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Multidimensional LC Determination of Proteins Using Monolithic WAX and RP Columns

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INTRODUCTION

The recent development of monolithic columns has significantly benefited the biopolymer analysis field. Monolithic columns offer several advantages over particulate columns due to their macroporous structure that facilitates fast mass transfer, low backpressure, and high resolution. Polystyrene divinylbenzene (PS-DVB) monolithic columns are chemically inert, which offers high pH stability and excellent chromatographic performance in reversed-phase liquid chromatography (LC).

This application highlights the use of weak anion-exchange (WAX) monolithic columns in combination RP monolithic columns in micro format, in an off-line 2-D LC setup.

Method flexibility and high throughput are achieved by performing fraction collection between the two chromatographic dimensions with fast flow rates. Off-line 2-D separation of proteins was performed on polymethacrylate based monolithic WAX and polystyrene divinylbenzene-based reversed-phase columns. The column performance, high peak capacity and throughput of the 2-D LC method are studied with a set of protein standards and a bacterial protein lysate.

EXPERIMENTAL

LC Conditions

LC System: UltiMate™ 3000 – Nano, Capillary, and Micro LC
Columns: ProSwift™ WAX -1S, 4.6 mm i.d. × 50 mm (P/N 064294)
ProSwift RP 1S, 4.6 mm i.d. × 50 mm (P/N 064297)
ProSwift RP 1.0 mm i.d. × 50 mm (prototype)
Samples: Five protein standard, 30 µg/mL
Cytochrome c digest (P/N 161089), 8 pmol/µL
Fractions from the *Escherichia coli* protein sample, 3 mg/mL

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First Dimension (WAX)

Mobile Phases: (A) 10 mM Tris (pH 7.6)
(B) 10 mM Tris, 1 M NaCl (pH 7.6)
Gradient: 0–750 mM NaCl in 9 min
Flow Rate: 2 mL/min
Fractions: 30 s
Injection: 10 µL
UV: 13 µL PDA flow cell, 214 nm

Second Dimension (RP)

Mobile Phases: (A) Water (100), 0.05% TFA
(B) Water/CH_3CN (50/50), 0.1% TFA
Gradients: 2.5–50% CH_3CN in 7.5 min for the protein and peptide standards
12.5–45% CH_3CN in 7.5 min for the *E. coli* fractions
Flow Rate: ProSwift RP-1S, (4.6 mm i.d.) 1,7 mL/min
ProSwift RP (1 mm i.d.) prototype 80 µL/min
UV: 280 nm

ESI-MS/MS on Digested Fractions

- Positive ion mode, mass range 200–2000 *m/z*, cycle time 0.12 min
- Mass tolerance of 1.0 Da for the parent ion and 0.5 Da for the fragment ions
- Proteins characterized by at least two peptides

RESULTS & DISCUSSION

ProSwift WAX Monolithic Columns

Functionalized polymethacrylate monolithic separation media offer several advantages over their microparticulate counterparts with regard to separation efficiency and analysis time.

A special technique of in-situ polymerization ensures the unique morphology of the polymeric rod characterized by large channels which allow fast mass transfer of large molecules. ProSwift WAX monoliths have a pore volume that is 60% of the total column volume, making them very permeable.

PITTCON 2007 Presentation

Due to the large column diameter, high percentage of open pores and channels, and absence of diffusion-controlled mass transfer phenomena, these columns feature large loading capacity, fast mass transfer, low backpressure, and high separation speed and resolution.

