Publikace I

# Enantioselective separation of unusual amino acids by high performance liquid chromatography

Kučerová, G., Vozka, J., Kalíková, K., Geryk, R., Plecitá, D., Pajpanova, T., Tesařová, E.

*Separation and Purification Technology* 2013, 119, 123-128

doi:10.1016/j.seppur.2013.09.010

Separation and Purification Technology 119 (2013) 123–128

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

# Enantioselective separation of unusual amino acids by high performance liquid chromatography

Gabriela Kučerová [a], Jiří Vozka [a], Květa Kalíková [a,*], Radim Geryk [a], Denisa Plecitá [a], Tamara Pajpanova [b], Eva Tesařová [a]

[a] Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University in Prague, Prague, Czech Republic
[b] Institute of Molecular Biology, Bulgarian Academy of Sciences, Sophia, Bulgaria

ARTICLE INFO

*Article history:*
Received 6 August 2013
Received in revised form 12 September 2013
Accepted 13 September 2013
Available online 26 September 2013

*Keywords:*
Unusual amino acid
Chiral separation
Teicoplanin-based chiral stationary phase
Polysaccharide-based chiral stationary phase

ABSTRACT

Unusual amino acids, *i.e.* amino acids not encoded by DNA, play fundamental roles in many scientific fields. Since the single enantiomeric form can cause different and often serious response of organisms, chiral separations of unusual amino acids are irreplaceable tools in their study and their employment. Two types of chiral stationary phases, two teicoplanin-based and four polysaccharide-based columns, were used. Separation conditions of reversed phase mode, polar organic mode and hydrophilic interaction chromatography were evaluated and compared. All columns used exhibited significantly different enantioselectivities. Teicoplanin-based chiral stationary phases, especially Chirobiotic T column, were able to separate almost all enantiomers tested, with the exception of *Z*-D,L-4-F-phenylalanine ethyl ester. No partial enantioseparation of this analyte was obtained on teicoplanin-based chiral stationary phases, while baseline enantioresolution was achieved on polysaccharide-based columns. Change of elution order of L- and D-enantiomers was proved regarding the chiral stationary phase used.



## 1. Introduction

Peptides with their functional variability belong undoubtedly to the most important chemical entities in nature. One of the widely used ways for study of peptides, their conformations and functions, is an employment of unusual amino acids (AAs), which are not encoded by DNA. Integration of such AAs into the peptide chain enables intentional changes, which provide a valuable insight into the mechanisms of peptide activity [*e.g.* 1–4]. This approach has already brought a lot of findings and enabled development of important drugs [*e.g.* 5–7]. Particularly, a variety of impressive applications of fluoro-phenylalanines can be found in the literature. For instance, their homogeneous antibody-drug complexes showed enhanced effectiveness against target cell types [8]. Fluoro-phenylalanines were also introduced as a class of promising carriers for *in vitro* cell cultures/drug delivery [9].

However, the use of unusual AAs brings also some difficulties. Since the unusual AAs are often synthesized as racemates, prepared substances may contain many impurities. Therefore, purification followed by enantioseparation of unusual AAs stays at the very beginning of peptide/potential drug design. HPLC as a widely used and reliable separation technique is a good choice for this challenge [10–16].

The aim of our work was to develop HPLC methods suitable for enantioseparation and possible purification of a set of unusual amino acids. The set contained fluorine derivatives of phenylalanine, D,L-4-Cl-phenylalanine and α-methyl D,L tyrosine. Other compounds in the set were *N*-protected fluorine derivatives of phenylalanine including *N*-blocked D,L-4-F-phenylalanine ethyl ester. As a protection group benzyloxycarbonyl (*Z*) substituent was used. Phenylalanine and tyrosine were used as reference standards. The complete set of AAs is depicted in Fig. 1.

Initially, chiral stationary phases (CSPs) based on the macrocyclic antibiotic teicoplanin, namely Chirobiotic T and T2 columns, were chosen since they are considered to have extraordinary enantioseparation potential for amino acids [17–19]. Reversed phase (RP) and polar organic (PO) modes were used. However, after the initial investigation four additional CSPs based on derivatized polysaccharides [20–23], namely Chiralpak IA, Chiralpak IB, Chiralpak IC and Chiralpak ID columns, were employed since no enantiosep-

*Abbreviations:* ACN, acetonitrile; AA, amino acid; CSP, chiral stationary phase; HOAc, acetic acid; HILIC, hydrophilic interaction liquid chromatography; MeOH, methanol; PO, polar organic; RP, reversed phase; TEA, triethylamine.

* Corresponding author. Address: Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University in Prague, Albertov 2030, 12843 Prague, Czech Republic. Fax: +420 224 919 752.
*E-mail address:* kveta.kalikova@centrum.cz (K. Kalíková).

1383-5866/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.seppur.2013.09.010

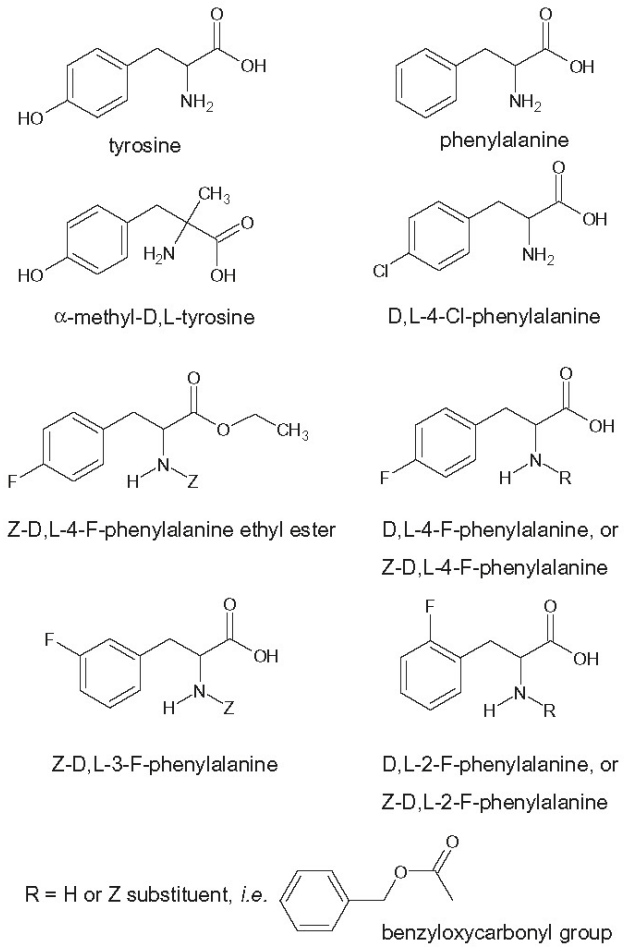


**Fig. 1.** Chemical structures of studied amino acids and *Z*-D,L-4-F-phenylalanine ethyl ester.

aration of *Z*-D,L-4-F-phenylalanine ethyl ester could be obtained on teicoplanin-based CSPs.

## 2. Experimental

### 2.1. Instrumentation

All chromatographic measurements were performed on Waters Alliance® system (Waters Corporation, Milford, USA) consisting of 2690 Separation Module, UV–VIS 2-channel detector 2487, 717 plus autosampler, and Alliance® Series column heater. Empower® software was used for data acquisition and analyses. Two teicoplanin-based CSPs were used, *i.e.* Astec Chirobiotic® T and Astec Chirobiotic® T2 columns (both column sizes 250 × 4.6 mm i.d., particle sizes 5 μm) from SUPELCO® (Bellefonte, USA). Other four polysaccharide CSPs used were based on (i) amylose tris(3,5-dimethylphenylcarbamate) – Chiralpak® IA column, (ii) cellulose tris(3,5-dimethylphenylcarbamate) – Chiralpak® IB column, (iii) cellulose tris(3,5-dichlorophenylcarbamate) – Chiralpak® IC column and (iv) amylose tris(3-chlorophenylcarbamate) – Chiralpak® ID column. The later CSPs were immobilized on 5 μm silica gel, columns sizes were 250 × 4.6 mm i.d., and they were obtained from Chiral Technologies Europe (Illkirch, France).

The columns were thermostated at 25 °C. The detection was performed at 254 nm. The flow rate was 1 mL/min and the injection volume was 10 μL in all experiments.

### 2.2. Chemicals and reagents

Methanol (MeOH, Chromasolv® for HPLC), acetonitrile (ACN, gradient grade), ammonium acetate (purity ⩾ 99%), triethylamine (TEA, purity ⩾ 99%), acetic acid (HOAc, purity > 99.8%) and formic acid (reagent grade, ⩾95%) were supplied by Sigma–Aldrich (St. Louis, USA). The deionized water used was purified by a Milli-Q water purification system from Millipore (Bedford, USA). D,L-tyrosine, D,L-phenylalanine, L-tyrosine, L-phenylalanine and potassium iodide were purchased from Sigma–Aldrich (St. Louis, USA). D,L-4-F-phenylalanine, *N*-benzyloxycarbonyl-D,L-4-F-phenylalanine, *N*-benzyloxycarbonyl-D,L-4-F-phenylalanine ethyl ester, D,L-4-Cl-phenylalanine, *N*-benzyloxycarbonyl-D,L-3-F-phenylalanine, D-2-F-phenylalanine, L-2-F-phenylalanine, *N*-benzyloxycarbonyl-D,L-2-F-phenylalanine, α-methyl-D,L-tyrosine, *N*-benzyloxycarbonyl-L-4-F-phenylalanine, *N*-benzyloxycarbonyl-D-3-F-phenylalanine, *N*-benzyloxycarbonyl-L-2-F-phenylalanine, L-4-Cl-phenylalanine and L-4-F-phenylalanine were prepared at the Institute of Molecular Biology, Bulgarian Academy of Sciences (Sophia, Bulgaria). Abbreviation *Z* stands for benzyloxycarbonyl group bonded to amino group in the following text.

### 2.3. Procedures

All measurements were performed in triplicates. Void volume was determined by injection of potassium iodide. Stock solutions of samples were prepared in concentration of 1 mg/mL using MeOH or mixture of MeOH and water as solvents.

Ammonium acetate buffer was prepared by dissolving appropriate amount of ammonium acetate in deionized water and adjusted with HOAc to reach the required pH value.

For teicoplanin-based CSPs, mobile phases in RP mode consisted of MeOH and deionized water or ammonium acetate buffer, while mobile phases in PO mode were composed of MeOH/HOAc/TEA in various volume ratios. For polysaccharide-based CSPs, MeOH or ACN and water or aqueous solution of formic acid, pH 2.20, in various volume ratios were used.

## 3. Results and discussion

### 3.1. Teicoplanin-based chiral stationary phases

#### 3.1.1. Enantioseparation in RP/HILIC mode

Initially, pH (3.00; 4.00; 5.00) and concentration (15 mM; 20 mM; 25 mM) of ammonium acetate buffer as an aqueous mobile phase component were tested for the separation of AAs enantiomers on teicoplanin-based CSPs. Organic part of the mobile phases was formed of MeOH. At first, two chiral columns (Chirobiotic T and T2), were used differing in higher teicoplanin coverage, longer spacer and presence of additional carbamate moiety of the later CSP [24,25]. Based on the obtained results 20 mM ammonium acetate buffer pH 4.00 was chosen for further investigation. Table 1 summarizes obtained results of enantioseparation, baseline or partial, in the optimized mobile phases. Injections of single enantiomers proved that L-enantiomers eluted first in all cases. By comparing the data in Table 1, it is obvious that Chirobiotic T and Chirobiotic T2 columns show different enantioseparation potential for the tested analytes under RP conditions. Chirobiotic T with lower teicoplanin coverage exhibits higher retention and enantioresolution for all AAs enantiomers, except for α-methyl-D,L-tyrosine at identical mobile phase composition. For illustration see Fig. 2. The substituent type on the benzene ring of phenylalanine has interesting influence on retention and resolution values on Chirobiotic T column (see Table 1). The highest resolution value was obtained for non substituted D,L-phenylalanine. D,L-phenylalanine substituted by chlorine had lower resolution value despite the

**Table 1**
Chromatographic data of the studied amino acids separated on Chirobiotic T and T2 columns in buffer methanolic and aqueous methanolic mobile phases. Retention factor of the first eluted enantiomer ($k_1$), resolution ($R$) and optimized mobile phase composition.

| Analyte | Column | $k_1$ | $R$ | Mobile phase composition |
|---|---|---|---|---|
| D,L-4-F-phenylalanine | T | 1.17 | 3.91 | MeOH/buffer 70/30 (v/v) |
| | T | 1.17 | 4.44 | MeOH/water 70/30 (v/v) |
| Z-D,L-4-F-phenylalanine | T | 0.13 | 2.37 | MeOH/buffer 80/20 (v/v) |
| Z-D,L-3-F-phenylalanine | T | 0.18 | 2.31 | MeOH/buffer 70/30 (v/v) |
| D,L-2-F-phenylalanine | T | 1.16 | 3.99 | MeOH/buffer 70/30 (v/v) |
| | T | 1.18 | 4.80 | MeOH/water 70/30 (v/v) |
| | T2 | 0.88 | 0.61 | MeOH/buffer 70/30 (v/v) |
| Z-D,L-2-F-phenylalanine | T | 0.19 | 1.88 | MeOH/buffer 70/30 (v/v) |
| D,L-4-Cl-phenylalanine | T | 1.32 | 3.46 | MeOH/buffer 70/30 (v/v) |
| | T | 1.36 | 4.14 | MeOH/water 70/30 (v/v) |
| D,L-phenylalanine | T | 1.24 | 4.24 | MeOH/buffer 70/30 (v/v) |
| | T | 1.22 | 4.19 | MeOH/water 60/40 (v/v) |
| D,L-tyrosine | T | 1.03 | 4.15 | MeOH/buffer 70/30 (v/v) |
| | T | 1.07 | 4.99 | MeOH/water 70/30 (v/v) |
| | T2 | 0.96 | 3.01 | MeOH/buffer 70/30 (v/v) |
| α-methyl-D,L-tyrosine | T2 | 0.68 | 1.04 | MeOH/buffer 60/40 (v/v) |

Buffer stands for 20 mM ammonium acetate buffer, pH 4.00.

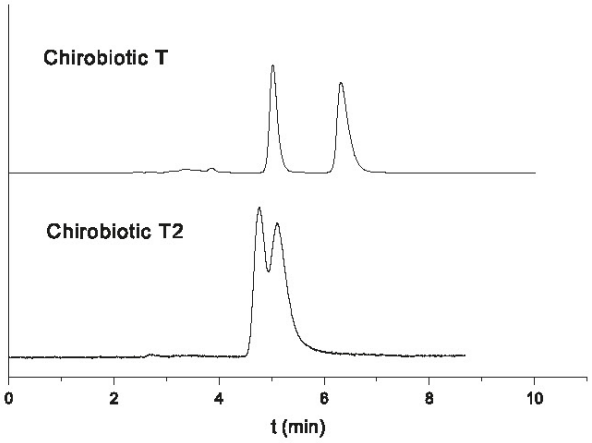


**Fig. 2.** Chromatograms of enantioseparation of D,L-2-F-phenylalanine on two teicoplanin-based CSPs. Mobile phase composition: ACN/20 mM ammonium acetate buffer, pH 4.00, 70/30 (v/v), flow rate: 1 mL/min, column temperature: 25 °C, UV detection: 254 nm.

fact that this analyte has the longest retention from the set of compounds. Both analytes with fluorine substituent in *ortho* or in *para* positions exhibit similarly lower retention and decrease in enantioresolution. The introduction of *Z*-group significantly decreases retention of the AAs. However, baseline resolution was preserved for some analytes. The effect of the amount of organic modifier on retention of AAs enantiomers was also tested. Generally, U-shaped dependencies of retention on the amount of organic modifier were obtained on both CSPs for non-blocked analytes, while for *N*-blocked AAs the retention just decreased with increasing MeOH content. This means that the less polar *N*-blocked AAs show a typical RP behavior. U-shaped dependencies indicate a change of the interaction/retention mechanism for the polar analytes. In the mobile phases with lower MeOH content teicoplanin-based CSPs work as RP columns, while in the mobile phases with high MeOH content as HILIC (hydrophilic interaction liquid chromatography) columns [24]. Thus, in the mobile phases with lower MeOH content non-polar interactions prevail, while at the higher MeOH content polar interactions become more important.

Use of simple mobile phases composed just of MeOH and deionized water also led to successful enantioseparation of non-blocked AAs on Chirobiotic T column – see Table 1. The majority of AAs enantiomers reached even higher resolution values while retention remained similar, however the peak shapes became worse than in the mobile phases with buffer. This behavior can be explained by the dissociation of carboxylic groups of AAs at different pH values. Dissociated carboxylic groups at the pH of water can interact as hydrogen-bond acceptors with amide groups of teicoplanin. This type of interaction has stereoselective character, as was already proved [26]. The use of ammonium acetate buffer, pH 4.00, suppresses the dissociation, which results in the decreased resolution values. The non-selective interactions remained unchanged. AAs with *Z*-group were not retained under these mobile phase compositions and eluted with the void volume.

### 3.1.2. Enantioseparation in PO mode

Suitable mobile phases used in PO mode were simply composed of MeOH with small additions of TEA and HOAc. The use of mobile phases composed of ACN/MeOH/HOAc/TEA had no positive effect on enantioseparation of the tested analytes. The optimized mobile phases for successful enantioseparations on Chirobiotic T and T2 columns with corresponding chromatographic parameters of individual AAs are summarized in Table 2. Chirobiotic T and T2 columns are complementary in PO mode. Chirobiotic T showed enantioselective potential only for *N*-blocked AAs, while Chirobiotic T2 for non-blocked AAs. Almost all *N*-blocked AAs were baseline

**Table 2**
Chromatographic data of the studied amino acids separated on Chirobiotic T and T2 columns in PO mode. Retention factor of the first eluted enantiomer ($k_1$), resolution ($R$) and optimized mobile phase composition.

| Analyte | Column | $k_1$ | $R$ | MeOH/HOAc/TEA (v/v/v) |
|---|---|---|---|---|
| D,L-4-F-phenylalanine | T2 | 1.72 | 1.94 | 100/0.5/0.1 |
| Z-D,L-4-F-phenylalanine | T | 0.43 | 2.46 | 100/0.1/0.5 |
| Z-D,L-3-F-phenylalanine | T | 0.43 | 2.21 | 100/0.1/0.3 |
| D,L-2-F-phenylalanine | T2 | 1.47 | 1.91 | 100/0.5/0.1 |
| Z-D,L-2-F-phenylalanine | T | 0.42 | 1.52 | 100/0.1/0.5 |
| D,L-4-Cl-phenylalanine | T2 | 2.10 | 1.61 | 100/0.5/0.1 |
| D,L-phenylalanine | T2 | 1.78 | 1.86 | 100/0.5/0.1 |
| D,L-tyrosine | T2 | 1.88 | 1.66 | 100/0.3/0.1 |
| α-methyl-D,L-tyrosine | T2 | 1.34 | 1.29 | 100/0.3/0.3 |

enantioresolved in PO mode on Chirobiotic T column, except for *Z*-D,L-4-F-phenylalanine ethyl ester. This seems to be a consequence of the presence of ethyl group instead of hydrogen on carboxylic group which results in the reduction of hydrogen bond interaction. Steric effects can also play a role. Non-blocked AAs showed no indication of enantioseparation, although they were more retained than the blocked ones under the same mobile phase composition. The best resolution of enantiomers of *N*-blocked AAs was obtained in mobile phases in which the amount of TEA was higher than that of HOAc. These results are comparable with those obtained in mobile phases with higher amounts of TEA and HOAc (volume ratio 1:1). Different results were obtained on Chirobiotic T2 column. All non-blocked AAs were baseline separated in short analysis times except for α-methyl-D,L-tyrosine that was just partially resolved ($R = 1.29$). Methyl group of this analyte probably rules out the proper steric fit with the chiral selector and thus decreases the enantioresolution. The best separations (comparing the *R* values) were obtained in mobile phases with higher amount of HOAc than TEA.

### 3.2. Polysaccharide-based chiral stationary phases

As no enantioseparation of *Z*-D,L-4-F-phenylalanine ethyl ester was obtained using teicoplanin-based CSPs, four polysaccharide-based CSPs, known for their good enantioselective separation abilities, were chosen for separation of *N*-blocked AAs and *N*-blocked ester. PO mode covering various mobile phase compositions was not suitable for enantioseparation of *N*-blocked AAs. The retention was very low with no sign of enantioseparation. RP mode mobile phases composed of ACN or MeOH as organic modifiers and aqueous solution of formic acid, pH 2.20, brought interesting results. Low buffer pH was used since enantiomers must be uncharged for successful enantioseparation on these CSPs [21]. Table 3 summarizes optimized mobile phase compositions for *N*-blocked AAs and the ester on Chiralpak columns. At least partial enantioseparation was obtained for all enantiomers on all four columns. The best column with respect to retention and resolution was Chiralpak IC. All analytes were baseline separated in significantly shorter analysis time as compared to the other Chiralpak columns. Both organic modifiers (ACN and MeOH) were proved to be suitable mobile phase components for this cellulose based column. Chiralpak IA column (amylose based CSP) provided better results with mobile phases composed of ACN as organic modifier rather than of MeOH. Nevertheless, only partial enantioseparations could be achieved. On the other hand, Chiralpak IB and ID columns gave better results with MeOH as organic modifier, except for enantioseparation of *Z*-D,L-3-F-phenylalanine on Chiralpak IB column where ACN was better mobile phase constituent. The enantioseparation of uncharged *Z*-D,L-4-F-phenylalanine ethyl ester is possible under very simple mobile phase compositions, ACN or MeOH and water, on all four Chiralpak columns. In separation systems with acidified aqueous part (pH 2.20) of mobile phases, hydrolysis of ester during separation occurred. The most suitable column for separation of the ester enantiomers was again Chiralpak IC. The baseline enantioseparation was obtained in the shortest analysis time – see Table 3. Fig. 3A and B compare enantioselectivity of individual Chiralpak columns under the same mobile phase compositions. Comparing amylose-based columns, Chiralpak IA and ID, we can summarize that the CSP without chlorine atom in the structure shows slightly higher enantioselectivity for all *N*-blocked AAs with the exception of *Z*-D,L-2-F-phenylalanine in mobile phase containing MeOH. In the case of cellulose-based columns, Chiralpak IB and IC, the situation is opposite. CSP with two chlorine atoms has significantly higher enantioselectivity for all *N*-blocked AAs in both mobile phase compositions. For illustration see Fig. 4.

The basic polysaccharide structure, amylose *vs.* cellulose, crucially influences the elution order of enantiomers. On Chiralpak IA, amylose tris(3,5-dimethylphenylcarbamate), L-enantiomers eluted first, while on Chiralpak IB, cellulose tris(3,5-dimethylphenylcarbamate), D-enantiomers eluted first. Introduction of chlorine atom(s) to the chiral selectors reverses the elution order in both cases. D-enantiomers eluted first using amylose based Chiralpak ID column and L-enantiomers eluted first using cellulose based Chiralpak IC column.

