Fast Agilent HPLC for Large Biomolecules

Technical Overview

Introduction

Agilent media for the analysis of large biomolecules is available in an array of pore sizes to maximize selectivity and capacity across the whole molecule size range, from di- and tri-peptides up to large proteins. Chromatographic media for biomolecule analysis require the maximum surface area to maximize selectivity and capacity, combined with sufficiently large pore sizes so that access is not restricted.

In the examples presented here, 1000Å and 4000Å materials are used, with reversed-phase PLRP-S and ion-exchange PL-SAX and PL-SCX columns from Agilent.

Agilent Technologies

Ion-exchange separation of ovalbumin and soyabean trypsin inhibitor using PL-SAX

PL-SAX is available in 1000Å and 4000Å pore sizes. For globular protein analysis and purification, the 1000Å material has the optimum pore size for maximum loading with low band broadening. The more open pore structure of the 4000Å is preferred for high resolution and high speed applications or for the separation of very large biomolecules.

The capability of PL-SAX is demonstrated by the separation of ovalbumin and soyabean trypsin inhibitor, in a comparison of the 1000Å and 4000Å materials using different flow rates with unmodified, conventional instrumentation[1]. Figure 1 shows separations at a high flow rate of 4.0 mL/min.

Conditions

Eluent A: 0.01 M Tris HCl, pH 8
Eluent B: A + 0.35 M NaCl, pH 8
Flow Rate: 4.0 mL/min
Detection: UV, 280 nm

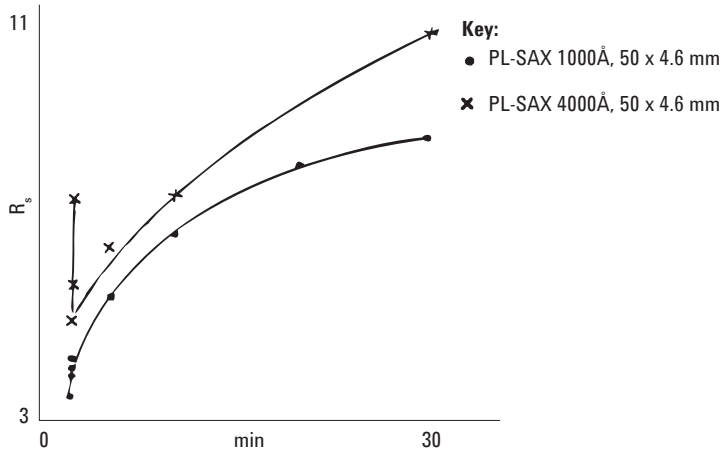

Figure 1. Separation of ovalbumin and soyabean trypsin inhibitor on different Agilent PL-SAX media.

Figure 2 compares separations obtained at 4.0 mL/min and 1.0 mL/min.

Conditions

Eluent A: 0.01 M Tris HCl, pH 8
Eluent B: A + 0.35 M NaCl, pH 8
Detection: UV, 280 nm

A) Column: PL-SAX 4000Å, 50 x 4.6 mm (p/n PL1551-1803)
Gradient: Linear 0-100% B in 5 min
Flow Rate: 4.0 mL/min

B) Column: PL-SAX 1000Å, 50 x 4.6 mm (p/n PL1551-1802)
Gradient: Linear 0-100% B in 20 min
Flow Rate: 1.0 mL/min

At high flow rates the efficiency of PL-SAX 4000Å increases even for large biomolecules, enabling resolution factors comparable to those obtained under standard gradient conditions to be achieved with short gradient times.

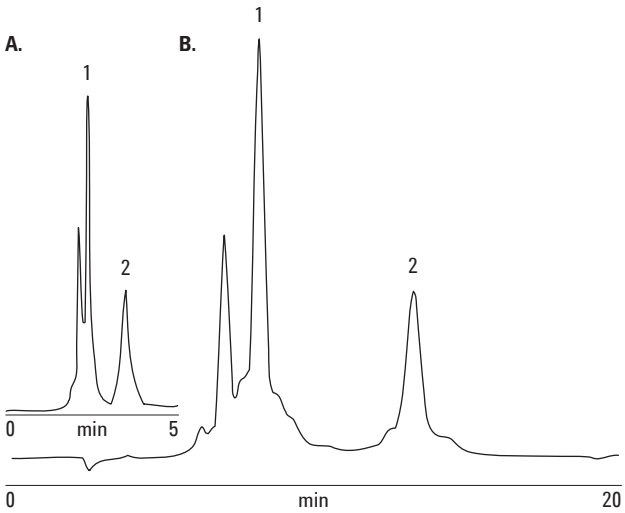

Figure 2. Separation of ovalbumin grade 3 (1) and soyabean trypsin inhibitor (2) in different Agilent PL-SAX media with different flow rates.

