Integrated Multi-Dimensional Capillary HPLC Coupled with Mass Spectrometry for Peptide and Protein Analyses

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Abstract

Proteome studies to examine total protein profiles typically begin with a 2-D gel electrophoresis separation step. The protein components are identified following enzymatic digestion of the excised protein spot. Difficulties in analyzing low copy number proteins, membrane proteins, highly acidic or basic proteins and other problems have encouraged development of high performance liquid phase separation schemes that omit gel electrophoresis.

Here we used a multi-dimensional capillary HPLC system to separate complex peptide mixtures. The peptides are captured on the cation exchange column. The fractions of peptides are then transferred to a reversed phase C18 column with steps of salt elution and are subsequently separated on the reversed phase C18 column. The peptides from the chromatographic separation are analyzed by a photo diode array detector or mass spectrometry. Proteins are identified by the sequence database. The integrated multi-dimensional capillary HPLC coupled with mass spectrometry system completely automates sample injection, separation, mass measurement, sequencing and database search.

The loading capacity of a strong cation exchange column (320 µm ID x 5 cm) was evaluated by increasing the loading of a bovine cytochrome c tryptic digest. The cation exchange elution step was optimized by comparing the performance of various salts. Using a strong salt such as KCl to elute peptides affects electrospray ionization and may block capillary tubing so that it is necessary to remove the salt prior to a mass spectrometric analysis. In contrast, volatile salts could be used online without prior removal. We compared the analysis and identification of peptides with two system configurations. One system used a 10-port valve. A pre-column C18 was added online after the cation exchange column to act as a desalting and trapping column, after which the trapped peptides were eluted onto an analytical column. Another system used a 6-port valve with a volatile salt for elution and did not require the additional desalting/trapping column between the ion-exchange column and the analytical reversed phase column. Both automated systems are capable of analyzing complex peptide mixtures with minimal sample cleanup.
The capillary LC coupled mass spectrometry provides broader sensitive applications for peptides and protein analysis.

General Experimental Conditions

LC/MS instrument: Waters CapLC™ and ZQ™ System
Strong cation exchange column (SCX): 0.32x50 mm, PolySULFOETHYL Aspartamide™ SCX, 5 µm, 300 Å (PolyLC Inc, Columbia, MD)
Analytical reversed-phase column (RPC): 0.32x50 mm, Symmetry® C18, 5 µm, 300 Å
Reversed-phase trapping column (RPC): 0.32x5 mm, Symmetry® C18, 5 µm, 300 Å
Mobile phase A: 0.1%TFA in water or 0.1% formic acid in water
Mobile phase B: 0.085% TFA in acetonitrile or 0.07% formic acid in acetonitrile
Loading buffer: 0.1% formic acid in water
Step elution buffer: a. 0, 25, 50, 100, 150, 200, 250, 300, 350 mM KCl in 5% ACN, 5 mM KH_2PO_4, pH 3; b. ammonium formate in 5% ACN
Flow rate: 15 µl/min for 50 mm length column
Gradient: 50 mm column, CYTC tryptic peptides: 3-43%B for 12 min and hold at 60% for 2 min, 5 protein tryptic peptides: 3-63%B for 18 min and hold at 80%B for 2 min
Detector: PDA and/or MS
Mass spectrometric conditions:
Voltages: capillary 3 kV, cone 45 V, extractor 3 V, RF lens 0.1 V
Temperatures: source 90 ^{0}C, desolvation 150 ^{0}C
Gas flow: desolvation 150 L/hr, cone 50 L/hr
Resolution and ion energy: LR 15, HR 15; Ion energy 0.1 V
Sample information:
Sample: tryptic digest of CYTC or BSA, BCA OVA, TRF, CYTC
Injection volume: 0.8 µl

Sample Preparation

CYTC peptides: 1 mg bovine cytochrome c (CYTC) in 0.2 ml of 6 M urea and 0.8 ml of 0.1 M NH_4HCO_3, pH 8.1, vortex for 1 min, add 20 µl of 1 mg/ml trypsin, incubate at 37 ^{0}C for 24 h.
Final solution: 1 ml of 1 mg/ml of Cytc or 80 pmol/µl, 1.2 M urea, 80 mM NH_4HCO_3, pH 8-8.1, Cytc:trypsin = 50:1 by weight. Diluted to 10 pmol/µl before injection.

