STUDY OF THE MASS TRANSFER AND ADSORPTION PROCESSES FOR HIGH-PERFORMANCE SEPARATION OF BIOMOLECULES

OTKA-ID: **K 75717**, 2009–2013

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FINAL REPORT

INTRODUCTION

The central part of our investigation was the study of the interaction of analyte molecules with stationary phases. We have employed a number of tools to characterize the solute or solvent adsorption on HPLC stationary phases, and the mass transfer properties of small and macromolecules in HPLC columns. We have employed the information we gained this way to develop methods for the analysis of samples of biological relevance.

EXCESS ISOTHERMS OF SOLVENTS IN HPLC [1–5]

The excess adsorption isotherms of organic modifiers were determined on a series of C18-bonded stationary phases. The coverage density of the ligands was varied over a wide range, and the adsorption properties of each stationary phase were determined. The excess adsorption isotherms of methanol, 2-propanol, acetonitrile, and tetrahydrofuran from water were measured on five silica-based packing materials by the minor disturbance method. The adsorbed amount of solvents was calculated per silica gel surface and per modeled surface of bonded phase which seem to give more correct data. Columns used in the study were either non-endcapped or endcapped. That set-up allowed investigating the influence of the density of residual silanols on solvent adsorption and on the adsorption properties of the surface. The stationary phases were also characterized by elemental analysis and low-temperature nitrogen adsorption. Geometric parameters of the stationary phases in the column were measured by inverse size exclusion chromatography. The relative adsorption of the organic solvents from water informs on the heterogeneity of the stationary phases and on the accessibility of the unreacted silanol groups to the mobile phase.

We determined that the adsorbed amount of organic solvent strongly depends on the coverage density of bonded ligands. The excess adsorbed amounts of solvent calculated per surface of the silica gel in the columns exhibit significant influence of the decrease of the stationary bonded phase surface with the coverage density. Although, calculations performed with modeled bonded ligand surface eliminate this problem. It seems that using the modeled surface area of bonded ligands gives a more correct explanation of the chromatographic processes. The number of adsorbed solvent molecules per one ligand decreases with the coverage density of bonded ligands. The thick structure of the bonded ligands makes the penetration of solvent and solute molecules from bulk mobile phase to silica gel surface difficult. The solvent adsorption governed by hydrophobic interactions may have important influence on the separation selectivity in the discussed systems. Regardless of the ligand coverage density, the stationary phase surface becomes heterogeneous. However, the surface heterogeneity depends on the coverage density of bonded ligands and on the organic modifier, which solvates the bonded ligands. The adsorption processes of organic modifiers on non-endcapped columns are governed by several properties. Interaction potential with polar groups (residual silanols) and the size of the hydrophobic part of the molecule gives the ability to form mono-, or multilayer adsorption through hydrophobic interactions on alkyl-ligands. With the comparison with similar measurements on endcapped columns, the range of the average minimum and maximum values of the heterogeneity parameter is much tighter than in the case of non-endcapped columns. This is because of the modification of the silica surface with TMS where the overall

apolar surface coverage is much higher than on non endcapped columns (~3.76 µmol/m^2 for every investigated column), so in the adsorption processes, the apolar interactions are much more dominant.

STUDY OF SOLVENT ADSORPTION ON CHEMICALLY BONDED STATIONARY PHASES BY MICROCALORIMETRY [6]

In order to facilitate the detailed, molecular-level description of the sorption mechanism in reversed-phase liquid chromatography solvent adsorption in the octadecyl stationary bonded phase was investigated by microcalorimetry. The results of the experimental measurement of the heat of immersion agree rather well with the excess isotherm measurement of solvents on the bonded stationary phases by the test solvents. The microcalorimetric measurements are a very useful method for determination of the solvation processes of the stationary phases. Changes of the heat of immersion provide information about the accessibility of the surface for interactions with solvent molecules. The increase of the stationary phase coverage density reduces free space between bonded chains. The following regularity was observed for all stationary phases: an increase in the coverage density of the stationary phase causes a decrease of the number of adsorbed molecules per one organic chain for methanol, acetonitrile, and hexane. This means that the penetration of solvent molecules through a dense film of bonded ligands is more difficult.

