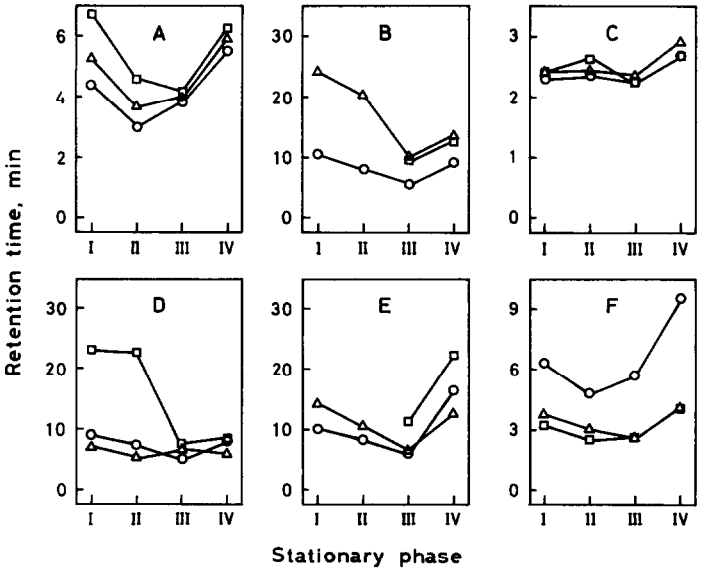

Fig. 2. Retention times of disubstituted benzene isomers, *ortho* (○), *meta* (△) and *para* (□), on Me-α-ens-Su-Silica (I), Me-α-en-Su-Silica (II), Me-β-en-Su-Silica (III) and Me-β-ens-Su-Silica (IV). Eluents: methanol–water (30:70) for I; methanol–water (20:80) for II–IV. Solutes: (A) cresol; (B) iodoaniline; (C) toluidine; (D) nitroaniline; (E) nitrophenol; (F) dinitrobenzene. *p*-Iodoaniline and *p*-nitrophenol were not eluted from I and II within 30 min.

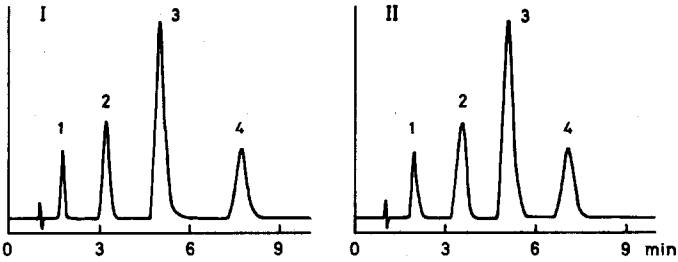

Fig. 3. Separation of antiepileptic drugs on Me-β-en-Su-Silica in methanol–water (20:80) (I) and on Me-β-ens-Su-Silica in methanol–water (30:70) (II). Solutes: 1 = primidone; 2 = phenobarbital; 3 = carbamazepine; 4 = phenytoin.

enediamine-monosubstituted per-O-methyl-CDs (Me-α-en-Su-Silica and Me-β-en-Su-Silica).

Fig. 2 shows the retention times of six disubstituted benzene derivatives on these four methylated CD stationary phases. It is apparent that the use of Me-α-ens or Me-β-ens results in no decrease in the selectivity. The solutes are retained on Me-α-ens-Su-Silica more strongly than on Me-α-en-Su-Silica. The same statement is true for the methylated β-CD stationary phases. This may be due to the extra ethylenediamine moieties in Me-α-ens or Me-β-ens. Further work is needed to explain this stronger retention.

Fig. 3 also gives an example showing no decrease in the selectivity in the separation of antiepileptic drugs.

It is shown that the use of Me-α-ens or Me-β-ens gives the methylated CD stationary phase much more readily without decrease in the selectivity, compared with the use of Me-α-en or Me-β-en.

Attempt to prepare methylated α-CD stationary phases with non-nitrogen-containing spacer arms

On our CD stationary phases, CDs are bonded to silica gel via a nitrogen-containing spacer arm. Consequently, a strong interaction with the spacer arm is observed for solutes having small pK_a values, as described above. In order to enhance the specificity of CD units, CD stationary phases with non-nitrogen-containing spacer arms are expected to be favourable. Armstrong and DeMond[4] first published results obtained with unmodified CD stationary phases having a non-nitrogen-con-

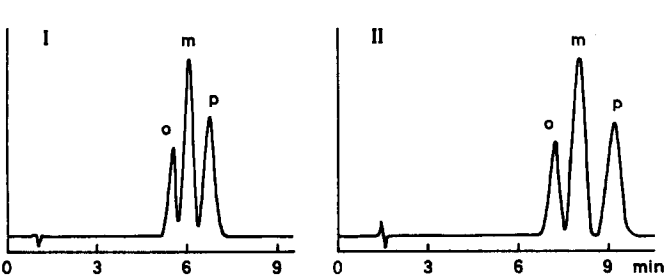

Fig. 4. Separation of cresol isomers on Me-α-pe-Silica (I) and on Me-α-pr-Silica (II) in methanol–water (30:70). *o*, *m* and *p* denote *ortho*, *meta* and *para* isomers, respectively.

taining spacer arm. On these stationary phases, CDs are coupled to a silica gel derivative with an active terminal group[14]. The existence of the unreacted, terminal group is unavoidable in this case, which is similar to our stationary phases as already mentioned.

We have, therefore, started to prepare CD stationary phases by reaction of bare silica gel with organohalogenosilanes incorporating CDs. Two trichlorosilanes, *i.e.*, mono[6-O-(5-trichlorosilylpentyl)]-per-O-methyl-α-CD and mono{6-O-[2-(3-trichlorosilylpropoxy)ethyl]}-per-O-methyl-a-CD, were synthesized and coupled to silica gel. The resulting stationary phases are denoted by Me-α-pe-Silica and Me-α-pr-Silica, respectively. The preliminary results for these stationary phases will be described briefly.

Fig. 4 shows typical liquid chromatograms for a mixture of *o*-, *m*- and *p*-cresol on these methylated α-CD stationary phases with non-nitrogen-containing spacer arms. The three isomers are well separated on both stationary phases and are eluted in the order *o*- < *m*- < *p*-. This elution order is consistent with the generally accepted order of stability for inclusion complex formation. On these stationary phases, as expected, aminobenzoic and nitrobenzoic acids can readily be eluted with eluents which do not contain phosphate buffer, which suggests no or little interaction between the solutes and the non-nitrogen-containing spacer arms. On the other hand, cresol, iodoaniline and nitroaniline were retained on Me-α-pe-Silica and Me-α-pr-Silica more strongly than on Me-α-en-Su-Silica. This may be ascribed to differences in the hydrophobicity of the spacer arms. Further work is now in progress, and the results together with the preparation procedures will be reported elsewhere.

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