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Automation of Nanoscale Microcapillary Liquid Chromatography−Tandem Mass Spectrometry with a Vented Column

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To fully automate the sample introduction step for nanoscale microcapillary liquid chromatography−tandem mass spectrometry (LC−MS/MS) analyses, 75 μm i.d. × 14 cm capillary columns were interfaced with a commercial autosampler instrument using a novel procedure which allowed dilute peptide samples to be transferred from the AS loop injector to the nanoscale column at flow rates up to 5 μL min^{-1}. On-column enrichment and desalting was demonstrated for large sample volumes (>40 μL) by constructing a vent 2 cm after the entrance to the packed bed of 5-μm ODS-AQ modified silica. Salts and nonretained solutes were removed via the vent, which allowed for column washing independent of the continuation of the bed into the electrospray source. Separations of test peptide mixtures demonstrated 50-nL elution peak volumes with low- to subfemtomole detection levels. In addition, a highly complex peptide mixture (outer membrane preparation from *Psuedemonas aeruginosa*) was efficiently separated with more than 100 proteins identified from a single reversed-phase LC−MS/MS analysis. Finally, the vented column (V-column) was utilized for online separations in a multidimensional chromatography/tandem MS experiment where large numbers of strong cation exchange chromatography fractions from a trypsinized yeast lysate were desalted, concentrated, and analyzed in a completely automated fashion. The procedures for constructing and using a V-column require minimal changes in current methods and equipment for nano-LC−MS analyses using columns of 100-μm diameter and smaller.

Tandem mass spectrometry (MS) has provided proteomics initiatives with highly selective methods for characterizing the dynamic state of protein mixtures responsible for biological function and specificity.[1−8] The throughput and sensitivity limits of MS-based methods for peptide sequence analyses are tested by comprehensively identifying and characterizing relevant proteins from crude cellular mixtures, thereby setting demanding requirements upon the initial stages of purification and separation. Progress toward meeting these requirements has been maintained by combining relatively large-volume methods developed to selectively isolate or resolve protein and peptide mixtures with low-volume capillary liquid chromatography methods performed on-line with automated MS/MS analyses for maximum sensitivity.[2,5,9] Often, excised gel bands from SDS−PAGE are converted to proteolytic mixtures prior to sequence analysis by LC−MS/MS techniques. Peptide sequence information (MS/MS spectra) is acquired with a high degree of automation via data-dependent software, which provides the ability to sequence five or more peptides independently following a single MS precursor scan to identify peptide parent ions.[7,10] This highly sophisticated data-dependent analysis can provide as many as 60 MS/MS acquisitions per minute for high throughput in mixture analyses where thousands of MS/MS identifications are needed.[3] Matching uninterrupted "raw" MS/MS spectra against protein and EST databases using Sequest or other database search algorithms is a rapid means to unambiguously identify peptide and protein sequences as well as many structural modifications to proteins.[11−13] However, achieving fully automated analyses is a challenging endeavor for nanoscale LC−MS/MS, which uses capillary columns of <100-μm i.d. that permit flow rates of ∼150 nL min^{-1}.

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Column miniaturization for on-line LC−MS/MS methods has proven essential for enhanced sensitivity and efficient separation of complex mixtures.[14−17] This is because the combined decrease in column cross section and lower volume flow rate increases the column efficiency proportionately.[18] In the low-nanoliter per minute range, electrospray (ES) ionization efficiency increases, providing additional enhancement to sensitivity.[14,19] While 75-μm-i.d. columns are preferred for these reasons, there is a need for automated procedures for on-column enrichment and desalting, to efficiently analyze dilute or large-volume samples. Automated methods for 75-μm columns have not been fully attainable because an unrealistically long duration is needed to load more than several microliters from an autosampler (AS). Typically, limited amounts of sample must be taken to dryness and redissolved in several microliters before loading the entire sample directly onto the nanocolumn via a pressure bomb. This minimizes sample losses that may result from dilution or from adsorption to surfaces by manually inserting the capillary column into the sample vial in order to pressure-inject the sample. The flow rate constraints for columns of <100-μm i.d. have led some research groups to resort to short precolumns (traps) which can be loaded at higher flow rates.[20,21] After the injection, a two-position valve switches the trap in-line with the resolving column, and the elution proceeds at a lower flow rate. While the use of a trap permits automated analyses, it represents a substantial compromise compared to manual loading via a pressure cell because (i) the elution flow rate (~150 nL min^{-1}) is nonoptimal for peptide elution from a wide cross-section trap, (ii) peptide peaks are diluted within the trap and in the ancillary tubing connections, and (iii) analysis times are significantly increased because of the extracolumn volume. We present here a simple method to perform rapid, automated sample introduction to 75-μm-i.d. capillary columns. A vent was constructed 2 cm after the head of the column in order to load samples at flow rates up to 5 μL min^{-1}.

