Automated injection of uncleaned samples using a ten-port switching valve and a strong cation-exchange trap column for proteome analysis

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Abstract

Nanoliter flow rate is optimum for separation in capillary column liquid chromatography coupled with tandem mass spectrometry (μ-HPLC–MS/MS). In order to develop a high-performance automated proteome analysis system allowing direct injection sample containing detergents, the influence of void volume varied from 0 to 5 μL on the separation performance and proteomic coverage of sample injection system using strong cation-exchange (SCX) trap column was investigated, it was found the void volume hardly affects the separation performance by using SCX trap column. Thus, a fully automated sample injection system using SCX trap column and ten-port switching valve was established for efficient shotgun proteome analysis. In this system, a nanoflow switching valve and a microtee were used to connect the SCX trap and analytical columns, and the uncleaned samples of proteolytic digests containing contaminants could be directly injected with minor influence on the separation performance, which was demonstrated to be a useful strategy in proteome analysis.

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Keywords: μ-HPLC–MS/MS; Shotgun proteome analysis; Automated sample injection; SCX trap column; Void volume insensitive; Multidimensional separation

1. Introduction

Mass spectrometry has been widely used as a highly reliable and sensitive technique for protein identification and characterization in proteomics [1–4]. In the approach of shotgun proteome analysis, the complex peptide mixture resulted from proteolytic digestion of the proteins is analyzed by HPLC–MS/MS [5,6], which has gained tremendous development in recent years. And sensitivity gives the driving force in the development of nanoflow HPLC (μ-HPLC) as electrospray mass spectrometry is a concentration-dependent detection method [7–9].

Manual injection of sample onto a nanoscale HPLC column in proteome analysis is achieved in previous reports [10,11,24]. This method is simple and practical. However, it is time consuming and labor intensive as it typically spends more than half hour to complete the sample loading manually. In order to overcome these limitations, automated sample injection for μ-HPLC–MS/MS is required [5]. The automation could be realized by using a short and larger inside diameter (I.D.) trap column coupled to an analytical column for rapid sample loading. Briefly, protein digest of big volume is firstly loaded onto the trap column at a high flow rate in short time and after equilibrium the adsorbed peptides are eluted from the trap column to a reversed-phase analytical column for separation. Two types of instrument configurations have been adopted for the automated sample injection in μ-HPLC–MS/MS using trap column. One is directly connecting the trap column and reversed-phase analytical column by a nanoflow switching valve. The flow through from trap column is directed to waste or analytical column during sample injection or separation by the switching valve [9,12–19]. In this type of system, proteolytic digest without prior purification could be directly injected and cleaned up on-line, which is very attractive in comprehensive proteome analysis. But the void volume introduced by switching valve would seriously degrade the separation performance. The other one is vented column system in which trap and analytical columns are directly connected via a microcross or microtee with an open/close switching valve [20–24]. In this type of system, the mobile phase for μ-HPLC
separation does not pass though the switching valve, so a regular six-port switching valve could be used instead of using nanoflow switching valve. In order to minimize the void volume resulted from the microcross or microtee, Licklider et al. [20] packed the open space of microcross with C18 particles. And Meiring et al. [21] drilled the microtee to 0.6 mm I.D. to fit a single micro sleeve having a V-shaped cut as a waste outlet, and the trap and analytical columns were butt-connected in this modified sleeve. However, these systems largely depend on experience and are not widely used. Another disadvantage of vented column system is that proteolytic digest containing denaturing agents cannot be loaded directly. This is because small portion of the sample solution will also enter the analytical column and contaminate the column during loading of sample onto trap column. So proteolytic digest must be desalted and cleaned before the automated sample introduction.

