

Novel Off-line Multidimensional LC Method for Separation and Tandem MS Detection of Tryptic Peptides

INTRODUCTION

Multidimensional LC separations are increasingly being used for the analysis of complex samples in proteomics and pharmaceutical biotechnology to increase resolution. The development of multidimensional LC methods involves optimization of various experimental parameters. Two of the foremost issues to be considered are 1) whether to use an on- or off-line approach, and 2) whether to perform the separation on the peptide or protein level.

Off-line multidimensional LC techniques have several advantages over on-line approaches: 1) more efficient separations, 2) higher flexibility with respect to column dimensions and mobile phase selection, 3) ability to perform re-injection of fractions. Here we present an LC instrument designed for fully-automated multidimensional LC, with both dimensions in the $\mu\text{L}/\text{min}$ flow range. The micro fractionation collection option (μFC) of the WPS-3000PL autosampler allows both injection and fractionation. The instrument's injection and fractionation performance is discussed in a fully automated 2-D LC peptide application.

INSTRUMENTAL SETUP

The UltiMate[®] 3000 Proteomics MDLC system equipped with the μFC option included the following modules:

- SRD-3600 Solvent Degasser
- DGP-3600M Dual Gradient Pump
- VWD-3400 UV Detector
- FLM-3100 Column Oven/Flow Manager with CAP splitter
- WPS-3000PL Autosampler with FC option

AUTOMATED OFF-LINE TWO-DIMENSIONAL LC OF PEPTIDES

The fractionation option of the WPS-3000PL allows automated off-line two-dimensional separations. In this work, both the first dimension, strong cation-exchange (SCX) separation, as well as the second dimension, reversed-phase (RP) separation, are performed at a capillary scale. The flow rates for the SCX and RP separation of 6.0 and 2.5 $\mu\text{L}/\text{min}$, respectively, are generated by active flow splitting using one flow manager with UltiFlow[™] technology. Two independent gradient pumps supply the mobile phase gradients for the ion-

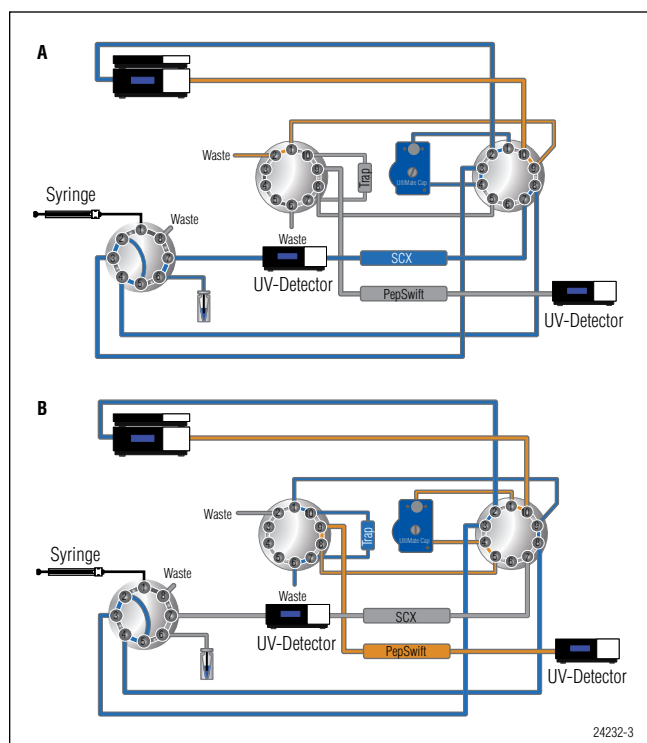


Figure 1. Fluidic setup. Top) 1st dimension separation. Bottom) 2nd dimension separation.

exchange and reversed phase separations. Figure 1 shows the schematic for automated off-line 2-D LC of peptides.

Peptides are separated on the capillary SCX column, fractionated through the injection needle into a 384 well plate, re-injected onto a monolithic trap column, and separated on a capillary PepSwift™ monolithic column.

Figures 1A and 1B show flow schematics for the first and second dimension separations, respectively. Table 1 lists the experimental parameters.