EXPERIMENTAL SECTION

Materials. High-purity acetonitrile (ACN), methanol, and acetic acid (HOAc) were purchased from VWR (Bridgeport, NJ). Heptafluorobutyric acid (HFBA) was from Pierce (Rockford, IL). Trypsin was from Promega (Madison, WI). MicroCrosses, FS unions, and a stainless steel screen microassembly, all of PEEK materials, were from Upchurch Scientific Inc. (Oak Harbor, WA). C18-bonded silica, 5 μm, 200 Å (Magic C18AQ), was from Michrom BioResources (Auburn, CA). A 100 μm i.d. × 365 μm o.d. FS containing a borosilicate frit (Integrafrit) was from New Objective Inc. (Cambridge, MA). Gold wire, 0.020-in. diameter, was from Scientific Instrument Services, Inc. (Rangoes, NJ). A gas torch kit (No. 14600) was from Alltech Associates (Deerfield, IL). A 10-port valve (C2-000EP) was from VICI (Houston, TX). A brass pressure cell (bomb) was fabricated in-house.

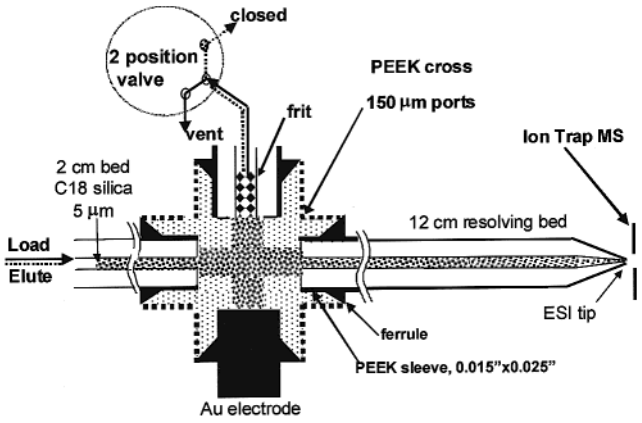

Figure 1. Schematic showing a cross-sectional view of a V-column. The vent is constructed within a commercial microcross union having 150-μm through-holes in PEEK. The fritted capillary on one arm of the cross union is connected on the other end to an open or closed port on a two-position valve. The microcross body is mounted on a three-axis positioning stage in front of the MS inlet. All procedures to manufacture and use the vented bed are described in the text.

Vented (V)-Column Assembly. FS tubing, 75 μm i.d. × 365 μm o.d. × 20 cm, was hung weighted with a small binder clamp. A needle tip was drawn above the weight by applying a torch flame to the FS until it separated. The needle tip was cut to a width of ~25 μm for later use as the ES emitter. The needle was packed with a methanolic slurry of C18-modified silica (5-μm, 200-Å pore size) to a length of 14.5 cm via a high-pressure cell (bomb).[15] Afterward, the column was flushed and stored in solvent A (5% ACN, 0.4% HOAc, 0.005% HFBA). To construct the vent, the column was carefully cut by by lightly scoring the FS with a carbide steel edge, holding it against a flat surface at a 90° edge, and gently bending to produce two packed segments of 2.5 and 12 cm, respectively. Each segment was inspected under a stereomicroscope to ensure of flat, 90° ends and then was placed into opposing arms of a PEEK cross (Figure 1). The fritted capillary was fitted into the cross with care taken to firmly tighten the fitting. Au wire was placed in the remaining arm. The fritted FS and the column were then connected to either of the flow path configurations as shown in Figure 2. An in-line stainless steel screen filter having 1-μm mesh (Upchurch Scientific) was inserted before the V-column. Except for the connections to the AS loop injector valve, FS tubing of 365 μm × 50 μm was used in place of PEEK tubing of 1/16 in. × 0.003 in. The stator on the inject/divert valve on the Deca LCQ was replaced with one (VICI Part No. C2-006) having smaller ports to prevent the FS entering the ports and damaging the valve rotor. After assembling all tubing connections, the valve position was set to vent, and solvent B (95% acetonitrile) was introduced at a pressure of 1000 psi (~8 μL min^{-1} solvent B) for 20 min. Afterward, the flow was halted by slowly bleeding out the pressure via the vent. The valve position was then switched to the "closed" position to allow the fluid pressure to be determined by flow rate through the 50-μm-i.d. FS on the waste arm of the gradient split tee. Solvent A was introduced at 1000 psi (~80 μL min^{-1} solvent A) for 20 min. This was followed

