Multidimensional Bioseparations Using Ion-Exchange and Reversed-Phase Monolith Columns

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INTRODUCTION

Historically protein analysis has relied on 2DE (two-dimensional electrophoresis) to isolate proteins of interest. However, the increased need for speed and sensitivity is pushing the limits of this technology. HPLC alternatives are being developed to overcome the present limitations of protein analysis. The advantages of using HPLC over 2DE are immense:

- No long gel runs
- No gel removal steps
- No gel interactions
- Fewer size limitations (small peptides to large proteins)
- No need for staining with in-line UV detection
- Greater sensitivity
- Ease of automation
- Multiplicity of HPLC separations (e.g. ion-exchange to reversed-phase)
- Direct interfacing to a variety of MS systems

The development of monolithic technology is expanding the boundaries of HPLC for both small molecule and bioseparations.

Using ProSwift™ monolith columns, the separation of biomolecules can be achieved at elevated linear velocities with little to no loss of resolution. This range of columns also provides high mass loading capacities, making them excellent for the first-dimension separation of crude samples. Alongside excellent batch-to-batch reproducibility, the unique monolith design and inert column housing provide the excellent pH stability required for column sterilization.

In proteomic analysis, sample availability is often limited. Improved sensitivity, high mass loading capabilities, and a variety of separating mechanisms are required to analyze low copy number, membrane, highly basic or acidic, or posttranslationally modified proteins.

Polymeric ion-exchange monoliths are designed to maintain good resolution at high loading capacities. Introduction of 1-mm analogues of the current 4.6-mm ion-exchange columns extends to capabilities of the product line by bridging the gap between analytical and micro-bore columns to provide increased sensitivity with lower solvent consumption while still allowing the use of standard analytical HPLC systems.

We will demonstrate here how ProSwift ion-exchange and reversed-phase columns can be used for the multidimensional separation of crude protein samples towards detection and identification by mass spectrometry.

INSTRUMENTS

ICS-3000 Dual Ion Chromatography System (Dionex Corp. Sunnyvale, CA) equipped with LPG DP-3000 pump, Detector/Chromatography module (DC), ICS-Series Variable Wavelength Detector (VWD), and AS Autosampler.

UltiMate® 3000 Chromatography System (Dionex Corp. Sunnyvale, CA) with LPG-3200A pump, TCC-3100 column oven, VWD-3400 variable wavelength detector, and WPS-3000 autosampler.

Software: Chromeleon® 6.8 Chromatography Management Software (Dionex, Sunnyvale, California) was used for system control and data processing.

Columns: ProSwift product line, Dionex, (Sunnyvale, California).

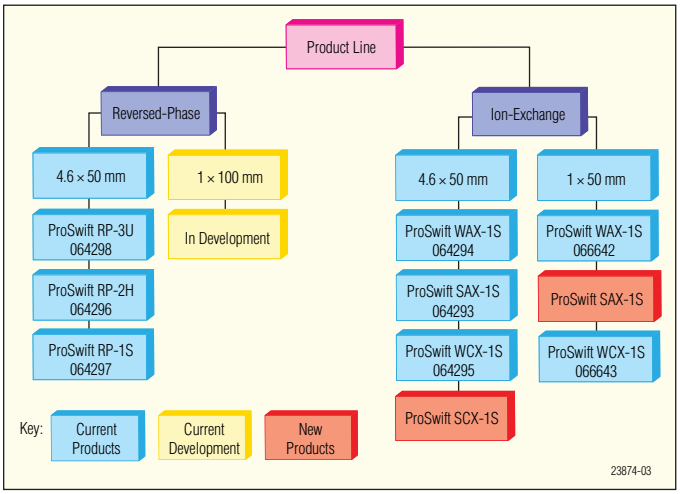

Figure 1. ProSwift family tree.

ION-EXCHANGE CHROMATOGRAPHY

Proteins are made up of acidic, basic, and neutral peptides. At a given pH, depending on the amount of acidic and basic peptides, the protein will become neutral. This pH is known as the isoelectric point, pI.

