

Introduction

A widely used method for protein identification incorporates two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by nanoelectrospray ionization mass spectrometry (nESI-MS). Nanospray, in combination with nanobore chromatography (< 100 μm i.d. columns), provides typical limits of detection in the subfemtomole to attomole range when combined with ion trap mass spectrometry.

Typically, an in-gel digested protein sample is injected onto a nanobore column by one of two methods: direct on-column injection using a pressure bomb, or on-line sample pre-concentration in a sample trap cartridge. The time intensive bomb injection method is highly sensitive, minimizing sample handling and maximizing sample utilization. Low back pressure trapping cartridges permit higher loading rates and compatibility with autosampler methods. Trap cartridges can also yield guard-column functionality, as in-line enrichment and desalting of samples increases nanobore column lifetime. Incorporating a trap cartridge with an alternative packing material may also be used modify the selectivity of a method.

Methods

Analysis were performed on an LC/MS system composed of an 1100 Cap LC (Agilent, Palo Alto, CA) interfaced with a LCQTM Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The LCQ Deca was outfitted with a PicoViewTM nanospray ion source (New Objective, Woburn, MA). (A in Figure 2) Chromatography was performed with a PicoFritTM column (New Objective) packed with ProteoPepTM C18 (5 μm particle with 300 \AA pore). (A in Figure 1) A PicoFrit column combines a nanobore LC column with a fritted electrospray emitter to eliminate post column analyte loss and band broadening. Two columns were analyzed each with different dimensions: a 75 μm i.d. column with a tip size of 15 μm packed with a 10 cm bed and a 20 μm i.d. column with a tip size of 10 μm packed with a 5 cm bed.

Experimental parameters:

Nanospray voltage: 1.7kV (75 i.d. μm column) and 1.0kV (20 μm i.d. column)

Tip Position: 2mm away from inlet

Heated capillary: 140^oC

Microscans: 3

HPLC Solvents: (A) Water, 0.1% Formic acid (B) Acetonitrile, 0.1% Formic acid

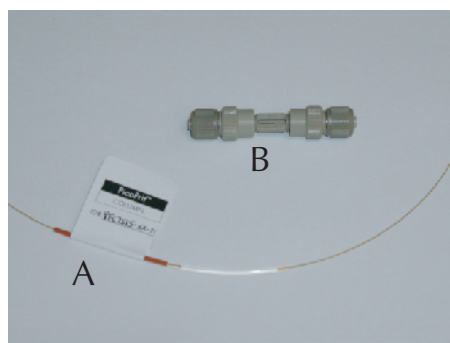


Figure 1 - PicoFritTM column (A) and capillary sample trap (B).

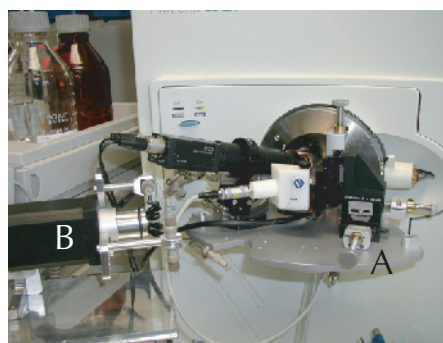


Figure 2 - The PicoViewTM nanospray source (A) with a 10 port Valco valve (B).

Injection With a Sample Trap

The capillary sample trap used in this study has a 25 mm bed length, compared to 1 mm of a traditional trap, and may be used on its own for fast separations. The capillary was packed with ProteoPep™ C18 (5 μm). Additional packing materials were also tested, including BioBasic™ C18 (5 μm) (Thermo Hypersil-Keystone). The sample trap was integrated into the HPLC system with a 10 port Valco micro-injection valve (Houston, TX).

Samples were injected into a 5 μl sample loop (position A in Figure 3) and subsequently flushed onto the trap column with 10 μl of solvent at a flow rate of 3 $\mu\text{L}/\text{min}$ (position B in Figure 3). This process takes up to **5 mins**; the flow rate can be increased to a maximum of 5 $\mu\text{L}/\text{min}$ with no loss in sensitivity.

After sample loading, a PEEK™ tee is incorporated in line during sample elution to supply a split flow LC gradient (40:1) resulting in a 200 nl/min flow through the 75 μm i.d. analytical column. The split flow was later modified to provide the necessary flow rate (<100 nl/min) for a 20 μm i.d. column. A second 6 port valve, supplied with the LCQ™, was used to control the flow splitter (Figure 3).

