IMPROVED HYDROPHOBIC PEPTIDE RECOVERY DURING ONLINE 2D NANOLC/MS/MS FOR PROTEOMIC ANALYSIS

Hongji Liu, Jeffrey W. Finch and John C. Gebler
Waters Corporation, 34 Maple St., Milford, MA 01757

INTRODUCTION

Despite wide applications of online 2D (SCX + RP) nanoLC/MS/MS methods for proteomic analysis, little in-depth investigation has been made to improve the 2D performance. In this presentation, we report a 2D method in which 2 major problems of conventional online 2D methods, namely the loss of hydrophobic peptides (caused by interaction with the hydrophobic SCX column surface) and tailing RP peaks (caused by the dead volume between the trap and the analytical RP columns), were solved. The application of this on-line 2D method is demonstrated with an *E. coli* digest.

METHODS

To elute hydrophobic peptides, a RP step gradient was applied to the SCX column after a salt step gradient. With the RP gradient, the hydrophobic peptides not eluted from the SCX column with the conventional salt gradient were eluted. The RP fractions only contained hydrophobic peptides, so the peptides could still be trapped on the RP trapping column even in the presence of up to 20% acetonitrile.

To minimize peak tailing, a band re-focusing method was developed. A RP analytical column which is more hydrophobic (relatively) than the RP trap column was used for RP separations. Thus, any dispersed peptide bands eluting from the trap column were re-focused on the analytical column.

EXPERIMENTAL

Sample: tryptic digests of enolase and *E. coli* and a mixture of MassPREP™ tryptic digests of 5 proteins (phosphorylase b, yeast enolase, bovine hemoglobin, yeast alcohol dehydrogenase and bovine serum albumin).

LC system: Waters nanoACQUITY UPLC™
MS system: Waters Q-Tof micro™

Trap column: 180 µm x 2 cm Symmetry® C_{18}, 5 µm

First dimension (SCX):

Column: Waters nanoACQUITY SCX (180 µM × 2.4 cm, packed with 5 µm PolySULFOETHYL Aspartamide material)

Salt Gradient: step gradient formed with plug injections (2 µl) of 40-200 mM ammonium formate (NH_4FA) containing 5% acetonitrile (ACN), pH3.20

Organic gradient: step gradient formed with plug injections of 5-20% ACN containing 200 mM NH_4FA, pH3.20

Second dimension (RP):

Column: 75 µm x 10 cm BEH C_{18}, 1.7 µm

Mobile phase: 0.1% formic acid in water (A) or ACN (B)

Gradient: 5-50% over 30 min at 250 nl/min

MS/MS conditions: DDA

Data processing: ProteinLynx Global Sever (PLGS) 2.0

System configuration (Figure 1)

