

Automated 1D to 6D LC MS/MS of Proteins and Peptides For Functional Proteomics Studies

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Introduction

Although mass spectrometry has become a key tool in proteomics research over the past decade, sample preparation remains a bottleneck when working with complex proteome samples. Proteomics researchers use a wide variety of techniques to separate both intact proteins and protein digests, but no one protocol works well for the broad range of proteomics applications which utilize MS detection.

The system developed in this study employs automated instrumentation, software and protocols to minimize sample handling and allow the versatility to adapt the dimensions of separation to the specific requirements of a given application. This system has been applied to a variety of different sample work flows including separation of intact proteins in human serum, 3D Mudpit fractionation of an E Coli whole cell lysate digest and isolation and identification of phosphopeptides in a yeast proteome sample. Since MDLC fractionation can generate a large number of samples, the robust ADVANCE “Plug and Play” high-sensitivity source was used for all of these high throughput applications.

Experimental

Paradigm Sample Processor



- Bio Cool Autosampler
- Fraction Collector
- MALDI Spotter
- Sample Diluter
- Xcalibur Control

Paradigm MS4-NC MDLC



- 4 PEEK Pumps
- 2 PEEK 10-Port Valves
- Dynamic Flow Splitter
- 50 nl/min – 5 ml/min
- Xcalibur Control

Thermo LTQ-Orbitrap MS



- ADVANCE Source
- Linear Ion Trap MS
- High Res Orbitrap MS
- CID – HCD
- Xcalibur Control

Automated MDLC MS/MS Work Flows

Human Serum

Buffer Exchange

HAP Affinity

Desalt

WAX/WCX

RPLC

Digest

nRPLC-MS/MS

Proteome Digest

Digest

Buffer Exchange

pH 12 RPLC

Buffer Exchange

SCX

Desalt

nRPLC-MS/MS

Phosphopeptides

Digest

Desalt

SCX

Desalt

IMAC

Desalt

nRPLC-MS/MS

ID of Low Abundance Proteins in Serum

The automated MDLC system was first used to isolate low abundance proteins from a human serum sample. A Sigma PP20 low pressure LC column was used to remove the high abundance proteins from serum. The Paradigm AS3 Sample Processor was used to equilibrate, load, wash and elute the PP20 column and the low abundance eluant was collected in a new vial on the AS3. This sample was then desalted online using a peptide macrotrap on the AS3 and collected to a third vial.

The desalted sample was then injected on a mixed bed ion exchange (MBIX) column and eluted using a salt gradient at pH 6.5 (with 6 fractions collected in a 96 well plate on the AS3). Each of the six MBIX fractions were then separated on a protein RPLC column and 8 RPLC fractions were collected for each run (48 total protein fractions).

The 48 MDLC intact protein fractions were reduced, alkylated and digested with trypsin and then analyzed by nRPLC-MS/MS in 24 hours. Over 1200 proteins were ID'd with 2 or more peptides using SEQUEST.

Automated MDLC of Serum Proteins

D2 MBIX
(WAX + WCX)

