Waters

Investigation of Protein Recovery and Memory Effects in Reversed-Phase and Ion-Exchange Chromatography

Amy E. Daly, Martin Gilar, and John C. Gebler
Waters Corporation, Life Sciences R&D, Milford, MA, USA

MICROMASS MS TECHNOLOGIES

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 provide for greater protein recovery than silica [4].

The separation of intact proteins was investigated by RP-HPLC and ion exchange chromatography using polymeric and silica-based sorbents (Fig. 1). Ribonuclease A, bovine serum albumin, beta-lactoglobulin A, and ovalbumin were chosen as a model sample for RP-HPLC. 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 (Fig. 2A).

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 (Fig. 3). We evaluated several column cleanup procedures to eliminate these memory effects (Fig. 4).

Carbonic anhydrase, conalbumin, ovalbumin, BSA, and soybean trypsin inhibitor were chosen as a model sample for anion exchange while myoglobin, alpha-chymotrypsinogen, cytochrome C, and lysozyme were chosen for cation exchange chromatography. Protein recovery was determined using an external calibration; recovery varied between 90-100 %. There was only a negligible memory effect observed for the ion exchangers (Fig. 2B).

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 (RP-HPLC)
20 mM Tris-HCl pH 7.8 (Anion exchange-HPLC)
20 mM sodium phosphate pH 7.0 (Cation exchange-HPLC)
B: 0.08% TFA in acetonitrile (RP-HPLC)
20 mM Tris-HCl pH 7.8 + 1 M NaCl (Anion exchange-HPLC)
20 mM sodium phosphate pH 7.0 + 1 M NaCl (Cation exchange-HPLC)

Gradient Conditions: From 20-65% B in 15 minutes (RP-HPLC)
0-50% B in 15 minutes (Anion exchange-HPLC)
0-70% B in 21 minutes (Cation exchange-HPLC)

Column Dimensions: 4.6 x 50 mm unless otherwise noted

Column Temperature: 40 °C (RP-HPLC)
Ambient (IE-HPLC)

Flow Rate: 0.75 ml/min

Injection Volume: 20 µl (Total protein load: 1.5 nmol) RP-HPLC
20 µl (Total protein load: 2.37 nmol) Anion exchange-HPLC
20 µl (Total protein load: 2.91 nmol) Cation exchange-HPLC

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.

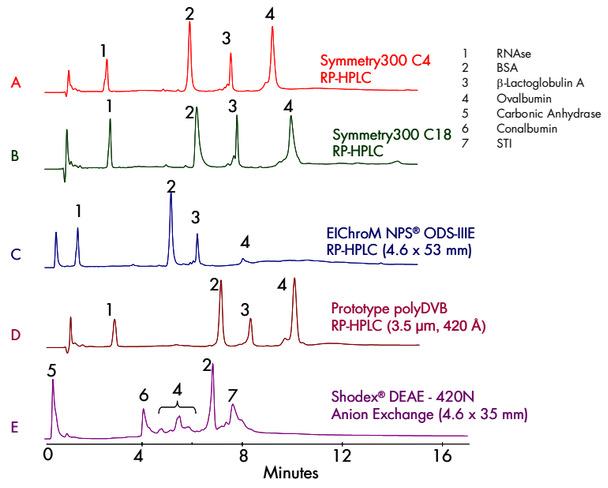
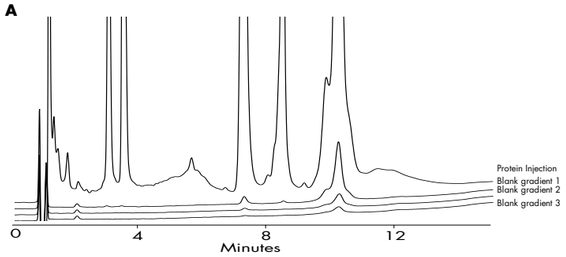


Figure 2: Memory effects of ovalbumin can be seen on all reversed-phase columns. The overlay labeled A shows one protein mixture injection followed by three blank injections onto the column. Ovalbumin can be seen eluting in all three blank injections. The overlay labeled B shows one protein mixture injection followed by one blank injection onto an anion exchange column. No ovalbumin memory effect was observed.



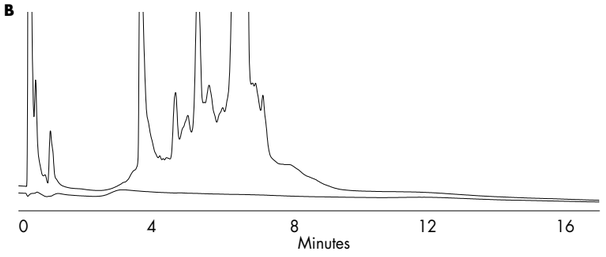


Figure 3: The memory effects of ovalbumin were calculated as the percentage of ovalbumin recovered from three blank injections (see figure 2), and compared to the original area of the ovalbumin peak (considered to be 100 %).

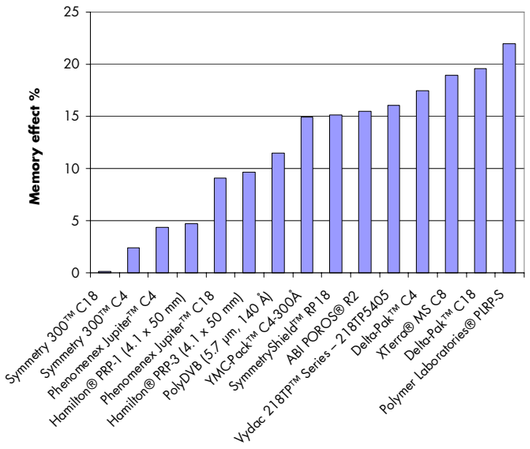
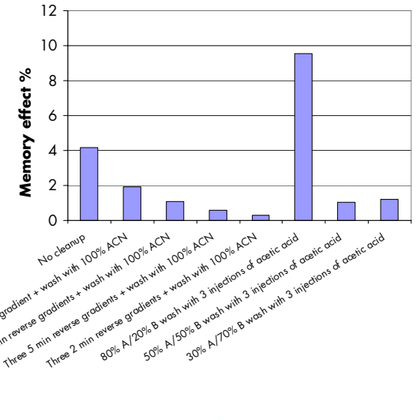


Figure 4: Effect of column cleanup on ovalbumin memory. Different cleaning procedures were investigated: reverse gradients, repetitive gradients, acetonitrile washes, and injections of concentrated acetic acid in between runs. The percentage of remaining ovalbumin after the column cleanup was measured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



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.
- Unlike RP, ion exchange HPLC allows for good protein recovery and exhibits minimal memory effects.
- Ion exchange (under these conditions) is not directly compatible with MS; a desalting step is required.
- PolyDVB columns and silica-based Delta-Pak columns were found to perform well with mass spectrometry compatible mobile phases (1% formic acid). Results not shown.

References

1. Burton, W.G., Nugent, K.D., Slattery, T.K., and Summers, B.R. *J. Chromatogr.*, 443 (1988) 363.
2. Nugent, K.D., Burton, W.G., Slattery, T.K., and Johnson, B.F. *J. Chromatogr.*, 443 (1988) 381.
3. Cooke, N.H.C., Archer, B.G., O'Hare, M.J., Nice, E.C., and Capp, M. *J. Chromatogr.*, 255 (1983) 115.
4. Lee, Dan P. *J. Chromatogr.*, 443 (1988) 143.