UNIVERSITY OF PÉCS

Doctoral School of Chemistry

Chemical and kinetic characterization of fast liquid chromatographic reversed-phase stationary phases

PhD thesis

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1. Introduction

Among separation techniques, liquid chromatography (LC) has become one of the most commonly used analytical methods, both in research and routine analyses. In recent years, thanks to a number of technical developments, the use of ultra-high performance liquid chromatography (UHPLC) has become increasingly widespread, in addition to high-performance liquid chromatography (HPLC) instruments available up to now. The fundamental difference between the two instruments is that while the upper pressure limit for HPLC was 400 bar, UHPLC instruments can operate up to 1000-1200 bar.

This step was necessary because when using so-called sub-2 μm (below 2 μm) stationary phases with smaller particle diameters, the required pressure will increase. To reduce the pressure drop, UHPLC systems usually use short columns.

Thus, the analysis time can be reduced during separations due to the shorter column design and the higher optimal linear velocities that can be applied, whereas the column efficiency is not necessarily increased. In order to increase the efficiency of the separation, longer columns are needed, which can provide a higher theoretical plate number, but due to the increase in pressure and the frictional heat generated by the flow of the mobile phase, one must consider the limitations of the column and the equipment.

In parallel with the evolution of the apparatus, manufacturers are constantly innovating in the field of stationary phases to meet the needs of users. In addition to the continuous reduction of particle sizes, it is important to mention the emergence of core-shell and monolithic stationary phases in addition to non-porous, perfusion and fully porous particles.

In addition to advances in the structure of stationary phases, great emphasis is also being placed on the ability to use them over a wider pH range, for which Waters was the first to develop a silica gel-based stationary phase (XTerra) that included methylene groups in the skeleton. These columns proved to be much more stable under basic conditions than earlier silica gels. Also, later, Waters used triethoxy silane coupled with ethylene to form the sol-gel. These columns were marketed as XBridge.

Kirkland and colleagues developed a special bonded phase using two adjacent silanol groups. This product is marketed by Agilent Technologies as Zorbax Extend and is available in a variety of column configurations. Phenomenex has also developed a process to make their stationary phases applicable over an extended pH range. In the final stage of silica production, a new composite particle is created with an organic layer.

This maintains the mechanical strength and efficiency of the particle while the organic shell protects the particle.

The possibility to separate basic compounds under reversed phase liquid chromatography conditions was also an important development criterion. For this purpose, so-called base-deactivated reversed phase stationary phases are now available, where the number of free silanol groups remaining after surface modification.

2. Motivation and problem statement

The main objectives of my research were:

1. To determine the hydrophobic retention (k_{AB}), surface coverage ($\alpha_{(CH2)}$), steric selectivity ($\alpha_{t/o}$) and hydrogen bonding capacity ($\alpha_{C/P}$) of reversed-phase fast liquid chromatography columns following the steps of the Tanaka test.
2. The effect of residual silanol groups on the stationary phases can be investigated using different test compounds. In conventional tests, these new types of base-deactivated stationary phases are producing increasingly better results, and it is therefore worthwhile to select more test components that are more sensitive to silanol groups. Retention (k) and asymmetry ($USP\ Tf$) values are determined for each column.
3. The obtained values are used to compare the different types of stationary phases.
4. 3-3 columns with (5, 10, 15 cm long) fully porous and core-shell packings are compared using flow-reversal method.
    - Firstly, the efficiency of the columns (H_{min}) is measured, the optimum flow rate is determined;
    - determination of the diffusion coefficient of the sample component (small molecule) using the peak parking method;
    - performing flow-reversal measurements at different "sections" of the columns;
    - comparison of the band broadening effects for different columns (heterogeneity of the packing bed, effect of column ends).
5. I am looking for a correlation between the effect of column packing procedure and the heterogeneity of the packing bed by a core-shell column. I will investigate whether the flow-reversal method is feasible with large molecules. This would help to partially eliminate the effect of intra-porous diffusion on the band broadening.
6. I will use an electron microscope to examine the frit of the columns used earlier to see if there is any destructive effect of the packing procedure on the frit and particle structure.

