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Characterization of Modern HPLC Columns and Their Application Potential

DISSERTATION THESIS

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This thesis summarizes the results obtained in the years 2007–2011 during my Ph.D. studies at the Department of Analytical Chemistry, Faculty of Science of the Charles University in Prague.

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I declare that all the results used and published in this thesis have been obtained by my own experimental work, supervised by Prof. RNDr. Eva Tesařová, CSc., all the references are properly cited and this thesis has not been applied to obtain the same or other academic degree.

Prague, 12.7.2011 Mgr. Lucie Janečková

DECLARATION OF CO-AUTHORS

On behalf of the co-authors I declare that Mgr. Lucie Janečková contributed substantially to Paper I entitled „Využití moderních reverzních stacionárních fází na bázi oxidu zirkoničitého pro analýzu bioaktivních peptidů." Her share was 90 %.

On behalf of the co-authors I declare that Mgr. Lucie Janečková contributed substantially to Paper II entitled „Study of interaction mechanisms on zirconia-based polystyrene HPLC column." Her share was 70 %.

On behalf of the co-authors I declare that Mgr. Lucie Janečková contributed substantially to Paper III entitled „Chiral separation of binaphthyl catalysts using new chiral stationary phases based on derivatized cyclofructans." Her share was 80 %.

On behalf of the co-authors I declare that Mgr. Lucie Janečková contributed substantially to Paper IV entitled „Characterization of cyclofructan-based chiral stationary phases by linear free energy relationship." Her share was 70 %.

On behalf of the co-authors I declare that Mgr. Lucie Janečková contributed substantially to Paper V entitled „Characterization of new *R*-naphthylethyl cyclofructan 6 chiral stationary phase and its comparison with *R*-naphthylethyl β-cyclodextrin-based column." Her share was 40 %.

Prague, July 2011 Prof. RNDr. Eva Tesařová, CSc.

ABSTRACT (EN)

The aim of the dissertation thesis is characterization of modern HPLC columns from the point of their interaction possibilities and demonstration of their application potential.

The first part of the thesis is focused on alternative reversed-phase HPLC columns based on zirconium dioxide. These packings offer excellent chemical stability and additional interactions that can be helpful in the development of analytical methods. A detailed study of the chromatographic behaviour of biologically active nonapeptides as model analytes confirmed the substantial effect of mobile phase composition on retention mechanism. Consequently, HPLC separation systems with zirconia-based polystyrene column were characterized by distinct approaches that allowed recognition of the interactions participating in the separation process. Empirically based chromatographic tests evaluated the fundamental properties of the system – hydrophobicity and polarity. The complex model of linear free energy relationship described the prevailing interactions in different separation systems. Application of a set of basic compounds revealed the contribution of ion-exchange interactions participating in the separation systems with zirconia-based column.

The second part of the thesis is devoted to new cyclofructan-based chiral stationary phases. Structurally different chiral compounds (binaphthyl derivatives and certain chiral pharmaceuticals) served for evaluation and comparison of the interaction and enantioseparation capabilities of three different cyclofructan-based chiral stationary phases. Some excellent enantioseparations were achieved. Furthermore, the linear free energy relationship model was used to reveal the dominant interactions affecting the retention and separation process on the cyclofructan-based columns in normal separation mode. Finally, two chiral stationary phases, i.e. cyclofructan- and cyclodextrin-based columns with the same substituent, were compared by the linear free energy relationship approach. The results showed the differences in the interaction mechanism on the two columns derived from their different basic structure.

ABSTRAKT (CZ)

Cílem předkládané disertační práce byla charakterizace moderních HPLC kolon z hlediska jejich interakčních možností a ukázka jejich aplikačního potenciálu.

První část disertační práce je zaměřena na alternativní reverzní HPLC kolony na bázi oxidu zirkoničitého. Tyto kolony vykazují vysokou chemickou stabilitu a rozšířený interakční mechanismus, který přináší řadu výhod při vývoji analytických metod. Detailní studie chromatografického chování biologicky aktivních nonapeptidů potvrdila značný vliv složení mobilní fáze na retenční mechanismus. Pro rozpoznání interakcí podílejících se na separačním procesu byly následně využity různé přístupy. Základní vlastnosti (hydrofobicita a polarita) kolony na bázi oxidu zirkoničitého s polystyrenovou stacionární fází byly zjištěny pomocí jednoduchých chromatografických testů. Model lineárních vztahů volných energií popsal interakce převládající v různých separačních systémech se zirkoniovou kolonou. Aplikace sady bazických látek vedla k odhalení iontově-výměnných interakcí, které se mohou významně podílet na separačním procesu v systémech se zirkoniovými kolonami.

Druhá část práce se věnuje novým chirálním stacionárním fázím na bázi derivatizovaných cyklofruktanů. Strukturně odlišné chirální látky (binaftylové deriváty a některá chiralní léčiva) byly využity pro porovnání interakčních a enantioseparačních možností tří různých cyklofruktanových chirálních stacionárních fází. Na těchto kolonách bylo dosaženo několika enantioseparací s vysokými hodnotami rozlišení. Následně byl využit model lineárních vztahů volných energií k určení interakcí významně ovlivňujících retenční a separační proces na cyklofruktanových kolonách v normálním separačním módu. Pomocí modelu lineárních vztahů volných energií byly také porovnány dvě chirální stacionární fáze – cyklofruktanová a cyklodextrinová se stejným substituentem. Tento přístup umožnil ukázat rozdíly v interakčních mechanismech na těchto dvou kolonách jako důsledek rozdílné základní struktury.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACN	acetonitrile
C.M.	complete LFER model
CD	cyclodextrin
CF	cyclofructan
CSP	chiral stationary phase
CZE	capillary zone electrophoresis
DETA	*N,N*-diethyl-*m*-toluamide
DMP-CF7	dimethylphenyl carbamoyl cyclofructan 7
hex	*n*-hexane
HPLC	high performance liquid chromatography
IPA	propane-2-ol
IP-CF6	isopropyl carbamoyl cyclofructan 6
IR	infrared
LFER	linear free energy relationships
MeOH	methanol
NMR	nuclear magnetic resonance
O.M.	optimal LFER model
PBD	polybutadiene
PCA	principal component analysis
PS	polystyrene
QSRR	quantitative structure retention relationships
RN-CD	*R*-naphtylethyl carbamoyl β-cyclodextrin
RN-CF6	*R*-naphtylethyl carbamoyl cyclofructan 6
RP-HPLC	reversed phase high performance liquid chromatography
TFA	trifluoroacetic acid
Zr-PBD	HPLC column based on zirconium dioxide with polybutadiene stationary phase
Zr-PS	HPLC column based on zirconium dioxide with polystyrene stationary phase

% (*v*)	volume percent
(*v/v*)	volume ratio
a	coefficient a of the LFER equation
A	effective or overall hydrogen bond acidity
b	coefficient b of the LFER equation
B	effective or overall hydrogen bond basicity
c	intercept in the LFER equation
e	coefficient e of the LFER equation
E	excess molar refraction
k	retention factor
K_a	dissociation constant
P	partition coefficient in *n*-octanol-water
R	resolution, enantioresolution
S	symmetry factor
s	coefficient s of the LFER equation
S	dipolarity/polarizibility
v	coefficient v of the LFER equation
V	McGowan characteristic volume of the solute
α	separation factor, enantioselectivity

1. INTRODUCTION AND SCOPE

High performance liquid chromatography (HPLC) is a widely established modern analytical method used as a powerful tool for the analysis of a variety of compounds. The advantages of HPLC lie in its high separation efficiency, reliability and wide choice of stationary phases with different selectivity. Despite the large offer and applicability of existing HPLC columns the research effort for the development of new and improved stationary phases still continues. It is a challenge for many research groups to develop new HPLC columns either for general use or for special applications.

Most of the HPLC stationary phases are based on silica gel possessing excellent mechanical but rather limited chemical and thermal stability. Certain metal oxides can serve as alternative carriers of stationary phases. Zirconium dioxide, apart from its exceptional chemical stability, offers additional interactions that can be helpful for separation of ionizable compounds. These features significantly enhance the possibilities of the optimization process.

Besides the need to separate complex mixtures of achiral compounds, mostly by reversed phase HPLC (RP-HPLC), the analysis of chiral compounds also is a very important part of modern analytical chemistry. HPLC with chiral stationary phases (CSPs) has become the most powerful method for separating racemic samples at analytical and preparative scales or determining enantiomeric purity. Many CSPs with various interaction mechanisms are routinely employed in the enantiomeric separation. Novel type of chiral selectors based on cyclofructan (CF) was introduced in 2009 and was shown to have promising enantioseparation capabilities.

The chemistry of the stationary phase, the property of the carrier and the mobile phase composition are the major factors affecting the complex separation mechanism in HPLC. Understanding the retention and separation process and revealing the interactions participating in the separation systems are important points that can be helpful in the development of new analytical methods as well as novel stationary phases.

Related to the facts mentioned above, the goal of this thesis is to contribute to the characterization of unconventional HPLC columns, i.e., ZrO_2-based RP-HPLC columns and cyclofructan-based chiral stationary phases (CF-CSPs) and to show their application possibilities.

The particular objectives were as follows:

- proof of the potential of ZrO_2-based columns on the separation of biologically active peptides as model analytes
- characterization of selected systems with a ZrO_2-based polystyrene HPLC column by different approaches and recognition of the interactions participating in the separation process

- demonstration of the enantioseparation possibilities of new HPLC chiral stationary phases based on derivatized cyclofructans and their comparison
- characterization and comparison of selected separation systems with CF-based CSP(s) by the model of linear free energy relationship (LFER)
- comparison of significant interactions affecting retention and enantioseparation on derivatized cyclofructan- *vs.* cyclodextrin-based CSPs

2. CHARACTERIZATION AND TESTING OF HPLC SEPARATION SYSTEMS

HPLC is a fundamental method in many fields of science, providing fast, efficient separations with high resolution [1]. The dynamic development of HPLC has been enabled by a wide offer of various column packings with different properties [2]. The selection of a suitable column is crucial in the process of solving a concrete analytical problem. Therefore, analysis of retention properties and characterization of interaction possibilities of HPLC separation media allows deeper understanding the complex retention process and can be helpful in the development of new analytical methods [3].

The extensive research on evaluation methods has followed the development of RP packings. Today, various testing procedures and their combinations can bring a more complex information about the properties of HPLC columns. Determination of physical properties of supports and stationary phases is necessary for reproducible synthesis of well-defined packings. The classical measurement methods evaluate the important physical properties such as particle size and shape, pore size and porosity, specific surface area or carbon load and have been discussed in many papers and books [4, 5, 6].

Spectroscopic techniques, namely infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, also play important role in evaluation and development of HPLC columns. IR spectroscopy is used for investigation of silica surface chemistry and revealing of silanol groups [7]. NMR techniques offer detailed information on the individual functional groups on the surface and on the bonded ligands as well. Distinct types of silanol groups can also be recognized by NMR [7]. However, these techniques are mostly destructive for the stationary phases and not always easy to perform. Statistical methods evaluating chromatographic data (mostly the principal component analysis, PCA) can be used for clustering the columns into groups of similar chromatographic properties [8, 9] and can simplify the selection of a column for a concrete purpose. Thermodynamically based evaluation methods investigate the transfer of solutes from the mobile to the stationary phase. Changes of enthalpies and entropies related to this process are calculated from the retention data emloying

the van't Hoff plots [10, 11, 12]. This procedure can serve for investigating changes in retention mechanism by altering the column temperature.

2.1. Empirically based chromatographic tests

Powerful procedures for characterization of column properties are the chromatographic evaluation methods. They characterize the column or the chromatographic system as a whole under the given conditions. Various tests have been proposed in numerous papers that have been recently reviewed [13, 14]. Generally, properties such as hydrophobicity (hydrophobic selectivity), silanol activity (also named hydrogen bond capacity), ion-exchange capacity, shape (steric) selectivity or the amount of metal impurities can be evaluated. Overall, 36 chromatographic parameters have been reported for characterizing stationary phases [15]. Although none of these tests has been widely accepted as a uniform method for evaluating HPLC columns, the evaluation procedures can help the analyst in the choice of a suitable column for a given purpose. Moreover, the test procedure can also be applied for verification of the column performance at any moment of its lifetime [16].

The main interest of empirically based evaluation methods are the basic properties of HPLC packings, which are hydrophobicity and silanol activity. These features are frequently tested by the referred procedures designed by Walters [17], Engelhardt [18], Tanaka [19], Galushko [20] and other researchers.

Hydrophobicity is related to the interactions between testing compounds and the bonded stationary phase. It is mostly determined by the retention of benzene derivatives and calculated from the retention factors of two closely related compounds [21]. Hydrophobicity can also be considered as methylene selectivity, i.e., the selectivity for specific molecular increment [16].

Evaluation of silanol activity is more complicated due to the existence of many types of interactions between the stationary phase and the analyte. Ion-ion (i.e., ion exchange) interactions and dipole-dipole interactions seem to be the most important [7]. Hydrogen bonding interactions of silanol groups facilitate the retention of polar compounds and electrostatic interactions affect the chromatographic behaviour of basic

compounds [14]. These interactions are undesirable because they cause the tailing peaks of basic analytes due to the slow exchange kinetics.

The testing procedures evaluate silanol interactions mostly by relative retention of two compounds where one compound is assumed to interact *via* hydrophobic and silanol interactions and the retention of the second testing solute is based solely on hydrophobic interaction. Certain tests take into account the peak symmetry of the testing compound to evaluate the ionic interactions [22]. Neue developed another test to evaluate the reproducibility of HPLC packing [23] and to clasify the commercially available columns [24]. His approach is based on relative retention of a basic compound and a hydrophobic solute, at acidic and neutral pH. Another test [25] combining the test methods by Engelhardt [18] and Tanaka [19] was designed for evaluation of column-to-column and batch-to-batch reproducibility.

2.2. Model of linear free energy relationship

Chromatographic properties of HPLC packings and generally separation systems can be evaluated using methods based on retention models. These protocols also study the retention of the test compounds. Moreover, the relationship between the retention and the structure (properties) of the test probes is employed for the characterization of the separation system. There are some notable methods based on a specific model. Horváth designed silanol scavenging model [26], Jandera proposed semi-empirical model of interaction indices [27] and the quantitative structure retention relationships (QSSR) model used by many researchers [28, 29, 30, 31, 32] seem to be the most important.

Quantitative structure retention relationships (QSRR) are the most studied applications of linear free energy relationships (LFER). They demonstrate statistically derived relationships between the structure of solutes and their chromatographic retention [33]. Using QSRR the chromatographic column can be considered as a „free energy convertor“, translating differences in chemical potentials of solutes, which are related to their different structures, to chromatographic retention. QSRRs can be utilized for prediction of retention of a new solute, identification of the most informative structural descriptors, revealing the molecular mechanisms of separation, evaluation

of complex physicochemical properties of solutes or estimation of biological activities of xenobiotic compounds [21, 34]. Certain approaches to QSSR can be applied for characterization of the chromatographic system. One uses the regression of log *k* values against 1-octanol-water partition coefficients (log *P*) [30]. Another approach describes log *k* values in terms of calculated molecular descriptors [35, 36], the other one employs the LFER-based empirical solute parameters based on series of experiments [37].

The advantages of the widely applicable LFER model lie in its ability to characterize and compare stationary phases, or more precisely the whole separation systems, and to describe (both qualify and quantify) the contributions of individual interaction types to the retention process. The LFER equation expresses the relationship between the retention characteristic (i.e., retention factor, *k*) determined for a representative set of analytes and the fundamental solute properties, as seen in the general form of the LFER equation [37]:

$$\log k = c + vV_X + a\sum\alpha_2^H + b\sum\beta_2^H + s\pi_2^H + rR_2 \quad (1)$$

This equation can be used in a following form to simplify the operation with the symbols [38]:

$$\log k = c + v\mathrm{V} + a\mathrm{A} + b\mathrm{B} + s\mathrm{S} + e\mathrm{E} \quad (2)$$

The independent variables in Eq. (2) are solute decriptors and specify certain solute properties. V is the McGowan characteristic volume reflecting hydrophobicity, A is the effective or overall hydrogen bond acidity, B refers to the effective or overall hydrogen bond basicity, S is the solute dipolarity/polarizibility parameter (a measure of the dipole-dipole interaction possibilities of the solute), E is the solute excess molar refraction modelling the solute ability to interact *via n*- and *π*-electron pairs [39, 40]. The desriptors characterize properties of the solute molecules and account for the differences among them. The selection of a representative set of analytes is essential for the system evaluation. The solutes should be structurally diverse and the distribution of the solutes desriptors should equally cover a wide range of interactions [41]. The coefficients in Eq. (2) are determined by multivariate

regression analysis and reflect the different types of molecular interactions in the studied system. In HPLC, the regression coefficients relate to the differences between the phases, i.e., the stationary phase and the concerned mobile phase. The *c* intercept in the LFER equation is obtained by the regression calculation but it does not reflect any special interaction type. All possible influences on retention that are not covered by the LFER equation are summarized in the *c* term [42]. The coefficient *v* reflects the difference in hydrophobicity between the stationary and the mobile phase; *a* represents the difference in hydrogen bond basicity; *b* refers to the difference in hydrogen bond acidity; *s* is equal to the difference in dipolarity/polarizibility between the phases; and *r* reflects the difference in ability of the stationary and the mobile phases to interact with solute *n*- and *π*-electron pairs. One remark on the *a* and *b* coefficients is proper to be stated to understand the interpretation of the LFER results better. The *a* and *b* regression coefficients are complementary to the molecular descriptors A and B in the LFER equation. Therefore, as A describes the hydrogen bond acidity of a test analyte, *a* accounts for the ability of the system to interact as a hydrogen bond accepting environment (i. e., show hydrogen bond basicity) [42]. The same explanation (in the opposite way) can be applied for the descriptor B and the corresponding coefficient *b*. Generally, the sign of the regression coefficient comprises another important information about the chromatographic system. A positive coefficient value shows that the given molecular interaction is stronger in the stationary phase and it increases retention of analytes. A negative value of the coefficient reflects stronger interaction in the mobile phase that decreases the retention.

Complete and optimal model parameters can be obtained from the multivariate regression analysis procedure. The accuracy of the LFER method can be improved using the optimal model that utilizes only the statistically significant regression coefficients. The complete model involves all the regression coefficients no matter what their probability level is. Statistically derived standardized coeficients equilibrate influences of the different units, their mean values are zero, and the standard deviations are the same for all of them. Therefore, the standardized coefficients can be used to analyze interactions within one separation system. However, the comparison of different separation systems cannot be done using standardized coefficients because of the zero mean values [43]. The LFER model characterizes the chromatographic

system as a whole. Therefore, comparison of different stationary phases must be done at the same mobile phase composition. However, the characterization of a HPLC system should be performed under various mobile phase compositions to obtain the complex information about the HPLC system because the mobile phase is an important factor in HPLC.

2.3. Interactions with basic compounds

The analysis of basic compounds by RP-HPLC is often hampered by undesirable ionic interactions of the basic analytes with residual silanol groups on the surface of silica gel. Compounds with a basic nitrogen atom in the structure often cause problems when analysed by RP-HPLC, they yield asymmetrical peaks and irreproducible retention. Some procedures described in the literature [16, 44, 45] can be applied to reveal and quantify these undesirable ion-exchange interactions. For instance, application of a set of basic compounds covering a wide range of hydrophobicity and pK_a constants [45] can be used for evaluation of the non-hydrophobic interaction involved in the retention mechanism of HPLC separation system.

Testing of the stationary phases usually brings large amounts of data. Then it is not easy to trace useful information on the tested packings. The combination of the different approaches discussed above is essential to get the complex information on the concrete stationary phase/separation system.

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3. HPLC COLUMNS BASED ON ZIRCONIUM DIOXIDE

High performance liquid chromatography (HPLC) is a widely established modern analytical method used as a powerful tool for the analysis of a variety of compounds. Most of the HPLC analyses are realized in the reversed phase mode (RP-HPLC) [1], which is best elaborated due to the availability of various RP columns along with many tools to control and optimize the separation.

Silica gel is the most common carrier of chemically bonded stationary phases. Its particles are mechanically stable and have large and reactive surface that can be easily modified. Silica-based HPLC columns have well-known chemical and temperature limitations [2, 3]. Siloxane bond (Si-O-Si) between silica gel and the functional organosilan is unstable in highly acidic solutions (pH below 2) which leads to the loss of the bonded phase. At higher (basic) pH values silica gel dissolves. Moreover, residual silanol group activity affects negatively the retention and peak symmetry of analytes [2], especially basic compounds. Increased separation temperature along with the extreme pH of the mobile phase cause the deterioration of silica-based columns. There are many approaches to improve the stability of silica gel that result in a wider offer of modern RP-HPLC stationary phases [4]. Polymer coated [5, 6], horizontally polymerized [7, 8], bidentate [9, 10], hybrid organic-inorganic [11] and also polar embedded [12] stationary phases are the examples of the new RP phases of improved stability.

3.1. Zirconium dioxide and its properties

The limitations of silica gel led to the investigation of other materials applicable as the supports for HPLC packings. Besides others, certain metal oxides have shown desirable properties [13]. Zirconium dioxide as the most promising material is chemically stable in the whole pH range and can be employed at high temperatures (up to 200 °C using special equipment) [13, 14]. It also offers additional interaction mechanism, which can be useful in the separation of ionizable compounds.

Zirconium dioxide possesses surface chemistry different from silica gel, based on the Lewis theory of acids and bases [13]. The atom of zirconium in the Zr-O-Zr

structure (similar to the structure of silica gel) has free *d*-orbitals that act as Lewis acid and can accept free electron pairs from the components of the mobile phase (e.g. phosphate, fluoride, acetate, carboxylic ions or hydroxyl groups) acting as Lewis bases. These components strongly adsorb on ZrO_2 surface by specific ligand-exchange interactions and the surface of the support is being dynamically modified. Consequently, these adsorbed components act as ion-exchange groups and they can contribute to the retention of the analytes by the ion-exchange interactions. Fig. 3.1. illustrates the described interaction possibilities of ZrO_2 surface.
One of the disadvantages of ZrO_2 particles (as well as other metal oxides used as a support in HPLC) is the impossibility of chemical modification using ordinary silane chemistry. Most of the hydroxyls on the zirconia surface exist in a bridged form and they cannot undergo silanization [15]. Therefore, commercially available reversed-phase zirconia-based columns are not based on silanization of the surface. The surface of zirconia can be modified dynamically, by addition of Lewis bases to the mobile phase, or permanently, by covering the surface with polymers or by depositing carbon. The decrease of polarity of ZrO_2 surface by coating with a polymeric stationary phase (mostly polybutadiene, PBD, and polystyrene, PS) brings a support possessing hydrophobicity and chemical selectivity similar to that of conventional reversed-phase silica-based columns [16] but with the advantageous stability at high pH and enhanced temperature. Generally, the retention and separation on the zirconia-based HPLC columns is based on hydrophobic interactions with the stationary phase and on ion-exchange interactions offered by the chemistry of the support and can be considered as mixed retention mode.

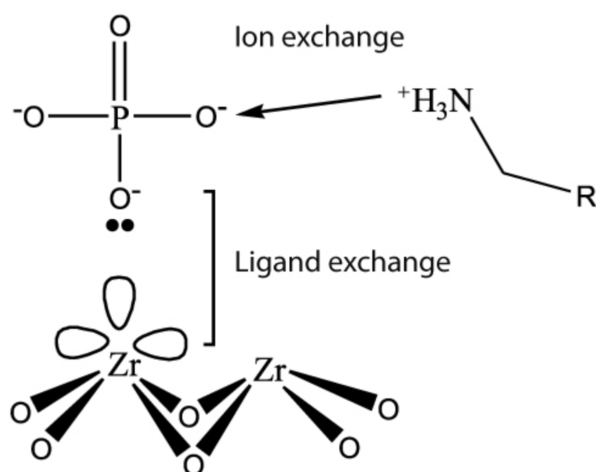

Fig. 3.1. Interaction possibilities on the surface of zirconium dioxide.

The ZrO_2-based stationary phases always offer mixed retention mode because no modification is known that would block all the Lewis sites on the surface [13]. Therefore, when employing zirconia-based columns, one must consider many factors affecting the behaviour of the separation system. The prevalence of the concrete type of retention mechanism depends on the type of the solute, pH, type of buffer used, total ionic strength and also on the amount of organic modifier present in the mobile phase. Polymeric modified ZrO_2 interacts with nonelectrolytes entirely by a reversed-phase mechanism. The retention of basic organic compounds (e.g. cationic amines) is based on mixed mode mechanism (ion-exchange/reversed-phase) strongly dependent on pH. Hard Lewis bases (e.g. organophosphates, phosphonates, carboxylates) interact *via* a ligand-exchange/reversed-phase mechanism [13].

There are many interesting applications of zirconia-based columns to separations of various analytes, e.g. the separation of peptides [17, 18, 19], proteins [19, 20, 21], natural phenolic antioxidants [22], antihistamine and antidepressant drugs of basic (cationic) character [23, 24], β-blockers [25], ibuprofen and its impurities [26, 27] or some other pharmaceuticals [28, 29].

3.2. Results and discussion – Separations of nonapeptides (Paper I)

Paper I presents the study of the separation behaviour of biologically active nonapeptides using modern separation media, PBD- and PS-coated zirconia-based stationary phases. Concerned nonapeptides were selected as model analytes to prove the potential of ZrO_2-based columns in the separations of compounds with basic functional groups and to emphasize the advantages and a wide range of possibilities of the optimization process if working with zirconia-based columns.

Nonapeptides, vasopressin, oxytocin and other structurally and functionally related peptides perform considerable biological activity. Although amino acid sequences of the four studied nonapeptides differ only in one or two amino acid residues, the individual physiological functions can vary substantially depending on the (animal) species where these substances perform.

Separation of a set of four nonapeptides (oxytocin, Arg-vasotocin, Arg-vasopressin, Lys-vasopressin) was studied on two stationary phases based on ZrO_2, i.e., Discovery Zr-PBD (polybutadiene modified zirconia) and Discovery Zr-PS (polystyrene modified zirconia). Optimization of the separation was performed by altering separation conditions. Changes of ionic strength, pH and composition of mobile phase and also temperature influence the separation process. Therefore effects of the buffer pH and concentration, the ratio of organic modifier in the mobile phase and separation temperature were studied in detail. Each optimization step included evaluation of retention factors k, resolution values R and symmetry of peaks A_S of the analytes.

Starting conditions on both zirconia-based RP columns were based on previous experiments with pentapeptides [17] that led to the following results. Firstly, a great difference between two common organic modifiers, methanol (MeOH) and acetonitrile (ACN), was observed for the separation of pentapeptides. ACN provided higher separation efficiency and shorter retention times, better peak shapes and lower backpressure than MeOH. Secondly, the effect of buffer type was studied, because the type of buffer strongly affects the interactions between analytes and ZrO_2 surface, as mentioned above. Acetate and phosphate buffers were investigated. The results showed that retention of the studied pentapeptides increased if phosphate buffer was

used. Phosphate is a stronger Lewis base than acetate and thus it enhances ion-exchange interactions offered by the ZrO_2 surface.

Following features that were observed on both columns showed their similarity. Chromatographic behaviour of the analytes did not alter much on the both stationary phases; trends in retention of the analytes with altering separation conditions were identical or very similar.

Optimization process started with the variation of buffer pH. Starting mobile phase was composed of ACN and 50 mM phosphate buffer in the ratio 20/80 (*v/v*). The effect of pH of the phosphate buffer was studied in the range 4–12 on the both columns. Higher pH led to lower retention of the analytes. This can be explained by higher deprotonization of the analytes leading to decreased ion-exchange interactions with adsorbed phosphate ions. Lower pH of the buffer led to increased retention and resolution improvement but also peaks symmetry deteriorated. The analytes carry higher charges in acidic solution and ion-exchange interactions are much stronger than at higher pH. Higher pH was thus found advantageous for separation of nonapeptides; the optimized pH value was 10.0 and 9.0 on Discovery Zr-PBD and Discovery Zr-PS, respectively.

Concentration of the buffer is an important factor that affects ion-exchange mechanism on ZrO_2 columns. Therefore, in the next step, the influence of phosphate buffer concentration in the range 10–100 mM was tested. The results showed that the increase of the buffer concentration led to decreased retention and better peak symmetry. Higher buffer concentration (abundance of phosphate ions) means competition between buffer and the molecules of analytes for the interaction sites on ZrO_2 surface. Application of lower buffer concentration led to the increase of retention, which was accompanied by an improvement of resolution of the analytes. ZrO_2 surface mostly interacts with buffer ions. This interaction has positive effect on ion-exchange mechanism. Suppression of the ion-exchange type of interaction makes the hydrophobic interaction a more important contribution to the retention mechanism.

Phosphate buffer concentrations of 40 mM (pH 10.0) and 50 mM (pH 9.0) were used on Discovery Zr-PBD and Discovery Zr-PS, respectively, in the experiments to assess the effect of organic modifier to buffer ratio. Lower content of ACN in the mobile phase means lower elution strength and this was the reason for higher retention of the analytes. They interacted more strongly with non-polar stationary phase.

In contrary, higher content of ACN led to lower retention of the analytes having then higher affinity to the mobile phase. Application of mobile phase containing only 5 % (*v*) of ACN on Discovery Zr-PS led to efficient separation of the studied analytes. This result confirmed the manufacturer's specification that this column can be used with nearly aqueous mobile phase.

Discovery Zr columns are designed to work at higher temperatures. Generally, elevated temperature decreases viscosity of mobile phase, it speeds up mass transfer during the separation process and thus it can positively influence the separation. The effect of temperature on separation of nonapeptides was studied in the range 25–75 °C. Higher temperature caused retention decrease, improvement of peak symmetry but resolution of some analytes deteriorated.

Optimized conditions for the separation of nonapeptides on Discovery Zr columns resulted from evaluation of all the optimized data. Discovery Zr-PBD column provided the best separation under these conditions: mobile phase ACN/40 mM phosphate buffer, pH 10.0, 18/82 (*v/v*), temperature 45 °C. The most suitable conditions for the separation of nonapeptides on Discovery Zr-PS column were following: mobile phase ACN/50 mM phosphate buffer, pH 9.0, 5/95 (*v/v*), temperature 45 °C. Figs. 3.2. and 3.3. show chromatograms obtained under optimized conditions on Discovery Zr-PBD and Discovery Zr-PS, respectively. The separation times did not exceed seven minutes.

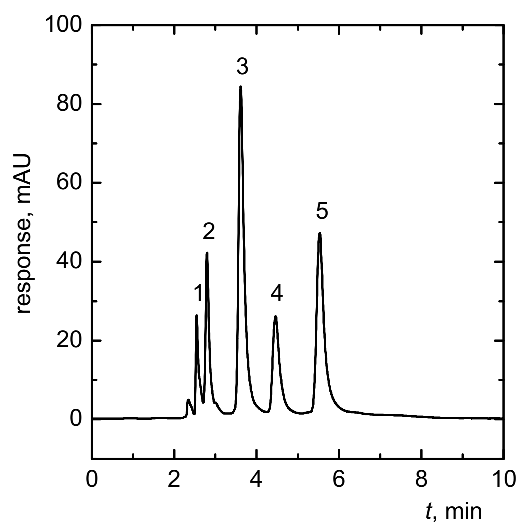

Fig. 3.2. Optimized separation of nonapeptides on Discovery Zr-PBD column; mobile phase ACN/40 mM phosphate buffer, pH 10.0, 18/82 (*v/v*); temperature 45 °C; flow rate 1 mL/min; detection 214 nm.
Peak identification: 1 – uracil, 2 – oxytocin, 3 – Arg-vasotocin, 4 – Arg-vasopressin, 5 – Lys-vasopressin.

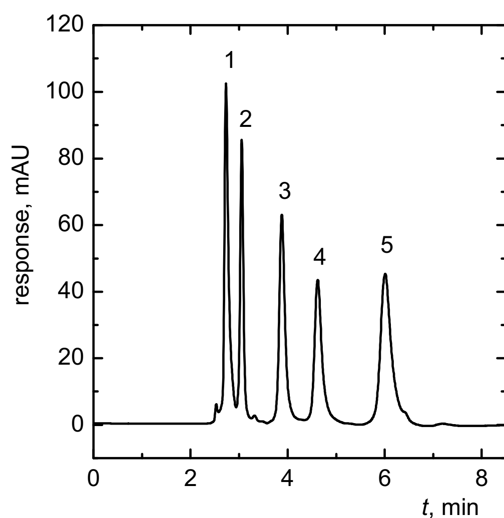

Fig. 3.3. Optimized separation of nonapeptides on Discovery Zr-PS column; mobile phase ACN/50 mM phosphate buffer, pH 9.0, 5/95 (*v/v*); temperature 45 °C; flow rate 1 mL/min; detection 214 nm.
Peak identification is the same as in Fig. 3.2.

