Modern stationary phases in reversed-phase liquid chromatography for analyzing peptides and proteins

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1. Introduction

In the field of high-performance liquid chromatographic analysis of peptides and proteins, with the development of the stationary phase technology several new columns filled with materials having different particle morphology and surface chemistry were marketed in the last decade. In practical terms, the characterization of the new columns is very important. In my Ph.D. work modern reversed-phase high performance liquid chromatographic stationary phases were tested and applied to real life samples.

The kinetic performance of chromatographic columns is often assessed based on van Deemter [1], where the height equivalent to a theoretical plate (HETP or H) is plotted against the linear solvent velocity (u). The A term of this model (Eddy diffusion) is related to the inhomogeneity of the packed bed, the B term (longitudinal diffusion) to the effective sample diffusivity along the packed bed, and the C term to the solid-liquid mass transfer across the particles, respectively. For the comparison of packing materials having different particle diameters, Knox introduced reduced parameters, the reduced plate height (h) and the reduced linear solvent velocity (ν) instead of H and u. This theory takes into account that the A term depends on the linear velocity and therefore the A term in the van Deemter equation needs to be replaced with A·ν^{1/3} [2].

It has to be noted that these models lack permeability considerations. Kinetic plots offer a possible solution to this problem. Using these forms of representation not only packing materials with different physical-chemical characteristics or stationary phase morphology, but also the kinetic performance of different separation techniques can easily be compared. In 2005, Desmet et al. introduced the theory called kinetic plot method, which also uses the H – u data pairs, used also in case of van Deemter or Knox representations, for constructing multiple types of kinetic plots [3].

However, peptides and proteins are generally separated using gradient chromatographic methods. The general concept of peak capacity, which is widely used for assessing the separation efficiency in gradient conditions, was introduced by Giddings and applied by

Horváth to gradient chromatography. This parameter is a special measure of separation efficiency which uses the whole separation range and takes the deviation in peak width over the entire chromatogram into account. The comparison of the efficiency of different packing materials for a specific linear solvent velocity can be assessed by plotting the peak capacity against the gradient time \( t_g \).

Similar representations to Knox’s separation impedance concept can be constructed by the calculation of the peak capacity per unit pressure and per unit time (PPT) [4]. The separation impedance of different columns for a specific linear velocity can be evaluated by plotting the PPT against the gradient steepness \( \beta \). However, kinetic efficiency of chromatographic columns for a specific gradient steepness can be evaluated using gradient kinetic plots, for the construction of which Desmet et al. introduced a simple method in 2010 [5], [6].

In addition to the assessment of the kinetic performance of stationary phases, the characterization of the mechanisms responsible for the retention of the different solutes on a column is also of great importance. In 1989 Tanaka et al. published a non-destructive method, which is also widely used nowadays, for the evaluation of the retention behaviour of packing materials. This method provides information regarding hydrophobicity, steric selectivity, hydrogen bonding and ion-exchange characteristics of a column based on the separation of small-molecular-weight model components. In this procedure, alkylbenzenes possessing different alkyl groups are used for the estimation of hydrophobicity. For evaluating steric selectivity a solution of triphenylene and o-terphenyl was used. Hydrogen bonding capacity is evaluated based on the selectivity between caffeine and phenol. Ion-exchange capacity is assessed using alkylamines (pK\(_{a}\) > 9) for example procainamide and N-acetylprocainamide (NAPA) or benzylamine [7].

In order to have symmetrical peak shape and narrow peaks, which corresponds to high kinetic performance, column overloading has to be determined. This parameter of a stationary phase can be estimated using general equations [8], [9]. Overload of stationary phases in RP-HPLC separations is always more significant with ionised compounds such as proteins or peptides.

Widepore stationary phases with 200 – 300 Å average pore size are widely used in RP-HPLC for separating peptides and proteins. Packing materials having smaller pore diameters can only be applied effectively in the separation of smaller proteins or peptides. Proteins can be injected directly into the chromatographic system (intact protein analysis) but several procedures contain some form of sample preparation (denaturation, oxidation, reduction, enzymatic cleavage), which has to be carried out prior to analysis. One of the most frequently applied methods in the field of protein analysis for the evaluation of the primary structure of proteins is peptide mapping, which is often carried out by means of RP-HPLC. During sample preparation certain peptide bonds are cleaved chemically or more frequently using enzymes. At the end of this procedure a mixture of peptide fragments arise, the complete separation of which is often almost impossible in one dimension. The situation is often similar in natural systems such as the protein content of wheat even without any sample preparation, because of the high number of different components. Therefore, in such cases it is essential to use methods providing very high resolution.

