UNIVERSITY OF PÉCS

Doctoral School for Chemistry

Separation of Biopolymers on Stationary Phases of Different Geometry in Reversed Phase Liquid Chromatography

PhD Thesis

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1. INTRODUCTION

The separation techniques have undergone considerable developments in the second half of the last century.
Nowadays, HPLC has found broad acceptance as the analytical technique of choice in many scientific and application oriented areas, like life science, food chemistry, and environmental chemistry. Developing liquid-phase separations is a complex task because several factors must be considered, such as the mobile phase, the stationary phase, the chemical structure and physical-chemical properties of samples, and parameters of detection.

There are different theories to envision the retention mechanism in reversed-phase chromatography (RP-HPLC). The most important ones are the well known hydrophobic interaction theory, the theory of partition and the adsorption theory. The RP-HPLC retention mechanism is still an important research area. Today, researchers seek to a better understanding of processes of retention and selectivity. The interpretation of these processes at the molecular level, as well as their connection with the physical-chemical properties of the stationary phases is particularly important.

The development of the chromatographic instrumentation was necessary in parallel with the development of stationary phases (of special packing materials) and columns. The developments and the availability of high-performance instrumentation and high-quality stationary phases have substantially supported the growth of HPLC researches and applications. In addition, new and also special instrumentation and columns for RPLC are continuously developed and marketed today. The stationary phases are the 'heart' of the chromatographic separations.

Probably, today's separations are mostly performed by HPLC. The reason for its popularity is the easy solubility and widespread applicability for the separation of small molecules and for biopolymers, as well. Selection of the chromatographic column for the separation of bioactive compounds requires special attention. Today, particles of less than 3 µm diameter are being increasingly applied in RP separations.

2. RESEARCH AIMS

In our work we aim to test and compare the different types (porous, superficially porous and non-porous) of reversed phase packing materials; taking into account both, the bioactive low molecular weight compounds and high-speed HPLC separations, and the determination of mass-transfer coefficients.

In our measurements we selected compounds, which are important in the field of life sciences.

Research goals are summarized as follows:

- In this study I aimed to investigate the chemical stability of Kovasil packing, by using strong acidic and basic pH of eluents.
- The aim of the measurements was to separate biopolymers within short time on non-porous reversed-phase packing. The HPLC separation of biopolymers (protein and peptide mixtures) is often a very important analytical task. These separations on porous packing require long analysis time.
- We ought to certify that the non-porous reversed-phase packing is excellent to use in the separation of biopolymers and in addition of low molecular weight (Mw = 100-500 Da) bioactive compounds (e.g. water- and fat-soluble vitamins).
- We also wanted to determine and compare the mass transfer coefficients by using different types of packing materials. In our measurements we used the porous Sun Fire C_{18} and the superficially porous Halo C_{18} stationary phase. For further calculations, we determined the porosity factors of these phases by applying inverse size exclusion chromatography.

  We used the following methods to define the mass transfer coefficients:
  - the general rate model;
  - the van Deemter equation;
  - the moment analysis;
  - and the stochastic model of chromatography.

3. MATERIALS AND METHODS

Among the different available HPLC separation methods, definitely reversed-phase liquid chromatography (RPLC) has taken and still takes a dominant position; presently about 80% of HPLC separations are performed using RPLC. The silica-based stationary phases (bonded alkyl groups) are the most widespread (~75%) among the reversed phase liquid chromatography packing materials.

Fast separation of bioactive molecules

A short, few-minute analysis of bioactive molecules was achieved by multistep gradient elution. The separations were carried out on Kovasil-MS-H and Kovasil-C_{14} columns (Chemie AG, Swiss Uetikon). The stationary phases are based on a high purity non-porous, monodisperse silica bed (d_p = 1.5 µm) chemically bound with C_6 and C_{14} chain, respectively.

HPLC separations were performed with ternary gradient pump, Rheodyne injector (Cotati CA, USA), 170S diode array detector with a Chromeleon Chromatographic Data System (Gynkotek, Germering, Germany). The columns were heated using a W6 Grant (Grant Instruments, Cambridge, England) circulating water bath.

Determination of the mass transfer coefficients

Two columns were used: Halo column (Advanced Materials Technology, Wilmington, DE) packed with shell particles (d_p = 2.7 µm and a shell thickness of 0.5 µm), and SunFire column (Waters Co., Milford, MA) with a fully porous silica particles (d_p = 3.5 µm). Both materials have an average mesopore size of 9 nm, prior to the bonding of the C_{18} alkyl chains.

The mobile phase flow rate was changed over the range 0.02–1.4 mL/min (p ~ 5-325 bar) at 20°C. Each measurement was executed with eluent containing 21 percent acetonitrile and 79 percent water (v/v), with 0.1 percent TFA. 1 mL sample containing 1 mg/mL bovine insulin (Mw = 5.5 kDa) was injected. The insulin was detected at 205 nm.