High speed separation of globular proteins on PL-SAX

PL-SAX 4000Å material permits the use of unmodified, conventional instrumentation. Using an isocratic system and high salt buffer (non-interactive eluent) plate count/efficiency measurements were carried out for three solutes of increasing molecular weight. As solute size in solution increases so the diffusion coefficient decreases, increasing the band spreading. However, comparing the 1000Å and 4000Å materials, it is evident that the diffusion is improved with increasing pore size (Figure 3). Thus, high speed/fast flow separations of large biomolecules would be possible with 4000Å pore packings[2].

Conditions

Columns: PL-SAX 1000Å 8 µm, 250 x 4.6 mm (p/n PL1551-3802)
PL-SAX 4000Å 8 µm, 250 x 4.6 mm (p/n PL1551-3803)
Eluent: 0.01 M Tris HCl + 0.5 M NaCl, pH 8
Detection: UV, 280 nm

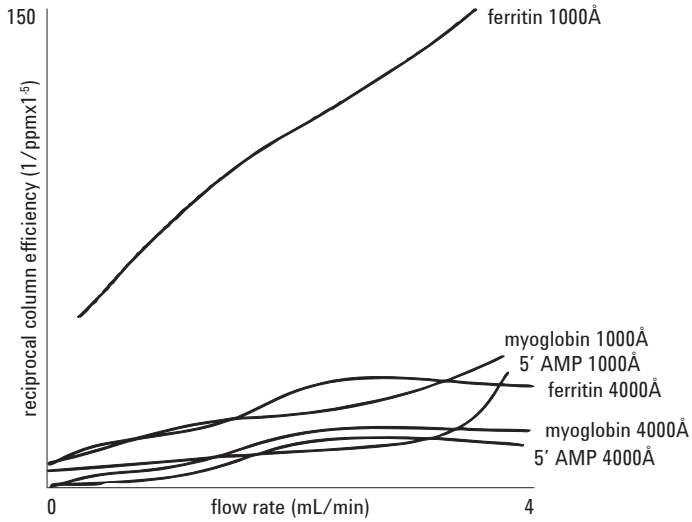

Figure 3. Influence of flow rate on efficiency using Agilent PL-SAX 1000Å and 4000Å packing material.

Fast separations of large biomolecules on PL-SCX

PL-SCX is a macroporous PS/DVB matrix with a very hydrophilic strong cation-exchange coating. For globular protein analysis and purification, the 1000Å material has the optimum pore size for maximum loading with low band broadening. The more open pore structure of the 4000Å is preferred for high resolution and high speed applications or for the separation of very large biomolecules (Figures 4 and 5).

Conditions

Eluent A: 0.02 M KH_2PO_4, pH 6
Eluent B: A + 0.5 M NaCl, pH 6
Detection: UV, 280 nm

Figure 4) Column: PL-SCX 4000Å 8 µm, 50 x 4.6 mm (p/n PL1545-1803)
Flow Rate: 4.0 mL/min

Figure 5) Column: PL-SCX 1000Å 8 µm, 50 x 4.6 mm (p/n PL1545-1802)
Gradient: Linear 0-100% B in 20 min
Flow Rate: 1.0 mL/min