Although above configurations enable the automated sample injection for proteome analysis by \( \mu \)HPLC–MS/MS, void volume resulted from the connections between trap and analytical capillary columns inevitably leads the degradation of separation performance. As further decreasing the void volume is a technique challenge, a good solution is to develop a void volume insensitive automated sample injection system for \( \mu \)HPLC–MS/MS analysis. Instead of using C18 trap column, we found recently that automation of sample injection using SCX trap column can alleviate the influence of void volume on separation in vented column system [24]. However, proteolytic digest sample must be cleaned before sample injection in order to eliminate the contamination of the analytical column. In this study, the influence of void volume between trap and analytical columns on the separation performance and proteomic coverage was systematically studied, and it was found the void volume (varied from 0 to 5 \( \mu L \)) hardly affect the separation performance by using SCX trap column. Thus an automated sample injection system with ten-port switching valve and SCX trap column was constructed, which allowed direct injection of proteolytic digest containing contaminants. This system allowed fast sample injection at a flow rate \(~2 \mu L/min\), and exhibited good separation performance as well as good proteomic coverage. Additionally, it could be applied to automated on-line multidimensional separation of complex peptides mixtures very conveniently.

2. Experimental

2.1. Materials

Daisogel ODS-AQ (5 \( \mu m \), 120 Å pore) was purchased from Daiso (Osaka, Japan), polysulfoethyl aspartamide (5 \( \mu m \), 200 Å pore) was from PolyLC (Columbia, MD, USA). Polyether ether ketone (PEEK) tubing, sleeves, microtee, microcross, zero dead volume (ZDV) union and mini microfilter assembly (with a filter capsule, porosity 2 \( \mu m \)) were obtained from Upchurch Scientific (Oak Harbor, WA, USA). Fused silica capillaries with 50 and 75 \( \mu m \) I.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA), and 100 \( \mu m \) I.D. from Yongnian Optical Fiber Factory (Hebei, China). All the water used in experiments was purified using a Mill-Q system from Millipore (Bedford, MA, USA). Trypsin was from Promega (Madison, WI, USA). Formic acid was obtained from Fluka (Buchs, Germany).

2.2. Sample preparation

The yeast protein extract was prepared in a denaturing buffer containing 50 mM Tris/HCl (pH 8.1) and 8 M urea as before [25]. The protein concentration was determined by BCA assay. The protein sample was reduced by dithiothreitol (DTT) at 37 °C for 2 h and alkylated by iodoacetamide in dark at room temperature for 40 min. Then the solution was diluted to 1 M urea with 50 mM Tris/HCl (pH 8.1). Finally, trypsin was added with weight ratio of trypsin to protein at 1/25 and incubated at 37 °C overnight. Then, half of tryptic digest was purified with a laboratory-made C18 solid-phase extraction (SPE) cartridge and exchanged into buffer A (0.1% formic acid water solution). The other half of tryptic digest was centrifuged at 25 000 \( \times g \) for 10 min to pellet the insoluble debris. All the samples were stored at \(-20^\circ C\) before usage.

2.3. Column preparation

Columns were packed using a homemade pneumatic pressure cell at constant nitrogen gas pressure of about 580 psi with a slurry packing method [22]. For the preparation of analytical column, one end of a 75 \( \mu m \) I.D. fused silica capillary was first manually pulled to a fine point of \(~5 \mu m\) with a flame torch, and then the C18 particles were packed until the packing section reached the length of 12 cm. Fused silica capillary with I.D. of 100 \( \mu m \) was used to prepare the trap column. Two types of frits were used to prepare trap columns in this study. One is the preparation of monolithic frit following the procedure previously described by Xie et al. [26–28]. The other one is to use a mini microfilter assembly (with a filter capsule, porosity 2 \( \mu m \)) as a frit. The trap columns were prepared by packing 2 cm length of SCX resin into the capillary with frit.

2.4. Mass spectrometric analysis

The temperature of the ion transfer capillary was set at 200 °C. The spray voltage was set at 1.82 kV and the normalized collision energy was set at 35.0%. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data dependent mode. The mass spectrometer was set that 1 full MS scan was followed by 6 MS/MS scans on the 6 most intense ions. The dynamic exclusion function was set as follows: repeat count 2, repeat duration 30 s, and exclusion duration 90 s. System control and data collection were done by Xcalibur software version 1.4 (Thermo).