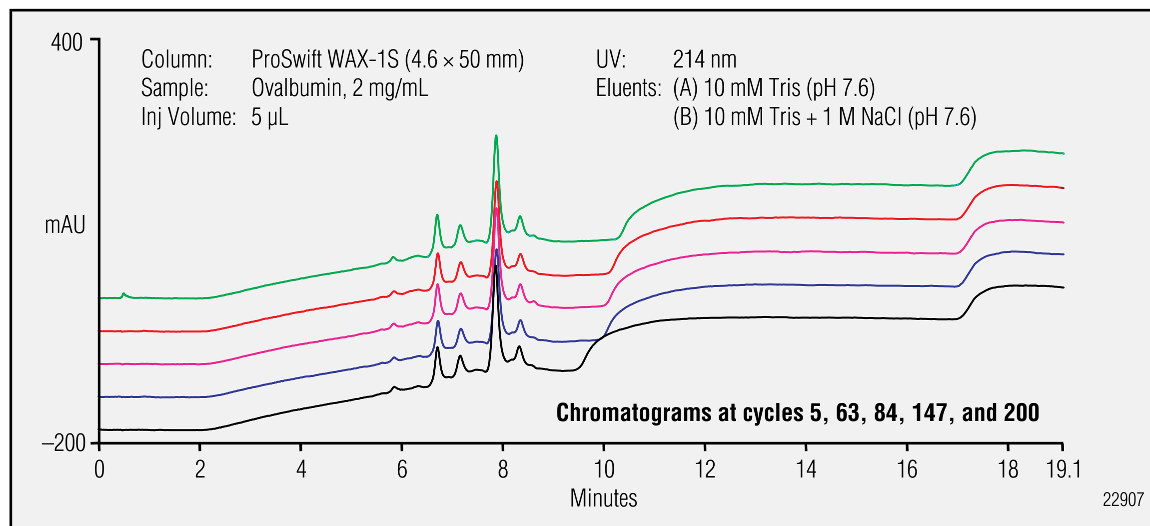


Figure 1. Ovalbumin separation on ProSwift WAX.

ProSwift RP-1S and 1.0 mm i.d. Prototype Monolithic Columns

The performance of the ProSwift RP-1S monolithic was compared to a prototype monolithic column 1 mm i.d. × 50 mm by separating a standard protein sample and a cytochrome c digest with the same gradient for both columns. The flow rate and amount of sample were adjusted to column dimensions. The results are shown in Figure 2.

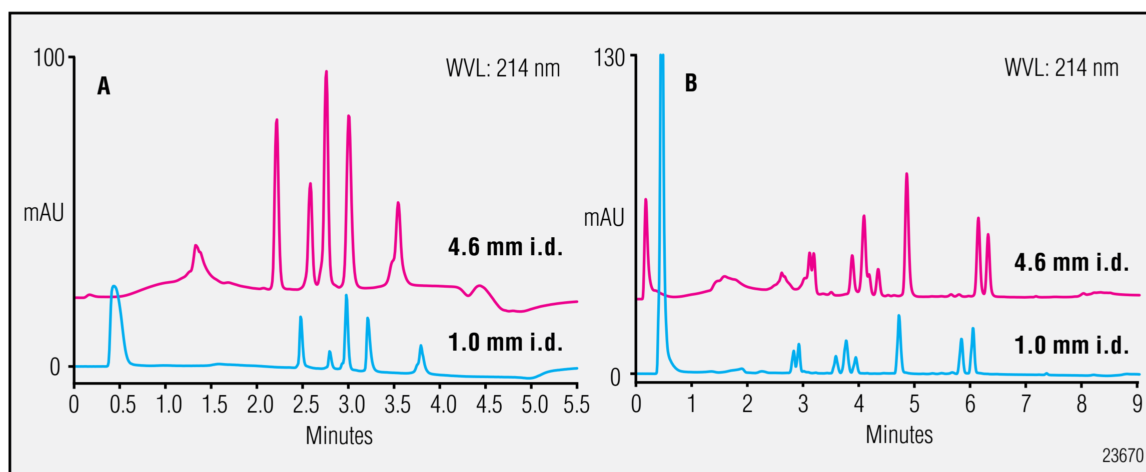


Figure 2. Comparison between ProSwift RP-1S 4.6 mm and 1 mm prototype monolithic column. Gradient: 2.5–50% CH_3CN in 3 min.

A. Protein Separation:

(1) RP, 1.0 mm i.d. × 50 mm, 80 µL/min, 15 ng (0.5 µL)
(2) RP-1S, 4.6 mm i.d. × 50 mm, 1.7 mL/min, 300 ng (10 µL)

B. Peptide Separation (cytochrome c digest):

(1) RP, 1.0 mm i.d. × 50 mm, 80 µL/min, 17 pmol (2.5 µL)
(2) RP-1S, 4.6 mm i.d. × 50 mm, 1.7 mL/min, 240 pmol (30 µL)

Multidimensional LC of *E. coli* Proteins

Intact proteins were collected upon elution from the 4.6 mm i.d. × 50 mm ProSwift WAX column in 20 fractions of 30 s in HPLC vials. Applying a 7.5-min gradient, 100 µL of each fraction was separated on a 1.0 mm i.d. × 50 mm PS-DVB monolithic column. Further fractionation of the RP fractions in a 384 low-bind well-plate, followed by in-well tryptic digestion, allows protein identification by LC-ESI/MS analysis of the resulting digests. The multi-dimensional approach for protein analysis by LC-MS is illustrated in Figure 3.