Peptide mixtures from 5 proteins (bovine serum albumin (BSA), ovalbumin (OVA), bovine carbonic anhydrase (BCA), human transferrin (TRF), bovine cytochrome c (CYTC)): 2 mg each BSA, OVA, BCA, TRF, CYTC in 0.2 ml of 6 M urea and 0.8 ml of 100 mM NH_4HCO_3 pH8.1, reduced with 50 mM diothiothretol at 65 ^{0}C for 30 min, alkylated with 120 mM iodoacetic acid for 40 min, dialyzed against 50 mM NH_4HCO_3 pH8.1 (10,000 MWCO), finally add 10 µl of 100 mM $CaCl_2$ and total 20 µl of 10 mg/ml trypsin (proteins:trypsin = 50:1 by weight) at 37 ^{0}C for 24 h.
Final solution: 1 ml of 2 mg/ml each protein or 30 pmol/µl based on BSA (66 kDa), 50 mM NH_4HCO_3 pH 8-8.1, 1 mM $CaCl_2$, Protein:trypsin = 50:1 by weight. Diluted 5 times before injection. e.g. 0.5 µl injection = 0.2 µg each protein or 1 µg of total proteins.

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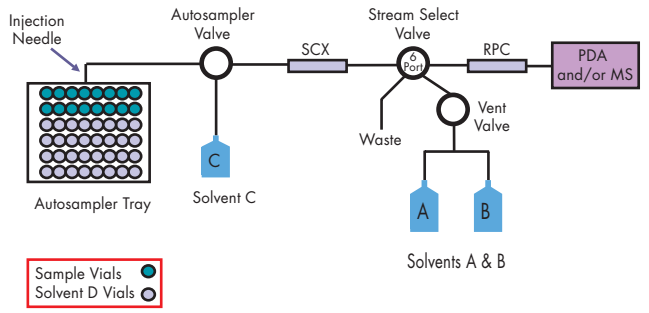
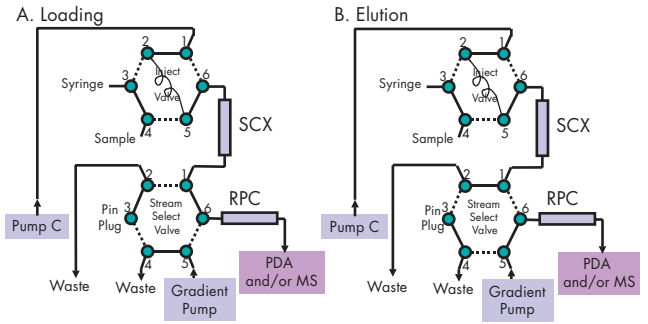
System I Configuration and Results

Schematic of 2-Dimensional Capillary HPLC System I



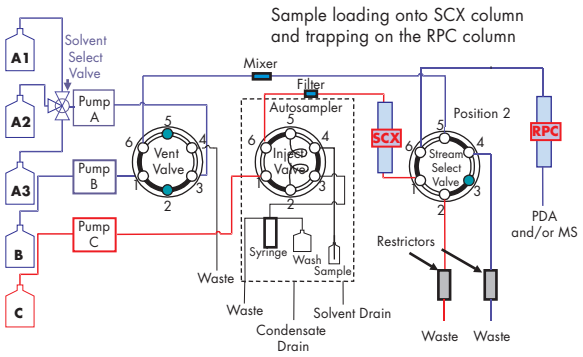
Six-Port Valve Action of 2D Capillary HPLC



A: Sample eluted from SCX and trapped on RPC. Valve action: (1) inject valve position 1 or inject (2) stream select valve position 2
B: Sample eluted from RPC. Valve action: (1) inject valve position 1 or inject (2) stream select valve position 1

This system permits automated 2D Analysis of complex mixtures. However, any salt used for elution from SCX column will be introduced into the MS unless the reversed-phase effluent is directed to waste for a period of time prior to running the RP gradient.

