High Performance Multidimensional Liquid Chromatography: Fundamental aspects, separation systems and data management

NKFIH project No. 104819

Final Report

Goal of the project

Most of the important problems that analysts must face nowadays involve the analysis of samples consisting of hundreds of components present in a very wide range of concentrations. Such problems frequently arise in proteomic, lipodomic and metabolomic researches. Separations by unidimensional chromatography are unable to provide acceptable analyses of these samples. For this reason, two-dimensional liquid chromatographic (2DLC) separations have attracted intense interest over the past years, due to the tremendous potential improvements in separation power that this method offers.

The efficient applicability of a two-dimensional separation depend on

- the retention mechanism and efficiency of the separations used at the two dimensions,
- the scheme used for implementing the coupling between dimensions,
- the robustness of the 2D method used, and
- the method for managing the large amount of data produced during 2DLC separations.

The aim of the work was the support of method development and routine analysis in two-dimensional liquid chromatography by investigating fundamental aspects and developing data management algorithms. The specific aims of the research project were to optimize 2DLC separations, quantify the robustness of 2DLC, study separation efficiency of multiprotic compounds, measure the isotherm of peptides, and produce an efficient data management algorithm for peak recognition and integration in 2DLC-DAD (DAD: diode array detection).

Results

In the summary bellow, references in square brackets refer to the corresponding publications presented in the *List of Publications* attached to the *Final Report* as a separate document.

Investigation of retention mechanism of multiprotic bioactive ions

The pressure of chromatographic separation affects the thermodynamic equilibrium of peptides and proteins due to the changes of their molecular volume during adsorption. Accordingly, in order to achieve the goal of this subproject, the pressure induced retention changes of model peptides possessing molecular weights between 1 and 4 kDa were studies experimentally and theoretically. Restrictor tubing was employed to generate pressures up to 1000 bar and experiments were conducted for mobile phase temperatures comprised between 30 and 80°C. It was found that the retention increased significantly with pressure, up to 200% for glucagon at around 1000 bar compared to 100 bar. The pressure induced change in retention was found to be temperature dependent and was more pronounced at 30°C than at 60 or 80°C. The obtained data were fitted to a theoretical model developed in this project. As a result, the fundamental thermodynamic parameters of adsorption (change of internal energy, molar volume and entropy) were determined. Our results allows to estimate easily the pressure induced change of equilibrium constant of adsorption under UHPLC conditions. Results were published in Ref. [1].

The effect of frictional heating was studied on retention behavior of solutes. Analysis of retention data revealed that the retention volumes were decreasing as the eluent flow rate increased. The results suggested that different axial temperature gradients formed in the column at different flow rates. The molar enthalpy and entropy change of solutes were determined from the linear van't Hoff plots under adiabatic conditions at low flow rate (0.05 ml/min). Based on these results, the Equilibrium Dispersive (ED) model of chromatography was solved in case of different temperature gradients under adiabatic conditions. Results of modeling confirmed the decrease of retention volumes. It was found, that the physical bands widths and release velocities increased as the steepness of axial temperature gradient increased. As a result of the sum of these effects, the apparent number of theoretical plates did not change as the slope of temperature gradient varied under adiabatic condition. It was shown however, that resolutions and peak capacities decreased due to the decrease of retention times. Results were published in Ref. [15].

The separation efficiencies of large biomolecules were analyzed under UHPLC conditions (large pressure drop in the column). The effect of pressure on the retention factor of solutes were integrated in the equilibrium dispersive model of chromatography that is solved by the Martin-Synge algorithm for different biomolecules. The calculated band profiles were analyzed. The effects of peak expansion and solute release processes on the efficiency were studied independently. It was shown that these two counteracting effects compensated each other and the overall chromatographic efficiency were independent of the magnitude of pressure drop. Results were published in Refs. [3]. Since large pressure drop always results in an increase of temperature, the effect of these factors should be studied simultaneously before publication in

a high impact journal.