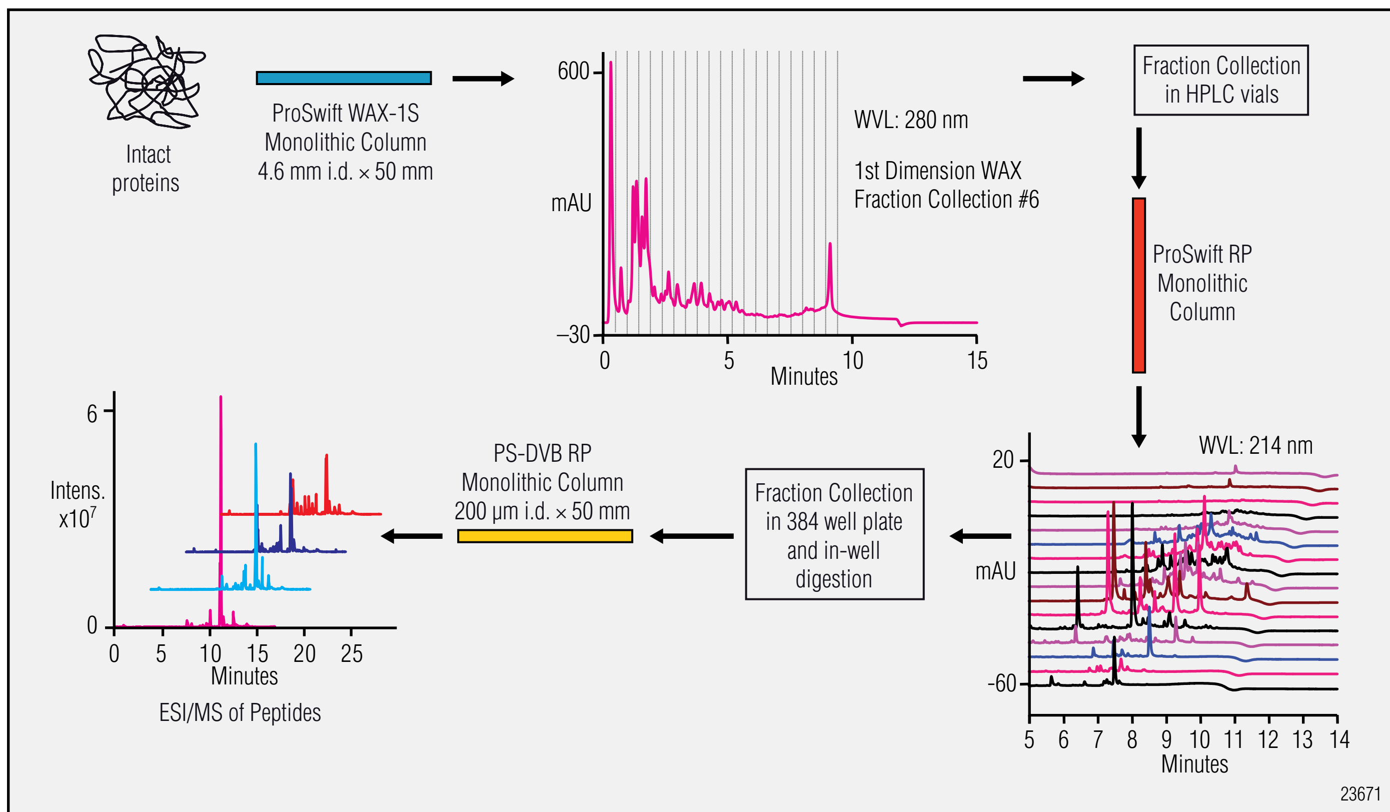
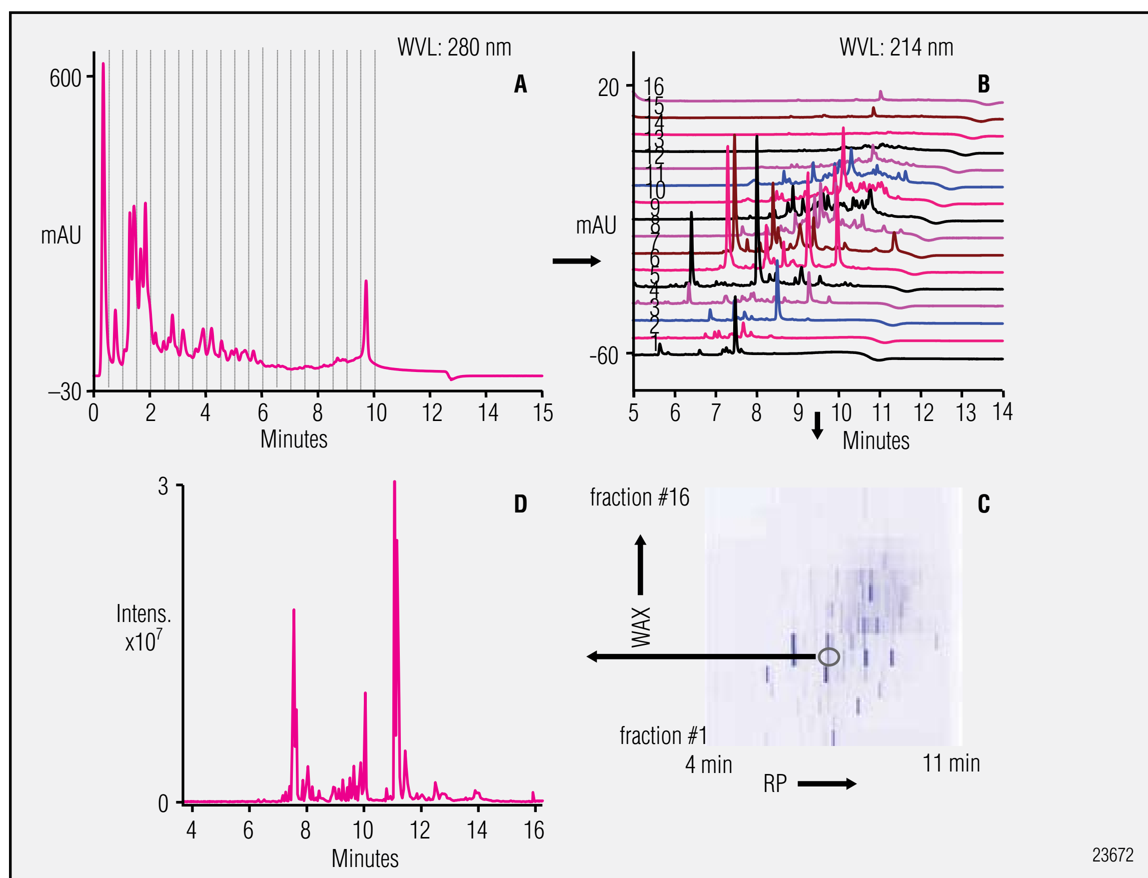