Peak Identification

1. Myoglobin
2. α-chymotrypsinogen A
3. Cytochrome C
4. Lysozyme

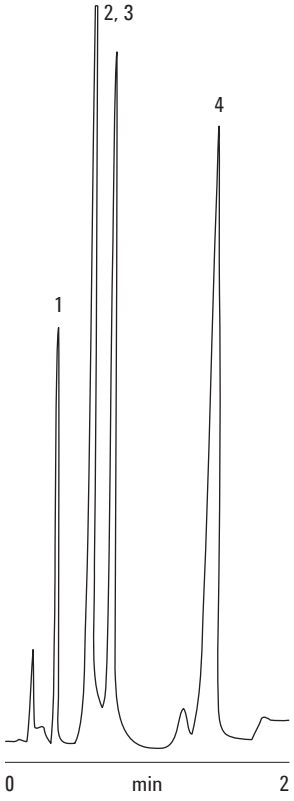

Figure 4. Separation of large molecules on Agilent PL-SCX 4000Å at 4.0 mL/min.

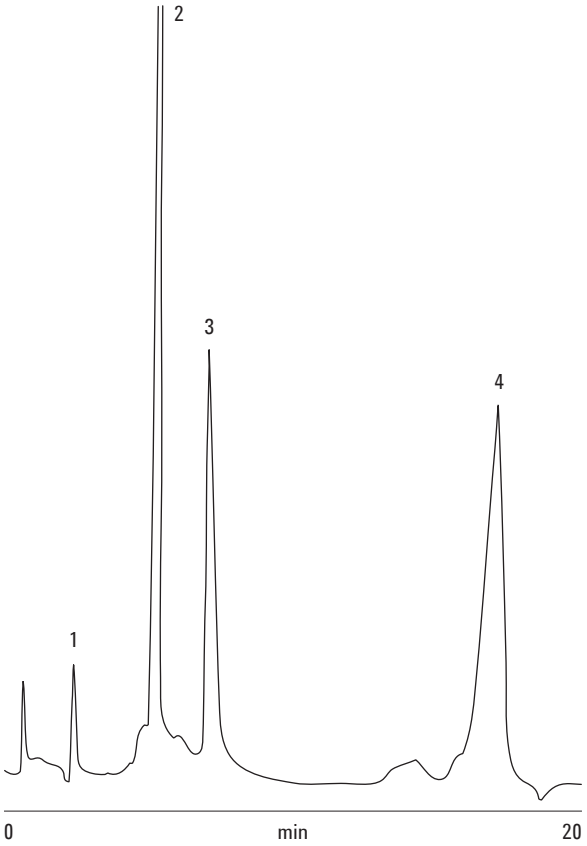

Figure 5. Separation of large molecules on Agilent PL-SCX 1000Å at 1.0 mL/min.

Very high speed reversed-phase protein analysis on PLRP-S

The structure of the gigaporous PLRP-S 4000Å 8 µm column permits the rapid reversed-phase separation of proteins. The six commonly used reversed-phase protein test probes are resolved in 60 s using this column (Figure 6). The separation is achieved at ambient temperature with a standard analytical HPLC UV detector with a 10 µL cell volume and 10 mm path length. The total mass of protein injected was 0.34 mg[3].

Conditions

Column: PLRP-S 4000Å 8 µm, 50 x 4.6 mm (p/n PL1512-1803)
Eluent A: 0.1% TFA in 5% ACN/95% water (v/v)
Eluent B: 0.1% TFA in 95% ACN/5% water (v/v)
Gradient: Linear 18-60% B in 60 s
Flow Rate: 4.0 mL/min
Detection: UV, 280 nm