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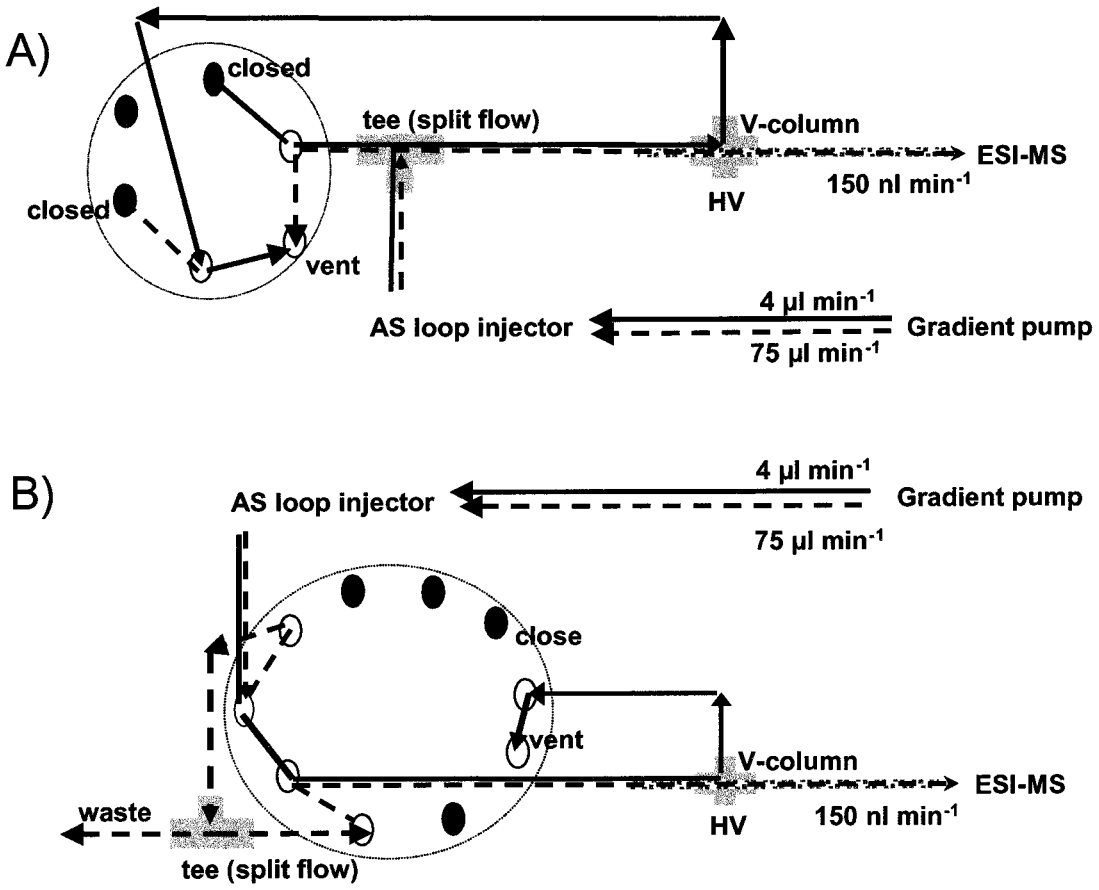

Figure 2. Schematic showing valve configuration and flow path for a V-column using either a (A) 6-port or (B) 10-port two-position valve. Shown are the tubing connections between the gradient pumps, autosampler (AS) loop injector valve, vented column, and the valve. Not shown are the AS syringe and AS tubing connections used to fill the loop from a sample vial. The sample is loaded and washed at a relatively high flow rate (~5 μL min^{-1}) because the vent is open. Following sample loading and washing, the valve position is switched and the vent is closed. Pump flow is then split down to ~150 nL min^{-1} for peptide gradient elution and analysis.

with solvent B again at 1000 psi for 20 min and by solvent A for another 10 min before stepping down pump flow over 1 min to bleed the pressure from the column. Alternating the solvents this way resulted in ~5-mm displacement of the vented bed into the union.

Sample Preparation. In-solution digestion of an outer membrane fraction (*Pseudomonas aeruginosa*) was accomplished by denaturing and reducing 1 mg of protein in 8 M urea, 10 mM DTT, 50 mM Tris-HCl, pH 8.5. Iodoacetamine (55 mM) was added to alkylate cysteine residues in the dark for 30 min. The volume was increased 4-fold, and trypsin was added. After overnight digestion, the digest was acidified with TFA. Salts and urea were removed using a C18 cartridge (Vydac). A fraction (20 μg) of the total digest was placed in the AS vial. A similar procedure reported elsewhere[22] was used to prepare trypsin digests of 1 mg of soluble yeast protein.

Analyses of Narrow-Bore SCX Fractions. Strong cation exchange chromatography was performed for 1 mg of yeast protein digest, and 200-μL fractions were collected each minute as described.[22] Eighty fractions were reduced in volume to 100 μL and placed in an autosampler. Injection volumes were between 10 and 100 μL, depending on the UV absorption obtained for each fraction, to maximize peptide loading.

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Nanoscale Microcapillary LC−MS/MS Analyses. A Surveyor MS gradient LC pumps, a Surveyor autosampler, and a LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) were used with the manufacturer's software (Xcalibur). Control over the AS, HPLC pumps, and V-column valve positions was written in the instrument setup method of Xcalibur. The V-column cross was placed on a three-axis micropositioning stage assembled in-house, and the emitter was placed within 2 mm of the heated metal capillary inlet of the LCQ. Sample injections began by transferring the entire contents of a conical sample vial into the sample loop. The loop injector valve put the loop in line with the V-column while pump flow entered the gradient split tee and the column valve position, Figure 2, was set to "closed". Immediately afterward, the valve position was switched to "vent". Flow rate across the 2-cm bed reached 4 μL min^{-1} over ~1 min. In the 10-port valve configuration (Figure 2B), contact closures to the valve actuator were delivered from the peripherals controls panel on the LCQ. To analyze strong cation exchange (SCX) fractions, the six-port configuration in Figure 2A was used. Salts were removed after loading SCX fractions by adding 10 min to wash the bed. No voltage was applied during the load method. Flow rate was set to zero to bleed pressure after each injection. Gradient delivery as shown in Figure 2 was done by switching the valve to close the vent and open the waste arm of the flow split tee. The waste arm held 50 μm i.d. × 80 cm FS, which produced ~1000 psi of

