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General approach for the development of preparative peptide separations

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Introduction

Preparative HPLC is ever gaining in importance as purification method in the pharmaceutical industry. A wide range of different peptides are purified successfully worldwide. As regulatory requirements increase, higher purity levels have to be met which opens for further growth of process scale HPLC applications.

In this presentation, the influence of the most important parameters such as pore size, particle size and surface modification of the packing material are elucidated, but also mobile phase aspects are explained and a simple step-by-step approach for the method development is presented.

The Stationary Phase

The stationary phase provides specific retention and adsorption capacity. It is characterized by pore size, particle size and surface modification.

Pore size: The pore size governs the surface area. The smaller the pore, the higher the specific surface area. The loadability is however controlled by the accessible surface area which depends upon the molecular weight of the solute (Fig. 1).

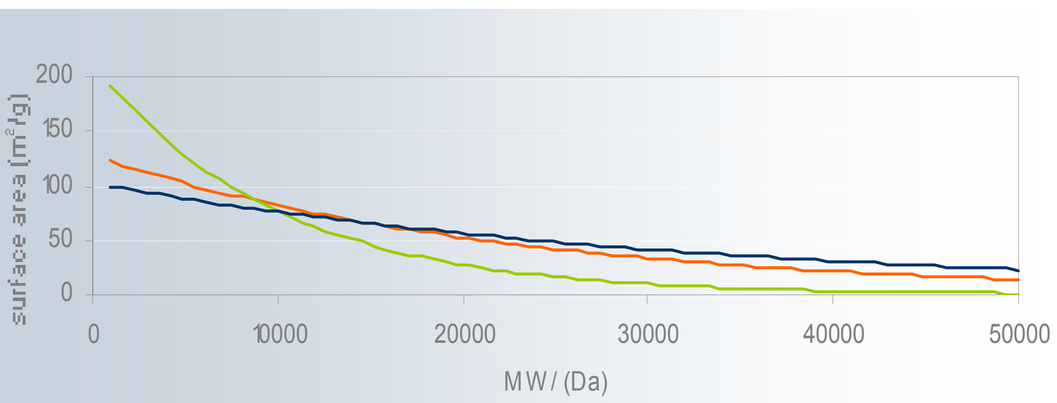


Fig. 1: Accessible surface area as function of the molecular weight of polystyrene standards. Green: 100Å; Orange: 200Å; Blue: 300Å

Particle size: The particle size controls the efficiency, but also the back pressure over the column. Increased efficiency with smaller particles results in higher resolution, while the selectivity obtained for a certain separation does not depend upon the particle size. The efficiency is inversely proportional to the particle size (1/dp) and the pressure drop is inversely proportional to the square of the particle size ($1/dp^2$).

Surface modification: Silica based RP-HPLC packing materials are generally modified by alkylsilanes (C4, C8, C18, Phenyl,…). The chain length influences the hydrophobicity of the stationary phase, but also the actual pore geometry. While C18 will reduce the actual pore diameter by ca 2x20Å, C4 brings along a reduction of 2x7Å. This aspect might play an important role for optimized mass transport of larger peptides in 100Å stationary phases.

The Mobile Phase

The mobile phase composition is the single most important parameter of an RP-HPLC peptide separation. As peptides are weak protolytes, buffers - or for analytical separations acidic additives – are added to the mobile phase in order to maintain a stable pH regime.

Buffer: The buffer has to fulfill two tasks: it has to ensure that the peptide exists in a uniform protonated state or it can act as counter-ion or ion-pairing agent for both the peptide and the residual silanol groups. Depending on the pH, and therewith the degree in protonation of the peptide (Fig. 2) and the silanol groups, the retention of the peptide might vary significantly.
As a rule of thumb, one should always operate at least 1 pH unit from the isoelectric point of the peptide in order to assure a stable regime.