Integrated 2D CapLC System Configuration

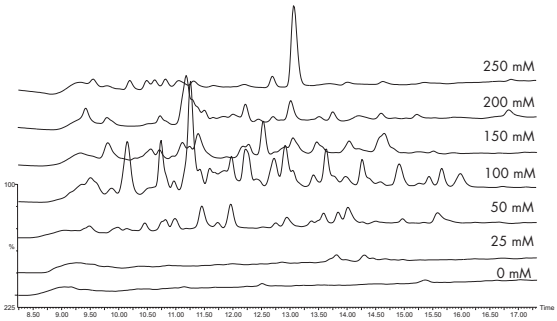


The restrictors are used for maintaining pressurization of the system when the stream select valve switches

Reversed-Phase Chromatogram of Peptide Mixtures After Elution With Ammonium Formate

System I: 6-port valve configuration
Column: 5 cm Symmetry® C18 column
Detection: 0.145 AU at 214 nm

Sample: tryptic peptides of 5 proteins
Injection: 0.8 µl, 0.32 µg each protein or 1.6 µg total
Loading buffer: 0.1% formic acid

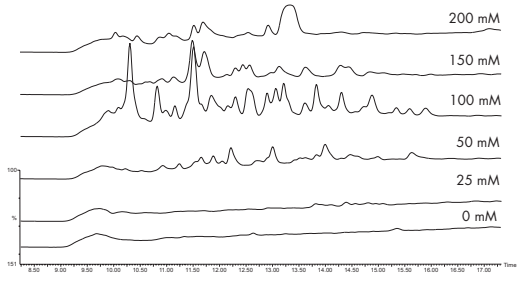


Most peptides eluted from SCX column between 50 mM and 250 mM

Reversed-Phase Chromatogram of Peptide Mixtures After Elution With KCl

System I: 6-port valve configuration
Column: 5 cm Symmetry® C18 column
Detection: 0.111 AU at 214 nm

Sample: tryptic peptides of 5 proteins
Injection: 0.8 µl, 0.32 µg each protein or 1.6 µg total
Loading buffer: 0.1% formic acid



Most peptides eluted from SCX column between 50 mM and 200 mM

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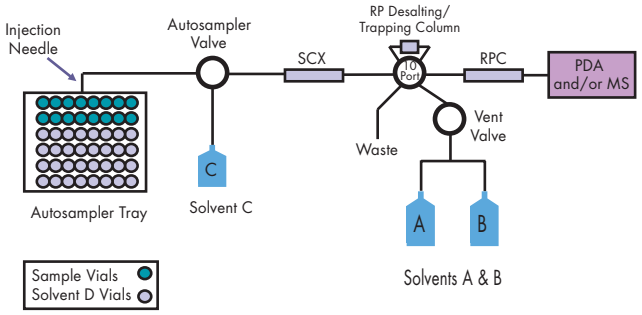
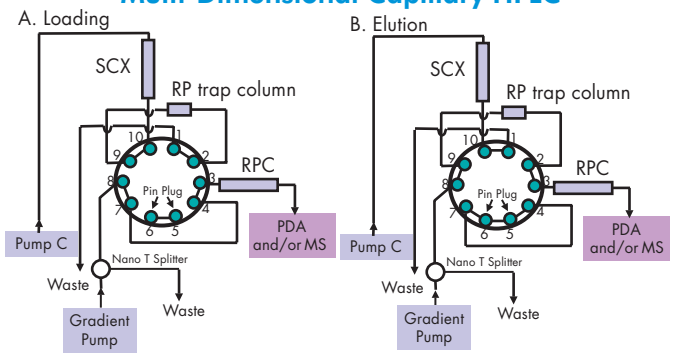
System II Configuration and Results