2.5. Chromatographic separation

The four buffer solutions used for the quaternary pump were 0.1% formic acid water solution (buffer A), acetonitrile (ACN) with 0.1% formic acid (buffer B), 500 mM NH₄Ac at pH 3 (buffer C), and 250 mM NH₄Ac at pH 3 (buffer D). The flow
rate after splitting was adjusted to optimize the separation performance \( \sim 200 \text{ nL/min} \). The binary gradient with buffer A and buffer B for reversed-phase separation was developed from 0 to 10% buffer B for 2 min, from 10 to 35% buffer B for 90 min and from 35 to 80% for 5 min. After flushing with 80% buffer B for 10 min, the separation system was equilibrated by buffer A again. In systems using SCX trap column, the peptides retained on SCX trap column were eluted onto the analytical column by flushing with buffer C (containing 500 mM NH\(_4\)Ac at pH 3) for 10 min. After the system was re-equilibrated with buffer A for appropriate time, the binary separation gradient was started. The data acquisition was begun immediately after the start of gradient separation.

### 2.6. Sample injection

Three configurations were adopted for sample injection in our experiments, and we classified them as off-line sample injection, on-line sample injection, and manual injection.

The first one was off-line sample injection system. During sample loading, the six-port valve was switched to close the split flow, and 0.95 µg SPE cleaned yeast protein digest (20 µL) was automatically injected at a flow rate of 2 µL/min. The mobile phase used was 0.1% formic acid (buffer A) [16]. After sample injection, the trap and analytical columns were manually connected by a ZDV union. In order to investigate the influence of void volume on separation, appropriate void volume was introduced on purpose between trap and analytical columns. Capillary with 150 µm I.D. was used to introduce appropriate void volume in this off-line sample injection system and two ZDV unions were applied to connect trap column, 150 µm I.D. open tubular capillary, and analytical column after sample injection. 5.7, 16.9, and 28.3 cm long 150 µm I.D. capillary were used to generate 1.3, and 5 µL void volume, respectively.

The configuration for the on-line sample injection was shown in Fig. 1. The trap column was connected to a C18 analytical column by a ten-port nanoflow switching valve and a microtee. During sample injection, 0.95 µg yeast protein tryptic digest without SPE pretreatment (20 µL) was automatically injected onto the trap column at a flow rate of 2 µL/min (Fig. 1 solid line mode). After sample injection, the switching valve was switched to activate the splitting flow and connect trap and analytical columns (Fig. 1 dashed line mode). The trap columns prepared by a mini microfilter assembly were used in the on-line sample injection system.

For manual injection, an open capillary filled with sample was connected between the microcross and the analytical column as previously reported [24,25]. The peptides in the open capillary was flushed by the buffer A and enriched onto the front of analytical column. After sample loading, the open capillary was removed, and the analytical column was directly connected to the microcross.

After sample was loaded by either of the three sample injection modes, a separation procedure described in Section 2.5 was used to separate the loaded peptides.

### 2.7. Multidimensional separation using SCX trap column

Nineteen microgram crude tryptic digest of yeast protein without SPE purification was automatically injected onto the SCX trap column (the tryptic digest was 0.238 µg/µL, 80 µL digest solution was automatically injected by four consecutive times, 20 µL per time). Then, a series stepwise elution with salt concentrations of 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 500 and 500 mM NH\(_4\)Ac was used to gradually elute peptides from trap column onto the analytical column [10,11,24]. Each salt step lasts 5 min except last two steps for 10 min by washing the column with 100% buffer C in order to elute all peptides. After each salt elution, the whole system was equilibrated for 25 min with buffer A. The binary gradient elution described in Section 2.5 was applied in each cycle to separate peptides prior to MS detection.