The examined alternative polymeric phases based on ZrO_2 were used successfully for the separation of biologically active nonapeptides. The experiments showed similar behaviour of the both columns, the trends in chromatographic behaviour of the analytes with altering separation conditions were almost the same. Zr-PS column offered efficient separation in the mobile phase containing only 5 % (*v*) of ACN. These separation conditions are gentle and environmentally friendly, the amount of organic modifier is reduced and biological activity of the studied peptides is maintained. These are the reasons why polystyrene modified ZrO_2 stationary phase is preferable. The big advantages of zirconia-based columns that should be emphasized are the wide possibilities in the optimization process, especially concerning pH and temperature.

The obtained results have shown that zirconia-based columns are a good alternative to silica-based columns, especially for separation of basic compounds. Different retention (interaction) mechanism, wide possibility of surface modification, high chemical and thermal stability are the main advantages, which can result in successful separations of various biomolecules.

3.3. Results and discussion – Study of interaction possibilities of a zirconia-based polystyrene HPLC column (Paper II)

The chemistry of the stationary phase, the character of its carrier and the experimental conditions (i.e., the mobile phase composition and temperature) are the major factors affecting the complex HPLC separation mechanism, where many types of interactions between the analyte, the stationary and the mobile phases need to be concerned. Zirconia-based HPLC columns are unique for the additional interaction mechanism strongly dependent on the nature of mobile phase components.

Some papers focus on chromatographic characterization of zirconia-based (or generally metal oxide-based) columns [30, 31, 32, 33] and their comparison to common silica-based packings. The aim of Paper II was to characterize the separation systems with polystyrene modified zirconia-based column, to reveal the interactions participating in these separation systems and to understand the complex retention process better. A combination of the evaluation methods introduced

in Chapters 2.1. – 2.3. resulted in a comprehensive description of the interaction possibilities of zirconia-based PS column.

3.3.1. Chromatographic tests of Zr-PS column

Empirically based chromatographic tests investigate the basic properties of HPLC columns. Silanol activity of zirconia surface cannot be obviously determined to provide the information about silanophilic interactions because no silanol groups appear on the surface. The parameters of silanol activity most likely relate to the polar interactions that are largely offered by the zirconia surface. For our purpose, three simple tests were chosen to evaluate hydrophobicity and polarity of the zirconia-based polystyrene column, trade named Discovery Zr-PS and purchased from Supelco, USA. Referred testing procedures designed by Walters [34], Engelhardt [35] and Galushko [36] were employed to provide rough information about the basic column properties.

Table 3.1. summarizes the definitions of the evaluated parameters, i.e., hydrophobicity and polarity, and the obtained values. Hydrophobicity values represent the selectivity for specific molecular increment, polarity can be considered as the selectivity between the compounds of different acid/base character.

Table 3.1. The Walters, Engelhardt and Galushko defined test conditions and the results for Discovery Zr-PS column; the values are measured/calculated for described pair of test solutes in the given mobile phase.

Definitions	Walters	Engelhardt	Galushko
Hydrophobicity	α (anthracene/benzene) in 65 % ACN	α (ethylbenzene/toluen) in 55 % MeOH	$1/2\ (k_{toluene} + k_{benzene})$ in 60 % MeOH
Polarity	α (DETA*/anthracene) in 100 % ACN	α (aniline/phenol) in 55 % MeOH	1+3 [α(aniline/phenol)-1] in 60 % MeOH

Results	Walters	Engelhardt	Galushko
Hydrophobicity	3.99	1.55	0.38
Polarity	2.93	0.72	0.17

* DETA – *N,N*-diethyl-*m*-toluamide

Hydrophobicity of the studied Zr-PS column defined by Walters and Engelhardt is comparable to common silica-based columns [30, 37]. Galushko parameter of hydrophobicity for the concerned zirconia-based column is lower than for silica-based packings, i.e., the studied Zr-PS column possesses lower methylene selectivity.

Discussing the polarity parameter some differences of Discovery Zr-PS column in comparison with silica-based ones arose from these simple experiments. *N,N*-diethyl-*m*-toluamid (DETA) as a testing compound of the Walters test performed strong retention on the Zr-PS column. As the mobile phase composed of pure ACN (in the Walters test) allows the solutes to interact primarily with the support, the strong retention of DETA confirms the interactions of active Zr support with free electrons of this test solute. The other two tests offered lower retention of aniline in comparison with phenol. Deactivated silica-based columns where free silanols are reduced provide this behaviour where aniline elutes before phenol [38]. Discussing this fact for zirconia-based column, phenol has more delocalized electrons than aniline. Therefore, it can better interact with free *d*-orbitals of Zr atoms on the surface of the carrier, and this fact probably causes higher retention of phenol than that of aniline.

Empirically based chromatographic tests showed some differences of the basic properties of zirconia-based column as compared with common silica-based ones. The different retention behaviour of some test solutes confirms the different interaction possibilities offered by the tested Zr packing.

3.3.2. LFER model applied to the systems with Zr-PS column

LFER is a powerful tool for characterization of HPLC separation systems. Some applications of the LFER model to the systems with zirconia-based columns can be found in the literature [30, 39, 40, 41]. In Paper II we used the LFER approach to describe and compare three separation systems consisting of the zirconia-based polystyrene column and three different mobile phase compositions. The selection of the chromatographic conditions for the LFER study was based on the results of our previous investigation of the chromatographic behaviour of nonapeptides (Paper I). As the retention mechanism on zirconia-based columns strongly depends on the constituents (components) of the mobile phase (viz. Chapter 3.1. of this thesis), the LFER model can reveal the effect of the type of buffer additive on the individual

interactions participating in the complex retention process. Therefore, three mobile phase compositions differing in the type of the aqueous component were applied in the LFER study to evaluate the differences between individual interaction types in the different separation systems. The tested mobile phases had a fixed amount of organic modifier, i.e., 20 % (*v*) of ACN; deionized water (pH=6.0), phosphate buffer (50 mM, pH=10.0) or ammonia solution (50 mM, pH=11.0) were chosen as the aqueous components of the mobile phases. A large set of test solutes with known solvation parameters was applied to obtain the regression coefficients of Eq. (2) of the complete and the optimal models of LFER. All these data can be found in Paper II in Tables 2 and 3. Correlation of the LFER data with experimental results (linear regression fit of the experimental log k against calculated/predicted log k) did not show any serious outliers with the exception of the separation system containing ammonia in the mobile phase, where poorer correlation was obtained. Lower p-values of the optimal model than those of the complete model indicate that the regression coefficients of the optimal model are more significant. Therefore, the optimal model was chosen for further evaluation.

The comparison of the regression coefficients of the optimal LFER model is shown in Fig. 3.4. The positive values for coefficients v and e indicate that the given molecular interaction is preferred in the stationary phase and contributes to the retention while negative values for coefficients a, b and s mean stronger interaction in the mobile phase.

The dominant contribution to retention in all the three studied systems is hydrophobicity, described by the coefficient v. Comparable values of coefficient v for the studied systems show little effect of the type of aqueous component on hydrophobic (dispersive) interactions. The coefficients a and b describing hydrogen bond interaction possibilities of the systems are preferred in the mobile phase. High values of coefficient b reflecting the difference in hydrogen bond acidity between the stationary and the mobile phases are connected with the prevalence of aqueous part (80 % (*v*)) in the mobile phase that has strong hydrogen bond donating property. Discussing the third system, ammonia strongly interacts with the stationary phase and thus decreases the difference in H-bond acidity between the two phases. Hydrogen bond basicity seems to be significant for the buffered systems only, but the values are quite small. Coefficient s describing the difference in dipolarity/polarizibility

is significant only for the system with ammonia where these interactions predominate in the mobile phase. Interactions with *n*- and π-electron pairs of the solute, described by the coefficient *e* are preferred in the stationary phases of the systems with water and phosphate buffer in the mobile phase.

The results obtained by the LFER model confirmed that the individual interactions participating in the separation systems with zirconia-based column strongly depend on the nature of mobile phase. The fact that ammonia solution acts as a strong eluting agent suppressing certain types of interactions was also confirmed.

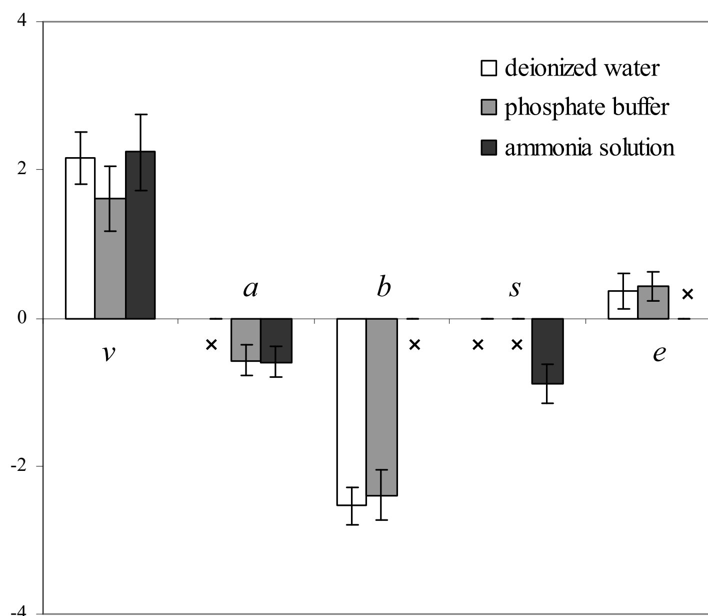

Fig. 3.4. Comparison of the regression coefficients (with their standard errors) of the optimal LFER model for the studied separation systems composed of the Zr-PS column and mobile phases composed of ACN/aqueous component (water, phosphate buffer or ammonia solution), 20/80 (*v/v*). Symbol × means insignificant interaction.

3.3.3. Application of a set of basic compounds

Ion-exchange interactions offered by zirconia-based HPLC packings can largely and positively affect the retention of ionizable compounds. Considering silica-based columns, residual silanol groups are able to interact *via* ion-exchange interactions which are undesirable and cause problems especially in the analysis of basic compounds. Methods that reveal and quantify the contributions of these interactions originally

designed for silica-based columns [37, 42, 43] can also be applied to zirconia-based stationary phases. Because ion-exchange interactions are not involved in the LFER model, the application of the basic compounds can supply an additive information about the interaction possibilities of the zirconia-based PS column.

For our purpose, a procedure applied by Sýkora [43] employing a set of basic compounds differing in hydrophobicity (log P) and dissociation constants, expressed as pK_a, was used. The selected basic compounds covering a wide range of hydrophobicity and pK_a constants are listed in Table 3.2., all the tested compounds can be found in Table 4 of Paper II. Three mobile phases applied were of the same composition as for the LFER study.

Table 3.2. Properties of selected basic compounds and their retention factors k.

Compound name	pK_a [a)]	log P [b)]	k		
			pH = 6.0 [c)]	pH = 10.0 [d)]	pH = 11.0 [e)]
3-Aminopyridine[f)]	5.25	0.20	0.20	0.05	0.08
4-Aminopyridine[f)]	8.61	0.26	2.55	0.10	0.40
2-Amino-4-picoline[f)]	7.67	1.02	2.08	0.20	0.23
3-Picoline	5.60	1.17	0.28	0.22	0.25
2,4,6-Collidine	7.61	2.21	1.29	0.57	0.60
N-Ethylaniline	5.50	2.13	1.59	1.47	1.56
Quinoline	4.64	2.05	0.99	0.87	0.94
Aniline	4.58	0.99	0.35	0.31	0.33

[a)] pK_a constants of protonated forms in aqueous solution at 25 °C, computed with software package PALLAS for prediction of pK_a constants [44]

[b)] partition coefficients in *n*-octanol-water computed with software package PALLAS for prediction of log P values [44]

[c)] mobile phase ACN/deionized water, 20/80 (*v/v*); pH corresponds to the aqueous component

[d)] mobile phase ACN/phosphate buffer (50 mM, pH = 10.0), 20/80 (*v/v*) ; pH corresponds to the aqueous component

[e)] mobile phase ACN/ammonia solution (50 mM, pH = 11.0), 20/80 (*v/v*) ; pH corresponds to the aqueous component

[f)] The lower dissociation constants of the protonated aminogroups of these analytes are not considered in context of this study because they are always deprotonated (-NH_2) in the pH range tested.

Evaluation of hydrophobic interaction possibilities of the separation system can be done by comparison of the retention behaviour of analytes with roughly the same pK_a constants and different hydrophobicities. Considering the analytes dissociated in all the mobile phases tested (e.g. 3-picoline and *N*-ethylaniline, or quinoline and aniline), their retentions do not alter with altering pH, and the differences between their retention correspond only to their different hydrophobicity (log *P* values). Test solutes with similar pK_a constants and different hydrophobicity, which are not fully dissociated in some of the applied mobile phases (e.g. 2-amino-4-picoline and 2,4,6-collidine), show different behaviour that can be explained by both hydrophobic and ion-exchange interactions. The main force affecting retention can also be revealed by this approach (Fig. 2 of Paper II).

Considering the compounds with comparable hydrophobicity (log *P*) and distinct dissociation constants (e.g. 3-aminopyridine and 4-aminopyridine, or 2-amino-4-picoline and 3-picoline), the presence of ion-exchange interactions in the tested separation systems can also be confirmed. Protonized analytes perform much higher retention in comparison with the non-protonized solutes of the same hydrophobicity. Also the partial dissociation of an analyte (e.g. 2-amino-4-picoline or 2,4,6-collidine) needs to be considered when dealing with the contribution of the discussed types of interactions to retention.

The study also confirmed the facts declared in [43] that great indicators of the ion-exchange interaction sites on the sorbent surface are 2-amino-4-picoline, 4-aminopyridine and 2,4,6-collidine, compounds with quite high and similar pK_a values and rather different log *P* values. The testing of stationary phases (HPLC systems) with basic compounds of different hydrophobicity mainly can reveal occurance of ion-exchange interactions (along with the hydrophobic ones) in the RP chromatographic system. If other types of interactions are involved in the separation environment some deviations of the experimental results from the expected data can be observed. Different nature of the aqueous part of the mobile phase and so, different solvation of the stationary phase (and the analyte) can also contribute to some deviations.

3.4. References II

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PAPER I

LABORATORNÍ PŘÍSTROJE A POSTUPY

VYUŽITÍ MODERNÍCH REVERZNÍCH STACIONÁRNÍCH FÁZÍ NA BÁZI OXIDU ZIRKONIČITÉHO PRO ANALÝZU BIOAKTIVNÍCH PEPTIDŮ

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Klíčová slova: HPLC, kolony na bázi oxidu zirkoničitého, bioaktivní peptidy

Úvod

Studium a analýza biologicky aktivních látek je předmětem výzkumu mnoha vědních oborů, zejména biochemie, analytické chemie, molekulární biologie, farmakologie a medicíny. Schopnosti detegovat, kvantifikovat a modelovat biologicky významné molekuly jsou nepostradatelné pro studium základních životních funkcí[1]. Z pohledu analytické chemie jde o vývoj selektivních a účinných separačních metod pro analýzy složitých směsí těchto látek. Vysokoúčinná kapalinová chromatografie tyto požadavky splňuje; poskytuje rychlé a účinné separace s vysokým rozlišením. Tuto metodu lze s úspěchem aplikovat na analýzu biologicky aktivních peptidů[2], látek slabého až výrazného bazického charakteru. Separace těchto látek o velmi nízkých koncentracích je umožněna vhodnou volbou stacionární a mobilní fáze.

V technice HPLC zaujímá dominantní pozici reverzní separační mód (RP-HPLC)[3–9]. Nejrozšířenějším nosičem stacionární fáze v RP-HPLC se stal silikagel, polymerní oxid křemičitý. Silikagelové částice jsou mechanicky stabilní a mají velký povrch, který lze modifikovat navázáním stacionární fáze a získat tak rozmanité systémy s různými mechanismy dělení. Hlavní nevýhodou silikagelu je jeho nestabilita při extrémních hodnotách pH a vyšších teplotách[10,11]. Při nízkých hodnotách pH (pH < 2) je siloxanová vazba nestabilní a dochází ke ztrátě navázané stacionární fáze; při vyšších hodnotách pH (pH > 8) se silikagel rozpouští. Omezení v hodnotách pH mobilní fáze je limitující faktor zvláště při separacích bazických látek. Zbytkové silanolové skupiny na povrchu silikagelu také značně komplikují separační proces[12]. Tyto nežádoucí vlastnosti silikagelu vedly k vývoji nových nosičů stacionární fáze, které by rozšířily pracovní rozsah hodnot pH a teploty a poskytly tak nové možnosti při vyvíjení analytických metod[13].

Výzkum vlastností některých kovů (Zr, Ti, Al) a jejich oxidů vedl k perspektivním výsledkům[13–15]. Oxid zirkoničitý (ZrO_2) se zdá být teplotně a chemicky nejstabilnější ze studovaných oxidů[16–21]. ZrO_2 je amfoterní oxid, vykazující kationtově- i aniontově-výměnné interakce, které závisejí na hodnotě pH a povaze použitého pufru[14,22,23]. Pro látky iontové povahy tedy existují sekundární interakce (iontově-výměnné) s povrchem nosiče, které významně ovlivňují selektivitu a retenci analytů. Atom Zr ve vazbě Zr-O-Zr má volné d-orbitaly, což ho činí akceptorem elektronového páru, je tedy Lewisovou kyselinou. To umožňuje silné ligandově-výměnné interakce s Lewisovými zásadami, mezi které patří např. fosforečnanové, fluoridové, octanové nebo karboxylové ionty či hydroxyskupiny[23–26]. Dochází k silné adsorpci těchto iontů z mobilní fáze na povrch částic ZrO_2, který je tak dynamicky modifikován. Adsorbovaná Lewisova zásada se pak chová jako iontově-výměnná skupina a může se účastnit iontově-výměnných interakcí s ionizovatelnými analyty.

Modifikací povrchu ZrO_2 organickými polymery (polybutadienem a polystyrenem) byly vyvinuty nové stacionární fáze[27] stabilní v celém rozsahu pH (1−14) a při teplotách do 200 °C, které lze využít v reverzním módu HPLC. Separace je založena jednak na hydrofobních interakcích molekul analytů se stacionární fází, a dále na iontově-výměnných interakcích s adsorbovanou Lewisovou bází. Tento smíšený retenční mód lze s úspěchem aplikovat na separaci peptidů[18].

Nonapeptidy, Arg-vasopresin, oxytocin a další strukturně i funkčně blízké peptidy, vykazují značnou biologickou aktivitu nejen v lidském organismu. Arg-vasopresin, známý pod označením antidiuretický hormon (ADH), má fyziologickou úlohu v ledvinách[28], reguluje retenci vody a zvyšuje její zpětné vstřebávání[29]. Vyvoláním vasokonstrikce také zvyšuje krevní tlak. Oxytocin má vliv na frekvenci a sílu kontrakce hladké svaloviny dělohy; toho se využívá v porodnictví. Nové poznatky v oblasti zkoumání fyziologických funkcí nonapeptidů se týkají procesů učení a paměti, sociálního a sexuálního chování, úzkosti, deprese a agresivity[30–34]. Arg-vasopresin mj. podporuje agresi a dominanci[34], charakteristické znaky samčího sociálního chování. Oxytocin má vliv na sexuální a sociální chování u obou pohlaví, ale hraje významnou roli v mateřství. Arg-vasotocin[31,32,35,36] je vývojově starší hormon, vyskytující se u nižších obratlovců. Působí antidiureticky jako Arg-vasopresin a má vliv na sexuální chování podobně jako oxytocin. Lys-vasopresin je syntetický derivát Arg-

vasopresinu s nižší antidiuretickou aktivitou.

V této práci bylo studováno chromatografické chování nonapeptidů na moderních separačních médiích s nosičem ZrO_2 a reverzními stacionárními fázemi polybutadienem (PBD) a polystyrenem (PS). Tyto stacionární fáze byly nejdříve charakterizovány Waltersovým testem, jedním z testů pro reverzní stacionární fáze. Dále byly na těchto stacionárních fázích optimalizovány separační podmínky, tzn. byl studován vliv složení mobilní fáze (typ, koncentrace a pH pufru, typ a množství organického modifikátoru) a vliv teploty na separační parametry (retenční faktor, účinnost, rozlišení, symetrie píků) jednotlivých analytů. V optimalizovaných separačních systémech byly určeny kvantifikační parametry – limity detekce a stanovitelnosti pro vybrané nonapeptidy.

Experimentální část

Přístroje a zařízení

Veškerá měření byla prováděna na kapalinovém chromatografu, který se skládal z gradientového čerpadla Ecom Beta 10, UV-VIS spektrofotometrického detektoru Sapphire 800 a vakuového odplyňovače mobilní fáze Ecom Vacuum Degasser DG 3014 (vše Ecom, Praha, ČR). K dávkování byl použit dávkovací ventil Rheodyne, model 7725i s 5 μl dávkovací smyčkou (Cotati, USA). Pro sběr dat a vyhodnocení chromatogramů byl použit program Clarity, verze 2.3.0.174 od firmy Data Apex (Praha, ČR).

Roztoky pufrů a peptidů byly připravovány odvážením příslušných množství na analytických vahách APX-100 (Denver Instruments, USA). Hodnota pH pufru byla kontrolována pH-metrem 3510 (Jenway, Velká Británie). Kolony byly termostatovány v termostatu Column Oven LCO 101 (Ecom, ČR).

K separaci byly použity kolony Discovery Zr-PBD a Discovery Zr-PS (Supelco, Bellefonte, USA). Obě kolony mají shodné parametry, tj. rozměry 25 cm × 4,6 mm, velikost částic 5 μm, velikost pórů 300 Å.

Chemikálie a vzorky

Acetonitril Chromasolv® pro HPLC byl získán od firmy Sigma-Aldrich (St. Louis, USA). Fosforečnanový pufr ($NaH_2PO_4 \cdot 2\ H_2O$, p.a.; $Na_2HPO_4 \cdot 12\ H_2O$, p.a.) byl produktem firmy Penta (Chrudim, Česká republika), kyselina fosforečná (p.a., 85%) a hydroxid sodný (p.a.) pro úpravu pH pufru byly od firmy Lach-Ner (Neratovice, Česká republika). Deionizovaná voda byla připravována v přístroji Milli Q (Millipore, Milford, USA).

Pro Waltersův test byly použity následující chemikálie: antracen (purum, >98%, HPLC; Fluka Chemie AG, Buchs, Švýcarsko), *N*,*N*-diethyl-*m*-toluamid (*N*,*N*-DETA; purum, >99%, GC, Fluka Chemie AG) a benzen (HPLC grade, Sigma-Aldrich).

Vzorky nonapeptidů byly komerční syntetické preparáty, produkty firmy Sigma-Aldrich. Jejich pořadí aminokyselin ukazuje tabulka I. Mezi cysteinovými zbytky v poloze 1 a 6 se tvoří disulfidický můstek. Bez dalších úprav byly tyto látky rozpuštěny v deionizované vodě a byly tak získány zásobní roztoky o koncentraci 1 mg ml^{-1}. Z těchto zásobních roztoků byla připravena směs peptidů pro jejich analýzu a sada roztoků pro kvantifikaci jednotlivých peptidů. K určení mrtvého času byl použit uracil (Sigma-Aldrich), jehož zásobní roztok měl koncentraci 0,1 mg ml^{-1}.

Tabulka I
Struktury studovaných nonapeptidů

Peptid	Sekvence aminokyselin
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly
Arg-vasotocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly
Arg-vasopresin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly
Lys-vasopresin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly

Výsledky a diskuse

Pro analýzu nonapeptidů byl použit systém reverzní vysokoúčinné kapalinové chromatografie (RP-HPLC). Byly použity dvě kolony se zirkoniovým nosičem modifikovaným polybutadienem (Discovery Zr-PBD) a polystyrenem (Discovery Zr-PS). Tyto stacionární fáze byly nejdříve otestovány Waltersovým testem, dále byly na těchto stacionárních fázích optimalizovány podmínky pro separaci čtyř biologicky aktivních nonapeptidů a pro optimalizované separační systémy byly určeny kvantifikační parametry – limity detekce a stanovitelnosti.

Na základě údajů z literatury[18,37] byly mobilní fáze pro optimalizaci separace nonapeptidů zpravidla složeny z acetonitrilu (ACN) a fosforečnanového pufru a pro detekci peptidů byla zvolena vlnová délka 214 nm.

Waltersův test – testování vlastností reverzních stacionárních fází

Z několika testů pro reverzní stacionární fáze byl vybrán test podle Walterse[38], určující index hydrofobnosti a silanolový index v prostředí acetonitrilu. Označení silanolový index není příliš vhodné pro stacionární fáze na bázi oxidu zirkoničitého, avšak jeho hodnota vypovídá o polárních interakcích analytů se stacionární fází a jejím nosičem. Silanolový index byl z těchto důvodů označen v této práci jako index polarity.

Index hydrofobnosti (HI) je podíl retenčních faktorů antracenu a benzenu, při použití mobilní fáze ACN/voda (65/35, v/v). Index polarity (SI) je podíl retenčních faktorů *N*,*N*-dietyl-*m*-toluamidu (*N*,*N*-DETA) a antracenu, při použití čistého ACN jako mobilní fáze. Mrtvý čas kolony je určen retenčním časem uracilu v mobilní fázi ACN/voda (65/35, v/v). Experimentální podmínky, předepsané pro tento test, jsou: průtoková rychlost 1 ml min^{-1}, dávkován

Tabulka II
Hodnoty indexů hydrofobnosti a polarity pro použité stacionární fáze

Kolona	Stacionární fáze	HI	SI
Discovery Zr-PBD	Zr-PBD	5,40	1,00
Discovery Zr-PS	Zr-PS	5,52	0,98

1 μl testovací směsi, UV detekce při 254 nm.

Z výsledků v tab. II je patrné, že obě kolony na bázi oxidu zirkoničitého mají velmi podobné separační vlastnosti pro nepolární analyty. Tyto kolony vykazují značně nepolární charakter, uplatňují se výrazné nepolární interakce stacionární fáze s analyty. Kolony se silikagelovým nosičem a reverzní stacionární fází poskytují hodnoty HI nižší; pro oktadecylové stacionární fáze (C_{18}) jsou běžně hodnoty HI menší nebo rovny 4, oktylové stacionární fáze (C_8) mají hodnoty HI menší nebo rovny 3 (cit.[2]).

Hodnota indexu polarity SI poskytuje informaci o polárních interakcích analytů se stacionární fází a jejím nosičem. Kolony se silikagelovým nosičem by měly mít hodnotu SI co nejmenší, protože pak nežádoucí interakce analytů a zbytkových silanolových skupin jsou minimální. U kolon na bázi ZrO_2 jsou hodnoty indexu polarity SI větší, což odpovídá předpokládanému mechanismu separace, kdy sekundární iontové interakce jsou žádoucí, jsou podporovány a navíc aktivovány dynamickou modifikací povrchu částic adsorbovanou Lewisovou zásadou.

Separace nonapeptidů na koloně Discovery Zr-PBD

Na koloně Discovery Zr-PBD bylo studováno chromatografické chování čtyř nonapeptidů. Změny iontové síly, pH a složení mobilní fáze a také pracovní teploty ovlivňují separaci a tyto vlivy byly během optimalizace studovány. Na základě údajů z literatury[18,37,39] byly mobilní fáze pro optimalizaci separace nonapeptidů složeny z ACN a fosforečnanového pufru a pro detekci peptidů byla zvolena vlnová délka 214 nm.

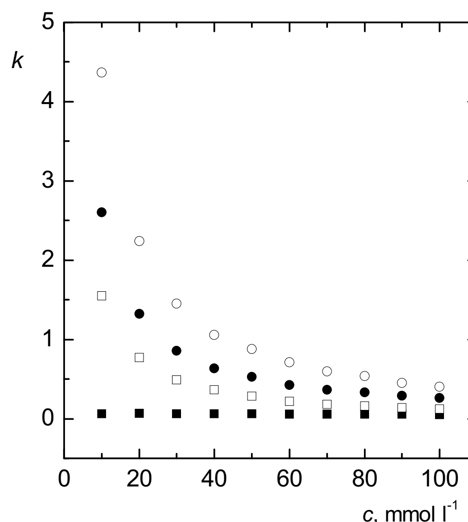
Vliv pH a koncentrace pufru na retenční chování nonapeptidů

Interakce mezi analyty a reverzními kolonami na bázi zirkonia silně závisejí na použitém pufru. Literatura[39] poukazuje na vhodnost použití fosforečnanového pufru vzhledem k jeho nízké absorbanci a možnosti použití v celém rozsahu pH.

Výchozí podmínky separace nonapeptidů na koloně Discovery Zr-PBD byly následující: mobilní fáze složená z 20 obj.% ACN a 80 obj.% fosforečnanového pufru; koncentrace fosforečnanového pufru byla 50 mmol l^{-1} a hodnota pH byla 12. Tyto podmínky byly zvoleny na základě údajů z literatury[37] a také hodnot isoelektrických bodů zkoumaných nonapeptidů, které se nacházejí v alkalické oblasti. Zároveň chemická stabilita stacionárních fází na bázi oxidu zirkoničitého dovoluje použití vyšších hodnot pH mobilní fáze než stacionární fáze na bázi silikagelu. Za těchto podmínek oxytocin eluoval s mrtvým časem, hodnoty rozlišení pro ostatní analyty byly uspokojivé, avšak eluční křivky všech analytů byly nesymetrické, hodnoty faktorů asymetrie byly větší než 2.

Vliv pH použitého pufru na chování analytů byl studován v rozmezí hodnot 4–12. Vyšší hodnoty pH vodné složky mobilní fáze vedly ke zkrácení doby analýzy, ale ne ke zhoršení rozlišení analytů. Retence analytů v kyselé oblasti byla větší, oxytocin vykazoval retenci při pH 4 a 5, kdy však doba analýzy byla větší než 25 min, píky ostatních analytů byly rozmyté a hodnoty rozlišení zbytečně vysoké. Píky analytů nevykazovaly uspokojivou symetrii. Pro další optimalizaci bylo vybráno pH vodné složky mobilní fáze 10. Za těchto podmínek sice oxytocin eluoval s mrtvým časem, analýzy ovšem byly krátké (do 6 min), poskytovaly rozlišení větší než 1,2 a faktory asymetrie kolem hodnoty 2.

V dalším optimalizačním kroku bylo sledováno chromatografické chování nonapeptidů v rozmezí koncentrace fosforečnanového pufru 10–100 mmol l^{-1}. Obr. 1 ukazuje závislost retenčních faktorů *k* jednotlivých analytů na koncentraci použitého pufru. Nižší koncentrace fosforečnanového pufru způsobovala větší retenci všech analytů. Nízká iontová síla pufru o koncentraci 10 mmol l^{-1} byla příčinou velké retence Lys-vasopresinu (*k*=4,4), kdy analýzy byly delší než 14 min a píky analytů rozmyté. Oxytocin při vyšších koncentracích pufru koeluoval s uracilem, při koncentracích menších než 50 mmol l^{-1} vykazoval malou retenci, která se ovšem při snižující se koncentraci pufru nezvyšovala. Retenční faktor oxytocinu pro koncentrace



Obr. 1. **Závislost retenčních faktorů *k* na koncentraci fosforečnanového pufru;** separace na koloně Discovery Zr-PBD; mobilní fáze ACN/fosforečnanový pufr, pH 10 (20/80, v/v); průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 μl; UV detekce při 214 nm; ■ oxytocin, □ Arg-vasotocin, ● Arg-vasopresin, ○ Lys-vasopresin

10–40 mmol l^{-1} byl 0,06. Hodnoty rozlišení ostatních analytů při koncentracích nižších než 50 mmol l^{-1} byly větší než 2,4, a tudíž dostatečné. Pro další optimalizaci byla tedy zvolena koncentrace fosforečnanového pufru 40 mmol l^{-1}. Při této koncentraci byla hodnota rozlišení oxytocinu nejvyšší, časy analýz byly kratší než 6 min a hodnoty faktorů asymetrie se stále pohybovaly kolem 2.