## 4. Conclusions

Enantiomers of all unusual AAs studied were baseline resolved except for α-methyl-D,L-tyrosine which was only partially separated on Chirobiotic T2 column. It was proved that teicoplanin-based CSPs work for these compounds as HILIC/RP mixed mode CSPs. Complementarity of teicoplanin-based CSPs with different

**Table 3**
Chromatographic data of the studied amino acids separated on Chiralpak columns in optimized mobile phases. Retention factor of the first eluted enantiomer ($k_1$), resolution ($R$) and optimized mobile phase composition.

| Analyte | Column | $k_1$ | $R$ | Mobile phase composition |
|---|---|---|---|---|
| *Z*-D,L-4-F-phenylalanine | CH IA | 3.11 | 1.27 | ACN/aq. part 40/60 (*v*/*v*) |
| | CH IB | 7.52 | 0.68 | MeOH/aq. part 55/45 (*v*/*v*) |
| | CH IC | 1.38 | 1.83 | ACN/aq. part 42/58 (*v*/*v*) |
| | | 1.33 | 1.92 | MeOH/aq. part 70/30 (*v*/*v*) |
| | CH ID | 4.10 | 1.63 | MeOH/aq. part 60/40 (*v*/*v*) |
| *Z*-D,L-3-F-phenylalanine | CH IA | 2.94 | 1.10 | ACN/aq. part 40/60 (*v*/*v*) |
| | CH IB | 10.52 | 0.67 | ACN/aq. part 30/70 (*v*/*v*) |
| | CH IC | 1.32 | 1.67 | ACN/aq. part 42/58 (*v*/*v*) |
| | | 1.26 | 1.74 | MeOH/aq. part 70/30 (*v*/*v*) |
| | CH ID | 4.27 | 0.82 | MeOH/aq. part 60/40 (*v*/*v*) |
| *Z*-D,L-2-F-phenylalanine | CH IA | 3.09 | 0.76 | ACN/aq. part 40/60 (*v*/*v*) |
| | CH IB | 6.41 | 0.91 | MeOH/aq. part 55/45 (*v*/*v*) |
| | CH IC | 1.63 | 1.73 | ACN/aq. part 40/60 (*v*/*v*) |
| | | 1.33 | 1.68 | MeOH/aq. part 70/30 (*v*/*v*) |
| | CH ID | 4.79 | 1.40 | MeOH/aq. part 60/40 (*v*/*v*) |
| *Z*-D,L-4-F-phenylalanine ethylester | CH IA | 13.81 | 3.24 | ACN/water 40/60 (*v*/*v*) |
| | CH IB | 10.19 | 1.69 | ACN/water 40/60 (*v*/*v*) |
| | CH IC | 6.22 | 4.19 | ACN/water 45/55 (*v*/*v*) |
| | CH ID | 23.59 | 1.41 | MeOH/water 60/40 (*v*/*v*) |

Aq. part means aqueous solution of HCOOH, pH 2.20.

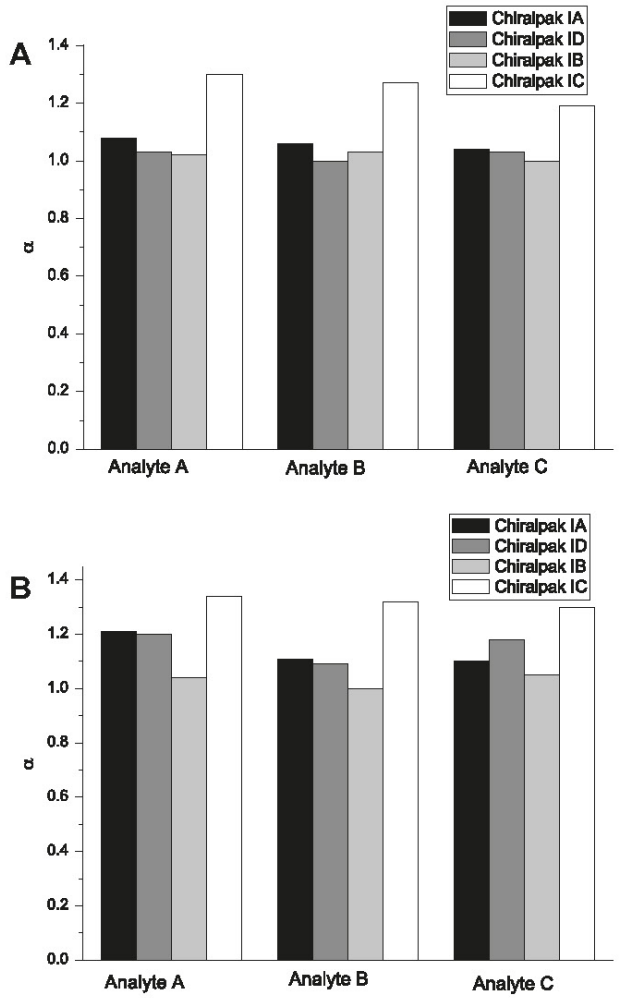


**Fig. 3.** The dependence of enantioselectivity (α) for *N*-blocked AAs on polysaccharide based CSPs. (A) mobile phase composition: ACN/aqueous solution of formic acid, pH 2.20, 30/70 (*v*/*v*); (B) mobile phase composition: MeOH/aqueous solution of formic acid, pH 2.20, 60/40 (*v*/*v*). Analyte A – *Z*-D,L-4F-phenylalanine; Analyte B – *Z*-D,L-3F-phenylalanine; Analyte C – *Z*-D,L-2F-phenylalanine.

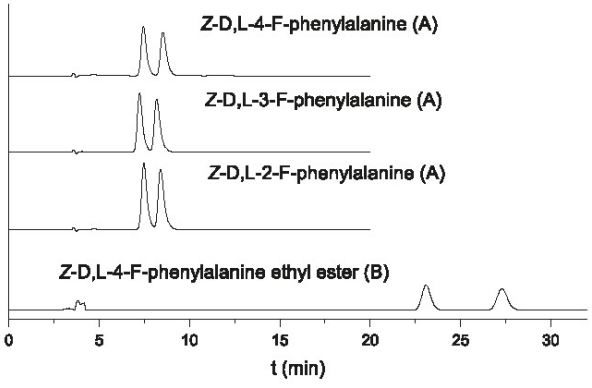


**Fig. 4.** Chromatograms of enantioseparation of *N*-blocked analytes on Chiralpak IC column. (A) Mobile phase composition: MeOH/aqueous solution of formic acid, pH 2.20, 70/30 (*v*/*v*); (B) mobile phase composition: ACN/water 45/55 (*v*/*v*). For more details see Experimental.

chiral selector coverage was observed. The best enantioseparation systems for non-blocked AAs are Chirobiotic T column in RP mode and/or Chirobiotic T2 column in PO mode. The most suitable conditions for enantioseparation of *N*-blocked AAs are either Chirobiotic T column in PO mode or Chiralpak IC column in RP mode. While L-enantiomers eluted first on teicoplanin-based CSPs, the elution order of enantiomers varied on polysaccharide-based CSPs depending on the subtle structural differences of the chiral selector. Chirobiotic T2 column was proved to be the only column suitable for enantioseparation of α-methyl-D,L-tyrosine. On the other hand, only polysaccharide-based columns are usable for separation of *Z*-D,L-4-F-phenylalanine ethyl ester enantiomers. The optimized HPLC methods are convenient for enantiomeric purity control and consequent sample purification.

## Acknowledgements

The authors wish to thank Dr. Pilar Franco for the Chiralpak columns. CEEPUS, Grant No. CIII-RO-0010-07-1213, KONTAKT AM 2010 Project LH11018, the Grant Agency of the Charles University in Prague, Project No. 356411, the Ministry of Education, Youth and Sports of the Czech Republic, and the long-term Project MSM0021620857 are gratefully acknowledged for the financial support. Furthermore, the authors want to express their gratitude to Renata Gilar for language corrections.

## References

[1] A. Hasuoka, Y. Nishikimi, Y. Nakayama, K. Kamiyama, M. Nakao, K. Miyagawa, O. Nishimura, M. Fujino, Total synthesis of novel antibiotics pyloricidin A, B and C and their application in the study of pyloricidin, J. Antibiot. 55 (2002) 322–336.
[2] L. Wang, P.G. Schultz, Expanding the genetic code, Angew. Chem. Int. Ed. Engl. 44 (2005) 34–66.
[3] M. Tanaka, Design and synthesis of chiral α, α/disubstituted amino acids and conformational study of their oligopeptides, Chem. Pharm. Bull. 55 (2007) 349–358.
[4] W. Leuchtenberger, K. Huthmacher, K. Drauz, Biotechnological production of amino acids and derivatives: current status and prospects, Appl. Microbiol. Biot. 69 (2005) 1–8.
[5] D. Venugopal, D. Klapper, A.H. Srouji, J.B. Bhonsle, R. Borschel, A. Mueller, A.L. Russell, B.C. Williams, R.P. Hicks, Novel antimicrobial peptides that exhibit activity against select agents and other drug resistant bacteria, Bioorg. Med. Chem. 18 (2010) 5137–5147.
[6] M.D. Seo, H.S. Won, J.H. Kim, T. Mishig-Ochir, B.J. Lee, Antimicrobial peptides for therapeutic applications: a review, Molecules 17 (2012) 12276–12286.
[7] R.P. Hicks, J.J. Abercrombie, R.K. Wong, K.P. Leung, Antimicrobial peptides containing unnatural amino acid exhibit potent bactericidal activity against ESKAPE pathogens, Bioorg. Med. Chem. 21 (2013) 205–214.
[8] W.H. Zhang, G. Otting, C.J. Jackson, Protein engineering with unnatural amino acids, Curr. Opin. Struct. Biol. 23 (2013) 581–587.
[9] Y. Wang, Z. Zhang, L. Xu, X. Li, H. Chen, Hydrogels of halogenated Fmoc-short peptides for potential application in tissue engineering, Colloids Surf. B 104 (2013) 163–168.
[10] X. Wang, Y. Liu, Ch.B. Ching, Kinetic and equilibrium study of enantioseparation of propranolol in preparative scale chromatography, Sep. Purif. Technol. 50 (2006) 204–211.
[11] H. Khan, T. Khan, J.K. Park, Separation of phenylalanine racemates using d-phenylalanine imprinted microbeads as HPLC stationary phase, Sep. Purif. Technol. 62 (2008) 363–369.
[12] S. Shi, Y. Ma, Y. Zhang, L. Liu, Q. Liu, M. Peng, X. Xiong, Systematic separation and purification of 18 antioxidants from *Pueraria lobata* flower using HSCCC target-guided by DPPH–HPLC experiment, Sep. Purif. Technol. 89 (2012) 225–233.
[13] I. Ilisz, A. Aranyi, A. Péter, Review: chiral derivatizations applied for the separation of unusual amino acids enantiomers by liquid chromatography and related techniques, J. Chromatogr. A 1296 (2013) 119–139.
[14] I. Ilisz, A. Aranyi, Z. Pataj, A. Péter, Recent advances in the direct and indirect liquid chromatographic enantioseparation of amino acids and related compounds: a review, J. Pharm. Biomed. Anal. 69 (2012) 28–41.
[15] K. Kalíková, M. Riesová, E. Tesařová, Recent chiral selectors for separation in HPLC and CE, Cent. Eur. J. Chem. 10 (2012) 450–471.
[16] A. Cavazzini, L. Pasti, A. Massi, N. Marchetti, F. Dondi, Recent applications in chiral high performance liquid chromatography: a review, Anal. Chim. Acta 706 (2011) 205–222.
[17] A. Berthod, Y. Liu, Ch. Bagwill, D.W. Armstrong, Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase, J. Chromatogr. A 731 (1996) 123–137.
[18] A. Péter, G. Török, D.W. Armstrong, High-performance liquid chromatographic separation of enantiomers of unusual amino acids on a teicoplanin chiral stationary phase, J. Chromatogr. A 793 (1998) 283–296.

[19] I. Ilisz, R. Berkecz, A. Péter, Review: retention mechanism of high-performance liquid chromatographic enantioseparation on macrocyclic glycopeptide-based chiral stationary phases, J. Chromatogr. A 1216 (2009) 1845–1860.
[20] T. Zhang, D. Nguyen, P. Franco, T. Murakami, A. Ohnishi, H. Kurosawa, Cellulose 3,5-dimethylphenylcarbamate immobilized on silica. A new chiral stationary phase for the analysis of enantiomers, Anal. Chim. Acta 557 (2006) 221–228.
[21] T. Zhang, D. Nguyen, P. Franco, Reversed-phase screening strategies for liquid chromatography on polysaccharide-derived chiral stationary phases, J. Chromatogr. A 1217 (2010) 1048–1055.
[22] T. Zhang, P. Franco, D. Nguyen, R. Hamasaki, S. Miyamoto, A. Ohnishi, T. Murakami, Complementary enantiorecognition patterns and specific method optimization aspects on immobilized polysaccharide-derived chiral stationary phases, J. Chromatogr. A 1269 (2012) 178–188.
[23] B. Chankvetadze, Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, J. Chromatogr. A 1269 (2012) 26–51.
[24] M. Honetschlägerová-Vadinská, S. Srkalová, Z. Bosáková, P. Coufal, E. Tesařová, Comparison of enantioselective HPLC separation of structurally diverse compounds on chiral stationary phases with different teicoplanin coverage and distinct linkage chemistry, J. Sep. Sci. 32 (2009) 1704–1711.
[25] Chirobiotic Handbook , Advanced Separation Technology, 5th ed., Whippany, New York, 2004.
[26] A. Cavazzini, G. Nadalini, F. Dondi, F. Gasparrini, A. Ciogli, C. Villani, Study of mechanisms of chiral discrimination of amino acids and their derivatives on a teicoplanin-based chiral stationary phase, J. Chromatogr. A 1031 (2004) 143–158.

Publikace II

# Chromatographic Characterization of a New Cationic β-CD Based Stationary Phase Prepared by Dynamic Coating

Kučerová, G, Kalíková, K., Procházková, H., Popr, M., Jindřich, J., Coufal, P., Tesařová, E.

*Chromatographia* 2016, 79, 529-536

doi:10.1007/s10337-016-3050-z

Chromatographia (2016) 79:529–536
DOI 10.1007/s10337-016-3050-z

ORIGINAL

# Chromatographic Characterization of a New Cationic β-CD Based Stationary Phase Prepared by Dynamic Coating

**Gabriela Kučerová[1] · Květa Kalíková[1] · Hana Procházková[1] · Martin Popr[2] · Jindřich Jindřich[2] · Pavel Coufal[3] · Eva Tesařová[1]**

Received: 8 October 2015 / Revised: 18 January 2016 / Accepted: 8 February 2016 / Published online: 6 April 2016


**Abstract** Cyclodextrin-based stationary phases are frequently used for separation of various isomers. The main reason for applying new cyclodextrin (CD) derivatives for HPLC stationary phases is the improvement of separation efficiency. This paper introduces a new stationary phase prepared by dynamic coating of ion-exchanger with a newly synthesized cationic derivative of β-cyclodextrin. This mono-substituted derivative of β-cyclodextrin contains two tetraalkylammonium groups in its side chain. Diverse sets of analytes were tested to reveal the separation potential of the derivatized stationary phase. This dynamically coated stationary phase exhibited mostly better selectivity and resolution than a commercial chemically bonded β-cyclodextrin stationary phase. Mixtures of analytes were baseline resolved in shorter analysis time. Furthermore, interactions participating in the retention mechanism were identified using a linear free energy relationship approach and ionic interaction study. Lower hydrophobicity and higher ability to interact as hydrogen bond donor affect retention on the coated stationary phase. Even though the ionic interaction study confirmed that the surface of the ion-exchanger was modified by dynamic coating, the ionic interactions still contributed to the retention mechanism on the coated stationary phase.

**Keywords** HPLC · Dynamic coating · $6^{I}$-Deoxy-$6^{I}$-(dimethyl(3-(trimethylammonio)propyl)ammonio)-β-cyclodextrin diiodide · Linear free energy relationship · Ionic interactions

## Introduction

Commercially available CD-based stationary phases (SPs) can be used for the separation of chiral but also a variety of non-chiral analytes. The glucopyranose units of CD are linked together and form hydrophobic cavity while the hydrophilic surface with free OH groups is available for derivatization [1–3]. SPs based on the derivatized β-CDs belong to the most popular ones [4, 5]. Separation techniques as HPLC, CEC or SFC use both native and derivatized CD-based SPs [6, 7]. Many papers deal with β-CD SPs for enantioselective separations but underivatized β-CD SPs exhibit quite low separation potential for chiral analytes [2, 8, 9]. On the other hand, the separation of sets of achiral analytes (for example homologues or isomers) can proceed efficiently due to the formation of inclusion complexes of different stability with the hydrophobic cavity of the β-CD and additional interactions with functional groups on the CD surface [7, 10].

Charged CD derivatives are mostly used as selectors in CE [5, 11–13]. The library of positively charged CDs continuously increases and their use is expanding also to chromatography as various SPs are developed [13–15]. These

**Electronic supplementary material** The online version of this article (doi:10.1007/s10337-016-3050-z) contains supplementary material, which is available to authorized users.

✉ Květa Kalíková
kveta.kalikova@centrum.cz; kalikova@natur.cuni.cz

[1] Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic

[2] Department of Organic Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic

[3] Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic

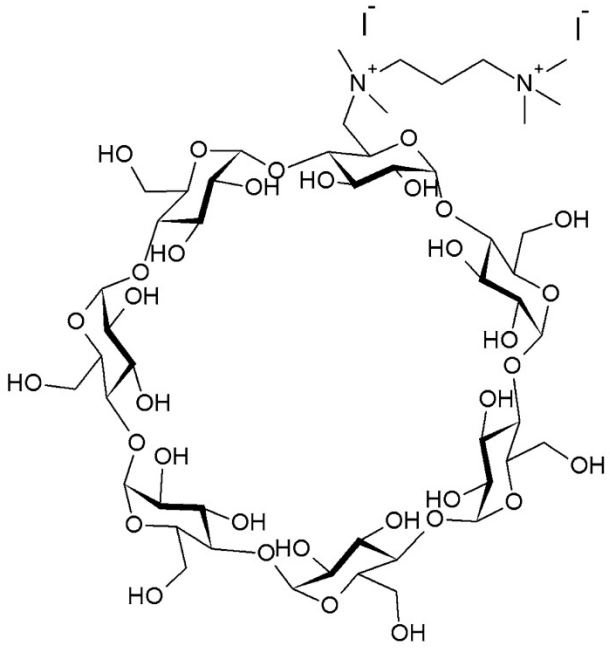

**Fig. 1** Structure of PEMPDA-β-CD

SPs are most often prepared by bonding of charged CDs onto silica gel [13]. Some cationic CD derivatives-based SPs were used for separation of structurally different analytes, with an emphasis on the nature of the selector [13]. For example imidazolium CD derivatives were used for separation of racemic aryl alcohols, flavanones, thiazides or dansyl-amino acids in HPLC [13, 16].

New mono-substituted tetraalkylammonium derivative of β–cyclodextrin, $6^{I}$-deoxy-$6^{I}$-(dimethyl(3-(trimethylammonio) propyl)ammonio)-β-cyclodextrin diiodide (PEMPDA-β-CD) was prepared as a promising cationic CD derivative (see Fig. 1) in our laboratories. Previously published paper by Popr et al. describes the synthesis of PEMPDA-β-CD in detail [17]. This CD derivative can be used as selector in CE or HPLC. Two permanent positive charges of PEMPDA-β-CD allow the preparation of SP by dynamic coating procedure.

In general, preparation of new SP comprises synthesis of the selector and its deposition on a SP support and high-pressure packing of the column in lab [13, 16, 18–20]. The most common are chemically bonded phases with selectors bonded on silica gel support. Dynamic coating used in this work represents another approach of selector deposition on a suitable carrier [2]. The latter approach can yield SPs stable for months against desorption of the selector as shown for example by dynamic coating of a monolithic SP with chiral selector [21]. However, change of the selector for another one is also possible, if suitable washing procedure is employed and followed by coating with a new selector. This possibility is one of the main benefits of the dynamic coating procedure.

This paper deals with description and characterization of a newly prepared charged CD derivative based SP for separation in HPLC. SP is prepared by dynamic coating of PEMPDA-β-CD on a suitable carrier, namely commercial ion-exchange SP (Luna 5u SCX 100Å). Main goal of this work is to evaluate the interaction capability of novel SP. Separations of diverse sets of analytes are performed to characterize the separation potential of the SP. Linear free energy relationship (LFER) method [22, 23] and ionic interactions study are employed to describe the interaction and retention properties of PEMPDA-β-CD SP and evaluate the interaction/separation mechanism. Commercial underivatized β-CD SP is used to compare the chromatographic characteristics.

## Experimental

### Apparatus and Chemicals

All chromatographic measurements were carried out on HPLC Waters Alliance® System with Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717 Plus, Waters Alliance® Series column heater, controlled by the Empower® software, Waters Corporation (Milford, MA, USA).

Two stationary phases were used, namely Astec Cyclobond™ I 2000 SP, native β-CD bonded on silica support from Supelco® (Bellefonte, PA, USA) and Luna 5u SCX 100Å, benzenesulfonic acid on silica support from Phenomenex (Torrance, PA, USA); the latter used for dynamic coating with PEMPDA-β-CD. Dimensions of chromatographic columns were the same, i.e., 250 × 4.6 mm i.d., silica particle size 5 µm. Temperature of columns and samples was kept at 25 °C. Injection volume was 10 µL. Flow rate was 1 mL $min^{-1}$. UV detection was performed at 254 nm. Void time was determined using solvent peak. All measurements were performed in triplicates.

Methanol (MeOH, Chromasolv® for HPLC), acetic acid (AAc, ReagentPlus®, 99 %), formic acid (FAc, reagent grade, ≥95 %), ammonium carbonate (for HPLC) and ammonium acetate (AMAC, 99 %) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Deionized water was purified with Rowapur and Ultrapur system from Watrex (Prague, Czech Republic). Sets of testing analytes (see Electronic Supplementary Material Fig. S1 for the structures) containing benzene [analytical standard (ACS)], naphthalene (ACS), anthracene (ACS), phenanthrene (98 %), pyrene (98 %), toluene (Chromasolv® for HPLC, 99.9 %), propylbenzene (ACS), butylbenzene

(ACS), pentylbenzene (ACS), aniline (ACS), *N*-methylaniline (*N*-MA, ACS), *N*-ethylaniline (*N*-EA, 98 %), *N*,*N*-diethylaniline (*N*,*N*-DIEA, purris. p.a., ≥99 %), cresols (*o*-, *m*-, *p*-, ACSs), xylenes (*o*-, *m*-, *p*-, ACSs), pyrocatechol (≥99 %), resorcinol (ACS reagent, ≥99 %), 2,3-dimethylphenol (PESTANAL®, ACS), 2,5-dimethylphenol (PESTANAL®, ACS), 2,6-dimethylphenol (PESTANAL®, ACS), benzenesulfonic acid (BSAC, ≥98 %), *p*-toluenesulfonic acid (*p*-TSAC, ACS reagent, ≥98.5 %) and *N*,*N*,*N*-trimethylanilinium chloride (TMA, ≥98 %) were purchased from Sigma–Aldrich (St. Louis, MO, USA). $6^{I}$-deoxy-$6^{I}$-(dimethyl(3-(trimethylammonio)propyl)ammonio)-β-cyclodextrin diiodide was synthesized by Popr et al. [17] with 98 % yield (see Fig. 1 for the structure).

## Procedures

### HPLC Procedure

Stock solutions of solid samples were prepared in concentration of 1 mg $mL^{-1}$ and stock solutions of liquid samples in concentration of 10 μL $mL^{-1}$ using MeOH as a solvent in most cases. Positional isomers of xylene, cresol and dimethylphenol were dissolved in mobile phase (MP) used for measurements. MeOH/10 mM AMAC buffer, pH 4.00 in various volume ratios was used as MPs. Seven sets of analytes were measured, i.e., group A containing benzene, naphthalene, anthracene, phenanthrene and pyrene; group B formed from toluene, propylbenzene, butylbenzene and pentylbenzene; group C composed of aniline, *N*-MA, *N*-EA and *N*,*N*-DIEA; groups D and E containing positional isomers of xylene and cresol, respectively; group F with pyrocatechol and resorcinol; and group G composed of positional isomers of dimethylphenol.

AMAC buffers were prepared by dissolving appropriate amount of AMAC in deionized water and adding the calculated amount of AAc to reach the required pH 4.00 in case of separations and LFER study. Measurements for ionic interactions study were carried out at pH 4.75. The compositions of AMAC buffers were: AMAC/AAc 10/52 mM, pH 4.00, ionic strength ($I$) = 10.1 mM or AMAC/AAc 5/9.7 mM, pH 4.75, $I$ = 5.0 mM, AMAC/AAc 10/19.1 mM, pH 4.75, $I$ = 10.0 mM, AMAC/AAc 15/28.4 mM, pH 4.75, $I$ = 15.0 mM and AMAC/AAc 20/37.6 mM, pH 4.75, $I$ = 20.0 mM. Aqueous solution of FAc was prepared by titration of water with diluted FAc to reach required pH 2.20 for dissolving PEMPDA-β-CD. Program PeakMaster [24] was used to calculate the concentration of AMAC, AAc and $I$. Minisart syringe filters 0.2 and 0.45 μm, Sartorius Stedim Biotech (Göttingen, Germany) were used for filtration of all the samples prepared and aqueous parts of MP, respectively.

