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Recovery and Memory Effects in Reversed-Phase Chromatography of Proteins

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Introduction

Reversed-phase high performance liquid chromatography (RP-HPLC) is a useful method for protein analysis. Due to its compatibility with mass spectrometry detection, it became an important method for the characterization of pharmaceutically important proteins (LC/MS).

Reversed-phase HPLC encounters problems with recovery of hydrophobic proteins and glycoproteins; in severe cases proteins do not elute from the column. Due to incomplete recovery, ghost peaks may appear in subsequent analyses (memory effect). Published reports have shown that C4 chemistry is preferred over C18 columns and provides better protein recovery [3]. Besides alkyl chain length, the pore size and the base sorbent also play a major role in protein recovery [3]. Polymer sorbents have been shown to allow for greater protein recovery than silica [4].

We investigated the separation of intact proteins by RP-HPLC using polymeric and silica-based sorbents. Ribonuclease A, bovine serum albumin, beta-lactoglobulin A, and ovalbumin were chosen as a model sample. Protein recovery was determined using an external calibration. The recovery of "good" proteins [1] like RNAse was found to be ~100%, "bad" proteins [1] like BSA ~90% and "ugly" proteins [1] such as ovalbumin and β-lactoglobulin A had an average of 30—60% recovery.

Residual amounts of protein were eluted by three successive blank gradients following the protein mixture injection. This column "memory effect" was observed to a varying degree for all columns tested. We evaluated several column cleanup procedures to eliminate these memory effects.

The goal of the study was to identify stationary phases with high recovery and low memory suitable for protein purification as well as LC/MS for protein analysis.

Experimental

HPLC System: Alliance® 2795 (Waters) with a 996 PDA detector
Mobile Phases: A: 0.1% TFA in water
B: 0.08% TFA in acetonitrile
Gradient Conditions: From 20-65% B in 15 minutes
Column Dimensions: 4.6 x 50 mm unless otherwise noted
Column Temperature: 40 °C
Flow Rate: 0.75 ml/min
Injection Volume: 20 µl (Total protein load: 1.5 nmol)

LC/MS System: CapLC® (Waters) with ESI-TOF mass spectrometer LCT™ (Micromass)
Mobile Phases: A: 1% formic acid in water
B: 0.65% formic acid in acetonitrile
Gradient Conditions: From 10-55% B in 15 minutes
Column Dimensions: 1 x 50 mm
Column Temperature: 40 °C
Flow Rate: 35.4 µl/min
Injection Volume: 4 µl (Total protein load: 0.3 nmol)

Figure 1: A protein mixture was chromatographed on selected reversed-phase and ion exchange columns. Recovery and memory effects were monitored for very hydrophobic proteins. For RP-HPLC conditions see experimental. Anion exchange conditions were 0-50% B in 15 minutes (mobile phase A: 20 mM Tris-HCl pH 7.8, mobile phase B: buffer A + 1 M NaCl). Symmetry300 C4 (A) shows slightly better recovery and peak shape then Symmetry300 C18 (B). Non-porous column (C) exhibits very poor recovery of ovalbumin. Prototype polyDVB column (D) has peak shape and recovery comparable to (A) and (B). Ion exchange (E) is able to separate both isoforms and glycoforms of ovalbumin. ~100% recovery was observed for ovalbumin.

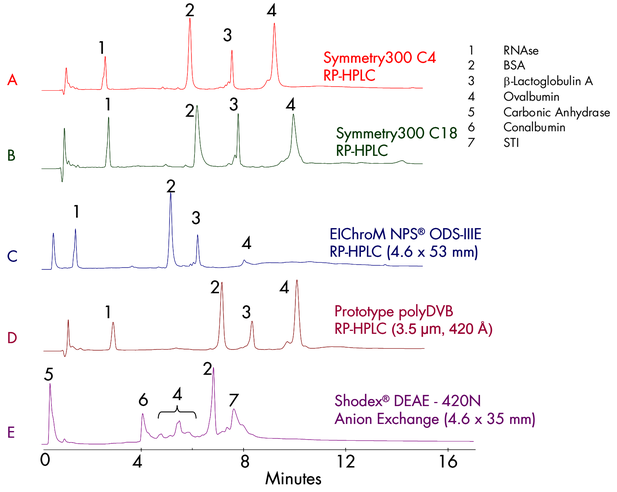


Figure 2: Memory effects of ovalbumin can be seen on all reversed-phase columns. This overlay shows one protein mixture injection followed by three blank injections onto the column. Ovalbumin can be seen eluting in all three blank injections.