- At a pH above the pI, a protein will have a negative net charge and below it, a positive net charge.
- The ion-exchange stationary phase is charged, either positive or negative. Ionized proteins will either be attracted or repelled depending on the charge of the protein.
- A cation-exchange column surface is negative and positively charged proteins will be retained.
- An anion-exchange column surface is positive and negatively charged proteins will be retained.
- Elution takes place in one of two ways:
  1. By the addition of counter ions which are attracted to the surface more strongly than the protein, taking its place on the surface and causing it to elute.
  2. By adjusting the pH of the system so that the protein is no longer ionized and will no longer be retained on the column.

Crude protein samples contain a mixture of proteins with very different pIs. The column and eluent used depend greatly on the mixture and protein of interest in the sample.

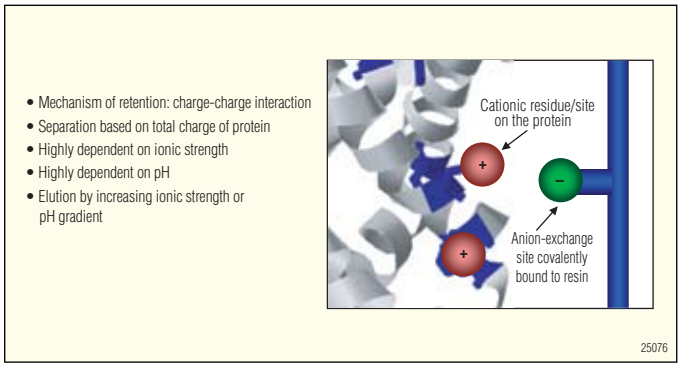

Figure 2. Protein interaction.

Table 1. Isoelectric Points and Molecular Weights of Studied Proteins

Protein Name	Mwt	pI	PN	Retention
Albumin, chicken egg	44287	4.54 (4.43-4.66)	A2512	SAX
Trypsin Inhibitor	21000	4.6	T1021	SAX
Lactoglobulin A, β	18363	4.83	L7880	SAX
holo-Transferrin	76-81 kDa	5	T4132	SAX
Lactoglobulin B, β	18726	5.31	L8005	SAX
Transferrin	79000	5.5	T3309	SCX
Albumin, bovine	66430	5.6	A0281	SAX
Albumin, Human	66478	5.85	A3782	SAX
apo-Transferrin	79000	6	T1428	SAX
Conalbumin	77776	6.85	C0755	SAX, SCX
Crystallin, β	80000	6.9	C5163	SAX, SCX
Chymotrypsinogen A, α	25600	8.97	C4879	SCX
Ribonuclease A	13700	9.6	R5500	SCX
Cytochrome C, Yeast	12588	9.86	C2436	SCX
Cytochrome C, Bovine	12200	10.37	C2037	SCX
Cytochrome C, Equine	12384	10-10.5	C7752	SCX
Lysozyme	14700	11.35	L6876	SCX

Protein pI information was gathered from Sigma Aldrich product info pages.

Swiss-Prot info was used as an estimation where data was not available from Sigma-Aldrich.

Retention mechanism obtained by anion and cation exchange chromatography.

At pH 6.0, the cation-exchange column will be negatively charged and retain protein with a pI above this pH. The anion-exchange column will be positively charged and will retain proteins with a pI below this pH. If the protein has a pI of 6 it will be neutral and unretained.