### Coating Procedure

The PEMPDA-β-CD selector dissolved in aqueous solution of FAc (pH 2.20, amount 1 mg–1 mL) was coated on a commercial ion-exchange Luna 5u SCX SP containing benzenesulfonate groups. The flow rate and time of dynamic coating procedure were optimized. The best conditions found were flow rate 1 mL $min^{-1}$ and coating time 1 h.

Gravimetric analysis was used to determine the amount of PEMPDA-β-CD coated on SP. The amount of $1.73 \cdot 10^{-5}$ mol (43.7 % of a total quantity) of PEMPDA-β-CD was deposited on the SP surface during this procedure. Prepared SP was stable after more than 400 injections and the measurements were repeatable.

To test the repeatability of column preparation and possible change of selector the following procedure was used: 10 % aqueous solution of ammonium carbonate was used to remove PEMPDA-β-CD from SP. Then, the column was washed with this solution for 14 h with flow rate of 0.2 mL $min^{-1}$. The original ion-exchange SP was obtained and prepared for another dynamic coating procedure. The same coating procedure of PEMPDA-β-CD was used again. RSDs of the retention times of tested compounds on prepared SPs were less than 2.2 %.

### Linear Free Energy Relationship Method

The LFER method applied is based on the most generally used Eq. 1 [25]:

$$\log k = eE + sS + aA + bB + vV + c \quad (1)$$

where $k$ is retention factor of a test solute. The independent variables are solute descriptors (see Electronic Supplementary Material Table S1 for individual values): $E$ is the excess molar refraction, $S$ is the dipolarity/polarizability, $A$ is the effective or overall hydrogen bond acidity, $B$ represents effective or overall hydrogen bond basicity, and $V$ refers to the McGowan characteristic volume [22, 25]. The complementary regression coefficients ($e$, $s$, $a$, $b$, $v$) can be calculated, if descriptors $E$, $S$, $A$, $B$ and $V$ of a set of compounds are known.

The regression coefficient $e$ reflects the difference in disposition of the SP and MP to interact with $n$- and $\pi$-electron pairs of the solutes; $s$ refers to the difference in dipolarity/polarizability, $a$ and $b$ are equal to the differences in hydrogen bond basicity and hydrogen bond acidity, respectively, and the coefficient $v$ represents the difference in dispersion interactions (in reversed phase mode considered as hydrophobicity) between these two phases. The intercept $c$ in the LFER equation does not reflect any interaction; $c$ is a characteristic of the given system [22, 23, 25, 26]. The regression coefficients of the LFER equation were obtained from series of retention measurements of a set of 36 structurally

different solutes. Model solutes with known descriptors (see Electronic Supplementary Material Table S1) were selected to cover a wide range of chemical and structural properties. Multivariate linear regression analysis of log *k* vs solute descriptors was performed using NCSS software, NCSS LLC (Kaysville, UT, USA) [27].

## Results and Discussion

### Separation Potential of PEMPDA-β-CD Dynamically Coated SP

Analyses of seven groups (A–G) of structurally different analytes were performed on PEMPDA-β-CD coated SP and commercially available β-CD SPs for comparison. The latter SP has the same hydrophobic cavity that plays important role in the separation mechanism in reversed-phase chromatographic mode. Chromatographic data obtained for all tested analytes under the same conditions on both SPs are summarized in Electronic Supplementary Material Table S3. The results obtained at the best MP compositions are summarized in Table 1. Analytes from groups B, D, E, F and G were baseline separated while those from group A only partially (phenanthrene and naphthalene were resolved with $R_S = 1.21$) using PEMPDA-β-CD coated SP. The optimal MP compositions were mostly different for the SPs under comparison, just for groups E and F were the same for the both SPs. Group A analytes (benzene, naphthalene, anthracene, phenanthrene and pyrene)

**Table 1** Chromatographic data *k*, $\alpha$ and $R_S$ of achiral sets of analytes on coated and bonded CD-based SPs obtained at the best MP composition

| Group/analyte | PEMPDA-β-CD coated SP | | | | Group/analyte | Commercial β-CD SP | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MP *(v/v)* | *k* | $\alpha$ | $R_S$ | | MP *(v/v)* | *k* | $\alpha$ | $R_S$ |
| *Group A* | 40/60 | | | | *Group A* | 30/70 | | | |
| Benzene | | 1.23 | | | Benzene | | 4.14 | | |
| Phenanthrene | | 1.72 | 1.40 | 2.40 | Naphthalene | | 9.34 | 2.26 | 13.77 |
| Naphthalene | | 2.03 | 1.18 | 1.21 | Pyrene | | 12.39 | 1.33 | 3.01 |
| Anthracene | | 3.51 | 1.73 | 4.46 | Phenanthrene | | 16.02 | 1.29 | 2.98 |
| Pyrene | | 9.04 | 2.58 | 8.98 | Anthracene | | 38.92 | 2.43 | 13.81 |
| *Group B* | 30/70 | | | | *Group B* | 40/60 | | | |
| Toluene | | 1.39 | | | Toluene | | 3.71 | | |
| Propylbenzene | | 4.79 | 3.35 | 8.69 | Propylbenzene | | 12.38 | 3.34 | 10.93 |
| Butylbenzene | | 8.73 | 1.82 | 5.25 | Butylbenzene | | 20.53 | 1.66 | 3.91 |
| Pentylbenzene | | 11.12 | 1.27 | 2.04 | Pentylbenzene | | 27.50 | 1.34 | 1.91 |
| *Group C* | 60/40 | | | | *Group C* | 30/70 | | | |
| Aniline | | 3.01 | | | *N*,*N*-Diethylaniline | | 0.21 | | |
| *N*-Methylaniline | | 3.84 | 1.28 | 4.06 | *N*-Ethylaniline | | 0.76 | 3.62 | 9.43 |
| *N*-Ethylaniline | | 7.68 | 2.00 | 14.75 | Aniline | | 0.92 | 1.21 | 3.25 |
| *N*,*N*-Diethylaniline | | 31.39 | 4.09 | 33.08 | *N*-Methylaniline | | 1.03 | 1.12 | 1.42 |
| *Group D* | 20/80 | | | | *Group D* | 40/60 | | | |
| *m*-Xylene | | 1.54 | | | *m*-Xylene | | 4.13 | | |
| *o*-Xylene | | 1.79 | 1.16 | 2.18 | *o*-Xylene | | 4.42 | 1.07 | 1.09 |
| *p*-Xylene | | 2.49 | 1.39 | 5.16 | *p*-Xylene | | 6.46 | 1.46 | 5.81 |
| *Group E* | 40/60 | | | | *Group E* | 40/60 | | | |
| *o*-Cresol | | 0.44 | | | *o*-Cresol | | 1.53 | | |
| *m*-Cresol | | 0.66 | 1.50 | 3.55 | *m*-Cresol | | 1.75 | 1.14 | 1.53 |
| *p*-Cresol | | 0.91 | 1.38 | 3.45 | *p*-Cresol | | 2.46 | 1.41 | 4.40 |
| *Group F* | 20/80 | | | | *Group F* | 20/80 | | | |
| Pyrocatechol | | 0.67 | | | Pyrocatechol | | 1.86 | | |
| Resorcinol | | 0.93 | 1.39 | 3.86 | Resorcinol | | 2.20 | 1.18 | 2.20 |
| *Group G* | 30/70 | | | | *Group G* | 40/60 | | | |
| 2,6-Dimethylphenol | | 0.51 | | | 2,6-Dimethylphenol | | 1.37 | | |
| 2,5-Dimethylphenol | | 0.72 | 1.41 | 3.72 | 2,5-Dimethylphenol | | 1.67 | 1.22 | 1.82 |
| 2,3-Dimethylphenol | | 1.02 | 1.42 | 4.28 | 2,3-Dimethylphenol | | 2.20 | 1.32 | 3.04 |

MP composition: MeOH/10 mM AMAC buffer, pH 4.00 *(v/v)*, *k* retention factor, $\alpha$ selectivity, $R_S$ resolution

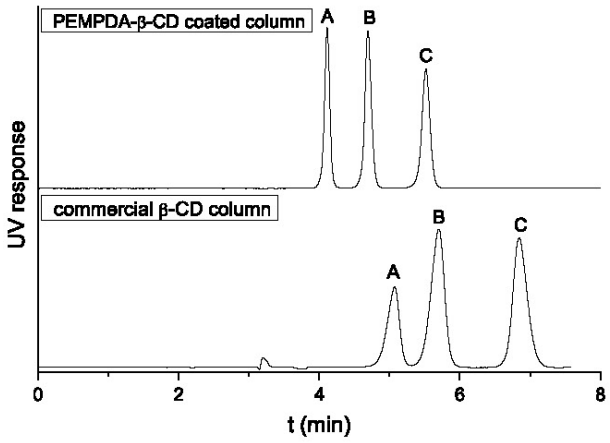


**Fig. 2** Chromatograms of separation of analytes of group G on both SPs in the optimal MP composed of MeOH/10 mM AMAC buffer, pH 4.00 30/70 *(v/v)* for PEMPDA-β-CD coated SP and MeOH/10 mM AMAC buffer, pH 4.00 40/60 *(v/v)* for commercial β-CD SP. *A* 2,6-dimethylphenol, *B* 2,5-dimethylphenol, *C* 2,3-dimethylphenol

exhibited different separation order on the SPs under comparison. Pyrene was the most retained on PEMPDA-β-CD coated SP with retention time 27.28 min in MP composed of MeOH/10 mM AMAC buffer, pH 4.00 40/60 *(v/v)*. On the other hand, the most retained analyte on the commercial β-CD SP was anthracene (retention time 50.13 min) in the same MP. As increasing retention did not correlate unequivocally with hydrophobicity that increased in the order—benzene (log $P = 2.3$), naphthalene (log $P = 3.4$), anthracene and phenanthrene (both log $P = 4.5$) and pyrene (log $P = 4.9$) [28, 29]. This behavior might be due to different steric hindrance caused by the different process of immobilization of CS to support.

The most important difference of prepared PEMPDA-β-CD dynamically coated SP is generally lower retention of all non-charged analytes in comparison with the commercial β-CD SP.

Different chromatographic behavior of the compared SPs was observed also in group C—aniline and its derivatives. The high retention on PEMPDA-β-CD SP can be explained by interactions of free amino group of the analytes with free benzenesulfonate groups that remain uncoated on SP surface. On the other hand, very low retention of aniline and its derivatives was observed in the system with commercial β-CD SP (under the same MP composition—all data not shown). Using MP composed of MeOH/10 mM AMAC buffer, pH 4.00 30/70 *(v/v)* lead to almost baseline separation of these analytes within 4.34 min. In addition, different elution order resulted from the compared SPs. *N*,*N*-DIEA was most strongly retained on the PEMPDA-β-CD SP whereas on the β-CD SP exhibited the lowest retention. This different behavior can be attributed mainly to ionic interactions participating in the retention mechanism on the PEMPDA-β-CD coated SP. For more detailed description of these interactions see chapter "Evaluation of ionic interactions".

Positional isomers of xylene (group D) were baseline separated on PEMPDA-β-CD coated SP in short analysis time (see Table 1). No baseline separation of *m*- and *o*-xylene in different MPs tested was achieved on the commercial SP. The best resolution value ($R_S = 1.09$) for these two isomers was obtained in MP composed of MeOH/10 mM AMAC buffer, pH 4.00 40/60 *(v/v)* on the commercial β-CD SP.

Baseline separation of analytes of group E (positional isomers of cresol) on both SPs was obtained in the same MP composed of MeOH/10 mM AMAC buffer, pH 4.00 40/60 *(v/v)* within 5.22 and 7.39 min on PEMPDA-β-CD SP and β-CD SP, respectively, as shown in Table 1. Additionally, resolution between *o*-cresol and *m*-cresol (and also selectivity) was significantly higher on the PEMPDA-β-CD coated SP ($R_S = 3.55$) than on the commercial β-CD SP ($R_S = 1.53$). Higher resolution value was achieved for another pair of isomers, *m*-cresol and *p*-cresol, on the commercial SP (see Table 1).

Pyrocatechol and resorcinol (group F) were baseline separated in MeOH/10 mM AMAC buffer, pH 4.00 20/80 *(v/v)* on the both tested SPs. Higher resolution as well as lower analysis time were observed in separation system with the coated SP.

**Table 2** Regression coefficients of the LFER equation, *SE* standard error values, *F*-test values and *R* correlation coefficient

| Column | Model | *e* | *s* | *a* | *b* | *v* | *c* | SE | *F* | *R* |
|---|---|---|---|---|---|---|---|---|---|---|
| PEMPDA-β-CD coated SP | CM | *x* | *x* | *x* | −0.68 | 1.24 | −1.13 | 0.56 | 29 | 0.91 |
| | *p* value | 0.36 | 0.62 | 0.98 | 0.00 | 0.00 | 0.00 | | | |
| | ±CI | | | | 0.25 | 0.35 | 0.35 | | | |
| Commercial β-CD SP | CM | *x* | *x* | *x* | −1.22 | 1.56 | −0.68 | 0.57 | 65 | 0.96 |
| | *p* value | 0.13 | 0.25 | 0.87 | 0.00 | 0.00 | 0.00 | | | |
| | ±CI | | | | 0.25 | 0.36 | 0.35 | | | |

MP used: MeOH/10 mM AMAC buffer, pH 4.00 40/60 *(v/v)*

*CM* Complete model, CI ±95 % confidence interval, *x* insignificant difference in interaction of the solute with the MP and SP, *p* statistical *p*-value, *SE* standard error in the estimate, *F* *F* ratio (*F*-test)

**Table 3** Results of evaluation of ionic interactions

| | Analyte ($pK_A$) [30, 31] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Benzene | Aniline (4.6) | *N*-EA (5.1) | *N*,*N*-DIEA (5.2) | BSAC (-2.8) | *p*-TSAC (-2.5) | TMA (-9.8) |
| | $k$ | $k_A/k_B$ | $k_A/k_B$ | $k_A/k_B$ | $k_A/k_B$ | $k_A/k_B$ | $k_A/k_B$ |
| 5 mM buffer | | | | | | | |
| PEMPDA-β-CD coated SP | 1.13 | 4.44 | 15.88 | 14.81 | ESP | ESP | NE |
| Luna 5u SCX 100Å column | 0.44 | 8.77 | 31.64 | 155.05 | ESP | ESP | NE |
| Commercial β-CD SP | 2.96 | 1.05 | 0.32 | 0.24 | 4.69 | 6.00 | ESP |
| 10 mM buffer | | | | | | | |
| PEMPDA-β-CD coated SP | 1.05 | 2.68 | 9.06 | 38.26 | ESP | ESP | NE |
| Luna 5u SCX 100Å column | 0.28 | 7.71 | 28.14 | 136.5 | ESP | ESP | 275.79 |
| Commercial β-CD SP | 2.53 | 1.04 | 0.27 | 0.19 | 2.87 | 3.52 | ESP |
| 15 mM buffer | | | | | | | |
| PEMPDA-β-CD coated SP | 0.70 | 2.99 | 9.70 | 42.39 | ESP | ESP | NE |
| Luna 5u SCX 100Å column | 0.27 | 6.63 | 19.89 | 87.00 | ESP | ESP | 189.89 |
| Commercial β-CD SP | 2.36 | 0.31 | 0.22 | 0.17 | 2.08 | 2.54 | ESP |
| 20 mM buffer | | | | | | | |
| PEMPDA-β-CD coated SP | 0.43 | 4.00 | 13.19 | 54.84 | ESP | ESP | 133.28 |
| Luna 5u SCX 100Å column | 0.22 | 6.00 | 17.77 | 77.73 | ESP | ESP | 169.46 |
| Commercial β-CD SP | 2.22 | 0.29 | 0.23 | 0.14 | 1.64 | 1.99 | ESP |

MP composition: MeOH/5, 10, 15 or 20 mM AMAC buffer, pH 4.75 40/60 *(v/v)*

$k$ Retention factor, $k_A/k_B$ retention factor of analyte related to retention factor of benzene, *NE* not eluted within 200 min, *ESP* elution with system peak

For illustration, chromatograms of separation of analytes of group G (positional isomers of dimethylphenol) on the both SPs under the optimal separation conditions are shown in Fig. 2. Certainly, the optimal MP composition is not the same. Peak symmetry of the group G analytes is better for system with coated SP than for the commercial β-CD SP. Values of symmetry factors (calculated at 5 % of peak height) for 2,6-dimethylphenol, 2,5-dimethylphenol and 2,3-dimethylphenol are 1.06, 0.91 and 1.01, respectively, for the PEMPDA-β-CD coated SP while 2,6-dimethylphenol and 2,5-dimethylphenol exhibit tailing peaks with symmetry factors 1.46 and 1.35, respectively, and 2,3-dimethylphenol is fronting with symmetry factor 0.78 on the commercial β-CD SP.

In general, for shorter, less expensive and "greener" analyses of compounds without basic group, the PEMPDA-β-CD coated SP is the first choice approach.

## Linear Free Energy Relationship Method

The LFER method was used for characterization and comparison of intermolecular interactions that dominate in the separation systems with MP composed of MeOH/10 mM AMAC buffer, pH 4.00 40/60 *(v/v)* and PEMPDA-β-CD coated SP or commercial β-CD SP. The LFER results are summarized in Table 2. The experimental vs. calculated log $k$ values show linear regression fit with correlation coefficients $R = 0.91$ and 0.96 for systems with PEMPA-β-CD coated SP and commercial β-CD SP, respectively.

The regression coefficients $e$ (interactions with $n$- and $\pi$-electrons), $s$ (dipolarity/polarizability) and $a$ (hydrogen bond basicity) are statistically insignificant in both chromatographic systems tested. This means that the corresponding interaction types are comparable in SP and MP in both systems tested and do not significantly affect retention of analytes.

The regression coefficient $b$ (hydrogen bond acidity) reaches negative values in both separation systems tested. So, this interaction type is preferred between analytes and MP. However, almost twice higher absolute value of coefficient $b$ obtained for system with commercial β-CD SP indicates that PEMPDA-β-CD SP coated possesses higher ability to interact as hydrogen bond donor than commercial β-CD SP.

System hydrophobicity is characterized by coefficient $v$. Values of this coefficient are positive in both systems and lower for the PEMPDA-β-CD coated SP. Thus, the dynamically coated SP shows lower hydrophobicity than the commercial one. This fact might be partially caused by bonded positively charged tetraalkylammonium chain on the PEMPDA-β-CD and probably by lower selector coverage on the support, which is connected with more significant

effect of the carrier. As a result, lower retention of analytes can be expected, which interact with the SP on the basis of their hydrophobicity.

### Evaluation of Ionic Interactions

Since the basic LFER equation does not include a term reflecting ionic interactions, an alternative approach had to be used to investigate this type of interactions.

Seven structurally different compounds (see Table 3) were employed for the study of ionic interactions participating in the interaction mechanism on the PEMPDA-β-CD coated SP, the ion-exchange SP used for coating of PEMPDA-β-CD and the compared commercial β-CD SP. Three compounds had primary amino group, one had charged tertiary amine, two were acidic with $SO_3H$ group and the last one was neutral. Strong acids, BSAC and *p*-TSAC, and strong base TMA are fully dissociated/protonated at the pH of the buffer constituent (pH 4.75) of the MP. Weak bases aniline, *N*-EA and *N*,*N*-DIEA (for $pK_A$ values see Electronic Supplementary Material Table S2) are partially protonated under the experimental conditions used [30, 31]. Benzene, which does not form ionic interactions, was selected for reference, i.e., retention factors of the solutes were related to the retention factor of benzene to minimize other effects, mainly hydrophobicity. Table 3 summarizes the obtained data: $k_A/k_B$ stands for retention factor of the analyte divided by retention factor of benzene obtained under the same chromatographic conditions.

Comparison of the $k_A/k_B$ values of weak bases obtained for the individual SPs shows the most important role of the ionic interactions (highest $k_A/k_B$ values) on the uncoated ion-exchange SP, which is in accord with the presumption. The lowest values obtained on the commercial β-CD SP show that this type of interaction is of less importance on this SP. Concerning the strong base (the fully protonated one) it was strongly retained on both ion-exchange and PEMPDA-β-CD coated SP while the fully dissociated acids were not retained at all, they eluted with system peak.

Decrease of the retention values with increasing ionic strength reflects the competition of the analyte with buffer components for ionic interactions with the SPs. This is unreserved true for the weak bases on the uncoated ion-exchange SP but only partly true on the PEMPDA-β-CD coated SP where other interactions than ionic ones participate in the retention mechanism. Very low retention values obtained on the commercial β-CD SP corresponds with the lowest contribution of the silica support to retention.

The evaluation of retention data obtained with PEMPDA-β-CD coated SP and with the uncoated Luna 5u SCX SP confirms that the original ion-exchange surface was partially modified by the coating with PEMPDA-β-CD and as the result the interaction mechanism can profit from the combination of the ionic interactions as well as from the other types of interactions revealed from the LFER results.

## Conclusion

New SP was prepared by dynamic coating of the ion-exchange SP Luna 5u SCX using the synthesized PEMPDA-β-CD selector. Separation potential study, LFER method and ionic interaction study were performed in order to describe properties, interaction mechanism and application possibility of the new SP. Results were compared with those obtained with the commercial β-CD SP Cyclobond™ I 2000. The PEMPDA-β-CD coated SP was shown to be suitable for separation of various compounds at shorter analysis times than if the commercially available β-CD SP was used. This fact was supported by LFER results concerning hydrophobicity. Lower hydrophobicity of PEMPDA-β-CD SP plays important role in retention mechanism on this SP with respect to hydrophobicity/polarity of analytes. Certainly, the effect of the ion-exchange carrier cannot be ignored. The benzenesulfonate groups of the ion exchanger affect retention of charged analytes more than the carrier of the commercial β-CD SP. The advantage of the coated SP is the possibility to simply change selector on the SP surface and in this way prepare new type of SP. Synthesis of the chiral selector used in this work was described in detail previously [17] and other derivatives will be prepared.

**Acknowledgments** The authors gratefully acknowledge financial support of the Grant Agency of the Charles University, Grant No. 790314 and the Grant Agency of the Czech Republic, Grant No. 13-01440S and P206/14-19278P.