![Fig. 1. Schematic diagram of the on-line sample injection system, solid line: loading sample onto trap column; dashed line: eluting sample onto analytical column and gradient µHPLC–MS/MS analysis.](image-url)
2.8. Data analysis

The acquired MS/MS spectra were searched on the database using the Turbo SEQUEST in the BioWorks 3.2 software suite (Thermo). The yeast database was downloaded from a website (ftp://genome-ftp.stanford.edu/yeast/data_download/sequence/genomic_sequence/orf_protein/orf_trans.fasta.gz). Revered sequences were appended to the database for the evaluation of false positive rate. Cysteine residues were searched as static modification of 57.0215 Da, and methionine residues as variable modification of +15.9949 Da. Peptides were searched using fully tryptic cleavage constraints and up to two internal cleavages sites were allowed for tryptic digestion. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. The peptides were considered as positive identification if the Xcorr were higher than 1.9 for singly charged peptide, 2.2 for doubly charged peptide and 3.75 for triply charged peptides, and ΔCn cutoff value was set to control the false positive rate <1%, determined by the calculation based on the reversed database search.

Grand average of hydrophobicity (GRAVY) value for each unique peptide was calculated according to the method of Kyte and Doolittle [29]. A visual-basic macro provided by DiagnoSwiss was used to calculate the peptides’ isoelectric points (pI).

3. Results and discussion

3.1. Effect of void volume on separation performance and proteomic coverage

In our previous work, Jiang et al. [24] used a microcross to connect the SCX trap and C18 analytical columns with vented column configuration to automate the sample introduction for µHPLC–MS/MS, and found the void volume introduced by the microcross has little influence on the separation performance. We would further develop an automated sample injection system allowing direct injection of uncleaned samples. To avoid the contamination of analytical column by the contaminants in the sample, a configuration with ten-port switching valve should be used as shown in Fig. 1. In this system, the total void volume resulted from the connecting capillary, microtee, microfilter assembly and switching valve was much bigger than that in the vented column system. Therefore, it is necessary to investigate the effect of relatively large void volume on separation performance by using SCX trap column system.

The number of unique peptides was the most important index to evaluate the performance of shotgun proteome analysis systems as the identification of proteins was achieved by the peptides identified from the µHPLC–MS/MS analysis. In Fig. 2 we could see that the number of identified unique peptides in the system using SCX trap column was hardly affected by the increase of void volume. That might be explained by the migration behavior of analytes in this system as discussed by Jiang et al. [24]. After sample was introduced onto the SCX trap column, the peptides bounded on SCX resin with electrostatic interaction were eluted onto the C18 analytical column by flushing with 500 mM NH₄Ac (pH 3, buffer C) for 10 min. When the binary gradient started for reversed-phase separation, there were no peptides eluted from SCX trap column to take part in separation. Therefore, the separation of peptides was only achieved on the analytical column. Similar to the number of unique peptides, the number of proteins identified on the system using SCX trap column was also not affected by the increase of void volume between trap and analytical columns (see Fig. 2).

In terms of separation, manual injection is an ideal sample injection mode. Its performance was used as a standard to evaluate the performance of the automated sample injection systems [24]. The procedures of manual injection were described in Section 2.6. After µHPLC–MS/MS analysis of 0.95 µg SPE cleaned tryptic digest of yeast proteins, 922 unique peptides (RSD = 7.9%, n = 3) from 349 distinct proteins (RSD = 8.8%, n = 3) were positively identified. Fig. 3A and B gave the pI and hydrophobicity distributions of peptides identified in manual injection system as well as off line sample injection system using SCX trap column with void volume of 5 µL. Obviously, the peptides’ pI and hydrophobicity distributions of system using SCX trap column are different to manual injection system. This is because the peptides are bounded on SCX trap column by electrostatic interaction in contrast to hydrophobic interaction in manual injection system. It can be found that SCX trap column more efficiently traps peptides in the range of pI > 4.5 and GRAVY < −0.5, which indicated that the system using SCX trap column has more efficiency to analyze basic and hydrophilic peptides.