Vliv poměru organického modifikátoru a fosforečnanového pufru na retenční chování nonapeptidů

Pro studium vlivu poměru organického modifikátoru a fosforečnanového pufru byly použity výsledné podmínky výše uvedené. Mobilní fáze byla složena z ACN a fosforečnanového pufru o koncentraci 40 mmol l^{-1} a hodnotě pH 10. Obsah ACN v mobilní fázi byl zkoušen v rozmezí 15–25 obj.% a byl měněn vždy po 1 %. Separace peptidů obecně je velmi citlivá na změnu obsahu organické složky v mobilní fázi, takže i malá změna v obsahu ACN, typicky jedno objemové procento, znamená významnou změnu v separačním chování těchto látek. Na obr. 2 je vynesena závislost retenčních faktorů *k* jednotlivých analytů na obsahu ACN v mobilní fázi.

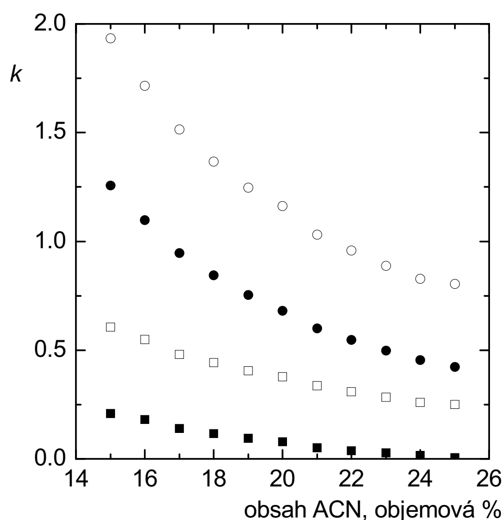
Ze získaných výsledků je patrné, že s rostoucím obsahem organického modifikátoru v mobilní fázi klesala retence všech studovaných látek. Větší eluční síla způsobovala nedostatečné rozlišení oxytocinu a jeho následnou eluci v mrtvém čase při použití mobilní fáze obsahující 23 a více obj.% ACN. Nižší obsah ACN sice vedl ke zlepšení rozlišení oxytocinu, ale rozlišení ostatních analytů se prakticky neměnilo. Faktory asymetrie se stále pohybovaly kolem 2. Při použití mobilní fáze obsahující 18 obj.% ACN byly hodnoty rozlišení všech analytů dostatečné (větší než 2) a doba analýzy byla kratší než 7 min. Proto byly tyto podmínky z hlediska složení mobilní fáze zvoleny jako optimální pro separaci vybraných nonapeptidů na koloně Discovery Zr-PBD.

Vliv separační teploty na retenční chování nonapeptidů

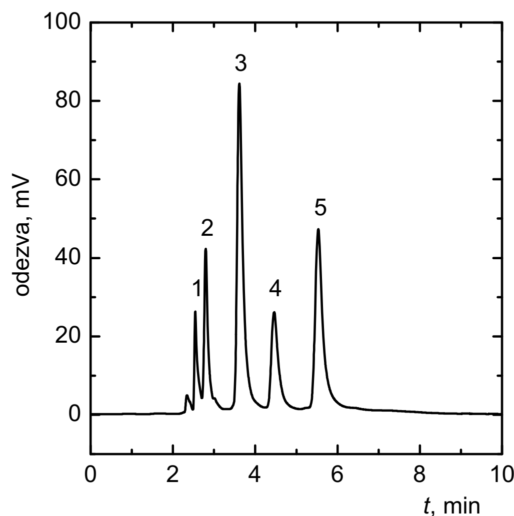
Poslední parametr, jehož vliv na separaci byl studován, byla teplota kolony při analýze. Obecně zvýšená separační teplota zrychluje přenos hmoty během separačního procesu, a tak může pozitivně ovlivnit analýzu. Kolony na bázi oxidu zirkoničitého umožňují použití vyšších pracovních teplot než kolony se silikagelovým nosičem. Použitá kolona Discovery Zr-PBD byla termostatována a byly provedeny analýzy při teplotách 25–75 °C. Získané výsledky jsou shrnuty v tab. III.

Vyšší separační teplota snižuje viskozitu mobilní fáze; analýzy za vyšších teplot obecně vedou ke snížení retence analytů a ke zlepšení faktorů asymetrie. Použití vyšších teplot při analýzách na koloně Discovery Zr-PBD sice vedlo ke snížení retence analytů, avšak hodnoty faktorů asymetrie se výrazně nezlepšily. Píky analytů sice změnily tvar, byly vyšší a užší, ale stále vykazovaly jistou asymetrii. Na základě hodnot faktorů asymetrie a s přihlédnutím k zachování biologické aktivity studovaných látek byla vybrána jako nejvhodnější teplota 45 °C.

Obr. 3 ukazuje optimalizovanou separaci nonapeptidů na koloně Discovery Zr-PBD.



Obr. 2. **Závislost retenčních faktorů *k* na obsahu ACN v mobilní fázi;** separace na koloně Discovery Zr-PBD; mobilní fáze ACN/40 mmol l^{-1} fosforečnanový pufr, pH 10; průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 µl; UV detekce při 214 nm; ■ oxytocin, □ Arg-vasotocin, ● Arg-vasopresin, ○ Lys-vasopresin



Obr. 3. **Separace směsi nonapeptidů na koloně Discovery Zr-PBD;** mobilní fáze ACN/40 mmol l^{-1} fosforečnanový pufr, pH 10 (18/82, v/v); pracovní teplota 45 °C; průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 µl; UV detekce při 214 nm, 1 – uracil, 2 – oxytocin, 3 – Arg-vasotocin, 4 – Arg-vasopresin, 5 – Lys-vasopresin

Tabulka III
Hodnoty retenčních faktorů k, rozlišení R_S a faktorů asymetrie A_F v závislosti na teplotě; separace na koloně Discovery Zr-PBD; mobilní fáze ACN/40 mmol l^{-1} fosforečnanový pufr, pH 10 (18/82, v/v); průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 μl; UV detekce při 214 nm

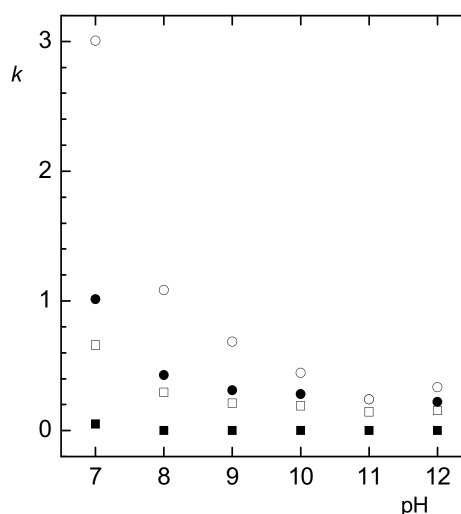
Veličina	Analyt	Teplota [°C]					
		25	35	45	55	65	75
k	oxytocin	0,12	0,11	0,10	0,08	0,07	0,06
	Arg-vasotocin	0,45	0,44	0,41	0,39	0,37	0,36
	Arg-vasopresin	0,85	0,80	0,75	0,68	0,63	0,58
	Lys-vasopresin	1,41	1,30	1,17	1,02	0,92	0,81
R_S	oxytocin	1,9	1,9	1,8	1,7	1,5	1,5
	Arg-vasotocin	3,5	3,6	4,0	4,2	4,4	4,7
	Arg-vasopresin	2,9	2,9	3,1	3,2	3,0	3,1
	Lys-vasopresin	3,5	3,5	3,5	3,3	3,0	2,6
A_F	oxytocin	2,3	1,7	1,4	1,4	1,8	1,8
	Arg-vasotocin	2,5	2,3	2,0	2,1	2,0	2,1
	Arg-vasopresin	2,1	1,7	1,8	1,9	1,8	2,0
	Lys-vasopresin	2,0	1,9	1,9	1,8	1,9	2,0

Separace nonapeptidů na koloně Discovery Zr-PS

Chromatografické chování vasopresinů bylo studováno také na koloně Discovery Zr-PS. Dle údajů výrobce má tato kolona nejméně hydrofobní stacionární fázi ze všech komerčních zirkoniových kolon a je kompatibilní s čistě vodnou mobilní fází. Proces optimalizace separace a výchozí podmínky byly stejné jako u kolony Discovery Zr-PBD. Mobilní fáze byla složena z 20 obj.% ACN a 80 obj.% fosforečnanového pufru; koncentrace pufru byla 50 mmol l^{-1} a hodnota pH byla 12. Oxytocin eluoval s mrtvým časem a ostatní analyty vykazovaly ještě nižší retenci než na koloně Discovery Zr-PBD, nedošlo k jejich rozdělení na základní linii. Bylo tedy přistoupeno k jednotlivým krokům optimalizace.

Vliv pH a koncentrace pufru na retenční chování nonapeptidů

Vliv pH fosforečnanového pufru byl studován v rozmezí 5–12. Obr. 4 ukazuje závislost retenčních faktorů k na pH vodné složky mobilní fáze. Z vypočtených hodnot retenčních faktorů k, rozlišení R_S a faktorů asymetrie A_F jednotlivých analytů vyplývá, že nižší pH vodné složky mobilní fáze vedlo ke zvýšení retence a rozlišení analytů, ale ke zhoršení tvaru píků (faktorů asymetrie A_F). Oxytocin vykazoval retenci až při hodnotě pH 5, kdy ale retenční faktor Lys-vasopresinu měl hodnotu 21,6; čas analýzy tak byl velmi dlouhý (60 min) a píky později eluujících analytů byly značně rozmyté. Použití fosforečnanového pufru o hodnotě pH 7 zkrátilo významně dobu analýzy (14 min), ale píky analytů stále vykazovaly značnou asymetrii. Při pH 11 došlo ke koeluci Arg-vasopresinu a Lys-vasopresinu. Pro další fázi optimalizace separace bylo tedy vybráno pH 9. Za těchto podmínek sice oxytocin nevyka-



Obr. 4. **Závislost retenčních faktorů k na hodnotě pH vodné složky mobilní fáze;** separace na koloně Discovery Zr-PS; mobilní fáze ACN/50 mmol l^{-1} fosforečnanový pufr (20/80, v/v); průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 μl; UV detekce při 214 nm; ■ oxytocin, □ Arg-vasotocin, ● Arg-vasopresin, ○ Lys-vasopresin

zoval retenci, hodnoty rozlišení ostatních analytů ale byly dostačující (v rozmezí hodnot 1,2–3,8) a faktory asymetrie lepší než pro hodnotu pH 8.

Vliv koncentrace použitého pufru na retenci analytů byl sledován v rozmezí 10–100 mmol l^{-1}. Bylo zjištěno, že iontová síla vodné složky mobilní fáze má velký vliv na chromatografické chování studovaných analytů. Nízká koncentrace fosforečnanového pufru (10 mmol l^{-1}) vedla k prodloužení doby analýzy na 15 min, bylo dosaženo nejlepšího rozlišení pro Arg-vasopresin, ale rozlišení Arg-vasotocinu a Lys-vasopresinu bylo zbytečně velké. Za těchto podmínek vykazovaly píky největší asymetrii. Zvýšením iontové síly, tzn. zvýšením koncentrace pufru na 100 mmol l^{-1} došlo sice ke zkrácení doby analýzy na 5 min, ale i k výraznému zhoršení rozlišení všech zadržovaných analytů, Arg-vasotocin a Arg-vasopresin byly neúplně rozdělené. Oxytocin stále nevykazoval retenci. Na základě hodnot faktorů asymetrie byla pro další experimenty vybrána koncentrace fosforečnanového pufru o hodnotě 50 mmol l^{-1}.

Vliv obsahu organického modifikátoru v mobilní fázi na retenční chování nonapeptidů

Pro tuto fázi optimalizace byly použity výše popsané výsledky. Mobilní fáze byla složena z ACN a fosforečnanového pufru o koncentraci 50 mmol l^{-1} a pH 9. Obsah ACN v mobilní fázi byl měněn v rozmezí 3–40 obj.%. Na obr. 5 je naměřená závislost retenčních faktorů *k* na obsahu ACN v mobilní fázi. Ze získaných dat vyplynulo, že vyšší obsah ACN v mobilní fázi (30 a 40 obj.%) vedl ke koeluci Arg-vasotocinu a Arg-vasopresinu a k eluci oxytocinu v mrtvém čase. Snižováním obsahu ACN v mobilní fázi se zvyšovala retence všech analytů, tzn. i oxytocinu, který doposud retenci nevykazoval. Použitím mobilní fáze obsahující 5 obj.% ACN bylo dosaženo uspokojivých hodnot rozlišení i faktorů asymetrie všech analytů. Rozlišení bylo v rozmezí 1,9–4,4 a faktory asymetrie v rozmezí 0,95–1,8, a proto byla tato mobilní fáze zvolena jako nejvhodnější pro separaci studovaných látek.

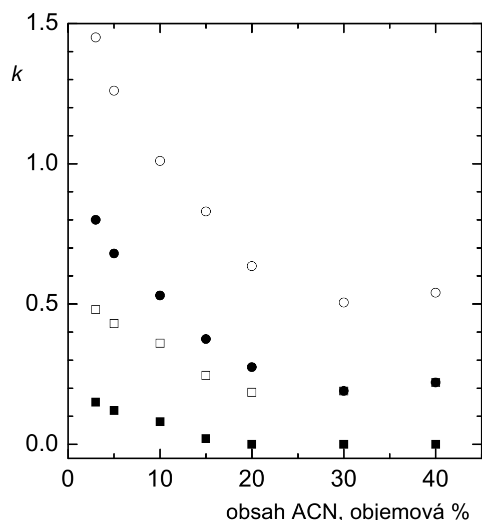
Vliv separační teploty na retenční chování nonapeptidů

Kolona Discovery Zr-PS byla termostatována a za optimalizovaných podmínek bylo sledováno retenční chování analytů při teplotách 25 °C, 35 °C, 45 °C a 55 °C. Tabulka IV shrnuje výsledky získané při tomto optimalizačním kroku. Analýzy za vyšších teplot vedly ke zlepšení faktorů asymetrie všech analytů, avšak zároveň se snížilo rozlišení s výjimkou Arg-vasotocinu. Při 55 °C se výrazně zhoršilo rozlišení oxytocinu. Z toho důvodu byla vybrána teplota 45 °C, při které je rozlišení všech analytů větší než 2 a faktory asymetrie menší než 1,6.

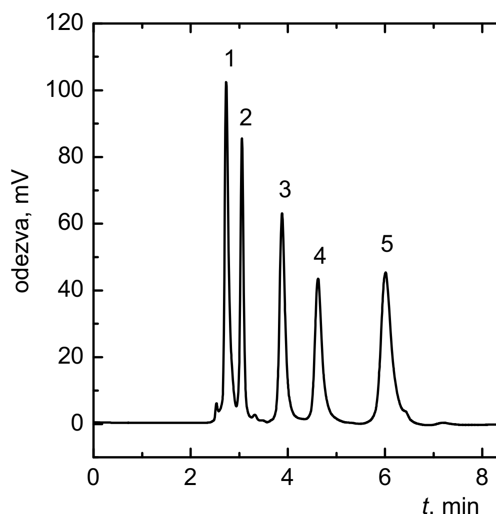
Chromatogram, ukazující separaci nonapeptidů na koloně Discovery Zr-PS za optimalizovaných podmínek, je na obr. 6.

Kvantifikace vybraných nonapeptidů

V optimalizovaných mobilních fázích na obou zirkoniových kolonách byly kvantifikovány tři vybrané nona-



Obr. 5. **Závislost retenčních faktorů *k* na obsahu ACN v mobilní fázi;** separace na koloně Discovery Zr-PS; mobilní fáze ACN/50 mmol l^{-1} fosforečnanový pufr, pH 9; průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 μl; UV detekce při 214 nm; ■ oxytocin, □ Arg-vasotocin, ● Arg-vasopresin, ○ Lys-vasopresin



Obr. 6. **Separace směsi nonapeptidů na koloně Discovery Zr-PS;** mobilní fáze ACN/50 mmol l^{-1} fosforečnanový pufr, pH 9 (5/95, v/v); pracovní teplota 45 °C; průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 μl; UV detekce při 214 nm; 1 – uracil, 2 – oxytocin, 3 – Arg-vasotocin, 4 – Arg-vasopresin, 5 – Lys-vasopresin

Tabulka IV
Hodnoty retenčních faktorů *k*, rozlišení R_S a faktorů asymetrie A_F v závislosti na teplotě; separace na koloně Discovery Zr-PS; mobilní fáze ACN/50 mmol l^{-1} fosforečnanový pufr, pH 9 (5/95, v/v); průtoková rychlost 1 ml min^{-1}; dávkovací smyčka 5 µl; UV detekce při 214 nm

Veličina	Analyt	Teplota [°C]			
		25	35	45	55
k	oxytocin	0,15	0,15	0,13	0,09
	Arg-vasotocin	0,46	0,47	0,44	0,36
	Arg-vasopresin	0,79	0,80	0,71	0,56
	Lys-vasopresin	1,45	1,40	1,23	0,95
R_S	oxytocin	2,5	2,5	2,4	1,6
	Arg-vasotocin	4,2	4,4	4,9	5,2
	Arg-vasopresin	3,2	3,2	3,1	2,7
	Lys-vasopresin	4,3	4,2	4,2	4,1
A_F	oxytocin	1,2	1,1	1,0	1,0
	Arg-vasotocin	1,7	1,6	1,5	1,2
	Arg-vasopresin	1,6	1,6	1,6	1,4
	Lys-vasopresin	1,8	1,9	1,6	1,4

peptidy, tj. Arg-vasotocin, Arg-vasopresin a Lys-vasopresin. Oxytocin nebyl kvantifikován vzhledem k eluci blízké mrtvému času. Byla proměřena závislost velikosti odezvy jednotlivých analytů na vlnové délce v rozmezí 210–220 nm. Při vlnové délce 214 nm měly odezvy analytů maximální hodnotu, tudíž byla tato vlnová délka použita pro kvantifikaci.

Na koloně Discovery Zr-PBD byly kalibrační závislosti proměřeny v koncentračním rozmezí $1{,}56 \cdot 10^{-4}$ až 1,00 mg ml^{-1}. Byly vyhodnoceny závislosti plochy (resp. výšky) píku na koncentraci příslušného analytu a určeny parametry normálních a logaritmických forem kalibračních křivek studovaných analytů. Směrnice logaritmických forem kalibračních křivek se pohybovaly v rozmezí 0,97 až 1,02. Korelační koeficienty ležely v intervalu <0,9988; 0,9999>, a proto mohly být kalibrační závislosti prohlášeny za lineární.

Kvantifikační studie byla také provedena na koloně Discovery Zr-PS při použití optimalizované mobilní fáze. Kalibrační závislosti byly proměřeny v koncentračním rozmezí $3{,}13 \cdot 10^{-4}$–1,00 mg l^{-1}. Směrnice logaritmických forem kalibračních křivek studovaných analytů ležely v intervalu <0,98;1,02>, korelační koeficienty se pohybovaly v rozmezí 0,9979–0,9999. Tato fakta svědčí o linearitě kalibračních závislostí.

Parametry kalibračních závislostí posloužily k výpočtu mezí detekce (LOD) a mezí stanovitelnosti (LOQ) studovaných nonapeptidů.

Mez detekce (LOD) odpovídá koncentraci, pro kterou je analytický signál statisticky významně odlišný od šumu. Pro odezvu meze detekce platí:

$$y_D = 3 \cdot h_{max}$$

kde h_{max} je maximální kolísání základní linie slepého pokusu v oblasti dané 20násobkem pološířky píku stanovovaného analytu.
Pro odezvu meze stanovitelnosti (LOQ) platí:

$$y_S = 10 \cdot h_{max}$$

Tabulka V
Meze detekce (LOD) a meze stanovitelnosti (LOQ) nonapeptidů na kolonách Discovery Zr-PBD a Discovery Zr-PS

Analyt	LOD [µg ml^{-1}]		LOQ [µg ml^{-1}]	
	Zr-PBD	Zr-PS	Zr-PBD	Zr-PS
Arg-vasotocin	1,0	2,4	3,3	8,0
Arg-vasopresin	2,4	6,7	7,9	22,3
Lys-vasopresin	1,8	5,6	5,8	18,7

Při výpočtu meze detekce a meze stanovitelnosti bylo využito závislosti výšky píku na koncentraci analytu.

Vypočtené hodnoty LOD a LOQ jednotlivých analytů na obou kolonách shrnuje tab. V.

Závěr

Chromatografické chování biologicky aktivních nonapeptidů bylo studováno na dvou reverzních kolonách na bázi ZrO_2, a to Discovery Zr-PBD s polybutadienovou stacionární fází a Discovery Zr-PS s polystyrenem jako stacionární fází. Ze získaných výsledků vyplývá velká podobnost použitých kolon. Chromatografické chování nonapeptidů se na obou kolonách výrazně nelišilo; trendy v retenci analytů při změnách separačních podmínek byly stejné či velmi podobné. Na koloně Discovery Zr-PS bylo dosaženo úspěšné separace při použití mobilní fáze složené z fosforečnanového pufru a obsahující pouze 5 obj.% ACN. Tyto podmínky separace jsou šetrné a umožňují zachování biologické aktivity analytů; zároveň přinášejí výhody v podobě úspory organického rozpouštědla. Lepší kvantifikační parametry ovšem poskytla kolona Discovery Zr-PBD.

Reverzní kolony na bázi ZrO_2 se ukázaly jako dobrá alternativa ke kolonám se silikagelovým nosičem pro separaci biologicky aktivních nonapeptidů. Odlišný retenční (interakční) mechanismus, široké možnosti modifikace povrchu ZrO_2 a možnost použití v celém rozsahu pH a při teplotách do 200 °C předurčují tyto stacionární fáze k dalším úspěšným aplikacím v separacích biomolekul.

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L. Janečková[a], J. Sobotníková[a], E. Tesařová[b], and Z. Bosáková[a] (*Charles University in Prague, Faculty of Science, [a]Department of Analytical Chemistry, [b]Department of Physical and Macromolecular Chemistry, Czech Republic*): **Application of Modern Reversed Phases Based on Zirconium Dioxide for the Analysis of Bioactive Peptides**

Separation of biologically active peptides was performed on polybutadiene (PBD) and polystyrene (PS) reversed phases based on zirconium dioxide. ZrO_2 as an alternative carrier to silicagel offers ion-exchange interactions, which are useful for separation of ionizable compounds. The parameters like buffer concentration and pH, the amount of organic modifier and temperature affected separation of nonapeptides. The retention characteristics (retention factor, resolution, peak symmetry, separation efficiency) were investigated. The systems consisting of acetonitrile and phosphate buffer of basic pH were found suitable for separation of vasopressin-related peptides.

PAPER II

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Research Article

Study of interaction mechanisms on zirconia-based polystyrene HPLC column

Separation systems with a zirconia-based polystyrene HPLC column were characterized by different approaches, which allowed the recognition of interactions participating in the separation environments. Zirconia-based HPLC columns as an alternative to silica-based ones offer unique interaction mechanism based on Lewis acid–base theory. Besides hydrophobic interactions with the modified surface of the zirconia carrier it includes ion-exchange and ligand-exchange interactions that are helpful in the separation of many bioactive compounds. Three distinct approaches were applied for description of the complex separation mechanism. General chromatographic tests by Walters, Engelhardt and Galushko were applied to evaluate the fundamental properties of the systems – hydrophobicity and polarity. The complex model of linear free energy relationship described the interactions from the qualitative and quantitative points of view more in detail. Application of a set of basic compounds revealed the contribution of ion-exchange interactions participating in the separation systems.

Keywords: Basic compounds / HPLC / Linear free energy relationship / Zirconia-based columns
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1 Introduction

RP HPLC has become a powerful analytical technique for the separation and analysis of a variety of compounds possessing different properties. This separation mode plays a dominant role in all the HPLC applications [1, 2] because of performing selective and efficient separations of high resolution. Silica gel is the most common carrier of the stationary phases used in RP HPLC because of its unique properties, *i.e.* excellent mechanical stability and large surface, which can be easily modified by bonding various stationary phases [3]. The major disadvantage of silica gel is its low chemical and thermal stability [4]. Restriction in pH range of the aqueous part of the mobile phase is a limiting factor especially in the separations of the basic compounds [5]. Increased separation temperature along with low pH of buffers as mobile phase constituents lead to gradual loss of the bonded phase. Moreover, residual silanol group activity affects negatively the retention and peak symmetry of the analytes [4], particularly basic compounds.

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Abbreviations: **LFER,** linear free energy relationship; **MeOH,** methanol; **PS,** polystyrene

The limits of silica gel led to increased interest in metal oxides as the supports for RP HPLC packings [6–8]. Zirconium dioxide has shown very promising quality [7–10]. It is chemically stable over the whole pH range (1–14) and can be employed at high temperatures (up to 200°C) [7]. Zirconia offers surface chemistry different from silica gel, based on Lewis theory of acids and bases [7]. Hard Lewis acid sites (free *d*-orbitals of Zr atom) on the surface of the carrier have a strong affinity for Lewis bases, components of the mobile phase (*e.g.* phosphate, hydroxylic, carboxylic and fluoride anions), which adsorb on the surface by specific ligand-exchange interactions and contribute to the retention of the analytes by the ion-exchange interactions. Zirconia can be coated with a polymeric stationary phase (polystyrene (PS), polybutadiene) [11] and offers different selectivity and retention mechanism from silica-based columns. Successful separations have been achieved on polymeric zirconia-based columns [10, 12, 13].

The chemistry of the stationary phase, the character of its carrier, the mobile phase composition and the temperature affect the complex separation mechanism, where many types of interaction forces among the analyte, the stationary phase and the mobile phase participate [6, 14, 15]. Deeper understanding of the complex separation process on zirconia-based columns and description of the interactions participating in the separation system are important and can be helpful in the development of new analytical methods. Many approaches for the characterization of separation systems can be applied. General characterization of the hydrophobicity and silanol activity of the RP columns is frequently performed by the widely known and referred

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testing procedures designed by Walters [16], Engelhardt and Jungheim [17], Tanaka [18] and Galushko [19]. These tests are mostly employed for comparative studies of the basic properties of the RP packings [15, 20]. Considering zirconia-based columns, the parameters of the silanol activity cannot obviously provide the information about the silanophilic interactions but most likely refer to other polar interactions on the surface of the zirconia carrier.

A comprehensive method for characterization and comparison of separation systems is the model of linear free energy relationship (LFER) [21], which can independently describe the contributions of individual interactions to the retention process. The LFER equation expresses the relationship between the retention characteristic (*i.e.* retention factor) determined for a representative set of analytes in a given separation system and the solute fundamental properties [22]:

$$\log k = c + vV_X + a\sum \alpha_2^H + b\sum \beta_2^H + s\pi_2^H + rR_2 \quad (1)$$

The independent variables in Eq. (1) are solute descriptors, where V_X is the McGowan characteristic volume [23], $\sum \alpha_2^H$ is the effective or overall hydrogen bond acidity (*i.e.* hydrogen bond donor ability) [24], $\sum \beta_2^H$ is the effective or overall hydrogen bond basicity (*i.e.* hydrogen bond acceptor ability) [24], π_2^H is the dipolarity/polarizibility (a measure of dipole–dipole interaction possibilities of the solute) [24] and R_2 is the excess molar refraction. The selection of a representative set of analytes is essential for the system evaluation. The solutes should be structurally diverse and the distribution of the solutes descriptors should equally cover a wide range of interactions [25]. Multivariate regression analysis is applied for the determination of the coefficients in Eq. (1) that reflect the different types of molecular interactions in the studied system. In HPLC, the regression coefficients relate to the differences in the properties of the stationary and mobile phases. The coefficient v represents the difference in hydrophobicity between the two phases; a reflects the difference in hydrogen bond basicity; b refers to the difference in hydrogen bond acidity; s is equal to the difference in dipolarity/polarizibility; and r reflects the difference in disposition of the stationary and mobile phases to interact with n- and π-electron pairs of the solutes. The c intercept in the LFER equation is characteristic of the given system but it does not reflect any interaction. This coefficient involves various parameters affecting retention that are not expressed by regression coefficients [26].

LFER seems to be a powerful tool for characterization of the separation systems with zirconia-based columns [15, 27, 28]. If the organic modifier content is kept constant in the mobile phase the LFER model can reveal the effect of the nature of the aqueous component, which plays a significant role in the retention process on zirconia-based columns, on the individual interactions participating in the complex retention process.

The ion-exchange interactions of zirconia support can be useful in the separations of ionizable compounds such as molecules with a basic nitrogen atom that often cause difficulties when analyzed on silica-based columns because of the interactions with residual silanol groups [5]. There are many procedures for description of the ion-exchange interactions on silica-based columns [29, 30]. Application of a set of basic compounds covering a wide range of hydrophobicity and pK_a constants is one of them. It can be used to reveal the non-hydrophobic interactions involved in the retention mechanism [30]. This approach could be applicable also on the zirconia-based columns.

The aim of this study was to characterize the separation systems with PS modified zirconia-based HPLC column using general chromatographic tests, the LFER model and a set of basic compounds. Combination of these approaches resulted in a complex description of the interaction possibilities of the zirconia-based PS column.

2 Materials and methods

2.1 Instrumentation

All chromatographic measurements were carried out by a Waters HPLC Breeze System (Milford, USA) consisting of an HPLC gradient pump 1525, an autosampler 717Plus, a column oven Jetstream 2 Plus and a UV–Vis dual absorbance detector 2487. For process control and data evaluation, Breeze software, version 3.30 SPA was used. Chromatographic column Discovery Zr-PS (column size 250 mm × 4.6 mm, particle size 5 μm, pore size 300 Å) with ZrO_2 support and PS as a stationary phase, manufactured by Supelco (Bellefonte, USA), was used for all measurements. The dead time was determined using the system peak. For all the analyses the sample volume of 5 μL was injected.

2.2 Chemicals and chromatographic conditions

Organic solvents (ACN and MeOH)) of HPLC grade were from Sigma-Aldrich (Steinheim, Germany). Sodium phosphate monohydrate (p.a.), sodium hydroxide (p.a.) and ammonia (aqueous solution, 25%) were obtained from Lach-ner (Neratovice, Czech Republic). Deionized water was prepared using a Milli-Q water purification equipment (Millipore, Milford, USA). The solutes for all the tests applied were of analytical grade purity and were purchased from Sigma-Aldrich (St. Louis, USA). 36 solutes for LFER study were chosen to cover a wide range of chemical properties. List of 36 solutes and their corresponding descriptors pertaining to Eq. (1) are shown in Table 2. A set of 15 basic compounds differing in pK_a constants and hydrophobicity was selected on the basis of the paper published by Sýkora *et al.* [30]. They are listed in Table 4.

The concentrations of stock solutions of solid and liquid samples were 1 mg/mL and 20 μL/mL, respectively. The analytes for the Walters, Engelhardt and Galushko tests

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were dissolved in the corresponding mobile phases. Pure ACN was used as a solvent of the analytes for the LFER study and in the experiments with basic compounds. All solutions were consequently diluted to obtain roughly equivalent detection signals of all the test compounds. The retention times of all the test compounds were measured in triplicate in all used mobile phases described below.

For the Walters, Engelhardt and Galushko tests experimental conditions were kept according to Refs. [16, 17, 19]. A flow rate of 1 mL/min was used, column temperature was maintained at 40°C and the detection wavelength was set to 254 nm. For the Walters test pure ACN and the mixture of ACN/deionized water, 65:35 v/v were used as the mobile phases. The Engelhardt and Galushko tests were performed in MeOH/deionized water, 55:45 v/v and MeOH/deionized water, 60:40 v/v, respectively.

Study of the LFER models and the experiments with the basic compounds were performed under these conditions: flow rate 1 mL/min, column temperature 25°C, detection wavelength 254 nm. Three mobile phases used in these studies were of the following compositions: ACN/deionized water (pH = 6.0), 20:80 v/v; ACN/sodium phosphate buffer (50 mM, pH = 10.0), 20:80 v/v; ACN/ammonia solution (50 mM, pH = 11.0), 20:80 v/v.

2.3 LFER procedure

The regression coefficients of the LFER equation were obtained from a series of measurements of the retention factors of the set of 36 solutes with known solvation parameters that are summarized in Table 2. The retention times of the analytes were measured in triplicate in three different mobile phases. The resulting coefficients were calculated for each separation system by multiple linear regression analysis of log k against the solute descriptors using the NCSS software (Kaysville, USA). The results were determined for both the complete model utilizing all regression coefficients and the optimal model employing just the statistically significant regression parameter values. The p-values in Table 3 express probability of the error that the individual coefficient does not contribute to the model. If p-values of the coefficients in the complete model are higher than 0.05 then the coefficients are not involved in the calculation of the optimal model that employs only statistically significant regression parameter values.