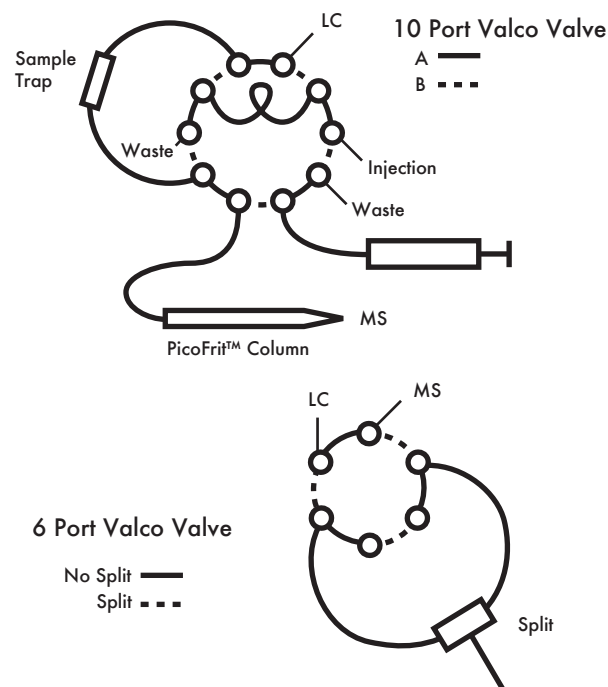


Figure 3 - Valve configuration for sample loading (upper left). A second valve was used to split the solvent flow from the LC (lower right).

Injection With a Pressure Bomb

For on-column injection, the entrance end of the column is removed from the high-voltage tee, taking care to leave the emitter end of the column undisturbed in the nanospray source. A sample, typically 2-5 μl , is placed in a glass vial and sealed in a pressure vessel (A in Figure 4). The column is inserted into the injector and 400 psi of helium is applied to the headspace above the vial, forcing the sample on to the column. The amount of sample injected, in this case 1 μl , is monitored by measuring mobile phase displacement from the column at the tip. After injection, the column is reattached and flushed with 2% acetonitrile for 5 mins to eliminate any possible air bubbles. The entire bomb injection process for a 75 μm column can take up to **20 mins**. The 20 μm column, which has a higher back pressure, may require more time for injection.

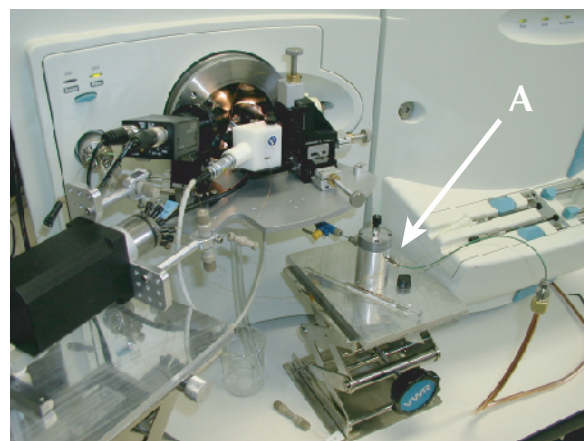


Figure 4 - Pressure Bomb (A)

Results

The performance characteristics of the capillary LC/MS system were assessed by the analysis of two standard samples; a test mix of five angiotensin variants and a tryptic digest of bovine serum albumin (BSA). Standards were purchased from Michrom BioResources (Auburn, CA) and were diluted using 2% acetonitrile and 0.1% formic acid. Figures of merit included limit of detection and sequence coverage.

Angiotensin Standard

Angiotensin was used to establish peak sensitivity of both injection methods. The acquisition method for the LCQ™ Deca involved one full scan followed by MS/MS scan for the precursor ion of m/z 459.5.

The limit of detection for an injection with a in-line sample trap was 25 fmol for one on the angiotensin variants with a S/N of 3 for a full scan MS scan (upper chromatogram in Figure 5a). For an on-column injection, all of the five angiotensin variants are easily detected at a limit of detection of 10 fmol (Figure 5b) with a S/N of 28. No consistent signal was detected with full scan MS at 1 fmol.

To establish the sensitivity limit for product ion monitoring the LCQ was set to acquire MS/MS spectra of the $[M+2H]^{2+}$ ion for angiotensin (m/z 459.5). (Lower chromatogram in Figure 5) The signal-to-noise ratio greatly improves with a MS/MS scan and the detection limits reached 100 amol for the in-line trap method and 30 amol for the on column injection. The extra plumbing of the in-line trapping system creates a small amount of peak tailing observable near the limit of detection (Figure 5a).