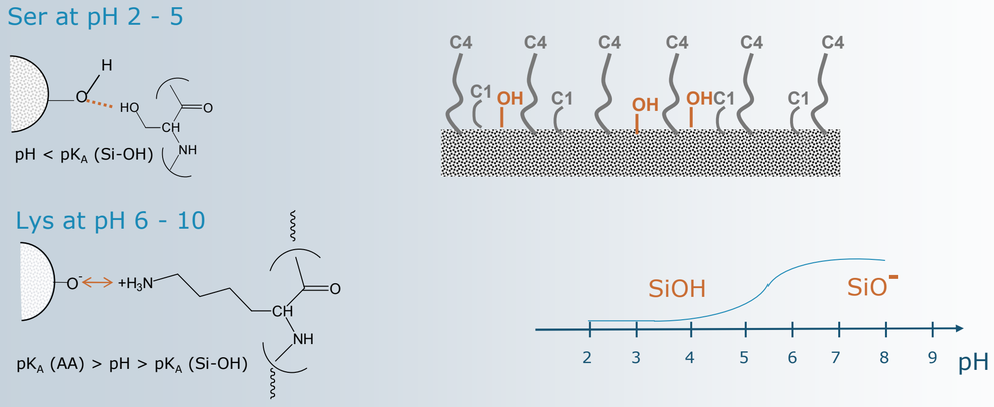


Fig. 2: Hydrogen bonding interaction at pH 2-5 and ion-exchange interaction at pH 6-10 between amino acid side chains and residual silanol groups of the stationary phase

Organic modifier: Compared to the buffer, the choice of organic modifier plays a subordinate role. Acetonitrile has the advantage of 2-3x lower viscosity upon mixing with water, compared to alcohols. This is beneficial both with respect to the obtained back pressure (→ max flow rate, productivity), but also with respect to the diffusivity (mass transport).

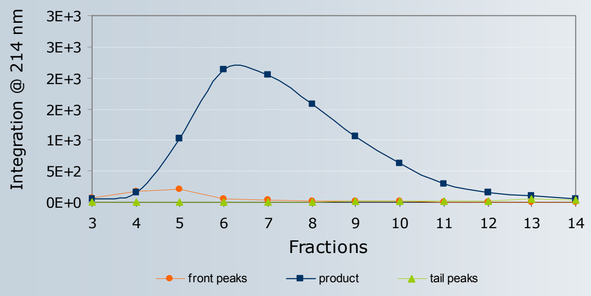
Gradient and flow rate: Gradients have a tremendous impact on both retention and resolution. Gradient elution enables the following features:

- Concentration step prior to elution → mandatory for large injection volumes
- Elution of substances that vary greatly in their hydrophobicity within a reasonable time span
- Column wash and reconditioning for next injection

A concentration step at the beginning of the separation is always recommended when dealing with preparative separations. The mobile phase composition is kept at a low content of organic modifier (5–10%) for ca 2–5 min in order to assure that the entire peptide load is adsorbed at the column inlet as a narrow band.

General Method Development Approach

1. Select pore size (100 or 300Å) depending on the molecular size
2. Select particle size (10µm is a good choice)
3. Select an initial surface modification (C18 for smaller peptides <5kDa, C4 for larger ones)
4. Run screening gradient (10–70% MeCN/45 min) to identify the approximate elution range We recommend to probe two different pH initially, pH 2.5 and pH 8.0 by means of a 100 mM phosphate buffer.
5. Adjust the gradient to a slope of 0.1–0.3%/min and judge the resolution under different pH values
6. For the best pH value, test alternative buffers and surface modifications, if the resolution is not complete.
7. Once satisfying separation is achieved with an analytical injection, overloaded injections should be conducted in order to see if the chosen buffer system also works under non-linear conditions. We recommend to increase the loading on a 4.6 x 250 mm column gradually from 1 mg to 20 mg. For the 20 mg injection we suggest collecting fractions and analyzing those in order to judge the separation performance by purity and yield results (Fig. 3).



Fractions	Purity [%]	Yield [%]
6-13	97.6	92.1
6-12	97.8	90.4
6-11	98.3	86.7

Fig. 3: Reconstructed elution profile based on results from fraction analysis. Product purity and yield are calculated for different fraction pools