2. Experimental

During the experimental work, the kinetic performance and retention behaviour of the Phenomenex Aeris WIDEPORE XB-C18 and C4 columns, filled with 3.6 μm core-shell particles, was evaluated first. Isocratic kinetic performance was assessed according to the theory of Knox (Eq. 1) using small-molecular-weight model components (estradiol and ivermectin) and a relatively small protein (insulin, M ~ 5.7 kDa):

\[ h = A \cdot \nu^{1/3} + \frac{B}{\nu} + C \cdot \nu \]

where \( h \) is the reduced plate number and \( \nu \) is the reduced linear velocity. The \( A \) is the Eddy diffusion term, \( B \) is the longitudinal diffusion term and \( C \) is the solid-liquid mass transfer resistance term of the Knox equation.

The Aeris WIDEPORE XB-C18 columns with different internal diameters (2.1 and 4.6 mm, respectively) were compared by means of \( h_{\text{min}} \) values.

In the next experimental section, the kinetic performance of the Aeris WIDEPORE XB-C18 and five other commercially available, 150 mm long narrow-bore columns was compared under gradient conditions. The comparison was carried out using peak capacity – gradient time, peak capacity per unit pressure and per unit time – gradient steepness and gradient kinetic plots. In the latter representation the column dead time was plotted against the peak capacity.

Peak capacity and PPT values were calculated according to Eq. 2 and Eq. 3, respectively:

\[ n_c = 1 + \frac{t_g}{1.7 \cdot W_{50\%}} \]  

\[ \text{PPT} = \frac{n_c}{t_g \cdot \Delta P} \]

where \( n_c \) is the peak capacity, \( t_g \) is the gradient time, \( W_{50\%} \) is the peak with measured at half height of the peaks, PPT is the peak capacity per unit pressure and per unit time, \( \Delta P \) is the backpressure, which arises in a column with \( L \) length when \( u \) linear velocity is applied.

For the construction of gradient kinetic plots the dead time of a column was calculated according to the procedure proposed by Desmet et al. (Eq. 4):

\[ t_0 = \frac{\Delta P_{\text{max}}}{\eta} \cdot \left( \frac{K_v}{u^2} \right) \]

where \( t_0 \) is the dead time of a column, \( K_v \) is the column permeability, \( u \) is the linear velocity, \( \eta \) is the maximum mobile phase viscosity during the gradient program and \( \Delta P_{\text{max}} \) is the maximum allowable backpressure of a column.
Later, the retention mechanisms on the Phenomenex Aeris WIDEPORE XB-C18 and C4 and on the Waters BEH300 C18 and C4, 150 mm long, narrow-bore columns was compared by means of the separation of small-molecular-weight model components (according to a modified version of the original Tanaka procedure) and test proteins.

The loading capacity of the Aeris WIDEPORE XB-C18 and C4 and that of the Waters BEH300 C18 and C4 columns was also evaluated by injecting different amounts of model proteins (M ~5.7 – 29.1 kDa). According to the predefined arbitrary criteria, overloading occurred when the relative change in peak width and tailing exceeded 10%.

After the characterisation of the Aeris WIDEPORE packing material, stationary phases (Ascentis Express Peptide ES-C18 (100 mm x 3 mm, 2.7 µm, 160 Å), Waters Acquity CSH C18 (100 mm x 3 mm, 1.7 µm, 130 Å) and Phenyl-Hexyl (100 mm x 3 mm, 1.7 µm, 130 Å) having moderate pore sizes were applied for the separation of tryptic digests of model proteins. The development and optimization of the chromatographic methods were carried out by means of the DryLab chromatographic software. Despite using optimized chromatographic methods, the co-elution of multiple peaks occurred in every case. It was also assessed whether in such a situation the peak heights or the peak areas are better candidates for representing the amounts of the different compounds.