**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

## References

1. Armstrong DW, Demond W (1984) J Chromatogr Sci 22:411–415
2. Bressolle F, Audran M, Pham T-N, Vallon J-J (1996) J Chromatogr B 687:303–336
3. Crini G, Torri G, Lekchiri Y, Martel B, Janus L, Morcellet M (1995) Chromatographia 41:424–430
4. Tanwar S, Bhushan R (2015) Chromatographia 78:1113–1134
5. Scriba GKE (2012) Chromatographia 75:815–838
6. Kalíková K, Šlechtová T, Vozka J, Tesařová E (2014) Anal Chim Acta 821:1–33
7. Kalíková K, Riesová M, Tesařová E (2012) Cent Eur J Chem 10:450–471
8. Kučerová G, Kalíková K, Riesová M, Tesařová E (2015) Chromatographia 79:917–921

9. Zhang X, Zhang C, Sun G, Xu X, Tan Y, Wu H, Cao R, Liu J, Wu J (2012) Instrum Sci Technol 40:194–215
10. Nguyen HP, Yang SH, Wigginton JG, Simpkins JW, Schug KA (2010) J Sep Sci 33:793–802
11. Tang W, Muderawan IW, Ong T-T (2007) Ng S-Ch. Tetrahedron Asymmetr 18:1548–1553
12. Dai Y, Wang S, Zhou J, Liu Y, Sun D, Tang J, Tang W (2012) J Chromatogr A 1246:98–102
13. Zhou J, Tang J, Tang W (2015) Trend Anal Chem 65:22–29
14. Zhou J, Liu Y, Lu Y, Tang J, Tang W (2015) RSC Adv 4:54512–54516
15. Zhang Q, Guo J, Xiao Y, Crommen J, Jiang Z (2015) J Sep Sci 38:1813–1821
16. Wang R-Q, Ong T-T, Tang W, Ng SC (2012) Anal Chim Acta 718:121–129
17. Popr M, Hybelbauerová S, Jindřich J (2014) Beilstein J Org Chem 10:1390–1396
18. Cavazzini A, Pasti L, Massi A, Marchetti N, Dondi F (2011) Anal Chim Acta 706:205–222
19. Zhou R-D, Li L-S, Cheng B-P, Nie G-Z, Zhang H-F (2014) Chin J Anal Chem 42:1002–1009
20. Guo J, Zhang Q, Yao Z, Zhao X, Ran D, Crommen J, Jiang Z (2014) J Sep Sci 37:1720–1727
21. Schmid MG, Schreiner K, Reisinger D, Gübitz G (2006) J Sep Sci 26:1470–1475
22. Abraham MH, McGowan J (1987) Chromatographia 23:243–246
23. Kalíková K, Lokajová J, Tesařová E (2006) J Sep Sci 29:1476–1485
24. Gaš B, Jaroš M, Hruška V, Zusková I, Štědrý M (2005) LC GC Eur 18:282–288
25. Abraham MH, Whiting GS, Doherty RM, Shuely W (1991) J Chromatogr 587:213–228
26. Kalíková K, Kozlík P, Gilar M, Tesařová E (2013) J Sep Sci 36:2421–2429
27. Hintze JL (2007) NCSS user's guide II. NCSS, Kaysville
28. Hansch C, Leo A (1979) Substituent constants for correlation analysis in chemistry and biology. Wiley, New York
29. Hansch C, Leo A, Hoekman DH (1995) Exploring QSAR, fundamentals and application in chemistry and biology. American Chemical Society, Washington DC
30. Guthrie JP (1978) Can J Chem 56:2342–2354
31. Perrin DD (1972) Dissociation constants of organic bases in aqueous solution, IUPAC chemical data series: supplement 1972. Butterworth, London

Publikace III

# Sulfobutylether-β-cyclodextrin as a chiral selector for separation of amino acids and dipeptides in chromatography

Kučerová, G., Procházková, H., Kalíková, K., Tesařová, E.

*Journal of Chromatography A* 2016, 1467, 356-362

doi:10.1016/j.chroma.2016.07.061

Journal of Chromatography A, 1467 (2016) 356–362

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Sulfobutylether-β-cyclodextrin as a chiral selector for separation of amino acids and dipeptides in chromatography

Gabriela Kučerová, Hana Procházková, Květa Kalíková *, Eva Tesařová

*Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic*

ARTICLE INFO

*Article history:*
Received 29 March 2016
Received in revised form 19 July 2016
Accepted 23 July 2016
Available online 25 July 2016

*Keywords:*
HPLC
Chiral selector
Sulfobutylether-β-cyclodextrin
Dynamic coating
Mobile phase additive

ABSTRACT

Various amino acids, dipeptides and their isomers were (enantio)separated using sulfobutylether-β-cyclodextrin as a chiral selector. Two different approaches were employed: first, dynamic coating of sulfobutylether-β-cyclodextrin onto a strong anion-exchange stationary phase and, second, use of sulfobutylether-β-cyclodextrin as a mobile phase additive in a separation system with a C18 column. Measurements were carried out using RP-HPLC and hydrophilic interaction liquid chromatography. Mobile phases composed of organic modifier (methanol) and four different aqueous parts: (i) deionized water, (ii) an aqueous solution of formic acid (pH 2.1), (iii) ammonium acetate buffer (pH 4.7), and (iv) ammonium acetate buffer (pH 8.8) in various volume ratios. Under these separation conditions, out of 23 chiral analytes, 9 were baseline enantio-resolved and 7 were partially separated. Of 9 mixtures of dipeptide isomers, 8 were baseline-separated. Sulfobutylether-β-cyclodextrin proved to be suitable for the separation of chiral and also achiral analytes. The use of sulfobutylether-β-cyclodextrin as a dynamic coating agent or as a mobile phase additive depends on the particular chromatographic system and analytes of interest.



## 1. Introduction

Cyclodextrins (CDs) and their derivatives as chiral selectors (CSs) in HPLC still represent a significant tool for the analysis of structurally different compounds in modern analytical chemistry [1–7]. The extensive use of CDs as CSs is due to their natural chirality and ability to form inclusion complexes with molecules *via* their hydrophobic cavity [8]. Because of the low solubility of native β-cyclodextrin (β-CD) in water and quite low enantioseparation efficiency [9], the synthesis of new derivatized neutral, anionic or cationic CDs is still continuing [3,7]. This fact contributes to further examination and application of CDs not only in HPLC or supercritical fluid chromatography (SFC), but mainly in CE [2,7,10]. Some possibilities exist for the preparation and utilization of the derivatized CDs in HPLC. For example, a CD-based stationary phase (SP) can be prepared by click chemistry or dynamic coating [3,11,12]. Another possibility is application of the CS as chiral mobile phase (MP) additive [3,7].

In this work we focused on CS sulfobutylether-β-CD (SBE-β-CD) with the commercial name Captisol®. The sulfobutylether substituent can be introduced at the 2, 3 and 6 positions in one or more glucopyranose units of CD. Captisol® is the hepta-substituted preparation, which was found to be a substance with very desirable drug-carrier properties [13]. Thus, Captisol® allows formation of complex with drug resulting in increased solubility and improved target delivery in the human body [14]. Consequently, SBE-β-CD is particularly favoured by pharmaceutical companies. However, as mentioned above, SBE-β-CD can also be used as CS in CE or HPLC. Several papers dealing with SBE-β-CD employed in CE measurements with very good results can be traced in the literature, *e.g.* [15,16]. Fillet et al. focused on CE enantioseparation of acidic drugs with both uncharged CD and charged SBE-β-CD as CSs [17]. Skanchy et al. studied the effect of the degree of substitution of SBE-β-CD on enantioseparations of drug mixtures by CE [18]. Perrin et al. used amino acid alkyl esters and *N*-amino acid derivatives for CE study of three derivatized CDs [19]. The different factors, *e.g.* CD type or pH of the background electrolyte, were examined [19]. SBE-β-CD was also employed in HPLC measurements as a chiral MP additive [20–22]. For example, amlodipine racemate was enantioresolved by dual chiral MP additives, where one of the additives was SBE-β-CD [22]. Peng et al. described the enantioseparation of the chiral drug citalopram using SBE-β-CD and a C18 column in RP-HPLC [23].

The analytes used in our study were chosen with respect to their nature (for the structures with marked chiral centers see the

* Corresponding author.
*E-mail addresses:* kalikova@natur.cuni.cz, kveta.kalikova@natur.cuni.cz (K. Kalíková).

http://dx.doi.org/10.1016/j.chroma.2016.07.061
0021-9673/

Electronic Supplementary material, Table S1). Chiral β-CD derivatives offer an opportunity to enantio-resolve chiral analytes, such as some underivatized and derivatized amino acids or dipeptides due to the possibility of binding host molecules into the CD cavity [8,19,24–27]. In general, amino acids and peptides are chemical compounds which play an important role in metabolism and nutrition [28]. Our work was focused on the amino acids Tyr, Trp, Phe and their derivatives and dipeptides derived from these amino acids. Protected amino acids could be used as precursors during peptide synthesis to protect the amino or carboxy group against racemization [29,30]. Dipeptide isomers form a unique group of analytes examined in this study. Their structures and purposes of their use are summarized in Table S2, in the Electronic Supplementary material. Dipeptides used for the mixtures have the same molecular formula but the sequence of amino acids constituting the dipeptide is reversed. Here we show that SBE-β-CD could also be used successfully for separation of these compounds.

The main advantage of SBE-β-CD lies in its polyanionic nature. The anionic species could be utilized during a coating procedure. Previously, we reported a study that describes a functional coating procedure of a charged CD to the oppositely charged strong cation exchange SP [12]. Based on this experience, the formation of ionic interactions between negatively charged SBE-β-CD and the positively charged surface of strong anion exchange SP can be expected. Thus, our main objectives of this work were to prepare SP based on SBE-β-CD by a coating procedure on a suitable strong anion exchange surface and further to discover and optimize the MP compositions for the separation of chiral underivatized and derivatized amino acids, dipeptides and mixtures of dipeptide isomers. However, as CSs could also serve as MP additives, we also tested SBE-β-CD in this manner. A C18 column was used for measurements with SBE-β-CD present in MP. Further these two different approaches, *i.e.* SBE-β-CD coated SP and SBE-β-CD as MP additive, were compared and discussed.

## 2. Materials and methods

### 2.1. Apparatus, reagents, analytes

All the chromatographic measurements were carried out on the Agilent Technologies HPLC System composed of 1200 series quaternary pump, 1260 Infinity autosampler, 1290 Infinity column and autosampler thermostats and 1260 Infinity diode array detector (Agilent Technologies, Waldbroon, Germany). The whole system was controlled by the OpenLab® software, Agilent Technologies (Waldbroon, Germany).

Two columns were used: (i) Spherisorb® column, strong anion exchange SP (containing a silica-based quaternary ammonium bonded sorbent), was used for dynamic coating with SBE-β-CD, (ii) XTerra™ MS C18 column. Both columns were purchased from Waters (Milford, MA, USA). The dimensions of both columns were 150 × 4.6 mm i.d.; silica particle size 5 μm.

Captisol® (SBE-β-CD) was kindly provided by Ligand Pharmaceuticals, Inc. (La Jolla, CA, USA). The degree of substitution of Captisol® is varied and can range from 4 to 8, where the manufacturer specifies a final substitution for each batch based on testing. Our Captisol® sample had degree of substitution 6.6 [13].

Acetic acid (AA, ReagentPlus®, 99%), ammonium acetate (AMAC, 99%), ammonium hydroxide solution (AHS, ≥30%), formic acid (FA, Reagent Grade, ≥95%) and methanol (MEOH, Chromasolv® for HPLC), were supplied by Sigma-Aldrich (St. Louis, MO, USA). The deionized water used was purified with a Rowapur and Ultrapur system from Watrex (Prague, Czech Republic).

Abbreviations *t-BOC* and *Z* in the names of the amino acids and dipeptides stand for *tert*-butyloxycarbonyl and benzyloxycarbonyl, respectively. The chiral analytes (for structures see Electronic Supplementary material, Table S1) namely *t-BOC*-DL-Phe, Gly-DL-Phe, Gly-DL-Trp were supplied by Santa Cruz Biotechnology Inc., (Heidelberg, Germany); *t-BOC*-DL-Trp, *t-BOC*-DL-Tyr, *t-BOC*-*p*-Cl-DL-Phe, DL-Phe, DL-Trp, DL-Trp benzylester, DL-Trp butylester, DL-Trp methylester hydrochloride, DL-Tyr, Phe-Pro, Tyr-Ala, Tyr-Phe, Val-Tyr and *Z*-Phe-Leu were obtained from Sigma-Aldrich (St. Louis, MO, USA). α-Methyl-DL-Tyr, DL-4-Cl-Phe, DL-4-F-Phe, *Z*-DL-2-F-Phe, *Z*-DL-3-F-Phe and *Z*-DL-4-F-Phe ethylester were prepared at the Institute of Molecular Biology, Bulgarian Academy of Sciences (Sophia, Bulgaria). The isomers of dipeptides (for structures see Electronic Supplementary material, Table S2), namely H-Tyr-Phe-OH and H-Phe-Tyr-OH, H-Ala-Tyr-OH and H-Tyr-Ala-OH, H-Phe-Ala-OH and H-Ala-Phe-OH, H-Ala-Trp-OH and H-Trp-Ala-OH, H-Phe-Trp-OH and H-Trp-Phe-OH, *Z*-Tyr-Ala-OH and *Z*-Ala-Tyr-OH, *Z*-Trp-Phe-OH and *Z*-Phe-Trp-OH, *Z*-Phe-Ala-OH and *Z*-Ala-Phe-OH, *Z*-Trp-Ala-OH and *Z*-Ala-Trp-OH were purchased from BACHEM AG (Bubendorf, Switzerland).

### 2.2. Procedures

#### 2.2.1. General conditions and procedures

The temperature of the columns and samples was kept at 25 °C and 20 °C, respectively. The range of injected volumes was 5–20 μL, depending on the detector response. The flow rate was 1 mL min$^{-1}$. Detection was performed in the UV region. The column void time was determined using the solvent peak. All the measurements were performed in triplicate.

Stock solutions of solid samples were prepared in a concentration of 1 mg mL$^{-1}$. MEOH was used as a solvent in the majority of cases. Table S1 in the Electronic Supplementary material gives different conditions for dissolution where required. If needed, the individual enantiomers of the amino acids were mixed at a concentration ratio of 1:1. Dipeptide samples were prepared, for example, by mixing H-Tyr-Phe-OH and H-Phe-Tyr-OH in a concentration ratio of 1:1. The other dipeptide mixtures were prepared in the same way. Mixtures of separated dipeptides are summarized in Table S2 in the Electronic Supplementary material.

FA (pH 2.1) was prepared by adding the appropriate amount of concentrated FA to deionized water. Buffers were prepared by dissolving the appropriate amount of AMAC in deionized water and adding the calculated amount of AA or AHS to reach the required pH 4.7 or 8.8, respectively. The buffer concentrations were: (i) AMAC/AA 20/60 mM and (ii) AMAC/AHS 10/23.2 mM. The PeakMaster program [31] was used to calculate the concentrations of FA, AMAC, AA and AHS. MEOH was used as an organic modifier. Measurements were carried out for MP composition MEOH/aqueous part 90/10 or 40/60 *(v/v)* to 10/90 *(v/v)* on the coated SP and MS C18 SP, respectively.

0.2 μm and 0.45 μm Minisart syringe filters, Sartorius Stedim Biotech (Göttingen, Germany) were used for filtration of all the prepared samples and aqueous parts of the MPs, respectively

#### 2.2.2. Coating procedure

The SBE-β-CD (Captisol®) dissolved in 40/60 *(v/v)* MEOH/deionized water at a concentration of 1 mg mL$^{-1}$ was used for coating the Spherisorb® commercial strong anion exchange column. The flow rate and time of the coating procedure were optimized. The best conditions found were: flow rate 0.6 mL min$^{-1}$ and coating time 2 h.

Gravimetric analysis was used for determination of the amount of SBE-β-CD-coated on the SP surface. An amount of 0.0034 g of SBE-β-CD was deposited on the SP surface during this procedure. To ensure system stability, a small amount (0.1 mg mL$^{-1}$) of SBE-β-CD was added to the MP during the whole measurements. The

prepared SP was stable after more than 500 injections and the measurements were repeatable.

#### 2.2.3. Mobile phase additive procedure

As the first step it was necessary to find a suitable amount of SBE-β-CD for (enantio)separations. Two different mass concentrations of SBE-β-CD were used, *i.e.* 0.5 mg mL$^{-1}$ and 1 mg mL$^{-1}$ in MP. CS was dissolved in the aqueous part of MP and mixed with the appropriate volume of organic modifier using HPLC equipment. We expressed the amount of SBE-β-CD in MP as "mg mL$^{-1}$" to facilitate comparison with the amount of SBE-β-CD coated on the strong anion exchange SP (see subsection above, 2.2.2 Coating procedure).

## 3. Results and discussion

The obtained results are divided into individual subsections according to the nature of the analytes for clarity of presentation and meaningful comparison. Separation results for chiral analytes are discussed first and followed by those for dipeptide isomers.

### 3.1. SBE-β-CD-coated SP

Simple MPs composed of MEOH and deionized water were used as the first option, especially for verification of the coating procedure. However, these conditions have already been found to be satisfactory for separation of 6 analytes and will be discussed in the text below. Three different aqueous parts of MP at different pH were used for the measurements – FA (pH 2.1), AMAC buffer (pH 4.7) and AMAC buffer (pH 8.8). MEOH was used as an organic modifier in all cases. These three pH values were used based on the nature of the analytes (charge dependence on the pH value).

#### 3.1.1. Separation of chiral analytes

Tested and optimized MP compositions for chiral analytes separated on the coated SP are summarized in Table 1. The use of AMAC buffer (pH 8.8) was not beneficial for any of the chiral analytes. They were mostly eluted at very short retention times without any indication of enantioseparation. Half of chiral analytes showed lower retention with decreasing content of MEOH in MP. The analytes with a free carboxyl group are negatively charged at pH 8.8. Therefore, there is no interaction between the negatively charged sulfo groups of CS coated on SP and the analyte. Moreover, interactions between negatively charged analytes and the positively charged original ion exchange surface of the SP do not contribute to retention, probably because of steric hindrance of SBE-β-CD-coated on SP surface. Using the FA (pH 2.1), AMAC buffer (pH 4.7) or deionized water yielded satisfactory results.

At least partial enantioseparations of *t-BOC* amino acids were achieved at various MP compositions – see Table 1. Appropriate MPs were composed of MEOH and FA (pH 2.1) or MEOH and AMAC buffer (pH 4.7) in different volume ratios. All the *t-BOC* amino acids exhibited typical RP behavior. *t-BOC*-DL-Trp, *t-BOC*-DL-Tyr and *t-BOC-p*-Cl-DL-Phe were baseline enantio-resolved. Higher resolution values were observed if FA (pH 2.1) was used instead of AMAC buffer (pH 4.7). Under these conditions (pH 2.1), *t*-BOC amino acids are uncharged. The enantiomeric elution order was determined for these analytes. D enantiomer was eluted first in all optimized MP compositions. The peak symmetry (calculated at 5% peak high) ranged from 0.76 to 1.30. Partial enantioseparation of *t-BOC*-DL-Phe with a resolution value of 0.76 was observed in MP MEOH/FA (pH 2.1) 10/90 *(v/v)*.

During optimization, Gly-DL-Phe and Gly-DL-Trp exhibited decreased retention at lower MEOH contents while the resolution increased. These two analytes were baseline enantioseparated in the MPs composed of MEOH/FA (pH 2.1) 10/90 and 20/80 *(v/v)*, respectively. Analysis times did not exceed 7 and 10 min, respectively. Dipeptides were positively charged under these conditions. Thus the positive charge of analytes probably supports the enantioseparation due to the forming an ionic interactions between CS and analyte. In addition, no enantioseparation was achieved at pH 4.7 (neutral charge) and at pH 8.8 (negative charge). For illustration, enantioseparation of Gly-DL-Phe is depicted in Fig. 1. For the first eluted enantiomer, the peak symmetry was 0.87 while for second eluted 0.83.

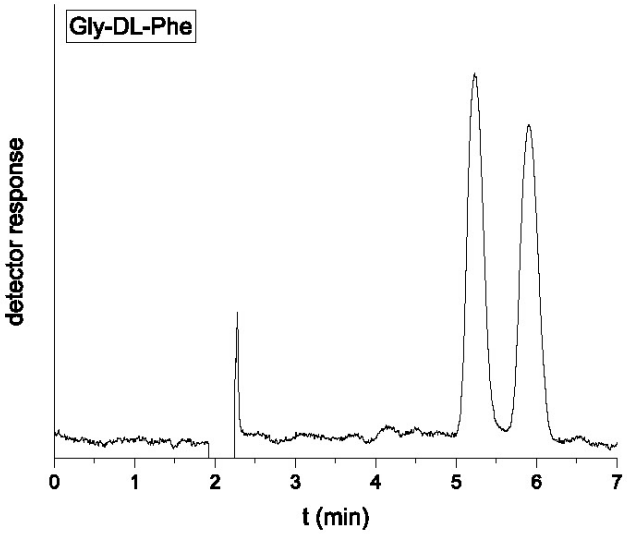


**Fig. 1.** Chromatogram of the enantioseparation of Gly-DL-Phe in MP composed of MEOH/FA (pH 2.1) 10/90 *(v/v)* on SBE-β-CD-coated SP ($R_S$ = 1.62).

Other chiral dipeptides, *i.e.* Phe-Pro, Tyr-Ala, Tyr-Phe, Val-Tyr and one *N*-blocked chiral dipeptide *Z*-Phe-Leu exhibited low retention on SBE-β-CD-coated SP in all the tested MPs. Despite the fact, that these analytes contain two chiral centers (see Electronic Supplementary material, Table S1) in their molecules, it was not possible to enantioseparate all the enantiomers in this separation system. Therefore only partial separations were achieved in the following MPs: MEOH/FA (pH 2.1) 10/90 *(v/v)* for positively charged Phe-Pro with resolution of 0.56 and MEOH/deionized water 10/90 *(v/v)* for Tyr-Ala and Val-Tyr with resolution values of 0.62 and 0.45, respectively. MP composed of MEOH and deionized water was the only one that exhibited at least partial separation of the latter dipeptides. Enantioseparation of the analytes Tyr-Phe and *Z*-Phe-Leu was not achieved in any of the tested MPs.

Enantiomers of α-Methyl-DL-Tyr were partially resolved in MP composed of MEOH/FA (pH 2.1) 10/90 *(v/v)*. This amino acid was positively charged under these conditions and showed lower retention with decreased content of MEOH in MP. The first and second eluted enantiomers exhibited peak symmetries of 0.83 and 0.90, respectively. Situation with charge dependence on pH is similar to glycyl derivatives of Phe and Trp (mentioned above). Separation mechanism is probably the same.

The *Z* derivatives of DL-Phe, *i.e.* Z-DL-2-F-Phe and Z-DL-4-F-Phe ethylester were baseline-separated and Z-DL-3-F-Phe was partially enantioseparated with resolution $R_S$ = 1.35. The compositions of the optimized MPs are listed in Table 1. All three derivatives of DL-Phe were uncharged in their best MP composition. On the other hand Z-DL-2-F-Phe and Z-DL-3-F-Phe were negatively charged at pH 4.7 and 8.8. Using MPs containing buffer with pH value 8.8 led to very low retention and no enantioseparations were observed. This is probably caused by repulsion interactions between CS and analytes. In addition, the position of the fluorine atom in the molecule probably negatively affected the enantioseparation of Z-DL-3-F-Phe.

