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Technical Notes

Device for the Reversed-Phase Separation and On-Target Deposition of Peptides Incorporating a Hydrophobic Sample Barrier for Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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The separation of peptide mixtures from proteolytic cleavage is often necessary prior to mass spectrometry (MS) to enhance sensitivity and peptide mapping coverage. When buffers, salts, and other higher abundance peptides/contaminants are present, competition for charge during the electrospray ionization and matrix-assisted laser desorption/ionization (MALDI) processes can lead to ion suppression for the targeted analyte(s). In this note, a simple reversed-phase microcolumn sample separation and deposition device (Sep-Dep) is described. The use of this device improves or renders possible the analysis of complex or contaminated peptide mixtures by MALDI-MS. The method is simple and inexpensive and utilizes single-use low-cost Geloader-type columns packed with reversed-phase material. The device described utilizes an open column, allowing for a gradient or narrow-step gradient to be applied by any solvent delivery system or manually with a pipet. A key feature of the device is a deposition chamber that can be custom-built to hold any MALDI target. The Sep-Dep device is attached directly to an in-house vacuum line and draws solvent from the open-ended LC column. The elution of separated peptides is performed directly onto a target that has been treated with a hydrophobic barrier. This barrier effectively isolates fractions and improves the quality and morphology of the matrix crystals. The method produces efficient separations of proteolytic peptides, significantly reducing signal suppression effects in MALDI.

Mass spectrometry (MS) has become a major analytical tool in proteomic and biological research. Most protein identification strategies involving MS analyze proteolytic peptides (e.g., tryptic digests) for mass fingerprinting in combination with tandem MS (MS/MS) to confirm amino acid sequence and the presence of various posttranslational modifications. In most proteome studies, proteins are separated on electrophoretic gels and in-gel digestion extracts are subjected to MS analysis. Although matrix-assisted laser desorption/ionization (MALDI) is very effective for screening high-abundance proteins in complex samples, lower abundance peptides often remain undetected.[1] Suppression effects are a common problem, arising from the presence of multiple analytes competing for protons during the ionization process. These effects are mostly pronounced when the abundance of analytes of interest decreases relative to more concentrated peptides or contaminants present in the sample matrix.[2−6] Autolytic cleavage of the protease and presence of peptides from human keratins also constitute potential problems.

Wilm and Mann[7] were the first to use microcolumns filled with reversed-phase media from solid-phase extraction (SPE) and direct elution/deposition into electrospray ionization needles. Gobom et al. used Geloader-type C18 columns to develop a method capable of concentrating femto- to attomoles of peptides using

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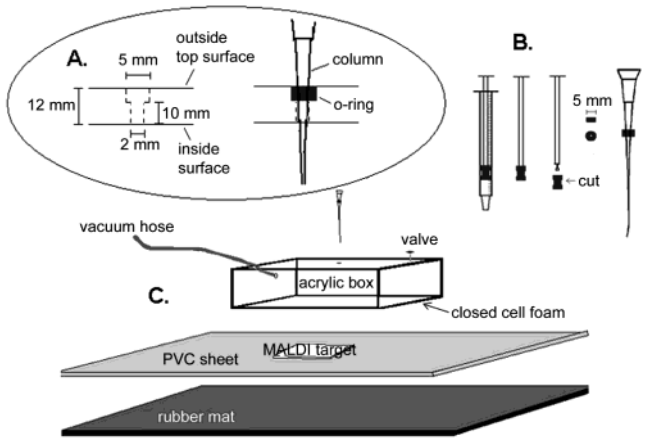

Figure 1. Components of the Sep-Dep device. (A) The column is fixed to the device by drilling a 2-mm-diameter hole through the center top panel of the deposition device. A larger 5-mm-diameter tap is centered onto the 2-mm hole. (B) A standard plastic 1-mL syringe is dismantled and the plunger is removed. The plunger is cut at the position indicated to form an O-ring. Geloader column is inserted through the O-ring (approximately 2.0−2.75 cm form the top of the column) and positioning is used to customize column distance from the target. The modified column is inserted into the center top hole of the device, creating a tight seal. (C) Components of the Sep-Dep device used in these experiments. A rubber mat is used to prevent slippage of the PVC sheet on the benchtop.

packing beds of 30−50 nL.[8] These methods have gained wide acceptance and recognition for their simplicity and efficiency for removing ionic salts, buffers, and detergents, thus enhancing the quality of MS analyses.