3.2. Automated injection of uncleaned samples

A ten-port nanoflow switching valve and a microtee was used to connect the SCX trap and C18 analytical columns and an online sample injection system was established (see Fig. 1). During sample injection, the switching valve is switched to close the splitting flow and the flow through from the SCX trap column is directed to waste (see Fig. 1 solid line mode). So peptides will be captured on the SCX trap column and contaminants in the sample are removed. As isolated with the tubes for sample
peptides are randomly selected across the elution, sample loading rate is 2 μL/min, and the separation conditions are the same as Fig. 4.

Table 1  
Run-to-run retention reproducibility of four consecutive analysis of crude tryptic digest of yeast proteins by on-line sample injection system using SCX trap column

<table>
<thead>
<tr>
<th>Mass</th>
<th>Retention time (min)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>29.82 30.99 33.62 31.73 31.54</td>
<td>4.4</td>
</tr>
<tr>
<td>Run 2</td>
<td>35.72 37.14 39.57 37.82 37.56</td>
<td>3.7</td>
</tr>
<tr>
<td>Run 3</td>
<td>42.85 44.20 46.50 45.00 44.64</td>
<td>3.0</td>
</tr>
<tr>
<td>Run 4</td>
<td>51.38 52.22 53.92 53.14 52.66</td>
<td>1.8</td>
</tr>
<tr>
<td>Av.</td>
<td>37.82 37.56 39.57 37.82 37.56</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Peptides are randomly selected across the elution, sample loading rate is 2 μL/min, and the separation conditions are the same as Fig. 4.

by 51% and 62%, respectively; the percentage of proteins identified by two or more peptides was about 10% lower by comparing with the usage of SCX trap column (see Supplementary Table S1).

The reproducibility of the automated on-line injection system using SCX trap column was investigated by four consecutive analyses of the tryptic digest of yeast proteins. The retention times of 15 peptides were determined and their relative standard deviations (RSDs) were calculated. As shown in Table 1, RSD for most peptides was less than 3.0%, which indicated reproducible separation could be obtained by using SCX trap column and ten-port switching valve. The run-to-run reproducibility of the same 15 peptides’ peak areas was also investigated and the RSD for most peptides was less than 30.0% with the average RSD was 21.9% (see Supplementary Table S2). The average numbers of unique peptides and distinct proteins identified in four consecutive runs were 1053 (RSD 6.0%) and 403 (RSD 2.9%), respectively. This also demonstrated that consistent proteome analysis results could be obtained by the system using SCX trap column for analysis of uncleaned samples.

3.3. Automated multidimensional μHPLC/MS/MS analysis

In the automated sample injection system using SCX trap column and ten-port switching valve, two-dimensional separation can be easily realized by using a series of salt stepwise gradient elution to fractionally elute peptides bounded on SCX trap column to the C18 analytical column for μHPLC/MS/MS analysis. Crude proteolytic digest (80 μL, 0.238 μg/μL) of yeast proteins was directly injected by autosampler, cleaned up and concentrated on-line by SCX trap column at the same time, which significantly reduced sample pretreatment time and sample loss and was extremely helpful to discover some low abundance proteins. After sample injection, 13 cycles of salt stepwise elution delivery, the analytical column will not be contaminated by the detergents in sample solution during sample loading. Therefore, this system should allow the direct analysis of proteolytic digest which has 1 M urea, 50 mM Tris, etc. without prior purification.