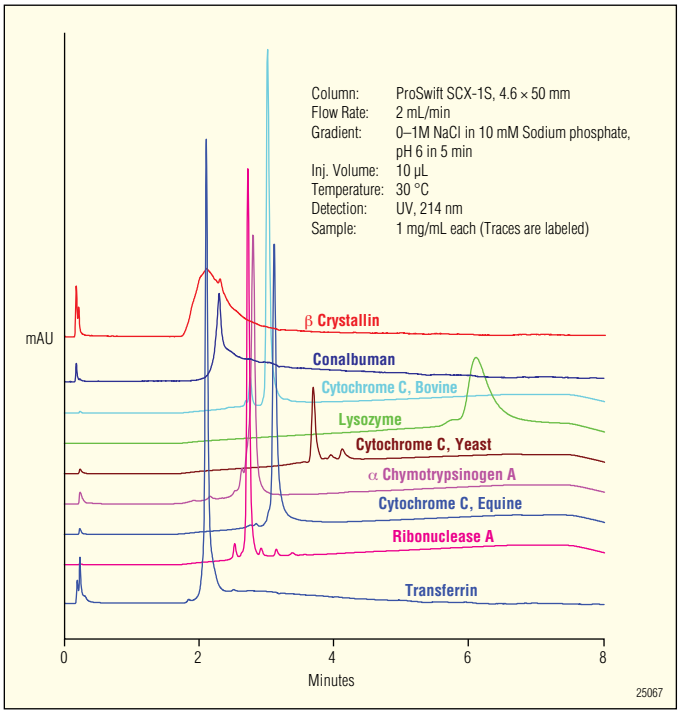

Figure 3. Proteins retained at pH 6.0 on strong cation-exchange column.

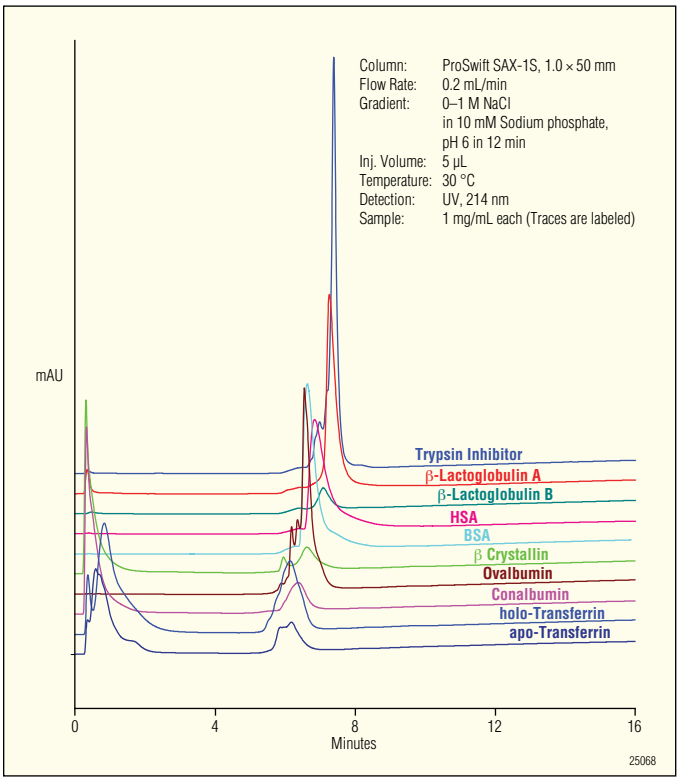

Figure 4. Proteins retained at pH 6.0 on strong anion-exchange column.

HIGH MASS LOADING AND FRACTIONATION

- ProSwift Ion-exchange monolith columns are designed to allow a high mass loading towards preparative analysis (Figure 5).
- The 1-mm format columns show high loading capacity (Figure 6) coupled with excellent mass sensitivity (Figure 7), making them an excellent choice for analysis where sample is limited or where a sample contains the protein of interest in low abundance. Large amounts of protein can be loaded without loss of resolution of this protein.
- Determination of dynamic loading capacity by 50% increase in PWHH can be deceiving. For a column with excellent resolution, far more protein than calculated can be loaded before resolution of the protein of interest is lost.

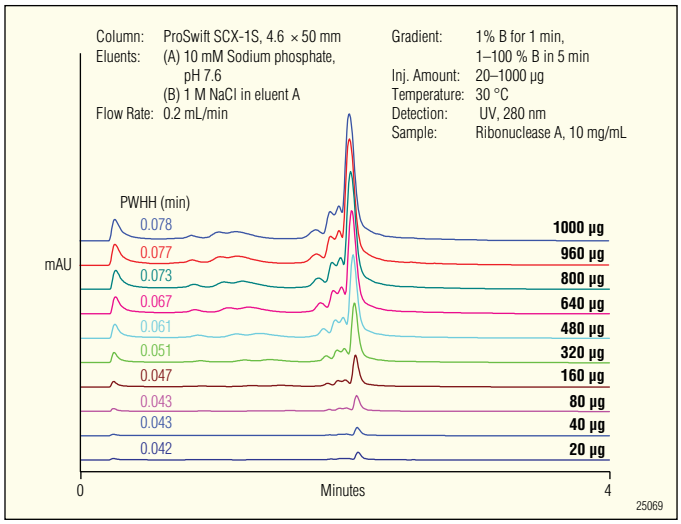

Figure 5. Dynamic loading of ProSwift SCX-1S, 4.6 × 50 mm.