3 Results and discussion

Distinct testing procedures were chosen to characterize the separation systems with Zr-PS column. Chromatographic tests by Walters, Engelhardt and Galushko brought the information about hydrophobicity and polarity of the Zr-PS column. Through LFER study it was possible to gain insight into the molecular mechanism of the separation in given chromatographic systems and reveal the individual interactions participating in the retention process. Application of a set of basic compounds indicated the ion-exchange interactions contributing to the retention.

3.1 Simple chromatographic tests

Characterization of separation systems can be performed by many different methods. Simple chromatographic tests by Walters, Engelhardt and Galushko operating with a few test compounds available in most laboratories are often employed for characterization of column hydrophobicity, shape selectivity and silanol activity. These tests are based on the relative retention of selected pairs of aromatic compounds (differing in hydrophobicity or acidity/basicity) using recommended aqueous-organic mobile phases. On silica-based columns free silanol groups on the surface cause undesirable interactions and thus the parameter testing the silanol activity should be as low as possible. When considering the "silanol activity" of metal oxide-based columns, the results cannot show the silanophilic interactions but most likely refer to the polar interactions on the surface of the carrier.

The hydrophobicity (HI) values represent selectivities for specific molecular increments. In the Walters and Engelhardt tests column hydrophobicity is calculated from the separation factor, α, of anthracene/benzene (in 65% ACN) and ethylbenzene/toluene (in 55% MeOH), respectively [16, 17]. Hydrophobicity defined by Galushko is obtained as a half of the sum of retention factors of toluene and benzene using 60% MeOH as a mobile phase [19].

Silanol activity (SI) parameters can be evaluated by a selectivity factor of two compounds of different acid/base characters. The Walters test defines this parameter as a separation factor of *N,N*-diethyl-*m*-toluamid/anthracene in pure ACN [16]. The Engelhardt procedure uses aniline to phenol retention factors ratio in 55% MeOH [17]. The silanol activity by Galushko is defined in 60% MeOH as $SI = 1+3\ [(k_{aniline}/k_{phenol})-1]$ [19].

The results of these tests applied to Discovery Zr-PS column are given in Table 1. The values of Walters and Engelhardt hydrophobicity parameters are comparable with common published data for silica-based columns [15, 20]. The hydrophobicity parameter by Galushko shows lower retention of the test compounds, *i.e.* lower methylene selectivity than common silica-based reversed columns possess [15, 20].

Some differences of the polarity parameters of the Zr-PS column in comparison with the silica-based columns were obtained. The Walters test showed strong retention of

Table 1. Results of the Walters, Engelhardt and Galushko chromatographic tests

	Walters	Engelhardt	Galushko
HI (hydrophobicity)	3.99	1.55	0.38
SI (polarity)	2.93	0.72	0.17

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Table 2. Test solutes for LFER model, corresponding solvation parameters and retention factors (k) in the three different mobile phases

Solute	V_X	$\sum \alpha_2^H$	$\sum \beta_2^H$	π_2^H	R_2	k		
						Water[a)]	Phosphate[b)]	Ammonia[c)]
Phenol	0.78	0.60	0.30	0.89	0.81	0.38	0.24	0.98
Benzamide	0.97	0.49	0.67	1.50	0.99	0.18	0.11	1.52
2-Naphthol	1.14	0.61	0.40	1.08	1.52	4.30	1.56	2.10
Resorcinol	0.83	1.10	0.58	1.00	0.98	0.58	0.02	0.70
Benzophenone	1.48	0.00	0.50	1.50	1.45	10.21	6.93	22.44
Hydroquinone	0.83	1.16	0.60	1.00	1.00	0.17	0.07	1.70
1,2-Cresol	0.92	0.52	0.31	0.86	0.84	0.88	0.54	1.59
Benzonitrile	0.87	0.00	0.33	1.11	0.74	0.87	0.40	2.98
1,3-Cresol	0.92	0.57	0.34	0.88	0.82	0.88	0.43	1.30
Benzylalcohol	0.92	0.33	0.56	0.87	0.80	0.26	×	1.73
Benzene	0.72	0.00	0.14	0.52	0.61	1.35	0.99	3.99
Naphthalene	1.09	0.00	0.20	0.92	1.34	12.86	5.78	20.78
Pyrocatechol	0.83	0.85	0.52	1.07	0.97	2.07	×	1.68
Dibenzothiophene	1.38	0.00	0.18	1.31	1.96	×	25.44	×
Nitrobenzene	0.89	0.00	0.28	1.11	0.87	1.53	1.12	4.39
Ethylbenzene	1.00	0.00	0.15	0.51	0.61	5.71	0.54	13.14
Benzaldehyde	0.87	0.00	0.39	1.00	0.82	0.68	0.48	2.65
Toluene	0.86	0.00	0.14	0.52	0.60	2.86	2.02	7.19
1,2-Toluidine	0.96	0.23	0.45	0.92	0.97	4.54	×	9.94
Biphenyl	1.32	0.00	0.22	0.99	1.36	34.34	8.13	67.78
Phenanthrene	1.45	0.00	0.26	1.29	2.06	×	41.46	×
1,2,3-Trichlorobenzene	1.08	0.00	0.00	0.86	1.03	×	9.52	×
1,2-Dichlorobenzene	0.96	0.00	0.04	0.78	0.87	9.01	6.50	20.65
3-Nitrotoluene	1.03	0.00	0.25	1.10	0.87	3.31	1.10	8.07
1,2-Xylene	1.00	0.00	0.16	0.56	0.66	5.41	3.81	12.82
Bromobenzene	0.89	0.00	0.09	0.73	0.88	5.39	3.99	12.98
2-Nitrotoluene	1.03	0.00	0.27	1.11	0.87	3.08	2.09	7.81
1,3-Xylene	1.00	0.00	0.16	0.52	0.62	5.96	4.11	13.86
Chlorobenzene	0.84	0.00	0.07	0.65	0.72	3.99	2.78	9.26
1,4-Xylene	1.00	0.00	0.16	0.52	0.61	5.86	4.04	12.99
2-Chlorophenol	0.90	0.32	0.31	0.88	0.85	1.89	0.72	0.42
3-Chlorophenol	0.90	0.69	0.15	1.06	0.91	2.47	1.18	0.63
4-Chlorophenol	0.90	0.67	0.21	1.08	0.92	2.35	0.85	0.93
2-Nitrophenol	0.95	0.05	0.37	1.05	1.02	3.13	0.88	0.35
4-Nitrophenol	0.95	0.82	0.26	1.72	1.07	3.40	0.63	0.35
3-Hydroxybenzaldehyde	0.93	0.74	0.40	1.38	0.99	0.80	0.34	0.48

The descriptors were obtained from the literature [22, 25]. McGowan characteristic volume was calculated from the atom and bond contributions according to [23]. × means no elution within 3 h.
a) Mobile phase ACN/H_2O, 20:80 v/v.
b) Mobile phase ACN/phosphate buffer (50 mM, pH = 10.0), 20:80 v/v.
c) Mobile phase ACN/ammonia solution (50 mM, pH = 11.0), 20:80 v/v.

N,N-diethyl-*m*-toluamid, which confirms the interactions of active Zr support with free electrons of the test compound. The other two tests yielded an interesting fact that aniline eluted before phenol. This retention behavior is specific for deactivated silica-based columns where silanophilic interactions are reduced [5]. Considering this fact on zirconia-based columns, the above described retention behavior can be explained by a larger number of delocalized electrons of phenol than aniline possesses. The electrons can interact with the free *d*-orbitals of Zr atoms on the surface of the carrier and thus the retention of phenol is higher than that of aniline.

Applied chromatographic tests, easy to perform, can roughly tell the chromatographer about the basic properties of the stationary phase. There are some deviations of the chromatographic behavior of the testing solutes on the Zr-PS column, which show the different interaction possibilities of zirconia-based columns.

3.2 LFER

The complex model of LFER offers the possibility to characterize the separation system by the description of

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individual molecular interactions participating in the separation process. LFER is often employed to characterize, compare and predict separation possibilities of various separation systems. Some papers applied the LFER model to the separation systems with zirconia-based columns [15, 27, 28]. We used the LFER model to describe and compare three separation systems consisting of the Zr-PS column and differing in mobile phase compositions. The choice of the chromatographic conditions was based on our previous study of chromatographic behavior of biologically active peptides [13, 31]. The goal was to describe the retention mechanism and to show the differences between individual interaction types using different aqueous components of the mobile phase. As mentioned above, the constituents of the mobile phase play a significant role in the specific ligand-exchange interactions with ZrO_2 support and can largely affect the retention of the ionizable compounds. The employed mobile phases had the same ratio of the organic modifier (ACN) and aqueous component, *i.e.* 20:80 v/v. Deionized water (pH = 6.0), phosphate buffer (50 mM, pH = 10.0) and ammonia solution (50 mM, pH = 11.0) were chosen as aqueous constituents of the mobile phases applied for the LFER study. The test solutes, their solvation parameters and retention factors are summarized in Table 2.

The differences of interactions of analytes between the stationary and the mobile phases are described by the regression coefficients in Eq. (1). The regression coefficients obtained from the complete and optimal models of LFER or the three separation systems (as well as the standardized coefficients of the optimal model) are summarized in Table 3. Plots of the experimental log k against calculated/predicted log k values for the first two systems (using water or phosphate buffer in the mobile phase) show no serious outliers and the correlation coefficients of linear regression fits were 0.94 and 0.97, respectively. This result indicates the strong correlation of the LFER model with the experimental data. Poorer correlation between the experimental and calculated results was found for the third separation system containing ammonia in the mobile phase, where the correlation coefficient of the linear regression fit was 0.84. It could be due to the fact that ammonia acts as a very strong eluting agent for the ZrO_2-based columns and so all other interactions on the stationary phase surface are suppressed (become less significant) and thus conditions under which the LFER is proposed are not fulfilled. The *p*-values in Table 3 express significance of the individual coefficients. The *p*-values are lower for the optimal model than for the complete one, *i.e.* the regression coefficients of the optimal model are more significant. Therefore, the optimal model was chosen for further evaluation. For the comparison of the types of interactions within one separation system the standardized coefficients should be used.

Table 3. Regression coefficients of the LFER equation in the separation systems with the different mobile phases – ACN/aqueous component, 20:80 v/v

Aqueous component of the mobile phase	Model	*v*	*a*	*b*	*s*	*r*	*c*	*R*
Water	CM	1.88	−0.14	−2.26	−0.24	0.65	−1.11	0.94
pH = 6.0	±95% CI	0.42	0.14	0.29	0.17	0.28	0.28	
	P	0.000	0.349	0.000	0.163	0.026	0.001	
	OM	2.16	×	−2.54	×	0.36	−1.30	0.94
	±95% CI	0.35	×	0.25	×	0.23	0.25	
	P	0.000	×	0.000	×	0.128	0.000	
	STD	0.58	×	−0.76	×	0.16	0.00	
Phosphate buffer	CM	1.61	−0.63	−2.15	−0.18	0.50	−1.07	0.97
50 mM, pH = 10.0	±95% CI	0.45	0.23	0.54	0.32	0.23	0.32	
	P	0.002	0.011	0.001	0.570	0.045	0.003	
	OM	1.61	−0.57	−2.39	×	0.43	−1.12	0.97
	±95% CI	0.45	0.20	0.35	×	0.20	0.31	
	P	0.001	0.009	0.000	×	0.040	0.001	
	STD	0.42	−0.24	−0.51	×	0.23	0.00	
Ammonia solution	CM	2.01	−0.62	−0.10	−0.92	0.22	−0.49	0.84
50 Mm, pH = 11.0	±95% CI	0.75	0.26	0.52	0.31	0.50	0.50	
	P	0.013	0.025	0.853	0.006	0.663	0.333	
	OM	2.24	−0.59	×	−0.89	×	−0.58	0.84
	±95% CI	0.51	0.21	×	0.26	×	0.44	
	P	0.000	0.009	×	0.002	×	0.205	
	STD	0.53	−0.36	×	−0.43	×	0.00	

CM is the complete model of the LFER equation involving all the regression coefficients; OM means the optimal model of the LFER equation utilizing just the statistically significant regression coefficients; *R* is the correlation coefficient; ±95% CI represents ±95% confidence interval; × means insignificant interaction; *p* is the statistical *p*-value; STD refers to the standardized coefficients of the optimal model.

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Figure 1 shows the comparison of the regression coefficients of the optimal LFER models for the three separation systems. A positive coefficient value shows that the given molecular interaction is stronger in the stationary phase and thus it increases retention of analytes. A negative value of the coefficient reflects stronger interaction with the mobile phase.

The dominant contribution to the retention increase in all the three studied systems is hydrophobicity. The positive values of the *v* coefficients of the individual systems indicate that this interaction is preferred in the stationary phase. The values are comparable, which shows little effect of the type of aqueous mobile phase component on the hydrophobic interactions. Nevertheless, the presence of phosphate buffer in the mobile phase seems to slightly reduce these interactions in the stationary phase.

The coefficients *a* and *b*, describing the ability of the system to interact through hydrogen bond interactions, are all negative, so they are preferred in the mobile phase but do not contribute to the retention in all the studied systems. The acidity of the investigated mobile phases is characterized by the solvation parameters of its components $\left(\sum \alpha_2^{\mathrm{H}}(\text{water}) = 1.17,\ \sum \alpha_2^{\mathrm{H}}(\text{ACN}) = 0.19\right)$, and it is affected also by other mobile phase components. The aqueous part of the mobile phase has strong hydrogen bond (donor) acidity [32] and as it prevails in the mobile phase it largely contributes to this type of interaction. This fact is obvious from the values of the coefficient *b* in the systems with water and phosphate buffer. The addition of the basic ammonia changes the situation substantially because ammonia interacts strongly with the stationary phase. Therefore, the acidity of the stationary phase is low and similar to that of the mobile phase for the third system. So the difference in this type of interaction between the stationary and mobile phases is insignificant and the coefficient *b* is not involved in the model.

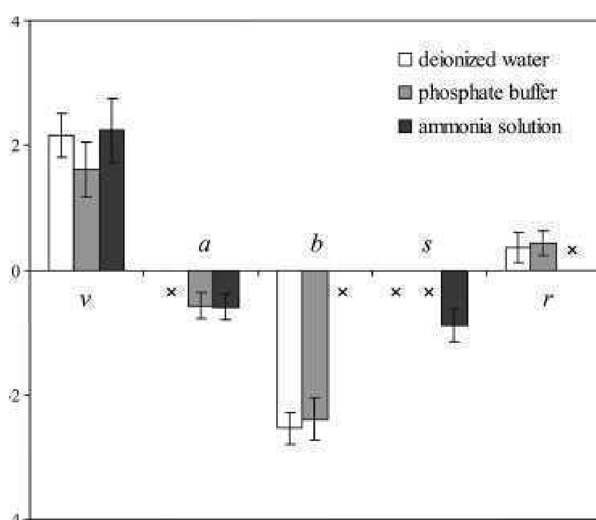

Figure 1. Comparison of the regression coefficients (with their standard errors) of the optimal LFER models for the three separation systems. Symbol × means insignificant interaction.

The coefficients *a*, reflecting the difference in the hydrogen bond (acceptor) basicity between the stationary and mobile phases, are significant in the systems with phosphate buffer and ammonia solution in the mobile phases. Their values are negative, *i.e.* this type of interaction is stronger in the mobile phase. Evaluating the system with water, the basicity of the stationary and mobile phases is comparable, so it does not contribute considerably to the retention process.

Comparison of the contributions of hydrogen bond acidity (*b*) and hydrogen bond basicity (*a*) to the retention mechanism clearly shows that the former reduces interactions with the stationary phase more significantly as the absolute values of *b* are higher than the values of *a* for the separation systems with water or phosphate buffer in the mobile phases.

Less significant contribution to the retention results from the ability to interact with *n*- and π-electron pairs of the solute, expressed by the coefficient *r*. This coefficient is not statistically significant in the LFER model for the system with ammonia. The positive values of the coefficient *r* for the two other systems show that the electron-involved interactions are preferred in the stationary phase. The aromatic rings of the PS stationary phase are capable of this type of interactions and can contribute to the retention process. Considering the system with ammonia, which strongly interacts with the zirconia-based stationary phase, the sorbent seems to be hardly accessible for the electron-involved interactions. Then the interactions with *n*- and π-electron pairs are comparable in the mobile and stationary phases, and the difference in this type of interaction between the stationary and mobile phases is not statistically significant.

The difference between the stationary and mobile phase dipolarity/polarizability is compared through the *s* coefficient. This coefficient is significant only for the mobile phase with ammonia. Whereas these interactions are similar in the stationary and mobile phases with phosphate or just with water they predominate in the aqueous solution of ammonia.

The described results confirm that the individual interactions participating in the retention are connected with the nature of both the mobile and the stationary phases. Some published data [15, 28] comparable with our results show similar trends that strongly depend on the experimental conditions.

3.3 Interactions of basic compounds

The columns based on ZrO_2 are interesting for the unique interaction mechanism, in which ion-exchange interactions of the support can largely and positively affect the retention of ionizable compounds. Considering silica-based columns, the undesirable ion-exchange interactions with residual

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Table 4. Properties of the studied basic compounds, their retention factors *k* and symmetry factors *S* in the three different mobile phases

Compound name	pK_a [a)]	log *P* [b)]	pH = 6.0 [c)]		pH = 10.0 [d)]		pH = 11.0 [e)]	
			k	*S*	*k*	*S*	*k*	*S*
2-Aminopyridine[f)]	6.84	0.50	0.77	1.45	0.10	1.44	0.13	1.28
3-Aminopyridine[f)]	5.25	0.20	0.20	1.27	0.05	1.46	0.08	1.32
4-Aminopyridine[f)]	8.61	0.26	2.55	0.82	0.10	1.56	0.40	1.35
2-Amino-4-picoline[f)]	7.67	1.02	2.08	1.02	0.20	1.45	0.23	1.29
2-Picoline	6.02	1.17	0.22	1.34	0.18	1.50	0.20	1.35
3-Picoline	5.60	1.17	0.28	1.40	0.22	1.50	0.25	1.36
2,6-Lutidine	6.78	1.69	0.41	0.94	0.29	1.54	0.30	1.37
2,4,6-Collidine	7.61	2.21	1.29	0.68	0.57	1.49	0.60	1.32
N-Ethylaniline	5.50	2.13	1.59	1.38	1.47	1.52	1.56	1.36
N,*N*-Dimethylaniline	5.06	2.18	2.33	1.42	2.12	1.54	2.25	1.37
N-Benzylmethylamine	9.52	1.24	0.72	1.27	0.68	1.45	×	×
2-Phenylethylamine	9.81	1.45	1.14	0.80	2.05	1.78	×	×
Quinoline	4.64	2.05	0.99	1.45	0.87	1.54	0.94	1.36
2,6-Dimethylquinoline	6.22	3.09	2.62	1.32	2.39	1.54	2.57	1.36
Aniline	4.58	0.99	0.35	1.45	0.31	1.55	0.33	1.31

a) pK_a constants of protonated forms in aqueous solution at 25°C, computed with software package PALLAS for prediction of pK_a constants [33].
b) Partition coefficients in *n*-octanol–water computed with software package PALLAS for prediction of log *P* values; CDR fragment database was used in this case [33].
c) Mobile phase ACN/deionized water, 20:80 v/v; pH corresponds to the aqueous component.
d) Mobile phase ACN/phosphate buffer (50 mM, pH = 10.0), 20:80 v/v; pH corresponds to the aqueous component.
e) Mobile phase ACN/ammonia solution (50 mM, pH = 11.0), 20:80 v/v; pH corresponds to the aqueous component.
f) The lower dissociation constants of the protonated amino groups of these analytes are not considered in context of this study because they are always deprotonated ($–NH_2$) in the pH range tested. × means no elution within 2 h.

silanol groups mostly cause tailing of the peaks and irreproducible retention of basic compounds. Therefore, some approaches have been described to reveal and quantify the contributions of these interactions [20, 29, 30]. Some of these methods can be applied also for the characterization of the ion-exchange interactions on ZrO_2 columns. An interesting procedure employing a set of basic compounds [30] was chosen for our purpose. This method was originally designed to reveal non-hydrophobic interaction involved in the interaction mechanism on silica-based RPs.

A set of 15 basic compounds that belong to the group of substituted pyridines (with some exceptions) and differ in hydrophobicity (log *P*) and dissociation constants, expressed as pK_a, was employed. Their properties as well as the obtained retention factors (*k*) are summarized in Table 4. It can be clearly seen that the chosen basic solutes cover a wide range of hydrophobicity and pK_a constants. They can be sorted into smaller groups with similar hydrophobicity and different pK_a constants and *vice versa* that enables the evaluation of the individual interactions contributing to the retention.

One of the possibilities is to compare the retention behavior of the analytes possessing roughly the same pK_a constants and differing in their hydrophobicity to evaluate the hydrophobic interactions. For example, 3-picoline and *N*-ethylaniline with similar pK_a constants and different log *P* values do not perform any outstanding behavior. They are both dissociated in all the mobile phases tested, so the contribution of the ion-exchange interactions to retention is the same and the main force affecting the retention is hydrophobicity. As a result, 3-picoline performs lower retention because of its lower hydrophobicity than *N*-ethylaniline, and this difference of their retention is almost the same in all the mobile phases. The similar behavior can be seen for quinoline and aniline. Considering the analytes that have almost the same pK_a constants but their values are higher than the lowest investigated pH value of the aqueous part of the mobile phase, the retention behavior is distinct from that described above. This fact is obvious from Fig. 2 illustrating the retention behavior of 2-amino-4-picoline and 2,4,6-collidine. In the first investigated mobile phase (pH 6.0), the hydrophobic and ion-exchange interactions participate in the retention process of these analytes. This correlates with their much higher retention factors if compared with the values obtained in the other mobile phases (pH of their aqueous components is 10.0 and 11.0). The highest retention of 2-amino-4-picoline can be explained by another amino group present in its molecule, which has low pK_a value ($pK_a = 2.17$). In the mobile phase of pH 6.0 this amino group is fully dissociated and can interact by its free electron pair and participate in the electron-involved interactions. The contribution of this type of interactions to the retention is also obvious from the results obtained by the LFER model (see the coefficient *r*). In the two other investigated systems (at higher pH) the ion-exchange interactions decrease and the hydrophobic interactions become the main force

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affecting the retention and reflecting the difference in the hydrophobicity of these two analytes (see Fig. 2).

If we consider the compounds with comparable hydrophobicity (log *P*) and distinct dissociation constants, other interesting results confirming the presence of ion-exchange interactions can be concluded. The retentions of two isomers, 3-aminopyridine and 4-aminopyridine, differing in pK_a constants do not vary substantially in the second (at pH 10.0) and the third (at pH 11.0) separation systems because

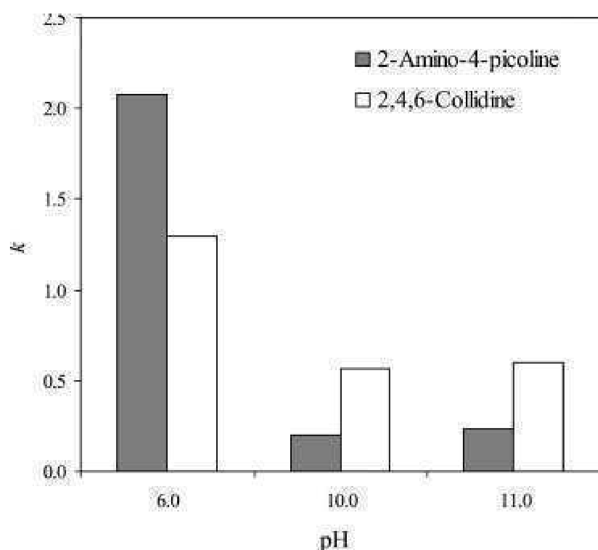

Figure 2. The dependence of retention factors of selected analytes with similar pK_a constants and different hydrophobicity. Discovery Zr-PS column; mobile phases composed of ACN/aqueous part 20:80 v/v, the pH values were measured before the addition of ACN, the values on the *x*-axis correspond to pH of deionized water, phosphate buffer and ammonia solution. For details see Section 2.

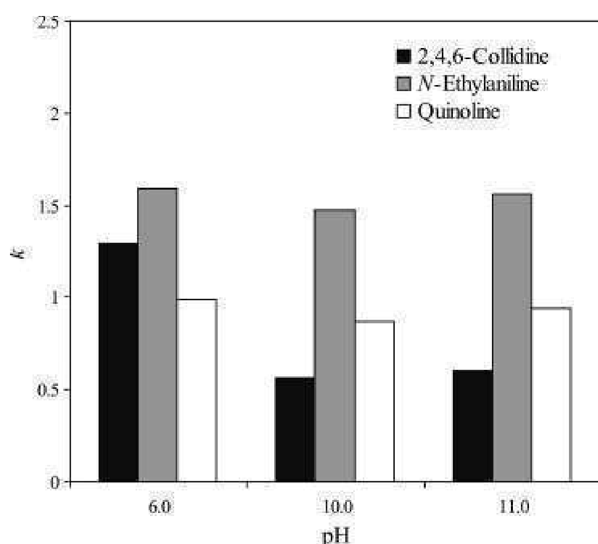

Figure 3. The dependence of the retention factors of selected analytes with similar hydrophobicity and different pK_a constants. For more information see caption of Fig. 2.

quite similar interactions participate there. As 3-aminopyridine is almost fully dissociated already at pH 6.0 its retention in this separation system is not much different from that in the previous ones. The ion-exchange interactions are rather limited. On the contrary, 4-aminopyridine with much higher pK_a value is strongly retained in this system of lower pH because the protonized analyte exhibits significant ion-exchange interactions. The same retention behavior can be found for 2-amino-4-picoline compared to 2-picoline or 3-picoline. Comparison of the retention behavior of 2,4,6-collidine, *N*-ethylaniline and quinoline in the three studied systems is shown in Fig. 3. *N*-Ethylaniline and quinoline have lower pK_a than the lowest investigated pH value of the aqueous component of the mobile phases, thus the ion-exchange interactions are constant in all the three separation systems and their retention is not affected by the pH. As these ion-exchange interactions are reduced, the retention of these compounds is mainly caused by hydrophobic interactions. 2,4,6-Collidine with higher pK_a value performs higher retention in the first system (pH 6.0) because the molecules are not fully dissociated and they can participate also in the ion-exchange interactions.

The results of these experiments with basic compounds performed on Discovery Zr-PS column confirmed some facts revealed by similar experiments performed with silica-based stationary phases [30]. Great indicators of the ion-exchange sites on the sorbent surface are 2-amino-4-pyridine, 4-aminopyridine and 2,4,6-collidine as can be seen from the results. Some deviations of the experimental data from the expected ones can be compared with the different aqueous component of each investigated mobile phase, and thus different solvation of both the analytes and the stationary phase. Other types of interaction can also be the factors causing some deviations in the chromatographic behavior of the basic compounds.

4 Concluding remarks

The separation systems with zirconia-based PS stationary phase offer various types of interactions that contribute to the complex retention mechanism. General characteristics of the Zr-PS stationary phase were evaluated. The effect of mobile phase composition was also considered. Simple tests by Walters, Engelhardt and Galushko revealed that the hydrophobicity of the PS modified zirconia-based stationary phase is comparable with the common silica-based RP columns; despite the different interaction mechanism on the zirconia support evidence of polar interactions was also given. The application of the LFER model to the separation systems differing in the aqueous component of the mobile phase resulted in the evaluation of the individual interaction types. The forces preferred in the stationary phase are hydrophobic interactions and electron-involved interactions, confirming the potency of PS stationary phase to interact through these types of interactions. The significant interactions prevalent in the mobile phases are the hydrogen bond interactions; their

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extent differs with the mobile phase composition, deionized water, phosphate buffer and ammonia solution used. Unfortunately, the ion-exchange interactions offered greatly by zirconia-based columns are not involved in the model used. Nevertheless, the application of the basic compounds revealed this type of interactions acting in the investigated systems and supplied the information about the complex separation mechanism on zirconia-based PS column. The results showed that the various tests used for the characterization of silica-based RP chromatographic columns can be successfully used also for zirconia-based stationary phases.

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4. CHIRAL STATIONARY PHASES BASED ON DERIVATIZED CYCLOFRUCTANS

4.1. Chiral stationary phases

Chirality, chiral recognition and enantiomeric differentiation are basic phenomena in nature and in chemical systems. These features greatly affect various chemical fields dealing with bioactive molecules such as pharmaceutical, agrochemical or food science [1]. Interest in enantioselective separation and development of chiral separation media has increased greatly in the past few decades due to the demand of the mentioned chemical fields. HPLC has become the most powerful method for separating racemic samples. This method can be used to separate enantiomers either indirectly using chiral derivatization before separation step or directly with chiral mobile phase additives or chiral stationary phases (CSPs) [2]. Indirect separation employs chiral derivatization reagents to form diastereoisomeric derivatives (pairs with enantiomers of analyte) that have different chemical and physical properties and therefore they can be separated on achiral stationary phases (in achiral environment). Chiral mobile phase additives are a simple and flexible alternative but they cannot always be applied. Due to high cunsumption of expensive chiral additives they are preferentially used in capillary systems. The most powerful direct approach to enantioselective separation is HPLC with chiral stationary phases that allows separation of racemic samples at analytical and preparative scales or determination of enantiomeric purity [3].

A variety of CSPs with complex interaction mechanisms have been developed for HPLC by many research groups, which dominate the works of Davankov, Pirkle, Okamoto, Blaschke, Allenmark, Hermansson, Armstrong, Gasparrini, and Lindner [3, 4, 5, 6, 7, 8]. Nowadays, chiral separation media can be easily classified according to their structure. The group of macromolecular selectors includes biopolymers (polysaccharide derivatives, proteins) and synthetic polymers (e.g. polyacrylamides). Macrocyclic selectors are cyclodextrins, macrocyclic antibiotics and chiral crown ethers. Low-molecular weight selectors are represented by Pirkle brush-type (π-donor and π-acceptor) selectors, chiral ion-exchange selectors and ligand exchange selectors

(chelating agents) [1]. Each of these selectors offers different enantiorecognition mechanism and thus different selectivity.

Polysaccharide-based CSPs are the mostly employed chiral separation media followed by the macrocyclic antibiotics and cyclodextrin CSPs [3]. Polysaccharide-based stationary phases show a very broad applicability to diverse compound classes. The development and applications of polysaccharide derivatives used as CSPs are reported in special reviews [8, 9, 10, 11]. The derivatives of cellulose, amylose and chitin as optically active natural polymers are the mostly employed polysaccharide-based chiral selectors and consequently CSPs [12]. They possess exceptional enantioselectivity due to many stereogenic centers and conformational chirality [1]. They are employed in analytical and preparative scale separations and offer the highest loading capacity.

Macrocyclic antibiotics, mainly teicoplanin, vancomycin and ristocetin A, belonging to the group of glycopeptides, are also commonly used for HPLC enantioseparations [13]. Macrocyclic antibiotics have many stereogenic centers and functional groups available for interactions with chiral analytes. The three selectors mentioned above perform a complementary selectivity [2]. Application of macrocyclic antiobiotics as chiral selectors both for HPLC and CZE can be found e.g. in the review by Ward and Fattis [14].

Cyclodextrins (CDs) are cyclic oligosaccharides consisted mostly of six to eight glucopyranose units that form a cone-shaped cavity [1]. Inclusion complexation into the chiral cavity is the main force of chiral recognition by CDs in reversed-phase separation systems [12]. Derivatization of native CDs enhances the enantioselectivity by introduction of new functionalities (functional groups) that are suitable for additional interactions with chiral analytes. CDs are employed both in the form of chiral mobile phase additives or chiral stationary phases in HPLC. They have been widely applied for the separation of enantiomeric drugs [15].