**Table 1**
Chromatographic data $k_1/k_2$ and $R_s$ on the coated SP in optimized MP composition.

| | Name of analyte/s | MP composition (*v/v*) MEOH/A or B or C or D | $k_1/k_2$ | $R_s$ | Mixed behavior♦ | RP behavior○ | LRLM behavior± |
|---|---|---|---|---|---|---|---|
| **Chiral analytes** | α-Methyl-DL-Tyr | 10/90 MEOH/A | 0.96/1.15 | 1.13 | xx | xx | A,B,C |
| | *t-BOC*-DL-Phe | 10/90 MEOH/A | 5.95/6.52 | 0.76 | xx | A,B | xx |
| | ***t-BOC***-DL-Trp | 40/60 MEOH/A | 1.52/2.62 | **3.05** | xx | A,B | xx |
| | | 30/70 MEOH/B | 1.31/1.70 | **1.67** | | | |
| | ***t-BOC***-DL-Tyr | 40/60 MEOH/A | 1.08/1.67 | **2.02** | xx | A,B | xx |
| | | 10/90 MEOH/B | 1.83/2.23 | **1.60** | | | |
| | ***t-BOC-p***-Cl-DL-Phe | 20/80 MEOH/A | 5.36/6.75 | **1.98** | xx | A | C |
| | DL-Phe | 10/90 MEOH/D | 0.19/0.24 | 0.47 | xx | xx | A |
| | **DL-Trp** | 20/80 MEOH/A | 1.78/2.17 | **1.72** | xx | xx | A,C |
| | DL-Trp benzylester | x | | | C | xx | A |
| | **DL-Trp butylester** | 60/40 MEOH/A | 4.12/5.52 | **2.57** | A,B,C | xx | xx |
| | | 70/30 MEOH/B | 1.56/1.99 | **2.40** | | | |
| | | 70/30 MEOH/D | 2.92/3.62 | **2.23** | | | |
| | DL-Trp methylester | x | | | B,C | xx | A |
| | DL-Tyr | x | | | xx | xx | A,B,C |
| | DL-4-Cl-Phe | x | | | xx | xx | A |
| | DL-4-F-Phe | x | | | xx | xx | A |
| | **Gly-DL-Phe** | 10/90 MEOH/A | 1.78/2.13 | **1.62** | xx | xx | A,C |
| | **Gly-DL-Trp** | 20/80 MEOH/A | 3.30/3.82 | **1.63** | xx | xx | A,C |
| | Phe-Pro | 10/90 MEOH/A | 1.25/1.38 | 0.56 | xx | A | C |
| | Tyr-Ala | 10/90 MEOH/D | 0.18/0.24 | 0.62 | xx | xx | A,B,C |
| | Tyr-Phe | x | | | B | xx | C |
| | Val-Tyr | 10/90 MEOH/D | 0.29/0.34 | 0.45 | xx | xx | C |
| | ***Z***-DL-2-F-Phe | 20/80 MEOH/A | 5.30/6.60 | **1.75** | A | B | xx |
| | Z-DL-3-F-Phe | 10/90 MEOH/A | 6.97/8.10 | 1.35 | A | B | xx |
| | ***Z***-DL-4-F-Phe ethylester | 20/80 MEOH/D | 3.33/4.08 | **1.58** | xx | A,B,C | xx |
| | Z-Phe-Leu | x | | | xx | A,B | xx |
| **Dipeptide isomers** | **1.H-Phe-Tyr-OH2.H-Tyr-Phe-OH** | 40/60 MEOH/A | 2.42/3.01 | 1.86 | A,B | xx | C |
| | **1.H-Ala-Tyr-OH 2.H-Tyr-Ala-OH** | 40/60 MEOH/A | 1.12/2.17 | 4.77 | xx | xx | A,B,C |
| | **1.H-Ala-Phe-OH2.H-Phe-Ala-OH** | 40/60 MEOH/A | 1.12/1.96 | 4.06 | xx | xx | A,B,C |
| | **1.H-Trp-Ala-OH** | 40/60 MEOH/A | 1.94/2.86 | 3.37 | A,B | xx | C |
| | **2.H-Ala-Trp-OH** | 90/10 MEOH/B | 1.00/1.42 | 2.54 | | | |
| | | 80/20 MEOH/D | 0.45/0.70 | 1.83 | | | |
| | **1.H-Phe-Trp-OH2.H-Trp-Phe-OH** | 40/60 MEOH/A | 4.22/4.92 | 1.56 | A,B | xx | C |
| | 1.Z-Ala-Tyr-OH 2.Z-Tyr-Ala-OH | 10/90 MEOH/B | 1.00/1.17 | 0.93 | xx | A,B | xx |
| | 1.Z-Phe-Trp-OH 2.Z-Trp-Phe-OH | 10/90 MEOH/A | 17.93/19.04 | 0.43 | xx | A,B | xx |
| | 1.Z-Ala-Phe-OH 2.Z-Phe-Ala-OH | 10/90 MEOH/A | 1.94/2.18 | 0.64 | xx | A,B | xx |
| | 1.Z-Ala-Trp-OH 2.Z-Trp-Ala-OH | 10/90 MEOH/B | 3.54/3.79 | 0.52 | xx | A,B | xx |

*t-BOC* – *tert*-butyloxycarbonyl, *Z* – benzyloxycarbonyl, $k_1/k_2$ – first, respectively second eluted enantiomer or isomer, x – no (enantio)separation, **Bold** indicates baseline separation.
A – FA (pH 2.1); B – AMAC buffer (pH 4.7); C – AMAC buffer (pH 8.8); D – deionized water.
♦,○,± evaluation of behavior of analytes is performed according to their retention on coated SP in MPs composed of: MEOH/A or B or C, in entire range, *i.e.* 90/10 to 10/90 *(v/v)*.
♦**Mixed behavior:** mixed HILIC and RP behavior, ○**RP behavior:** higher retention with lower content of MEOH in MP, ±**LRLM behavior:** lower retention with lower content of MEOH in MP, **xx:** no mixed or RP or LRLM behavior observed, **1.** and **2.** indicates first and second eluted isomer, respectively.

The hydrophobicity of the analytes also plays an important role. Blocking of the $NH_2$ group of AAs has a positive effect on the enantioseparation, regardless of whether a *t-BOC* or *Z* group is present. On the other hand, no enantioseparation was observed for DL-4-Cl-Phe and DL-4-F-Phe in any of the tested MP. Mixed retention behavior – hydrophilic interaction liquid chromatography (HILIC) *vs.* RP behavior, was observed for the analytes Z-DL-2-F-Phe and Z-DL-3-F-Phe while the *Z*-blocked ethylester of DL-Phe exhibited typical RP behavior (increasing retention with decreasing MEOH in MP). This behavior can be explained as follows: *Z*-blocked amino acids have a free COOH group, and thus their molecules are more polar than the *Z*-blocked ester, due to blocking of the $NH_2$ group and also the COOH group of the latter analyte. The peak symmetry of the *Z*-blocked analytes ranged between 0.71 and 1.13.

Interesting retention and separation behavior was observed for three esters of DL-Trp. While no enantioseparation of the methylester and benzylester was achieved, three optimal MP compositions were found for the DL-Trp butylester. The DL-Trp butylester was at least partially positively charged under these conditions. Resolutions were higher than 2.2 and the analysis time did not exceed 15 min. This analyte also exhibited mixed retention behavior – see Table 1. The different behavior of the butylester of DL-Trp can probably be attributed to its higher hydrophobicity compared to the methylester of DL-Trp. The steric effect can negatively influence the enantioseparation of the DL-Trp benzylester. Good peak symmetry was observed for the DL-Trp butylester, *e.g.* 0.98 and 0.88 for the first and second eluted enantiomers, respectively, in MP MEOH/AMAC buffer (pH 4.7) 70/30 *(v/v)*.

Native amino acids, *i.e.* DL-Phe, DL-Tyr and DL-Trp, were poorly enantioseparated. Enantioresolution higher than 1.5 was achieved only for positively charged DL-Trp in MP MEOH/FA (pH 2.1) 20/80 *(v/v)* (see Table 1). DL-Phe was partially enantioseparated ($R_s$ = 0.47) in MP MEOH/deionized water 10/90 *(v/v)* within 3 min. No enantioseparation of DL-Tyr was obtained. The charges of these analytes depend on the pH values (2.1, 4.7 and 8.8) as follows – positively charged, neutral and negatively charged, but this is not the main factor that should be taken into account for successful enantioseparation according to obtained results.

### 3.1.2. Dipeptide separation with coated stationary phase

MPs composed of MEOH and FA (pH 2.1) or AMAC buffer (pH 4.7) or deionized water were suitable for the separation of dipep-

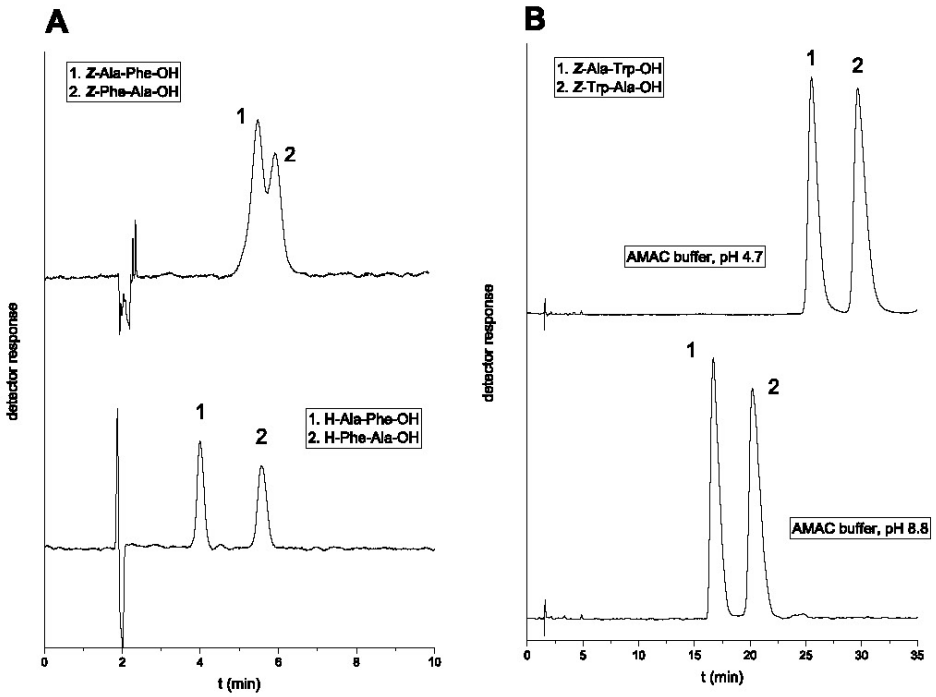


**Fig. 2.** A) Chromatograms of the separation of Z-Ala-Phe-OH/Z-Phe-Ala-OH and H-Ala-Phe-OH/H-Phe-Ala-OH in MP composed of MEOH/FA (pH 2.1) 10/90 *(v/v)* on SBE-β-CD-coated SP. B) Chromatograms of the separation of Z-Ala-Trp-OH/Z-Trp-Ala-OH in two optimized MPs composed of MEOH/AMAC buffer (pH 8.8) or AMAC buffer (pH 4.7) 40/60 *(v/v)*, on MS C18 SP with SBE-β-CD as MP additive, respectively.

tide isomer mixtures. The best results are listed in Table 1. Using AMAC buffer (pH 8.8), no separation was achieved and the retention time of the analytes was only about 2 min. The retention behavior could be explained in the same way as for the chiral analytes discussed in chapter 3.1.1, because all the dipeptides are negatively charged at pH 8.8. The mixture of dipeptides that differed in the sequence of amino acids H-Trp-Ala-OH (eluted first) and H-Ala-Trp-OH was baseline-separated even in three different aqueous parts of MPs in a short analysis time. In addition, these analytes exhibited mixed retention behavior (HILIC *vs.* RP) in MPs composed of MEOH and FA (pH 2.1) or AMAC buffer (pH 4.7). The other mixtures of dipeptide isomers without a *Z* group were baseline-separated in MP MEOH/FA (pH 2.1) 40/60 *(v/v)* with resolutions higher than 1.56 and analysis times of less than 15 min. The individual pairs of isomers of dipeptides were analysed separately. The presence of a benzyloxycarbonyl group caused worsening of the separation of the dipeptide analogues (see Table 1). This effect is depicted in Fig. 2: A). All the *Z*-substituted dipeptides exhibited RP behavior in MPs composed of MEOH and FA (pH 2.1) or AMAC buffer (pH 4.7). The highest resolutions were observed in MP MEOH/aqueous part 10/90 *(v/v)*. While the resolutions and retention times of all the *Z*-blocked dipeptide isomers were quite similar, the Z-Phe-Trp-OH/Z-Trp-Phe-OH pair (first/second eluted isomer) exhibited extremely high retentions. Its non-blocked analogues H-Phe-Trp-OH/H-Trp-Phe-OH (first/second eluted isomer) exhibited the highest retentions of the entire group of non-blocked dipeptides. Nevertheless, the difference was not very distinctive. This behavior occurs because these dipeptides contain two benzene rings in their molecule and a non-polar *Z* substituent, which causes higher hydrophobicity and thus higher retention. A peak symmetry higher than 1.00 was observed for only two isomers (Z-Phe-Trp-OH and Z-Ala-Trp-OH in optimized MPs). Slightly fronting peaks are more common for this separation system than slightly tailing peaks.

### 3.2. SBE-β-CD as MP additive

A sufficient amount of SBE-β-CD in MP was 0.5 mg mL$^{-1}$. No significant difference was observed between 1 mg mL$^{-1}$ and 0.5 mg mL$^{-1}$ of CS in MP for the chosen tested analytes. Thus the data presented in this part of the work were obtained with 0.5 mg mL$^{-1}$ of the CD derivative in the MP. Measurements were carried out with MPs of the same composition as used previously. However, MPs composed of MEOH and deionized water with SBE-β-CD as MP additive were not used for measurements on MS C18 SP because this MP composition was primarily used only for verification of the coating procedure. In addition, it is generally better to use a well-defined aqueous part of MP (*e.g.* buffer) to avoid possible pH fluctuations. The measured volume ratios of MEOH/aqueous part ranged from 40/60 to 10/90. At higher MEOH content no satisfactory and meaningful results were expected on the non-polar SP.

#### 3.2.1. Separation of chiral analytes with the MP additive

Chromatographic data obtained for this part of the work are summarized in Table 2. The section of Table 2, that contains the results of the analysis of chiral analytes on MS C18 SP with SBE-β-CD as MP additive is substantially shortened and lists only three analytes. The rest of the chiral amino acids and dipeptides did not yield satisfactory results and no enantioseparation was obtained.

DL-Trp butylester was enantioseparated with resolutions values of 2.88 and 2.24 in MPs composed of MEOH/FA (pH 2.1) or AMAC buffer (pH 4.7) 30/70, respectively – see Table 2. Analysis times equaled 40 and 55 min at pH 2.1 and 4.7, respectively. This implies that the charge of the analyte affects mainly the retention but probably has no impact on the enantioresolution if SBE-β-CD is used as a MP additive. A much lower analysis time was observed for baseline enantioseparation of the DL-Trp butylester using SBE-β-CD-coated SP. Different values of the peak symmetry were observed in the two different optimized MPs. Slightly tailing peaks were observed

**Table 2**
Chromatographic data $k_1/k_2$ and $R_s$ on the C18 SP with the SBE-β-CD as MP additive in optimized MP composition.

| | Name of analyte/s | MP composition (v/v)MEOH/A or B or C | $k_1/k_2$ | $R_s$ |
|---|---|---|---|---|
| CA | **DL-Trp butylester** | 30/70 MEOH/A | 14.9/17.5 | **2.88** |
| | | 30/70 MEOH/B | 24.8/28.6 | **2.24** |
| | Gly-DL-Trp | 10/90 MEOH/A | 19.8/20.6 | 0.58 |
| | Val-Tyr | 10/90 MEOH/B | 0.81/0.94 | 0.35 |
| **Dipeptide isomers** | **1.H-Phe-Tyr-OH** | 30/70 MEOH/A | 0.80/1.42 | **3.10** |
| | **2.H-Tyr-Phe-OH** | 30/70 MEOH/B | 0.65/1.29 | **2.46** |
| | | 20/80 MEOH/C | 10.0/11.6 | **2.04** |
| | 1.H-Ala-Tyr-OH | 20/80 MEOH/A | 0.20/0.42 | 1.38 |
| | 2.H-Tyr-Ala-OH | 10/90 MEOH/C | 0.64/1.04 | 1.06 |
| | **1.H-Ala-Phe-OH** | 30/70 MEOH/A | 0.30/0.70 | **2.98** |
| | **2.H-Phe-Ala-OH** | 10/90 MEOH/B | 1.55/2.22 | **1.55** |
| | | 10/90 MEOH/C | 4.08/5.27 | **2.54** |
| | **1.H-Trp-Ala-OH2.H-Ala-Trp-OH** | 30/70 MEOH/A | 0.47/0.95 | **2.66** |
| | **1.H-Phe-Trp-OH** | 40/60 MEOH/A | 1.04/1.50 | **1.90** |
| | **2.H-Trp-Phe-OH** | 40/60 MEOH/B | 1.32/2.19 | **2.90** |
| | | 40/60 MEOH/C | 4.40/5.52 | **2.06** |
| | **1.Z-Ala-Tyr-OH** | 30/70 MEOH/B | 10.3/12.4 | **2.46** |
| | **2.Z-Tyr-Ala-OH** | 30/70 MEOH/C | 6.25/7.75 | **2.67** |
| | Z-Phe-Trp-OH Z-Trp-Phe-OH | NE in all pH values and volume ratios tested | | |
| | **1.Z-Ala-Phe-OH** | 40/60 MEOH/A | 26.18/29.74 | **2.46** |
| | **2.Z-Phe-Ala-OH** | 40/60 MEOH/C | 9.40/11.96 | **2.47** |
| | **1.Z-Ala-Trp-OH** | 40/60 MEOH/B | 15.11/17.71 | **2.39** |
| | **2.Z-Trp-Ala-OH** | 40/60 MEOH/C | 9.53/11.75 | **2.13** |

CA – chiral analytes, $k_1/k_2$ – first, respectively second eluted enantiomer or isomer, NE – not eluted until 60 min, A – FA (pH 2.1); B – AMAC buffer (pH 4.7); C – AMAC buffer (pH 8.8), **Bold** indicates baseline separation, **1.** and **2.** indicates first and second eluted isomer, respectively.

at pH 2.1 while slightly fronting at pH 4.7. Partial enantioseparations were also achieved for Gly-DL-Trp and Val-Tyr in MEOH/FA (pH 2.1) and AMAC buffer (pH 4.7), respectively. Positively charged Gly-DL-Trp at pH 2.1 was separated within 40 min.

According to the obtained results, it is evident (see Table 2), that a separation system composed of MS C18 SP with SBE-β-CD as MP additive is not suitable for the majority of these analytes.

### 3.2.2. *Dipeptide separations with MP additive*

The results of measurements of dipeptide isomers are summarized in Table 2. The MS C18 SP with SBE-β-CD as MP additive was suited for separation of these analytes. Very promising data and optimal MP compositions for almost all the mixtures of dipeptide isomers were found. One pair of dipeptides (Z-Trp-Phe-OH/Z-Phe-Trp-OH) could not be separated in any of the separation systems used within 60 min. The retention was very high and thus no elution was observed within 60 min. It is anticipated that strong interaction occurs with the C18 column due to high hydrophobicity.

Non-blocked dipeptide isomers were baseline separated with resolution values in the range from 1.55 to 3.10. Up to three optimal aqueous parts of MPs were found for each of three dipeptide mixtures. Only partial separation ($R_S$ = 1.38) was achieved for the H-Ala-Tyr-OH/H-Tyr-Ala-OH pair (first/second eluted isomer). There is no direct relationship between the retention and resolution, as can be seen from Table 2. The charges of non-blocked dipeptide isomers were also changed with changing MP compositions, *i.e.* buffer pH. Positive charge at pH 2.1 and negative at pH 8.8 of analytes did not significantly affect the separation – see Table 2. As an example, H-Phe-Tyr-OH/H-Tyr-Phe-OH (first/second eluted isomer) was separated in MPs with three different aqueous parts. The highest retention resulted in MP with AMAC buffer (pH 8.8) but this corresponded to the lowest resolution value. In general, non-blocked isomers of dipeptides had lower retention than the more hydrophobic Z-blocked analogues. This behavior can be explained by the hydrophobic nature of MS C18 SP; therefore, more hydrophobic analytes are retained strongly. The correlation between the retention and hydrophobicity (presence of a Z-blocking group) and a certain correlation between the charge of the analytes and the retention was observed. All the Z-blocked dipeptides were neutral at pH 2.1, partially negatively charged at pH 4.7 and negatively charged at pH 8.8. Z-blocked dipeptide isomers were retained less at pH 8.8 (both negatively charged CS in MP and the analyte caused reduced retention) than at pH 2.1 or 4.7. The effect of the buffer pH on the separation of Z-Trp-Ala-OH/Z-Ala-Trp-OH is depicted in Fig. 2: B). The peak symmetry at pH 8.8 was mostly less than 1 (0.35–0.92) while it was close to 1.00 at pH 4.7. The largest fluctuation of the peak symmetry was observed at pH 2.1, where both fronting and tailing peaks were observed.

## 4. Concluding remarks

Novel CD-based chiral SP was successfully prepared by coating SBE-β-CD on the strong anion exchange SP surface. Its (enantio)separation properties were evaluated under HILIC and RP conditions. The results were compared with those obtained on C18 SP with SBE-β-CD used as MP additive. The coated SP was suitable for enantioseparation of 9 chiral analytes, with resolution higher than 1.5 and analysis times not exceeding 17 min. Seven other analytes were partially enantioseparated with resolution ranging between 0.45 and 1.35. The optimized MP compositions differ depending on the analyte properties/structures. However, for the majority of the analytes, the most appropriate MPs consisted of MEOH/FA (pH 2.1). Under these conditions the majority of analytes are positively charged, which enables ionic interactions with chiral selector coated on SP. The coated SP was also suitable for separation of the dipeptide isomers. Five mixtures were baseline-separated and four were partially separated with resolution ranging between 0.43 and 0.93. The partially separated dipeptide isomers contained a hydrophobic Z substituent blocking the amino group, which seemed to have a negative effect on the separation. The most

suitable MP compositions for separation of these dipeptide isomers were MEOH/FA (pH 2.1) in various volume ratios. The analytes bring positive charge in these MP compositions.

Different behavior of the analytes was observed if SBE-β-CD was employed as an MP additive. First, this system exhibited very low separation ability towards chiral analytes, where only one compound was baseline-resolved and two were partially resolved. On the other hand, the opposite situation was obtained when dipeptide isomer mixtures were employed. The percentage success of MS C18 SP with SBE-β-CD as a MP additive was more than 70%. Seven mixtures of dipeptide isomers were baseline-separated ($R_S > 1.5$). One mixture of dipeptide isomers was partially separated ($R_S = 1.38$).

To conclude, SBE-β-CD-coated SP is more suitable for separation of chiral analytes than MS C18 SP with SBE-β-CD as MP additive. On the other hand, the latter is more appropriate for non-chiral separations of mixtures of dipeptide isomers. SBE-β-CD-coated SP enables very efficient separation ability of structurally different analytes. Finally, we should emphasize the advantage of the coating procedure of SBE-β-CD compared to the MP additive procedure due to the significantly lower consumption of CS.

## Conflict of interest

The authors declare that there is no conflict of interest.

## Acknowledgements

The authors gratefully acknowledge the financial support of the Grant Agency of Charles University, Grant No. 790314 and the Czech Science Foundation, Grants Nos. P206/14-19278P, and 16-05942S.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.07.061.