The limit of detection can be further improved to 1 fmol in full scan (Figure 6) with a column i.d. of 20 μm (S/N of 22). Lowering the column volume increases the relative concentration of the analyte within the column (Table 1). In this case the improvement is over an order of magnitude.

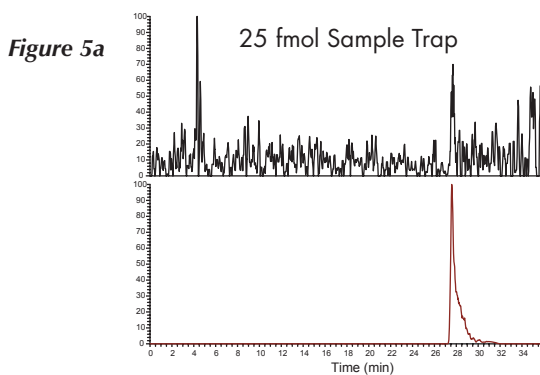


Figure 5a

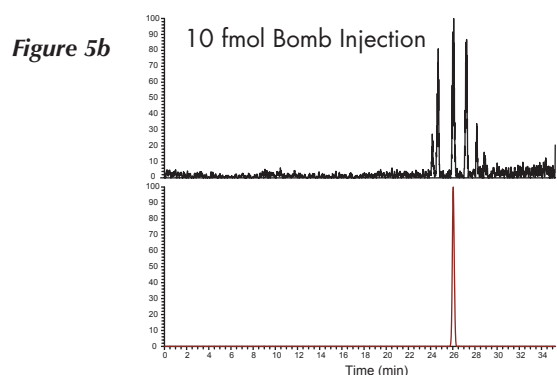


Figure 5b

Figure 5 - (a) Injection of 25 fmol of angiotensin with the sample trap and (b) 10 fmol with an on-column injection Top: Base peak reconstructed ion chromatogram for Full scan MS data

Figure 6 (top) - Injection of 1 fmol of angiotensin with an on-column injection. The standard only included 4 angiotensin variants. (bottom) Single ion chromatogram for the MS/MS of m/z 459.5

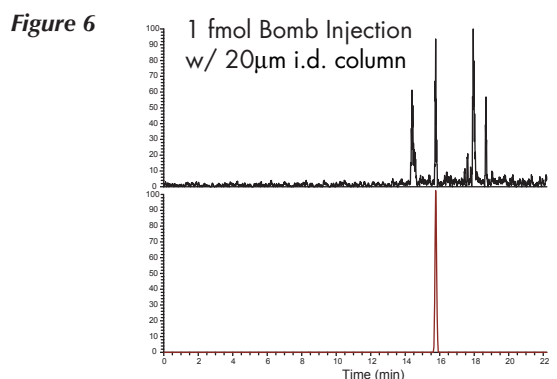


Figure 6

Column	ID	Flow Rate	Relative [C]
Standard	4.6 mm	1 ml/min	1
Microbore	1 mm	50 $\mu\text{L}/\text{min}$	21
Capillary	320 μm	5 $\mu\text{L}/\text{min}$	206
Nanobore	75 μm	250 nl/min	3760
Nanobore	20 μm	100 nl/min	52910

Table 1 - Relative concentration of analyte using different chromatographic column formats

Adapted from Tomer & Moseley, *Mass. Spec. Rev.*, 1994, 13, 431

Bovine Serum Albumin Tryptic Digest

BSA was used to test differences in sequence coverage for the two injection methods. The acquisition method for the LCQ™ Deca involved one MS precursor scan from 350 to 1500 amu followed by three data-dependent MS/MS scans (isolation width 3 amu, 30% collision energy) on the three most abundant ions in the MS scan. The resultant reconstructed base peak ion chromatograms for a trap injection and an on-column injection are shown in Figure 7(a) and (b) respectively. More early elution, hydrophilic peptides were observed for the on-column injection.

The tandem MS data were used to search sequence databases using the Sequest™ algorithm. The database search resulted in an amino acid coverage of 64.20% and 74.35% for a trap injection and an on-column injection respectively, a difference of 10.15%.