Although SPE methods allow for the removal of ionic and polar components from the sample matrix, suppression effects resulting from highly abundant peptides are often problematic. The batch elution of peptide extracts using increasing percentages of organic solvent (sequential elution with, for example, 25, 50, and 75% acetonitrile in water) helps to limit suppression, but in the case of complex samples, a significant number of peptides may elute over a given solvent batch range. The use of narrow solvent percentage increments may solve these problems but can be time-consuming and not well adapted to direct on-target deposition for MALDI-MS analysis.

The removal of interfering compounds from peptide mixtures by continuous-flow liquid chromatography (LC) or capillary electrophoresis (CE) is common practice to minimize ion suppression. The separation of peptide mixtures by LC or CE also allows resolution of isobaric species and isoforms that otherwise may be difficult to detect or distinguish. LC separation prior to MS improves sequence coverage[9−15] and enhances the quality of the analysis,[16,17] while allowing the identification of larger peptide fragments that were not detectable in the presence of smaller ones.[18,19]

In this work, the advantages of coupling LC separations with MALDI have been exploited in the design and development of a simple and inexpensive device for the collection of peptide fractions. This device serves the function of a micro-LC system by separating peptides using reversed-phase material packed in a Geloader-type column.[20,21] The design of this device includes a clear acrylic vacuum chamber to house the MALDI target (Figure 1). This chamber is an open bottom (5-sided) box placed onto a smooth poly(vinyl chloride) (PVC) sheet. The box has a connection to an in-house vacuum line with rubber tubing. The center of the top panel has a small hole that securely fastens the column. The top panel also has a "thumb-controlled" release valve used to position the deposition device, to control the flow rate, and to facilitate target removal. It is important to note that the MALDI target and PVC sheet are stationary to the benchtop and that the clear acrylic box (including column) is positioned for sample deposition. When the chamber is evacuated, solvent is drawn through the column and peptides are eluted directly onto the target located within the Sep-Dep device. Features of the device allow the use of a narrow-step gradient with a pipet. The volume deposited per spot can be controlled by changing the initial distance (when the chamber is at atmosphere) between the end of the column and the target or by controlling vacuum rates within the chamber using the thumb valve.

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Sample deposition for MALDI is aided by the preapplication of a hydrophobic barrier with a commercially available pen. This highly hydrophobic barrier is composed of polybutadiene (butadiene rubber) and is easily applied onto the target using a marker-type applicator. The barrier is reusable and remains highly hydrophobic for several application/wash cycles. It also increases the uniformity of the peptide dispersed within the MALDI matrix, reducing edge effects. Using the barrier, 2,5-dihydroxybenzoic acid (DHB) matrix can be preapplied and dried onto a 384-target spot in less than 4 min prior to the deposition of peptide samples.

The typical volume-per-spot deposited on the target was between 1.0 and 1.5 μL. Collection of 200−344 fraction spots was completed within 30−45 min using a 0−80% acetonitrile/H_2O (0.1% TFA) narrow-step gradient. The cost of construction for this Sep-Dep device, not including disposables, was under $70.00 U.S. All materials were readily available from local hardware and scientific suppliers.

For the purposes of this article, the Sep-Dep device was tested first to demonstrate its utility in reducing MALDI ion suppression effects. The device was then used to map tryptic peptides from citrate synthase and phosphorylated β-casein.