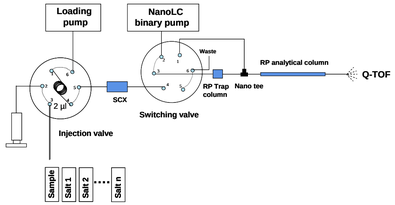

Figure 1. 2D SCX/RP nanoLC/MS/MS system

RESULTS AND DISCUSSION

- The band re-focusing effect took place with a RP trap column less hydrophobic (Symmetry C_{18}) than the RP analytical column used (BEH C_{18}). Any dispersed peptide bands eluted from the trap column were re-focused at the inlet of the analytical column. As shown in Figure 2, the added complexity to the LC system by the use of the trap column and connections did not degrade the analytical column performance.
- Excellent peak shapes were observed when the 2D LC/MS system was employed for a 5-protein digest mixture (Figures 3 and 4) and an E coli digest (Figures 5 and 6). As indicated in Figure 4, the 2D peaks were equivalent to those observed with a1D no-trap-column RP separation (direct load) under the same 30 min gradient.
- As shown in Figures 3 and 5, many peptides were contained in the SCX fractions obtained with the RP gradient applied to the SCX column. As indicated by their retention times, these compounds were generally more hydrophobic than those contained in other fractions.
- The peaks obtained with a 2D run (30 min gradient for each SCX fraction) were sharper than those obtained with a 1D run using a 300 min gradient, both of which were carried out with approximately the same total run time (Figure 6). Greater peak heights were observed for the 2D peaks, providing possibilities to identify peptides at lower quantities.
- The developed 2D LC/MS/MS method was applied for the analysis of an *E. coli* digest (chromatograms not shown). Figure 7 describes a processed MS/MS spectrum of an *E coli* peptide. There are 235 *E. coli* peptide identified and validated, 169 of which were unique.
- Table 1 lists the numbers of peptides uniquely identified from each SCX fraction. There were no peptides in the first fraction identified as an *E. coli* peptide, demonstrating the effectiveness of the SCX loading condition: all peptides of interest were initially retained on the SCX column for further fractionation.
- There were 27 *E. coli* peptides (16% of all identified with 2D) uniquely recognized from the 2 RP fractions (Fractions #6 and #7). Due to hydrophobic interaction with the SCX column, these peptides had not eluted from the SCX column with the salt gradient alone. The identification of these peptides with 2D LC/MS/MS would have been impossible if the RP gradient had not been applied to the SCX column. Table 2 lists all peptides uniquely identified from one RP fraction (fraction #7)

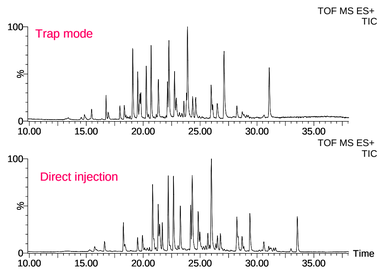

Figure 2. Comparison of performance of RP analytical column with (trap mode) and without (direct injection) a trap column
See Figure 1 for trap mode configuration (SCX column removed).

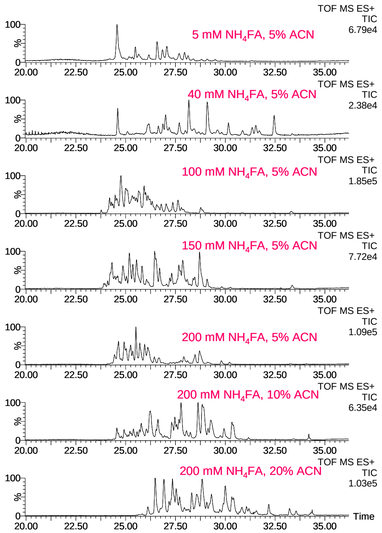

Figure 3. Online 2D nanoLC/MS of a 5-protein digest mixture (100 fmol per protein).

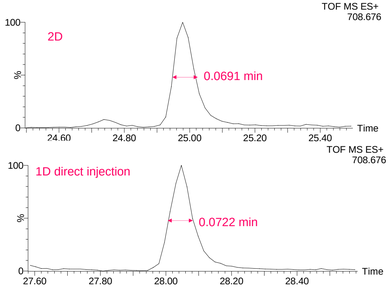

Figure 4. Comparison of peak shapes of the same peptide extracted from 1D (no trap column) and 2D (SCX/RP) chromatograms

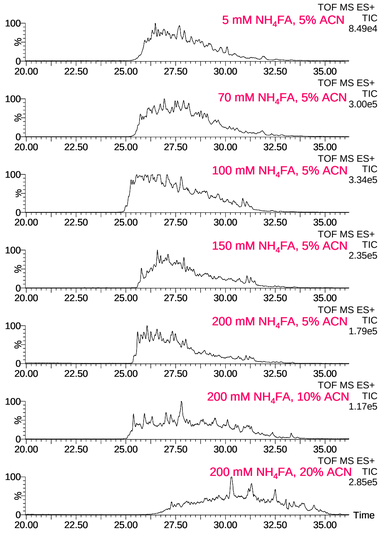

Figure 5. Online 2D nanoLC/MS of 200 ng *E. coli* digest.

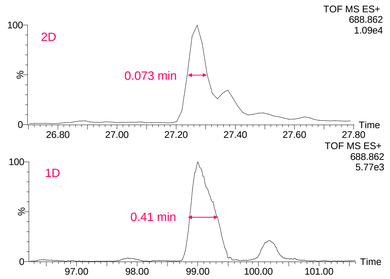

Figure 6. Comparison of peak widths and heights of the same *E. coli* peptide extracted from 2D (30 min RP gradient) and 1D (300 min RP gradient) chromatograms. Sample amount: 200 ng. The peak(s) eluted after the major peak related to other peptides with similar m/z.