An Agilent 1100 HPLC instrument (Agilent Technologies, Palo Alto, CA), equipped with a diode array detector, high-pressure multisolvent delivery system, column thermostat compartment, and a computer data station with Chemstation software was used in all measurements.

4. RESULTS

In my dissertation, I studied the applicability of non-porous hydrophobic silica-based stationary phases in high performance liquid chromatography for the fast separation of low molecular weight bioactive compounds (Mw = 100-500 Da) and biopolymers
In the selection of samples, the preferred groups of compounds were those, which are important in life science, medical and biological research (e.g. peptides, proteins, water- and fat-soluble vitamins). The selected compounds were diverse from the point of view of chromatographic separations, since among them there were highly polar, weakly polar, acidic and basic compounds. I verified the comprehensive applicability of non-porous, monodisperse packing by the separation of these different compounds.

1. The behavior of Kovasil packings by using highly acidic and alkaline pH of eluent were characterized by the chemical stability. In the stability test, eluents at pH 1.9 and pH 8.5 were pumped through the column at 80°C. The retention of the test compounds proved to be stable during the nearly 400 hours of continuous usage. The chemical stability of the packing was excellent, ensuring the reproducibility of results over a long period of time.

I developed a new super-fast separation procedure for the analysis of low molecular weight (water and fat soluble vitamins) and high molecular weight (peptides, polypeptides) of bioactive compounds using a short, non-porous column (33 mm × 4.6 mm), routine liquid chromatography equipment and gradient elution technique.

2. The separation of six water soluble vitamins (acidic and basic components) required 1.1 minutes at room temperature, using gradient elution with quick ion-pair separation, the solvent strength and the pH of the eluent were changed, too.
3. I studied eight fat soluble vitamins. The analysis time of the vitamins was 2.5 minutes. In most cases the detection limit of the separated compounds was low, typically 10^{-12} mole.
4. I developed the method for separation of enzymatic digest of proteins and peptides using high-temperature (70°C), high flow speed and multi-step gradient. The digestion of high molecular weight bovine serum albumin (Mw = 68,000 Da) with trypsin resulting in an average number of 7-12 of fairly short fragments gives rise to a complex mixture of 79 fragments. Although Cytochrome c is a relatively small molecular weight protein (Mw = 13 000) for

the separation of the 13 peptide fragments, good resolution and reproducibility was necessary. The analysis time is less than 3 minutes, which is one tenth (or even less) of that measured on porous stationary phases.

5. The efficiency, the short analysis times, and sensitivities were demonstrated by the separation of different proteins, where the recovery was almost complete. (This could explain the long lifetime of the non-porous columns in protein separation – no protein precipitation occurs on the surface of the particles.)
6. I presented the separation of β-lactoglobulin B and β-lactoglobulin A. The analysis was carried out at high temperature (95°C), thus due to a reduction in the viscosity of the eluent, the flow rate (3.6 mL/min) could be increased, consequently a very fast (0.2 min) separation could be carried out. The separation efficiency is improved at higher temperatures because the viscosity of the system decreases due to decrease in the mass transfer resistance.

I applied macroscopic and microscopic methods to evaluate the experiments. I determined the mass transfer coefficients of insulin on totally porous (Sun Fire) and superficially porous (Halo) stationary phases.

7. In case of insulin and other large molecules I found that the flow rate and the pressure strongly influence the retention. I determined the axial dispersion (D_L), the external mass transfer coefficient (k_{ext}) and the internal diffusion coefficient (D_p) using the general rate model.
8. With the stochastic analysis, I defined the molecular retention characteristics of insulin such as the number of the adsorption-desorption steps (n), the average stationary phase sojourn time (τ_s) and the average mobile phase sojourn time (τ_m). At high flow rates, the average stationary phase residence times are found at 150 ms for the superficially porous stationary phase, whereas they are above 100 ms for the totally porous stationary phase. The retention factor of insulin is two times higher for the superficially porous stationary phase than for the totally porous one. The stationary phase sojourn time is directly proportional to the retention factor, since $k' = \tau_s / \tau_m$. If the mobile phase composition were changed so that the retention factors are equal on the two columns, the average residence time in the shell particle would be three quarters of that in the totally porous one.

5. RESULTS OF THE POSSIBILITIES OF EXPLOITATION

In our work we studied and presented the properties of the stationary phases, and we supported the results by our measurements. The conclusions are useful for the users and manufacturers, and help them better understand the separation processes, as well as help to solve difficult separations, as well as to plan separation methods.

7. PUBLICATIONS, PRESENTATIONS

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