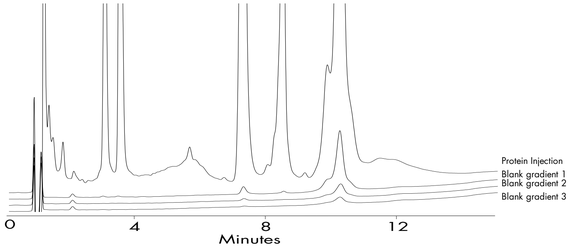


Figure 3: The memory effects of ovalbumin were calculated as the percentage of ovalbumin recovered from three blank injections (see figure 2), and compared to the original area of the ovalbumin peak (considered to be 100%).

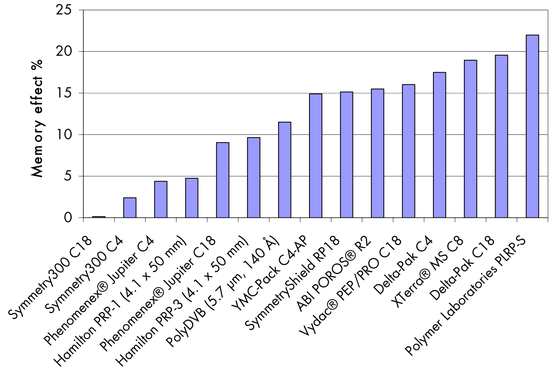


Figure 4: Effect of column cleanup on ovalbumin memory. Different cleaning procedures were investigated: reverse gradients, repetitive gradients, acetonitrile washes, and injections of concentrated acetic acid in between runs. The percentage of remaining ovalbumin after the column cleanup was measured using blank gradient elution (refer to figure 3).

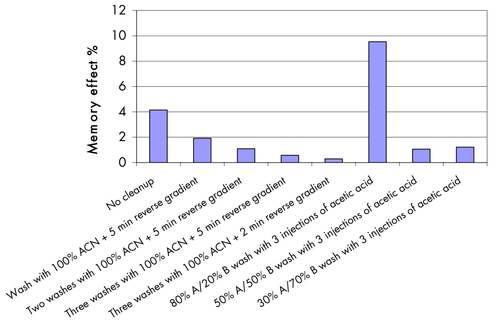
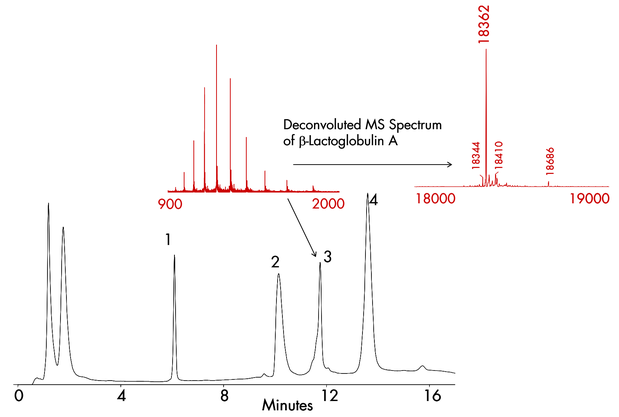


Figure 5: LC/MS of a protein mixture with formic acid as an ion pairing agent. For the sake of sensitive mass spectrometry detection, formic acid is preferred over TFA, however, protein peak shape was compromised on most columns evaluated. Polymer-based sorbents usually perform well, but the best performance was achieved using Delta-Pak C4 (shown below). The combined spectra from β-Lactoglobulin A is shown below; spectra were deconvoluted using MaxEnt 1 software. In peak 3 we observed a signal of β-Lactoglobulin A and some of its glycoforms.



Conclusions

- Limited recovery and memory effect of proteins was found to a varying degree on all tested RP-HPLC columns.
- Memory effect can be greatly reduced by effective column cleanup.
- PolyDVB columns and silica-based Delta-Pak columns were found to perform well with mass spectrometry compatible mobile phases (1% formic acid).
- Unlike RP, ion exchange HPLC allows for good protein recovery and exhibits minimal memory effects.

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