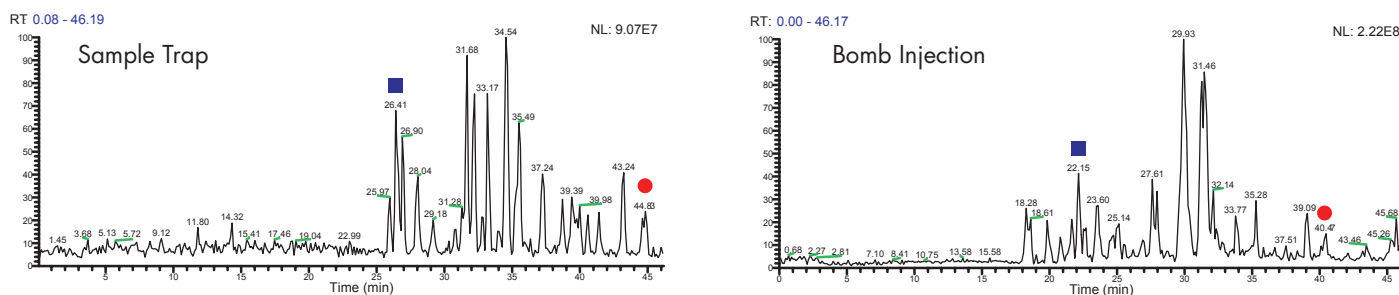


Figure 7 - Base peak reconstructed ion chromatogram of 100 fmol BSA tryptic digest for (a) an injection with a sample trap and (b) an on column injection. The two colored shapes are associated with identical peptides on the two different chromatograms.

DTHKSEIAHR	FKDLGEEHFK	GLVLIAFSQY	LQQCPFDEHV	KLVNELTEFA
KTCVADESHA	GCEKSLHTLF	GDELCKVASL	RETYGDMADC	CEKEQPERNE
CFLSHKDDSP	DLPKLPDPN	TLCDEFKADE	KKFWGKLYE	IARRHPYFYA
PELLYANKYN	GVFQECQAA	DKGACLLPKI	ETMREKVLTS	SARQLRCAS
IQKFGERALK	AWSVARLSQK	FPKAEFVEVT	KLVTDLTKVH	KECCHGDLE
CADDRADLAK	YICBBZBTIS	SKLKECKDPC	LLEKSHCIAE	VEKDAIPEDL
PPLTADFAED	KDVCKNYQEA	KDAFLGSFLY	EYSRRHPEYA	VSVLLRLAKE
YEATLEECCA	KDDPHACYTS	VFDKLGHLVD	EPQNLIKZBC	BZFEKLGEXX
XXALIVRYTR	KVPQVSTPTL	VEVSRSLGKV	GTRCCTKPES	ERMPCTEDYL
SLILNRLCVL	HEKTPVESKV	TKCCTESLVN	RRPCFSALTP	DETYVPKAFD
EKLFTFHADI	CTLPDTEKQI	KKQTALVELL	KHKPKATEEQ	LKTMENFVA
FVDKCCAADD	KEACFAVEGP	KLVVSTQTAL	A	

Figure 8a - Sequence coverage for the trap injection of the BSA tryptic digest.

DTHKSEIAHR	FKDLGEEHFK	GLVLIAFSQY	LQQCPFDEHV	KLVNELTEFA
KTCVADESHA	GCEKSLHTLF	GDELCKVASL	RETYGDMADC	CEKEQPERNE
CFLSHKDDSP	DLPKLPDPN	TLCDEFKADE	KKFWGKLYE	IARRHPYFYA
PELLYANKYN	GVFQECQAA	DKGACLLPKI	ETMREKVLTS	SARQLRCAS
IQKFGERALK	AWSVARLSQK	FPKAEFVEVT	KLVTDLTKVH	KECCHGDLE
CADDRADLAK	YICBBZBTIS	SKLKECKDPC	LLEKSHCIAE	VEKDAIPEDL
PPLTADFAED	KDVCKNYQEA	KDAFLGSFLY	EYSRRHPEYA	VSVLLRLAKE
YEATLEECCA	KDDPHACYTS	VFDKLGHLVD	EPQNLIKZBC	BZFEKLGEXX
XXALIVRYTR	KVPQVSTPTL	VEVSRSLGKV	GTRCCTKPES	ERMPCTEDYL
SLILNRLCVL	HEKTPVESKV	TKCCTESLVN	RRPCFSALTP	DETYVPKAFD
EKLFTFHADI	CTLPDTEKQI	KKQTALVELL	KHKPKATEEQ	LKTMENFVA
FVDKCCAADD	KEACFAVEGP	KLVVSTQTAL	A	

Figure 8b - Sequence coverage for the on-column injection of the BSA tryptic digest.

