CHARLES UNIVERSITY IN PRAGUE

FACULTY OF SCIENCE

Study programme: Analytical chemistry

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Characterization of Modern HPLC Columns
and Their Application Potential

DISSERTATION THESIS

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Prague, 2011

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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]:

$$\log k = c + vV_{X} + a\sum \alpha_2^{H} + b\sum \beta_2^{H} + s\pi_2^{H} + rR_2 \tag{1}$$

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

$$\log k = c + vV + aA + bB + sS + eE \tag{2}$$

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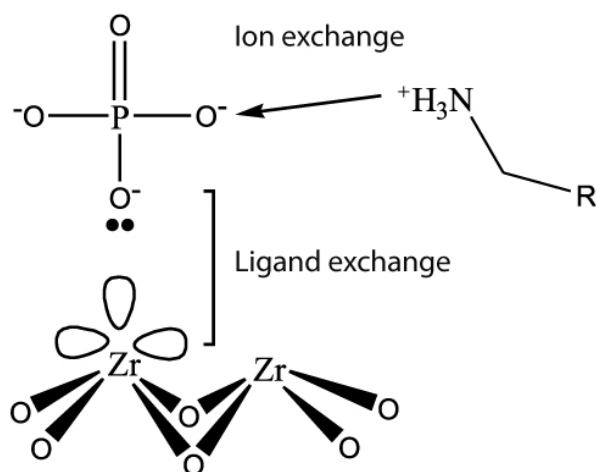
Fig. 3.1. Interaction possibilities on the surface of zirconium dioxide.

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.

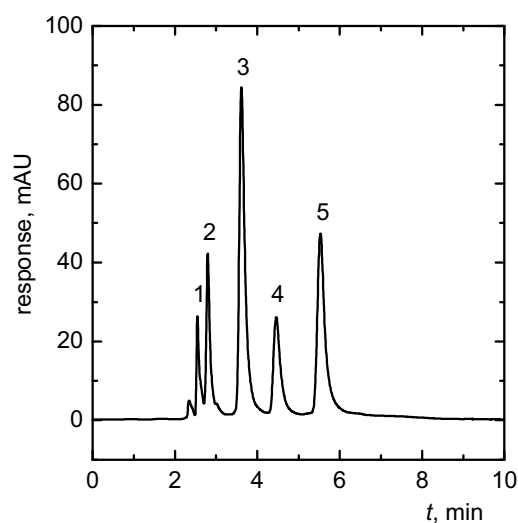Peak identification: 1 – uracil, 2 – oxytocin, 3 – Arg-vasotocin, 4 – Arg-vasopressin, 5 – Lys-vasopressin.
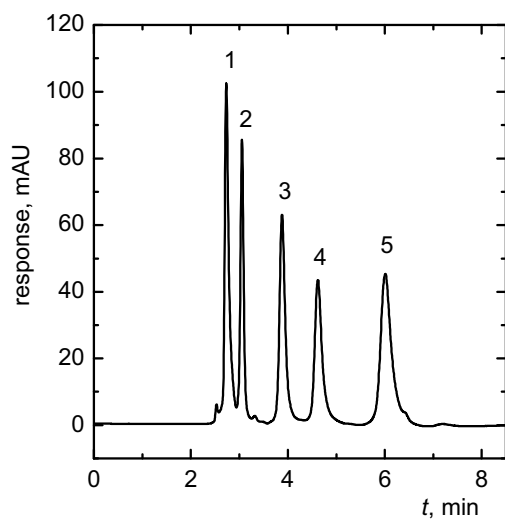

Fig. 3.3. Optimized separation of nonapeptides on Discovery Zr-PS column; mobile phase ACN/50 mM phosphate buffer, pH 9.0, 5/95 (*v/v*); temperature 45 °C; flow rate 1 mL/min; detection 214 nm.