RESULTS AND DISCUSSION

The precision of the fully-automated 2-D LC system was validated by performing consecutive analyses. A mixture of tryptic peptides from six proteins (Table 2) was used as a test sample.

| Table 1. Experimental Conditions for Fully Automated Off-line 2-D LC | | |
|----------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|
| | First Dimension: SCX | Second Dimension: RP |
| Column | PolySULFOETHYL, 300 m i.d. × 15 cm, 5 μm, 300Å | PepSwift Monolith, 200 μm i.d. × 5 cm; PepSwift Monolithic trap 200 μm i.d. × 5 mm |
| Mobile Phase A | 95/5 water/CH ₃ CN, 5 mMol NaH ₂ PO ₄ buffer, pH = 3 | Water + 0.05% TFA |
| Mobile Phase B | A + 1 M NaCl | 20/80 water/CH ₃ CN + 0.04% TFA |
| Loading Solvent | | Water + 0.05% HFBA |
| Flow Rate | 6 μL/min | Analytical 2.5 μL/min Loading 20 μL/min for 5 min |
| Gradient | 0% to 60% B in 20 min, 100% B for 5 min. 20 min equilibration | 0% to 35% B in 10 min, 90% B for 2 min: 6 min equilibration |
| UV Detection | 214 nm (45 nL flow cell) | 214 nm (3 nL flow cell) |
| Sample | 1 pmol/μL tryptic digest of cytochrome c, lysozyme, alcohol dehydrogenase, bovine serum albumin, apo-transferrin, β-galactosidase | SCX fractions |
| Injection Volume | 10 μL | 6 μL |
| Fractionation | 20 fractions of 1 min | |
| Oven Temperature | ambient | 60 °C |

Figures 2A and B show the chromatograms obtained for the SCX separations and RP separations of SCX fraction #13, respectively, from the three consecutive 2-D LC experiments. The chromatograms were obtained with a time interval of 12 hours, corresponding to the duration of one 2-D LC experiment.

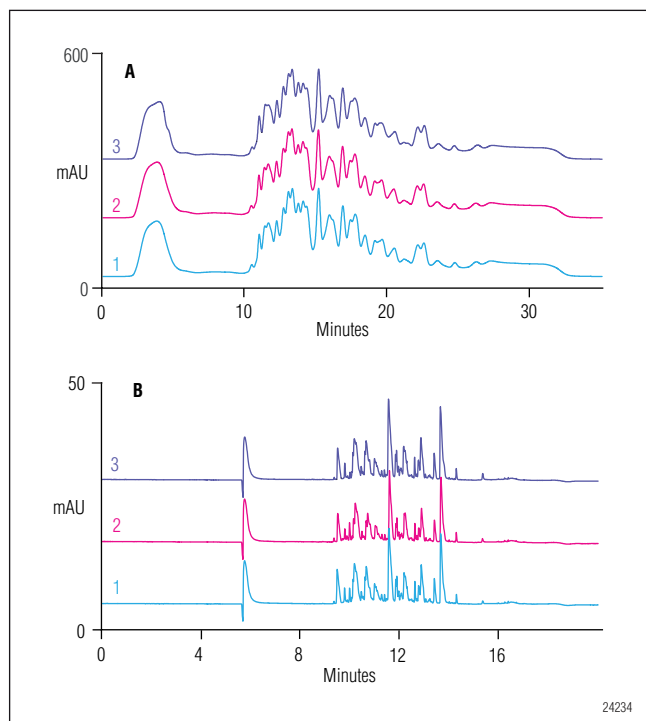


Figure 2. Second dimension: injection, RP separation, and detection of SCX

The chromatographic data obtained with the automated off-line 2-D LC system is highly repeatable. Excellent precision of retention times is achieved for both separation dimensions; retention time RSD is 0.07% for the SCX and 0.08% for the RP separations.

The capillary scale PS-DVB monolithic column yields high resolution separations for tryptic peptides with a peak capacity of around 150. The 2-D LC plot in Figure 3 shows the large number of separated peptides.

The excellent retention time precision is also evident from Figure 4. Here the MS and MS/MS spectra for a tryptic peptide of bovine serum albumin with the sequence TYGDMADCCEKQPER are shown for the three consecutive 2-D LC experiments. The retention time, mass accuracy, and intensities show excellent precision over the 24 h experimental window.

