DEVELOPMENT OF AN AUTOMATED RP/RP 2D NANOLC/MS METHOD FOR PROTEOMIC ANALYSIS

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OVERVIEW

- An automated RP/RP 2D nanoLC/MS method was developed
- The orthogonality of the two RP (Reverse Phase) dimensions was realized by running the separations at sharply different pH
- The automation of the system was made possible by using a novel online dilution method: the organic mobile phase eluting from the first dimension was diluted with an aqueous mobile phase before being collected for separation on the second dimension
- More efficient fractionation was observed with RP than with SCX
- Highly reproducible protein identification results were obtained with the system

METHODS

LC/MS System: nanoACQUITY UPLC®/ Q-Tof micro® or Q-Tof Premier®

***E coli* Sample:** 200 ng/µl MassPREP *E. coli tryptic* digest

First dimension (Fig. 1a)
Column: 100 µm x 10 cm XBridge® C_{18} (5 µm, high pH tolerant)
Gradient formation: Step gradient using plug injection
Plug solutions: 20 mM ammonium formate, pH 10.0 in 10-40% ACN
Plug loading: 2 µl loaded at 2.5 µl/min for 5 min
Online dilution flow rate: 25 µl/min aqueous

Second dimension (Fig. 1b)
Column: 75 µm x 10 cm Bridged Ethyl Hybrid C_{18} (1.7 µm)
Gradient: 5-40% B for 60 min at 300 nl/min
Eluent A: 0.1% formic acid in water
Eluent B: 0.1% formic acid in acetonitrile

Peptide fragment data collection: MS^E

MS Data processing : MS^E data from the individual chromatograms were processed separately with Identity^E using an *E. coli* database (*E. coli* peptide sequencing) combined with a reversed *E. coli* database (false positive rate calculation). The results for a protein from individual fractions were combined to calculate a protein sequencing score.

Fluidic Design: When an organic-containing fraction is eluted from an XBridge C_{18} column with an isocratic pump, an aqueous flow is delivered into the system with a binary pump, and mixed with the eluted fraction to reduce its organic content (Fig. 1a). When the fraction is collected onto the Symmetry C_{18} trap column, the organic concentration in the fraction is lowered enough to allow the peptides present in the fraction to be retained on the trap column. This eliminates the manual removal of the organic component in the fractions, allowing the fractions to be automatically run with the RP column of the second dimension. The effectiveness of online dilution is shown in Fig. 2

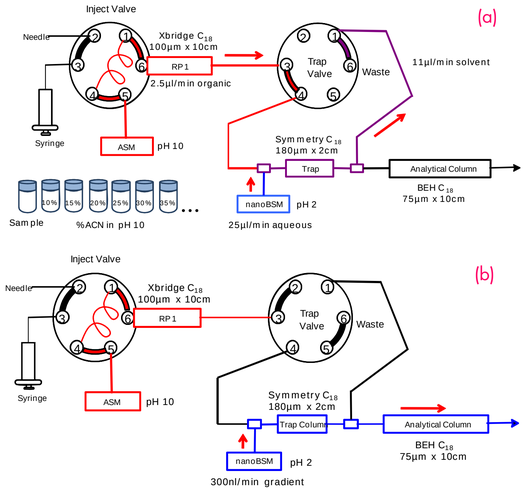


Fig. 1. Configuration of an automated RP/RP 2D LC/MS with online dilution (a): First dimension with online dilution (b): Second dimension

RESULTS

Effectiveness of online dilution: A 1D RP LC/MS system modified from the configuration of Fig. 1 was employed. The modification was made by replacing the XBridge column with a piece of capillary tubing. 100 fmol enolase digest containing 0-60% ACN was injected onto the trap column. During each injection, an aqueous mobile phase was delivered from the binary pump. The flow rate combination was varied so that the ACN in the organic-containing samples would be diluted to 4% .