3. Experimental and methods

During my research, chromatographic measurements were performed on a Waters Acquity I Class UPLC.

Tanaka test

The method introduced by Tanaka and co-workers can be used to characterize C_{18} reversed phase stationary phases. The method can be used to estimate the amount of alkyl chains, hydrophobicity, steric selectivity and hydrogen bonding capacity and the amount of free silanol groups remaining on the silica surface. This test can be performed by injecting different test compounds without destroying the stationary phase. The retention coefficients, separation factors and asymmetry factors of the chromatographic peaks of the sample components provide information on the above-mentioned properties of the stationary phases.

Peak parking measurement

By peak parking measurements an unretained component is injected into the column. As soon as the sample band reaches the center of the column, the flow is stopped for a given time (parking time), during which the sample is free to diffuse in the axial direction. A linear relationship between the parking time and the peak width is observed and the effective diffusion coefficient of the sample can be calculated from the slope of the plotted line.

The flow-reversal method

The flow-reversal method is used with aim of determination of axial heterogeneity of the packing bed and thus the differences between the column ends. By the measurements, a sample of an unretained marker (thiourea, insulin) was injected from a given direction into the column. As the sample band travelled a given distance in the column, the flow was stopped, and the column was inverted. After reversal, the flow was restarted, and the sample eluted at the same end of the column as it entered. During the flow-reversal, the sample band diffuses freely in the axial direction, so the variance values of the flow-reversal must be corrected for the calculated variance from the peak parking measurements.

4. Results

The results obtained in the Tanaka test show that the tested C18 reversed phase columns (InertSustain, InertSustainSwift, InertCore, MotoTower, Kinetex, Kixetex EVO) have rather similar properties (in terms of alkyl chain amount, hydrophobicity, steric selectivity, hydrogen bonding capacity), although the stationary phases are fundamentally different - such as monolithic, core-shell or fully porous particles. The higher $\alpha_{(C/P)}$ value indicate the presence of free silanol groups remaining on the silica gel surface, but this value was also quite similar for the tested columns. In order to correctly determine the amount of silanol groups, more sensitive test compounds had to be used. These measurements allowed us to observe differences between the columns. The best peak shapes were obtained with InertSustain and InertSustainSwift, which have completely porous particles. The Monotower column also gave excellent results with the more sensitive test compounds. This is probably due to its unique pore structure. The solid-core/core-shell type columns such as InertCore, Kinetex and Kinetex EVO proved to be the least successful in the tests.

In all cases, flow-reversal measurements showed that the peaks become more symmetrical and narrower than the peaks obtained without reversing the flow. This is due to the elimination of the multipath dispersion effect and the heterogeneity of the radial flow profile.

The column end efficiency and the packed bed efficiency - i.e. the local plate heights – show differences; the columns are heterogeneous, but the difference is negligible and we cannot be sure whether the column inlet or outlet performs better. The value of the local plate height increases as the length of the column increases. By comparing these local plate heights to the total plate height values for the column, we can conclude the effect of frit and column end structure near the frit is greater for Cortecs columns. In addition, we can conclude that shorter columns can be packed with better efficiency. The results show that column length affects heterogeneity, and the shorter the column, the more significant the effect of frit. Accordingly, although shorter columns have a more homogeneous packing bed and lower plate height values than longer columns, the overall efficiency of short columns is worse than that of long columns, since the relative contribution of frit is more significant for short columns.

The molecular diffusion of macromolecules is much smaller than of small molecules. Thus, radial heterogeneity in the packed bed has less influence on their migration and consequently a larger peak compression effect is observed for macromolecules when the flow is reversed. The flow-reversal experiment with macromolecules also allowed to measure the difference between the column ends and the difference between the local plate height values depending on the direction in which the column was used. However, due to the Taylor-Aris dispersion, the experimental conditions had to be significantly changed compared to those with small molecules, so the experiments were much longer than with thiourea or other small molecule markers.