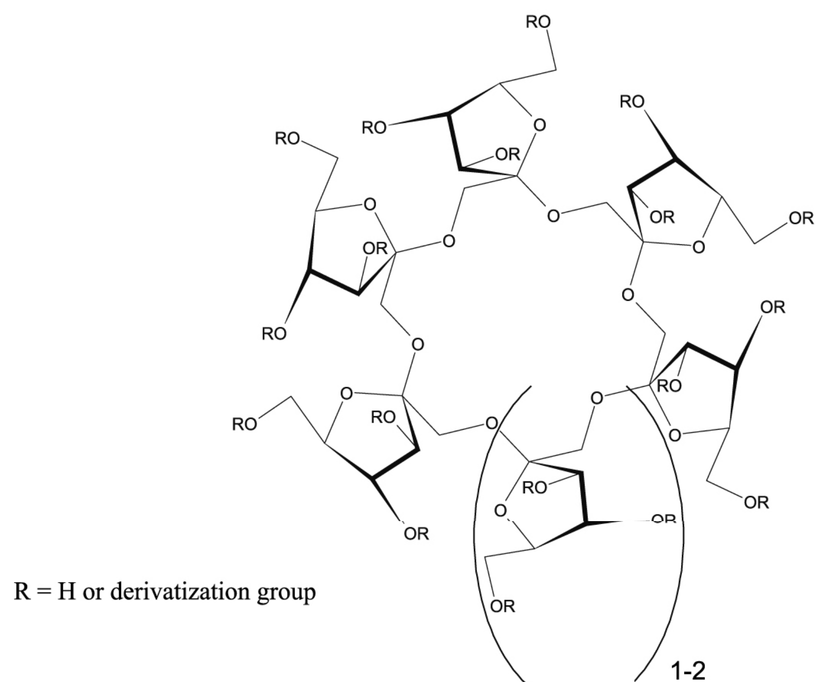
Protein-based chiral selectors are used both as mobile phase additives and CSPs. In biological systems these macromolecules are responsible for the chiral discrimination of nutrients and drugs [12]. They posses a number of chiral centers and binding sites. However, they are very sensitive to experimental conditions. Development of protein-based CSPs and their application are the subjects of the review by Haginaka [16].

Other types of chiral selectors are used in smaller extent and are often developed and applied for special applications. Ligand exchange selectors are discussed in detail in [17].Crown ether-based CSPs, their development and applications are reported in Ref. [18]. Pirkle-type (π-donor and π-acceptor) selectors are comprehensively reviewed in Ref. [19, 20]. A review on the synthesis and application of chiral synthetic polymers as CSPs was published by Nakano [21].

4.2. Cyclofructans and their properties

Despite the applicability and broad selectivity of existing CSPs briefly discussed in the previous chapter the research effort for the development of new and improved chiral selectors continues. In 2009 a novel class of CSPs based on cyclofructans was introduced by Armstrong [22]. This group of chiral selectors was shown to have potential both for HPLC [22, 23, 24, 25, 26] and CZE [27]. Cyclofructans (CFs) are macrocyclic oligosaccharides as cyclodextrins. However, cyclofructans are quite different in both their structure and behaviour. They consist of six or more β-(2→1) linked D-fructofuranose units [28, 29]. Their abbreviations CF6, CF7, CF8 etc. indicate the number of fructofuranose units in the macrocyclic ring. Each fructofuranose unit contains four stereogenic centers and three hydroxyl groups, which can be utilized for derivatization. CF6 is the most studied member of this unique group. It consists of an 18-crown-6 ether core, six fructofuranose units are arranged in spiral fashion. CF6 presents a clear "front/back" regionalization of hydrophilic and hydrophobic groups, i.e., three oxygen atoms and two CH_2 groups, respectively [30]. Consequently, one side of the CF molecule is hydrophilic and the other side is hydrophobic. Moreover, CF6–CF8 do not possess central hydrophobic cavities, as do cyclodextrins [31]. Stable intramolecular hydrogen bonds formed in native CF molecules block their core structure from possible interactions. If some of the hydrogen bonding groups are blocked or derivatized, the molecular structure „relaxes", the cavity becomes more open and that exposes the central crown ether core and/or other previously inaccessible interaction sites. Therefore, while native CFs have rather limited enantioselectivity in HPLC [22] their derivatized forms show improved and unique chiral recognition abilities for a wide range of analytes [22, 23, 25, 32]. Aliphatic- or aromatic-functionalization of a native

chiral selector is a common strategy used to develop new chiral stationary phases and improve enantioseparation performance. Fig. 4.1. shows the molecular structure of cyclofructans CF6 and CF7 and the derivatization groups studied in this work. Aliphatic-derivatized CF6s with a low substitution degree demonstrated exceptional capability for separating racemic primary amines [22, 23]. IP-CF6 CSP utilizing isopropyl-carbamoyl CF6 as the chiral selector was considered to be the most broadly applicable CSP for primary amino group-containing analytes [23]. Larger derivatization groups, e.g., aromatic moieties, can reduce or even block the access to the molecular core but provide other interaction sites about its periphery. Aromatic-functionalized CFs performed complementary selectivity to aliphatic-derivatized CFs in some cases. RN-CF6 CSP with *R*-naphthylethyl carbamate as a derivatization group provided good enantioselectivity toward a variety of chiral compounds that were not primary amines [22, 23]. DMP-CF7 was developed as a 3,5-dimethylphenylcarbamoyl cyclofructan containing seven fructofuranose units. It can provide unique and somewhat complementary enantioselectivity to RN-CF6. CF-based columns are compatible with all common organic solvents as the chiral selector is covalently bonded to the silica gel support. These CSPs can be operated in all common separation modes (normal, reversed phase and polar organic) but mostly higher selectivity was obtained in the normal phase mode [22].



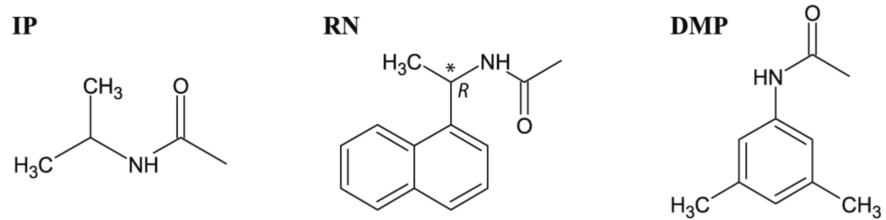


Fig. 4.1. Molecular structure of cyclofructan CF6 and CF7 and the derivatization groups.

4.3. Results and discussion – Enantioseparation potential of CF-based CSPs

The selectivity of cyclofructan-based CSPs is dependent on the nature and spatial arrangement of the cyclofructan molecule as well as on the type and degree of its substitution [22]. An important feature of CF6 and CF7 affecting the interaction possibilities offered by these chiral selectors is the size of the central crown ether core, which increases with increasing number of fructofuranose units [30]. The size of the derivatization group and the degree of the substitution also play an important role. These features, crucial in the enantiorecognition mechanism, are considered and discussed in this part of the thesis investigating enantioseparation capabilities of the CF-based CSPs.

The chiral recognition capabilities of three CF-based CSPs, i.e., isopropyl carbamoyl cyclofructan 6 (IP-CF6), *R*-naphthylethyl carbamoyl cyclofructan 6 (RN-CF6) and dimethylphenyl carbamoyl cyclofructan 7 (DMP-CF7) were evaluated by injection of racemic compounds either exhibiting axial chirality (binaphthyl catalysts) or containing stereogenic centers (certain chiral pharmaceuticals). It should be noted that the separation conditions were not optimized. The mobile phase compositions examined were *n*-hexane/propane-2-ol (hex/IPA) in the volume ratios 80/20 and 60/40, the addition of trifluoroacetic acid (TFA, at concentrations of 0.1 % and 0.5 %) was also examined. Overall, six different mobile phases were tested.

4.3.1. Chiral separations of binaphthyl derivatives (Paper III)

A set of binaphthyl derivatives (see Fig. 4.2.) was chosen in order to evaluate the chiral recognition capabilities of the CF-based CSPs. These compounds were synthesized as racemates at the Department of Organic and Nuclear Chemistry, Faculty of Science, Charles University in Prague and have been described in detail in Refs. [33, 34, 35]. Despite of the similar basic axial chirality of the binaphthyl derivatives the type and positions of the substituents substantially affect their properties and chromatographic behaviour. Enantiomeric separations of analogous compounds have been reported on CSPs based on cyclodextrin, polysaccharide and synthetic

polymers [33, 36, 37]. The binaphthyl structures appear to be well suited for interactions with the aromatic moieties of the CF-based CSPs (i.e., RN-CF6 and DMP-CF7 CSPs). Mobile phases composed of hex/IPA 60/40 (*v/v*) produced very little retention of the analytes on the IP-CF6 CSP. Table 4.1. summarizes chromatographic data for the binaphthyl derivatives obtained on the three CF-based columns in the mobile phases, which provided higher retention and/or higher values of enantioresolution (hex/IPA 80/20 (*v/v*) without and with TFA).

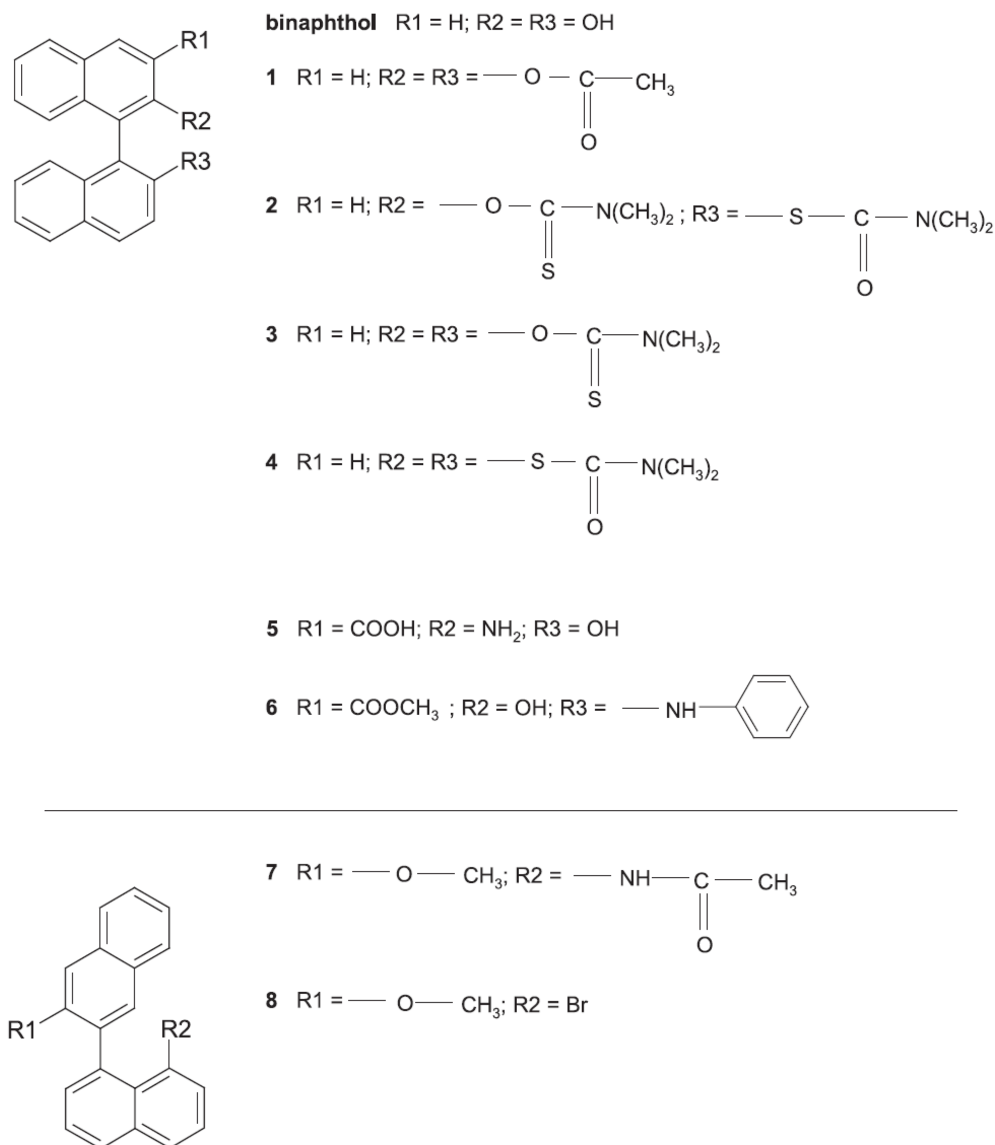


Fig. 4.2. The structures of the binaphthyl derivatives studied in this work.

Table 4.1. The chromatographic parameters of binaphthyl derivatives using the three CF-based CSPs; k_1, retention factor of the first eluted atropisomer; α , enantioselectivity; R , enantioresolution.

Mobile phase	Analyte	IP-CF6			RN-CF6			DMP-CF7		
		k_1	α	R	k_1	α	R	k_1	α	R
hex/IPA	Binaphthol	1.26	1.08	0.84	1.38	1.08	1.08	1.47	1.43	3.44
80/20	1	0.64	1.22	2.43	0.94	1.10	1.32	1.14	1.04	0.50
(*v/v*)	2	1.12	1.07	1.00	2.17	1.07	0.98	2.46	1.27	2.95
	3	0.72	1.17	1.95	1.35	1.16	1.95	1.45	2.65	10.73
	4	3.28	1.00	0.00	6.34	1.00	0.00	7.40	1.00	0.00
	5	1.52	1.11	1.35	2.14	1.05	0.14	12.35	1.00	0.00
	6	0.51	1.00	0.00	0.89	1.00	0.00	1.22	1.03	0.39
	7	2.04	1.00	0.00	3.19	1.00	0.00	4.83	1.00	0.00
	8	0.30	1.00	0.00	0.43	1.00	0.00	0.62	1.00	0.00
hex/IPA/TFA	Binaphthol	1.25	1.07	0.69	1.47	1.08	1.04	1.69	1.35	3.59
80/20/0.5	1	0.63	1.21	2.26	0.89	1.10	1.44	1.11	1.05	0.62
(*v/v/v*)	2	1.04	1.07	0.92	2.02	1.07	0.95	2.34	1.30	3.11
	3	0.68	1.19	1.84	1.29	1.17	1.87	1.50	2.51	9.49
	4	2.86	1.00	0.00*	5.38	1.00	0.00	5.65	1.00	0.00
	5	1.60	1.11	1.66	1.76	1.10	1.46	1.87	1.00	0.00
	6	0.52	1.00	0.00	0.88	1.00	0.00	1.16	1.10	1.14
	7	1.72	1.03	0.21	2.76	1.00	0.00	4.13	1.00	0.00
	8	0.30	1.00	0.00	0.44	1.00	0.00	0.59	1.00	0.00

* slight indication of enantioseparation

Some general conclusions can be drawn from the data in Table 4.1. The retention behaviour of binaphthyl derivatives correlates with the size of the crown ether core and the substituents on the cyclofructan. DMP-CF7 having the largest core and smaller substituents than RN-CF6 provided the highest resolution values for atropisomers of binaphthol analytes 2, 3 and 6. There is probably lower steric hindrance of the substituent groups of DMP-CF7. Remarkably high resolution values, R = 10.73 and 9.49 (without and with the addition of TFA, respectively), were achieved for atropisomers of analyte 3. Comparing the enantioseparation capabilities of the employed columns, the similarity of the IP-CF6 and RN-CF6 columns is obvious. The RN-CF6 column separated atropisomers of the same analytes (binaphthol, analytes 1, 2, 3 and 5) as IP-CF6 column with comparable values of enantioresolution.

For most analytes the retention increased in the sequence IP-CF6 < RN-CF6 < DMP-CF7 while the trend of enantioresolution values seemed to be as follows: RN-CF6 ≤ IP-CF6 << DMP-CF7.

It is not surprising that the substituent on the binaphthyl moiety affects the analytes chromatographic behaviour (Table 4.1.). Analytes 2, 3 and 4 are interesting to compare (for structures see Fig. 4.2.). In all cases analyte 4 had the greatest retention, but its atropisomers were not separated. Conversely the atropisomers of analyte 3 were least retained and best separated, while atropisomers of analyte 2 were intermediate in both respects. It appears that the carbonyl substituent of analyte 4 contributes only to the retention, while the thiocarbonyl substituent of analyte 3 has positive effects on chiral recognition. Both contributions can be observed on the chromatographic behaviour of analyte 2, which contains one each of the two substituent types. Relating the chromatographic behaviour to the structures of binaphthyl derivatives (analytes 1, 2 and 3) carbonyl or thiocarbonyl groups seem to have a substantial impact on the interaction mechanism. This could be the reason why atropisomers of analytes 1, 2 and 3 exhibited successful separations.

Atropisomers of analytes 4, 7 and 8 were not separated in any of the chromatographic systems tested with the exception of rather limited separation of atropisomers of analyte 7 offered by IP-CF6 column in the mobile phase hex/IPA/TFA 80/20/0.5 (*v/v/v*).

Addition of the acid to the mobile phase had almost negligible effect on retention of the majority of analytes; mostly slightly reduced retention was

accompanied by somewhat decreased resolution. Exceptional chromatographic behaviour was observed for analyte 5. Its retention increased and resolution of its atropisomers improved on the IP-CF6 CSP in the acidified mobile phase. On the RN-CF6 CSP the resolution value of atropisomers of analyte 5 also increased while retention decreased in the mobile phase with TFA. Furthermore, analyte 5 showed very high retention on DMP-CF7 CSP in hex/IPA 80/20 (*v/v*). These results can be attributed to the role of the accessible ionizable groups (carboxylic, amino and hydroxyl functional groups) of analyte 5 in the interaction mechanism, i.e., forming of hydrogen bonds with free hydroxyl groups of the cyclofructan molecule. Addition of the acid caused dramatic decrease of retention of analyte 5 because TFA, as an ion-pairing agent, hamper the interactions of the analyte with the CF molecule. The influence of acidification of the mobile phase with TFA positively affected also the resolution of atropisomers of analyte 7 on the IP-CF6 CSP and analyte 6 on DMP-CF7 CSP; partial separation was made possible and resolution was improved after TFA was added to the mobile phase in the former and the latter case.

The CF-based CSPs performed good separation capabilities with some excellent resolutions of atropisomers of the binaphthyl derivatives. They can be considered suitable for the chiral separation of this type of compounds because the structure of binaphthyls seems to be compatible with the arrangement of the CF molecule. The performance of the tested columns can be improved by tuning up the separation conditions. The IP-CF6 column was thought to be mainly or primarily a column for chiral primary amines [22, 23]. This work shows that it also has selectivity for another class of compounds. The selectivity of the IP-CF6 CSP seems to be exceptional and broader compared to the aromatic-functionalized CF-based CSPs. However, high values of resolution of atropisomers of binaphthol and analyte 3 offered by DMP-CF7 CSP enhances the application of this stationary phase to semipreparative scale – see the chromatograms in Fig. 4.3.

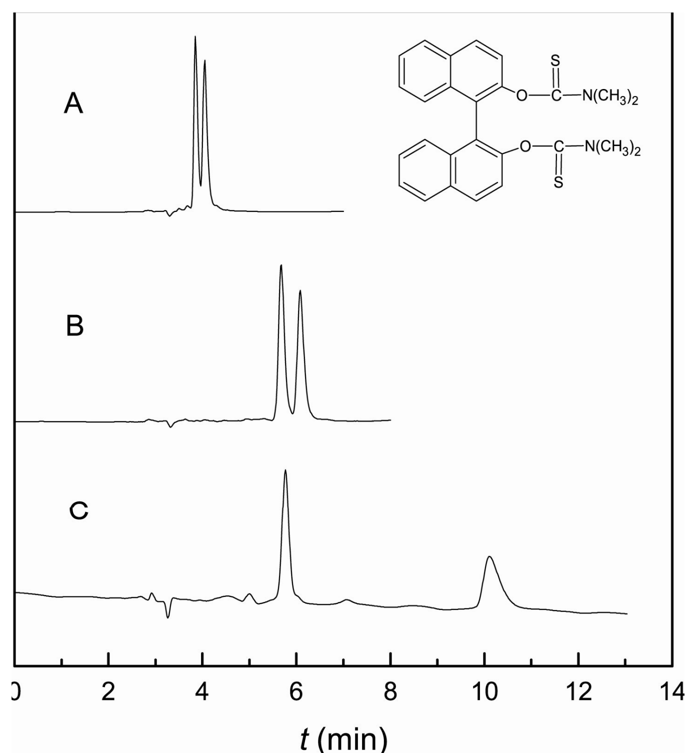


Fig. 4.3. Chiral separation of atropisomers of analyte 3 on the three CF-based chiral stationary phases. A: IP-CF6 column; B: RN-CF6 column; C: DMP-CF7 column. Mobile phase hex/IPA/TFA 60/40/0.5 (*v/v/v*); temperature: 25 °C; flow rate: 1 mL/min; UV detection: 254 nm.

4.3.2. Chromatographic behaviour of chiral pharmaceuticals

The chiral recognition capabilities of the CF-based CSPs were extended by injection of a series of racemic neutral, acidic and basic drugs. While the binaphthyl derivatives exhibited axial chirality, all of these compounds contained stereogenic centers. The testing set was composed of pairs of analytes with similar structures (with the exception of catechin and thalidomide) from the groups of β-blockers, calcium channel blockers or non-steroidal anti-inflammatory drugs. Besides the aromatic or heterocyclic moieties these molecules possess functional groups such as hydroxyl, carboxyl, amino and substituted amino groups, amide, ester or nitro groups. These groups can contribute to the multiple interactions between analyte and chiral selector, i.e., hydrogen bonds, dipolar interaction, steric interaction, which are responsible for chiral recognition. The separation systems tested were the same

as for binaphthyl derivatives, i.e., the separation conditions were not optimized and six different mobile phase compositions were tested.

Table 4.2. summarizes the best results of the separation of the racemic pharmaceuticals. Most of the analytes were at least partially separated in the six tested mobile phases. An increase in the amount of hexane in the mobile phase increased the retention of all analytes, which is a typical normal phase chromatographic behaviour. For some compounds, the retention of the analytes was so high that no elution was observed within 3 hours. TFA as an additive decreased retention and induced the separation of enantiomers in some cases. Also, higher amounts of TFA in the mobile phase usually improved the resolution of partially separated enantiomers.

Table 4.2. The chromatographic data of the chiral pharmaceuticals separated on the three CF-based chiral stationary phases. For explanation of symbols see caption of Table 4.1.

Analyte	Column	Mobile phase hex/IPA/TFA (v/v/v)	k_1	α	R
BP766	IP-CF6	80/20/0.5	2.42	1.05	0.55
	RN-CF6	80/20/0.5	2.76	1.03	0.47
BP34	IP-CF6	60/40/0.5	16.64	1.38	2.36
	RN-CF6	80/20/0.5	47.02	1.33	2.91
	DMP-CF7	60/40/0.5	7.25	1.26	0.88
Alprenolol	IP-CF6	60/40/0.1	3.10	1.08	0.51
	RN-CF6	80/20/0.5	4.67	1.02	0.30
Oxprenolol	IP-CF6	80/20/0.1	19.66	1.03	0.32
	RN-CF6	80/20/0.5	9.53	1.07	0.89
Flobufen	DMP-CF7	80/20/0.5	1.23	1.05	0.59
Tiaprofenic acid	IP-CF6	80/20/0.5	1.01	1.05	0.52
	DMP-CF7	80/20/0.0	2.32	1.28	2.83
Amlodipine	IP-CF6	60/40/0.5	23.26	1.02	0.20
	RN-CF6	60/40/0.5	15.57	1.09	0.71
Catechin	IP-CF6	80/20/0.1	18.58	1.03	0.47
Thalidomide	RN-CF6	80/20/0.1	15.78	1.02	0.41
	DMP-CF7	80/20/0.1	23.84	1.05	0.58

Considering the data in Table 4.2. the IP-CF6 column was the most broadly useful CSP. The DMP-CF7 CSP was the least effective for these particular analytes, although it offered the best separation for racemic tiaprofenic acid (R = 2.83, see Table 4.2.). Other baseline separation was achieved for analyte BP34 on IP-CF6 and RN-CF6 CSPs.

Analyte BP34 [38] possessing free amino group was retained strongly by all the CSPs tested and no elution was observed in some of the separation systems with higher amount of *n*-hexane in the mobile phase unless TFA was added. TFA also decreased the retention of BP34 and improved resolution. BP766 is the trifluoroacetylated derivative of BP34 (for structures see Fig. 4.4.). It was only partial separated on the IP-CF6 and RN-CF6 columns in the mobile phase hex/IPA/TFA 80/20/0.5 (*v/v/v*) (see Table 4.2.). The importance of the free amino group close to the chiral center is apparent and supports previous results of the dominant separations of racemic primary amines on the CF-based CSPs [22, 23].

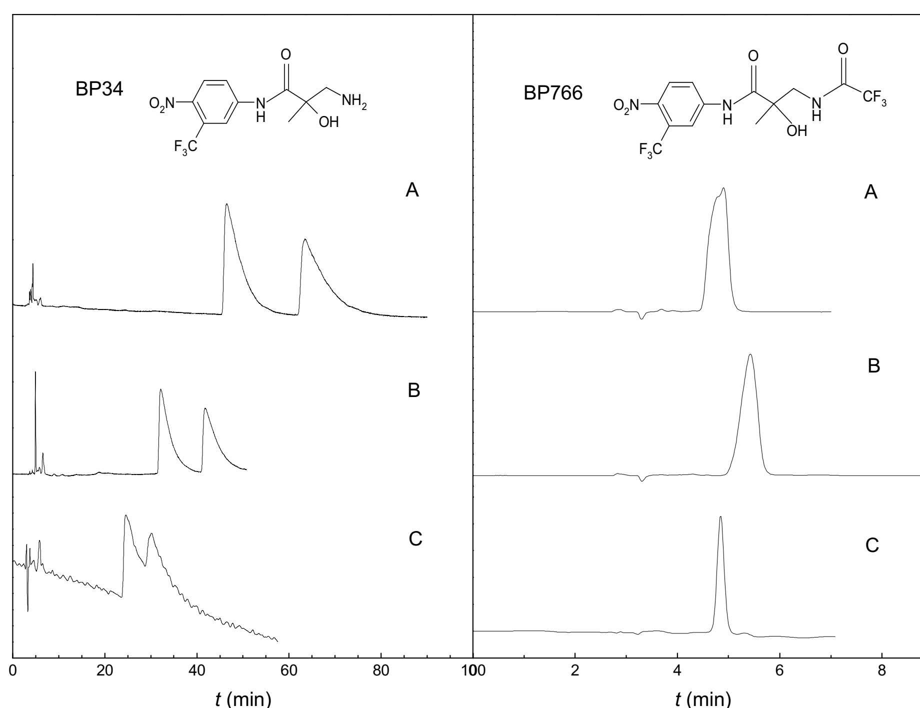


Fig. 4.4. Chromatographic behaviour of BP34 and BP766 on the three CF-based chiral stationary phases. A: IP-CF6 column; B: RN-CF6 column; C: DMP-CF7 column. Mobile phase hex/IPA/TFA 60/40/0.5 (*v/v/v*); temperature: 25 °C; flow rate: 1 mL/min; UV detection: 254 nm.

Alprenolol and oxprenolol are secondary amines with non-polar side chains. The highest retention of these analytes was observed on IP-CF6 in the mobile phases hex/IPA 80/20 (*v/v*) and hex/IPA 60/40 (*v/v*). Considering the two CF6 columns, the addition of TFA lowered the retention substantially and in most cases initiated at least partial separation – see Table 4.2. One important remark on the structures of BP34 and β-blockers is the presence of hydroxyl group directly bonded to the stereogenic center. The ability to interact *via* hydrogen bonds is most probably employed in the retention and chiral recognition process.

Amlodipine has a primary amino group but it is situated far away from the stereogenic center. Therefore, it can contribute to high retention (viz. Table 4.2.) but it participates less in the enantiomeric discrimination interactions. Amlodipine interacted so strongly with all three chiral stationary phases, that no elution was observed within 3 hours in the mobile phases hex/IPA 80/20 (*v/v*) even with the addition of TFA. The CF6-based stationary phases provided partial enantioseparation in the acidifed mobile phases. In comparison with amlodipine, its structure analogue nitrendipine showed lower retention (about 12 minutes) in all the systems tested and no enantioseparation was observed. This big difference caused most probably absence of free animo group in the structure of nitrendipine.

Catechin has multiple hydroxyl groups. Its highest retention and only a partial separation was obtained on the IP-CF6 column. The small isopropyl substituent does not block the non-substituted OH groups of cyclofructan and thus they are available for hydrogen bonding with the functional groups of catechin.

Thalidomide with aromatic and heterocyclic moieties in its molecule was partially separated in the systems with aromatic-derivatized CF-based CSPs, i.e., RN-CF6 and DMP-CF7 columns. DMP-CF7 offered the highest retention and resolution in all the mobile phases tested. It can be concluded that the aromatic derivatization groups (RN and DMP) considerably participate in the interactions with the molecule of thalidomide.

Tiaprofenic acid showed suitable retention on IP-CP6 and DMP-CF7 columns. Reduction of interaction of this compound by addition of TFA was important to reach at least partial resolution on the IP-CF6 CSP where stronger hydrogen bonding interactions between the carboxyl group of this analyte and carbamate or hydroxyl group of the cyclofructan can be expected. Mobile phase without TFA was suitable

for baseline separation of these enantiomers on DMP-CF7 CSP, on which the carbamate or hydroxyl groups are less accessible. The behaviour of flobufen, also possessing a carboxyl group, differed from that of the tiaprofenic acid on the DMP-CF7 CSP. This result corresponds with the structure of flobufen where other interaction groups important for chiral recognition are farther away from the stereogenic center.

If we compare the selectivity of the employed stationary phases, the similarity of IP-CF6 CSP and RN-CF6 CSP is evident from Table 4.2. These CSPs partially separated the same analytes (with some exceptions). DMP-CF7 performed lower separation capabilities for the selected analytes in the applied mobile phases. The separation performance can certainly be improved by optimizing the separation conditions or maybe by application of polar-organic mode.

4.4. Results and discussion – LFER as a tool for characterization of chiral stationary phases

LFER is used for understanding the types and relative strengths of the chemical interactions that control retention and selectivity in the various modes of liquid chromatography and other separation methods [39]. Introduction to LFER is given in Chapter 2.2. of this thesis. LFER analyses have been extensively used to evaluate and compare LC stationary phases. The application of the LFER model to chiral separation systems is not explicit as the model has little solute shape recognition ability [40], i.e., no chiral term is involved in the equation. Nevertheless, this approach can be useful for estimation of the interactions participating in the enantiorecognition process and for understanding the separation mechanism more in detail.

4.4.1. Characterization of cyclofructan-based CSPs by LFER (Paper IV)

This chapter deals with characterization and comparison of interaction abilities of cyclofructan-based chiral stationary phases, introduced in Chapter 4.2., in normal phase separation mode using the LFER model.

The LFER method was applied to the systems with three different CF-based CSPs under two mobile phase compositions, i.e., six separation systems were investigated. A set of 44 solutes of different properties was used for the measurements. Test solutes with their solvation parameters are listed in Table 1 of Paper IV. Effect of acidification of hex/IPA mobile phase was also examined.

Table 4.3. summarizes the LFER data obtained for the investigated separation systems. The optimal model was chosen for the comparison of the individual separation systems because it includes only significant interactions. Plots of the experimental values of log k against calculated/predicted log k values of test solutes show linear dependencies with correlation coefficients higher than 0.95 in all cases. That indicates strong correlation of the LFER model with the experimental data.

Table 4.3. Regression coefficients of the LFER equation and correlation coefficient R.

