Optimization of Sample Loading Capacity for Absolute Peptide Quantification by Nanobore LC-MS/MS

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INTRODUCTION

A dominant workflow for qualitative proteomics has been "GeLC-MS," a combination of 1- (or 2-D) gel electrophoresis with reverse-phase nanoflow liquid chromatography mass spectrometry (nLC-MS/MS). The limited protein quantity isolated from a single gel band and column loading capacity necessitate the use of 75 um inside diameter (ID) packed columns for optimal sensitivity. However, limitations on sample injection volume, gradient and flow characteristics, and excessive delay volume hinder quantitative applications. Novel solution phase tube-gel fractionation yields a greater than 10-fold increase in gel capacity. This increase permits the effective use of larger diameter columns (150 to 200 um). These larger columns prove effective for absolute quantification method development, reducing analytical cycle time by 4-fold.

MATERIALS AND METHODS

Preparation of Samples
- Phosphorylase B (Sigma), BSA (Thermo Pierce), and Lysozyme (Sigma) were mixed at equimolar ratio.
- In-gel digestion was performed according to Shevchenko A., et. al. Nature Protocols 1 (6), 2006, 2856-2860.
- 1D Gels – 10 lane 10-20% Tris-Glycine Gels (Invitrogen).
- Gelfree Fractionation – Whole cell lysate was prepared from Yeast (Fluka) and 500 µg was loaded into a single channel of a Gelfree™ 8100 Mid Mass Cartridge (Protein Discovery, Inc.). Gelfree was operated according to standard fractionation protocols using the Gelfree™ 8100 Fractionation System (Protein Discovery, Inc.).

Instrumentation
- 3-D ion-trap mass spectrometer (LCQ Deca, Thermo Fisher)
- Customized nanospray source (Digital PicoView, New Objective)
- nano LC·2D pump (Eksigent)
- Autosampler (Leap HTC Pal) equipped with 6-port micro-valve (VICI) containing 1.0 µl loop (for BSA standard) and 2.0 µl loop (for GelFree samples)
- Columns:
  - # 1 - PicoFrit column (360µm OD x 75µm ID x 15µm tip) packed with 10 cm ProteoPep II (5µm, 300 Å, C18, New Objective)
  - # 2 - PicoFrit column (360µm OD x 150µm ID x 15µm tip) packed with 10 cm ProteoPep II (5µm 300 Å, C18, New Objective)

Reagents
- BSA Digest (MassPrep, Waters)
- 0.1% Formic Acid in Water (JT Baker)
- 0.1% Formic Acid in Acetonitrile (JT Baker)

LC Conditions
- Gradient: 30 minutes 2-50% B
  - Mobile Phase A = 0.1% Formic Acid in Water
  - Mobile Phase B = 0.1% Formic Acid in Acetonitrile
- Flow rate: 250 nl/min (75µm ID PicoFrit) or 1000 nl/min (150µm ID PicoFrit)
- On-column injection: variable concentrations

RESULTS

The Problem…

In GeLC-MS, 75 µm columns have become the standard. This format provides exquisite sensitivity and performance when sample is limited. Due to the limited loading capacity of 1D gels, there is not enough sample available in a gel band to permit the use of larger diameter columns. The advantages of larger diameter columns include increased robustness and faster analysis times, requirements for quantitative assays.

How Much Can I Load on My Column?

Column capacity can be defined in three ways, depending on the application.

1. Optimal capacity
   - Analytical separations
   - Consistent peak width
   - Excellent resolution
2. Practical capacity
   - Preparative separations
   - Good peak shape
   - 10 – 50X optimal capacity
3. Maximum capacity
   - Purification

Table 1: Optimal flow rates and sample capacity for capillary columns. The sample capacity in µg is the quantity of polypeptide which can be loaded onto a column without compromising the resolution, peak shape and peak width.

Column Diameter (µm)	Flow Rate (µl/min)	Sample Capacity (µg)
75	0.25	0.05
150	1.00	0.20
300	5.00	1.00
500	10.00	2.00

Adapted from David Carr. *The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC*, 3rd ed., Hesperia, CA: Grace Vydac Technical Support Group, 2002.

Figure 1: Two different size columns were evaluated for optimum performance. Photo of a) 75µm ID x 15µm tip PicoFrit column and b) 150µm ID x 15µm tip PicoFrit column

Figure 2: Chromatographic comparison of 75µm ID *vs.* 150µm ID PicoFrit column at 50 and 200 ng on-column injection of BSA. The increased flow rate used for the 150µm ID column (1000 nl/min) demonstrates a definitive advantage in cycle time while providing better chromatographic performance.

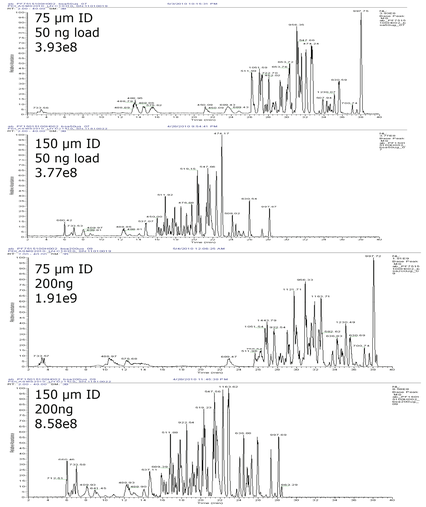


Figure 3: Sample Capacity Curve for Two Different PicoFrit Columns: 75µm ID *vs.* 150µm ID. Loading capacity is reached when the peak width increases by 10%. Peak width increase is observed at 50 ng on the 75 µm ID column and 200 ng on the 150 µm ID column. The average peak width was calculated for a set of six XIC BSA peaks.