EXPERIMENTAL SECTION

Materials. Geloader tips were purchased from Eppendorf (Hamburg, Germany). Trifluoroacetic acid (TFA), phosphorylated bovine β-casein, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TCPK)-treated bovine pancreatic trypsin, DHB, and ammonium bicarbonate were purchased from Sigma Chemicals (St. Louis, MO). Connexin-43 peptide corresponding to sequence CDQRPSS-RASSRASSRPR was synthesized by Anaspec (San Jose, CA). The 1-mL syringes used to fabricate O-rings were obtained from Becton Dickinson (Franklin Lakes, NJ). Fluoropore PTFE membrane filter disks with 0.45-μm pore size were obtained from Millipore (Bedford, MA). Widepore (15 μm, 300 Å pore size) C18 packing material used in these experiments was purchased from J.T. Baker (Phillipsburg, NJ). HPLC grade acetonitrile (MeCN) and methanol (MeOH) were obtained from Fisher Scientific (Fair Lawn, NJ). Deionized, distilled water was from a Barnstead Nanopure (Dubuque, IA) water filtration/deionization system supplied by a reversed-osmosis feedstock. All solutions were degassed by sonication under vacuum for at least 10 min.

Peptides Used for Ion Suppression Study. Connexin-43 peptide (2 μg, 1 nmol) was dissolved in 10 μL of 20 mM aqueous ammonium bicarbonate. The solution was digested by the addition of 0.5 μL of trypsin (1.0 mg/mL) for 1 h at 37 °C. Digestion was halted by the addition of TFA to a final concentration of 2.5%. The solution was then spiked with 5.0 μL of angiotensin I in water (3.85×10^{-3} M) and mixed thoroughly. The solution (2.0 μL) was then transferred to the column for separation.

Digestion of β-Casein. Phosphorylated bovine β-casein (2.5 mg, 10 nmol) was dissolved in 500 μL of aqueous 20 mM ammonium bicarbonate. TCPK-treated trypsin (1.0 mg/mL) in 20 mM ammonium bicarbonate was then added to the solution, to a final relative mass ratio of 50:1 (β-casein/trypsin protein) and the solution was allowed to incubate for 14 h at 37 °C. Digestion was stopped by adding TFA to a final concentration of 2.5%, and the resultant mixture was stored at −20 °C until application to the reversed-phase column. Once the RP column was prepared, 2.5 μL of the β-casein digest (2.5 nmol) was loaded onto the column and separated as described further.

Digestion of *Escherichia coli* Citrate Synthase. A stock solution of citrate synthase (257 mg/mL) in 20 mM Tris-Cl, 1 mM EDTA, and 50 mM KCl was prepared as described by Ayed et al.[22] Citrate synthase stock solution (3 μL) was then diluted to 50 μL with 20 mM ammonium bicarbonate to a concentration of 3.2×10^{-4} M. The protein was digested by adding 15 μL of 1 mg/mL trypsin and incubating for 4 h at 37 °C. Digestion was halted by adding 3 μL of TFA, and the solution was stored at −20 °C. The peptide solution (4.0 μL) was then loaded onto the RP column for on-target deposition of separated peptides using the Sep-Dep device.

Sep-Dep Device. The chamber was constructed out of clear acrylic plastic. The open-bottom acrylic box measures *L* 25.0 cm × *W* 25.0 cm × *H* 4.5 cm, is held together with screws, and is sealed with general-purpose household silicone. The open edge of the box is lined with closed-cell weather stripping (0.6 × 1.0 cm) to provide a good seal when placed upon a smooth PVC sheet (54 cm × 30 cm; Figure 1). Both acrylic and PVC sheets are 1.2 cm thick and provide sufficient rigidity when placed under maximum house vacuum (0.2 atm; University of Manitoba, Department of Chemistry). The column is inserted into the center-top surface and sealed with an O-ring constructed from that of a 1-mL syringe (Figure 1). Vacuum within the chamber is established using a screw-in tapered hose connector and standard laboratory quality rubber tubing. A screw-in stainless steel tube (thumb valve) was installed near the corner of the top surface of the device.