6 Salt Steps



D3 RPLC
Separations

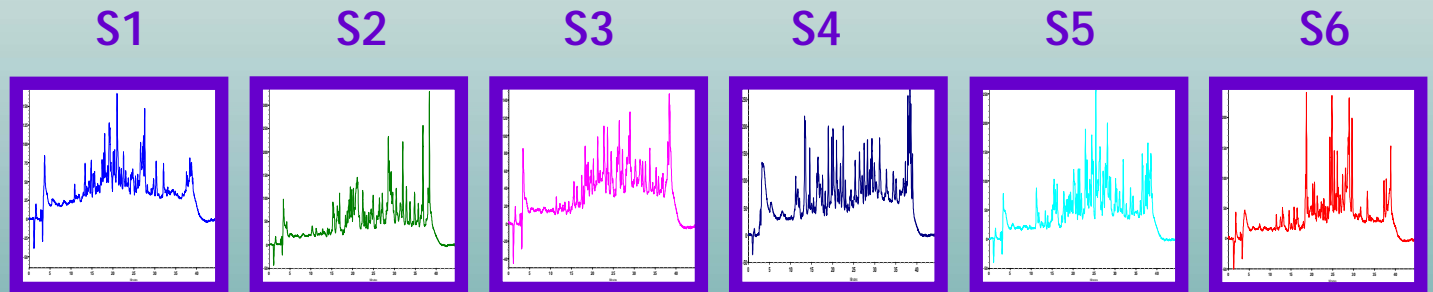
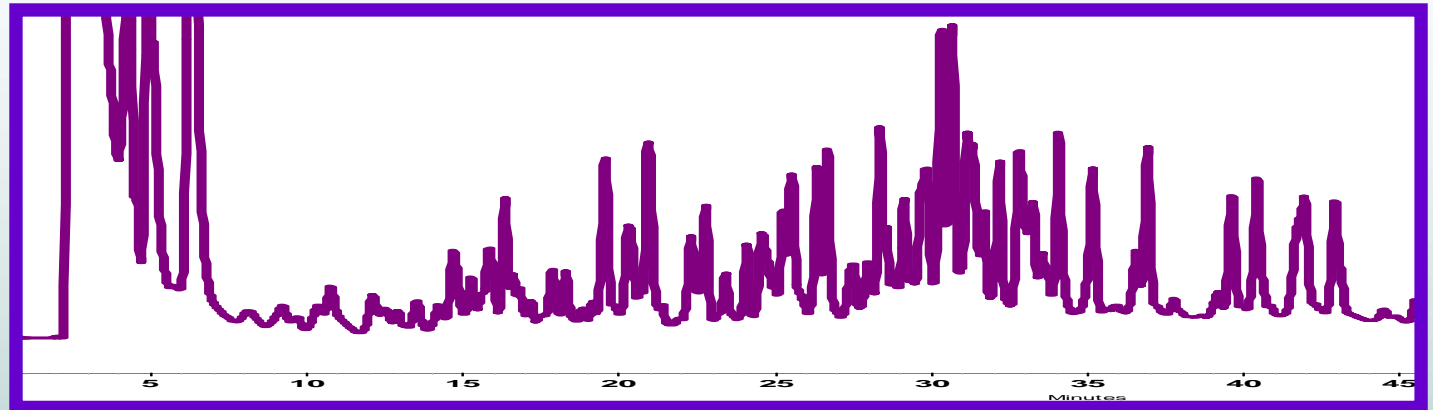
8 Fractions
For Each RPLC