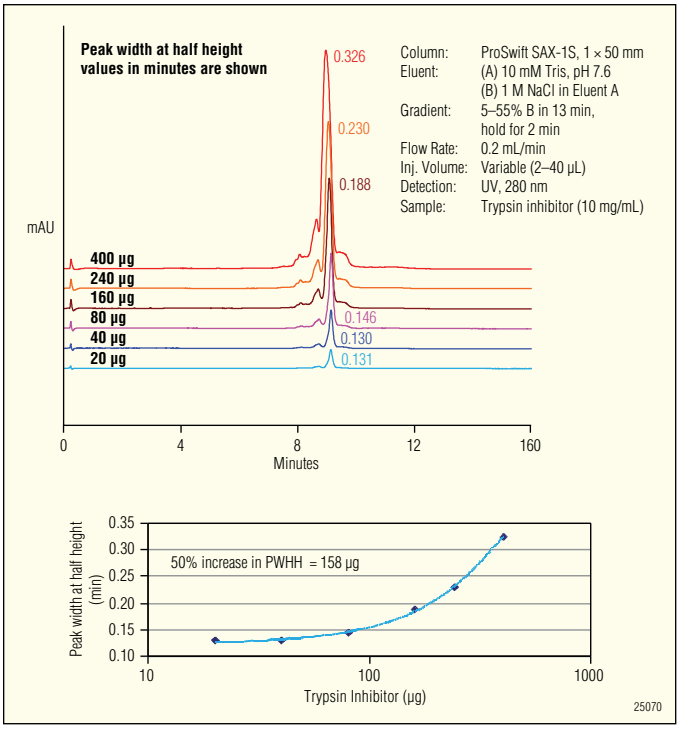

Figure 6. Dynamic Loading of ProSwift SAX-1S, 1 × 50 mm.

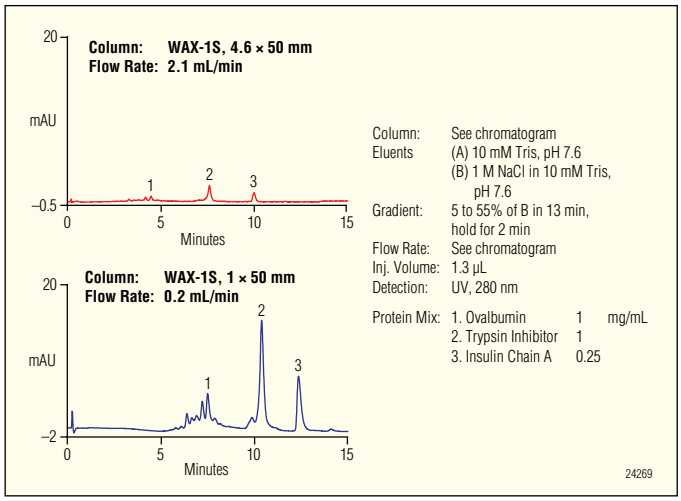

Figure 7. Sensitivity of 1-mm column vs 4.6-mm column.