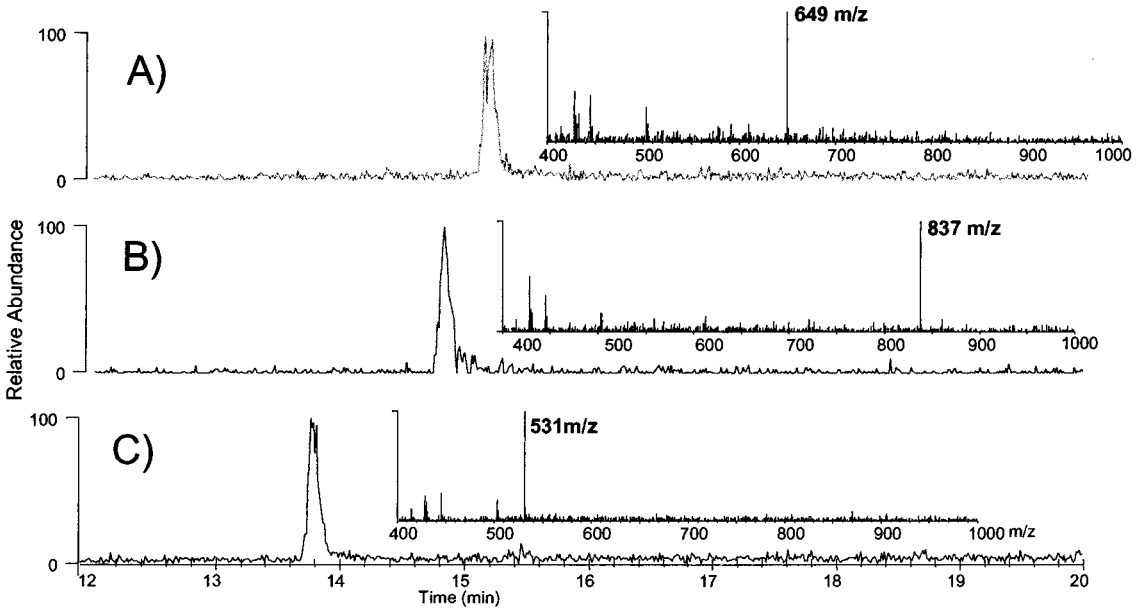

Figure 3. Nanoscale chromatography analysis of standard peptide mixture at the 5-fmol level. Shown are the selected ion chromatograms for (A) angiotensin $[M + 2H]^{2+}$, 649 *m*/*z*; (B) neurotensin $[M + 2H]^{2+}$, 837 *m*/*z*; and (C) bradykinin $[M + 2H]^{2+}$, 531 *m*/*z*. The AS transferred 10 μL of the peptide standard (500 amol μL^{-1}) to the sample loop injector. The flow path used was that from Figure 2B with a 75-μm V-column. One full MS scan (inset) at the peak maximum is shown for each peptide to demonstrate the signal-to-noise ratio.

pressure for a pump setting of 80 μL min^{-1} in solvent A. During the gradient to 35% solvent B, the pumps were set to deliver 50 μL min^{-1} to the split tee and ~150 nL min^{-1} to the V-column. Flow rates were measured using calibrated 5-μL micropipets (VWR). The potential applied to the Au electrode was 1.8 kV. The acquisition method for the LCQ Deca involved one MS precursor scan from 450 to 1700 amu (3 microscans) followed by four data-dependent MS/MS scans (5 microscans each, isolation width 3 amu, 35% normalized collision energy) on the top four most abundant ions in the MS survey scan. The analysis of 100 amol of angiotensin was done with 3 microscans and then repeated with 1 microscan per MS/MS scan for a precursor ion of 649 *m*/*z*. Tandem MS data were used to search sequence databases using the Sequest algorithm.[11] Sequest search results were displayed, filtered, and manipulated using custom software written in-house in the Perl language. Filtering was accomplished by applying the following constraints:[23] (i) a minimum Xcorr score of 1.9, 2.2, and 3.75 for charge states of 1^+, 2^+, and 3^+, respectively; (ii) a minimum ΔCn score of 0.1; and (iii) matched sequences have fully tryptic ends.