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	$1/2\,(k_{\text{toluene}} + k_{\text{benzene}})$ in 60 % MeOH
Polarity	α (DETA*/anthracene) in 100 % ACN	α (aniline/phenol) in 55 % MeOH	$1+3\,[\alpha(\text{aniline/phenol})-1]$ 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.
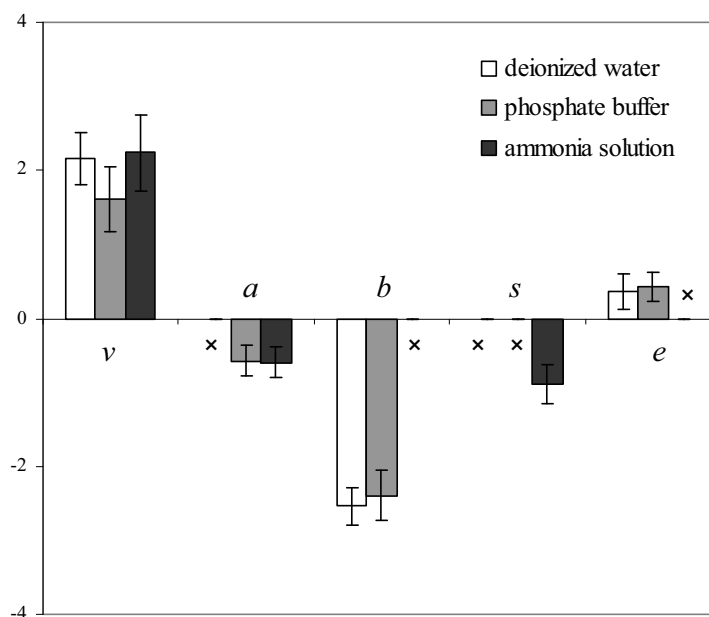
Fig. 3.4. Comparison of the regression coefficients (with their standard errors) of the optimal LFER model for the studied separation systems composed of the Zr-PS column and mobile phases composed of ACN/aqueous component (water, phosphate buffer or ammonia solution), 20/80 (v/v). Symbol $\times$ means 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

PAPER II

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].
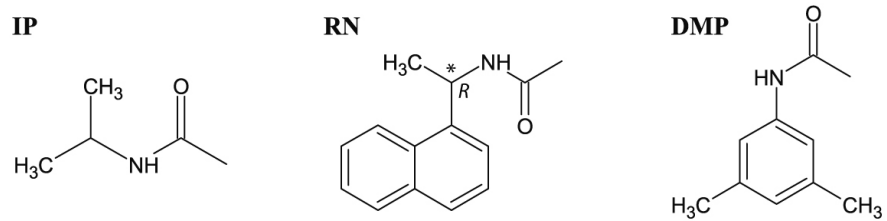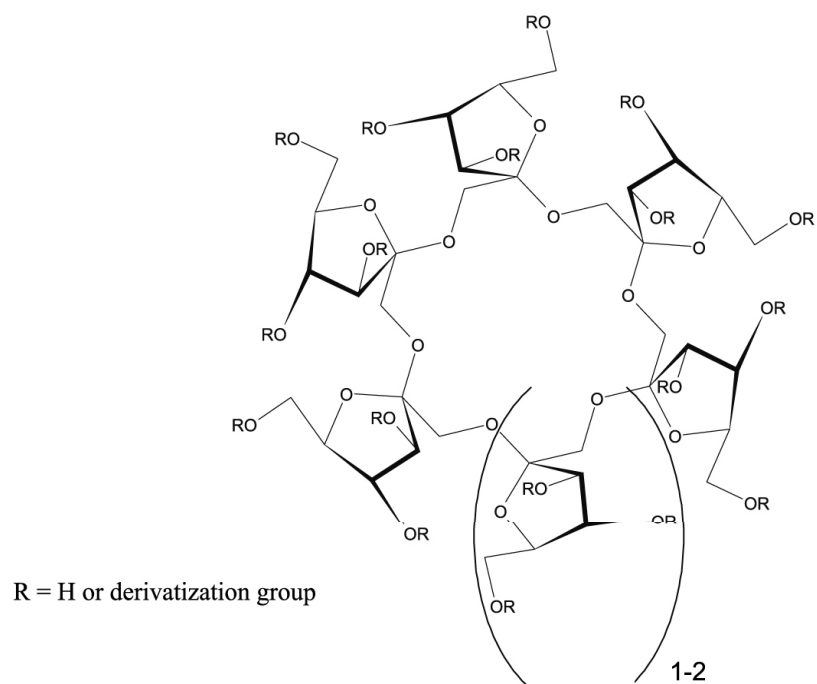
R = H or derivatization group

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).