Schematic of Multi-Dimensional Capillary HPLC System II



Ten-Port Valve Action of Multi-Dimensional Capillary HPLC



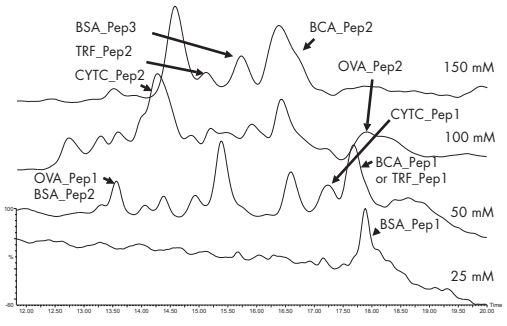
A: Valve position 1. Sample eluted from SCX and trapped on RP trapping column
B: Valve position 2. Sample eluted from RPC

This configuration permits desalting of peptides eluted from the SCX column without introducing salt into the MS interface. Thus, the peptides desorbed from the SCX column are captured by the trapping column, and the salt is flushed to waste prior to switching the valve to run the reversed-phased separation for LC/MS/MS analysis

Reversed-Phase Chromatogram of Peptide Mixtures After Elution With Ammonium Formate

System II: 10-port valve configuration
Column: 5 cm Symmetry® C18 column
Detection: 0.113 AU at 214 nm

Sample: tryptic peptides of 5 proteins
Injection: 0.8 µl, 0.32 µg each protein or 1.6 µg total
Loading buffer: 0.1% formic acid



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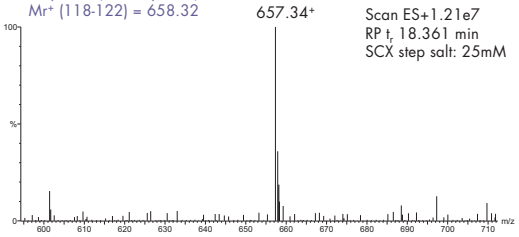
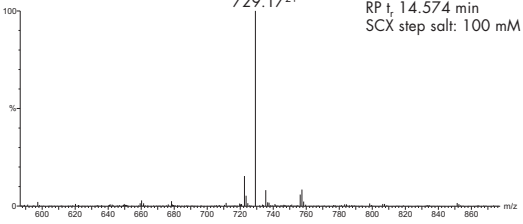
Mass Spectra for Multi-Dimensional Separation

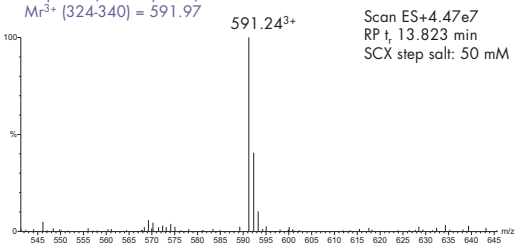
Sequence (BSA Peptide1): QEPER
Mr^{+} (118-122) = 658.32
657.34+
Scan ES+1.21e7
RP t_r 18.361 min
SCX step salt: 25mM



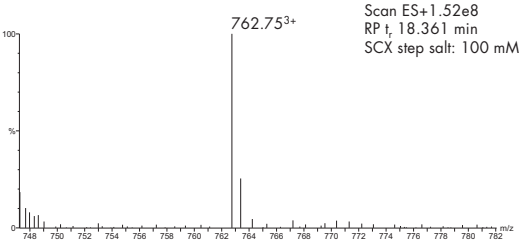
Sequence (CYTC Peptide2): TGQAPGFSYTDANK
Mr^{2+} (40-53) = 728.84
729.17^{2+}
Scan ES+7.74e8
RP t_r 14.574 min
SCX step salt: 100 mM