Isotherm parameters of proteins were determined by the inverse method. In order to determine the adsorption isotherm, the overloaded band profiles of proteins were recorded after gradient elution. Taking into account the effect of pressure on the retention, a software was developed in C++ language for the calculations. The method based on the Martin-Synge algorithm. The software was validated by simulated band profiles. Isotherm data were being collected for insulin, bovine serum albumin and myoglobin. Isotherms of these proteins were determined from the overloaded band profiles. Results were published in Ref. [5].

Study of efficiency of stationary phases in liquid chromatography

The influence of width of particle size distribution (PSD) of HPLC stationary phases on chromatographic efficiency was studied. A theoretical framework was developed for calculation of chromatographic stationary phases with different particle size distributions of fully and superficially porous phases. The general rate model of chromatography was extended in order to calculate moments of peaks and heights equivalent to a theoretical plate in case of different PSDs. Our calculations demonstrated and verified that wide particle size distributions have significant effect on the separation efficiency of molecules. The differences of fully porous and coreshell phases regarding the influence of width of PSD were significant. The efficiencies of bimodal phases were also calculated. The results showed that these packings did not have any advantage over unimodal phases. Results were published in Refs. [6, 8].

The growing interest in the pharmaceutical industry for preparative chromatography that permits the purification of significant amounts of drug intermediates, peptides or proteins by eliminating closely related but unwanted compounds and impurities has made the optimization of the experimental conditions in preparative liquid chromatography a topic of serious concern. Recently, core-shell columns have been introduced to the market for preparative separations, which calls for further studies in this area. The use of coreshell phases in preparative separations was studied. The preparative separations were optimized for two compounds having bi-Langmuir isotherms. The differential mass balance equation of chromatography was solved by the Rouchon algorithm. Our results showed that as the size of the core increased, larger particles could be used in separations, resulting in higher applicable flow rates, and shorter cycle times. Due to the decreasing volume of porous layer, loadability of the column dropped significantly. As a result, the productivity and economy of the separation decreased. It was shown that the use of coreshell phases proved to be advantageous when the goal is to build preparative column for general purposes. Results were published in Ref. [13].

A new numerical method was introduced for the estimation of the surface heterogeneity below an adsorbed multilayer of the analyte in liquid chromatography. The calculation procedure based on the raw adsorption isotherm data points derived from frontal analysis exper-

iments. To permit the mapping of the nature of the analyte-surface interaction, a numerical procedure was used to pre-estimate the adsorbate-adsorbate interactions occurring during the adsorption process. The surface heterogeneity estimations were carried out using the affinity-energy distribution calculations with local BET isotherm. In the local BET isotherm the pre-estimated adsorbate-adsorbate interaction constant was used, and the surface heterogeneity was described. After the test of the numerical method with artificial isotherms, the algorithm was tested on several experimental isotherms. The isotherms were measured using phenol as test molecule on reversed phase adsorbents, with different surface coverages of the octadecyl ligands. Results show that surface heterogeneity is connected with the accessible residual silanol groups. The surface of non-end-capped stationary phases showed detectable heterogeneity during the adsorption process of the test molecule. Results were published in Ref. [16].

Studies of 2DLC separations

By the means of 2DLC techniques, very complex samples can be resolved when unidimensional methods cannot yield the desired resolution in a reasonable time. Since two different chromatographic methods are connected, many parameters affect the separation power of the whole system, such as the operating parameters of the first and the second dimensions, the retention mechanisms used in the two dimensions, and the number of collected fractions. The widespread use of 2DLC needs the investigation and evaluation of its capacity to remain unaffected by variations in method parameters. The robustness of two-dimensional liquid chromatographic methods were studied. The resolutions of pairs of randomly distributed compounds were calculated after varying the eluent concentrations in each dimensions and volume of collected fractions. The effect of change of mobile phase composition was simulated by the linear solvent strength model. It was shown that the uncertainty of separations increased by adding one more dimension. The fraction collection, however, eased the effect of random changes of separation parameters of first dimension. Results were published in Ref. [7]. Publication in a high-impact journal is expected in 2017.