Column	Mobile phase	Model	v	a	b	s	e	c	R
RN-CF6	hex/IPA/TFA	C.M.	-1.623	-0.035	1.669	0.995	0.278	-0.659	0.957
	80/20/0.0	±95% CI	0.704	0.247	0.312	0.364	0.328	0.475	
	(*v/v/v*)	*p*	0.000	0.776	0.000	0.000	0.092	0.008	
		O.M.	**-1.169**	**x**	**1.596**	**1.128**	**x**	**-0.944**	**0.953**
		±95% CI	0.382		0.302	0.298		0.307	
		p	0.000		0.000	0.000		0.000	
IP-CF6	hex/IPA/TFA	C.M.	-0.849	0.040	1.571	0.492	0.001	-0.423	0.963
	80/20/0.0	±95% CI	0.530	0.194	0.219	0.284	0.253	0.358	
	(*v/v/v*)	*p*	0.003	0.675	0.000	0.001	0.991	0.022	
		O.M.	**-0.887**	**x**	**1.573**	**0.518**	**x**	**-0.401**	**0.963**
		±95% CI	0.282		0.211	0.228		0.232	
		p	0.000		0.000	0.000		0.001	
DMP-CF7	hex/IPA/TFA	C.M.	-1.252	-0.222	1.781	0.835	0.211	-0.710	0.954
	80/20/0.0	±95% CI	0.737	0.263	0.289	0.368	0.329	0.499	
	(*v/v/v*)	*p*	0.002	0.096	0.000	0.000	0.203	0.007	
		O.M.	**-0.703**	**x**	**1.729**	**0.803**	**x**	**-1.048**	**0.950**
		±95% CI	0.391		0.287	0.305		0.315	
		p	0.001		0.000	0.000		0.000	
RN-CF6	hex/IPA/TFA	C.M.	-1.019	0.092	1.556	0.759	0.130	-0.823	0.967
	80/20/0.5	±95% CI	0.623	0.216	0.237	0.299	0.275	0.421	
	(*v/v/v*)	*p*	0.002	0.395	0.000	0.000	0.342	0.000	
		O.M.	**-0.918**	**x**	**1.535**	**0.891**	**x**	**-0.892**	**0.965**
		±95% CI	0.316		0.231	0.244		0.254	
		p	0.000		0.000	0.000		0.000	
IP-CF6	hex/IPA/TFA	C.M.	-0.651	0.178	1.480	0.467	0.014	-0.654	0.961
	80/20/0.5	±95% CI	0.600	0.212	0.228	0.293	0.266	0.411	
	(*v/v/v*)	*p*	0.034	0.097	0.000	0.003	0.916	0.003	
		O.M.	**-0.821**	**x**	**1.486**	**0.594**	**x**	**-0.553**	**0.957**
		±95% CI	0.311		0.226	0.242		0.254	
		p	0.000		0.000	0.000		0.000	
DMP-CF7	hex/IPA/TFA	C.M.	-1.100	-0.099	1.654	0.910	0.153	-0.900	0.958
	80/20/0.5	±95% CI	0.731	0.259	0.278	0.358	0.325	0.500	
	(*v/v/v*)	*p*	0.004	0.445	0.000	0.000	0.347	0.001	
		O.M.	**-0.765**	**x**	**1.622**	**0.927**	**x**	**-1.110**	**0.956**
		±95% CI	0.368		0.267	0.287		0.300	
		p	0.000		0.000	0.000		0.000	

CI represents ±95% confidence interval; x, insignificant interaction; C.M., complete model of the LFER equation; O.M., optimal model of the LFER equation; *p*, statistical *p*-value. The *p*-values express probability of the error that the individual coefficient does not contribute to the model, i.e., *p*-values represent the significance of the individual coefficients.

The dominant contribution to retention is described by the coefficient *b*, which represents the difference between the stationary and the mobile phases in hydrogen bond donating properties. Positive values of the coefficient *b* denote that this type of interaction is preferred in the stationary phase. While hydrogen donating groups are available on the stationary phases they are not present in the mobile phase composed of hex/IPA. Addition of a low amount of TFA to the mobile phase causes small decrease of the *b*-values in the separation systems with any of the CF-based CSPs because the acidified mobile phase can contribute to the H-donating interactions. Comparison of the acidity of the individual columns evidenced the sequence IP-CF6 ≤ RN-CF6 < DMP-CF7. The difference in the H-donating properties between the two CF6 columns (RN-CF6 and IP-CF6) is very small. Due to its larger basic structure (core), DMP-CF7 offers more hydrogen donating groups and so, its ability to interact *via* H-bonds is the highest.

The regression coefficient *v*, reflecting the difference in hydrophobicity between the stationary and the mobile phases, is negative in all cases. This is consistent with the applied normal separation mode where, in principle, the mobile phase is less polar than the stationary phase. The lowest absolute values of coefficient *v* were obtained for the systems with DMP-CF7 column. Comparing the hydrophobicity of the two CF6 columns that have the same core size, much smaller isopropyl derivatization group has better accessibility to the OH groups of cyclofructan while the derivatization seems to be more difficult with the bigger naphthylethyl substituent. Therefore, the biggest difference in hydrophobicity between the stationary and the mobile phases was observed for the systems with RN-CF6 CSP. The addition of TFA increases polarity of the mobile phase. So, the difference in hydrophobicity between the stationary and the mobile phases is reduced and the absolute values of coefficient *v* can decrease. This fact is significant mainly for the RN-CF6 column. While evaluating the LFER results one must take into account that the properties of the stationary phase can also be affected by sorption of the components of the mobile phase on the surface of stationary phase.

The difference in dipolarity/polarizibility between the stationary and the mobile phase, described by the coefficient *s*, is positive for all the investigated separation systems. Polarizibility of the attached derivatization groups clearly increases in the sequence: isopropyl- < dimethylphenyl- < naphthylethyl-. In a more rigorous

approach, the core size of cyclofructan must also be considered. Addition of TFA to the mobile phase affects the *s* values of IP-CF6 and DMP-CF7, and RN-CF6 in a different way. While the dipolarity/polarizibility coefficients increased for the two former columns lower *s* value was obtained for the latter CSP. The interaction of the acid with the stationary phase (cyclofructan) takes place most easily if cyclofructan is substituted with the biggest naphthylethyl group, which is sterically less convenient to reach the cyclofructan basic structure.

The *e* coefficient describing the difference in the propensity of the stationary and the mobile phases to interact with *n*- and *π*-electron pairs of the solute and the regression coefficient *a* relating to the difference in H-bond basicity are statistically insignificant for all studied separation systems. This denotes that the ability of the stationary and the mobile phases to participate in these types of interactions is comparable.

The regression coefficient *a* is statistically insignificant in all the studied systems. It means that the hydrogen bond basicity (ability to accept protons) of the stationary and the mobile phases is comparable.

The results of LFER indicate that significant interactions affecting retention on all three CF-based columns studied in this work are the same, namely H-donating interactions and dipolarity/polarizibility (with positive regression coefficient values) and hydrophobicity (with negative values).

Concerning enantioseparation the LFER results indicate that analytes should offer H-accepting groups and polarizable moieties near the stereogenic center and low hydrophobicity. These facts are partly supported by the results reported in Chapter 4.3.2.

4.4.2. Comparison of *R*-naphthylethyl carbamoyl cyclofructan 6 CSP with *R*-naphthylethyl carbamoyl β-cyclodextrin CSP by LFER (Paper V)

This chapter is focused on comparison of *R*-naphthylethyl carbamoyl CF6 (RN-CF6) chiral stationary phase with a well-established *R*-naphthylethyl carbamoyl β-cyclodextrin (RN-CD) chiral stationary phase using LFER method. These CSPs have the same substituent, *R*-naphthylethyl carbamate group, and isomeric saccharide units, six fructofuranose and seven glucopyranose units in CF6 and β-CD, respectively [41, 42]. Cyclodextrins possess a hydrophilic surface and a truncated cone with a hydrophobic cavity [2], which make them different from CFs.

Two mobile phase compositions were applied, i.e., hex/IPA/TFA 80/20/0.0 (*v/v/v*) and hex/IPA/TFA 80/20/0.5 (*v/v/v*). The LFER data obtained for the separation systems are summarized in Table 4.4. that shows the regression coefficients obtained from the complete and the optimal models. Correlation of the LFER data with experimental results (plot of the experimental log *k* against calculated log *k*) achieved for the set of the test solutes on the both CSPs did not show any serious outliers, correlation coefficients of linear regression fits were always higher than 0.93. Due to the fact that insignificant interactions are also included in the complete LFER model, the optimal model offers a better tool for comparison of the chromatographic systems studied in this work.

Table 4.4. Regression coefficients of the LFER equation and correlation coefficient R.

Column	Mobile phase	Model	v	a	b	s	e	c	R
RN-CF6	hex/IPA/TFA	C.M.	-1.623	-0.035	1.669	0.995	0.278	-0.659	0.957
	80/20/0.0	±95% CI	0.704	0.247	0.312	0.364	0.328	0.475	
	(*v/v/v*)	p	0.000	0.776	0.000	0.000	0.092	0.008	
		O.M.	**-1.169**	**x**	**1.596**	**1.128**	**x**	**-0.944**	**0.953**
		±95% CI	0.382		0.302	0.298		0.307	
		p	0.000		0.000	0.000		0.000	
	hex/IPA/TFA	C.M.	-1.019	0.092	1.556	0.759	0.130	-0.823	0.967
	80/20/0.5	±95% CI	0.623	0.216	0.237	0.299	0.275	0.421	
	(*v/v/v*)	p	0.002	0.395	0.000	0.000	0.342	0.000	
		O.M.	**-0.918**	**x**	**1.535**	**0.891**	**x**	**-0.892**	**0.965**
		±95% CI	0.316		0.231	0.244		0.254	
		p	0.000		0.000	0.000		0.000	
RN-CD	hex/IPA/TFA	C.M.	-1.381	0.506	0.928	1.033	0.211	-0.342	0.931
	80/20/0.0	±95% CI	0.898	0.337	0.331	0.503	0.416	0.647	
	(*v/v/v*)	p	0.004	0.004	0.000	0.000	0.309	0.290	
		O.M.	**-1.037**	**0.553**	**0.901**	**1.148**	**x**	**-0.581**	**0.929**
		±95% CI	0.591	0.324	0.327	0.449		0.443	
		p	0.001	0.001	0.000	0.000		0.012	
	hex/IPA/TFA	C.M.	-0.963	0.284	1.455	0.831	0.194	-0.896	0.966
	80/20/0.5	±95% CI	0.600	0.211	0.255	0.293	0.265	0.414	
	(*v/v/v*)	p	0.002	0.010	0.000	0.000	0.147	0.000	
		O.M.	**-0.623**	**0.330**	**1.421**	**0.906**	**x**	**-1.114**	**0.964**
		±95% CI	0.382	0.204	0.255	0.279		0.291	
		p	0.002	0.002	0.000	0.000		0.000	

CI represents ±95% confidence interval; x, insignificant interaction; C.M., complete model of the LFER equation; O.M., optimal model of the LFER equation; p, statistical p-value. The p-values express probability of the error that the individual coefficient does not contribute to the model, i.e., p -v represent the significance of the individual coefficients.

Negative values of the regression coefficient v (representing difference in hydrophobicity between the stationary and the mobile phases) obtained for all four separation systems show that hydrophobic interactions are preferred in the mobile phase. This is legitimate in a normal separation mode. After addition of TFA to the mobile phase the absolute v values decrease on the both CSPs. Considering the same mobile phase composition the difference in hydrophobicity between the stationary and the mobile phase is higher for the system with RN-CF6 column. This confirms the fact that cyclofructans do not possess hydrophobic cavity as do cyclodextrins.

Discussing the regression coefficient *a* (relating to the difference in hydrogen bond basicity), it is statistically insignificant for RN-CF6 column in the both mobile phases tested. This means that the ability of this stationary phase to accept protons is similar to that of the mobile phase. For the two systems with RN-CD column the coefficient *a* is significant. Positive values indicate that this type of interaction contributes to retention. Lower value of the coefficient *a* was observed in the system with acidified mobile phase. TFA can occupy some of the proton accepting sites on the stationary phase and in this way reduce their availability to the analytes.

The regression coefficients *b* (describing the difference in hydrogen bond acidity) are positive in all chromatographic systems studied, i.e., the hydrogen bond acidity of the both CSPs is higher than that of the applied mobile phases. RN-CF6 column possesses higher hydrogen bond donating ability than RN-CD column. The addition of TFA to the mobile phase had almost negligible effect on the system with RN-CF6 column. Interestingly, the coefficient *b* significantly increases in the system with cyclodextrin-based column after the addition of TFA. The interaction possibilities are greatly influenced by the sorption of mobile phase components. TFA as a hydrogen donor can increase the *b* values if sorbed on the stationary phase. This corresponds to the decrease of coefficient *a* (hydrogen bond basicity) on this column after addition of TFA.

The *s* regression coefficient (describing difference of polarity/polarizibility) is positive for all the studied separation systems because many polar and polarizable groups are available on the both CSPs. The value of this coefficient decreases by addition of TFA to the mobile phase for the both chiral stationary phases to a similar extent. The acid competes with the analytes for the interaction sites of this type on the stationary phases and in this way decreases their retention.

The *e* coefficient is statistically insignificant in all the chromatographic systems tested. The ability of the stationary and the mobile phases to interact with solute *n*- and *π*-electron pairs is equal. It can be even further deduced that this type of interaction is related to the same substituent on CF or CD and has equal effects in all the separation systems compared.

The LFER results showed that the main impact on the interaction mechanism on the RN-CF6 column have hydrogen bond acidity and polarity/polarizibility, while dispersion interactions are preffered in the mobile phase and hydrogen bond basicity

and interactions with n- and π-electron pairs seem to be insignificant. Furthermore, the LFER model revealed some differences between the RN-CF6 and RN-CD CSPs. RN-CD column can be considered less polar but has significant hydrogen bond acidity compared to RN-CF6 packing in the tested mobile phases.

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PAPER III

Chiral Separation of Binaphthyl Catalysts Using New Chiral Stationary Phases Based on Derivatized Cyclofructans

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Abstract

Cyclofructans as a completely new and promising class of chiral selectors have been introduced last year for application in separation techniques such as liquid chromatography and capillary electrophoresis. Mainly derivatives of cyclofructans perform interesting separation possibilities for a variety of compounds. The aromatic derivatized cyclofructans composed of six D-fructofuranose units (CF6) have exhibited the most interesting properties and unique enantioselectivity.

In this work, two derivatized cyclofructan-based chiral stationary phases, RN-CF6 (*R*-naphthylethyl carbamate CF6) and DMP-CF7 (dimethylphenyl carbamate CF7) were used for enantioseparation of substituted binaphtyl catalysts, widely used to control asymmetric processes. Normal separation mode, i.e. mobile phase composed of hexane and propane-2-ol in different ratios was applied and the enantioselectivity of the employed stationary phases was compared.

Keywords
chiral stationary phase
cyclofructans
enantioseparation
HPLC
substituted binaphthyls

1. Introduction

Chiral separations have been given great attention over the past few decades due to their wide range of applications in pharmaceutical and food industry or agriculture. High performance liquid chromatography (HPLC) employing chiral stationary phases (CSPs) has become one of the most common and powerful techniques in enantioselective separations at both analytical and preparative scales. Variety of CSPs, usually bonded to silica gel, with complex interaction mechanisms have been reported [1]. Some classes of chiral selectors dominate the enantiomeric separations, i.e. cyclodextrins (CDs), polysaccharides and their derivatives or macrocyclic antibiotics such as vancomycin, teicoplanin or ristocetin A. Many applications of these chiral selectors have been published [1–4].

A unique class of CSPs based on cyclofructan has been introduced last year with the expectation of great separation potential both for HPLC [5] and CZE [6]. Cyclofructans (CFs) belong to a group of macrocyclic oligosaccharides with a crown ether skeleton. They consist of six or more β-(2→1) linked D-fructofuranose units (see Figure 1) and each fructofuranose unit contains four stereogenic centers and three hydroxyl groups, which can be mostly derivatized. The abbreviations such as CF6, CF7, CF8 etc. indicate the number of fructose units in the macrocyclic ring. CF6 has been largely studied due to its highly defined geometry and availability in pure form [5].

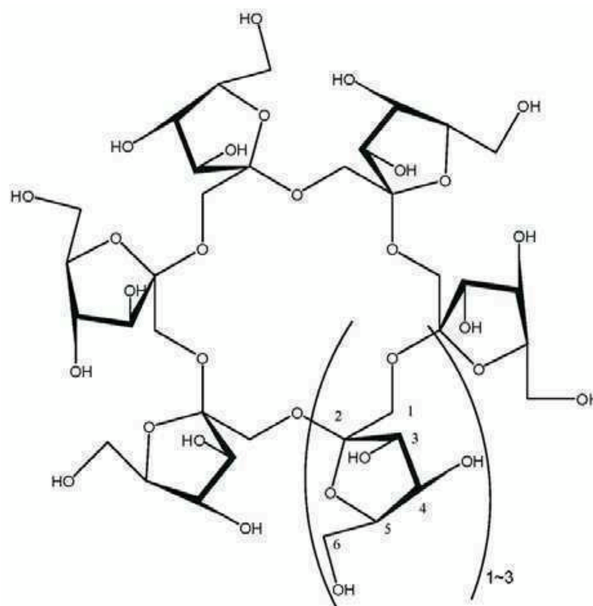

Fig. 1. Molecular structure of cyclofructan (CF6, CF7 and CF8).

Native CFs perform rather limited enantioselectivity but their derivatized (aliphatic or aromatic functionalized) forms show improved and unique separation possibilities over a wide range of analytes.

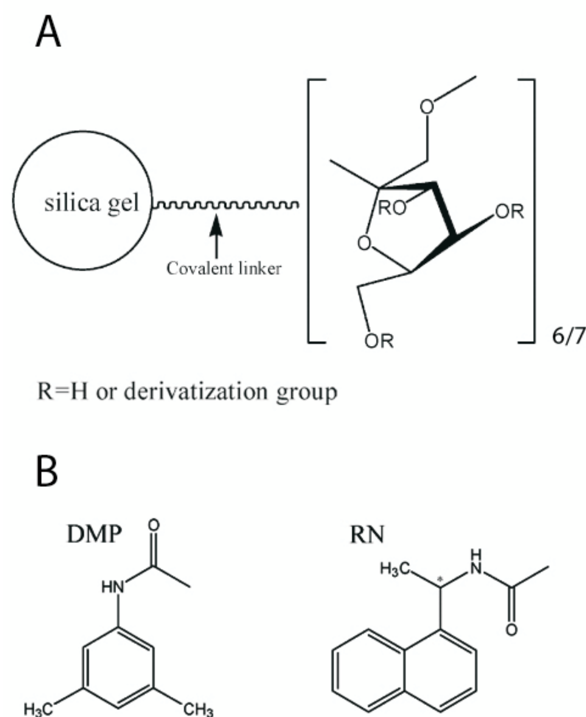

Fig. 2. Scheme of (A) chemically-bonded CF6/CF7 stationary phase, and (B) chemical structures of the derivatizing groups.

Figure 2 shows two CF derivatives studied in this work. RN-CF6 utilizes *R*-naphthylethyl-functionalized CF6 as the chiral selector. It proves excellent enantioselectivity toward various types of analytes including acids, secondary and tertiary amines, alcohols, and many neutral compounds. As the chiral selector is covalently bonded to the silica gel carrier this CSP is compatible with all common organic solvents creating a wide range of compound types that can be separated [7]. DMP-CF7 was developed as a 3,5-dimethylphenyl functionalized CF7. This column also provides chiral recognition toward a broad variety of compounds. In addition, it demonstrates complementary enantioselectivity when compared to RN-CF6 [7]. Both CSPs can operate in all three separation modes (normal-, reversed-phase and polar-organic) but mostly higher selectivity can be obtained in normal-phase mode.

Binaphthyl derivatives have been extensively used to control asymmetric processes and have demonstrated excellent chiral discrimination properties, due to their unique features derived from their chirality, spatial arrangement and rigidity. The chirality of these molecules is caused by restricted rotation around the single bond in the binaphthyl skeleton [8]. Although the basic structures of the binaphthyl derivatives are similar, the substituents and their position significantly affect their properties.

Most papers on binaphthyls derivatives deal with their synthesis. Enantioselective HPLC has been used to control the enantiomeric purity or the yield of individual final products. Just a few studies of enantioselective interactions of these compounds with CSPs can be found in the literature [9–11]. That is why we included these analytes into a set of testing compounds for the evaluation of the enantioseparation abilities of the newly developed cyclofructan-based CSPs. We worked in the normal-phase separation system with hexane and propane-2-ol as mobile phase constituents. The effect of the solvents ratio and addition of trifluoracetic acid (TFA) was studied. We show here just our preliminary results as the work is currently in progress.

2. Experimental

Organic solvents of HPLC grade, *n*-hexane (HEX) and propane-2-ol (IPA), were purchased from Sigma Aldrich (St. Louis, USA). Trifluoracetic acid (TFA), 98% purity, was from Fluka Chemie (Buchs, Germany).

The studied analytes have been synthesized as racemates at the Department of Organic and Nuclear Chemistry, Faculty of Science, Charles University in Prague. The synthesis procedure has been described in detail in Ref. [10]. The chromatographic behavior of nine compounds, i.e. binaphthol, analyte **1**, **4**, **5**, **6**, **7**, **9**, **12**, **13**, was studied in this work. Figure 3 (on next page) shows the structures of these analytes.

The chromatographic measurements were carried out on two HPLC systems (Waters, Milford, USA): Waters HPLC Breeze System (consisting of HPLC Gradient Pump 1525, an autosampler 717Plus, a column heater Jetstream 2 Plus and a UV-Vis dual absorbance detector 2487; Breeze software) and Waters Alliance System (Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717Plus and a Waters Alliance Series column heater; Empower software). Chromatographic columns RN-CF6 and DMP-CF7 (column size 250 × 4.6 mm) with silica gel (particle size 5 μm) as a carrier of the CSPs were used. The chiral selectors bonded to the support were naphtyl ethyl substituted cyclofructan with 6 D-fructose units for RN-CF6 column and dimethyl phenyl derivatized cyclofructan with 7 D-fructose units for DMP-CF7 CSP. These columns have been prepared at the Department of Chemistry and Biochemistry, University of Texas at Arlington, Arlington, Texas.

Normal-phase mode was applied, i.e. mobile phase was composed of HEX/IPA in various volume ratios, the addition of TFA was also tested. The flow rate was 1 mL min^{-1}, the temperature was 25 °C and the detection wavelength was 254 nm.

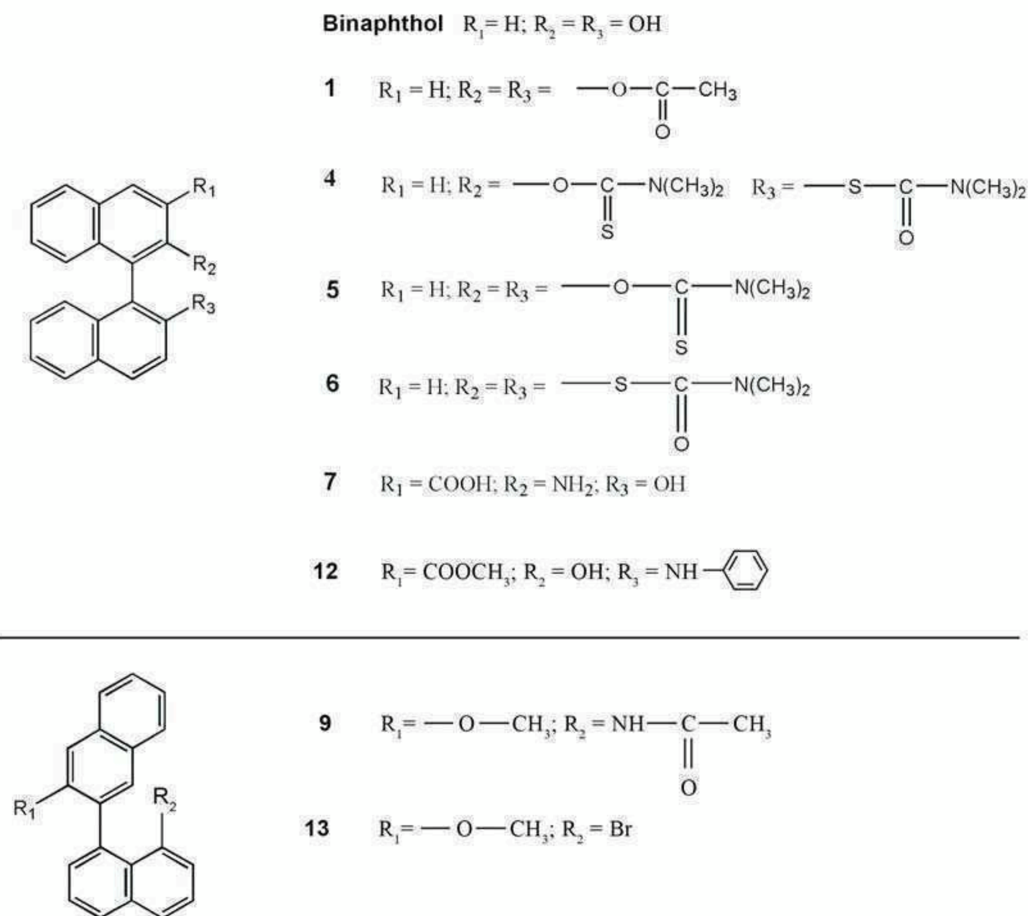

Fig. 3. Structures of the binaphthyl derivatives, studied in this work.

3. Results and Discussion

Two novel CSPs based on cyclofructans working in normal-phase mode were tested and successful enantioseparations of some binaphthyl derivatives were achieved so far. Other testing compounds (e.g. blockers, profens and other chiral pharmaceuticals of diverse structures) were also used to evaluate the separation properties of these CSPs. These experiments are not finished and thus we deal only with binaphthyls in this paper.

RN-CF6 column was evaluated using seven mobile phases differing in volume ratios of HEX/IPA and/or the addition of TFA. Enantioseparation of binaphthol, analyte **1** and analyte **4** was achieved in all the mobile phases but the resolution did not exceed 1.5. Sample **5** performed the best enantioseparation in HEX/IPA, 90/10 (v/v), and its enantioresolution was sufficient in all the mobile phases studied. Other analytes (**6**, **9**, **12** and **13**) were not separated in the tested systems. The addition of TFA to the mobile phase did not have significant impact on retention and resolution of the enantiomers with the exception of analyte **7** that has accesible ionizable groups. The addition of TFA significantly improved its enantioresolution, due to enhanced efficiency of the TFA modified separation system, which is obvious from Figure 4.

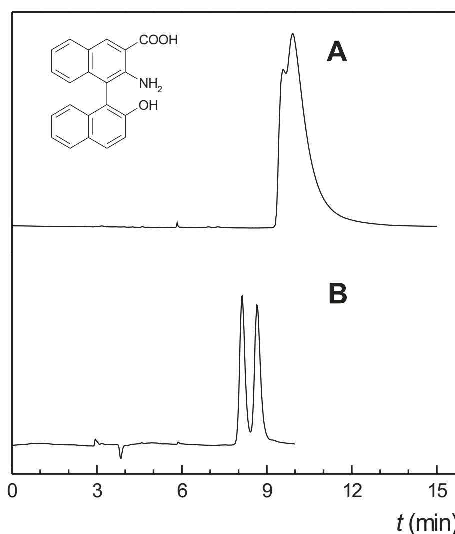

Fig. 4. Separation of analyte **7**; column RN-CF6; mobile phase: (A) HEX/IPA, 80/20 (v/v), (B) HEX/IPA/TFA, 80/20/0.5 (v/v/v); temperature: 25 °C, flow rate: 1 mL min^{-1}, UV detection: 254 nm.

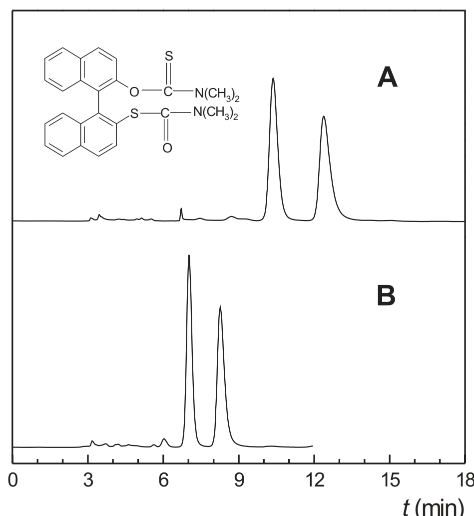

Fig. 5. Separation of analyte **4**; column DMP-CF7; mobile phase: (A) HEX/IPA, 80/20 (v/v), (B) HEX/IPA, 60/40 (v/v); temperature: 25 °C, flow rate: 1 mL min^{-1}, UV detection: 254 nm

DMP-CF7 column was tested using two mobile phases, based on the results obtained with RN-CF6 CSP. The mobile phases were composed of HEX/IPA, 80/20 (v/v) and HEX/IPA, 60/40 (v/v). In these two systems enantioseparations with excellent resolution values were achieved for binaphthol, analyte **4** and analyte **5**. Figure 5 illustrates excellent separation of analyte **4** in the studied mobile phases. Samples **1** and **12** performed partial separation. Four other compounds studied (analyte **6**, **7**, **9** and **13**) did not show any enantioseparation in the mobile phases studied as on RN-CF6 column. Increased amount of IPA in the mobile phase slightly decreased the retention of the analytes and improved enantioresolution. The addition of TFA is being currently tested, hence the results cannot be shown here. Figure 5 illustrates excellent separation of analyte **4** in the studied mobile phases.

4. Conclusion

Novel CSPs based on derivatized cyclofructan were tested for enantioseparation of binaphthyl derivatives, which are widely employed as catalysts of asymmetric processes. The experiments showed good separation abilities of the both cyclofructan-based CSPs in normal chromatographic mode. DMP-CF7 column offered much higher enantioresolution of some binaphthyl derivatives than did RN-CF6. The enantioselectivity of the employed CSPs seems to be slightly different, because of their different structures. The addition of TFA can help to improve the resolution.

Acknowledgments

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PAPER IV

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Characterization of cyclofructan-based chiral stationary phases by linear free energy relationship

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Keywords:	cyclofructan-based chiral stationary phases, HPLC, LFER, normal phase mode

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Characterization of cyclofructan-based chiral stationary phases by linear free energy relationship

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Running title: Characterization of cyclofructan-based CSPs

Abbreviations: CF, cyclofructan; CSP, chiral stationary phase; DMP-CF7, dimethylphenyl carbamate cyclofructan 7; IP-CF6, isopropyl carbamate cyclofructan 6; IPA, propane-2-ol; hex, *n*-hexane; LFER, linear free energy relationship; RN-CF6, *R*-naphthylethyl carbamate cyclofructan 6

Keywords: cyclofructan-based chiral stationary phases, HPLC, LFER, normal phase mode.

Abstract

Cyclofructans, a new class of chiral selectors, have been recently introduced for application in liquid chromatography and capillary electrophoresis. So far, derivatized cyclofructans have performed interesting separation possibilities for a variety of compounds. The current work is focused on characterization of three different cyclofructan-based chiral stationary phases (CF-based CSPs), i.e., isopropyl carbamate cyclofructan 6 (IP-CF6), *R*-naphthylethyl carbamate cyclofructan 6 (RN-CF6) and dimethylphenyl carbamate cyclofructan 7 (DMP-CF7). The LFER model was used to reveal the dominant interactions participating in the complex retention mechanism. A set of 44 different test solutes, with known solvation parameters, was used to determine the regression coefficients of the LFER equation under two mobile phase compositions in normal separation mode. The LFER results showed that hydrogen bond acidity, hydrophobicity and dipolarity/polarizibility mostly affect the retention and separation process on the CF-based columns in the studied separation systems.

1. Introduction

Interest in the field of the enantiomeric separation and development of chiral separation media (chiral selectors, chiral stationary phases) has increased greatly in the past few decades due to the demand of pharmaceutical, agrochemical and food analysis. HPLC with chiral stationary phases (CSPs) has become the most powerful method for separating racemic samples at analytical and preparative scales and/or determining enantiomeric purity. A variety of CSPs with complex interaction mechanisms have been reported by many research groups, which dominate the works of Davankov, Pirkle, Okamoto, Blaschke, Allenmark, Hermansson, Armstrong, Gasparrini, and Lindner [1, 2, 3, 4, 5]. Numerous applications have been reported that involve different chiral selectors chemically bonded to silica gel (or polymeric support) such as polysaccharides [6, 7], proteins [8], macrocyclic antibiotics [9, 10], crown ethers [11] and cyclodextrins [12].

Despite the applicability and broad selectivity of many existing CSPs the research effort for the development of new or improved chiral selectors continues. In 2009 a novel class of CSPs based on cyclofructans was introduced by Armstrong [13]. This group of chiral selectors was shown to have potential both for HPLC [13, 14, 15, 16, 17] and CZE [18]. Cyclofructans (CFs) are macrocyclic oligosaccharides as cyclodextrins. However, cyclofructans are quite different in both their structure and behavior. They consist of six or more β-(2→1) linked D-fructofuranose units [19, 20]. Their abbreviations CF6, CF7, CF8 etc. indicate the number of fructofuranose units in the macrocyclic ring. Each fructofuranose unit contains four stereogenic centers and three hydroxyl groups, which can be utilized for derivatization. While native CFs have rather limited enantioselectivity in HPLC [13] their derivatized forms show improved and unique chiral recognition abilities for a wide range of analytes [13, 14, 16, 21]. Aliphatic or aromatic functionalization of a native chiral selector is a common strategy used to develop new chiral stationary phases and improve enantioseparation performance. Fig. 1 shows the molecular structure of cyclofructans CF6 and CF7 and the

derivatization groups studied in this work. These CSPs can be operated in all common separation modes (normal, reversed phase and polar organic) but mostly higher selectivity was obtained in the normal phase mode.