- All hydrophilic peptides were observed even in presence of 60% ACN
- Hydrophobic peptide recovery was enhanced with the presence of ACN. This is likely due to improved solubility or reduced surface interaction (vial, loop, tubing, etc)

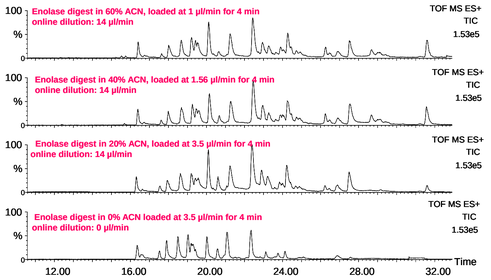


Fig. 2. Effectiveness of online dilution

RP/RP 2D Method: Seven fractions were produced from the first dimension. The fractionation was carried out at pH 10.0 with a step gradient formed with plug injection (plug size: 2 µl). See each chromatogram for the ACN concentrations in the plugs injected to produce the fractions. An aqueous dilution flow was delivered with the binary pump at 25 µl/min to dilute the ACN concentration in the plug eluted from the XBridge column. The second dimension was performed at pH 2.6 with a linear ACN gradient of 5-40%

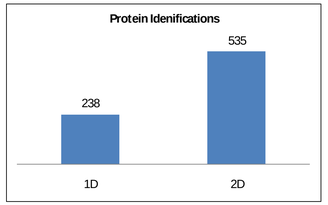
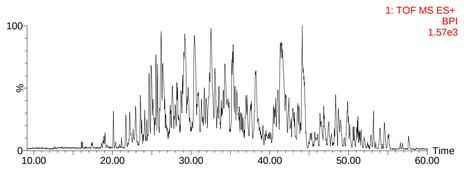


Fig. 3. Protein identifications from 500 ng *E. coli* digest



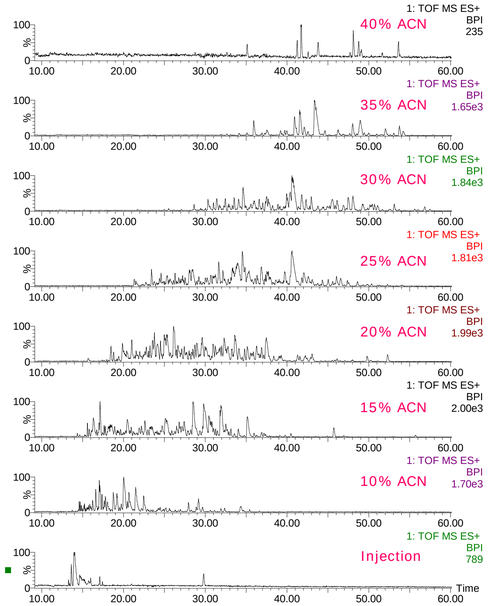


Fig. 5. A set of RP/RP 2D nanoLC/MS chromatograms of 500 ng *E. coli* digest

- Peptides fractionated at a 5% ACN window were spread out to a ~20% ACN window, indicating good orthogonality between 2 RP dimensions (Fig. 5)

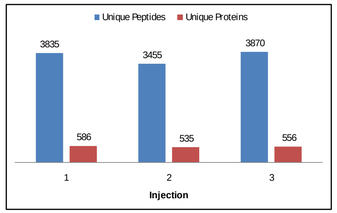


Fig. 6. Reproducibility of *E. coli* protein identification with RP/RP 2D LC/MS (n=3)

Fraction Number	%ACN in Step	Number of Uniquely Identified Peptides	Number of Peptides Identified in other Fraction(s)	Number of False Positively Identified Peptides
Injection	0	16	4	0
1	10	225	12	2
2	15	755	35	5
3	20	1115	54	8
4	25	979	45	6
5	30	590	26	5
6	35	137	7	0
7	40	30	2	0

Table 1. Peptide sequencing results from MS data of a set of RP/RP 2D LC/MS chromatograms of 500 ng *E coli* digest

CONCLUSION

- The *E. coli* peptides were fractionated with good distribution (Table 1)
- There were only 11.4% peptides with multiple times of identification from adjacent fractions as compared to 33% with SCX used for first dimension. This indicates a high-quality fractionation, mainly resulted from the use a RP fractionation column, which was more efficient to separate peptides than an SCX column
- The false positive rate of identification was 0.67% at peptide level, and was 3.3% at protein level
- There was only 20 mM salt (ammonium formate) involved in the RP/RP 2D system, making it more compatible with MS
- The 500 ng *E .coli* digest was analyzed 3 times. The RSD of the number of proteins and the number of peptides identified from the runs were 4.6% and 6.2%, respectively (Fig. 6)
- 95% of the E. coli peptides went into the same fractions each time during the 3 times of fragmentation
- The average standard deviation of the 3 retention times of a peptide during the 3 runs was 0.092 min
- The highly reproducible 2D data were due to the use of RP for both dimensions. The highly reproducible fractionation was also resulted from the use of plug injection

References

Gilar, M., Olivova, P., Daly, A., Gebler, J. J. Sep. Sci. 2005, *28*, 1694-1703

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