Collect into
48 Wells



Trypsin Digest



S1 1-8

S2 1-8

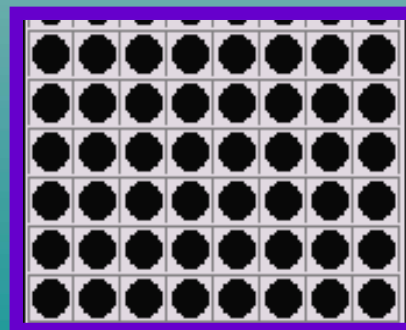
S3 1-8

S4 1-8

S5 1-8

S6 1-8

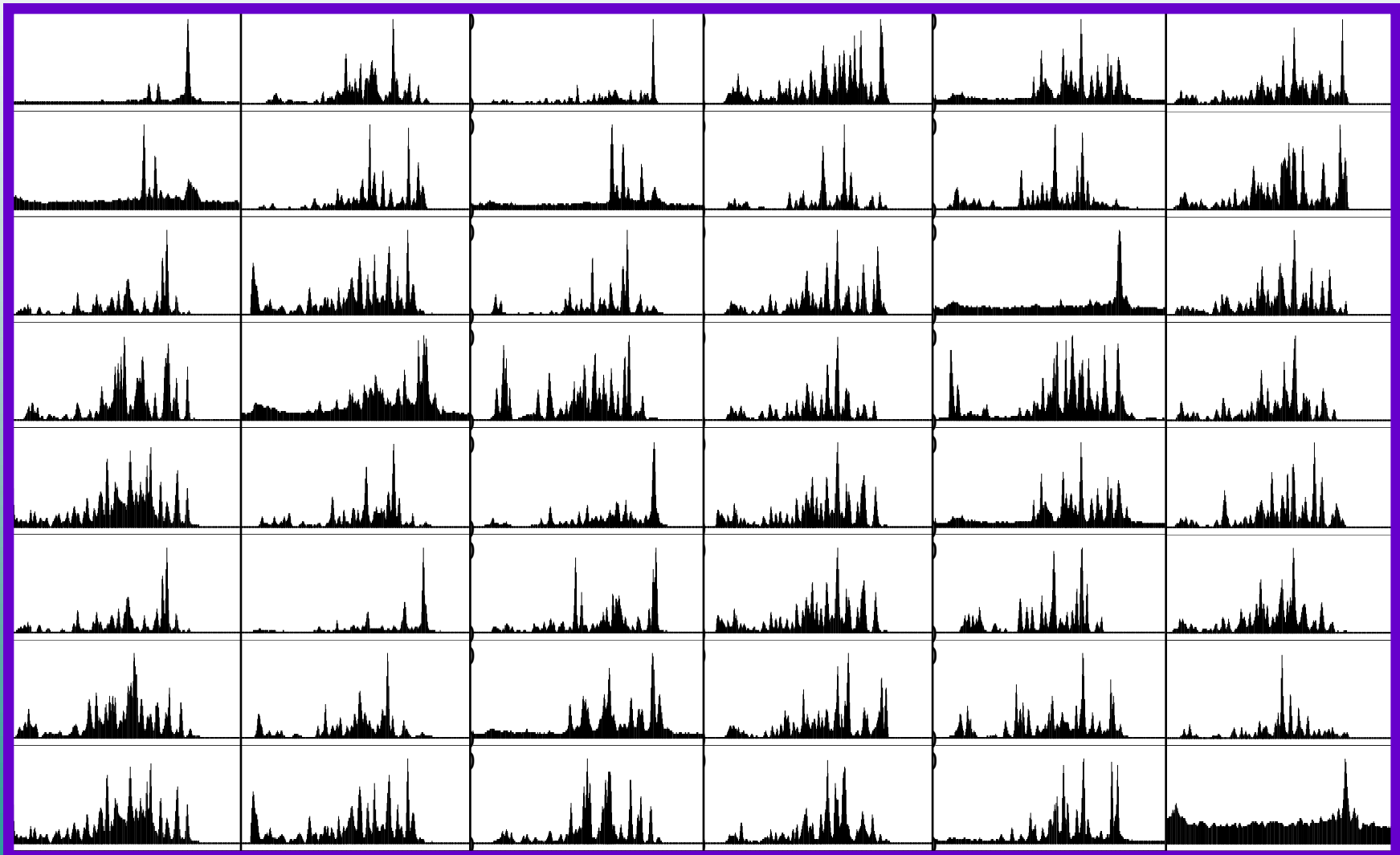
S1
S2
S3
S4
S5
S6



LC-MS/MS of 48 Serum Fraction Digests

48 x 30 min AS3/MS4-NC/ADVANCE/LTQ BPT MS runs in 24 hours

5-45%B in 20 min at 500nl/min on 0.1x150mm Halo C18 Column



Peptide MDLC-MS/MS of E. coli Digest

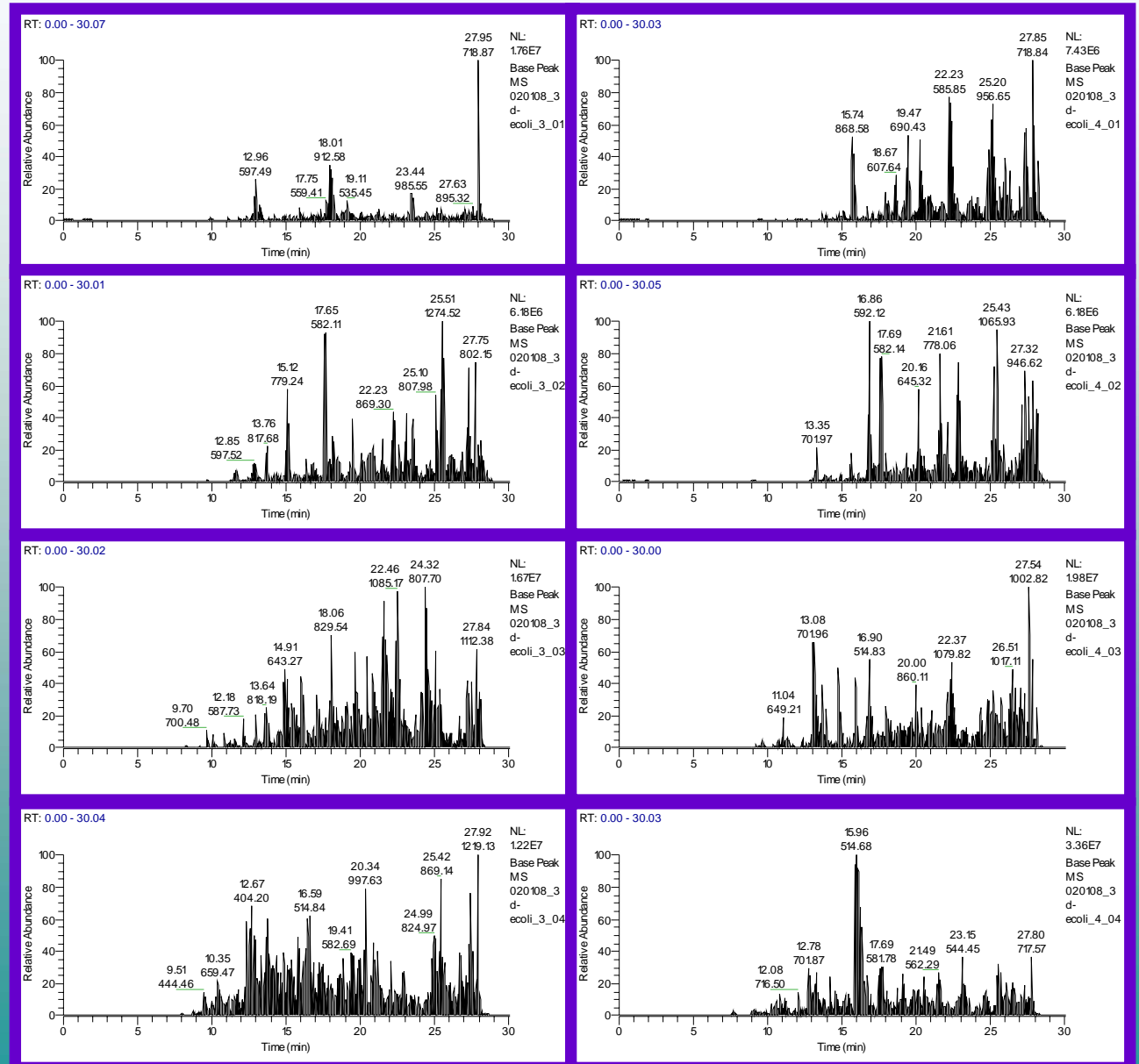
Although MudPIT (SCX/RP LC-MS/MS) has become a routine tool in many proteomics labs, it still lacks sufficient resolution for very complex proteome samples. High pH RP LC has been shown to be orthogonal to both SCX LC and low pH RP LC for peptide separations, so a protocol was developed to include all three modes of LC to separate very complex proteome digests. Since protein digests require desalting prior to SCX separation, we chose high pH RP LC for the first dimension separation.

100ug of E. coli whole cell lysate digest was injected and separated by pH 12 RPLC on a 2x150mm PLRP-S column and 8 fractions were collected into vials on the AS3 containing TFA/H₂O to adjust the pH to 3. Each D1 fraction was then injected on to an SCX microtrap column and four salt steps were used to elute the peptides, which were then desalted on a peptide captrap prior to pH 3 RP LC-MS/MS (8D1x4D2x30minD3 = 16 hours of total MS/MS time). Since this large sample was fractionated on high capacity D1/D2 columns, the D3 separations had 1-4ug of peptides loaded on a 0.2x150mm column.