## References

[1] P.K. Owens, A.F. Fell, M.W. Coleman, J.C. Berridge, Chiral recognition in liquid chromatography utilising chargeable cyclodextrins for resolution of doxazosin enantiomers, Chirality 9 (1997) 184–190.
[2] R.-Q. Wang, T.-T. Ong, S.-C. Ng, Synthesis of cationic beta-cyclodextrin derivatives and their applications as chiral stationary phases for high-performance liquid chromatography and supercritical fluid chromatography, J. Chromatogr. A 1203 (2008) 185–192.
[3] Y. Xiao, S.-C. Ng, T.T.Y. Tan, Y. Wang, Recent development of cyclodextrin chiral stationary phases and their applications in chromatography, J. Chromatogr. A 1269 (2012) 52–68.
[4] D.W. Armstrong, W. Demond, Cyclodextrin bonded phases for the liquid-chromatographic separation of optical geometrical, and structural isomers, J. Chromatogr. Sci. 22 (1984) 411–415.
[5] R. Furuta, H. Nakazawa, Optical resolution mechanisms of uniconazole and structurally related-compounds on cyclodextrin-bonded columns, Chromatographia 35 (1993) 555–559.
[6] J. Debowski, J. Jurczak, D. Sybilska, Resolution of some chiral mandelic-acid derivatives into enantiomers by reversed-phase high-performance liquid-chromatography via alpha-cyclodextrin and beta-cyclodextrin inclusion complexes, J. Chromatogr. 282 (1983) 83–88.
[7] J. Zhou, J. Tang, W. Tang, Recent development of cationic cyclodextrins for chiral separation, TrAC-Trends Anal. Chem. 65 (2015) 22–29.
[8] X. Lai, W. Tang, S.-C. Ng, Novel cyclodextrin chiral stationary phases for high performance liquid chromatography enantioseparation: effect of cyclodextrin type, J. Chromatogr. A 1218 (2011) 5597–5601.
[9] G. Kucerova, K. Kalikova, M. Riesova, E. Tesarova, Effect of buffer constituents on retention and separation in achiral and chiral HPLC systems with beta-cyclodextrin-based stationary phase, Chromatographia 79 (2015) 917–921.
[10] K. Kalikova, T. Slechtova, J. Vozka, E. Tesarova, Supercritical fluid chromatography as a tool for enantioselective separation; a review, Anal. Chim. Acta 821 (2014) 1–33.
[11] J. Guo, Q. Zhang, Z. Yao, X. Zhao, D. Ran, J. Crommen, Z. Jiang, One-step strategy for the synthesis of a derivatized cyclodextrin-based monolithic column, J. Sep. Sci. 37 (2014) 1720–1727.
[12] G. Kucerova, K. Kalikova, H. Prochazkova, M. Popr, J. Jindrich, P. Coufal, E. Tesarova, Chromatographic characterization of a new cationic β-CD based stationary phase prepared by dynamic coating, Chromatographia 79 (2016) 529–536.
[13] I. Ligand, Pharmaceuticals, What is captisol? in: I. CyDex Pharmaceuticals (Ed.), 2015, pp. 1–8.
[14] J.D.P. Vincent, D. Antle, Considering modified cyclodextrins as a tool for solving parenteral solubility challenges, Pharm. Qual. 14 (2012) 26–29.
[15] F. Suss, W. Poppitz, C.E. Sanger-van de Griend, G.K.E. Scriba, Influence of the amino acid sequence and nature of the cyclodextrin on the separation of small peptide enantiomers by capillary electrophoresis using randomly substituted and single isomer sulfated and sulfonated cyclodextrins, Electrophoresis 22 (2001) 2416–2423.
[16] B. Chankvetadze, G. Endresz, G. Blaschke, About some aspects of the use of charged cyclodextrins for capillary electrophoresis enantioseparation, Electrophoresis 15 (1994) 804–807.
[17] M. Fillet, I. Bechet, G. Schomburg, P. Hubert, J. Crommen, Enantiomeric separation of acidic drugs by capillary electrophoresis using a combination of charged and uncharged beta-cyclodextrins as chiral selectors, HRC-J. High Resolut. Chromatogr. 19 (1996) 669–673.
[18] D.J. Skanchy, G.H. Xie, R.J. Tait, E. Luna, C. Demarest, J.F. Stobaugh, Application of sulfobutylether-beta-cyclodextrin with specific degrees of substitution for the enantioseparation of pharmaceutical mixtures by capillary electrophoresis, Electrophoresis 20 (1999) 2638–2649.
[19] C. Perrin, M.G. Vargas, Y. Vander Heyden, M. Maftouh, D.L. Massart, Fast development of separation methods for the chiral analysis of amino acid derivatives using capillary electrophoresis and experimental designs, J. Chromatogr. A 883 (2000) 249–265.
[20] P.K. Owens, A.F. Fell, M.W. Coleman, J.C. Berridge, Effect of charged and uncharged chiral additives on the resolution of amlodipine enantiomers in liquid chromatography and capillary electrophoresis, J. Chromatogr. A 797 (1998) 187–195.
[21] J. Guan, J. Yang, Y. Bi, S. Shi, F. Yan, F. Li, Determination of the enantiomeric impurity in S-(−)pantoprazole using high performance liquid chromatography with sulfobutylether-beta-cyclodextrin as chiral additive, J. Sep. Sci. 31 (2008) 288–293.
[22] J. Xie, Q. Tan, L. Yang, S. Lai, S. Tang, C. Cai, X. Chen, A simple and rapid method for chiral separation of amlodipine using dual chiral mobile phase additives, Anal. Methods 6 (2014) 4408–4413.
[23] Y. Peng, Q.S. He, J. Cai, Enantioseparation of citalopram by RP-HPLC, using sulfobutyl ether-beta-cyclodextrin as a chiral mobile phase additive, Int. J. Anal. Chem. (2016), article ID 1231386.
[24] C. Desiderio, S. Fanali, Use of negatively charged sulfobutyl ether-beta-cyclodextrin for enantiomeric separation by capillary electrophoresis, J. Chromatogr. A 716 (1995) 183–196.
[25] M. Riesova, R. Geryk, K. Kalikova, T. Slechtova, M. Voborna, M. Martinkova, A. Bydzovska, E. Tesarova, Direct CE and HPLC methods for enantioseparation of tryptophan and its unnatural derivatives, Sep. Purif. Technol. 158 (2016) 24–30.
[26] Y. Chen, J. Zhang, L. Zhang, G. Chen, Separation of dipeptides with two chiral centres using 2-hydroxypropyl-β-CD-modified MEKC, Electrophoresis 31 (2010) 1493–1497.
[27] I. Ali, M. Suhail, Z. Al-Othman, A. Alwarthan, H.Y. Aboul-Enein, Enantiomeric resolution of multiple chiral centres racemates by capillary electrophoresis, Biomed. Chromatogr. 30 (2016) 683–694.
[28] D. Kumar, M.A. Rub, M. Akram, D. Kabir-ud, Interaction between dipeptide (glycyl-phenylalanine) and ninhydrin: role of CTAB and gemini (16-s-16 s = 4,5,6) surfactant micelles, J. Colloid Interface Sci. 418 (2014) 324–329.
[29] E. Tesarova, Z. Bosakova, V. Pecakova, Comparison of enantioselective separation of *n-tert.*-butyloxycarbonyl amino acids and their non-blocked analogues on teicoplanin-based chiral stationary phase, J. Chromatogr. A 838 (1999) 121–129.
[30] G. Kucerova, J. Vozka, K. Kalikova, R. Geryk, D. Plecita, T. Pajpanova, E. Tesarova, Enantioselective separation of unusual amino acids by high performance liquid chromatography, Sep. Purif. Technol. 119 (2013) 123–128.
[31] B. Gas, M. Jaros, V. Hruska, I. Zuskova, M. Stedry, Peakmaster – a freeware simulator of capillary zone electrophoresis, LCGC Eur. 18 (2005) 282.

Publikace IV

# Effect of Buffer Constituents on Retention and Separation in Achiral and Chiral HPLC systems with β-Cyclodextrin-Based Stationary Phase

Kučerová, G., Kalíková, K., Riesová, M., Tesařová, E.

*Chromatographia* 2014, 78, 917-921

doi:10.1007/s10337-014-2834-2

Chromatographia (2015) 78:917–921
DOI 10.1007/s10337-014-2834-2

SHORT COMMUNICATION

# Effect of Buffer Constituents on Retention and Separation in Achiral and Chiral HPLC Systems with β-Cyclodextrin-Based Stationary Phase

**Gabriela Kučerová · Květa Kalíková · Martina Riesová · Eva Tesařová**

Received: 30 October 2014 / Revised: 8 December 2014 / Accepted: 9 December 2014 / Published online: 21 December 2014


**Abstract** In HPLC, cyclodextrin-based stationary phases allow separation of both achiral and chiral analytes. Complexation of analytes into the cyclodextrin cavity is a very important part of the interaction mechanism. However, possible complexation of buffer constituents must be also considered. This fact should not be overlooked in method development procedures. In this study, β-cyclodextrin-based stationary phase (CYCLOBOND I 2000) was used, and two different buffers, namely aqueous solutions composed of lithium hydroxide/benzoic acid 10.0/24.5 mM, pH 4.00 and lithium hydroxide/acetic acid 9.9/60.0 mM, pH 4.01 of the same ionic strength were compared. Methanol was added as organic modifier in a methanol/buffer volume ratio 40/60. The linear free energy relationship method was used for evaluation of interactions contributing to chromatographic performance of the separation system. Strong complexation of benzoic acid with cyclodextrin was obvious from the results. This effect was clearly reflected in reduced retention and restricted (enantio) separation ability of the separation system containing benzoic acid in the buffer. On the other hand, complexing buffer constituents of mobile phases can offer a worthy alternative, to less "green"organic solvents, for reduction of the analysis time.

Published in the topical collection *20th International Symposium on Separation Sciences* in Prague with guest editors Aleš Horna and Pavel Jandera.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10337-014-2834-2) contains supplementary material, which is available to authorized users.

G. Kučerová · K. Kalíková (✉) · M. Riesová · E. Tesařová
Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic
e-mail: kveta.kalikova@centrum.cz; kalikova@natur.cuni.cz

**Keywords** HPLC · Cyclodextrin · Linear free energy relationship · Complexation · Chiral separation

## Introduction

Cyclodextrins (CDs) are well-established complex-forming agents widely used in separation science. Chiral selectors based on CDs became very popular for separation of stereoisomers in HPLC and also in CE, CEC, GC and SFC [1–4]. It is generally known that these cyclic oligosaccharides form inclusion complexes with a wide range of substances of suitable size and hydrophobicity. This property of CDs is the subject of research and applications in science and technology [3, 5]. In analytical and/or preparative separations, this complexation plays an irreplaceable role [6]. However, CDs can form complexes not only with analytes. Riesová et al. and Beneš et al. [7, 8] have recently shown both theoretically and experimentally that the complex-forming ability of CDs could have also negative consequences in CE. This is in the case that constituents of background electrolyte (BGE) also interact with CD (enter the CD cavity). Then separation of analytes can substantially deteriorate because of changes of pH values, ionic strength and conductivity values of BGE.

The question arises if similar effects can be observed also in HPLC, i.e., if possible complexation of mobile phase (MP) constituents with CD-based chiral stationary phase (CSP) can influence retention and enantioseparation there. This possibility is often neglected; just simplified separation systems are considered where only the complexation equilibria that take place between the CDs and analytes are taken into account [3].

One of the comprehensive and broadly applicable methods that can help in the characterization of biphasic

systems is linear free energy relationship (LFER) [9, 10]. The LFER can reveal interaction mechanism, i.e., identify interactions that participate in retention and separation.

Our work is aimed at describing possible complexation of β-CD CSP with components of MP, benzoic acid (BAc) and acetic acid (AAc) for comparison, and on clarification of the effect of complexation on chromatographic data, retention, selectivity and resolution. To explore the impact of the overlooked complexation more closely, the LFER method, which has never been applied for such purpose before, is used in this study.

## Experimental

### Instrumentation

All chromatographic measurements were carried out on HPLC Waters Alliance® System with Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717Plus, Waters Alliance® Series column heater, controlled by the Empower® software (Waters Corporation, Milford, USA).

Astec Cyclobond™ I 2000 column, native β-CD bonded on silica support from SUPELCO® (Bellefonte, USA), was used. Dimensions of the column were 250 × 4.6 mm i.d., silica particle size 5 μm. Temperature of the column and samples was kept at 25 °C. Injection volume was 5 or 20 μL (higher volume due to low response of analytes when BAc was used as MP component). Flow rate was 1 mL $min^{-1}$. Detection was performed in the range 190–400 nm, depending on presence of BAc in MP. Column dead time was determined using system peaks. All measurements were performed in triplicates.

### Chemical and Reagents

Methanol (Chromasolv® for HPLC), acetic acid (ReagentPlus®, ≥99 %), benzoic acid (puriss p.a.), formic acid (ReagentGrade, ≥95 %) and lithium hydroxide monohydrate (puriss p.a.) were supplied by Sigma-Aldrich (St. Louis, USA). The deionized water used was purified with a Rowapur and Ultrapur system from Watrex (Prague, Czech Republic). Achiral analytes containing toluene, naphthalene and anthracene were purchased from Sigma-Aldrich (St. Louis, USA). Chiral analytes (see supporting information Fig. S1 for the structures) were *R,S*-10-[2-(1-methyl-2-piperidyl)ethyl]-2-(methylthio)-10*H*-phenothiazine hydrochloride (thioridazine, ≥99 %), *R,S*-10-[2-(dimethylamino) propyl]phenothiazine hydrochloride (promethazine, ≥98 %), *R,S*-7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-1,4(2*H*)-benzodiazepin-2-one (oxazepam, ≥98 %), *R,S*-7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one (lorazepam) and atropisomers (*R*)-(−)-1,1′-binaphthalene-2,2′-diyl hydrogen phosphate and (*S*)-(+)-1,1′-binaphthalene-2,2′-diyl hydrogen phosphate (BNP, ≥98 %; 97 %), which were purchased from Sigma-Aldrich (St. Louis, USA). The compounds for LFER measurements were of analytical grade purity and purchased from Sigma-Aldrich (St. Louis, USA). The list of 32 achiral solutes for LFER measurement with their corresponding descriptors is presented in supporting information Table S1.

### Procedures

Stock solutions of solid samples were prepared in concentration of 1 mg $mL^{-1}$ and stock solutions of liquid samples in concentration of 10 μL $mL^{-1}$ using methanol (MeOH) as a solvent. Individual atropisomers of analyte BNP were mixed in a unit ratio. Lithium acetate, lithium formate and lithium benzoate buffers were prepared by dissolving appropriate amounts of LiOH in deionised water and adding the calculated amount (calculated by PeakMaster) of AAc, formic acid (FAc) or BAc to reach the required pH 4.01, resp. 4.00 for benzoate buffer. The concentration of buffers were: LiOH/AAc 9.90/60.0 mM, ionic strength (*I*) 10.0 mM, LiOH/FAc 10.0/15.2 mM, $I = 10.1$ mM and LiOH/BAc 10.0/24.5 mM, $I = 10.1$ mM. LiOH was used for maintaining similarity with previously used CE conditions [7, 8]. All the prepared samples, resp. buffers were filtered with Minisart syringe filters (0.2 μm, resp. 0.45 μm, Sartorius, Stedim Biotech, Germany). MPs for all measurements were composed of MeOH and acetate buffer, pH 4.01, MeOH and formate buffer, pH 4.01 or MeOH with benzoate buffer, pH 4.00, at different volume ratios (v/v) before the final value 40/60 was set. The same MeOH content in MP was used to consider only effect of buffer type.

### Linear Free Energy Relationship Method

The LFER method applied is based on the most generally used Eq. 1 [11]:

$$\log k = eE + sS + aA + bB + vV + c, \quad (1)$$

where *k* is retention factor of a test solute. The independent variables are solute descriptors (see supporting information Table S1 for individual values): E is the excess molar refraction, S is the dipolarity/polarizability, A represents the effective or overall hydrogen bond acidity, B is the effective or overall hydrogen bond basicity, and V refers to the McGowan characteristic volume [9, 11]. If these descriptors are known, then complementary regression coefficients (*e*, *s*, *a*, *b*, *v*) can be calculated.

The regression coefficient *e* reflects the difference in disposition of the stationary phase (SP) and MP to interact with n- and π-electron pairs of the solutes; *s* is equal

to the difference in dipolarity/polarizability, $a$ and $b$ refer to the differences in hydrogen bond basicity and hydrogen bond acidity, respectively, and the coefficient $v$ represents the difference in dispersion interactions (mostly considered as hydrophobicity in RP systems) between the two phases. The intercept $c$ in the LFER equation is characteristic of the given system but it does not reflect any interaction [9–13]. The regression coefficients of the LFER equation were obtained from a series of retention measurements of the set of 32 structurally different solutes (they were selected to cover a wide range of chemical and structural properties) with known descriptors (see supporting information Table S1). A multiple linear regression analysis of log $k$ vs solute descriptors was performed. NCSS software (Kaysville, USA) was used for calculation of appropriate regression coefficients that characterize the chromatographic separation systems under comparison.

## Results and Discussion

### The Effect of Buffer Constituents on Retention of Achiral Analytes

The influence of buffer type on retention of selected achiral analytes on β-CD CSP was tested first. We regard benzoate buffer a complexing MP component, as inclusion of BAc into β-CD cavity was described previously [14]. On the contrary, we tested acetate and formate buffers (both pH 4.01) as possible noncomplexing MP components. In the course of our preliminary measurements, we obtained very similar retention values of the tested achiral analytes in both acetate and formate containing MPs (data not shown). Based on these preliminary results, we verified the both buffers as "noninteracting", safe MP components. So, we have chosen acetate buffer for further investigation and comparison with benzoate buffer. Retention factors of the analytes in two different MP compositions concerning the buffer used are shown in Table 1. As could be expected, lower retentions were observed in MP containing benzoate buffer (pH 4.00) while MP composed of acetate buffer (pH 4.01) in the same volume ratio to MeOH 60/40 (v/v) showed significantly higher retentions. The reductions of retentions (expressed as retention factors) in MP containing BAc for toluene, naphthalene and anthracene were about 28, 66 and 71 %, respectively. Reduction of retention of analytes in RP-HPLC is generally achieved by increasing organic modifier contents in MPs. Therefore, such MPs become more expensive and less "green". From this point of view, complexing MPs can offer a worthy alternative for reduction of the analysis time.

Clearly, the presence of BAc in MP substantially influences the interaction abilities of the β-CD CSP with analytes. To investigate which particular interactions are more or less pronounced in the chromatographic system containing benzoate buffer in comparison with acetate in the MPs we carried out the LFER measurements.

**Table 1** Chromatographic data ($k_1$, $\alpha$) of achiral and chiral analytes on β-CD CSP in two different MPs

| Analyte | MP composition | | | |
|---|---|---|---|---|
| | MeOH/acetate buffer 40/60 (v/v) | | MeOH/benzoate buffer 40/60 (v/v) | |
| | $k_1$ | $\alpha$ | $k_1$ | $\alpha$ |
| Achiral | | | | |
| Toluene | 2.19 | * | 1.58 | * |
| Naphthalene | 13.36 | * | 4.56 | * |
| Anthracene | 44.14 | * | 12.78 | * |
| Chiral | | | | |
| Thioridazine | 2.88 | 1.16 | 2.06 | 1.00 |
| Promethazine | 0.38 | 1.00 | 0.28 | 1.00 |
| Oxazepam | 1.50 | 1.50 | 1.02 | 1.14 |
| Lorazepam | 1.49 | 1.00 | 1.05 | 1.00 |
| BNP | 64.86 | 1.17 | 31.10 | 1.07 |

$k_1$—retention factor of achiral analytes resp. retention factor of the first eluted enantiomer/atropisomer of chiral analytes

$\alpha$-selectivity calculated as $k_2/k_1$ for the chiral analytes (*not calculated for achiral analytes)

### The Effect of Buffer Type on LFER Regression Coefficients

The results of the previous chapter showed that the buffer type significantly influences retention of analytes on β-CD CSP. Therefore, the LFER method was used for the characterization and comparison of intermolecular interactions that dominate the chromatographic systems with β-CD column and MPs composed of MeOH/acetate buffer, pH 4.01 or MeOH/benzoate buffer, pH 4.00, 40/60 (v/v). The LFER results are summarized in Table 2. The experimental log $k$ values show linear correlation with calculated log $k$ values with correlation coefficients $R = 0.95$ and 0.96 for systems with acetate and benzoate buffers, respectively. The similar values of regression coefficient $e$ for the both chromatographic systems show negligible effect of buffer type on interactions with $n$- and π-electrons. The positive $e$ values for both systems indicate that this interaction type is preferred between analyte and SP. This coefficient characterizes other interactions than interaction with β-CD cavity. So, the inclusion of BAc to the β-CD cavity does not influence the value of coefficient $e$ and thus $n$- and π-electron interactions contribute to retention of analytes in the same extent, no matter what buffer was used. The regression coefficients $s$ (characterizes dipolarity/polarizability) and $a$ (characterizes hydrogen bond basicity) are statistically

**Table 2** Regression coefficients of the LFER equation, *SE* standard error values, *F* test values and *R* correlation coefficient

| Aqueous part of MP | Model | *e* | *s* | *a* | *b* | *v* | *c* | *SE* | *F* | *R* |
|---|---|---|---|---|---|---|---|---|---|---|
| Benzoate buffer | CM | 0.42 | x | x | −0.89 | 0.78 | −0.67 | 0.18 | 64 | 0.96 |
| | *p* value | 0.00 | 0.91 | 0.30 | 0.00 | 0.00 | 0.00 | | | |
| | ±CI | 0.15 | | | 0.16 | 0.27 | 0.23 | | | |
| | CM | 0.40 | x | x | −1.41 | 1.32 | −0.62 | 0.52 | 44 | 0.95 |
| Acetate buffer | *p* value | 0.00 | 0.70 | 0.80 | 0.00 | 0.00 | 0.00 | | | |
| | ±CI | 0.26 | | | 0.28 | 0.46 | 0.39 | | | |

CI represents ±95 % confidence interval; *x* insignificant difference in interaction of the solute with the MP and SP; *p*, statistical *p* value, *SE*, standard error in the estimate, *F–F* ratio (*F* test)

*MP* MeOH/benzoate buffer (LiOH/BAc 10.0/24.5 mM), pH 4.00 40/60 (v/v)

MeOH/acetate buffer (LiOH/AAc 9.9/60.0 mM), pH 4.01 40/60 (v/v)

insignificant in the both chromatographic systems tested. This means that the corresponding interaction types are comparable in SP and MP and do not significantly affect retention of analytes. The buffer type significantly influences interactions described by regression coefficients *b* and *v*. The *b* coefficient attains negative values in both separation systems as this interaction is preferred in the MP. Nevertheless, the difference between SP and MP in hydrogen bond acidity is higher for system with acetate buffer (higher negative value of regression coefficient *b*). Thus, BAc can lower this difference of complexation with the β-CD CSP and in this way increasing hydrogen-donating capability of this CSP. The regression coefficient *v* reaches lower positive value in system with benzoate buffer than with the acetate one, i.e., the difference in hydrophobicity between phases is smaller in the former buffer. This phenomenon can be attributed to significantly higher hydrophobicity of BAc (log P[1] = 1.79) [15] than AAc (log P = −0.30) [15], thus incorporation of BAc into the hydrophobic β-CD cavity can partially disable this type of interaction for analytes. The hydrophobic β-CD cavity can better contribute to retention of analytes if there is no competition of this type of interaction with the components of MP.

In summary, substantive impact of the buffer composition in the MP on retention was observed and simultaneously confirmed by regression coefficient values of LFER equation.

## The Effect of Buffer Constituents on Separation of Chiral Analytes

Based on the results obtained in the previous chapters, we investigated different buffers' effects on retention and separation of chiral analytes. Five chiral analytes were tested, namely thioridazine, promethazine, oxazepam, lorazepam and BNP. The results are summarized in Table 1.

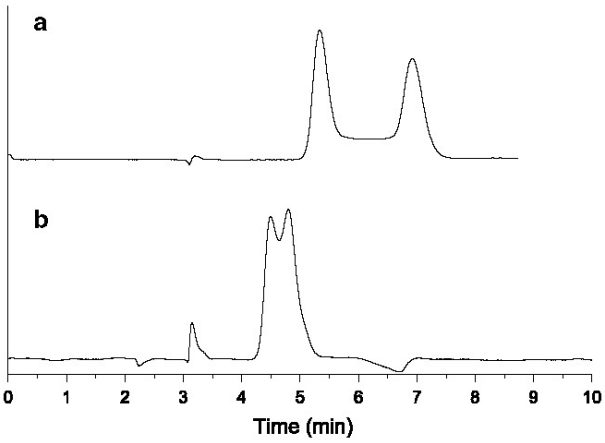


**Fig. 1** Chromatogram of separation of enantiomers of oxazepam on β-CD CSP in *A* MP composed of MeOH/acetate buffer, pH 4.01, 40/60 (v/v) and *B* MP composed of MeOH/benzoate buffer, pH 4.00, 40/60 (v/v)

The chiral analytes clearly showed the same influence of MP composition on their retention as the achiral analytes did. This fact again indicated that analytes compete with BAc for β-CD cavity. Let us compare, for example, the two different MPs used for analysis of BNP; see the data in Table 1. The selectivity of the systems is different, substantially better for MP with acetate buffer. The presence of BAc in the MP deteriorated the enantioseparation but brings an important benefit—much shorter analysis time. However, the reduced interaction of analytes with β-CD cavity is mostly insufficient for achieving enantioresolution. This can be illustrated on enantioseparation of oxazepam (Fig. 1) in MPs composed of MeOH/benzoate buffer and MeOH/acetate buffer both 40/60 (v/v) with enantioresolution values 1.06 and 3.00, respectively.

[1] log P = partition coefficient between *n*-octanol and water [15].