The base peak chromatogram for HPLC–MS/MS analysis of 0.95 g uncleaned tryptic digest of yeast proteins was shown in Fig. 4. The gradient delay in this system was ∼30 min, which indicated there was ∼3 L void volume between SCX trap and C18 analytical columns by comparing to the gradient delay in off-line sample injection system. It was found that the peak intensity of peptides on this system was much higher than that in off-line sample injection system even with 0 μL void volume between trap and analytical columns (data not shown). After database search, a total of 403 distinct proteins were identified through assignment of the 1077 unique peptides. Therefore, it was obvious that on-line injection of sample without prior SPE purification (containing 1 M urea, 50 mM Tris, etc.) was a useful strategy to obtain high proteomic coverage in shotgun proteome analysis. However, if the C18 trap column was used in the same system, the number of proteins and peptides identified decreased by 51% and 62%, respectively; the percentage of proteins identified by two or more peptides was about 10% lower by comparing with the usage of SCX trap column (see Supplementary Table S1).

The reproducibility of the automated on-line injection system using SCX trap column was investigated by four consecutive analyses of the tryptic digest of yeast proteins. The retention times of 15 peptides were determined and their relative standard deviations (RSDs) were calculated. As shown in Table 1, RSD for most peptides was less than 3.0%, which indicated reproducible separation could be obtained by using SCX trap column and ten-port switching valve. The run-to-run reproducibility of the same 15 peptides’ peak areas was also investigated and the RSD for most peptides was less than 30.0% with the average RSD was 21.9% (see Supplementary Table S2). The average numbers of unique peptides and distinct proteins identified in four consecutive runs were 1053 (RSD 6.0%) and 403 (RSD 2.9%), respectively. This also demonstrated that consistent proteome analysis results could be obtained by the system using SCX trap column for analysis of uncleaned samples.

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of peptides from SCX trap to analytical columns followed by 
μHPLC/MS/MS analysis were automatically conducted. After 
database search, 2388 unique peptides were identified from 870 
different proteins. Not only the identified protein number was 
increased, but also the peptides’ pI and hydrophobicity dis-
tribution range expanded reasonably (data not shown), which 
demonstrated the advantage of this sample injection system 
using SCX trap column in automated multidimensional pro-
teome analysis.

3.4. Discussion

In automated sample injection systems, void volume is 
inevitably introduced by the connection components used for 
sample collection and transfer. Void volume will result in serious 
degradation of separation efficiency in μHPLC if C18 trap col-
umn was adopted. We presented an alternative way to establish a 
void volume insensitive on-line sample injection system allow-
ging direct injection of sample containing detergents. Though 
∼3 μL void volume was introduced by 75 μm I.D. transferring 
capillary, microfilter assembly, switching valve, and microtee 
between trap and analytical columns, good separation perfor-
mance and good proteomic coverage were still obtained 
when SCX trap column was used in the on-line sample injection 
system. Correspondingly, serious degradation in the separation 
efficiency and proteomic coverage was observed when the SCX 
trap column was replaced with C18 trap column.

There are many reports on proteome analysis by using 
SCX-RP based multidimensional separation, in which the SCX 
column was used as the first dimension to fractionate proteins 
or peptides mixture. In our case, the SCX-RP based proteome 
analysis system is established for automatic sample injection at 
a flow rate of ∼2 μL/min and both one- and multi-dimensional 
separation can be performed. Besides, as ten-port switching 
valve is used, proteolytic digest containing contaminants such 
as salt, denaturant, etc. can be directly injected and the analytes 
are cleaned and concentrated onto the SCX trap column at a high 
flow rate.

4. Conclusions

To avoid labor intensive operations and obtain highly repro-
ducible results, it is necessary to automate the μHPLC–MS/MS 
system in shotgun proteome analysis. We systematically stud-
ied the influence of void volume between trap and analytical 
columns on the separation performance and proteomic cover-
age in sample injection system using SCX trap column. And the 
sample injection system with combination of ten-port switching 
valve and SCX trap column was developed for alleviating the 
influence of void volume and direct injection of uncleaned sam-
ple. This on-line system exhibits good separation performance 
as well as good proteomic coverage in both one- and multi-
dimensional separation, and represents a convenient, useful, and 
reliable approach for routine shotgun proteome analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2007.09.048.

References