Introduction

Coupling columns of same or different resin materials is often employed in complex proteomic digest analysis. Despite enhanced separation, these multidimensional columns are costly, time-consuming to produce, and initiate post-column loss by dead-volume introduction. Confounding factors of column-coupling can be eliminated via transparent, true zero-dead-volume (ZDV) unions that achieve flush connections and rapid swap-out facility during system maintenance. In the current investigation, two conventional 10 cm-bedded columns were coupled and connected to the bed terminus of a third 10 cm nanobore column with integrally fritted tip. Analytical merit of this extended column was compared with a single 30 cm-bedded column with integrally fritted tip and the same resin material. These novel unions supported chromatographic data collection with zero dead-volume, negligible resolution loss, and comparable caliber as the single 30 cm-bedded column. The performance of a 35 cm column fabricated from 15- and 20-columns is also detailed.

Methods & Materials

Instrumentation & Components

- Ion-trap mass spectrometer (LCQ Deca™, Thermo Fisher Scientific)
- Customized nanospray source (Digital PicoView® 150, New Objective, Inc.)
- NanoLC Pump (Eksigent™)
- Six-port automatic nano-valve (Scivex) with 0.5µL sample loop
- PicoFrit® columns (360 µm OD, 75 µm ID, 15 µm tip ID, New Objective), each containing ProteoPep™ II (New Objective) 5.0 µm-diameter particles packed to 10 cm-, 20-, and 30 cm-bed lengths
- IntegraFrit™ Columns (360 µm OD, 75 µm ID, New Objective), containing ProteoPep™ II (New Objective) 5.0 µm-diameter particles packed to 10 cm-, 15-, and 20 cm-bed lengths

Sample Preparation

- A commercially available bovine serum albumin (BSA) standard was diluted to 200, 100, and 20 fmol/µL in an aqueous solvent of 2% ACN, 0.1% formic acid
- A commercially available mixture of 5 angiotensins was diluted to 0.1 ng/peptide concentration with 2% ACN, 0.1% formic acid aqueous solvent
- Samples were analyzed via online nanobore ESI-MS in positive-ion-mode
Results

All column combinations were employed in analyzing the angiotensin standard. Data collected using the 30 cm ProteoPep™ II (PP2)-packed PicoFrit® Column resulted in FWHMs between 8.4 – 10.2 seconds. The 20 cm IntegraFrit™ column + 10 cm PicoFrit column combination displayed FWHMs between 13.2 – 14.4 seconds. The two 10 cm IntegraFrit column + 10 cm PicoFrit displayed FWHMs between 12.6 – 14.4 seconds. Figure 4 illustrates three chromatograms from each column combination for analyzing the angiotensin standard; 0.25 ng total peptide were subjected to a 300 nL/min. flow rate over a 70 min. gradient from 2% - 50% organic modifier concentration.

Figure 5 illustrates the three chromatograms produced in the BSA digest analysis through each column combination; 100 fmol BSA was subjected to a gradient identical to that used for angiotensin. Data collected using the 30 cm ProteoPep II (PP2)-packed PicoFrit column allowed 71.8% sequence coverage. The 20 cm IntegraFrit column + 10 cm PicoFrit column supported 58.6% sequence coverage. The two 10 cm IntegraFrit column + 10 cm PicoFrit column yielded 65.1% sequence coverage. Figure 6 shows the rich data possible when combining a long column (35 cm) with a long gradient (90 min.) Narrow peak widths (FWHM 10-14 sec.) yield an average peak capacity of 239 (Figure 10).

![Figure 4](image1.png)

**Figure 4** Angiotensin chromatographic data collected with each column-union configuration A) Chromatogram collected with a 30 cm PicoFrit® Column, B) chromatogram collected with 20 cm IntegraFrit™ Column + 10 cm PicoFrit Column, and C) chromatogram collected using two 10 cm IntegraFrit Columns + 10 cm PicoFrit column

Injection: 0.25 ng total peptide, Flow rate: 300 nL/min., Gradient: 2% - 50% B over 70 min.