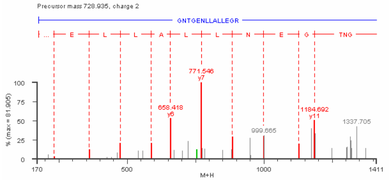

Figure 7. A MS/MS spectrum of an *E. coli* peptide processed with PLGS. The spectrum was acquired with 2D online nanoLC/MS/MS (DDA). E. coli sample amount: 200 ng.

Table 1. Peptides uniquely identified with 2D online nanoLC/MS/MS

SCX Fraction	SCX conditions NH_4FA, ACN	No. of peptides identified*	No. of peptides and their charges
1	5 mM, 5%	0 (0)	-
2	70 mM, 5%	65 (65)	65 (+2)
3	100 mM, 5%	50 (35)	50 (+2)
4	150 mM, 5%	36 (25)	24 (+2), 12 (+3)
5	200 mM, 5%	32 (17)	28 (+2), 4 (+3)
6	200 mM, 10%	16 (3)	13 (+2), 3 (+3)
7	200 mM, 20%	35 (24)	26 (+2), 9 (+3)

*The numbers in parentheses indicate unique identifications. If identification of the same peptide was observed in multiple fractions, only the identification for the first occurrence was counted as unique.

Table 2. Peptides uniquely identified from 7th SCX fraction with 2D online nanoLC/MS/MS

Sequence	Protein ID	Charge
ATGLDALFDATIK	P07651	+2
IATDPFVGNLTFFR	P02996	+2
ILELAGFLDSYIPEPER	P02990	+2
LLPWIDGLLDAGEK	P11604	+2
LVVATDTAFVPFEFK	P10344	+2
STAESIVYSALETLAQR	P02359	+2
STQVYGQDVWLPAETLDLIR	P08200	+2
SVEELNTELLNLLR	P02429	+2
TAIESALTALETALK	P04475	+2
VQNASYQVAAYLADEIAK	P80063	+2
AAVEEGVVAGGGVALJR	P06139	+2
ALDAIIASVTESLK	P02341	+2
DDVAFQIINDELYLDGNAR	P80063	+2
FGGYAQSGLLAEITPDK	P02928	+2
FTGWYDVDLSEK	P31217	+2
DKPEDAVLDVQGIATVTPAIVQACTQDK	P26604	+3
FADVACAGPLLAAELDALGK	P11665	+3
IELSSAQQTDVNLPYITADATGPK	P04475	+3
LGPYEFICTGRPDEGIPAVCFK	P80063	+3
LLPHIPADQFPAQALACELYK	P00913	+3
TQTAPVATPQELADYDAIIFGTPTR	P30849	+3
ADAVLHDTPNILYFIK	P10344	+3
AGVPAGVFNVVTGSAGAVGNELTSNPLVR	P25526	+3
GVTPVHFDSANDGVAAASEAVNLLR	P21599	+3

CONCLUSION

- **A 2D (SCX/RP) nanoLC/MS/MS system has been developed with improved separation efficiency and peptide recovery**
- **The 2D separation was improved with an band re-focusing effect, made possible with the use of an analytical column which was more hydrophobic than the trap column**
- **The chromatographic peaks acquired with the developed 2D method were equivalent in shape to those obtained with a 1D direct injection method run with the same conditions**
- **Many hydrophobic peptides were recovered with the use of a RP step gradient applied to the SCX column after a conventional salt gradient. Due to stronger hydrophobic interaction with the SCX column, these peptides could have not been eluted from the SCX column, and therefore not characterized with 2D without the application of the RP step gradient**
- **The developed 2D method can be employed as an effective tool to characterize peptides with improved chromatographic resolution, MS sensitivity and sequence coverage.**

TO DOWNLOAD A COPY OF THIS POSTER VISIT WWW.WATERS.COM/POSTERS

720001722EN