Trap/Analytical Column Combinations

Sample trapping permits the use different chemistries within an analytical method. To examine the change in retention characteristics a sample trap filled with BioBasic™ C18 was used in conjunction with a ProtepPep™ analytical column with 5 cm of packing and compared with a ProtepPep sample trap. The combination of the BioBasic/ProtepPep resulted in wider peak widths and lower resolution in the analysis of the five angiotensin variants (Figure 9) and a mix of different retention characteristics in the analysis of the BSA digest (figure 10)

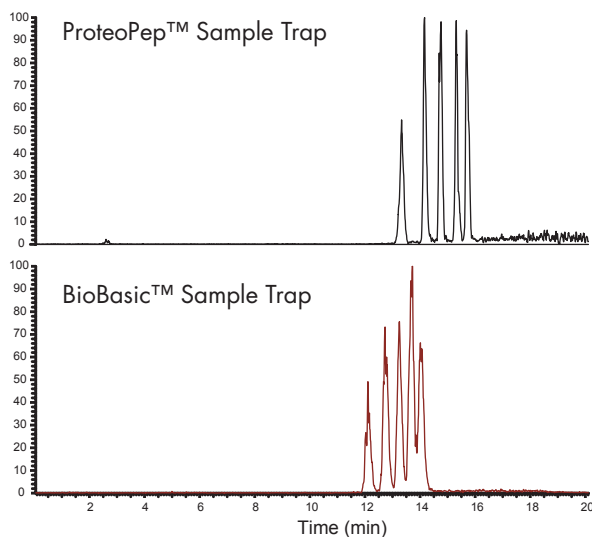


Figure 9 - Base peak reconstructed ion chromatogram for full scan MS data of 5 angiotensin variants on a ProtepPep™ PicoFrit™ column with a ProtepPep sample trap (Top) and a BioBasic™ sample trap (Bottom)

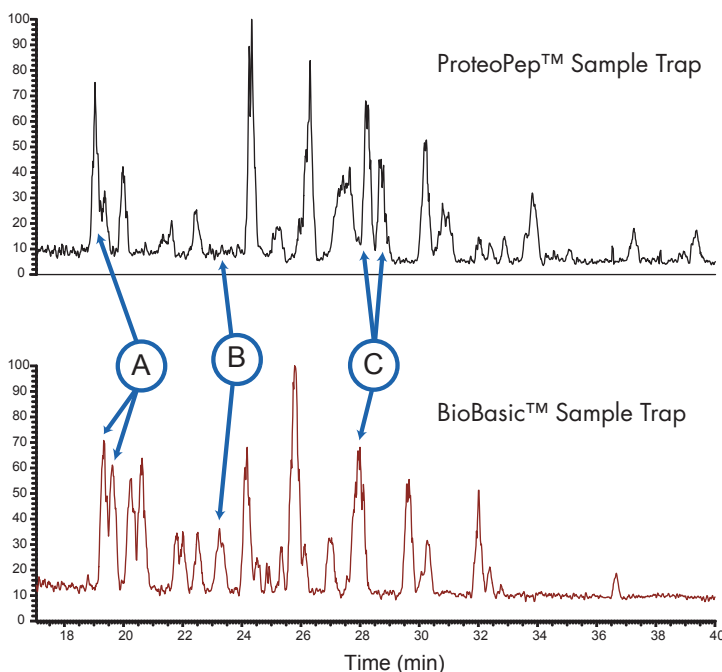


Figure 10 - Base peak reconstructed ion chromatogram for full scan MS data of 100 fmol of BSA tryptic digest on a ProtepPep™ PicoFrit™ column with a ProtepPep sample trap (Top) and a BioBasic™ sample trap (Bottom).

(A) Shows two co-eluting peptides (top) are separated with the BioBasic/ProtepPep combination (bottom)

(B) Shows a peptide not identified with the ProtepPep/ProtepPep combination (top) was identified using a BioBasic sample trap (bottom)

(C) Shows two peptides which are separated (top) co-elute with a BioBasic sample trap (bottom)

Conclusions

On-column injection yields optimal sensitivity, providing a lower detection limit with greater sequence coverage for tryptic peptides. Sample loading, however is fully manual proves to be very time consuming. Detection limits can be further improved with the use of smaller i.d. columns, a 20 μm i.d. column improved over an order of magnitude compared to a 75 μm i.d. column.

Sample trap injection provides reasonably high sensitivity, with the added advantage of autosampler compatibility. Even though these initial results suggest a limit of detection that is 2-3 fold compromised, at the 100 fmol level sequence coverage is nearly comparable.

Trap chemistries with different retention characteristics from the analytical column can be used to provide alternate selectivity for a method.