Figure 3. Off-line 2D-LC work flow of intact proteins on ProSwift monolithic columns.



A. First dimension. Separation of 300 µg proteins on a ProSwift WAX-1S with 0–750 mM NaCl in 9 min.

B. Second-dimension: Separation of 16 WAX fractions on a 1.0 mm i.d. monolithic column. Gradient: 12.5–45% CH_3CN in 7.5 min

C. Reconstructed 2-D plot of E. coli *proteins from the WAX and RP separations.*

D. BPC-All MS of selected fraction after in-well digestion with trypsin. The digest was run on a 200 µm i.d. × 50 mm monolith and analyzed on-line using ESI-MS/MS.

The entire protein separation was achieved in just 4 h, after which an in well digestion was performed on the reversed-phase fractions. Table 1 lists significant protein identifcation of four adjacent wells with average peak intensity and their sequence coverage.

Table 1: Significant Identified Proteins in In-well Digested Protein Fractions (3rd Dimension)

Protein	Molecular Weight (Da)	Score	Seq. cov. (%)
Leucine-specific binding protein precursor [*E. coli* UTI89]	41717	156	31
High-affinity zinc transporter periplasmic component [*E. coli* K12]	33872	140	23
COG3443: Predicted periplasmic or secreted protein [*E. coli* E24377A]	24883	70	14
Peptidyl-prolyl *cis/trans* isomerase B (rotamase B) [*E. coli* K12]	18258	58	12

Peak Capacity of the 2-D LC Method on Monolithic Columns

A column's peak capacity for a given gradient equals the ratio between the gradient (separation) time and the peak width at 4 s (13.4% of peak height) plus 1.

The 9-min gradient on the WAX monolithic column 4.6 mm i.d. × 50 mm (first dimension) allows peak capacities of 50. In the second dimension (monolithic column 1.0 mm i.d. × 50 mm), average peak capacities of 90 are attained. Assuming that the two LC dimensions used in the experiment provide true orthogonal separation selectivity and that a fraction has been collected for each peak from the WAX separation, the peak capacity values can be multiplied. Therefore, a maximum peak capacity of 4500 is achieved when a 7.5-min gradient is employed in the second dimension (RP). However, in this particular case, the number of fractions collected from the first dimension is 16, which gives a maximum peak capacity of 1440 for the *E. coli* intact proteins.

CONCLUSIONS

1. ProSwift WAX columns were used for ion-exchange separation of proteins with high efficiencyand reproducibility.

2. ProSwift RP monolithic columns 4.6 mm i.d. × 50 mm allow for the fast separation of a large quantity of proteins, with excellent resolution.

3. Resolution and peak shape of peptides separated on ProSwift RP-1S and the 1.0 mm i.d. prototype monolithic columns are very good and comparable to each other.

4. This off-line 2D-LC method provides a fast and high-resolution separation of intact *E. coli* proteins. When carried out with 4.6 mm i.d. columns in both dimensions, the method allows for HPLC analysis of large protein amounts in a relatively short time frame.

5. Using monolithic columns for intact protein separations is an attractive technique because LC-based protein prefractionation can be achieved in comparable or less time than gel electrophoresis.

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