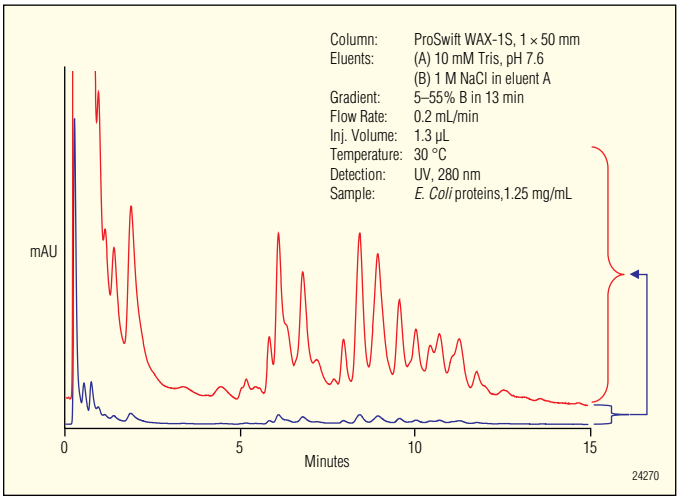

Figure 8. Separation of E. coli *proteins on the ProSwift WAX-1S, 1 × 50 mm.*

FRACTIONATION OF CRUDE PROTEIN SAMPLES

E. coli protein sample (BioRad, Hercules, CA)

- About 200 intact proteins can be isolated from *E. coli* K-12 (strain MG1655), resulting in over 200 modular units.
- A modular unit is a protein element of at least 83 amino acids that has an independent biological function. Most *E. coli* proteins contain one module. Proteins encoded by a gene that has undergone a gene fusion event contain two or more modules.
- In order to study the structure of each protein fractionation, separation and subsequent analysis by MS is required.
- Separation and isolation by 2DE is labor intensive and unsuitable for instances of low copy number.
- Separation using ion exchange allows the first-dimensional separation by protein pI.
- The majority of *E. coli* proteins are anionic at pH 7. Therefore anion-exchange chromatography is desirable for the isolation and detection of the widest range of proteins.
- Separation by cation exchange at low pH allows the specific isolation of the few cationic proteins.

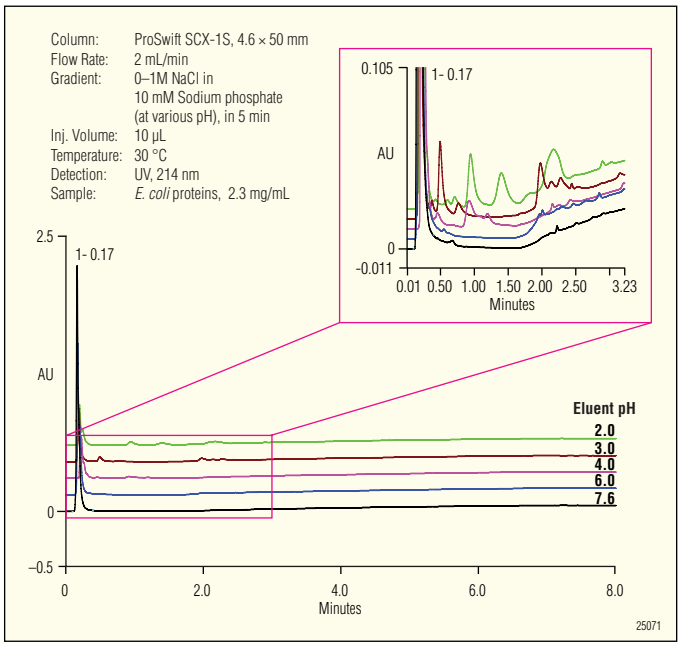

Figure 9. Separation of E. coli *by strong cation exchange at various pHs.*

E. coli proteins (200 µg) were loaded onto a 1-mm ProSwift SAX-1S column and fractions were collected at 2 minute intervals (Figure 10). Each fraction was then separated using a prototype 1-mm monolithic RP column (Figure 11). This column is housed in inert housing, ensuring stability at high pH to enable sterilization often required for bioapplications. Fraction 1 was then separated by cation exchange at low pH (Figure 12).