In the last section, the development and application of a coupled-column chromatographic method, which uses two Aeris WIDEPORE XB-C18 (150 mm x 4,6 mm, 3,6 µm, 300 Å, ρ = 0,87) and an Aeris WIDEPORE C4 (150 mm x 4,6 mm, 3,6 µm, 300 Å, ρ = 0,87) connected in series for the separation of gliadin protein fraction of wheat proteins, is presented. In these experiments samples of ten different wheat varieties cultivated on three different plots of land separately were involved. The chromatographic data were processed using multivariate exploratory data analysis algorithms (principal component analysis, hierarchical clustering and nearest mean classification). It was evaluated whether wheat varieties having different gliadin protein profiles can be differentiated from each other based on the results of the coupled-column chromatographic method.
3. Results

The Aeris WIDEPORE XB-C18 stationary phase consists of 3.6 µm, superficially porous particles. The thickness of the porous layer is ~0.2 µm, which means that the volume fraction of the porous shell in these particles is about 34%.

Knox plots of the standard-bore and narrow-bore, 150 mm long Aeris WIDEPORE XB-C18 columns were constructed based on the experimental data coming from isocratic measurements of small-molecular-weight components (estradiol and ivermectin). The obtained $h_{\text{min}}$ values were equal to 1.4 and 1.7-1.8 for the 4.6 and 2.1 mm internal diameter columns, respectively. These values are close to those reported in the literature for shell particles. The achievable lowest $h$ value for insulin (~5.7 kDa) measured at 35 °C and 50 °C were below 2 whatever the column internal diameter and temperature. The global minimum of the Knox plot for insulin was not reached in the applied linear velocity range.

Real-life protein separations are always carried out under gradient conditions. Therefore, the kinetic performance of six narrow-bore, 150 mm long columns (two columns packed with wide pore conventional fully porous particles (Agilent Zorbax 300SB-C18, 5 µm, 300 Å; Phenomenex Jupiter C18, 3 µm, 300 Å), two columns packed with sub-2 µm fully porous particles (Waters Acquity BEH300 C18, 1.7 µm, 300 Å; Hypersil Gold C18, 1.9 µm, 175 Å), one column packed with the new 3.6 µm wide-pore core-shell particles (Aeris WIDEPORE XB-C18) and one column of 2.7 µm 160 Å core-shell particles (Ascentis Express Peptide ES-C18)) was evaluated in gradient separation of model proteins (insulin, cytochrome c, myoglobin, bovine serum albumin (BSA)). In a first instance, peak capacity values calculated at 0.25 mL/min and 0.4 mL/min flow rates for 10 – 110 min gradient times were compared. The Aeris WIDEPORE and Acquity BEH300 materials showed higher separation power than the other columns for large proteins but provided similar efficiency for small proteins such as insulin. At elevated flow rates or with long gradient times, the Acquity material often outperformed the other columns. However, due to the very low backpressure generated by the 3.6 µm core–shell particles, the Aeris WIDEPORE material surpassed the other columns based on peak capacity per time and pressure unit, irrespectively of the applied mobile phase flow rate and gradient steepness.

The effect of mobile phase velocity on peak capacity was assessed using gradient kinetic plots. Similar performance was obtained in case of the different columns for insulin, but the difference between columns became more evident with increasing size of the model proteins. The Acquity BEH300 C18, the Aeris WIDEPORE XB-C18 and the Jupiter C18 columns
proved to be significantly better for the separation of larger proteins cytochrome c (12.4 kDa) and BSA (66.8 kDa) than the other stationary phases.

These 150 mm long narrow-bore columns were also evaluated for the separation of real-life samples of filgrastim (18.8 kDa) and related proteins (e.g.: oxidized and reduced forms). The performance of these columns could be ranked in the following order: Aeris WIDEPORE XB-C18 > Acquity BEH300 C18 > Peptide ES-C18 > Jupiter C18 > Zorbax 300 SB-C18 > Hypersil Gold C18. This result confirmed the theoretical investigations previously described. The Aeris WIDEPORE XB-C18 column was successfully applied also for the separation of reduced and digested (papain) monoclonal antibody.