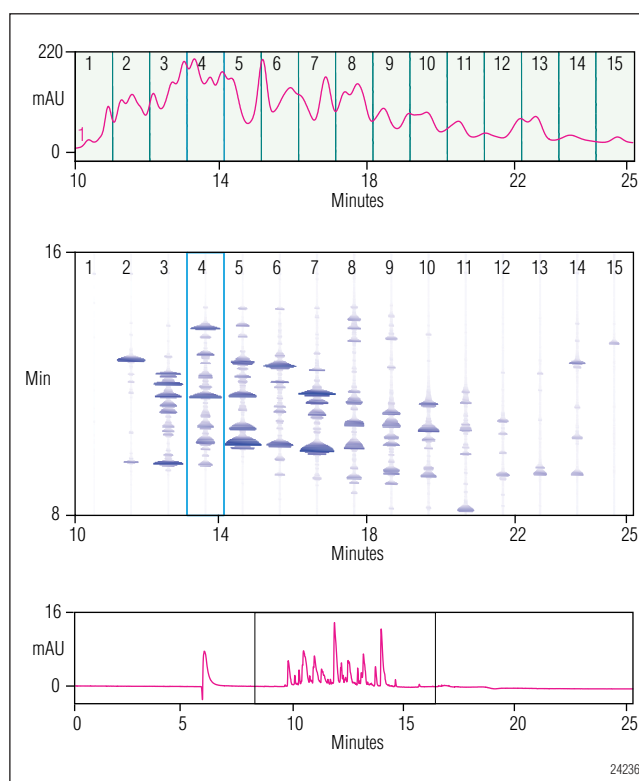


Figure 3. 2-D retention map of the second-dimension (PS-DVB monolithic column) separation.

The mass spectrometric detection of the peptides enables assessment of the SCX separation quality. The elution behaviour of peptides on the SCX was verified by examining the occurrence of 200 peptides in the collected fractions. For that purpose peptide fragmentation MS spectra were matched with a database. In Figure 5, the peptide elution is categorized by the number of SCX fractions in which a peptide was detected.

The majority of peptides (84%) eluted in one or two adjacent fractions. Only 16% eluted in three fractions or more. Elution in two adjacent fractions is inevitable due to peak splitting. Post column dispersion in the injection valve was measured and was found to be negligible.