Figure 1.

A comprehesive method for characterization and comparison of separation systems is the model of linear free energy relationship (LFER) [22, 23, 24, 25, 26], which can independently describe the contributions of individual interactions to the retention process. The LFER equation expresses relationship between the retention characteristic (i.e., retention factor k) determined for a representative set of analytes in a given separation system and the solute fundamental properties described by its descriptors [27]:

$$\log k = c + e\mathrm{E} + s\mathrm{S} + a\mathrm{A} + b\mathrm{B} + v\mathrm{V} \quad (1)$$

The independent variables in Eq. (1) are solute descriptors, where E is the solute excess molar refraction modelling the solute ability to interact *via* n- and/or π-electron pairs, S is the solute dipolarity/polarizibility, A is the effective or overall hydrogen bond acidity, B is the effective or overall hydrogen bond basicity and V is the McGowan`s characteristic molecular volume reflecting hydrophobicity [28, 29, 30]. The selection of a representative set of structurally diverse analytes is essential for aquiring reliable results [31]. Multivariate regression analysis is applied for the determination of the coefficients in Eq. (1) that reflect the different types of molecular interactions in the studied system. In HPLC, the regression coefficients relate to the differences in the properties of the stationary and the mobile phases. The c intercept in the LFER equation is characteristic of the given system but it does not reflect any interaction [32]. The coefficient e reflects the difference in disposition of the stationary and the mobile phases to interact with n- and π-electron pairs of the solutes, s is equal to the difference in dipolarity/polarizibility, a reflects the difference in hydrogen bond

basicity, b refers to the difference in hydrogen bond acidity and the coefficient v represents the difference in hydrophobicity between the two phases.

This work is focused on characterization and comparison of interaction abilities of three different cyclofructan-based chiral stationary phases in normal phase separation mode using the LFER model. These CSPs differ in the substituents and/or the CF core size. Effect of acidification of *n*-hexane/propane-2-ol mobile phase is also examined. Description of the interactions revealed by LFER can serve as a tool for prediction of analytes' retention. Although the application of the LFER model to chiral separations is not explicit, because no chiral term is involved in the equation, this approach can be useful for estimation of the interactions participating in the enantiorecognition process.

2. Experimental

2.1. Chemicals and materials

Organic solvents of HPLC grade, *n*-hexane (hex), propane-2-ol (isopropanol, IPA) and methanol, were purchased from Sigma-Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA; 99.8% purity) was product of Merck (Darmstadt, Germany). The test solutes for LFER were of analytical grade purity and were purchased from Sigma-Aldrich (Steinheim, Germany). The solutes with their descriptors, are listed in Table 1.

Chromatographic columns (Larihc CF6-P, Larihc CF6-RN and Larihc CF7-DMP, AZYP, Arlington, TX, USA) which contain IP-CF6, RN-CF6 and DMP-CF7 with silica gel as a support were used in this study. The chiral selectors were bonded to the silica gel surface. IP-CF6 utilizes isopropyl carbamate CF6 as the chiral selector, RN-CF6 is *R*-naphthylethyl carbamate modified CF6 and DMP-CF7 represents 3,5-dimethylphenyl carbamate

functionalized CF7. The dimensions of these columns were 250 mm × 4.6 mm i.d.; particle size 5 μm. The synthesis procedure has been described previously [13].

The concentrations of stock solutions of solid and liquid samples were 1 mg/mL and 20 μL/mL, respectively. Methanol was used for preparation of all sample solutions.

2.2. HPLC method

All chromatographic measurements were carried out on two HPLC systems (Waters, Milford, USA): (1) Waters HPLC Breeze System consisting of HPLC Gradient Pump 1525, an autosampler 717Plus, a column heater Jetstream 2 Plus and a UV-Vis dual absorbance detector 2487, handled by Breeze software; (2) Waters Alliance System with Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717Plus, Waters Alliance Series column heater, controlled by Empower software.

The temperature of the columns and samples was kept at 25 °C. The injection volume was 10 μL and the flow rate was 1 mL/min. The detection was performed at 254 nm. Normal separation mode was chosen for the whole study due to higher selectivity reported in the literature [13, 14]. Mobile phases were composed of *n*-hexane and propane-2-ol in various volume ratios, small additions of TFA were also tested. System peaks obtained by injection of *n*-hexane to the studied separation systems served for determination of the dead time.

2.3. LFER procedure

The regression coefficients of the LFER equation were obtained from a series of measurements of the retention times of the set of 44 solutes with known solvation parameters [27, 28, 31] that are summarized in Table 1. The retention times were measured in triplicates and from these data retention factors were calculated. The resulting regression coefficients

were obtained for each separation system by multiple linear regression analysis of log *k* against the solutes' descriptors using the NCSS software (Kaysville, USA) [33].

3. Results and Discussion

The LFER model is often used for characterization and comparison of separation systems. This approach can describe individual molecular interactions participating in the retention and separation process. In this work, the LFER method was applied to the systems with three different CF-based CSPs under two mobile phase compositions, i.e., six separation systems were investigated. A set of 44 analytes of different properties, i.e., with different descriptors, was used for the measurements. The retention data (log *k*) are displayed in Table 1.

Table 1.

Table 2 summarizes the LFER data calculated for the investigated separation systems. Plots of the experimental values of log *k* against the calculated/predicted log *k* values of the test solutes show linear dependencies with correlation coefficients higher than 0.95 in all cases. That indicates strong correlation of the LFER model with the experimental data. The optimal model was chosen for the comparison of the individual separation systems (see Fig. 2) because it includes only significant interactions while the complete model involves all the interactions no matter what their statistical significance is.

Table 2.

Figure 2 A, B.

The dominant contribution to retention is described by the coefficient *b*, which reaches the highest values in all the systems investigated. This coefficient represents the difference between the stationary and the mobile phases in hydrogen bond donating properties. Positive values of the coefficient *b* denote that this type of interaction is preferred in the stationary phase. While hydrogen donating groups are available on the stationary phases they are not present in the mobile phase composed of hex/IPA. Addition of a low amount of TFA to the mobile phase causes small decrease of the *b*-values in the separation systems with any of the CF-based CSPs because the acidified mobile phase can contribute to the H-donating interactions. Comparison of the acidity of the individual columns evidenced the sequence IP-CF6 ≤ RN-CF6 < DMP-CF7. The difference in the H-donating properties between the two CF6 columns (RN-CF6 and IP-CF6) is very small, the stationary phases under the concerned conditions are comparable from the point of their acidity. Due to its larger basic structure (core), DMP-CF7 offers more derivatization (hydrogen donating) groups and so, its ability to interact *via* H-bonds is the highest.

The regression coefficient *a* is statistically insignificant in all the studied systems. It means that the hydrogen bond basicity (ability to accept protons) of the stationary and the mobile phases is comparable.

The regression coefficient *v*, reflecting the difference in hydrophobicity between the stationary and the mobile phases, is negative in all cases. This is consistent with the applied normal separation mode where, in principle, the mobile phase is less polar than the stationary phase. The lowest absolute values of coefficient *v* were obtained for the systems with DMP-CF7 column. Based on these results this stationary phase can be considered the most non-polar from the three tested columns. Comparing the hydrophobicity of the two CF6 columns that have the same core size, much smaller isopropyl derivatization group has better accessibility to the OH groups of cyclofructan while the derivatization is more difficult with the bigger naphthylethyl substituent. Due to steric reasons more underivatized OH groups remain on the cyclofructan in the latter case and increase the polarity of the RN-CF6

stationary phase. Therefore, the biggest difference in hydrophobicity between the stationary and the mobile phases (i.e., the highest absolute value of coefficient *v*) was observed for the systems with RN-CF6 CSP. By addition of TFA the mobile phase becomes more polar so, the difference in hydrophobicity between the stationary and the mobile phases is reduced and the absolute values of coefficient *v* can decrease. This fact is significant mainly for the RN-CF6 column. The absolute value of coefficient *v* for the system with DMP-CF7 slightly increases after the addition of TFA. The properties of the stationary phase can be affected by sorption of the components of the mobile phase [34].

The difference between the stationary and the mobile phase dipolarity/polarizibility is described by the coefficient *s*, which is positive for all the investigated separation systems. Polarizibility of the attached derivatization groups clearly increases in the sequence: isopropyl- < dimethylphenyl- < naphthylethyl-. In a more rigorous approach, the core size of cyclofructan must also be considered. Addition of TFA to the mobile phase affects the *s* values of IP-CF6 and DMP-CF7, and RN-CF6 in a different way. While the dipolarity/polarizibility coefficients increased for the two former columns lower *s* value was obtained for the latter CSP. The interaction of the acid with the stationary phase (cyclofructan) takes place most easily if cyclofructan is substituted with the biggest naphthylethyl group, which is sterically less convenient to reach the cyclofructan basic structure.

The *e* coefficient describing the difference in the propensity of the stationary and the mobile phases to interact with *n*- and *π*-electron pairs of the solute is statistically insignificant for all studied separation systems. This denotes that the ability of the stationary and the mobile phases to participate in this type of interactions is comparable.

The results of LFER indicate that significant interactions affecting retention in all separation systems studied in this work are the same, namely H-donating interactions and dipolarity/polarizibility (with positive regression coefficient values) and hydrophobicity (with negative values).

The LFER approach characterizes the prevailing interactions in the separation system but it does not relate to information on enantioselective behavior of analytes. Based on the LFER results retention of analytes can be estimated or even predicted if molecular descriptors are known. Concerning enantioseparation the LFER results indicate that analytes should offer H-accepting groups and polarizable moieties near the stereogenic center and low hydrophobicity.

4. Concluding remarks

The LFER model was used to describe interactions participating in the retention and separation process on the newly developed cyclofructan-based chiral stationary phases RN-CF6, IP-CF6 and DMP-CF7 in normal separation mode. Although LFER does not take into account chirality and spatial arrangement of analytes, it proved which forces take part in the interaction mechanism. The same types of interactions in a different extent were shown to be preferred by all three stationary phases, i.e., hydrogen bond acidity and dipolarity/polarizibility. Also the effect of hydrophobicity as the retention reducing factor plays a role with all tested CF-based CSPs. Hydrogen bond basicity and interactions with *n*- and π-electron pairs seemed to be insignificant. Some differences of the concerned stationary phases due to different cyclofructan core size and/or the substituents were shown by the LFER model.

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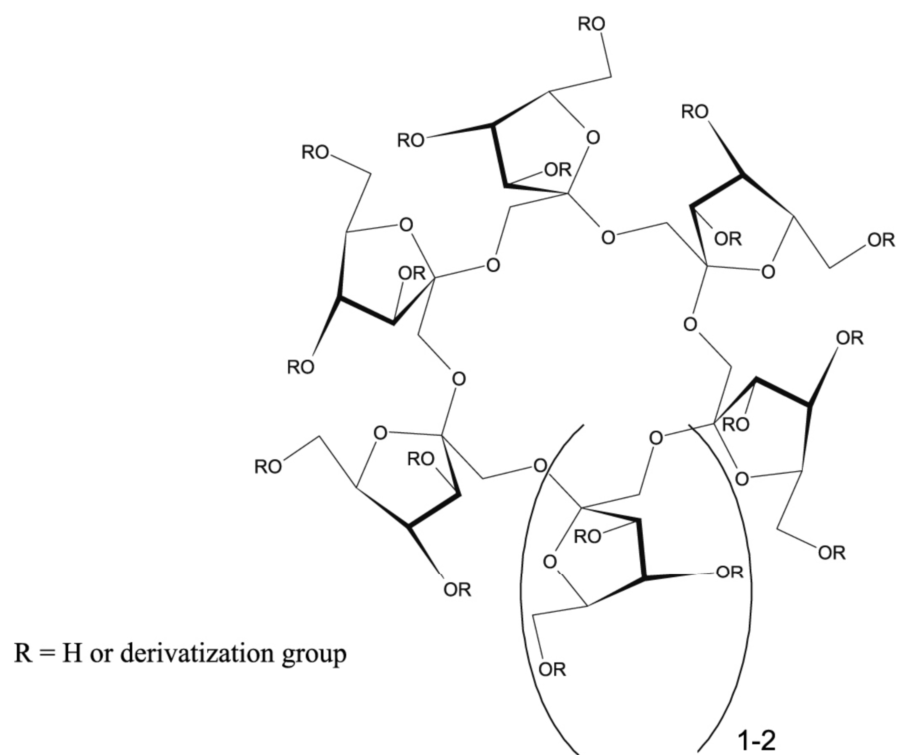
Captions to figures

Figure 1. Molecular structure of cyclofructan CF6 and CF7 and the derivatization groups studied in this work.

Figure 2. Comparison of the regression coefficient values (with their standard errors) obtained from the optimal LFER models for the three CF-based CSPs in the mobile phases: (A) hex/IPA/TFA 80/20/0.0 (*v/v/v*); (B) hex/IPA/TFA 80/20/0.5 (*v/v/v)*.

Figure 1.



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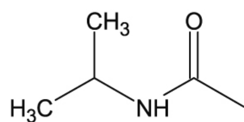

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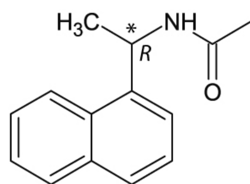

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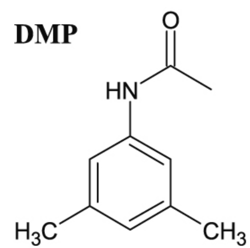

Figure 2A.

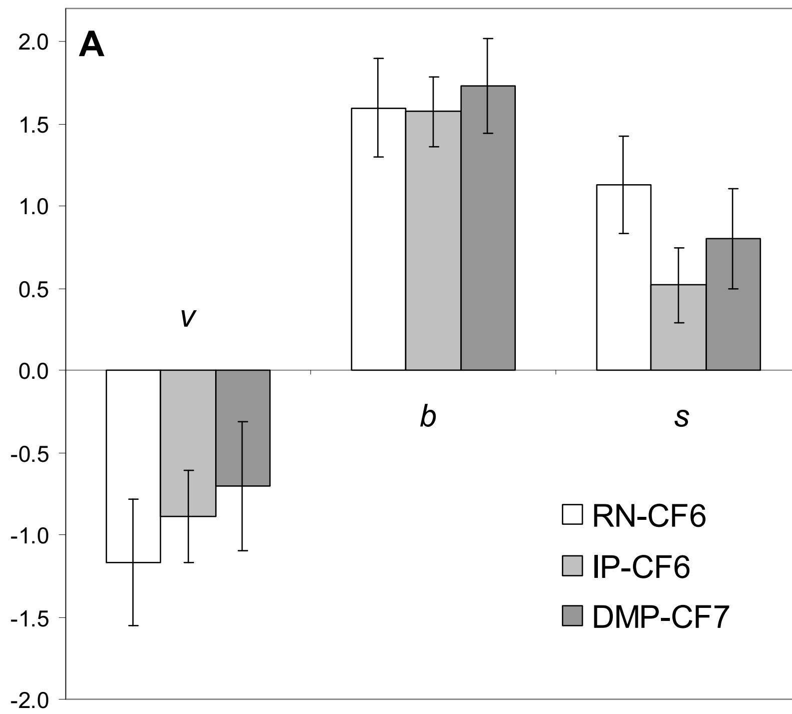

Figure 2B.

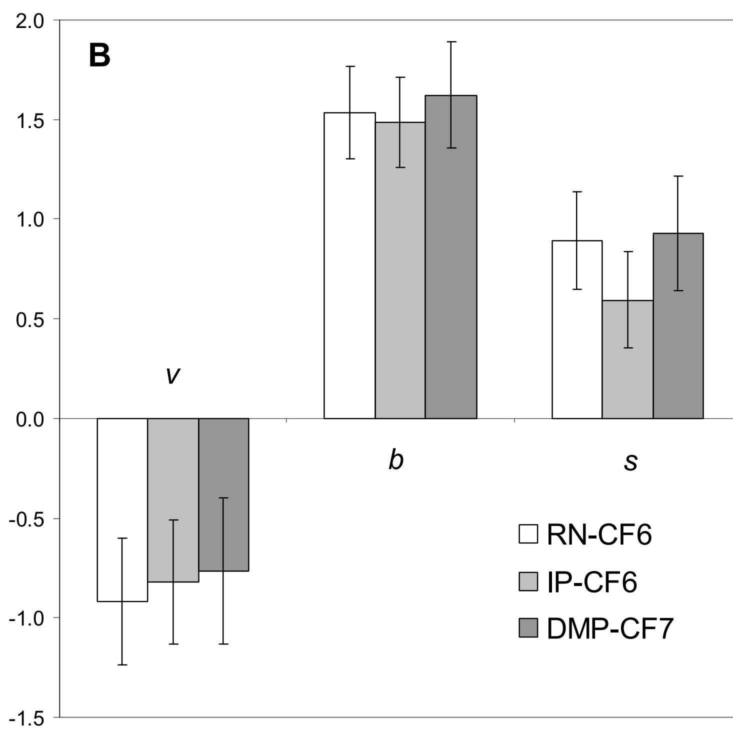

Table 1. Set of test solutes, their solvation parameters and obtained retention data.

Analyte	Solvation parameters					log *k*					
						hex/IPA/TFA 80/20/0.0 (*v/v/v*)			hex/IPA/TFA 80/20/0.5 (*v/v/v*)		
	E	S	A	B	V	RN-CF6	IP-CF6	DMP-CF7	RN-CF6	IP-CF6	DMP-CF7
Benzamide	0.99	1.50	0.49	0.67	0.973	0.550	0.634	0.674	0.533	0.609	0.665
2-Naphthol	1.52	1.08	0.61	0.40	1.144	-0.210	-0.123	-0.278	-0.176	-0.139	-0.253
Resorcinol	0.98	1.00	1.10	0.58	0.834	0.139	0.261	0.030	0.176	0.265	0.070
Benzophenone	1.45	1.50	0.00	0.50	1.481	-0.336	-0.348	-0.266	-0.330	-0.359	-0.286
Hydroquinone	1.00	1.00	1.16	0.60	0.834	0.245	0.362	0.143	0.277	0.392	0.185
1,2-Cresol	0.84	0.86	0.52	0.31	0.916	-0.379	-0.232	-0.485	-0.341	-0.246	-0.430
Benzonitrile	0.74	1.11	0.00	0.33	0.871	-0.199	-0.177	-0.150	-0.199	-0.198	-0.124
1,3-Cresol	0.82	0.88	0.57	0.34	0.916	-0.345	-0.203	-0.426	-0.311	-0.207	-0.378
Benzylalcohol	0.80	0.87	0.33	0.56	0.916	-0.147	-0.033	-0.093	-0.128	-0.038	-0.094
Benzene	0.61	0.52	0.00	0.14	0.716	-1.159	-0.728	-1.038	-1.022	-0.737	-1.004
Naphthalene	1.34	0.92	0.00	0.20	1.085	-0.889	-0.677	-0.804	-0.821	-0.699	-0.764
Pyrocatechol	0.97	1.07	0.85	0.52	0.834	-0.006	0.104	-0.080	0.030	0.121	-0.020
Dibenzothiophene	1.96	1.31	0.00	0.18	1.379	-0.670	-0.617	-0.499	-0.625	-0.617	-0.568
Ethylbenzene	0.61	0.51	0.00	0.15	0.998	-1.447	-0.822	-0.987	-1.229	-0.823	-1.262
Benzaldehyde	0.82	1.00	0.00	0.39	0.873	-0.284	-0.252	-0.212	-0.276	-0.260	-0.198
Toluene	0.60	0.52	0.00	0.14	0.857	-1.304	-0.785	-0.916	-1.137	-0.788	-1.103
1,2-Toluidine	0.97	0.92	0.23	0.45	0.957	0.910	0.918	1.041	1.562	×	1.629
Biphenyl	1.36	0.99	0.00	0.22	1.324	-0.925	-0.725	-0.667	-0.900	-0.726	-0.796
Phenanthrene	2.06	1.29	0.00	0.26	1.454	-0.666	-0.621	-0.553	-0.625	-0.623	-0.529
1,2,3-Trichlorobenzene	1.03	0.86	0.00	0.00	1.084	-0.775	-0.624	-0.682	-0.731	-0.633	-0.661
3-Nitrotoluene	0.87	1.10	0.00	0.25	1.032	-0.398	-0.380	-0.313	-0.379	-0.387	-0.360
1,2-Xylene	0.66	0.56	0.00	0.16	0.998	-1.377	-0.802	-1.196	-0.893	-0.812	-1.311
Bromobenzene	0.88	0.73	0.00	0.09	0.891	-0.971	-0.692	-0.872	-0.879	-0.691	-0.937
2-Nitrotoluene	0.87	1.11	0.00	0.27	1.032	-0.394	-0.366	-0.325	-0.376	-0.378	-0.341
1,3-Xylene	0.62	0.52	0.00	0.16	0.998	-1.482	-0.822	-1.300	-1.229	-0.841	-1.411
Chlorobenzene	0.72	0.65	0.00	0.07	0.839	-1.000	-0.697	-0.916	-0.910	-0.703	-0.966
1,4-Xylene	0.61	0.52	0.00	0.16	0.998	-1.495	-0.849	-1.334	-1.276	-0.838	-1.240
2-Chlorophenol	0.85	0.88	0.32	0.31	0.898	-0.355	-0.233	-0.464	-0.328	-0.233	-0.425
3-Chlorophenol	0.91	1.06	0.69	0.15	0.898	-0.335	-0.197	-0.465	-0.309	-0.204	-0.427
4-Chlorophenol	0.92	1.08	0.67	0.21	0.898	-0.294	-0.163	-0.418	-0.271	-0.172	-0.383
2-Nitrophenol	1.02	1.05	0.05	0.37	0.949	-0.358	-0.327	-0.202	-0.340	-0.329	-0.309
4-Nitrophenol	1.07	1.72	0.82	0.26	0.949	-0.038	0.025	-0.104	-0.026	0.034	-0.085
3-Hydroxybenzaldehyde	0.99	1.38	0.74	0.40	0.932	-0.032	0.004	-0.100	-0.052	0.020	-0.072
Acetone	0.18	0.70	0.04	0.49	0.547	-0.031	0.098	0.125	-0.024	0.090	0.094
Aniline	0.96	0.96	0.26	0.41	0.816	0.171	0.250	0.288	0.709	0.902	×
Anthracene	2.29	1.34	0.00	0.26	1.454	-0.670	-0.633	-0.571	-0.642	-0.636	-0.615
Tetrachlorobenzene	1.18	0.92	0.00	0.00	1.206	-0.824	-0.702	-0.585	-0.788	-0.709	-0.802
Pyrene	2.81	1.71	0.00	0.29	1.585	-0.567	-0.577	-0.458	-0.541	-0.581	-0.463
Caffeine	1.50	1.60	0.00	1.33	1.364	1.737	1.679	1.951	1.688	1.635	1.889
1,4-Toluidine	0.92	0.95	0.23	0.45	0.957	0.172	0.252	0.304	0.790	1.022	-0.570
Theophylline	1.50	1.60	0.54	1.34	1.222	1.130	1.125	1.351	1.131	1.121	1.309
Thymine	0.80	1.00	0.44	1.83	0.893	0.841	0.927	0.920	0.855	0.946	0.990
Ethylacetate	0.11	0.62	0.00	0.45	0.747	-0.815	-0.671	-0.850	-0.849	-0.692	-0.863
Uracil	0.81	1.00	0.44	1.00	0.752	1.027	1.181	1.222	1.038	1.191	1.235
Phenol	0.81	0.89	0.60	0.30	0.775	-0.304	-0.179	-0.391	-0.268	-0.177	-0.343

× no elution within 300 minutes

Table 2. Regression coefficients of the LFER equation and correlation coefficient *R*.

Column	Mobile phase	Model	*v*	*a*	*b*	*s*	*e*	*c*	*R*
RN CF6	hex/IPA/TFA	C.M.	-1.623	-0.035	1.669	0.995	0.278	-0.659	0.957
	80/20/0.0	±95% CI	0.704	0.247	0.312	0.364	0.328	0.475	
	(*v/v/v*)	*p*	0.000	0.776	0.000	0.000	0.092	0.008	
		O.M.	**-1.169**	**x**	**1.596**	**1.128**	**x**	**-0.944**	**0.953**
		±95% CI	0.382		0.302	0.298		0.307	
		p	0.000		0.000	0.000		0.000	
IP CF6	hex/IPA/TFA	C.M.	-0.849	0.040	1.571	0.492	0.001	-0.423	0.963
	80/20/0.0	±95% CI	0.530	0.194	0.219	0.284	0.253	0.358	
	(*v/v/v*)	*p*	0.003	0.675	0.000	0.001	0.991	0.022	
		O.M.	**-0.887**	**x**	**1.573**	**0.518**	**x**	**-0.401**	**0.963**
		±95% CI	0.282		0.211	0.228		0.232	
		p	0.000		0.000	0.000		0.001	
DMP CF7	hex/IPA/TFA	C.M.	-1.252	-0.222	1.781	0.835	0.211	-0.710	0.954
	80/20/0.0	±95% CI	0.737	0.263	0.289	0.368	0.329	0.499	
	(*v/v/v*)	*p*	0.002	0.096	0.000	0.000	0.203	0.007	
		O.M.	**-0.703**	**x**	**1.729**	**0.803**	**x**	**-1.048**	**0.950**
		±95% CI	0.391		0.287	0.305		0.315	
		p	0.001		0.000	0.000		0.000	
RN CF6	hex/IPA/TFA	C.M.	-1.019	0.092	1.556	0.759	0.130	-0.823	0.967
	80/20/0.5	±95% CI	0.623	0.216	0.237	0.299	0.275	0.421	
	(*v/v/v*)	*p*	0.002	0.395	0.000	0.000	0.342	0.000	
		O.M.	**-0.918**	**x**	**1.535**	**0.891**	**x**	**-0.892**	**0.965**
		±95% CI	0.316		0.231	0.244		0.254	
		p	0.000		0.000	0.000		0.000	
IP CF6	hex/IPA/TFA	C.M.	-0.651	0.178	1.480	0.467	0.014	-0.654	0.961
	80/20/0.5	±95% CI	0.600	0.212	0.228	0.293	0.266	0.411	
	(*v/v/v*)	*p*	0.034	0.097	0.000	0.003	0.916	0.003	
		O.M.	**-0.821**	**x**	**1.486**	**0.594**	**x**	**-0.553**	**0.957**
		±95% CI	0.311		0.226	0.242		0.254	
		p	0.000		0.000	0.000		0.000	
DMP CF7	hex/IPA/TFA	C.M.	-1.100	-0.099	1.654	0.910	0.153	-0.900	0.958
	80/20/0.5	±95% CI	0.731	0.259	0.278	0.358	0.325	0.500	
	(*v/v/v*)	*p*	0.004	0.445	0.000	0.000	0.347	0.001	
		O.M.	**-0.765**	**x**	**1.622**	**0.927**	**x**	**-1.110**	**0.956**
		±95% CI	0.368		0.267	0.287		0.300	
		p	0.000		0.000	0.000		0.000	

CI represents ±95% confidence interval; x, insignificant interaction; C.M., complete model of the LFER equation; O.M., optimal model of the LFER equation; *p*, statistical *p*-value. The *p*-values express probability of the error that the individual coefficient does not contribute to the model, i.e., *p*-values represent the significance of the individual coefficients.

PAPER V

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Characterization of new *R*-naphthylethyl cyclofructan 6 chiral stationary phase and its comparison with *R*-naphthylethyl β-cyclodextrin-based column

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ABSTRACT

Derivatized cyclofructans have been recently introduced as a new class of chiral selectors with great application potential. In this study, a *R*-naphthylethyl-functionalized cyclofructan 6 based chiral stationary phase (RN CF6 CSP) was used for separation of substituted binaphthyl catalysts in the normal phase HPLC mode. Dominant interaction types that play a role in the separation mechanism were revealed by a linear free energy relationship (LFER) method. In order to evaluate the contribution of the substituent on the cyclofructan structure to retention, the *R*-naphthylethyl-functionalized β-cyclodextrin (RN CD) CSP was chosen for comparison. Retention factors of 46 widely different solutes, with known solvation parameters, were determined on each of the columns under the same mobile phase compositions used for the enantiomeric separations. The LFER results showed that hydrogen bond acidity and polarity/polarizibility have the greatest impact on retention and enantioresolution on the RN CF6 CSP. The equal influence of the naphthylethyl substituent on the both CSPs was also confirmed while the effects of the basic cyclofructan *versus* cyclodextrin structures were different. The addition of trifluoroacetic acid to the hexane/propane-2-ol mobile phase was negligible on the RN CF6 CSP for the majority of atropoisomers except for one with ionizable functional groups. The RN CF6 column was shown to be more suitable for enantioseparation of the binaphthyl catalysts than the RN CD column. Higher retention offered by the latter CSP had no positive effect on the enantioresolution.

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1. Introduction

Enantiomeric separations have been the focus of considerable attention for nearly three decades due to their importance especially in areas of pharmaceutical, agrochemical and food science. HPLC with chiral stationary phases (CSPs) is far and away the most powerful and widely used technique for enantioselective separations at both the analytical and preparative scales. A variety of CSPs usually bonded or adsorbed to silica gel have been reported [1]. A new class of chiral selectors based on cyclofructan was introduced in 2009 and shown to have potential both for HPLC [2–4] and CZE [5]. Cyclofructans (CFs) refer to a group of macrocyclic oligosaccharides that consist of six or more β-(2→1) linked D-fructofuranose units [6,7]. Each fructofuranose unit contains four stereogenic centers and three hydroxyl groups. Native CFs have rather limited enantioselectivity in HPLC [2]. The CF hydroxyl groups can be derivatized with aliphatic or aromatic groups. These functionalized forms of CFs show improved and unique separation abilities over a wide range of analytes. Derivatization of native chiral molecules with aromatic moieties is a common strategy used to enhance their chiral recognition abilities [4]. The recently introduced RN CF6 column utilizes *R*-naphthylethyl-functionalized cyclofructan 6 (CF6, contains six fructofuranose units) as the chiral selector. This new CSP shows very good enantioselectivity toward a variety of enantiomers except for primary amines. As the chiral selector is covalently bonded to the silica gel support, this CSP is compatible with all common organic solvents. In principle it can be operated in all three modes – normal, reversed phase and polar organic. However, better resolution was achieved in the normal phase mode, due to higher selectivity, which also offers the potential for preparative separations [2].

Binaphthyl derivatives have been extensively used to control asymmetric processes. Their outstanding chiral discrimination abilities are derived from their rigidity and spatial arrangement [8,9]. The chirality of these compounds is caused by restricted rotation around the single bond in the binaphthyl skeleton [10,11]. Although the basic structure of the binaphthyl derivatives is similar, the substituents and their position significantly affect their properties. For more information about these compounds see Refs. [9,12–14].

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One of the comprehensive methods that allow characterization of stationary phase/separation systems and allows a better understanding of the relevant intermolecular interactions, which play a role in the separation processes, is the linear free energy relationship (LFER) [15]. The LFER can independently describe the contributions of individual interactions to the retention. The overall applicability of the LFER model has been presented in numerous reports in recent years (e.g. [16–21]).

One of the more widely accepted representations of the LFER was proposed by Abraham et al. [22] and now it is used in the following form:

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

where k is the solute retention factor. The independent variables in Eq. (1) are solute descriptors and denote specific solute properties: E is the solute excess molar refraction modeling the solute polarizability due to n- and/or π-electron pairs, S is the solute dipolarity/polarizibility parameter, A is the effective or overall hydrogen bond acidity, B is the effective or overall hydrogen bond basicity and V is the McGowan's characteristic molecular volume calculated from the solute structure [23–26]. The descriptors characterize properties of the solute molecules and account for the differences among them. A representative series of analytes must be selected to evaluate the chromatographic system. These compounds should be structurally diverse and the distribution of the individual descriptors should equally cover the whole range of interactions [27,28]. The coefficients in Eq. (1) are determined by multivariate regression analysis and reflect the individual types of molecular interactions acting in the given separation system. Since in HPLC Eq. (1) is applied to the distribution between two phases, the regression coefficients refer to differences between the phases, i.e., a given stationary phase and a fixed composition of the mobile phase. The c constant is the intercept obtained in the regression calculation; it depends on the separation system used but it does not reflect any interaction [29]. The value e reflects the difference in propensity of the stationary and the mobile phases to interact with solute n- and π-electron pairs; s reflects difference in dipolarity/polarizability between the phases; a refers to the difference in hydrogen bond basicity between the stationary and the mobile phases; b is equal to the difference in hydrogen bond donating properties and v reflects the difference in hydrophobicity between the stationary and the mobile phases.