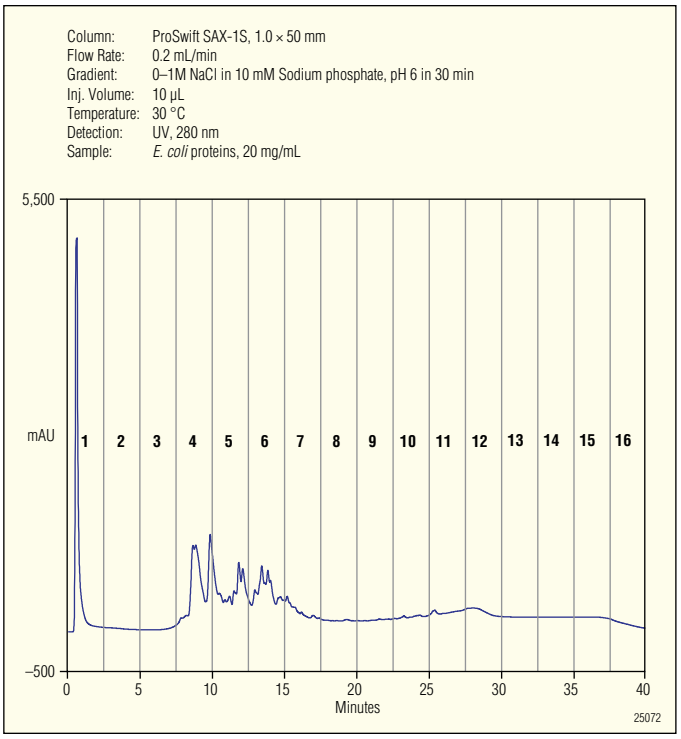

Figure 10. High mass loading of E. coli *on ProSwift SAX-1S, 1 × 50 mm.*

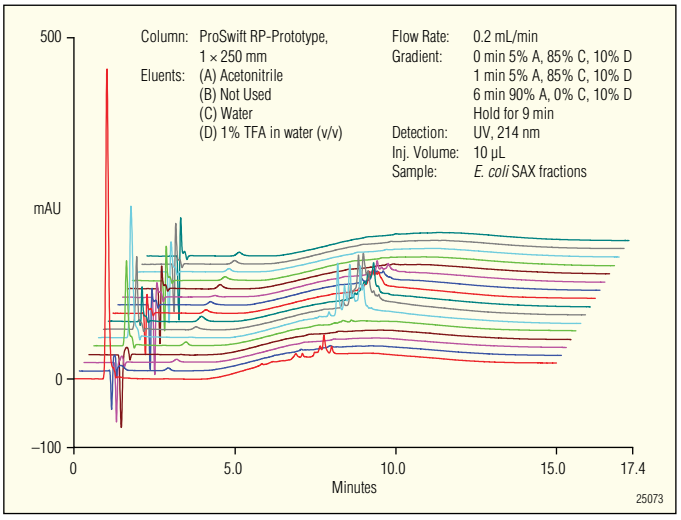

Figure 11. Reversed-phase analysis of SAX protein fractions.

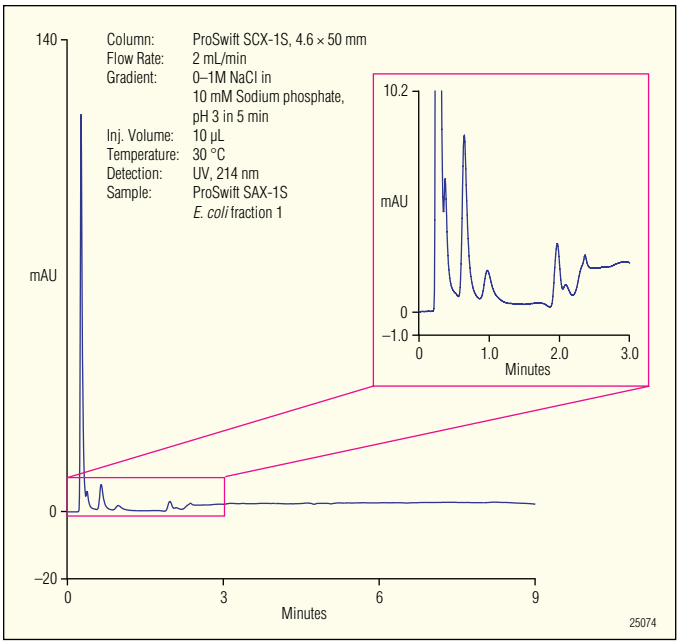

Figure 12. Fraction 1 by Strong Cation Exchange. Fraction 1 contains proteins with a pI < 6.

SUMMARY

- ProSwift monoliths are a new family of columns developed for protein separations. They offer high speed, high resolution, and high loading capacity.
- Monolith technology allows excellent scalability, reproducibility, and overall performance.
- New techniques have been successfully employed to allow preparation of formats >500 nm.
- ProSwift (1-mm) columns allow increased sensitivity and bridge the gap between analytical and microbore columns.
- ProSwift ion-exchange columns now include SCX-1S, available in 4.6 × 50 mm format, and a SAX-1S in a 1 × 50 mm format.
- Due to the high capacity of ProSwift IEX columns, they are ideally suited for the first dimension in a multidimensional chromatography separation.

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