MALDI Time-of-Flight (TOF) MS Target Preparation. The hydrophobic barrier was applied to a stainless steel Bruker Daltonics 384 target. The Super HT Pap Pen, with a 4-mm tapered point, was manufactured by Daido Sangyo Co. Ltd. (Tokyo, Japan). A 4-mm-wide barrier was "drawn" onto the target using parallel lines to separate the rows (A−P) and columns (1−24). The target was then rinsed with ∼200 mL of water followed by 200 mL of MeOH. The new surface was then allowed to cure at room temperature for at least 24 h under vacuum. The target was then stored in a dust-free environment to prevent surface contamination until sample deposition.

Tilt-and-Tap Prespotting Technique. With rows and columns partitioned by hydrophobic polybutadiene, the MALDI target was tilted 60−70° from horizontal and 27 μL of matrix solution (DHB, 60 mg in 500 μL of 50:50 MeCN/H_2O, 0.1% TFA) were added over the upper positions (1 and 2) of row A. The target was then lightly tapped onto the benchtop while the angle was maintained, causing the solution to run down its assigned row. Excess matrix solution was then allowed to run off the end of the target plate and wiped off with a damp lint-free paper tissue. The prespotting procedure was then repeated for each row (B−P). The estimated volume of matrix solution per MALDI sample spot was 0.8−1.0 μL. The square matrix spots were allowed to dry under ambient conditions prior to sample deposition.

Packing of Reversed-Phase Microcolumns. A column frit was made by carefully cutting the Fluoropore filter disk to

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Figure 2. Square spots prepared using the polybutadiene hydrophobic sample barrier. MALDI matrix (DHB) was prespotted using the tilt-and-tap technique. Peptides were deposited using the Sep-Dep device.

approximately 0.25−0.50 mm^2 using a pair of tweezers and scissors. The small square frit was then transferred to the top end of the Geloader pipet tip and carefully positioned in the lower portion of the column with the application of pressure using a 6-cm-long, 290-μm-o.d. steel capillary. Frit flow-through was tested by positioning the column on the device and applying a slight vacuum while adding a small volume of MeOH to the top end of the column. Flow through the frit was adequate to clear 10−20 μL of MeOH within 20 s under full or near full vacuum. The column was then partially filled with MeOH and bubbles were removed by gently tapping its side or by suction. Once all air bubbles were removed, and C18 material in MeOH was added and allowed to partially settle before vacuum was reapplied to pack the stationary phase. Packing material was continually added until the final volume was between 3 and 5 μL. The column was then washed three times with 30 μL of 0.1% TFA using full vacuum. The final wash was allowed to reside ~2 mm above the LC bed for sample loading.

Sample Loading and Separation. Eight 60-μL aliquots of solvents (10, 20, 30, 40, 50, 60, 70, and 80% MeCN/H_2O, 0.1% TFA) were prepared. Peptide samples were loaded into the 2 mm of remaining wash solution. House vacuum was applied and the thumb valve was closed to obtain a chamber pressure of 0.3−0.5 atm, drawing the mobile phase through the column. Once the sample load resided ~0.5−1.0 mm from the top of the separating medium, vacuum was shut off and 5 μL of 0.1% TFA was added. Vacuum was then applied until the solution resided just above the chromatographic bed, followed by the addition of 45 μL of H_2O (0.1% TFA). The 45-μL solvent level was marked on the side of the column using a fine felt-tipped pen, followed by the addition of 15 μL of H_2O (0.1% TFA) to yield a total column volume of 60 μL. The target was then positioned within the chamber for the collection of the first fraction. As vacuum was established, eluent was drawn through the column and deposited on to the MALDI target. Repositioning of the device was achieved by reducing the internal vacuum. As the solvent level reached the lower mark, 15 μL of 10% MeCN was added to the column until the full 60-μL aliquot was consumed. As the deposition process continued, each solvent aliquot was added in 15-μL increments, in order of increasing MeCN concentration until elution was complete.