RESULTS AND DISCUSSION

Vent Construction. The utility of the vented column to automate on-column enrichment and desalting of samples hinges upon the ability to perform the gradient elution step without sacrificing peak sensitivity. The column vent, as described schematically in Figure 1, was constructed 2 cm into the packed bed. A peptide-trapping column of 2-cm length was chosen for samples that will typically contain large amounts of salts and hydrophilic contaminants from PAGE. To ensure that hydrophilic peptides were trapped, the maximum loading flow rate was examined and found experimentally to be ~5 μL min^{-1}. Tests using a peptide standard mixture showed that the base peak ion intensity for bradykinin, a relatively hydrophilic peptide, began to decrease at flow rates of >5 μL min^{-1} (data not shown). Very hydrophilic trypsin-autolytic peptides have routinely been analyzed after loading the sample at 4 μL min^{-1}. The 2-cm bed before the vent resulted in pressures of ~1000 psi at 4 μL min^{-1} of solvent A. Nearly all of the flow-through from the trap bed was diverted to waste, rather than to the ESI source, as is feasible with more-sophisticated column-switching procedures.[20] The proportion of the volume transferred from the AS, which exits from the vent, can be calculated from the ratio (distance to the ES tip/distance to the frit), multiplied by the ratio (radius of bed in cross)^2/(radius of bed after cross)^2. This gave the vented volume as >99.9% of the transfer volume. Less than 1 nL of each microliter transferred to the V-column entered the 120-mm-bed segment which ended inside the ES tip. The length of this bed segment was also effective in resolving complex peptide mixtures. A single two-position valve (Figure 2) sufficed to vent the column and to integrate the V-column with the gradient flow splitter tee in order to perform the gradient elution step. Sample holdup, gradient delay time, and reequilibration time between analyses were minimal for each of the flow paths, as the swept volume after the loop injector was <1 μL, and column bed volume before the vent was <40 nL.

Constructing the vent by connecting a fritted FS capillary directly to the column bed was done to preserve the high chromatographic efficiency demonstrated with 75-μm columns. The diagram in Figure 1 shows a commercial cross union having 150-μm-diameter ports with ferruled sleeves that fasten the fritted FS, a Au electrode, and each segment of the 75-μm-i.d. column. Following the manufacturer's recommendations for attaching fittings in the union was essential in order to prevent void spaces around the ports. Commercial fritted FS (Integral Frit) were

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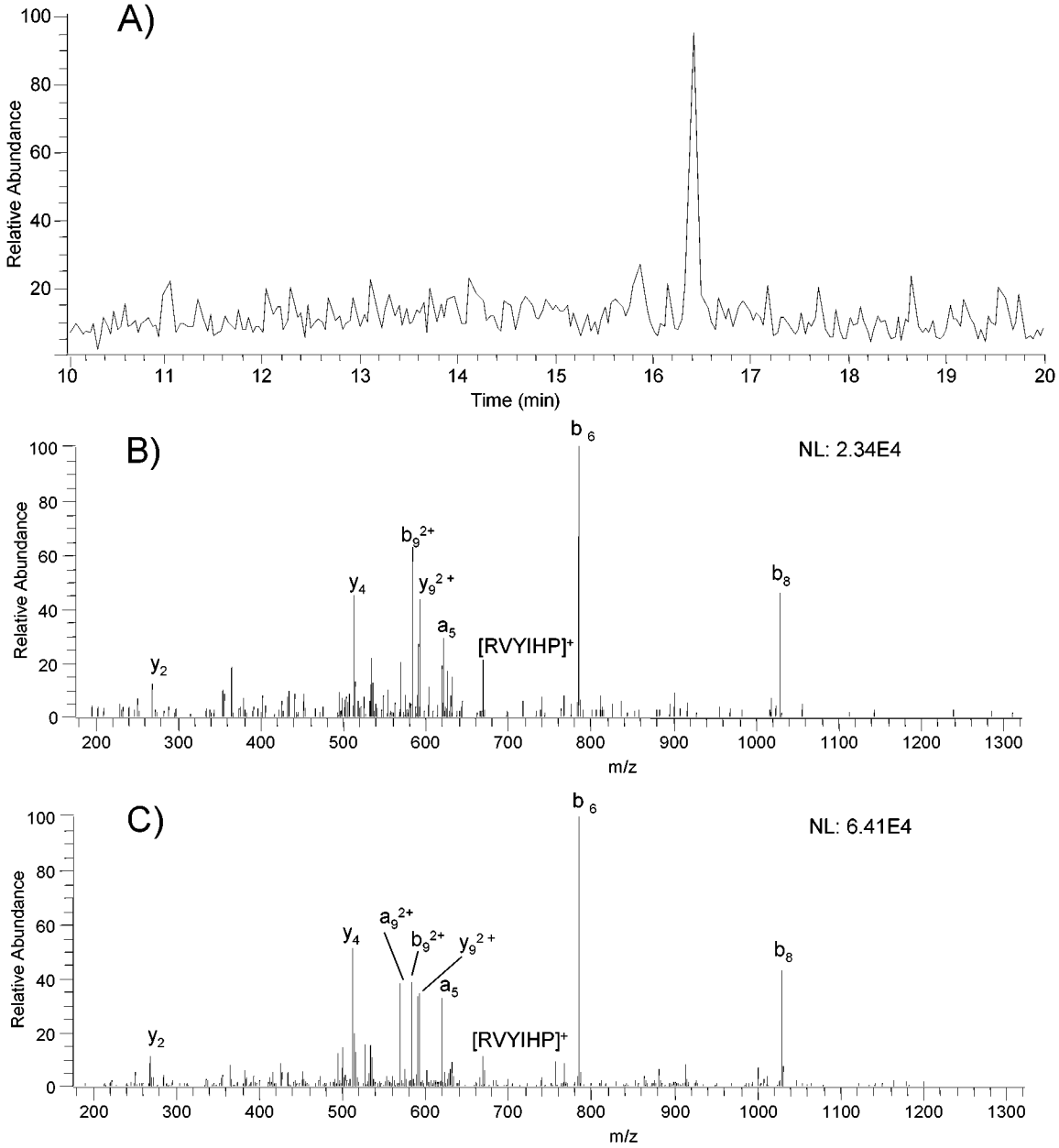