Electron microscopic measurements of the column frits showed no evidence of damage to their structure. However, it was clearly visible how heterogeneous this sintered frit structure was, into which the packing particles could penetrate, increasing the wall effect in the measurements, which enhances the band broadening effect of the frit.

5. Thesis points

1. Based on the results of the Tanaka test, we conclude that different types of stationary phases show significant differences when using sensitive test compounds. Differences were observed in the presence of silanol groups and other useful sensitive test compounds should be identified in the future. I have also found that due to differences in the silica gels used for stationary phases (carbon content, specific surface area, porosity) between manufacturers, I do not see the possibility of developing an universal testing method in the future.

2. I observed peak compression effects when measuring with UHPLC columns. The peaks observed with the reversed flow were always narrower and more symmetric than the peaks obtained without reversing the flow. This is due to the compensation of the multipath dispersion effect and the radial heterogeneity of the flow

3. After the flow reversal measurements, comparing the local plate heights with the total plate height values for the column, I found that the effect of frit and the column end structure near the frit was larger for the Cortecs columns. Furthermore, one can conclude that shorter columns can be packed with better bed efficiency. The results show that the column length has an influence on heterogeneity and the shorter the column, the more significant the effect of the frits is.

   Accordingly, although the packed beds in shorter columns are more homogeneous and the local plate heights are smaller for short than for long columns, the overall efficiency of the short columns is worse than that of the long columns since the relative contribution of the frits is more substantial for short columns. The results also show that 70-80% of the peak broadening is due to the contribution of the system and the column ends, while the remaining 20-30% of the broadening is due to the effect of the packing bed (with the exception of the XBridge 15 cm column, where this ratio is around 50-50%)

4. The flow-reversal experiment with macromolecules was also used to measure the difference between the column ends and the local plate height values, depending on the direction in which the column was used. I found that the design of the column input end (fit, frit, and fill structure at the end of the column) contributed more to the variance

than the column output end. This difference is probably due to the column packing procedure, as the particle density and the structural design near the ends of the column should be different. Furthermore, the band broadening caused by the two column ends was greater than the band broadening effect of the packing bed. The magnitude of the peak compression was found to be significant, but the Taylor-Aris dispersion made the measurements much more complex and longer than for small molecules, so it is still preferable to choose the small molecule option for future flow-reversal measurements.

5. Electron microscopy was used to investigate whether column packing causes any damage to the frit and particle structure. I did not detect any damage in the frit material itself or in the packing particles, but it was clearly visible that the packing particles penetrate into the frit structure, increasing the wall effect and thus the band broadening.

6. Publications

Publications related to this thesis

1. D. Zelenyánszki, A. Mester, A. Felinger
   Flow-Reversal experiments with macromolecules to measure column end efficiency and bed heterogeneity
   Chromatographia, 82 (2019), 1303–1309 **IF: 1,596**

2. D. Zelenyánszki, N. Lambert, F. Gritti, A. Felinger
   The effect of column packing procedure on column end efficiency and on bed heterogeneity – experiments with flow-reversal
   J. Chromatogr. A 1603 (2019), 412 – 416. **IF: 4,049**

3. D. Zelenyánszki, N. Lambert, N. Tanaka, A. Felinger
   Chemical characterization of stationary phases for fast liquid chromatography – with focus on the residual silanol groups
   J. Chromatogr. A - under submission **IF: 4,759**

4. D. Zelenyánszki, A. Felinger
   The impact of column hardware on efficiency in liquid chromatography
   LCGC Europe 33 (2020), 498-504 **IF: 0,529**

Posters and presentations related to this thesis

1. Zelenyánszki D., Lambert N., Tanaka N., Felinger A.
   A szilanol-hatás vizsgálata fordított fázisú folyadékkromatográfiában alkalmazott állófázisok esetében
   V. Interdiszciplináris Doktorandusz Konferencia 2016. Pécs; Magyarország 198-199. o.