3DLC-MS/MS Data for E. Coli Digest

Base peak traces (BPT) from MS/MS analyses in the left hand column are for the 4 salt steps of the third RP₁₂ fraction, while the BPT in the right hand column are for the 4 salt steps of the fourth RP₁₂ fraction.

The chromatograms show that peptides are well distributed in the 3D separation and each fraction contains a different peptide population.



Comparison of MDLC-MS/MS Results

The table below shows the number of high confidence protein IDs obtained in 16 hours of MS/MS data acquisition using 1D, 2D and 3D LC separation protocols. The automated 3D method identified 30% more proteins than the 2D method and 80% more proteins than the 1D method. Many of these additional proteins are low abundance proteins that may have biological significance. Since the three separation modes appear to be orthogonal, this 3D protocol could be expanded to include higher fractionation (no time limit) or be used for isolating a single fraction to characterize and/or quantify specific protein biomarkers.

<u>MDLC Protocol</u>	<u>Fractions</u>	<u>MS/MS Time</u>	<u>Protein IDs</u>
1D RP ₃	1	16 hours	674
2D SCX/RP ₃	32	16 hours	931
3D RP ₁₂ /SCX/RP ₃	32	16 hours	1172

Automated MDLC For Functional Proteomics

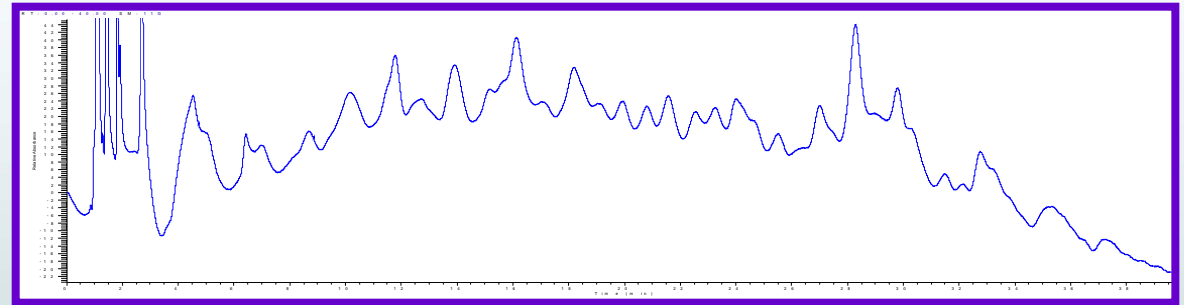
Post translational modifications (PTMs) of proteins have biological significance and their isolation, characterization and quantification is of great interest. Since protein phosphorylation is one of the most important PTMs, the automated MDLC system was also used to isolate and identify phosphopeptides in a yeast digest sample.

Since the phosphoproteome of bakers yeast (*S. cerevisiae*) has been well characterized, we decided to use it as a final test for the auto MDLC system. 100 ug of yeast whole cell lysate was reduced, alkylated and digested with trypsin. The yeast digest was desalted online in the AS3 and then separated into 48 SCX fractions on a 2x150mm PolySulfoEthyl A column using a gradient from 0-500 mM NaCl (pH 3).

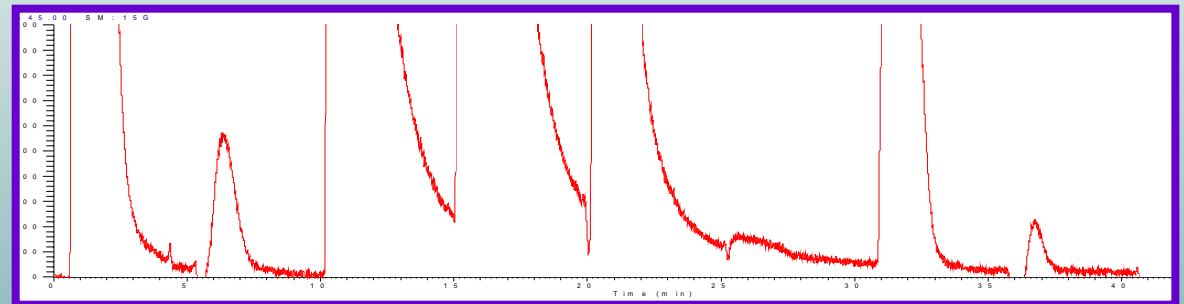
Each SCX fraction was desalted online by the AS3 and then step separated on a Fe³⁺ activated 0.3x150mm Poros-20MC IMAC column. The flow through and the eluant (enriched phosphopeptides) from each SCX fraction was collected for final LC-MS/MS analysis.