![Figure 5](image2.png)

**Figure 5** Expanded regions of BSA tryptic digest chromatographic peaks. A) Chromatographic region, as collected with 30 cm PicoFrit® Column, B) Chromatographic region, as collected with 20 cm IntegraFrit™ + 10 cm PicoFrit, and C) Chromatographic region, as collected using two 10 cm IntegraFrit Columns coupled to a 10 cm PicoFrit column.

Injection: 100 fmol BSA, Flow rate: 300 nL/min., Gradient: 2% - 50% B over 70 min.
Figure 6 Long gradient (90 min to 50% B) base-peak chromatogram (A) obtained using a 35 cm x 75 µm ID column fabricated by coupling a 20 cm long PicoFrit® column to a 15 cm long IntegraFrit™ Column through a PicoClear™ connector. Packing material was 5 µm ProteoFep II C18 (300 Å). Mobile phase flow rate was 400 nL/min. The sample was a four protein tryptic digest (50 fmol/peptide on-column). Chromatograms (B-E) are base-peak reconstructions for the mass ranges indicated in the right hand column.

Figure 7 Conditions as in Figure 6. Here the gradient is fast (5.4 min to 50% B). Base peak (A) and selected ion current of three marker analyte ions are shown in (B). Full width at half height is labeled in (B).

Figure 8 Same column as in Figure 6 running a BSA digest (100 fmol on-column) with a 25 min gradient to 50% B. The selected ion current of 8 marker ions, along with respective peak widths, is shown in (B).

Figure 9 Same run as in Figure 6 showing the selected ion current of the marker ions used in Figure 7.
Efficient Multi-Column Coupling for Enhanced nLC-ESI-MS Separation

Table 2 5-Angiotensin composition

<table>
<thead>
<tr>
<th>Angiotensin</th>
<th>MW</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ile7]-Angiotensin III</td>
<td>897.1</td>
<td>RYVHPF</td>
</tr>
<tr>
<td>[Val9]-Angiotensin III</td>
<td>917.1</td>
<td>RYVHPF</td>
</tr>
<tr>
<td>[Asn1,Val5]-Angiotensin II</td>
<td>1,031.0</td>
<td>NRVYHPF</td>
</tr>
<tr>
<td>[Val5]-Angiotensin I</td>
<td>1,282.5</td>
<td>DRYVHPFLA</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>1,296.0</td>
<td>DRYVHPFL</td>
</tr>
</tbody>
</table>

Figure 10  Summary of peak width (baseline) and peak-capacity measurements from the data obtained in Figures 7, 8, 9. (B) Plot of peak capacity vs. gradient elution time (to 50% B) for the 35 cm long column.

Conclusions

- Minimal resolution loss and post-column loss were observed for columns combined using transparent, true ZDV unions.
- Long 75 µm ID nanobore columns (30+ cm) are easily fabricated.
- Negligible sequence coverage differences were recorded between each column, although the integral 30 cm column provided the best overall score.
- Coupling columns enables peak capacities >200 when running longer gradients.
- Transparent, true zero-dead-volume (ZDV) unions ensure clean connections between columns without dead volume.
- Connecting columns containing different resins will be explored in future work.
- Nanobore columns having “semi-disposable” integral guard columns are a viable next step.

Observations & Conclusions

- Switching the high voltage simultaneously with emitter position is a viable method for switched-column nanobore LC-MS.
- Carryover is reduced to that inherent to the memory effect of the heated-capillary interface as spray is only possible from a single channel at a time.
- Nearly identical analyte-ion current (within 10%) was obtained by fine-tuning each channel’s emitter position using continuous infusion.
- Channel-switching yielded a dead time of approximately 6 seconds for the stage to locate and the emitter to re-establish spray.
- Column-switching can greatly reduce the time lost to column-rinsing and conditioning.
- Method development requires careful optimization of conditioning to ensure high reproducibility between column(s) and injections.
- The two channels in gradient-LC mode yielded MS signal to better than 50% of each other (as determined by analyte SIC’s). Given that each channel possessed different vendor LCs, each with different delay volumes, gradient-mixing schemes, and injection valves, the results are promising.