2. D. Zelenyánszki , N. Lambert, N. Tanaka, A. Felinger
   Chemical characterization of stationary phases for fast liquid chromatography
   16th ISSSB (CEEPUS) International Symposium and Summer School on Bioanalysis 2016. Varsó; Lengyelország, P-29

3. Zelenyánszki D., Lambert N., Tanaka N., Felinger A.
   Fordított fázisú folyadékkromatográfiás állófázisok jellemzése
   Elválasztástudományi Vándorgyűlés 2016. Kecskemét; Magyarország, P-42

4. D. Zelenyánszki , N. Lambert, N. Tanaka, A. Felinger
   Chemical characterization of stationary phases for fast liquid chromatography
   17th ISSSB (CEEPUS) International Symposium and Summer School on Bioanalysis 2017. Ohrid; Macedónia, O-09

5. A. Mester, D. Zelenyánszki, A. Felinger
   Comparison of various reversed phase stationary phases by manual and automatized flow-reversal method
   17th ISSSB (CEEPUS) International Symposium and Summer School on Bioanalysis 2017. Ohrid; Macedónia, O-17

6. A. Mester, D. Zelenyánszki, A. Felinger
   Comparison of various reversed phase stationary phases by flow-reversal method
   XI. Balaton Symposium on High Performance Separation Methods 2017. Siófok; Magyarország, P-46

7. N. Lambert, D. Zelenyánszki, A. Felinger
   Characterization of stationary phases for fast liquid chromatography
   XI. Balaton Symposium on High Performance Separation Methods 2017. Siófok; Magyarország, L-42

8. D. Zelenyánszki, N. Lambert, F. Gritti, A. Felinger
   Effect of column packing procedure on the column end structure and bed heterogeneity – experiments with flow-reversal
   32nd International Symposium on Chromatography (ISC) 2018. Cannes-Mandelieu, Franciaország, PS-07-15

9. Zelenyánszki D., Lambert N., Gritti F., Felinger A.
   Az oszloptöltés hatása gyors folyadékkromatográfiás állófázisok heterogenitására – kísérletek „oszlopforgatásos” módszerrel
   Elválasztástudományi Vándorgyűlés 2018. Tapolca; Magyarország, E-28

10. D. Zelenyánszki, A. Mester, A. Felinger
    Flow-reversal experiments with macromolecules to measure column end efficiency and bed heterogeneity
    V. Interdiszciplináris Doktorandusz Konferencia 2016. Pécs; Magyarország, 170. o.

11. D. Zelenyánszki, N. Lambert, F. Gritti, A. Felinger
    Effect of column packing procedure on the column ends structure and on bed heterogeneity - experiments with flow-reversal
    International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC) 2019. Milánó; Olaszország, P-440

12. D. Zelenyánszki, A. Felinger
    Efficiency, bed heterogeneity and column and structure of segmented columns – experiments with flow-reversal
    XII. Balaton Symposium on High Performance Separation Methods 2019. Siófok; Magyarország, P-14

Posters and presentations not related to this thesis

5. Simon J., Rédei Cs., Zelenyánszki D., Felinger A.
   Komplex mintakeverékből származó komponensek eredetének meghatározása és vizsgálata alterációs analízissel
   Elválasztástudományi Vándorgyűlés 2018. Tapolca; Magyarország, E-22

6. Simon J., Rédei Cs., Zelenyánszki D., Felinger A.
   Two-dimensional correlation and alteration analysis is chromatography International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC) 2019. Milánó; Olaszország, OC-154

7. Simon J., Rédei Cs., Zelenyánszki D., Felinger A.
   Exploring two-and three-dimensional chromatographic data with alteration analysis
   XII. Balaton Symposium on High Performance Separation Methods 2019. Siófok; Magyarország, L-16