EFFECT OF POLAR INTERACTIONS ON SOLUTE ADSORPTION AND RETENTION [5,7–8]

The effect of the coverage density and the activity of the unreacted silanol groups of the non-endcapped octadecyl bonded phase on the adsorption properties were investigated. The adsorption of two polar low molecular weight compounds with weak acidic (phenol) and with basic (aniline) character was measured. Adsorption data were acquired by frontal analysis from methanol-water and acetonitrile-water solutions to investigate the influence of the type of the organic modifier on the solute retention mechanism and adsorption under nonlinear conditions. The adsorption behavior of phenol changes from monolayer Langmuir type (from aqueous mixture of methanol) to multilayer BET type (from aqueous mixture of acetonitrile).
The adsorption of aniline becomes BET-type, regardless of the mobile phase composition in the tested range of the surface coverage of octadecyl ligands. The nature of the organic modifier significantly affects the retention mechanism and the shape of the overloaded elution bands of the studied compounds.
The experimental conditions under which the adsorption isotherms were measured correspond to those preferred in HPLC analyses. A series of non-endcapped octadecyl bonded phases was used to investigate the role of the residual silanols in solute adsorption and retention mechanism. Acetonitrile is the stronger eluent and it significantly reduces the column saturation capacities as well as the retention factor of the analytes at high surface coverage. At this range of the surface coverage, the hydrophobic interactions are predominant in the comparison while the polar interactions do not have such significant effect.
The residual silanols are uncovered and accessible for interaction with aniline, whereas when methanol is used as organic modifier, the residual silanols may be covered by a mixture of methanol and water. Using aqueous acetonitrile as a mobile phase, the silanol groups are covered by a much more water-rich layer. This more polar layer cannot shield effectively the silanol groups and they will be accessible much more for polar interaction. This phenomenon modifies the shape and the position of the overloaded elution bands.

ADSORPTION EQUILIBRIA OF PROLINE IN HYDROPHILIC INTERACTION CHROMATOGRAPHY [9]

The adsorption behavior of proline under hydrophilic interaction chromatography (HILIC) conditions was investigated from aqueous solution of acetonitrile. Proline adsorption isotherms were recorded at a number of mobile phase compositions by frontal analysis and inverse method. The extended liquid–solid BET model was found to be the best choice to describe the nonlinear behavior of proline adsorption under hydrophilic interaction chromatography conditions. The multilayer adsorption becomes stronger with the increase of the acetonitrile concentration. The fronting of the overloaded bands becomes more pronounced from less polar mobile phases.

The excess isotherm of waters was also determined. The column saturation capacity changes parallel with the excess adsorption of water. The partition of proline between the bulk mobile phase and the adsorbed water-rich layer is the most favorable in 20% (v/v) water–80% (v/v) acetonitrile mobile phase composition.

COMPARISON OF THE MASS TRANSFER IN TOTALLY POROUS AND SUPERFICIALLY POROUS STATIONARY PHASES IN LIQUID CHROMATOGRAPHY [10–12]

The characterization of mass-transfer processes in a chromatographic column during a separation process is essential, since the influence of the mass-transfer kinetics on the shape of the chromatographic band profiles and on the efficiency of the separation is crucial. Several sources of mass transfer in a chromatographic bed have been identified and studied: the axial dispersion in the stream of mobile phase, the external mass-transfer resistance, intra-particle diffusion, and the kinetics of adsorption–desorption. We measured and compared the characteristics and performance of insulin on a novel brand of shell particles and those of a conventional brand of totally porous silica particles. The shell stationary phase was made of 2.7-µm superficially porous particles (a 1.7-µm solid core is covered with a 0.5-µm-thick shell of porous silica). The other material consisted of totally porous particles of conventional 3.5-µm commercial silica.

The strong change of the retention parameters of insulin with the flow rate can be attributed to the fact that the partial molar volume of insulin is smaller when it is adsorbed than when it is in the mobile phase. Therefore, at higher pressures the adsorption of insulin on the stationary phase is more preferential than at lower pressures. For the case of large molecules, the intraparticle pore diffusion process is the major band-broadening factor. Since the mobile phase flow rate strongly affects the retention factor, the fitting of the plate height equation and accordingly the calculated numerical values of the axial dispersion and the intra-particle diffusion coefficients are prone to error since the fitting of the plate height equation provides a single value of axial dispersion coefficient and pore diffusivity for the range of flow rates applied. We concluded that although the calculated intraparticle diffusion coefficients are somewhat smaller for the superficially porous stationary phase, they are in the same range for both stationary phases. This is in agreement with the nearly identical pore diameter of the two stationary phases (90 Å). The fact that flow-rate dependent intraparticle diffusion coefficients were found – when flow-rate-dependent axial dispersion was used in the calculations – is most probably associated with the fact that the retention factor is proportional to the flow rate. When the retention factor increases, surface diffusion may become dominant and that may result in a higher apparent pore diffusivity coefficient.
The average stationary phase residence times calculated with the stochastic analysis showed that in the case of the superficially porous particles, the stationary phase residence times are slightly higher than they are for the totally porous stationary phase. At low flow rates, the calculations are affected by some flow-rate-dependent systematic error, but at high flow rates, the average stationary phase residence times were found at 150 ms for the superficially porous stationary phase, whereas they were around 100 ms for the totally porous stationary phase.