SCX & IMAC of Yeast Whole Cell Digest

100 ug of yeast digest
2x150mm SCX column
0-500mM NaCl in 50 min
200ul/min - UV 214nm
48 x 1 min fractions



48 desalted SCX fractions
0.3x150mm Poros20MC
Reagent steps from AS3
40ul/min - UV 214nm



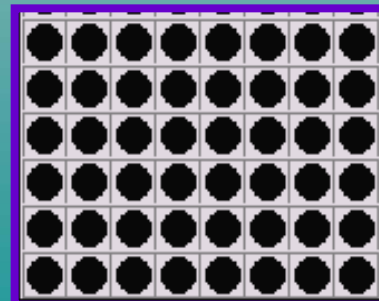
Wash Rinse EDTA FeCl₃ Rinse Load(FT) Wash Elute(PP)

AS3 collect of 48x IMAC

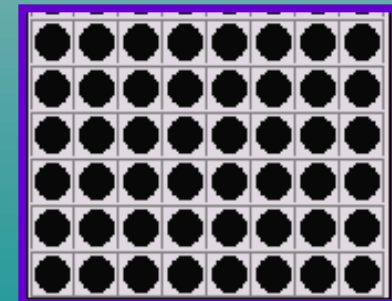
FT (flow through)

PP (enriched phosphopeptides)

SCX 1-48 Load (FT)



SCX 1-48 Elute (PP)



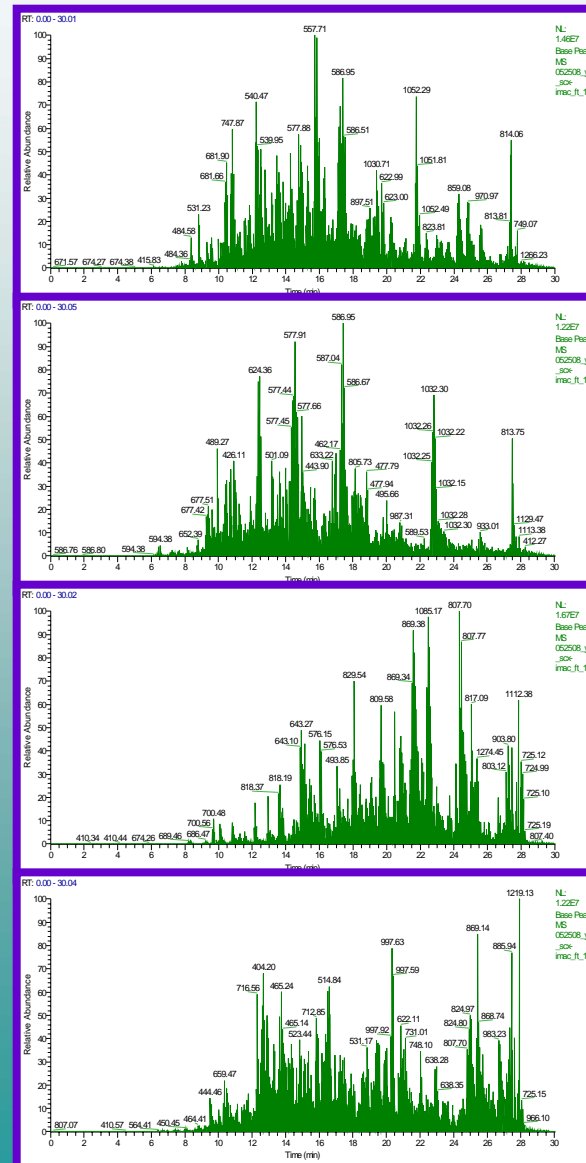
nLC-MS/MS of Yeast Phosphoproteome

The traces at the right show 4 of 48 fractions (SCX 11-14) of the IMAC flow through (FT) and phosphopeptide (PP) elutions.