The goal of the next study was the characterization of the Aeris WIDEPORE XB-C18 and C4 stationary phases (i.e. particle size, particle size distribution, ligand density, silanol activity, hydrophobic selectivity) and the evaluation of their loading capacity and retention behaviour with model proteins and real-life protein samples. The tests were also carried out using the Acquity BEH300 C18 and C4 packing materials. Finally, the results of the four 150 mm long narrow-bore columns were compared. Using the Tanaka test and model proteins it was demonstrated that strong interaction mechanisms (ion exchange) were predominant on the Aeris WIDEPORE while hydrophobic interaction was the driving force for retention on the Acquity BEH300. This is a reasonable explanation to the fact that, despite the lower pore volume of the Aeris WIDEPORE material, the retention factor of proteins possessing both hydrophobic and charged amino acid residues was very close on the investigated columns. In accordance with the previous results, the kinetic performance of the Aeris WIDEPORE was also found to be similar to that of the Acquity BEH300 column. This is surprising since the strong interactions such as ion exchange and hydrogen bonding should contribute strongly to band broadening because of the slow kinetics of these secondary interactions. This might be compensated by the excellent mass transfer characteristics afforded by the thin porous layer at the surface of the superficially porous particles in case of the Aeris WIDEPORE stationary phase (~0.2 µm for a 3.6 µm particle).

The loading capacity of the Aeris WIDEPORE XB-C18 and C4 and that of the Acquity BEH300 C18 and C4 was evaluated by injecting different amounts of model proteins of varying sizes and charge states. There is a variety of factors that could explain the differences in loading capacity for proteins between columns (e.g. silanol activity, ligand density, carbon load, pore size, total pore volume). The experimental results showed that the Aeris WIDEPORE material possesses on average 2 to 4-fold lower loading capacity compared to the Acquity BEH300 columns. This corresponds approximately to the difference in stationary
phase volume between the two sorbents. It has to be noted that in the normal operating range the Aeris WIDEPORE stationary phases provided better absolute kinetic performance in several cases.

The Aeris WIDEPORE and Acquity BEH300 packing materials were designed for the separation of proteins. Stationary phases with moderate pore sizes on the other hand can only be considered for the separation of peptides and smaller proteins. Therefore, the Ascentis Express Peptide ES-C18 (100 mm x 3 mm, 2.7 µm, 160 Å), Waters Acquity CSH C18 (100 mm x 3 mm, 1.7 µm, 130 Å) and Phenyl-Hexyl (100 mm x 3 mm, 1.7 µm, 130 Å) columns were tested in RP-HPLC peptide mapping. Based on the theory of Guiochon et al., the effect of power functions on the comparison of the chromatographic results of the peptide separations was investigated. Both peak heights and areas were used for representing the amounts of the different components. Similarity of samples was evaluated by means of linear regression on the data set, in which the peak heights or areas of the different components in a sample were plotted against those of an other. The measure of similarity was the value of the coefficient of determination ($r^2$).

Efficient separations of tryptic digests of proteins were carried out using all the three columns involved in this study. The Ascentis Express Peptide ES-C18 reached $n_c = 176$, the Waters CSH C18 and Phenyl-Hexyl $n_c = 160$ and $n_c = 151$, respectively. The separations were optimized using the DryLab method development software. The peak tracking was carried out based on the results of the ten most intense peaks. The value of $r^2$ increased monotonically with the power of the function used for the transformation of the original data when peak areas were used for representing the amounts of the different components. The improvement of the observed resolution is a possible explanation to this result since the integration can be carried out much easier for better resolved peaks. On the other hand, this tendency was not observed using peak heights. The value of $r^2$ was not dependent in this case on the type of the power function. Based on these results peak heights are better candidates for representing the amounts of the different components in peptide separations when there are multiple critical peak pairs present on the chromatograms (the use of power function is not needed).

