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COMPARISON OF PEAK PARKING VERSUS AUTOMATED FRACTION ANALYSIS OF A COMPLEX PROTEIN MIXTURE

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Introduction

The study of protein expression allows a greater understanding of biological function and can now routinely be performed using mass spectrometry. However, analysis of the post-translational modifications present on identified proteins is a more challenging problem, often due to the level of complexity or low abundance. Novel methods allowing the mass spectrometer to investigate the low level peptides could increase coverage or dynamic range, and possibly identify additional post-translational modifications.

If an MS/MS spectrum is not matched to a sequence in a database, the quality of the spectrum will facilitate the determination of sequence. MS/MS spectra with poor fragmentation can be matched to a database, but *de novo* sequencing of similar spectra is difficult, thus the quality of the spectra from the different acquisition methods is compared.

Sample Preparation

Yeast Ribosomal Protein Collection and Digestion

- Reference:

  Hongji Lu, Scott J. Berger, Asish B. Chakraborty, Robert S. Plumb, Steven A. Cohen J. Chromatography B, 782 (2002) 267-289

  - Cell Growth and Harvesting
  - Cell Lysis by French Press
  - Preparation of Cleared Lysate and Pelleting of Ribosomes
  - Resuspension of Ribosomal Proteins
  - Digestion with RapiGest™

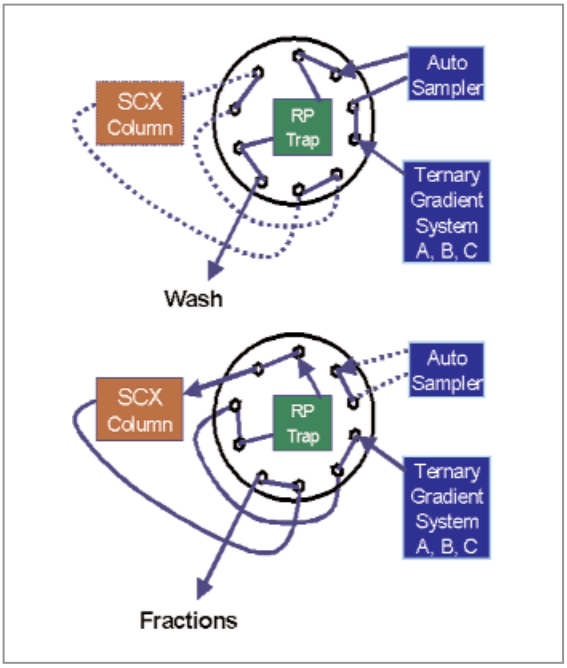
Thank you to Scott Berger of Waters for supplying the yeast ribosomal digest.

SCX Fractionation and Reverse Phase Fractionation

- Sample
  - 50 µL (assumed to be 1 mg/mL protein) of digested ribosomal peptides and treated with 100 µL of formic acid to remove the RNA
- Instrumentation
  - Waters® CapLC® configured as a ternary system
  - 10-port switching valve controlled as a stream select valve
- Columns
  - Strong cation exchange column (SCX): PolyLC PolySULFOETHYL A, 1.0 x 50 mm
  - Reverse phase trapping column (Trap): Waters Sentry™ Guard cartridge, Symmetry® C_{18}, 2.1 x 10 mm
- Mobile phases
  - A = 5% acetonitrile, 0.1% formic acid
  - B = acetonitrile, 0.1% formic acid
  - C = 300 mM ammonium formate, natural pH (AmF), 5% acetonitrile
  - Flow rate 20 µL/min during sample loading, 40 µL/min for the gradient
- Injection
  - 125 µL of the treated peptide sample was loaded at 10 µL/min onto the trapping column equilibrated in A mobile phase

SCX Plumbing

- Load and cleanup
  - Load sample onto RP trap column
  - Wash to remove salts from sample
- Fractionation
  - Back flush peptides from RP trap column to SCX column
  - Wash SCX column
  - Elute peptides from SCX column with ammonium formate



Fraction	Flow rate µL/min	%C	AmF mM	%B	% ACN	Collected µL
1. Load, 125 µL + 25 µL A	20	0	0	0	5	150
2. Wash trap	40	0	0	0	5	200
3. Transfer from RP to SCX	40	0	0	50	55	200
4. Wash SCX	40	1	3	15	20	200
5. Gradient elution 5 minutes	40	1-5	3-15	15	20	200
6. Gradient elution 5 minutes	40	5-10	15-30	15	20	200
7. Gradient elution 5 minutes	40	10-15	30-45	15	20	200
8. Gradient elution 5 minutes	40	15-30	45-90	15	20	200
9. Gradient elution 5 minutes	40	30-60	90-180	15	20	200
10. Gradient elution 5 minutes	40	60-85	180-225	15	20	200
11. Gradient elution 5 minutes	40	85	225	15	20	200

Sample volume reduced in SpeedVac to about 20 mL and then diluted to 150 mL in A.