Figure 4. V-column sequence analysis of 100 amol of angiotensin. The mass spectrometer was set to continuously acquire MS/MS scans of the $[M + 2H]^{2+}$ ion for angiotensin, 649 *m*/*z*. (A) Total ion chromatogram showing the elution of the angiotensin peak. (B) Averaged MS/MS spectrum showing peptide sequence information acquired at three microscans per MS/MS scan during the peak elution. (C) Repeat analysis showing averaged MS/MS spectrum acquired at one microscan per MS/MS scan during the peak elution. The increased sampling across the peak at one microscan per MS scan provided superior signal-to-noise ratios. Detected peptide fragmentation ions (a-, b-, and y-type) are labeled.

visually inspected periodically over several thousand hours of use in such unions and were found to be free of deterioration. Stability of the short bed before the union was examined over repeated gradient analyses. Changes in bed length of several millimeters were observed over repeated gradients, although the changes had no substantial effect on peptide elution relative to that obtained for unvented columns. However, formation of a defined bed length was done by subsequent modification of the V-column construction. This involved connecting one segment of prepacked FS having the ESI tip to an empty cross and then connecting each of the other arms of the cross before injecting packing slurry to fill at the same time both the cross interior and the short bed before the cross.

As expected with a 75-μm cross section, insoluble material deposited at the front of the bed has led to variable pressure increases. An in-line stainless steel screen (1-μm mesh) installed immediately before the bed helped to secure the flow path and subsequently was found not to contribute to holdup of test peptides. However, the accumulation of stain residues from PAGE gel-derived samples resulted in flow rate changes and required replacement of the vented bed after more than 20 analyses (manuscript in preparation). To allow for easy replacement of the vented bed and for the reuse of the end containing the ES tip, an empty FS was connected to the cross in place of the used bed. The vial of slurry packing was connected in the pressure bomb to the empty FS, and the progress in filling the FS was monitored.

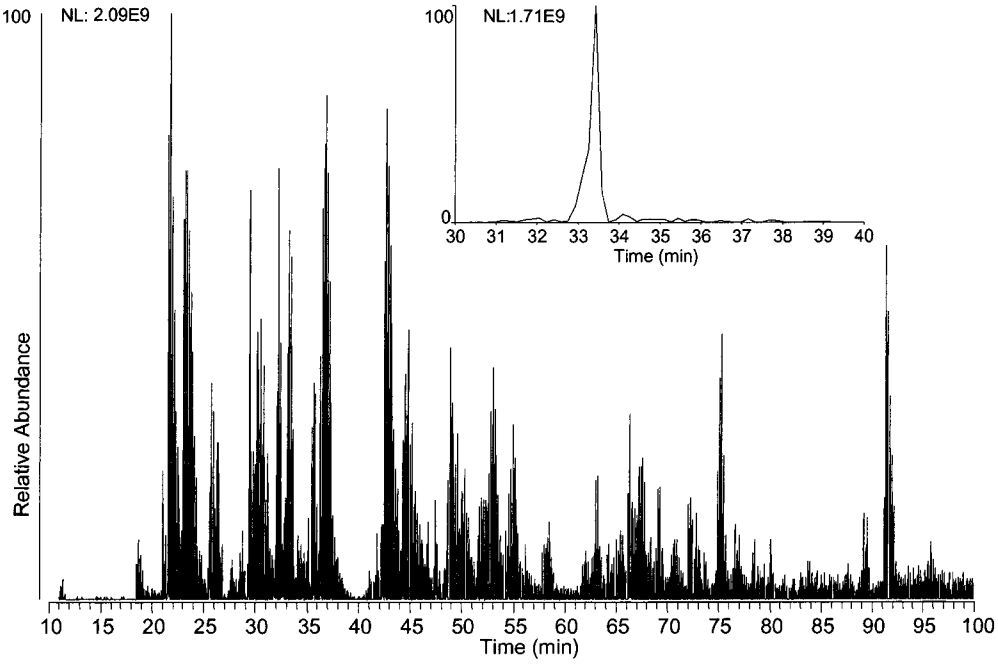

Figure 5. Automated direct LC−MS/MS analysis of a highly complex peptide mixture from purified outer membrane proteins from *P. aeruginosa*. The autosampler injected 30 μL (20 μg) of the peptide mixture onto a 75-μm V-column via the flow path diagrammed in Figure 2B. The number of MS/MS scans automatically acquired during the 90-min separation was 1955. More than 100 proteins were unambiguously identified by database searching. Inset: selected ion chromatogram for the $[M + H]^+$ peak for a trypsin autolytic peptide, 843 *m*/*z*. Normalized peak intensity values are shown to emphasize base peak intensities for many of the peptides exceeded that for background ions by 3 orders of magnitude.