As the LFER model characterizes the chromatographic system as a whole, comparisons of different stationary phases must be done at the same mobile phase composition. However, the mobile phase is an important factor affecting separation, therefore the characterization of a HPLC separation system should be performed under various mobile phase compositions. Complete or optimal model parameters can be obtained from the multivariate regression analysis. The complete model involves all the regression coefficients while the optimal model utilizes just the statistically significant values. Ordinary regression coefficients serve well for comparison of different stationary phases at the same mobile phase composition. Statistically derived standardized coefficients equilibrate influences of the different units, their mean values are zero, and the standard deviations (SDs) are the same for all of them. Therefore, the standardized coefficients are well-suited to analyze the various interactions within one separation system, composed of a given stationary phase and a mobile phase [30].

This work is focused on a study of separation properties of the new cyclofructan-based chiral stationary phase – *R*-naphthylethyl-functionalized cyclofructan 6 (RN CF6) CSP, and comparison of its separation abilities with *R*-naphthylethyl carbamoyl β-cyclodextrin (RN CD) CSP, using LFER method. These CSPs have the same substituent, *R*-naphthylethyl carbamate group, and isomeric saccharide units, six fructofuranose and seven glucopyranose units in CF6 and β-CD, respectively [31,32]. The paper is aimed at elucidating the molecular interaction mechanisms, i.e., revealing the types of interactions responsible for retention. The application of LFER to enantiomeric separations is not explicit as no chiral term is involved in the equation but the calculated regression coefficients can serve as a tool for estimation of the interactions "useful" for chiral discrimination. Separation performance of the new RN CF6 CSP is demonstrated on binaphthyl catalysts. Their structure seems to be well-suited for interaction with the naphthylethyl substituent of the chiral selector.

2. Experimental

2.1. Instrumentation

All chromatographic measurements were performed on Waters Alliance system (Waters Chromatography, Milford, MA, USA) consisting of a Waters 2695 Separation Module, a Waters 2996 Photodiode Array Detector, a Waters 717 plus Autosampler, and a Waters Alliance Series column heater. Empower software was used for process control and data handling. Chromatographic columns RN CF6 (*R*-naphthylethyl carbamate cyclofructan 6 CSP bonded to silica gel) and Cyclobond I 2000 RN (RN CD; *R*-naphthylethyl carbamate β-cyclodextrin CSP bonded to silica gel) were used in this work. The dimensions of both columns were 250 mm × 4.6 mm i.d.; particle size 5 μm. RN CF6 column has been prepared at the Department of Chemistry and Biochemistry, University of Texas at Arlington (Arlington, TX, USA). Cyclobond I 2000 RN column is a product of ASTEC (Whippany, NJ, USA). The columns and samples were thermostated at 25 °C. Detection was performed at 254 nm. The flow rate was 1 mL/min for all measurements.

2.2. Chemicals

Organic solvents of HPLC grade, n-hexane (hex), propane-2-ol (isopropanol, IPA) and methanol were products of Sigma–Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA; 99.8% purity) was purchased from Merck (Darmstadt, Germany). The solutes for LFER were of analytical grade purity and were purchased from Sigma–Aldrich (St. Louis, MO, USA). They were selected to cover a wide range of chemical properties. The list of the 46 solutes used and their corresponding solvation parameters are summarized in Table 1. The chiral compounds (binaphthyl catalysts) have been synthesized as racemates at the Department of Organic and Nuclear Chemistry, Faculty of Science, Charles University in Prague (Prague, Czech Republic) [8,9]. The structures of binaphthyl catalysts are shown in Fig. 1.

Stock solutions of solid test compounds were prepared in concentration of 1 mg/mL and stock solutions of liquid samples were diluted to obtain 20 μL/mL using methanol as a solvent.

Mobile phases were composed of hexane and propane-2-ol in various ratios and/or hexane and propane-2-ol mixtures with the small additions of trifluoroacetic acid.

2.3. Procedures

The retention times of the test solutes were measured in triplicates in all the chromatographic systems studied. The void volumes were determined using system peaks obtained by injection of n-hexane to individual separation systems. The retention times were calculated from the peak maxima. As the detector responses of the test analytes were kept rather low, the effect of peak shape was not critical in the evaluation of retention times used for the calculations. The average SD of sequential measurements of the retention factor did not exceed 1.5%. The regression coefficients of the LFER equation were obtained from a series of measurements of the retention

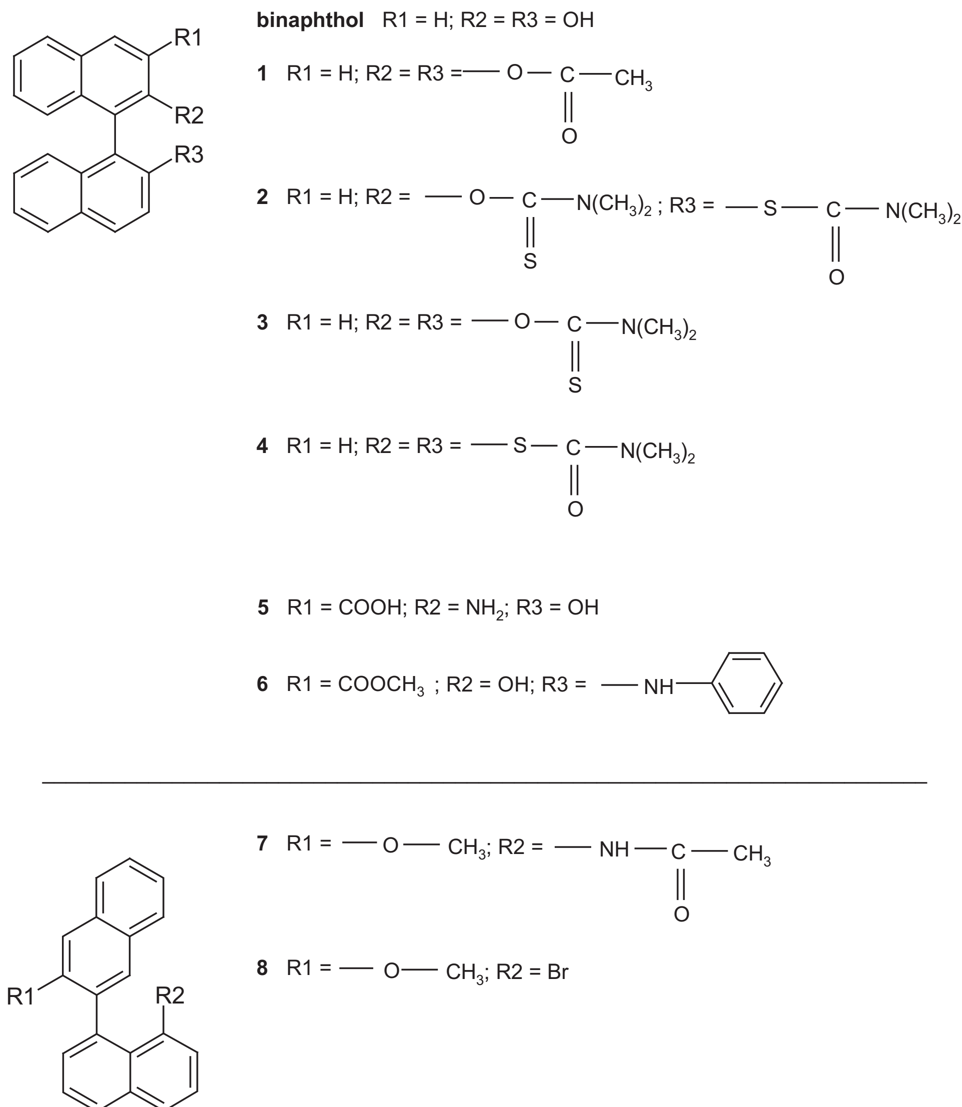

Fig. 1. Structures of the binaphthyl derivatives.

data of the set of 46 structurally different test solutes with known solvation parameters [23,28,33] that are shown in Table 1. The coefficient values were calculated for each separation system, i.e., CSP and mobile phase composition, by multiple linear regression analysis of log *k* against the solute descriptors using NCSS software (NCSS, Kaysville, UT, USA) [34]. The results were determined for both the complete model utilizing all regression coefficients and the optimal model handling just the statistically significant regression parameter values.

3. Results and discussion

3.1. Enantiomeric separation of binaphthyl catalysts

The cyclofructan-based RN CF6 column, a representative of this novel class of CSPs, was chosen for enantioseparation of substituted binaphthyl catalysts in the normal phase separation mode. The column was selected because the structure of the chiral selector, the cyclofructan derivative (*R*-naphthylethyl group), seemed to be compatible with the structure of the binaphthyl derivatives. Mobile phases were composed of hexane and propane-2-ol in various ratios and also the addition of trifluoroacetic acid into these mobile phases was tested. The influence of the addition of the acid to the hex/IPA 80/20 (*v*/*v*) mobile phase is obvious from the results summarized in Table 2. The presence of TFA in the mobile phase did not have significant effect on the retention and separation of the majority of the analytes and their atropoisomers. The acidified mobile phase just slightly reduced the retention values.

One exception to this was analyte 5 which exhibited an exceptional behavior (Fig. 2). This binaphthyl derivative has accessible ionizable groups, and so the addition of TFA significantly improved its enantioresolution (the *R*s increased from 0.14 to 1.43 without and with the acid, respectively, even though retention was reduced in the latter case). In addition, baseline resolution was achieved for atropoisomers of derivative 3 and partial separation was obtained for atropoisomers of binaphthol and solutes 1 and 2 (see Table 2) under both mobile phase compositions. Atropoisomers of analytes 4, 6, 7 and 8 were not separated in any chromatographic system tested. A comparison of the retention and separation of atropoisomers of compounds 2, 3 and 4 is interesting given the similarity of their substituents (see Fig. 1). Analytes 3 and 4 have their oxygen and sulfur moieties reversed. Analyte 2 has one substituent the same as analyte 3 and one substituent the same as analyte 4. Analyte 3 has the lowest retention factor and the highest enantioresolution.

Analyte 4 had the greatest retention among all these analytes but its atropoisomers were not separated. The retention factor of

Table 1
Set of test analytes and their solvation parameters.

Analyte	*E*	*S*	*A*	*B*	*V*
Benzamide	0.99	1.50	0.49	0.67	0.973
2-Naphthol	1.52	1.08	0.61	0.40	1.144
Resorcinol	0.98	1.00	1.10	0.58	0.834
Benzophenone	1.45	1.50	0.00	0.50	1.481
Hydroquinone	1.00	1.00	1.16	0.60	0.834
1,2-Cresol	0.84	0.86	0.52	0.31	0.916
Benzonitrile	0.74	1.11	0.00	0.33	0.871
1,3-Cresol	0.82	0.88	0.57	0.34	0.916
Benzylalcohol	0.80	0.87	0.33	0.56	0.916
Benzene	0.61	0.52	0.00	0.14	0.716
Naphthalene	1.34	0.92	0.00	0.20	1.085
Pyrocatechol	0.97	1.07	0.85	0.52	0.834
Dibenzothiophene	1.96	1.31	0.00	0.18	1.379
Ethylbenzene	0.61	0.51	0.00	0.15	0.998
Benzaldehyde	0.82	1.00	0.00	0.39	0.873
Toluene	0.60	0.52	0.00	0.14	0.857
1,2-Toluidine	0.97	0.92	0.23	0.45	0.957
Biphenyl	1.36	0.99	0.00	0.22	1.324
Phenanthrene	2.06	1.29	0.00	0.26	1.454
1,2,3-Trichlorobenzene	1.03	0.86	0.00	0.00	1.084
3-Nitrotoluene	0.87	1.10	0.00	0.25	1.032
1,2-Xylene	0.66	0.56	0.00	0.16	0.998
Bromobenzene	0.88	0.73	0.00	0.09	0.891
2-Nitrotoluene	0.87	1.11	0.00	0.27	1.032
1,3-Xylene	0.62	0.52	0.00	0.16	0.998
Chlorobenzene	0.72	0.65	0.00	0.07	0.839
1,4-Xylene	0.61	0.52	0.00	0.16	0.998
2-Chlorophenol	0.85	0.88	0.32	0.31	0.898
3-Chlorophenol	0.91	1.06	0.69	0.15	0.898
4-Chlorophenol	0.92	1.08	0.67	0.21	0.898
2-Nitrophenol	1.02	1.05	0.05	0.37	0.949
4-Nitrophenol	1.07	1.72	0.82	0.26	0.949
3-Hydroxybenzaldehyde	0.99	1.38	0.74	0.40	0.932
Acetone	0.18	0.70	0.04	0.49	0.547
Aniline	0.96	0.96	0.26	0.41	0.816
Anthracene	2.29	1.34	0.00	0.26	1.454
Tetrachlorobenzene	1.18	0.92	0.00	0.00	1.206
Pyrene	2.81	1.71	0.00	0.29	1.585
Caffeine	1.50	1.60	0.00	1.33	1.364
1,4-toluidine	0.92	0.95	0.23	0.45	0.957
Pyridine	0.63	0.84	0.00	0.52	0.675
Theophylline	1.50	1.60	0.54	1.34	1.222
Thymine	0.80	1.00	0.44	1.83	0.893
Ethylacetate	0.11	0.62	0.00	0.45	0.747
Uracil	0.81	1.00	0.44	1.00	0.752
Phenol	0.81	0.89	0.60	0.30	0.775

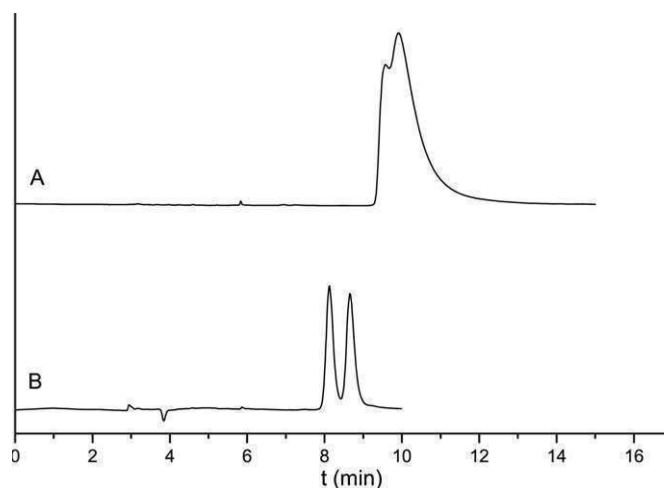

Fig. 2. Chiral separation of analyte 5 atropoisomers on the RN CF6 column. Mobile phase compositions: A: n-hexane/IPA 80/20 (v/v); B: n-hexane/IPA/TFA 80/20/0.5 (v/v/v); column and sample temperatures: 25 °C; flow rate: 1 mL/min; UV detection: 254 nm.

analyte 2 is between those of analytes 3 and 4, and the resolution of its atropoisomers is higher than that of analyte 4 ($R = 0$) and lower than that of analyte 3. It can be concluded that the substituent type of analyte 3 has a positive effect on the chiral discrimination process whereas the substituent of analyte 4 has the opposite effect.

β-Cyclodextrin based RN CD column also was tested for the separation of these compounds with mobile phases of the same compositions as those used with the cyclofructan-based CSP (Table 2). The RN CD CSP was selected because the β-cyclodextrin derivative contains the same substituent (naphthylethyl carbamoyl group) on an oligosaccharide base as the RN CF6 chiral selector. A strong effect of the addition of TFA to the mobile phase on retention of the binaphthyl derivatives was observed on the RN CD column. The retention was substantially reduced with the acidified mobile phase. Unfortunately, no significant enantioseparation of the atropoisomers was observed on this CSP in the normal separation mode. Only atropisomers of compound 5 were partly resolved in the mobile phase with TFA (Fig. 3).

A comparison of the results obtained on these two chiral stationary phases shows that the RN CF6 column is more suitable for enantioseparation of the binaphthyl catalysts. The retention of

Table 2
The chromatographic parameters of the chiral analytes using CF6 RN and CD RN columns; k_1, retention factor of the first eluted atropoisomer; α, selectivity; R, resolution.

Mobile phase	Analyte	CF6 RN			CD RN		
		k_1	α	R	k_1	α	R
hex/IPA/TFA 80/20/0.0 (v/v/v)	Binaphthol	1.38	1.08	1.08	7.67	1.00	0.00
	1	0.94	1.10	1.32	1.56	1.00	0.00
	2	2.17	1.07	0.98	4.02	1.00	0.00
	3	1.35	1.16	1.95	3.09	1.00	0.00
	4	6.34	1.00	0.00	8.26	1.00	0.00
	5	2.17	1.05	0.14	8.55	1.00	0.00
	6	3.19	1.00	0.00	5.52	1.00	0.00
	7	0.89	1.00	0.00	2.17	1.00	0.00
	8	0.43	1.00	0.00	0.85	1.00	0.00
hex/IPA/TFA 80/20/0.5 (v/v/v)	Binaphthol	1.47	1.08	1.04	3.11	1.00	0.00[a]
	1	0.89	1.10	1.44	1.26	1.00	0.00
	2	2.02	1.07	0.95	3.04	1.00	0.00
	3	1.29	1.17	1.87	2.42	1.00	0.00
	4	5.38	1.00	0.00	5.83	1.00	0.00
	5	1.76	1.10	1.43	3.44	1.05	0.31
	6	2.76	1.00	0.00	4.23	1.00	0.00
	7	0.88	1.00	0.00	1.61	1.00	0.00
	8	0.44	1.00	0.00	0.56	1.00	0.00

[a] Slight indication of enantioseparation.

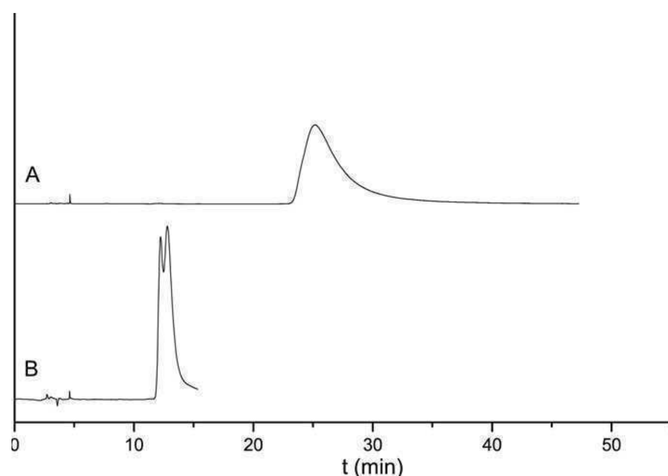

Fig. 3. Chiral separation of analyte 5 atropoisomers on the RN CD column. Mobile phase compositions: A: n-hexane/IPA 80/20 (v/v); B: n-hexane/IPA/TFA 80/20/0.5 (v/v/v). For other experimental conditions see caption to Fig. 2.

all the tested analytes was much higher if the RN CD column was used. Nevertheless, the higher retention had no positive impact on enantioresolution.

3.2. Comparison of RN CF6 and RN CD columns using LFER model

The LFER model was used in order to better understand the interactions involved in the retention and separation of the substituted binaphthyl derivatives on the two chiral stationary phases. This approach can reveal contributions of the individual interaction types, obtained from regression coefficients v, a, b, s and e of Eq. (1).

The LFER data for the separation systems previously discussed in Section 3.1 are summarized in Table 3. The table shows the regression coefficients obtained from the complete and the optimal models of LFER and also the standardized coefficients of the optimal model. Correlation of the LFER data with experimental results (plot of the experimental $\log k$ against calculated $\log k$) achieved for the set of 46 structurally diverse test solutes on the both CSPs did not show any serious outliers, correlation coefficients of linear regression fits were always higher than 0.93. Lower p-values of the optimal model than those of the complete model (see Table 3) show that the regression coefficients of the former model are more significant [34]. Due to the fact that insignificant interactions are also included in the complete LFER model, the optimal model offers a better tool for comparison of the chromatographic systems studied in this work.

Negative values of the regression coefficient v (representing difference in hydrophobicity between the stationary and the mobile phases) obtained for all four separation systems show that hydrophobic interactions are preferred in the mobile phase. This is legitimate in a normal separation mode where the hydrophobicity of a mobile phase is higher than that of a stationary phase. The v values show a clearly defined trend, i.e., the absolute v value decreases if TFA is added to the mobile phase no matter what CSP is used. If we compare the results for the tested columns obtained in the separation systems with the same mobile phase the difference in hydrophobicity between the stationary and the mobile phases is higher for the system employing RN CF6 column. Thus, the RN CF6 stationary phase can be considered more polar than the RN CD column. Obtained results (v values) correspond with the retention of binaphthyl derivatives (Table 2), i.e., their retention factors are higher in chromatographic systems with the RN CD column. This confirms the general idea that cyclofructans do not possess central hydrophobic cavity as do cyclodextrins [35,36]. The regression coefficient a (describing difference in hydrogen bond basicity) is statistically insignificant for RN CF6 column in the both mobile phases tested. So this type of interaction is not involved in the optimal model. That means that the basicity (ability to accept protons) of this stationary phase is low and similar to that of the

Table 3
Regression coefficients of the LFER equation and correlation coefficient R.

Column	Mobile phase	Model	v	a	b	s	e	c	R
RN CF6	hex/IPA/TFA 80/20/0.0 (v/v/v)	C.M.	−1.623	−0.035	1.669	0.995	0.278	−0.659	0.957
		±95% CI	0.704	0.247	0.312	0.364	0.328	0.475	
		p	0.000	0.776	0.000	0.000	0.092	0.008	
		O.M.	−1.169	x	1.596	1.128	x	−0.944	0.953
		±95% CI	0.382		0.302	0.298		0.307	
		p	0.000		0.000	0.000		0.000	
		STD	−0.407	x	0.638	0.572	x	0.000	
	hex/IPA/TFA 80/20/0.5 (v/v/v)	C.M.	−1.019	0.092	1.556	0.759	0.130	−0.823	0.967
		±95% CI	0.623	0.216	0.237	0.299	0.275	0.421	
		p	0.002	0.395	0.000	0.000	0.342	0.000	
		O.M.	−0.918	x	1.535	0.891	x	−0.892	0.965
		±95% CI	0.316		0.231	0.244		0.254	
		p	0.000		0.000	0.000		0.000	
		STD	−0.338	x	0.705	0.482	x	0.000	
RN CD	hex/IPA/TFA 80/20/0.0 (v/v/v)	C.M.	−1.381	0.506	0.928	1.033	0.211	−0.342	0.931
		±95% CI	0.898	0.337	0.331	0.503	0.416	0.647	
		p	0.004	0.004	0.000	0.000	0.309	0.290	
		O.M.	−1.037	0.553	0.901	1.148	x	−0.581	0.929
		±95% CI	0.591	0.324	0.327	0.449		0.443	
		p	0.001	0.001	0.000	0.000		0.012	
		STD	−0.365	0.280	0.434	0.519	x	0.000	
	hex/IPA/TFA 80/20/0.5 (v/v/v)	C.M.	−0.963	0.284	1.455	0.831	0.194	−0.896	0.966
		±95% CI	0.600	0.211	0.255	0.293	0.265	0.414	
		p	0.002	0.010	0.000	0.000	0.147	0.000	
		O.M.	−0.623	0.330	1.421	0.906	x	−1.114	0.964
		±95% CI	0.382	0.204	0.255	0.279		0.291	
		p	0.002	0.002	0.000	0.000		0.000	
		STD	−0.234	0.189	0.590	0.484	x	0.000	

CI represents ±95% confidence interval. x, insignificant interaction; C.M., complete model of the LFER equation; O.M., optimal model of the LFER equation; STD, standardized coefficients of the optimal LFER equation; p, statistical p-value. The p-values express probability of the error that the individual coefficient does not contribute to the model, i.e., p-values represent the significance of the individual coefficients.

mobile phases. On the other hand, the regression coefficients *a* are significant in both the systems with the RN CD column. The *a* values are positive, i.e., this type of interaction contributes to the retention. Lower value of the coefficient *a* was observed in the system with TFA in the mobile phase. TFA can occupy some of the proton accepting sites on the stationary phase and in this way reduce their availability to the analytes. The regression coefficients *b* (expressing hydrogen bond acidity difference) are positive in all chromatographic systems studied. The hydrogen bond acidity of the RN CF6 and RN CD CSPs is always higher than that of the both mobile phases used. Moreover, RN CF6 CSP has higher hydrogen bond donating properties than RN CD CSP, or groups that can exhibit this type of interaction may be better accessible on the former CSP. The addition of TFA to the mobile phase has an interesting effect on the values of coefficient *b*. While this is almost negligible in the system with RN CF6 CSP, the H-bond acidity increases significantly on RN CD column if TFA is present in the mobile phase. This result correlates with the retention values of binaphthyl derivatives in Table 2. Sorption of mobile phase components on the surface of a stationary phase substantially influences interaction possibilities offered by the stationary phase. TFA is a hydrogen donor and as such it can increase the *b* values if sorbed on the stationary phase. The obtained results indicate that sorption of TFA is much higher on the RN CD CSP. This corresponds to the decrease of hydrogen bond basicity (coefficient *a* values) observed on this column after addition of TFA.

The *s* regression coefficient (describing difference of polarity/polarizibility) is positive for all the studied separation systems because many polar and polarizable groups are available on the both CSPs. The value of this coefficient decreases by addition of TFA to the mobile phase for the both chiral stationary phases to a similar extent. The acid competes with the analytes for the interaction sites of this type on the stationary phases and in this way decreases their retention.

The *e* coefficient is statistically insignificant in all the chromatographic systems tested. That means that propensity of the stationary and the mobile phases to interact with solute n- and π-electron pairs is equal. It can be even further deduced that this type of interaction is related to the same substituent on CF or CD and has equal effects in all the separation systems compared in this work.

4. Conclusions

A new naphthylethyl substituted cyclofructan-based chiral stationary phase was investigated in the normal phase separation mode. Advantageous enantiodiscrimination capabilities of this CSP over a cyclodextrin based column with the same substituent (bonded to a different oligosaccharide structure) were demonstrated on a group of binaphthyl catalysts. Addition of trifluoroacetic acid to the mobile phase composed of hexane and propane-2-ol did not affect retention or enantioresolution of the analytes to a great extent with the exception of compound 5 (with ionizable functional groups) which exhibited lower retention but higher resolution of its atropoisomers in the system with TFA.

The interactions participating in the retention and enantioseparation mechanism were identified using LFER. As mentioned above the LFER model cannot reveal directly the difference between interactions of individual enantiomers that would be related to their different spatial arrangements. However, the LFER results denoted that the main impact on the interaction mechanism on the RN CF6 CSP have hydrogen bond acidity and polarity/polarizibility, while hydrogen bond basicity and interactions with n- and π-electron pairs seem to be insignificant and dispersion interactions are preffered in the mobile phase. The negligible effect of the addition of TFA to the mobile phase on the contribution of hydrogen bond acidity to the interaction mechanism also was confirmed by LFER.

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5. CONCLUDING REMARKS

The dissertation thesis is focused on characterization of interaction possibilities of modern unconventional HPLC columns and their application potential. The thesis is a commented collection of four papers published in respected journals with impact factors and one as a contribution to international conference proceedings.

The first part of the thesis is devoted to RP-HPLC columns based on zirconium dioxide. The methods for efficient separation of biologically active nonapeptides were developed successfully and proved the separation potential and the stability of ZrO_2-based columns. The substantial effect of mobile phase composition on the separation on zirconia-based columns was confirmed by a detailed study of the chromatographic behaviour of model analytes. Furthermore, HPLC separation systems with ZrO_2-based polystyrene packing were characterized by different approaches, which provided the possibility to recognize interactions participating in the separation process. Empirically based chromatographic tests described the basic properties of the systems – hydrophobicity and polarity. The application of the LFER model to the separation systems that differed in the aqueous component of the mobile phase resulted in the evaluation of individual interaction types. It was shown that the interactions involved in the separation systems with zirconia-based column strongly depend on the constituents of the mobile phase. The utilization of a set of basic compounds covering a wide range of hydrophobicity and pKa constants revealed the contribution of ion-exchange interactions participating in the separation systems with Zr-PS column.

The results showed that the various tests used for the characterization of silica-based RP chromatographic columns can also be used successfully for ZrO_2-based stationary phases.

Newly developed cyclofructan-based chiral stationary phases are the subject matter of the second part of the thesis. Three different CF-based CSPs, i.e., IP-CF6, RN-CF6 and DMP-CF7, were investigated from the point of their enantioseparation capabilities. Effects of the derivatization groups on cyclofructan and its core size, structure of the analyte and also the mobile phase composition on retention

and enantiorecognition were studied. The evaluation and comparison of the tested chiral packings were done by injection of structurally diverse chiral compounds, i.e., binaphthyl derivatives possessing axial chirality and certain chiral pharmaceuticals with different functional groups. Some efficient enantioseparations of binaphthyl derivatives were achieved on the three CF-based CSPs. DMP-CF7 performed exceptional enantioselectivity toward this group of analytes. The application of the CF-based CSPs to enantioseparations of selected chiral pharmaceuticals gave insight into the complex retention and enantiorecognition process. The LFER model gave evidence of the forces affecting the interaction mechanism on the CF-based CSPs. The same types of interaction in a different extent were shown to be preferred, i.e., hydrogen bond acidity and dipolarity/polarizibility, increasing the retention, and hydrophobicity as a retention reducing factor. Some differences of the concerned stationary phases due to different cyclofructan core size and/or the substituents were also shown by the LFER model. Application of LFER to separation systems with RN-CF6 CSP and RN-CD CSP revealed the fundamental difference derived from the different basic structure of CF *vs.* CD and the consequences on the interaction mechanism.

The LFER model was shown to have potential to characterize the prevailing interactions in HPLC separation systems. Based on the LFER results retention of analytes can be estimated or even predicted if molecular descriptors are known. Although the application of the LFER model to chiral separations is not explicit, as it does not relate to information on enantioselective behaviour of analytes, this approach can be useful for estimation of the interactions participating in the enantiorecognition process.

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LIST OF PUBLICATIONS

I. Janečková, L., Sobotníková, J., Tesařová, E., Bosáková, Z.: Využití moderních reverzních stacionárních fází na bázi oxidu zirkoničitého pro analýzu bioaktivních peptidů. *Chemické listy* 104 (2010) 334–342.

II. Janečková, L., Kalíková, K., Bosáková, Z., Tesařová, E.: Study of interaction mechanisms on zirconia-based polystyrene HPLC column. *Journal of Separation Science* 33 (2010) 3043–3051.

III. Janečková, L., Kalíková, K., Bosáková, Z., Tesařová, E.: Chiral separation of binaphthyl catalysts using new chiral stationary phases based on derivatized cyclofructans. *Book of Proceedings, 6th International Students Conference - Modern Analytical Chemistry*, p. 62–65. Charles University in Prague, Faculty of Science, 2010. ISBN 978-80-7444-005-2.

IV. Janečková, L., Kalíková, K., Vozka, J., Armstrong, D. W., Bosáková, Z., Tesařová, E.: Characterization of cyclofructan-based chiral stationary phases by linear free energy relationship. *Journal of Separation Science*, accepted for publication (July 2011).

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LIST OF PRESENTATIONS

Oral contributions:

Janečková, L.: Využití zirkoniových kolon pro separace biologicky aktivních peptidů; Workshop SPE and HPLC organized by Sigma-Aldrich, Prague, Czech Republic, 3.6.2008.

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Janečková, L.: Separations of biologically active peptides on zirconia-based columns; 5th International Students Conference – Modern Analytical Chemistry, Prague, Czech Republic, 21. – 22.9.2009.

Janečková, L.: HPLC columns based on zirconium dioxide and their applications; 10th International Symposium and Summer School on Bioanalysis, CEEPUS, Zagreb, Croatia, 7. – 14.7.2010.

Janečková, L.: Chiral separation of binaphthyl catalysts using new chiral stationary phases based on derivatized cyclofructans; 6th International Students Conference – Modern Analytical Chemistry, Prague, Czech Republic, 23. – 24.9.2010.

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