After the evaluation of the Aeris WIDEPORE XB-C18 and C4 stationary phases, they were applied for the separation of the gliadin fraction of wheat proteins. Ten wheat varieties were involved in this study, which were each cultivated on three separated plots of land simultaneously (60 samples in total considering also the repeated sample preparations). Two Aeris WIDEPORE XB-C18 (150 mm x 4.6 mm, 3.6 µm, 300 Å, $\rho = 0.87$) and a single Aeris WIDEPORE C4 (150 mm x 4.6 mm, 3.6 µm, 300 Å, $\rho = 0.87$) columns were connected in
series in order to increase the resolution. The separation was optimized using only a single Aeris WIDEPORE XB-C18 column after the comparison of the selectivity of the applied stationary phases by varying the separation temperature and the gradient time. In accordance with the previous results, there were no significant differences observed between the selectivity of the Aeris WIDEPORE XB-C18 and C4 phases. In the initial experiments gradient times of 10 and 30 min, and column temperatures of 30 °C and 50 °C were applied. The measurements of the real-life gliadin protein samples with the coupled-column chromatographic method were carried out using 60 min gradient time (3 times 20 min for a single column) and 40 °C oven temperature. In addition to the method of analysis of variances, multivariate exploratory data analysis algorithms were used to evaluate the chromatographic data. Based on the results of the coupled-column chromatographic method, even the wheat varieties having only minor differences in their gliadin profiles were successfully differentiated.

4. Thesis

1. The kinetic performance of the Aeris WIDEPORE XB-C18 stationary phase was first evaluated in this work. For the construction of Knox plots, small-molecular-weight (M < 1000 Da) components (estradiol and ivermectin) and insulin were applied. Under gradient conditions using model proteins (insulin, cytochrome c, myoglobin, bovine serum albumin) the peak capacity – gradient time, peak capacity per unit pressure and per unit time – gradient steepness and gradient kinetic plots were used for the evaluation of the kinetic efficiency of this packing material. It was demonstrated that very efficient and fast protein separations can be carried out using the Aeris WIDEPORE XB-C18 stationary phase. Due to the high permeability it often outperforms several commercially stationary phases in terms of kinetic efficiency [1].

2. The physical-chemical properties, retention behaviour and loading capacity of the Aeris WIDEPORE XB-C18 and C4 stationary phases were assessed. The results were compared with those of the Acquity BEH300 C18 and C4 materials. It was demonstrated that the strong hydrophilic interactions were predominant on the Aeris WIDEPORE XB-C18 and C4 phases, while the hydrophobic interaction was the main driving force of the retention on the Acquity BEH300 C18 and C4 columns [2].
3. The loading capacity of the Aeris WIDEPORE XB-C18 and C4, and the Acquity BEH300 C18 and C4 was compared using model 5.7 – 29.1 kDa proteins. The Aeris WIDEPORE XB-C18 and C4 stationary phases showed to possess 2 – 4 times lower loading capacity compared to the Acquity BEH300 columns [2].

4. The Ascentis Express Peptide ES-C18, the Waters Acquity CSH C18 and the Waters Acquity CSH Phenyl-Hexyl stationary phases were first compared in a peptide mapping application. Peak capacities of \( n_{c,ES-C18} = 176 \), \( n_{c,CSH C18} = 160 \) and \( n_{c,CSH Phenyl-Hexyl} = 151 \) were achieved using optimized (DryLab) chromatographic methods. These results show the potential of these columns for the separation of peptides [3].

5. It was demonstrated for the first time that in peptide separations having numerous critical peak pairs, peak heights are better candidates for the representation of amounts of the different components than peak areas. This principle was also applied in the separation of wheat proteins [3].

6. It was the first time when a coupled-column chromatographic method was used for the separation of gliadin fraction of wheat proteins. Applying two Aeris WIDEPORE XB-C18 and an Aeris WIDEPORE C4 columns connected in series, highly detailed chromatographic results were generated, which were evaluated using univariate and multivariate data analysis algorithms. Even the wheat varieties having only small differences in gliadin protein patter were successfully differentiated [4].

5. Application of the results

The results of the characterisation of the Aeris WIDEPORE stationary phases in terms of kinetic performance, retention behaviour and loading capacity can be used in the development of protein separations.

The coupled-column chromatographic method, which uses two Aeris WIDEPORE XB-C18 and an Aeris WIDEPORE C4 columns connected in series, could effectively be used in the bread making industry for the characterisation of wheat cultivars. Using multivariate data analysis algorithms for the evaluation of the results of this high-resolution method may be a useful tool in the process of wheat varietal identification.
6. Publications

Scientific papers (connected to the topic of the thesis)


Oral presentation (connected to the topic of the thesis)


Scientific papers (not connected to the topic of the thesis)


*Oral presentation (not connected to the topic of the thesis)*