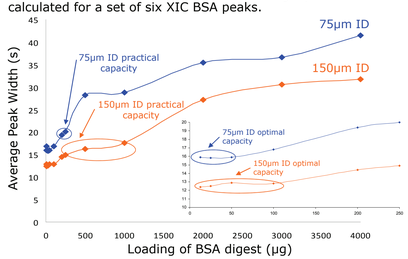
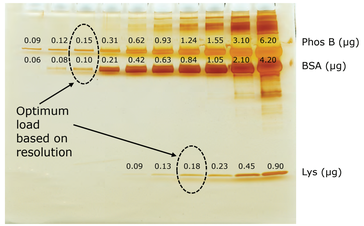


Figure 4: 1D gel loading capacity evaluated for standard proteins. The loading capacity for 1mm, tris-glycine gels was found to be 0.1-0.2 µg for the three protein standards: lysozyme, albumin, and phosphorylase B.



CONCLUSION: Unless extraction of peptides from in-gel digest can be performed at quantitative efficiency, it is not possible to load the 150 µg column to capacity without degrading 1D gel resolution.

Figure 5: Schematic of the Gelfree 8100 fractionation technology. The system uses high performance, high capacity tubular SDS-PAGE to solubilize and separate analytes based on molecular weight. As size-based fractions elute from the end of the gel, they are entrapped in a 150 µL liquid layer, permitting high efficiency liquid phase recovery of intact proteins.

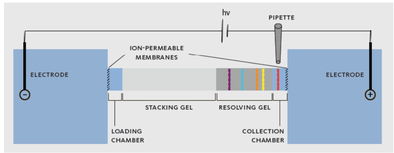


Figure 6: Gelfree 8100 fractionation of 500 µg of *S. Cerevisiae* cell lysate. 3 µL of each fraction was analyzed by 1D gel and silver stained. Selected fractions analyzed via LC-MS.

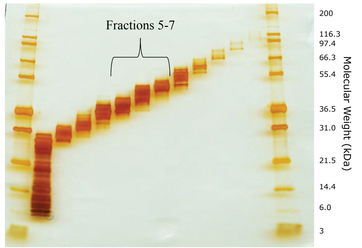
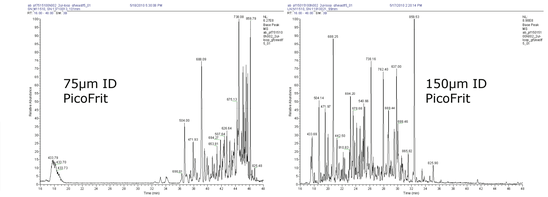
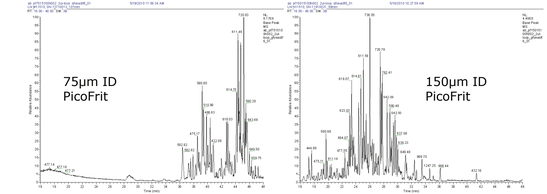


Figure 7: Chromatographic comparison of three yeast fractions analyzed using 75 and 150 µm columns. The estimated load was 4 µg of total protein. Significant degradation of chromatographic resolution is observed using the 75 µm column. The increased loading capacity and recovery of Gelfree 8100 provides significantly more material, making it possible to use 150 µm columns without sacrificing data quality.

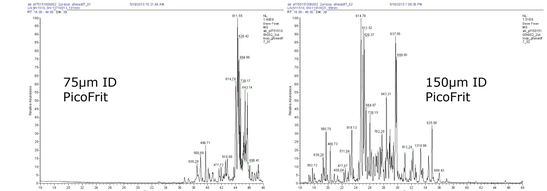
Gelfree 8100
Yeast Fraction 5



Gelfree 8100
Yeast Fraction 6



Gelfree 8100
Yeast Fraction 7



CONCLUSIONS

- Traditionally, narrow bore columns have been a requirement for many applications in proteomics because sample amounts isolated from a single 1D gel band are limited.
- The disadvantage of these columns include slower analytical cycle times and a lack of robustness. In spite of the advantages of higher sensitivity, these problems have prevented these formats from being widely adopted in higher-throughput, quantitative applications.
- Increase of column ID from 75 to 150 µg was observed to decrease RT by 20%, indicating increased cycle time, while providing the sensitivity of smaller bore columns.
- The loading capacity was determined to increase from 50 ng to 200 ng of standard protein upon increasing the column ID from 75 to 150 µm. A 4-fold increase in loading capacity was realized.
- Many methods of sample preparation, including 1D gels, do not yield enough total protein to take advantage of the benefits of 150 µm columns.
- The loading capacity of 1mm tris-glycine gels was 0.1-0.2 µg of standard proteins. Higher loadings compromised resolution.
- The high loading capacity (500 µg total protein) and high recovery (>90%) of the Gelfree 8100 technology yields a greater than 10-fold increase in total protein per fraction relative to 1D gels.
- Utilizing the Gelfree 8100 technology in tandem with 150 µm PicoFrit columns provided a 30% reduction in time of analysis with no apparent impact on data quality.
- Analysis of yeast lysate fractions prepared using Gelfree 8100 demonstrated the performance benefits of speed and resolution of the workflow described.

FUTURE DIRECTIONS

- Further optimization of Gelfree 8100 Fractionation System and PicoFrit columns for the quantitation of proteins.
- The goal is to provide a method for the quantitation of proteins from complex proteomes that takes advantage of the sensitivity of nanoESI while providing the throughput and robustness required for routine quantitative analytical assays.

ACKNOWLEDGEMENTS

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