Reverse Phase Subfractionation

- Sample
  - Peptide-rich SCX fractions
- Instrumentation
  - Waters CapLC configured as a ternary system
  - 10-port switching valve controlled as a stream select valve
- Columns
  - Symmetry300™ C_{18} 0.32x150 mm, 5 µm, 40 °C
- Mobile phases
  - A = 5% acetonitrile, 0.1% formic acid
  - B = acetonitrile, 0.1% formic acid
  - Flow rate 10 µL/min during sample loading or 40 µL/min for the gradient
- Injection
  - 45 µL from the 150 µL worked-up fraction

Reverse Phase Gradient / Subfraction Collection

Time min	%A	%B	Flow µL/min
0	99	1	10
5	99	1	10
65	45	55	10
75	20	80	10
76	99	1	10

SCX Fractions	Time min	Well #	Collected µL/well
1,6,7,8	1-10	A1, A2	50
	10-70	B1-F12	10
	70-80	G1, G2	50

Preparation of subfractions for NanoMate™ 100

- 96-well microtitre plates containing subfractionated samples were centrifuged prior to removal of cover. The wells were then evaporated to dryness. Samples in wells A1 through G2 were reconstituted in 5 µL of 50% methanol, 0.1% acetic acid.

Sample Analysis

Reverse Phase Chromatography LC Methods

- Instrumentation
  - Waters CapLC configured as an auxiliary system (A & B for gradient mobile phases, C for sample delivery)
  - 10-port switching valve controlled as a stream select valve
- Columns
  - Trapping Column: LC Packings 300 µm id, 5 mm PepMap™
  - Analytical Column: Waters Symmetry300™ C_{18} 75 µm id NanoEase™
- Mobile phases
  - A = 3% acetonitrile,0.1% formic acid
  - B = 97% acetonitrile,0.1% formic acid
  - C = 3% acetonitrile,0.1% formic acid
  - Flow rate = 20 µL/min during sample loading, 400 nL/min at initial conditions for the gradient after being split

Unfractionated Sample (240 min)

Time	%A	%B
0	95	5
3	95	5
153	60	40
173	40	60
175	10	90
195	10	90
200	95	5

SCX Fractions (120 min)

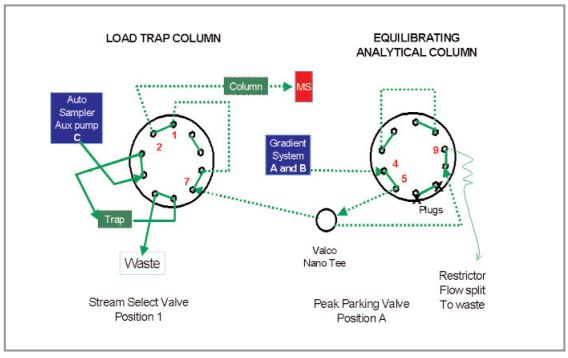
Time	%A	%B
0	95	5
3	95	5
73	60	40
83	40	60
85	10	90
95	10	90
100	95	5

Variable Flow (100 min)

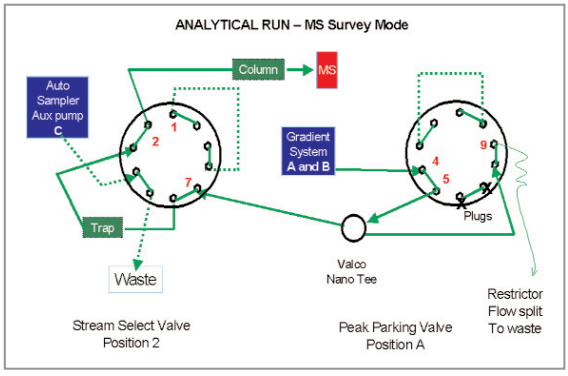
Time	%A	%B
0	95	5
4	95	5
64	60	40
74	40	60
75	10	90
85	10	90
90	95	5

Flow rate = 8.5 µL/min of the combined A & B buffers at the mixing tee.