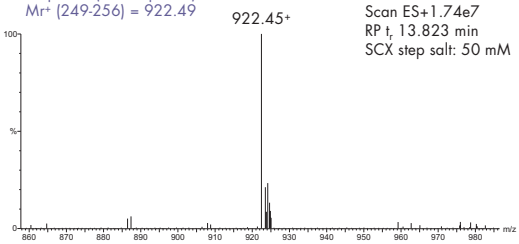
Sequence (OVA Peptide1): ISQAVHAAHAEINEAGR
Mr^{3+} (324-340) = 591.97
591.24^{3+}
Scan ES+4.47e7
RP t_r 13.823 min
SCX step salt: 50 mM



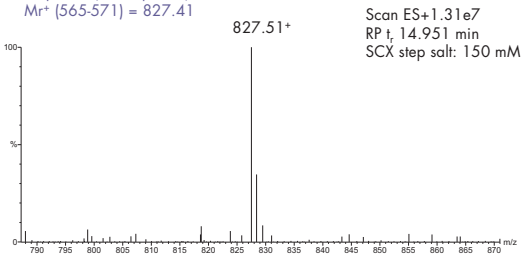
Sequence (OVA Peptide2): VTEQESKPVQMMYQIGIGLFR
Mr^{3+} (201-219) = 762.05
762.75^{3+}
Scan ES+1.52e8
RP t_r 18.361 min
SCX step salt: 100 mM



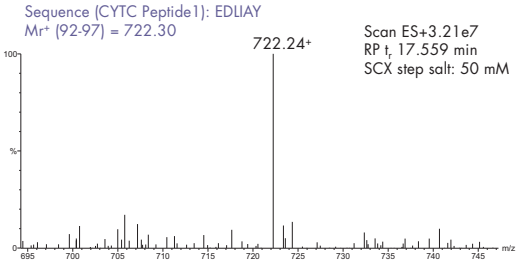
Sequence (BSA Peptide2): AEFVEVTK
Mr^{+} (249-256) = 922.49
922.45+
Scan ES+1.74e7
RP t_r 13.823 min
SCX step salt: 50 mM



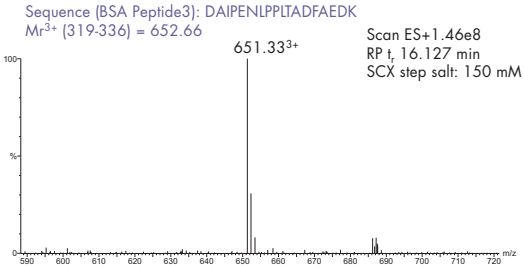
Sequence (TRF Peptide2): NPDPWAK
Mr^{+} (565-571) = 827.41
827.51+
Scan ES+1.31e7
RP t_r 14.951 min
SCX step salt: 150 mM



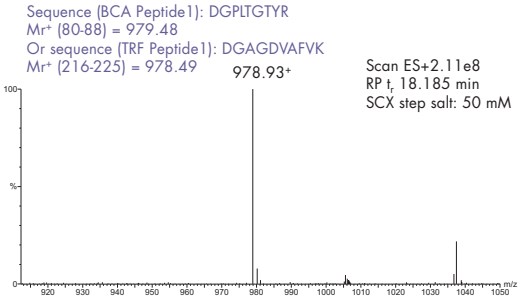
Sequence (CYTC Peptide1): EDLIAY
Mr^{+} (92-97) = 722.30
722.24+
Scan ES+3.21e7
RP t_r 17.559 min
SCX step salt: 50 mM