Off-line two dimensional separation method was developed for the separation of protein digests. Orthogonality of retention of tryptic digest of bovine serum albumin under different separation mechanisms (reversed phase, hydrophilic interaction, ion exchange chromatography) have been evaluated. The ion-exchange – reversed phase mechanisms were found to be the most orthogonal. An algorithm was developed for the minimization of consumption of organic solvent in comprehensive two-dimensional liquid chromatography. It was shown that one can reach higher peak capacities only by using more eluent. The equilibration volume of the second dimension, however, did not affect the solvent consumption significantly. Calculations confirmed that the same target peak capacity could be achieved by consuming significantly different volume of organic modifier depending on the number of fractions analyzed in the second

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dimension suggesting that 2D separations can be optimized for eluent consumption. It was shown that minimization of eluent usage requires the use of small and efficient columns in the second dimension. A simple equation was derived for the calculation of the optimal number of collected fractions from the first dimension that allowed the minimization of eluent usage, cost and environmental impact of comprehensive 2DLC separations. Published in Ref. [12].

One of the goals of this research project was to develop a suitable algorithm for peak recognition and integration for 2D chromatograms recorded by diode array UV-Vis detection. The evolving factor analysis was adopted for analyzing 2D chromatograms. The method was developed on data sets obtained during the 2DLC analyses of amino acids (AA). By using AAs as the test molecules, it was always known which peak on the 2D chromatogram belonged to which component. The number of compounds found by factor analysis was determined (1) from the single chromatograms obtained during the second dimension analysis, and (2) when the adjacent chromatograms were combined. The developed factor analysis were unable to find extra peaks after the combination of adjacent peaks. Accordingly, results were not published.

HPLC method development

High performance anion chromatography (HPIC) method was developed for the simultaneous separation and analysis of ionic Gd chelates ($[GdDTPA^{2-}]$, $[GdEDTA^-]$, $[GdDCTA^-]$) and free, organic and inorganic anions. Gd-based contrast agents are used widely in MRI imaging. Since Gd chelates are among the most important emerging environmental contaminants, analysis of these compounds are fundamental. The developed method based on a systematic retention study and complex formation equilibria of metal chelates. At alkaline pHs, polydentate complexing agents such as ethylene-diamine-tetraacetate (EDTA), the diethylene-triamine pentaacetate (DTPA) and trans-1,2-diamine-cyclohexane-tetraacetate (DCTA) tend to form stable Gd chelate anions and can be separated by anion exchange. Separations were studied in the simple isocratic chromatographic run over the wide range of pH and concentration of carbonate eluent using suppressed conductivity detection. The ion exchange and complex forming equilibria were quantitatively described and demonstrated in order to understand major factors in the control of selectivity of Gd chelates. Parameters of optimized resolution between concurrent ions were presented on a 3D resolution surface. The applicability of the developed method is represented by the simultaneous analysis of Gd chelates and organic / inorganic anions. ICP-AES (inductively coupled plasma atomic emission spectroscopy) analysis was used for confirmation of HPIC results for Gd. Collection protocols for the heart-cutting procedure of chromatograms were applied. SPE procedures were also developed not only to extract traces of free gadolinium ions from samples, but also to remove the high level of interfering anions of the complex matrices. The limit of detection, recoverability and linearity of the method were also determined. Results were published in Ref. [14].

Ultra-high performance, reversed phase and hydrophilic interaction liquid chromatography methods were developed and applied successfully in degradation studies of non-ionic detergents. The developed methods allowed the complete separation and analysis of the detergents and the degradation byproduct allowing the elucidation of degradation pathways. The effects of ozonation and photocatalysis using peroxodisulfate as other AOPs were examined by the means of the developed UHPLC methods. Results were published in Refs. [2, 4, 9–11, 17].

Training of novel generation of researchers

Besides the scientific importance of the results, one PhD program was elaborated and 17 BSc/MSc degrees (2 in average in each semester) were defended successfully by the financial and infrastructural support of the research project.