Peak Identification

1. Ribonuclease A
2. Cytochrome c
3. Lysozyme
4. BSA
5. Myoglobin
6. Ovalbumin

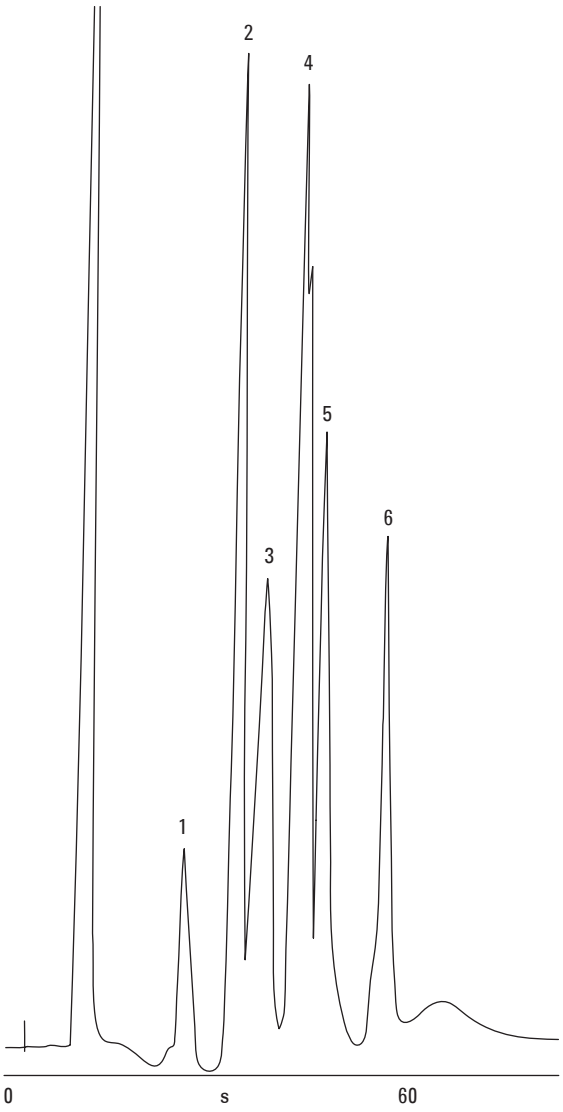

Figure 6. Separation of six protein test probes in less than sixty seconds on an Agilent PLRP-S 4000Å column.

High speed peptide analysis on PLRP-S

As a single column, PLRP-S operates across the entire range of HPLC eluents. Because of the stability and physical robustness of PLRP-S, it is possible to switch between organic modifiers such as ACN and tetrahydrofuran, and eluent pH 0 to 14.

The capability of the gigaporous PLRP-S 4000Å material is demonstrated in high speed separations of small peptides, the neurotensins, and the larger protein, myoglobin, with no loss in resolution (Figure 7).

Conditions

Column: PLRP-S 4000Å 8 µm, 50 x 4.6 mm (p/n PL1512-1803)
Eluent A: 0.1% TFA in 1% ACN/99% water
Eluent B: 0.1% TFA in 99% ACN/1% water
Gradient: Linear 10-60 % B in 2 min
Flow Rate: 4.0 mL/min
Detection: UV, 220 nm

Peak Identification

1. Neurotensin fragment 1-8
2. Neurotensin fragment 8-13
3. Neurotensin
4. Myoglobin

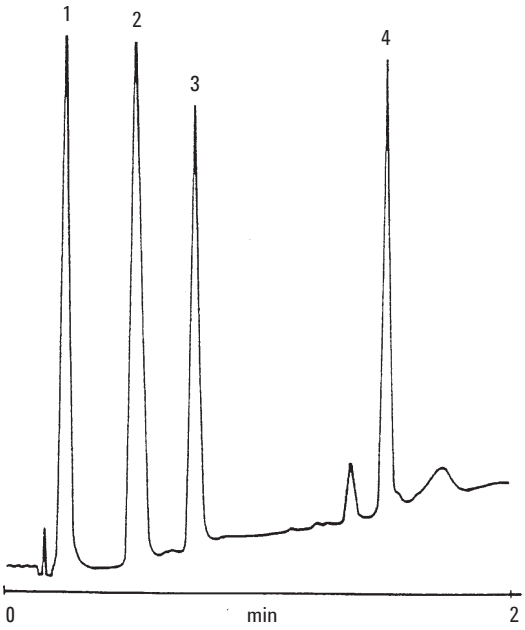

Figure 7. High speed separation of peptides and protein on Agilent PLRP-S 4000Å.

References

[1] Linda L. Lloyd and Frank P. Warner (1991) High speed analytical and preparative separation of biological macromolecules. In: D L Pyle (Ed.) *Separations for Biotechnology.* Elsevier Applied Science.

[2] Linda L Lloyd (1991) Rigid macroporous copolymers as stationary phases in high performance liquid chromatography. *J. Chrom.*, 544, 201-217.

[3] L. L. Lloyd and F. P. Warner (1990) Preparative high performance liquid chromatography on a unique high-speed macroporous resin. *J. Chrom.*, 512, 365-376.

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