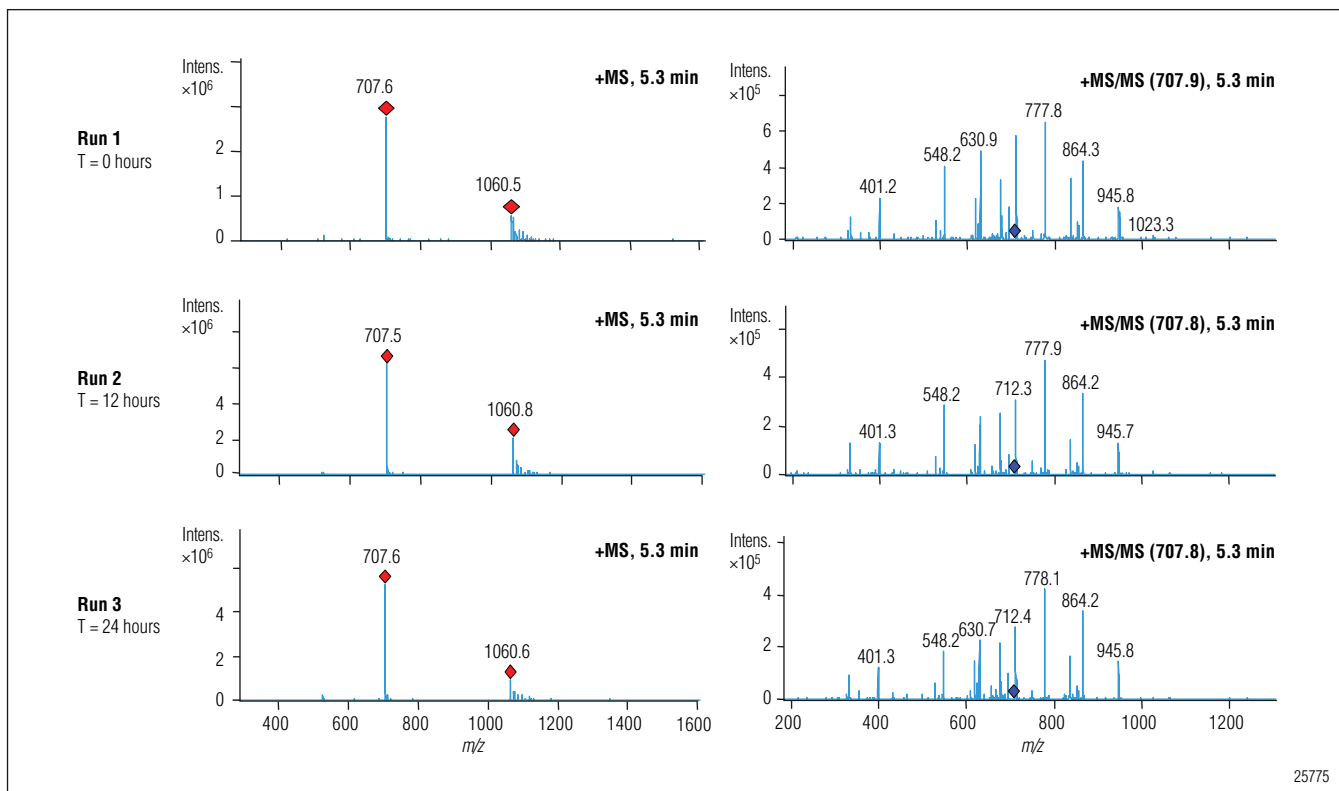


Figure 4. MS and MS/MS spectra for tryptic peptide ETYGDMDACCEKQEPER.

The peptide fragmentation data of each 2-D LC experiment was searched against an in-house protein database using Mascot[®]. Table 2 shows the sequence coverage obtained for the six proteins for three automated off-line 2-D LC experiments.

CONCLUSIONS

- An autosampler with combined injection and fractionation capabilities has been developed for fractionation of capillary-scale separations.
- The instrument supports fully-automated off-line 2-D LC, i.e., sample injection, fraction collection, and re-injection of collected fractions on a second column.
- No detrimental effect on the injection performance is observed.
- The automated off-line 2-D LC method shows excellent repeatability for retention time, peak area (UV) and peptide sequencing.

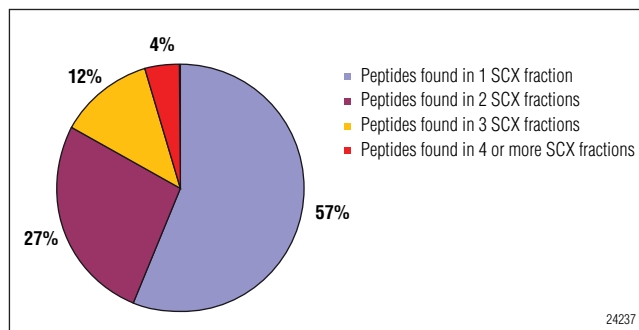


Figure 5. Distribution of peptides over SCX fractions.

| Table 2. Protein Sequence Coverage Over All Fractions | | | |
|-------------------------------------------------------|-------------|-------------|-------------|
| Protein, (Mw, kDa) | Sequence #1 | Sequence #2 | Sequence #3 |
| Cytochrome-C (11.7) | 58% | 57% | 57% |
| Lysozyme (14.8) | 58% | 75% | 75% |
| Alcohol dehydrogenase (37.3) | 53% | 45% | 57% |
| Bovine serum albumin (71.3) | 71% | 71% | 71% |
| Serotransferrin (80.0) | 68% | 66% | 66% |
| β-Galactosidase (117.4) | 45% | 43% | 39% |

MATERIALS FOR AUTOMATED OFF-LINE 2-D PEPTIDE SEPARATION

To order, use the following part numbers and contact your local Dionex office or distributor nearest you. In the U.S., call (800) 346-6390. In other regions, refer to the phone numbers below.

| Description | Part Number |
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| Off-line 2-D LC kit for peptides, polysulfoethyl ASP 300 µm i.d./PepSwift 200 µm i.d. | 6720.0100 |
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| Splitter Cartridge 1:100 (CAP) | 6720.3150 |
| Splitter Cartridge 1:100, biocompatible (CAP) | 6721.3150 |
| UZ-View™ flow cell for VWD 3x00, 10 mm path length, volume 45 nL | 6074.0280 |
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LPN 2155 PDF 2/09
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