The vent ensured that the slurry transferred rapidly into the cross, requiring only several minutes to replace a 2-cm bed. To demonstrate the robustness of the vent construction, 75-μm V-column analyses have been continuously performed over 8 months time and have a success rate of nearly 100%. Typically, each sample analysis from in-gel digestions has been followed by a "blank gradient", to eliminate contamination arising from superabundant samples and from acrylamide or coomassie-stain contaminants. The problem of carry-over between V-column analyses of SCX column fractions has been avoided by judiciously choosing the amount injected according to the absorbance determined in the SCX chromatogram. Column performance has deteriorated after sudden changes in the bed pressure. This has resulted when the direction of force was suddenly reversed after switching the valve at high pressures. This resulted in fragmentation and dispersal of the bed. This situation can be avoided by gradually decreasing the flow rate over 1 min, prior to switching the valve. The sample load method (isocratic flow rates of ~ 4 μL min^{-1}) should be performed apart from the gradient analysis method. Otherwise, long delays to "home" the pumps have resulted in the low-pressure gradient mixing design used in this work.

Chromatographic Performance. The peptide test standard was used to demonstrate the peak sensitivity obtainable with 75-μm V-columns. A single analysis of 5 fmol of a standard peptide mixture (10-μL injection of 500 amol μL^{-1}) is shown in Figure 3. Each selected ion chromatogram is displayed to allow for inspection of the peak volumes obtained. Each single full MS spectrum (inset) demonstrates that the signal-to-noise ratio for each peptide was >20, which suggests the selection of each peptide ion for data-dependent peptide sequencing can be accomplished at the 1−5-fmol level in complex mixtures.

To establish the sensitivity limit for product ion monitoring, the MS was set to continuously acquire MS/MS scans of the $[M + 2H]^{2+}$ ion for angiotensin (649 m/z) over the entire analysis. A 100-amol injection (5 μL of a 20 amol μL^{-1} solution) was analyzed twice with the MS set to acquire either one or three microscans per MS/MS scan. A representative result is shown in Figure 4. The ion current increased when the angiotensin peptide eluted from the V-column to provide a signal-to-noise ratio of 5 (Figure 4A). Averaged MS/MS scans obtained during peak elution for either three or one microscan per scan is shown in Figure 4B and C, respectively. Although the information content is very similar, the signal-to-noise ratio for 100 amol improved with the increase in the peak sampling rate using 1 microscan per MS/MS scan.

Complex Mixture Analysis. The utility of the 75-μm V-column for data-dependent analyses of highly complex mixtures was examined by a direct analysis of a trypsin digestion generated from a purified outer membrane fraction of *P. aeruginosa*. The 30-μL AS injection (20 μg of total peptides) at 4 μL min^{-1} was followed by a shallow gradient of 10−40% solvent B, with ~150 nL min^{-1} passing through the V-column. The base peak ion chromatogram is shown in Figure 5and the protein sequence analysis is shown in Figure 6. Excellent column efficiency allowed for over 100 min of useful MS/MS acquisition during elution of the peptides. The column performance for a relatively hydrophilic autolytic peptide ($[M + H]^+ = 842.5$ m/z) is shown in the selected ion chromatogram (inset) by its peak response. The presence of many more-hydrophilic peptides, some having their peak intensity at least 3 orders of magnitude greater than background levels, demonstrated that the sample loading capacity may not be a limitation of the 75-μm-i.d. V-column. The total number of MS/