binaphthol   R1 = H; R2 = R3 = OH

1 R1 = H; R2 = R3 = — O — C(=O) — CH₃

2 R1 = H; R2 = — O — C(=S) — N(CH₃)₂ ; R3 = — S — C(=O) — N(CH₃)₂

3 R1 = H; R2 = R3 = — O — C(=S) — N(CH₃)₂

4 R1 = H; R2 = R3 = — S — C(=O) — N(CH₃)₂

5 R1 = COOH; R2 = NH₂; R3 = OH

6 R1 = COOCH₃ ; R2 = OH; R3 = — NH — (phenyl)

7 R1 = — O — CH₃; R2 = — NH — C(=O) — CH₃

8 R1 = — O — CH₃; R2 = Br

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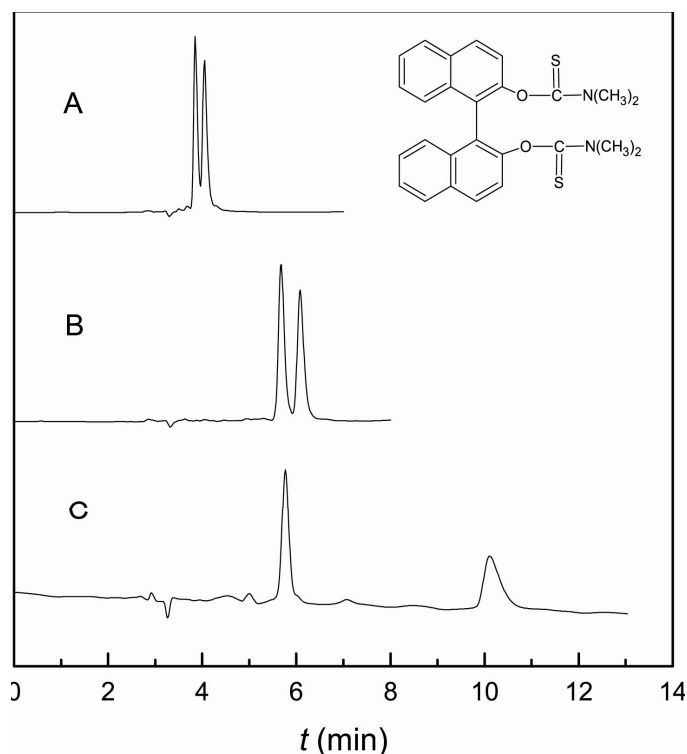
Fig. 4.3. Chiral separation of atropisomers of analyte 3 on the three CF-based chiral stationary phases. A: IP-CF6 column; B: RN-CF6 column; C: DMP-CF7 column. Mobile phase hex/IPA/TFA 60/40/0.5 (*v/v/v*); temperature: 25 °C; flow rate: 1 mL/min; UV detection: 254 nm.

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].
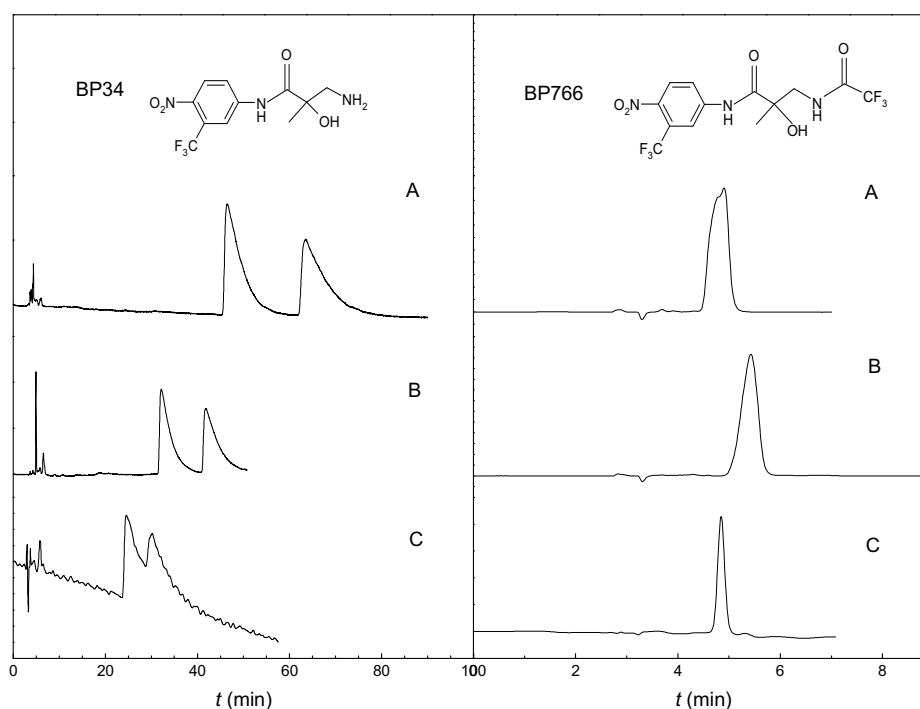

Fig. 4.4. Chromatographic behaviour of BP34 and BP766 on the three CF-based chiral stationary phases. A: IP-CF6 column; B: RN-CF6 column; C: DMP-CF7 column. Mobile phase hex/IPA/TFA 60/40/0.5 (*v/v/v*); temperature: 25 °C; flow rate: 1 mL/min; UV detection: 254 nm.

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.

4.5. References III

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PAPER III

PAPER IV

PAPER V

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.

ACKNOWLEDGEMENTS

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).

V. Kalíková, K., Janečková, L., Armstrong, D. W., Tesařová, E.: Characterization of new *R*-naphthylethyl cyclofructan 6 chiral stationary phase and its comparison with *R*-naphthylethyl β-cyclodextrin-based column. *Journal of Chromatography A* 1218 (2011) 1393–1398.

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.: 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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Poster presentations:

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