## Conclusion

It is generally known that the structure of analytes, namely their possibility to enter the hydrophobic cavity of CDs, plays a significant role in separation mechanism on CD-based CSPs in HPLC. What is mostly overlooked is the possibility of complexation of constituents of buffer/MP with CDs and so the resulting influence of buffer components on retention and (enantio) separation. This study demonstrates that it is necessary to consider responsibly the composition of the buffer used, with regard to the nature of SP. Two extreme cases were taken into consideration: "safe" acetate buffer, with low or negligible interaction with the CD cavity, and benzoate buffer that strongly complexes with the CD cavity. As the real separation system lies mostly somewhere in between, the interaction of buffer constituents with CSP must be taken into account and can not be underestimated.

**Acknowledgments** The authors gratefully acknowledge financial support of the Grant Agency of the Charles University, Grant No. 790314 and the Grant Agency of the Czech Republic, Grant No. P206/14-19278P.

**Conflict of interest** The authors declare no conflict of interest.

## References

1. Armstrong DW, Demond W (1984) J Chromatogr Sci 22:411–415
2. Kalíková K, Riesová M, Tesařová E (2012) Cent Eur J Chem 10:450–471
3. Scriba GKE (2012) Chromatographia 75:815–838
4. Kalíková K, Šlechtová T, Vozka J, Tesařová E (2014) Anal Chim Acta 821:1–33
5. Nagy ZM, Molnár M, Fekete-Kertész I, Molnár-Perl I, Fenyvesi É, Gruiz K (2014) Sci Tot Env 485–486:711–719
6. Chankvetadze B, Burjanadze N, Santi M, Massolini G, Blaschke G (2002) J Sep Sci 25:733–740
7. Riesová M, Svobodová J, Tošner Z, Beneš M, Tesařová E, Gaš B (2013) Anal Chem 85:8518–8525
8. Beneš M, Riesová M, Svobodová J, Tesařová E, Dubský P, Gaš B (2013) Anal Chem 85:8526–8534
9. Abraham MH, McGowan J (1987) Chromatographia 23:243–246
10. Kalíková K, Lokajová J, Tesařová E (2006) J Sep Sci 29:1476–1485
11. Abraham MH, Whiting GS, Doherty RM, Shuely W (1991) J Chromatogr 587:213–228
12. Ahn J, Bones J, Yu YQ, Rudd PM, Gilar M (2010) J Chromatogr B 878:403–408
13. Kalíková K, Kozlík P, Gilar M, Tesařová E (2013) J Sep Sci 36:2421–2429
14. Pitchumani K, Vellayappan M (1992) J Inclusion Phenom Mol Recognit Chem 14:157–162
15. Klopman G, Li J-Y, Wang S, Dimayuga M (1994) J Chem Inf Comput Sci 34:752–781

Publikace V

# Enantioselective potential of polysccharide-based chiral stationary phases in supercritical fluid chromatography

Kucerova, G., Kalikova, K., Tesarova, E.

*Chirality 2017, 29, 239-246*

doi:10.1002/chir.22701

Received: 14 November 2016 | Revised: 14 February 2017 | Accepted: 21 February 2017

DOI: 10.1002/chir.22701

SPECIAL ISSUE ARTICLE

WILEY

# Enantioselective potential of polysaccharide-based chiral stationary phases in supercritical fluid chromatography

Gabriela Kucerova | Kveta Kalikova | Eva Tesarova

Department of Physical and Macromolecular Chemistry, Charles University, Prague, Czech Republic

**Correspondence**
Kveta Kalikova, Department of Physical and Macromolecular Chemistry, Charles University, Albertov 6, Prague 2, 12843, Czech Republic.
Email: kveta.kalikova@natur.cuni.cz

**Funding information**
Czech Science Foundation, Grant/Award Number: 16-05942S

**Abstract**

The enantioselective potential of two polysaccharide-based chiral stationary phases for analysis of chiral structurally diverse biologically active compounds was evaluated in supercritical fluid chromatography using a set of 52 analytes. The chiral selectors immobilized on 2.5 μm silica particles were tris-(3,5-dimethylphenylcarmabate) derivatives of cellulose or amylose. The influence of the polysaccharide backbone, different organic modifiers, and different mobile phase additives on retention and enantioseparation was monitored. Conditions for fast baseline enantioseparation were found for the majority of the compounds. The success rate of baseline and partial enantioseparation with cellulose-based chiral stationary phase was 51.9% and 15.4%, respectively. Using amylose-based chiral stationary phase we obtained 76.9% of baseline enantioseparations and 9.6% of partial enantioseparations of the tested compounds. The best results on cellulose-based chiral stationary phase were achieved particularly with propane-2-ol and a mixture of isopropylamine and trifluoroacetic acid as organic modifier and additive to $CO_2$, respectively. Methanol and basic additive isopropylamine were preferred on amylose-based chiral stationary phase. The complementary enantioselectivity of the cellulose- and amylose-based chiral stationary phases allows separation of the majority of the tested structurally different compounds. Separation systems were found to be directly applicable for analyses of biologically active compounds of interest.

**KEYWORDS**

amylose, biologically active compounds, cellulose, chiral separation, chiral stationary phase, enantioselectivity, supercritical fluid chromatography

## 1 | INTRODUCTION

Supercritical fluid chromatography (SFC) has become a very successful technique for fast and efficient achiral or chiral separations of diverse compounds in the past few years. SFC is becoming a method of first choice in pharmaceutical applications concerning enantioseparations and/or purifications.[1-8] This is largely thanks to the commercialization of new-generation SFC systems, which offer enhanced sensitivity, robustness, and quantitative performance.[9] Supercritical $CO_2$ as the main part of the mobile phase (MP) is referred to as a "green solvent" and a further desirable MP component thanks to its properties such as density, solvating power, or viscosity.[1,10] From this point of view, SFC offers benefits such as higher throughput or lower analysis times than the conventional high-performance liquid chromatography (HPLC) technique.[1,11-13]

[This article is part of the Special Issue: Proceedings of 28th International Symposium on Molecular Chirality, Heidelberg 2016. See the first articles for this special issue previously published in Volumes 29:1, 29:3–4, and 29:5. More special articles will be found in this issue as well as in those to come.]

  

On the market, there are a number of chiral stationary phases (CSPs) including many of HPLC CSPs, that are suitable for enantioseparation in SFC. Regarding HPLC as well as SFC enantioseparations, polysaccharide-based CSPs are classified as the versatile ones and are extensively used.[5,14-24] New-generation SFC systems offer better compatibility with modern stationary phases (SPs), such as those packed with sub-2 μm fully porous particles or sub-3 μm superficially porous particles.[9,25] In fact, SFC columns with 2.5 μm silica particles containing tris-(3,5-dimethylphenylcarbamate) of cellulose or amylose as chiral selectors (CSs) used in this study are recent products fully compatible with commercial UPC$^2$ (ultra-performance convergence chromatography) systems.

Organic modifiers, e.g., some alcohols, and basic or acidic additives added to the main MP component $CO_2$ are used to modulate the separation ability of the SFC system. A study has been performed on the effect of different alcohols in MP in separation systems with immobilized polysaccharide-based CSPs.[26] The authors demonstrated complementary separations of pharmaceuticals in MPs containing MEOH and propane-2-ol. However, in general the separation success rate for the studied pharmaceuticals was not very high in their work. Concerning the addition of basic isopropylamine (IPAM) and/or acidic trifluoroacetic acid (TFA) additives, the combination of both was reported to reduce nonspecific interactions and so to increase enantioselectivity. The dual addition also led to minimization of the memory effect of SP.[27] However, higher concentration of these additives could result in undesirable precipitation of the forming salt complexes.[28]

Nowadays the SFC method is used for separation of neutral, acidic and also basic compounds.[29-31] Nevertheless, separation of basic compounds can be hampered by forming of ionic interactions with residual silanol-carrier groups.[1,32,33] Among basic, acidic, bifunctional, and neutral biologically active compounds (BACs) used in this study, we classify very well-known drugs like profens, thiazide diuretics, flavanone derivatives, and calcium channel blockers or phenothiazines and β-blockers.[22,34,35] Moreover, newly synthesized drugs called "legal highs" belong likewise to BACs.[36] The "legal highs" used in this work are derivatives of amphetamine or benzofuran. Mostly, they are "abused" similarly as prohibited addictive substances, with the difference that there is insufficient legislation that would punish this permitted activity. Recently, BACs based on amphetamine or benzofuran were successfully enantioseparated using amylose-based CSP in SFC.[1] Some enantiomers of β-blockers used in this study were previously separated on two different polysaccharide-based CSPs (Chiralpak IB-3 and Chiralpak AD columns) by SFC.[37,38] Almost 20 years ago, Berger and Wilson enantioseparated several phenothiazine substances using packed column SFC.[39]

The aim of this work was to find out and compare the enantioselective potential of new short polysaccharide-based columns (50 mm long), i.e., ACQUITY UPC$^2$ Trefoil CEL1 and ACQUITY UPC$^2$ Trefoil AMY1. The CSs are immobilized on 2.5 μm silica particles. The goal was to show differences in the chromatographic behavior between these two columns, as they differ in the nature of the polysaccharide backbone. For this purpose, a set of 52 structurally different BACs was tested under diverse SFC separation conditions, namely, different MP compositions, to examine the enantioseparation abilities and differences of these two columns. The other objective was to find the best/optimal mobile phases for enantioseparation of the tested chiral compounds.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and analytes

Methanol (MEOH, Chromasolv, gradient grade, ≥99.9%), propane-2-ol (PROH, Chromasolv for HPLC, ≥99.8%), isopropylamine (IPAM, ≥99.5%), trifluoroacetic acid (TFA, 99%), and tetrahydrofuran (THF, Chromasolv for HPLC) were supplied by Sigma-Aldrich (St. Louis, MO). Pressurized liquid $CO_2$ 4.5 grade (99.995%) was purchased from Messer (Prague, Czech Republic). Chiral analytes: profen derivatives (PF1, ibuprofen; PF2, indoprofen; PF3, flurbiprofen; PF4, tiaprofenic acid; PF5, carprofen, PF6, suprofen; PF7, ketoprofen; PF8, fenoprofen), flavanone derivatives (F1, 6-hydroxyflavanone; F2, 7-hydroxyflavanone), thiazide diuretics (TD1, butizide; TD2, mefruside; TD3, chlorthalidone; TD4, trichlormethiazide; TD5, bendroflumethiazide), calcium channel blockers (CB1, amlodipine; CB2, nimodipine; CB3, nitrendipine; CB4, nicardipine; CB5, verapamil; CB6, nisoldipine), phenothiazines (PH1, thioridazine; PH2, promethazine), amphetamine derivatives (A1, 4-fluoromethcathinone; A2, 4-fluoroamphetamine; A3, 4-bromomethcathinone; A4, buphedrone; A5, ethylone; A6, 3-fluoroamphetamine; A7, 2-fluoromethcathinone; A8, methylendioxypyrovalerone), benzofury derivatives (B1, 5-(2-aminopropyl)benzofuran; B2, 6-(2-aminopropyl)benzofuran; B3, 5-(2-aminopropyl)-2,3-dihydrobenzofuran; B4, 6-(2-aminopropyl)-2,3-dihydrobenzofuran; B5, 1-(benzofuran-5-yl)-*N*-ethylpropan-2-amine; B6, 1-(benzofuran-6-yl)-*N*-ethylpropan-2-amine; B7, 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine), β-blockers (BB1, propranolol; BB2, oxprenolol; BB3, metoprolol; BB4, metipranolol; BB5, acebutolol; BB6, pindolol; BB7, bopindolol; BB8, atenolol; BB9, alprenolol) and others (O1, BP34; O2, BP766; O3, thalidomide; O4, tramadol; O5, lorazepam) were purchased from Sigma-Aldrich or kindly donated from M.G. Schmid from Institute of Pharmaceutical Chemistry and Pharmaceutical Technology, Karl Franzens University, Graz, Austria. See Figures S1 in the Supporting Information for the structures of the compounds.

## 2.2 | SFC instrumentation and columns

The Waters Acquity Ultra Performance Convergence Chromatography ($UPC^2$) system was equipped with a binary solvent delivery pump (MP flow rates up to 4 mL $min^{-1}$, pressures up to 6000 psi), an autosampler which included a partial loop volume injection system, a back-pressure (BP) regulator, a column oven, and a photodiode array detector (Waters, Milford, MA). The Empower 3 software was used for system control and data acquisition. Both columns: ACQUITY $UPC^2$ Trefoil CEL1 (CEL1) and ACQUITY $UPC^2$ Trefoil AMY1 (AMY1) were obtained from Waters. The CSs immobilized on 2.5 μm silica particles were tris-(3,5-dimethylphenylcarbamate) derivatives of cellulose (CEL1) or amylose (AMY1). The dimensions of both columns were 3.0 × 50 mm.

## 2.3 | General conditions

The chromatographic measurements were performed at a flow rate 2.5 mL $min^{-1}$ based on our previous experience[1] and our preliminary measurements in this work (measurements were carried out in the range 1.5–3 mL $min^{-1}$). Both retention and resolution increased at lower flow rate. Thus, the best separation conditions were considered as a compromise between resolution and short analysis time. The column temperature was 35 °C, BP of 2000 psi and UV detection at 254, 260 and 280 nm. Void volume was determined using the solvent peak. Injection volume was in the range 0.6–1.0 μL depending on the detector response. Sample temperature was 10 °C. All measurements were performed in triplicate.

## 2.4 | Sample preparation

The stock solutions of profen derivatives, flavanones, thiazide diuretics, calcium channel blockers (except for CB6), phenothiazines, β-blockers, others (except for O3), and 3-fluoroamphetamine (A7) were prepared in MEOH at a concentration of 1.0 mg $mL^{-1}$. Amphetamine derivatives (except for A5, A6, and A7) were dissolved in MEOH at a concentration of 0.5 mg $mL^{-1}$. The stock solutions of benzofury derivatives were prepared at a concentration of 0.25 mg $mL^{-1}$ in MeOH/THF 50/50 *(v/v)*. CB6 was dissolved in MEOH/THF 80/20 *(v/v)* at a concentration of 1.0 mg $mL^{-1}$ and O3 in MEOH/THF 75/25 *(v/v)* at a concentration of 1.0 mg $mL^{-1}$.

## 2.5 | MP compositions

MPs composed of $CO_2$ and organic modifiers MEOH or PROH with the addition of basic additive IPAM and/or acidic additive TFA was prepared in various volume ratios. See Table 1 for exact MP compositions used in this work.

**TABLE 1** MP compositions used for the enantioseparation of BACs on CEL1 and AMY1 CSPs

| MP compositions: | Volume ratios *(v/v/v(/v))*: | | |
|---|---|---|---|
| $CO_2$/MEOH/IPAM | 90/10/0.1 | 95/5/0.1 | 98/2/0.1 |
| $CO_2$/PROH/IPAM | 90/10/0.1 | 95/5/0.1 | 98/2/0.1 |
| $CO_2$/MEOH/IPAM/TFA | 90/10/0.1/0.1 | 95/5/0.1/0.1 | 98/2/0.1/0.1 |
| $CO_2$/PROH/IPAM/TFA | 90/10/0.1/0.1 | 95/5/0.1/0.1 | 98/2/0.1/0.1 |
| $CO_2$/MEOH/TFA | 90/10/0.1 | 95/5/0.1 | 98/2/0.1 |
| $CO_2$/PROH/TFA | 90/10/0.1 | 95/5/0.1 | 98/2/0.1 |

# 3 | RESULTS AND DISCUSSION

The columns CEL1 and AMY1 were investigated under various MP compositions in order to evaluate their enantioselective ability for separation of acidic, basic, bifunctional, and neutral compounds. Temperature and BP were kept constant during measurements in order to monitor the impact of the CSP type and MP compositions. The best/optimized separation conditions were found for the majority of the studied compounds even without the additional optimization of temperature and BP. Some of the baseline separated compounds could be resolved under several MP compositions. However, other suitable MP compositions are not shown, particularly due to a higher duration of analysis that is claimed to be as short as possible. Chromatographic data collected from the measurements on the both CSPs at different MP compositions are summarized in Table S1 in the Supporting Information for better understanding and comparison. In general, a higher retention of analytes was obtained in MPs containing more hydrophobic PROH than those containing MEOH (comparing the same volume ratios). We did not observe the general effect of enhanced and decreased enantioselectivity on the cellulose-based and amylose-based CSPs, respectively, caused with the dual additives in the SFC separation systems that were reported by other authors.[28] It is obvious from Table 2 and Table S1 that different groups of tested analytes prefer different CSPs-polysaccharide backbone compared in this work.

## 3.1 | Profen derivatives

From the set of eight profen derivatives, seven were baseline separated under optimized conditions on the CSP with amylose backbone AMY1, while just three exhibited a resolution higher than 1.5 on CEL1 CSP. Enantiomers of ibuprofen (PF1) could not be resolved on any of these CSPs. Despite the general observation that better enantioseparation of profens can be achieved on the amylose-based column, certain separations show opposite results, as demonstrated in Figure 1B. Comparison of the separation of flurbiprofen

**TABLE 2** Chromatographic data and the best MP compositions for enantioseparation of studied compounds on CEL1 and AMY1 CSPs

| | | CEL1 CSP | | | | | AMY1 CSP | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Compounds | | $t_{r,1}$ (min) | $k_1$ | $\alpha$ | $R_s$ | MP composition (v/v/v(/v)) | $t_{r,1}$ (min) | $k_1$ | $\alpha$ | $R_s$ | MP composition (v/v/v(/v)) |
| **Profen derivatives** | **PF1** | X | X | X | X | X | X | X | X | X | X |
| | **PF2** | 1.41 | 9.71 | 1.19 | **2.25** | **C/M/T 90/10/0.1** | 15.08 | 134 | 1.22 | **2.73** | C/P/T 90/10/0.1 |
| | **PF3** | 3.95 | 32.50 | 1.13 | **1.52** | C/P/T 98/2/0.1 | 0.39 | 2.53 | 1.35 | **2.89** | **C/M/T 90/10/0.1** |
| | **PF4** | 14.29 | 120 | 1.10 | 1.36 | C/P/T 98/2/0.1 | 3.24 | 28.50 | 1.86 | **6.71** | **C/P/I/T 95/5/0.1/0.1** |
| | **PF5** | 3.38 | 24.60 | 1.23 | **2.07** | **C/P/I/T 90/10/0.1/0.1** | 5.42 | 46.97 | 1.20 | **2.34** | C/M/I/T 90/10/0.1/0.1 |
| | **PF6** | 2.53 | 21.02 | 1.11 | 1.27 | C/P/T 95/5/0.1 | 1.17 | 9.42 | 1.26 | **2.55** | **C/M/T 90/10/0.1** |
| | **PF7** | X | X | X | X | X | 1.36 | 11.37 | 1.17 | 1.47 | **C/P/I/T 95/5/0.1/0.1** |
| | **PF8** | X | X | X | X | X | 5.61 | 41.80 | 1.17 | **2.13** | **C/P/T 98/2/0.1** |
| **Flavanones** | **F1** | 1.61 | 11.18 | 1.11 | **1.51** | C/M/I/T 95/5/0.1/0.1 | 1.73 | 14.99 | 1.48 | **3.86** | **C/P/I/T 90/10/0.1/0.1** |
| | **F2** | X | X | X | X | X | 1.55 | 13.38 | 1.18 | **1.80** | **C/P/I/T 90/10/0.1/0.1** |
| **Thiazide diuretics** | **TD1** | 2.69 | 19.36 | 1.52 | **4.73** | **C/M/I/T 90/10/0.1/0.1** | 15.17 | 133 | 1.24 | **1.81** | C/P/I/T 90/10/0.1/0.1 |
| | **TD2** | X | X | X | X | X | 2.63 | 22.86 | 1.70 | **3.85** | **C/M/I 90/10/0.1** |
| | **TD3** | 3.40 | 24.83 | 1.23 | **2.53** | **C/M/I/T 90/10/0.1/0.1** | 12.18 | 87.91 | 1.36 | **2.64** | C/M/I 90/10/0.1 |
| | **TD4** | 5.75 | 49.41 | 1.11 | **1.51** | **C/M/T 90/10/0.1** | 18.74 | 165 | 1.13 | **8.68** | C/P/I/T 90/10/0.1/0.1 |
| | **TD5** | 17.39 | 130 | 1.09 | 1.05 | C/P/I 90/10/0.1 | 3.36 | 28.69 | 1.26 | **2.03** | **C/M/I/T 90/10/0.1/0.1** |
| **Calcium channel blockers** | **CB1** | 7.16 | 52.75 | 1.24 | **1.70** | **C/P/I/T 95/5/0.1/0.1** | X | X | X | X | X |
| | **CB2** | 10.72 | 89.83 | 1.09 | 1.05 | C/P/T 98/2/0.1 | 0.72 | 5.43 | 3.37 | **4.23** | **C/P/T 90/10/0.1** |
| | **CB3** | X | X | X | X | X | X | X | X | X | X |
| | **CB4** | X | X | X | X | X | X | X | X | X | X |
| | **CB5** | 0.73 | 4.50 | 1.28 | **1.87** | **C/M/I/T 90/10/0.1/0.1** | 0.85 | 6.11 | 1.14 | 0.84 | C/P/I 90/10/0.1 |
| | **CB6** | 8.00 | 58.84 | 1.09 | 1.23 | **C/P/I 98/2/0.1** | X | X | X | X | X |
| **Phenothiazines** | **PH1** | X | X | X | X | X | 2.85 | 22.73 | 1.24 | **2.04** | **C/P/I 90/10/0.1** |
| | **PH2** | 1.41 | 9.73 | 1.10 | 0.93 | C/P/I 98/2/0.1 | 1.95 | 12.73 | 1.26 | **1.56** | **C/P/I 98/2/0.1** |
| **Amphetamine derivatives** | **A1** | X | X | X | X | X | 1.65 | 12.85 | 1.30 | 1.20 | **C/P/T 95/5/0.1** |
| | **A2** | X | X | X | X | X | 10.53 | 88.23 | 1.15 | **1.83** | **C/P/I 98/2/0.1** |
| | **A3** | X | X | X | X | X | 0.43 | 2.80 | 1.51 | **1.78** | **C/P/I/T 90/10/0.1/0.1** |
| | **A4** | 2.25 | 18.72 | 1.16 | 1.43 | C/M/T 98/2/0.1 | 3.05 | 25.07 | 1.35 | **2.68** | **C/P/I 95/5/0.1** |
| | **A5** | X | X | X | X | X | 1.75 | 14.92 | 1.66 | **2.54** | **C/P/I/T 95/5/0.1/0.1** |
| | **A6** | X | X | X | X | X | 3.06 | 25.14 | 1.27 | **2.15** | **C/P/I 95/5/0.1** |
| | **A7** | X | X | X | X | X | 3.06 | 25.50 | 1.26 | **2.66** | **C/P/I 95/5/0.1** |
| | **A8** | 1.34 | 9.12 | 1.27 | **2.25** | C/P/I/T 95/5/0.1/0.1 | 0.56 | 3.80 | 1.24 | **1.84** | **C/P/I 95/5/0.1** |
| **Benzofury derivatives** | **B1** | 1.45 | 9.99 | 1.24 | **1.80** | **C/P/I/T 95/5/0.1/0.1** | 7.25 | 60.97 | 1.31 | **3.72** | C/P/I 95/5/0.1 |
| | **B2** | 1.45 | 9.93 | 1.23 | **1.68** | **C/P/I/T 95/5/0.1/0.1** | 7.28 | 61.18 | 1.32 | **3.48** | C/P/I 95/5/0.1 |
| | **B3** | 2.71 | 19.70 | 1.92 | **7.33** | **C/M/I 98/2/0.1** | 7.58 | 63.79 | 1.11 | 1.37 | C/P/I 95/5/0.1 |
| | **B4** | 0.54 | 3.05 | 1.50 | **2.42** | **C/P/I/T 90/10/0.1/0.1** | 0.80 | 5.94 | 1.35 | **1.97** | C/M/I/T 95/5/0.1/0.1 |
| | **B5** | X | X | X | X | X | 1.99 | 16.04 | 1.24 | **2.38** | **C/P/I 95/5/0.1** |
| | **B6** | X | X | X | X | X | 2.08 | 16.81 | 1.13 | 1.42 | **C/P/I 95/5/0.1** |
| | **B7** | 3.27 | 23.78 | 1.12 | 1.01 | C/P/I 98/2/0.1 | 2.92 | 23.96 | 1.19 | **2.07** | **C/P/I 95/5/0.1** |
| **β-blockers** | **BB1** | 0.80 | 5.09 | 2.00 | **6.36** | **C/M/I/T 90/10/0.1/0.1** | 2.48 | 21.37 | 1.50 | **2.92** | C/P/I 90/10/0.1 |
| | **BB2** | 0.31 | 1.35 | 2.44 | **4.14** | **C/M/I/T 90/10/0.1/0.1** | 1.25 | 10.25 | 1.28 | **1.63** | C/P/I 90/10/0.1 |
| | **BB3** | 0.28 | 1.13 | 4.02 | **5.00** | **C/M/I/T 90/10/0.1/0.1** | 1.83 | 15.46 | 1.34 | **1.94** | C/P/I 90/10/0.1 |
| | **BB4** | 0.60 | 3.54 | 1.30 | **2.26** | **C/M/I/T 95/5/0.1/0.1** | 13.58 | 115 | 1.20 | **2.92** | C/P/I 95/5/0.1 |
| | **BB5** | 5.43 | 40.10 | 1.15 | **1.71** | **C/M/I/T 95/5/0.1/0.1** | 15.69 | 140 | 1.26 | **1.62** | C/P/I 90/10/0.1 |
| | **BB6** | 2.01 | 14.25 | 4.46 | **14.2** | **C/M/I/T 90/10/0.1/0.1** | X | X | X | X | X |
| | **BB7** | 1.76 | 12.25 | 1.72 | **5.66** | **C/M/I/T 90/10/0.1/0.1** | 5.56 | 47.4 | 1.86 | **5.52** | C/M/I/ 95/5/0.1 |
| | **BB8** | 1.55 | 10.61 | 2.31 | **8.62** | **C/M/I/T 90/10/0.1/0.1** | X | X | X | X | X |
| | **BB9** | 0.23 | 0.76 | 1.79 | **2.05** | **C/M/I/T 90/10/0.1/0.1** | 0.93 | 7.35 | 1.41 | **2.07** | C/P/I 90/10/0.1 |
| **Others** | **O1** | 2.70 | 19.41 | 1.41 | **1.92** | **C/P/I/T 95/5/0.1/0.1** | 3.46 | 27.83 | 1.40 | **1.68** | C/P/I 90/10/0.1 |
| | **O2** | X | X | X | X | X | 2.02 | 15.97 | 1.31 | **1.58** | **C/P/T 95/5/0.1** |