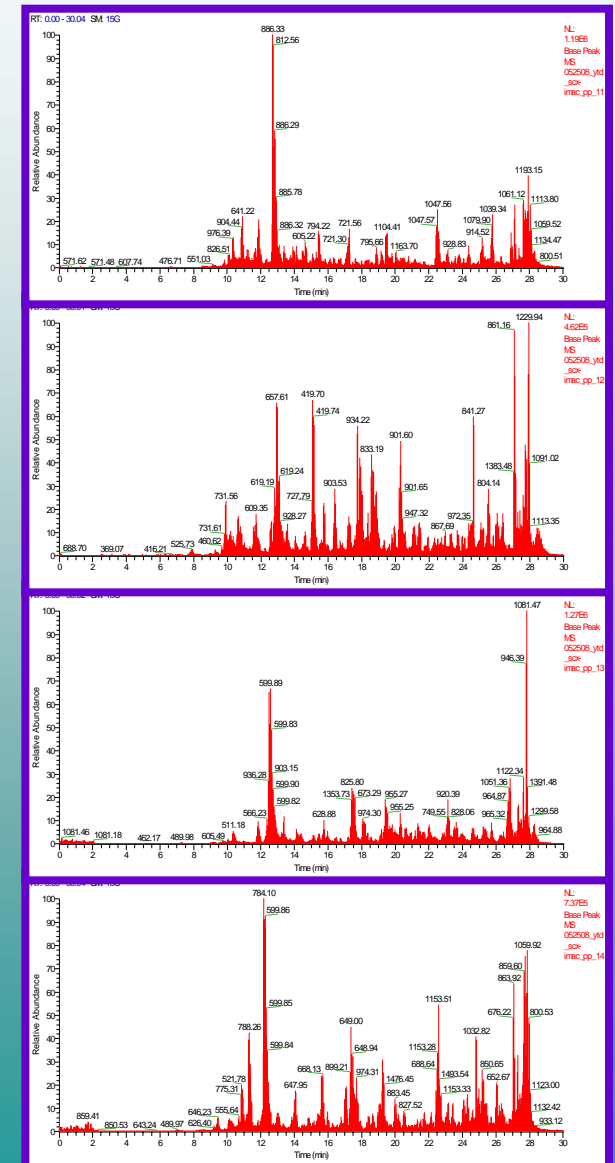
There was minimal overlap of identified peptides between the fractions on either the SCX or IMAC runs.

Over 95% of the 987 phosphopeptide IDs were from the PP eluant fractions.

FT (nonPP – PP ratio > 99:1)



PP (nonPP – PP ratio < 2:1)



Summary

Although many researchers utilize MDLC to separate complex proteome samples, these systems are often application specific and difficult to implement and run routinely. In this study, we developed a versatile, automated MDLC system which includes hardware, software and protocols for running a variety of proteomics LC-MS applications.

This system has been applied to three challenging applications (low abundance proteins in serum, a whole proteome digest and a phosphoproteome), all of which require a different MDLC work flow to achieve the desired results. Although the chemistries and protocols of each of these applications is different, they could all be run on the same automated hardware/software platform.

The software allows users to run as many dimensions as necessary for each sample (i.e. 1D for a 2D Gel Spot, 2D for a IP sample and 3D for a whole cell lysate), while providing online desalting/buffer exchange between separations to maximize separation and minimize losses. This automated platform coupled with the ADVANCE source provide a robust sample preparation and analysis system to maximize the information that can be obtained from today's powerful mass spectrometers.

Conclusions

- An automated 1D to 6D LC system has been developed
- Degree of separation depends on sample complexity
- Modes of separation can be adapted to varied applications
- Fractionates intact proteins for top down proteomics
- Separates proteome digests for bottom up proteomics
- Enriches specific peptide classes for functional proteomics
- Automates sample preparation for biomarker quantitation
- Provides a robust “front end” to maximize MS performance