We used a microscopic random walk model of diffusion to calculate the mean diffusion time in fully porous and shell-type packing materials. The calculations show that the relative decrease of the diffusion time is rather significant with the currently available core–shell packing materials. The diffusion times calculated on the basis of the microscopic random walk model are equivalent with the appropriate intraparticle mass-transfer kinetics terms. As long as the diffusion time is considered, the 2.7-µm Halo and Poroshell 120 phases are equivalent to a 1.8-µm fully porous packing material. The 2.6-µm Kinetex packing material is equivalent to a 1.34-µm fully porous stationary phase.
Hindered diffusion – which is observed when the size of the analyte macromolecules becomes comparable to the pore diameter – affects both fully porous and core–shell packing materials in the same manner. It will, however, cause a much more momentous band broadening in fully porous particles where diffusion path as well as diffusion time are longer than in core–shell particles.

PEAK SHAPE ANALYSIS [13]

The accurate determination of the shape parameters of chromatographic peaks is of utmost importance when mass-transfer properties are studied. The physical models of flow systems or chromatography are often solved in Fourier of Laplace domain in closed form. The Fourier or Hartley transform of the experimentally recorded signals can be calculated, and the models can directly be fitted. In this case, information from the entire peak shape is utilized. We have shown using various models of extra-column broadening the importance of the exact physical model. Also the stochastic model of chromatography is rather convenient to fit in the Fourier domain, since the characteristic function of the peak shape is expressed analytically.
We have demonstrated that the parameter estimation in frequency domain gives results identical to the time domain curve fitting; the accuracy of the frequency domain estimation is excellent. One observes, however, that the precision of the parameter estimation is somewhat worse in frequency domain. That occurs in spite of the fact that Fourier transform separates the low-frequency signal and the high frequency noise. The noise pattern of the UV detector signals exhibits a brown ($1/f^2$) or pink ($1/f$) spectrum. Thus, the low-frequency component of the noise is more dominant than the high-frequency one and it overlaps with the low-frequency signal in the Fourier or Hartley domain, affecting the precision of the parameter estimation.

BIOANALYTICAL APPLICATIONS [14–18]

A high performance liquid chromatography method coupled with electrospray mass spectrometric detection has been developed for the determination of atropine and scopolamine in *Datura* plant extracts. The validation characteristics tested showed low quantitation limits, allowing the determination of the alkaloids in solutions and in extracts at the concentration levels of sub-ng mL^{-1}. The detection limits for both alkaloids are rather good: 100 pg mL^{-1} for scopolamine and 50 pg mL^{-1} for atropine. The method requires a rather simple sample preparation, and it could be also applied to the analysis of nearly any type of plant material containing atropine and scopolamine alkaloids. The results showed that the technique is repeatable and accurate; the limit of detection is rather low. The separation performed on core-shell RP column and the employment of UFLC instrumentation coupled with mass spectrometer result in a rapid analysis.

An LC-MS method was developed for the determination of fifteen polyphenolic compounds in *Thymus* extract sample. The method requires a very simple sample preparation prior to analysis. The results showed that the technique is very sensitive, reproducible, and accurate. The LOD range for this method using ESI-MS detection is 0.84-66.79 ng mL^{-1} for the polyphenolic compounds. The flavonoid profiles found in species of *Thymus* provide useful additional taxonomic characters at different levels of classification.

HPLC proved to be an efficient tool for the analysis of leaves of different *Bergenia* samples. The evaluated validation characteristics (repeatability and intermediate precision, LOD, LOQ, calibration range and recovery) showed that the technique is very sensitive, reproducible and accurate.
Results of validation showed low quantitation limits allowing the determination of arbutin, bergenin and gallic acid in solutions and in extracts at the concentration levels of sub-5-µg mL^{-1}. The detection limits for measured compounds are rather good: 1.0, 1.5 and 1.0 µg mL^{-1} for arbutin, bergenin and gallic acid, respectively. A further advantage of the method is that it requires a very simple sample preparation prior to analysis, which could be applied to nearly any type of plant material containing arbutin, bergenin and gallic acid. The separation performed on a reversed phase HPLC column and the employment of UFLC instrumentation coupled with DAD result in a rapid analysis.

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