(*Continues*)

**TABLE 2** (Continued)

| Compounds | | CEL1 CSP $t_{r,1}$ (min) | $k_1$ | $\alpha$ | $R_s$ | MP composition (v/v/v(/v)) | AMY1 CSP $t_{r,1}$ (min) | $k_1$ | $\alpha$ | $R_s$ | MP composition (v/v/v(/v)) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **O3** | 2.43 | 20.29 | 1.13 | **1.70** | **C/M/T 95/5/0.1** | 3.40 | 29.11 | 1.30 | **2.34** | C/P/I/T 90/10/0.1/0.1 |
| | **O4** | 0.53 | 2.99 | 1.18 | **1.56** | C/M/I/ 95/5/0.1 | 0.38 | 2.13 | 1.29 | **1.58** | **C/P/I 90/10/0.1** |
| | **O5** | 1.46 | 10.07 | 1.27 | **2.72** | **C/M/I/T 90/10/0.1/0.1** | 1.96 | 13.77 | 1.30 | **2.36** | C/M/I/T 90/10/0.1/0.1 |

In the case of baseline separation, the optimized chromatographic data are reported with respect to the shortest analysis time; resolution value in bold indicates baseline separation; MP composition in bold indicates the best chromatographic conditions (including CSP) for enantioseparation, C: $CO_2$, M: MEOH, P: PROH, I: IPAM, T: TFA; $t_{R,1}$: retention time of the first eluted enantiomer, $k_1$: retention factor of the first eluted enantiomer, $\alpha$: selectivity, $R_S$: resolution of the two enantiomers, X: no indication of enantioseparation.

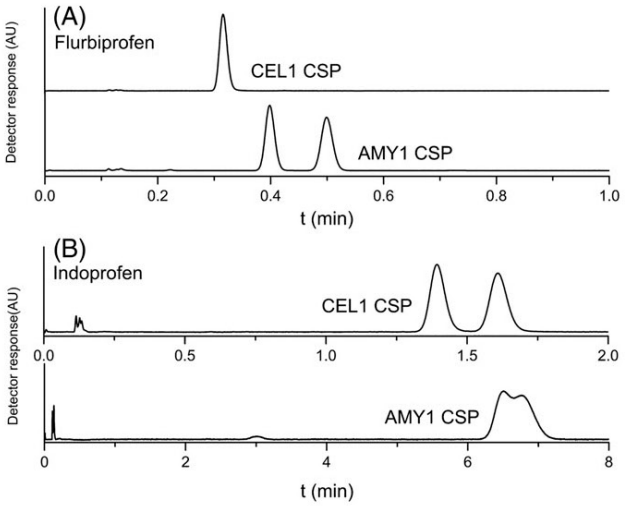


**FIGURE 1** Analyses of flurbiprofen **A**, and indoprofen **B**, on the polysaccharide-based CSPs. MP composition: $CO_2$/MEOH/TFA 90/10/0.1 *(v/v/v)*

and indoprofen in the same MP composed of $CO_2$/MEOH/TFA 90/10/0.1 *(v/v/v)* supports this statement, i.e., better result of indoprofen enantioseparation obtained on CEL1 CSP. Moreover, indoprofen (PF2) was baseline enantioseparated in all the MPs tested using CEL1 CSP (Table S1 in the Supporting Information). MPs with basic IPAM additive were not suitable for enantioseparation of profen derivatives. The basic additive increases "dissociation" of acidic profens, while compounds to be separated on the polysaccharide-based CSPs are nondissociated. Thus, addition of TFA or a combination of IPAM and TFA resulted in improved enantioseparation. Certain complementarity of the enantioseparation ability of the compared CSPs is clearly seen from the obtained results (Figure 1).

## 3.2 | Flavanones

The two flavanone derivatives with very similar structures (the difference lies only in the position of the hydroxy group in their molecule) were successfully enantioseparated using AMY1 CSP and MP composed of $CO_2$/PROH/IPAM/TFA 90/10/0.1/0.1 *(v/v/v/v)* (Table 2). The combination of both MP additives (IPAM and TFA) was very supportive for fast enantioseparation of both analytes on AMY1 CSP. On the other hand, they were not baseline enantioresolved on CEL1 CSP under the above-mentioned MP. However, enantioresolution $R_S = 1.5$ for F1 enantiomers was achieved on CEL1 CSP in MP with higher $CO_2$ content. The representative chromatograms of analysis of 7-hydroxyflavanone on both columns in the same MP composition are depicted in Figure 2.

## 3.3 | Thiazide diuretics

All the enantiomers of analytes from the group of thiazide diuretics could be baseline separated under optimized separation conditions on AMY1 CSP. However, their retention and thus analysis time was too long for practical purposes. On the other hand, butizide (TD1), chlorthalidone (TD3), and trichlormethiazide (TD4) enantiomers could be baseline separated on CEL1 CSP in a significantly shorter analysis time. Concerning the MPs, the most suitable composition varies by CSP used and analyte of interest (Table 2).

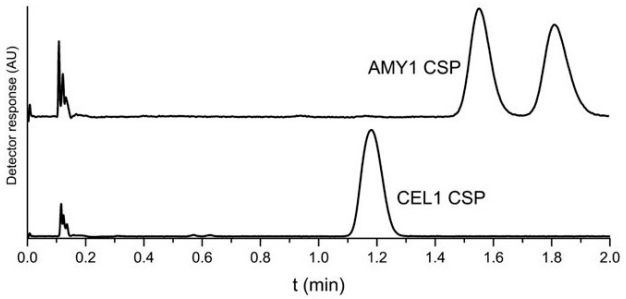


**FIGURE 2** Analyses of 7-hydroxyflavanone on the polysaccharide-based CSPs. MP composition: $CO_2$/PROH/IPAM/TFA 90/10/0.1/0.1 *(v/v/v/v)*

### 3.4 | Calcium channel blockers

Neither AMY1 nor CEL1 CSPs were suitable for enantioseparation of this group of analytes. Despite the fact that calcium channel blockers possess in their molecules structurally similar motifs, we observed rather different results for diverse derivatives. Whereas amlodipine, verapamil, and nisoldipine enantiomers were baseline separated on CEL1 CSP, nimodipine could be enantioseparated on AMY1 CSP. No CSP could be preferred; moreover, the best MP composition for analysis of the calcium channel blockers differed in the type of organic modifier as well as in MP additive.

### 3.5 | Phenothiazines

AMY1 CSP better suited for separation of phenothiazine enantiomers than CEL1 CSP. Thioridazine (PH1) and promethazine (PH2) were both baseline resolved in MP containing $CO_2$, PROH, and IPAM (Table 2). A better resolution value was achieved for PH1 than for PH2 with amylose-based column, while the opposite result was observed on cellulose-based CSP. On the latter, partial enantioseparation was obtained for PH2, while no enantioseparation was achieved for PH1.

### 3.6 | Amphetamine derivatives

All amphetamine derivatives were baseline enantioseparated, except of 4-F-methcathinone (A1) enantiomers, which were partially separated using AMY1 CSP. PROH was a better organic modifier for separation of all the analytes from this group and also the best MP composition was mostly the same, i.e., $CO_2$/PROH/TFA 95/5/0.1 *(v/v/v)* (Table 2). Using CEL1 CSP, all these analytes eluted in very short retention times and no enantioseparation was observed (except for A4 and A8) (Table 2 and Table S1 in the Supporting Information). Only one amphetamine derivative, A8, was baseline enantioresolved using CEL1 CSP. Nevertheless, faster baseline separation of A8 was observed using AMY1 CSP. Comparing the observed results AMY1 CSP is definitely the better choice for the enantioseparation of amphetamine derivatives in SFC.

### 3.7 | Benzofury derivatives

The measurements of the group of benzofury derivatives brought interesting results. No general trends could be related to the structure of these analytes, to the structure of the polysaccharide backbone of the CSP, or to MP composition. On the one hand, enantiomers of B1, B2, B3, and B4 were baseline enantioseparated using CEL1 CSP. On the other hand, no enantioseparation of B5 and B6 was observed in any of the MPs tested on CEL1 CSP, and just partial enantioseparation was achieved for B7 with this CSP. The use of MP composed of PROH and both basic IPAM and acidic TFA additives seemed to be advantageous. On the other hand, MP consisted of $CO_2$, PROH and just the basic additive, mostly in the volume ratio $CO_2$/PROH/IPAM 95/5/0.1 *(v/v/v)*, was a better choice if AMY1 CSP was used. A special result was observed with compound B3 (Table 2). The highest resolution value ($R_S$ = 7.33) of all the compounds of this group was achieved with cellulose-based CSP, while on an amylose-based column the lowest resolution value ($R_S$ = 1.37) of B3 was obtained under the "best" MP compositions for a given system.

### 3.8 | β-blockers

It is obvious from Table 2 as well as from Table S1 that for separation of enantiomers of β-blockers cellulose-based CSP should be considered the column of first choice. All β–blockers were baseline enantioseparated with very high resolution values in very short retention times (except for BB5) using CEL1 CSP (Table 2). The best MP compositions for the enantioseparation of β-blockers on the CEL1 CSP were: $CO_2$/MEOH/IPAM/TFA 90/10/0.1/0.1 or 95/5/0.1/0.1 *(v/v/v/v)*. MPs suited for enantioseparation of β–blockers on AMY1 CSP contained PROH as organic modifier (except for BB7). The presence of the less polar alcohol PROH instead of MEOH in MP caused longer analyses on AMY1 than on CEL1 CSP (Table S1). Illustrative chromatograms of enantioseparation of metoprolol (BB3) are shown in Figure 3.

### 3.9 | Others

The last tested analytes are structurally less similar compounds than those in the other groups. Thus, any general discussion cannot be performed. However, we show these results that can be helpful to those who have to carry out separations of these enantiomers because these are compounds of interest (thalidomide, tramadol, lorazepam) for analyses

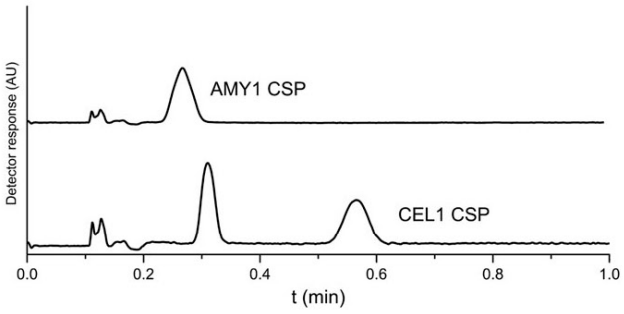


**FIGURE 3** Analyses of metoprolol on the polysaccharide-based CSPs. MP composition: $CO_2$/MEOH/IPAM/TFA 90/10/0.1/0.1 *(v/v/v/v)*

in clinical or pharmaceutical laboratories. As can be seen from Table 2, both columns are applicable for fast enantioseparation of thalidomide, tramadol, as well as lorazepam enantiomers.

## 4 | CONCLUSION

A set of 52 structurally different chiral BACs were used to reveal the enantioselective potential of two polysaccharide-based CSs immobilized on 2.5 μm silica particles, i.e., CEL1 and AMY1 CSPs in SFC. We monitored the influence of the type of CS backbone, the type and amount of organic modifier, as well as MP additives on enantioresolution of the studied compounds. MPs were composed of $CO_2$, organic modifier, i.e., MEOH or PROH and MP additive, i.e., IPAM and/or TFA. The results showed that the tris-(3,5-dimethylphenylcarbamate) derivatives of amylose and cellulose show very broad and complementary enantiorecognition abilities. In general, tris-(3,5-dimethylphenylcarbamate) of amylose was more suitable for enantioseparation of the studied compounds than tris-(3,5-dimethylphenylcarbamate) of cellulose. However, certain enantiomers could be better resolved using the CSP with cellulose backbone. We obtained baseline and partial enantioseparations of 45 and 4 tested compounds, respectively, even without further BP and temperature optimization. Three compounds were not enantioseparated under any conditions used.

In summary, 27 analytes were baseline enatioresolved on CEL1 CSP, whereas 40 on AMY1 CSP. Furthermore, eight and five analytes were partially enantioseparated on CEL1 and AMY1 CSPs, respectively. We were not able to achieve enantioseparation of 17 analytes on CEL1 CSP, while seven on AMY1 CSP. Some complementary behavior of the CSPs was observed. Thus, the combination of these two CSPs offers a powerful tool for enantioseparation of different types of BACs in SFC.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of the Czech Science Foundation, Grant No. 16-05942S.

## REFERENCES

1. Geryk R, Kalikova K, Schmid MG, Tesarova E. Enantioselective separation of biologically active basic compounds in ultra-performance supercritical fluid chromatography. *Anal Chim Acta*. 2016; 932:98-105.
2. Desfontaine V, Guillarme D, Francotte E, Novakova L. Supercritical fluid chromatography in pharmaceutical analysis. *J Pharmaceut Biomed*. 2015;113:56-71.
3. De Klerck K, Mangelings D, Heyden YV. Supercritical fluid chromatography for the enantioseparation of pharmaceuticals. *J Pharmaceut Biomed*. 2012;69:77-92.
4. Wang RQ, Ong TT, Tang WH, Siu-Choon N. Recent advances in pharmaceutical separations with supercritical fluid chromatography using chiral stationary phases. *Trac-Trend Anal Chem*. 2012;37:83-100.
5. Lee J, Lee JT, Watts WL, et al. On the method development of immobilized polysaccharide chiral stationary phases in supercritical fluid chromatography using an extended range of modifiers. *J Chromatogr A*. 2014;1374:238-246.
6. Lemasson E, Bertin S, West C. Use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification. *J Sep Sci*. 2016;39:212-233.
7. West C, Cieslikiewicz-Bouet M, Lewinski K, Gillaizeau I. Enantiomeric separation of original heterocyclic organophosphorus compounds in supercritical fluid chromatography. *Chirality*. 2013;25:230-237.
8. Nováková L, Douša M. General screening and optimization strategy for fast chiral separations in modern supercritical fluid chromatography. *Anal Chim Acta*. 2017;950:199-210.
9. Guillarme D. SFC star. *Anal Sci*. 2015;09:27
10. De Klerck K, Parewyck G, Mangelings D, Vander HY. Enantioselectivity of polysaccharide-based chiral stationary phases in supercritical fluid chromatography using methanolcontaining carbon dioxide mobile phases. *J Chromatogr A*. 2012;1269:336-345.
11. Kalikova K, Slechtova T, Vozka J, Tesarova E. Supercritical fluid chromatography as a tool for enantioselective separation; a review. *Anal Chim Acta*. 2014;821:1-33.
12. West C. Enantioselective separations with supercritical fluids — Review. *Curr Anal Chem*. 2014;10:99-120.
13. Novakova L, Desfontaine V, Ponzetto F, et al. Fast and sensitive supercritical fluid chromatography — Tandem mass spectrometry multi-class screening method for the determination of doping agents in urine. *Anal Chim Acta*. 2016;915:102-110.
14. Toribio L, del Nozal MJ, Bernal JL, Bernal J, Martin MT. Study of the enantiomeric separation of an acetamide intermediate by using supercritical fluid chromatography and several polysaccharide based chiral stationary phases. *J Chromatogr A*. 2011;1218:4886-4891.
15. Franco P, Zhang T. Common approaches for efficient method development with immobilised polysaccharide-derived chiral stationary phases. *J Chromatogr B*. 2008;875:48-56.
16. Vozka J, Kalikova K, Tesarova E. Rapid supercritical fluid chromatography method for separation of chlorthalidone enantiomers. *Anal Lett*. 2013;46:2860-2869.
17. Geryk R, Kalikova K, Vozka J, Plecita D, Schmid MG, Tesarova E. Enantioselective potential of chiral stationary phases based on immobilized polysaccharides in reversed phase mode. *J Chromatogr A*. 2014;1363:155-161.
18. Geryk R, Vozka J, Kalikova K, Tesarova E. HPLC method for chiral separation and quantification of antidepressant citalopram and its precursor citadiol. *Chromatographia*. 2013;76:483-489.
19. Lesellier E, West C. The many faces of packed column supercritical fluid chromatography — A critical review. *J Chromatogr A*. 2015;1382:2-46.
20. Khater S, West C. Insights into chiral recognition mechanisms in supercritical fluid chromatography. V. Effect of the nature and proportion of alcohol mobile phase modifier with amylose and

cellulose tris-(3,5-dimethylphenylcarbamate) stationary phases. *J Chromatogr A*. 2014;1373:197-210.

21. Bargmannleyder N, Tambute A, Caude M. A comparison of LC and SFC for cellulose-derived and amylose-derived chiral stationary phases. *Chirality*. 1995;7:311-325.
22. Gyllenhaal O, Stefansson M. Reversal of elution order for profen acid enantiomers in packed-column SFC on Chiralpak AD. *Chirality*. 2005;17:257-265.
23. Gumustas M, Ozkan SA, Chankvetadze B. Separation and elution order of the enantiomers of some beta-agonists using polysaccharide-based chiral columns and normal phase eluents by high-performance liquid chromatography. *J Chromatogr A*. 2016;1467:297-305.
24. Matarashvili I, Shvangiradze I, Chankvetadze L, et al. High-performance liquid chromatographic separations of stereoisomers of chiral basic agrochemicals with polysaccharide-based chiral columns and polar organic mobile phases. *J Sep Sci*. 2015; 38:4173-4179.
25. West C, Lemasson E, Bertin S, Hennig P, Lesellier E. An improved classification of stationary phases for ultra-high performance supercritical fluid chromatography. *J Chromatogr A*. 2016;1440:212-228.
26. De Klerck K, Vander Heyden Y, Mangelings D. Pharmaceutical-enantiomers resolution using immobilized polysaccharide-based chiral stationary phases in supercritical fluid chromatography. *J Chromatogr A*. 2014;1328:85-97.
27. Matthijs N, Maftouh M, Vander HY. Screening approach for chiral separation of pharmaceuticals. IV. Polar organic solvent chromatography. *J Chromatogr A*. 2006;1111:48-61.
28. De Klerck K, Mangelings D, Clicq D, De Boever F, Vander HY. Combined use of isopropylamine and trifluoroacetic acid in methanol-containing mobile phases for chiral supercritical fluid chromatography. *J Chromatogr A*. 2012;1234:72-79.
29. Pell R, Lindner W. Potential of chiral anion-exchangers operated in various subcritical fluid chromatography modes for resolution of chiral acids. *J Chromatogr A*. 2012;1245:175-182.
30. Wolrab D, Macíková P, Boras M, Kohout M, Lindner W. Strong cation exchange chiral stationary phase — A comparative study in high-performance liquid chromatography and subcritical fluid chromatography. *J Chromatogr A*. 2013;1317:59-66.
31. Wolrab D, Kohout M, Boras M, Lindner W. Strong cation exchange-type chiral stationary phase for enantioseparation of chiral amines in subcritical fluid chromatography. *J Chromatogr A*. 2013;1289:94-104.
32. Novakova L, Perrenoud AGG, Francois I, West C, Lesellier E, Guillarme D. Modern analytical supercritical fluid chromatography using columns packed with sub-2 μm particles: A tutorial. *Anal Chim Acta*. 2014;824:18-35.
33. Perrenoud AGG, Boccard J, Veuthey JL, Guillarme D. Analysis of basic compounds by supercritical fluid chromatography: Attempts to improve peak shape and maintain mass spectrometry compatibility. *J Chromatogr A*. 2012;1262:205-213.
34. Morante-Zarcero S, Sierra I. Simultaneous enantiomeric determination of propranolol, metoprolol, pindolol and atenolol in natural waters by HPLC on new polysaccharide-based stationary phase using a highly selective molecularly imprinted polymer extraction. *Chirality*. 2012;24:860-866.
35. Fanali C, Fanali S, Chankvetadze B. HPLC separation of enantiomers of some flavanone derivatives using polysaccharide-based chiral selectors covalently immobilized on silica. *Chromatographia*. 2016;79:119-124.
36. Albals D, Vander Heyden Y, Schmid MG, Chankvetadze B, Mangelings D. Chiral separations of cathinone and amphetamine-derivatives: Comparative study between capillary electrochromatography, supercritical fluid chromatography and three liquid chromatographic modes. *J Pharmaceut Biomed*. 2016;121:232-243.
37. Svan A, Hedeland M, Arvidsson T, Jasper JT, Sedlak DL, Pettersson CE. Rapid chiral separation of atenolol, metoprolol, propranolol and the zwitterionic metoprolol acid using supercritical fluid chromatography-tandem mass spectrometry — Application to wetland microcosms. *J Chromatogr A*. 2015;1409:251-258.
38. Toribio L, Alonso C, del Nozal MJ, Bernal JL, Martin MT. Semipreparative enantiomeric separation of omeprazole by supercritical fluid chromatography. *J Chromatogr A*. 2006; 1137:30-35.
39. Berger TA, Wilson WH. Separation of drugs by packed column supercritical-fluid chromatography. 1. Phenothiazine antipsychotics. *J Pharm Sci*. 1994;83:281-286.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article.

**How to cite this article:** Kucerova G, Kalikova K, Tesarova E. Enantioselective potential of polysaccharide-based chiral stationary phases in supercritical fluid chromatography. *Chirality*. 2017; 29:239–246. https://doi.org/10.1002/chir.22701