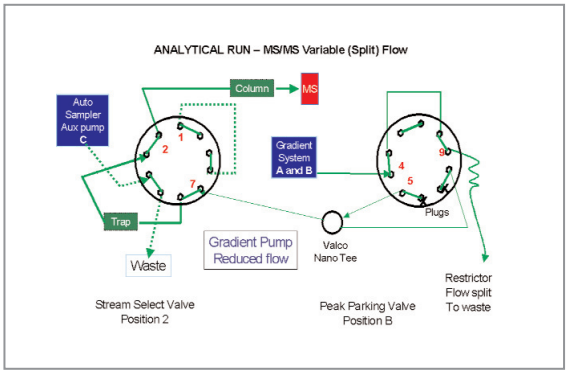
Variable Flow – Split Mode 1



Variable Flow – Split Mode 2

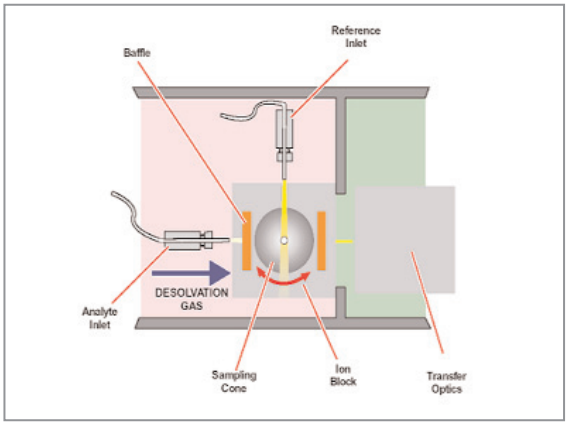


Variable Flow – Split Mode 3

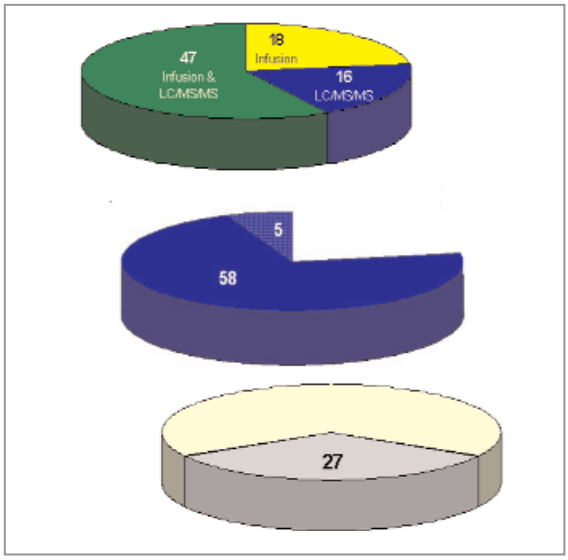


MS Methods

- Instrumentation
  - Waters Micromass® Q-Tof micro™ NanoLockSpray™ or NanoMate™ 100 (Advion)
  - Data Directed Analysis (DDA™)
  - Collision Energies Applied via Charge State Recognition
- Bioinformatics



- ProteinLynx™ Global SERVER 2.0



- *81 yeast ribosomal proteins were identified using two complimentary techniques.*
- *63 identified with LC/MS/MS techniques*
- *65 identified with infusion (NanoMate)*

- *58 of the 63 proteins identified via LC/MS/MS were found with both configurations.*
- *61 identified with the standard flow LC/MS/MS*
- *60 identified with the variable flow LC/MS/MS*

- *81 proteins were found with SCX pre-fractionation, while 54 were identified analyzing the unfractionated digest*

Yeast Ribosomal Proteins Identified

The error reported on the mass of the parent was less than 5 ppm RMS for the online LC/MS, whereas the non-lockmass corrected data (NanoMate) was sub 20 ppm.

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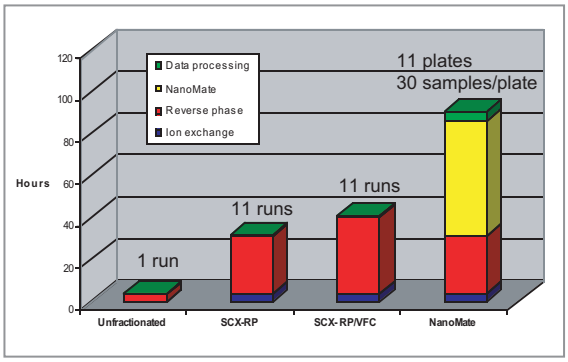
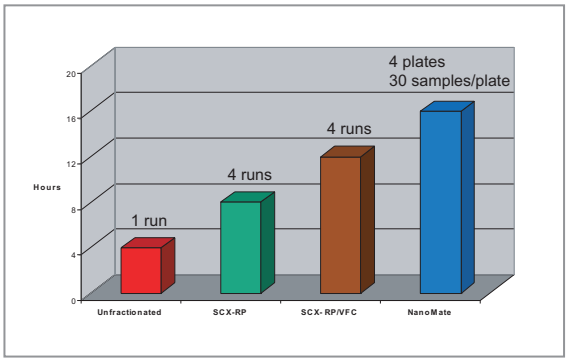
Total Analysis Time