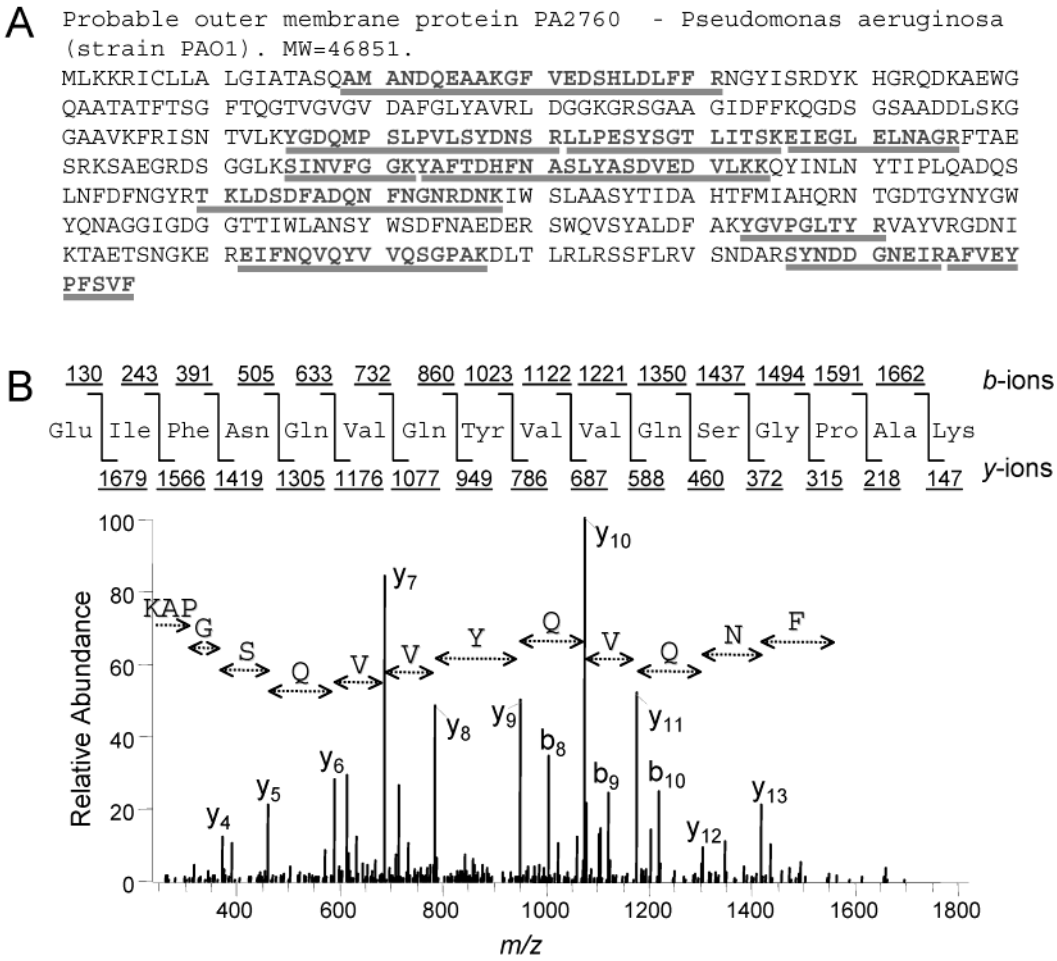

Figure 6. Protein sequence analysis of *P. aeruginosa* protein PA2760. This protein was identified from an analysis in Figure 5. (A) Twelve peptides containing 37% of the predicted sequence coverage were identified including the C terminus and the potential N terminus. (B) Tandem mass spectrum for one of the peptides identified. Shown are the predicted b- and y-type fragment ions. A total of 101 other proteins (307 peptides) were also identified from the analysis in Figure 5.

MS acquisitions acquired over the gradient elution was 1955. The total number of unique peptides matched to database sequences using established Sequest scoring parameters[22,23] was 319, resulting in 102 proteins being identified. Automated analyses of complex cellular extracts from PAGE gels using 75-μm V-columns (manuscript in preparation) have demonstrated chromatographic performance comparable to that in Figure 5.

Analysis of Large-Volume Cation-Exchange Fractions. The V-column capabilities to remove salts and to provide the enrichment needed for relatively large sample volumes were demonstrated to automate data-dependent analyses of narrow-bore SCX chromatography fractions. To generate the fractions, a tryptic mixture derived from 1 mg of soluble whole yeast lysate was separated on a 2.1-mm-diameter SCX column using a gradient to 250 mM KCl at 200 μL min^{-1}. Eighty fractions were placed in the autosampler for automated LC−MS/MS sequence analyses. These analyses were done with flow rates ~3 orders of magnitude below that of the narrow-bore separation. Table 1 shows the combined data from an unattended overnight run of fractions 33−37. The acquisition of 11 753 MS/MS acquisitions led to 861 unique peptides and 568 proteins being identified from these five fractions.

Table 1. Automated LC−MS/MS Analysis of Five SCX Fractions from a Tryptic Digest of Whole Yeast Lysate with 2-h Gradients

fraction no.	no. of MS/MS acquired	no. of peptides identified[a]			no. of proteins identified[a]	
		total	unique[b,d]	unique 2^{+} [c,d]	total[d]	by 2^{+} [c,d]
33	2336	618	284	278	231	226
34	2366	576	278	273	230	225
35	2303	592	289	287	237	237
36	2421	738	380	373	292	289
37	2327	766	375	363	295	288
total	11753	3297	861	852	568	559

[a] Criteria for identifications by Sequest algorithm are described in the Experimental Section. [b] Unique peptides are defined as uniquely identified sequences (i.e., a 2+ and a 3+ ion from the same peptide are not unique). [c] Only peptide and protein identifications from doubly charged peptide ion precursors are included. [d] In "total" row, the number of unique identifications was drawn from combined fractions.

As shown in the table, nearly all peptide matches were from doubly charged peptide ions because of the stringent criteria for Sequest.[22] The combined data for the entire eighty fractions will be presented elsewhere (J. Peng et al. Manuscript in preparation).

CONCLUSIONS

Integration of a 75-μm nanocolumn with an autosampler instrument provided capabilities for completely automated peptide amino acid sequence analyses without sacrificing column performance. The chromatographic performance was examined for low flow rate ($\sim$150 nL min^{-1}) analyses and found to be comparable to that of nonsegmented columns, in terms of the peak volumes, sensitivity, and loading capacity demonstrated with the V-column. The on-column enrichment and sample desalting capabilities make the V-column a practical tool for LC−MS/MS and LC/LC−MS/MS experiments aimed at mining sample complexity encountered in proteome-wide analyses.

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