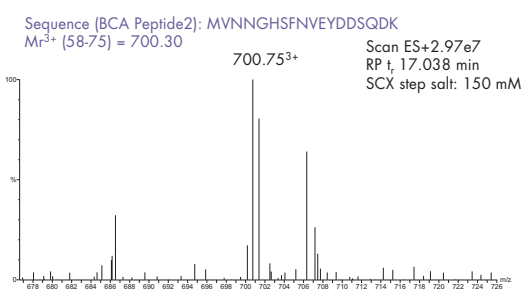
Sequence (BSA Peptide3): DAIPENLPPLTADFAEDK
Mr^{3+} (319-336) = 652.66
651.33^{3+}
Scan ES+1.46e8
RP t_r 16.127 min
SCX step salt: 150 mM



Sequence (BCA Peptide1): DGPLTGTYR
Mr^{+} (80-88) = 979.48
Or sequence (TRF Peptide1): DGAGDVAFVK
Mr^{+} (216-225) = 978.49
978.93+
Scan ES+2.11e8
RP t_r 18.185 min
SCX step salt: 50 mM



Sequence (BCA Peptide2): MVNNGHSFNVEYDDSQDK
Mr^{3+} (58-75) = 700.30
700.75^{3+}
Scan ES+2.97e7
RP t_r 17.038 min
SCX step salt: 150 mM



In this case, since there are two possible assignments based solely on relative mass, fragmentation is needed to sequence the peptide for identification of a protein

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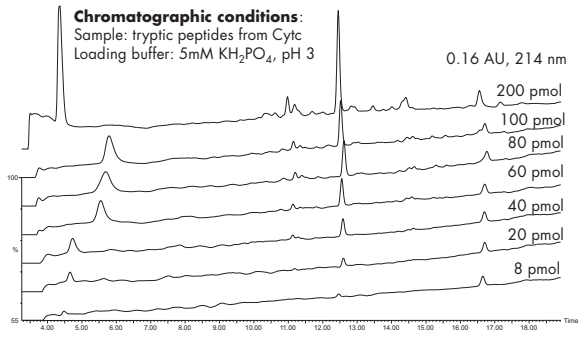
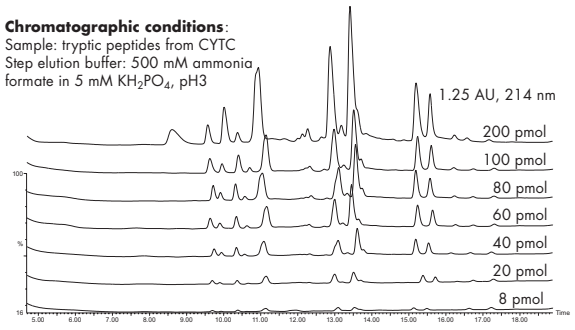
System I Loading Experiment Results

RP Chromatogram of Peptides at Loading Step
- Elution of Unretained Peptides on SCX



In this step, acidic peptides that are not retained on SCX column are eluted on RPC column at all loading levels. When the amount loaded exceeds the maximum capacity of the SCX column, new peaks will appear in the reversed-phase separation that are not present when a lower mass is loaded.

RP Chromatogram of Peptides after High Salt Elution
- Elution of Retained Peptides on SCX



Loading a CYTC digest mixture on a 320 µm ID x 50 mm L (CV = 4 µl) column up to 2 µg does not exceed the maximum loading capacity of the SCX column.

Summary

- An integrated multi-dimensional capillary HPLC coupled with mass spectrometry can be used to fractionate complex peptide mixtures for peptide and protein analyses.
- Two-dimensional capillary HPLC with a 6-port valve system can be used for volatile salt elution of peptides in the first dimension when coupled to mass spectrometer; multi-dimensional capillary HPLC with a 10-port valve system can be used to remove salt after first dimension elution of peptides prior to a mass spectrometric analysis.
- The Multi-dimensional system using the ten-port valve permits on-line desalting for LC/MS(MS) analysis and peptide identification.

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