- *Standard LC/MS run is 150 min with peptides eluting for 80 min.*
- *VFC LC/MS run is 200 min with peptides eluting for 150 min.*

Mass Spectrometer Analysis Time

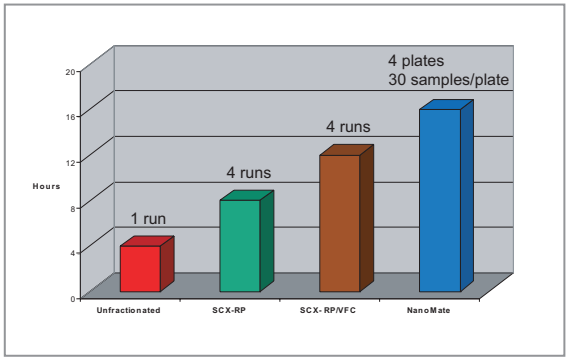


Protein ID from Unfractionated Digest

In the unfractionated digest 4 peptides were identified from the 40 S Ribosomal protein S16 RP.

The plate color changes based on the presence or absence of protein identification. SCX fractions 1-11 and the unfractionated sample.

The MS/MS data can be searched as a fraction or as a merged set of 11 fractions.



Protein ID from SCX fraction 01(NanoMate)

Protein identification from a single SCX fraction. Peptides 546 and 708 eluted in two different fractions, but ProteinLynx Global SERVER 2.0 merged all 74 data files and searched it as one file.

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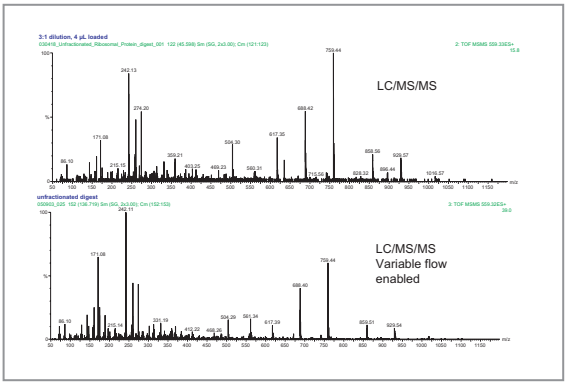
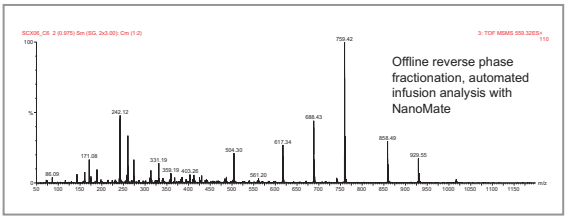
Standard vs Variable flow MS/MS Spectra



NanoMate MS/MS Spectra



The quality of the spectra obtained from the three modes of acquisition is presented. The MS/MS spectral quality is increased with increased time of acquisition. Each MS/MS spectrum in the online experiments is 2.0 sec. The standard experiment averaged 1-2 MS/MS spectra per ion, whereas the variable flow experiment averaged 3-4 MS/MS spectra per ion. The NanoMate data was collected at 5 sec/spectrum, averaging 2-3 MS/MS spectra per peptide.

Summary

A series of yeast ribosomal proteins have been identified and the coverage compared between the methods. 71 of the 75 large and small ribosomal proteins (excluding isoforms) were identified utilizing the three configurations of inlets. The NanoMate is able to spray each fraction for an extended period of time (between 20 and 30 minutes) to allow the collection of multiple lower level MS/MS spectra in each fraction. However, the data obtained from the variable flow LC/MS/MS experiment is able to identify the majority of the ribosomal proteins present in the sample.

Additional work is needed to identify one system to return all of the protein ID's, currently these two techniques are quite complimentary. There is a tradeoff between quality of MS/MS spectra and the time of analysis. Online LC/MS/MS identified 63 proteins with a total analysis time of about 41 hours. The infusion system identified 65 proteins, with a total analysis time of about 80 hours. The total analysis time for separation and identification can be optimized for more specific sample sets. Additional control of the software between the NanoMate and Q-Tof micro will also facilitate decreasing the time of analysis for the infusions. The increased quality of the spectra (variable flow and NanoMate) facilitated the automated *de novo* sequencing of ions not reported here.

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