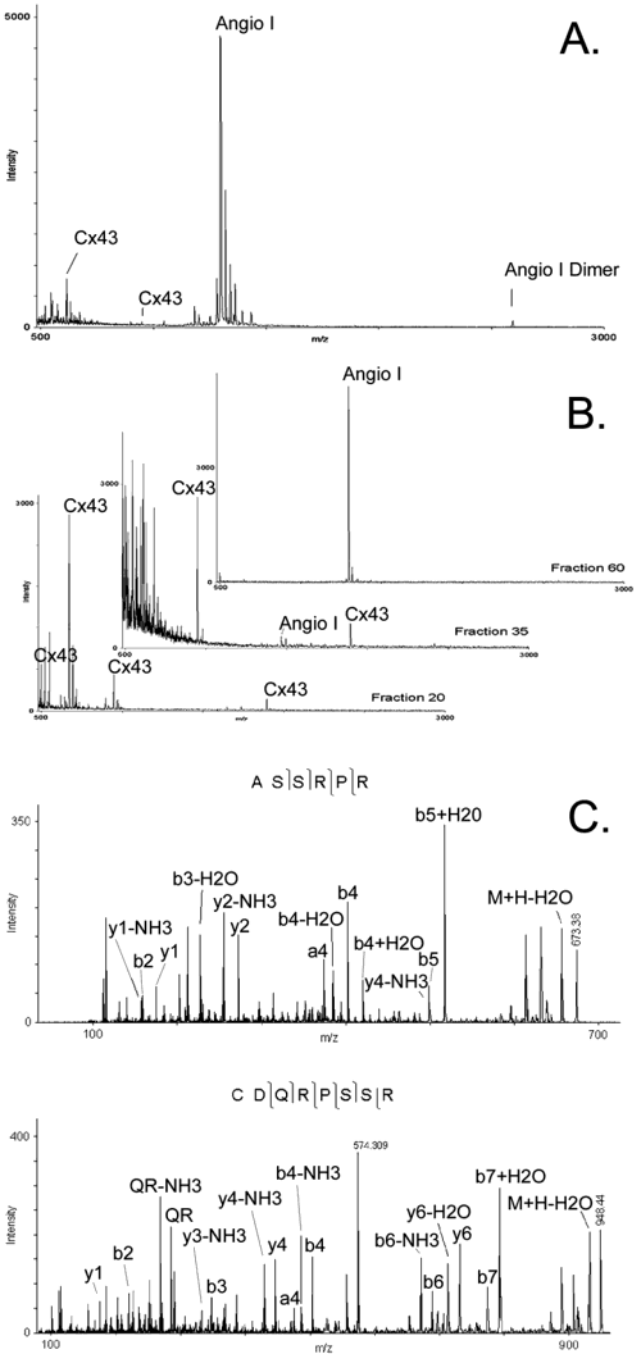

Figure 3. Reduction of suppression effects. (A) Suppression of Cx43 peptides by a 19-fold of angiotensin I peptide and Na^+ ions. Only two out of the possible four Cx43 peptides can be observed. (B) Sample partitioning using the Sep-Dep device, using a narrow-step MeCN/H2O gradient and resolving all Cx43 peptide *m*/*z* values. (C) MS/MS of Cx43 peptides, *m*/*z* 948.44 and 673.38. Both spectra were obtained from fraction 25.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Spectra were obtained on a Bruker Daltonics Biflex-IV instrument (Billerica, MA) with delayed extraction, operated in reflectron mode, using 16- (lens 1) and 19-V (lens 2) acceleration voltages. The instrument was calibrated externally using angiotensin I, bombesin, substance P, and ACTH 18−39 with target columns 1, 12, and 24 reserved for calibration. Spectra were acquired using the AutoExecute (Bruker Daltonics) interface with a maximum of 150 shots accumulated using 30−50% relative laser

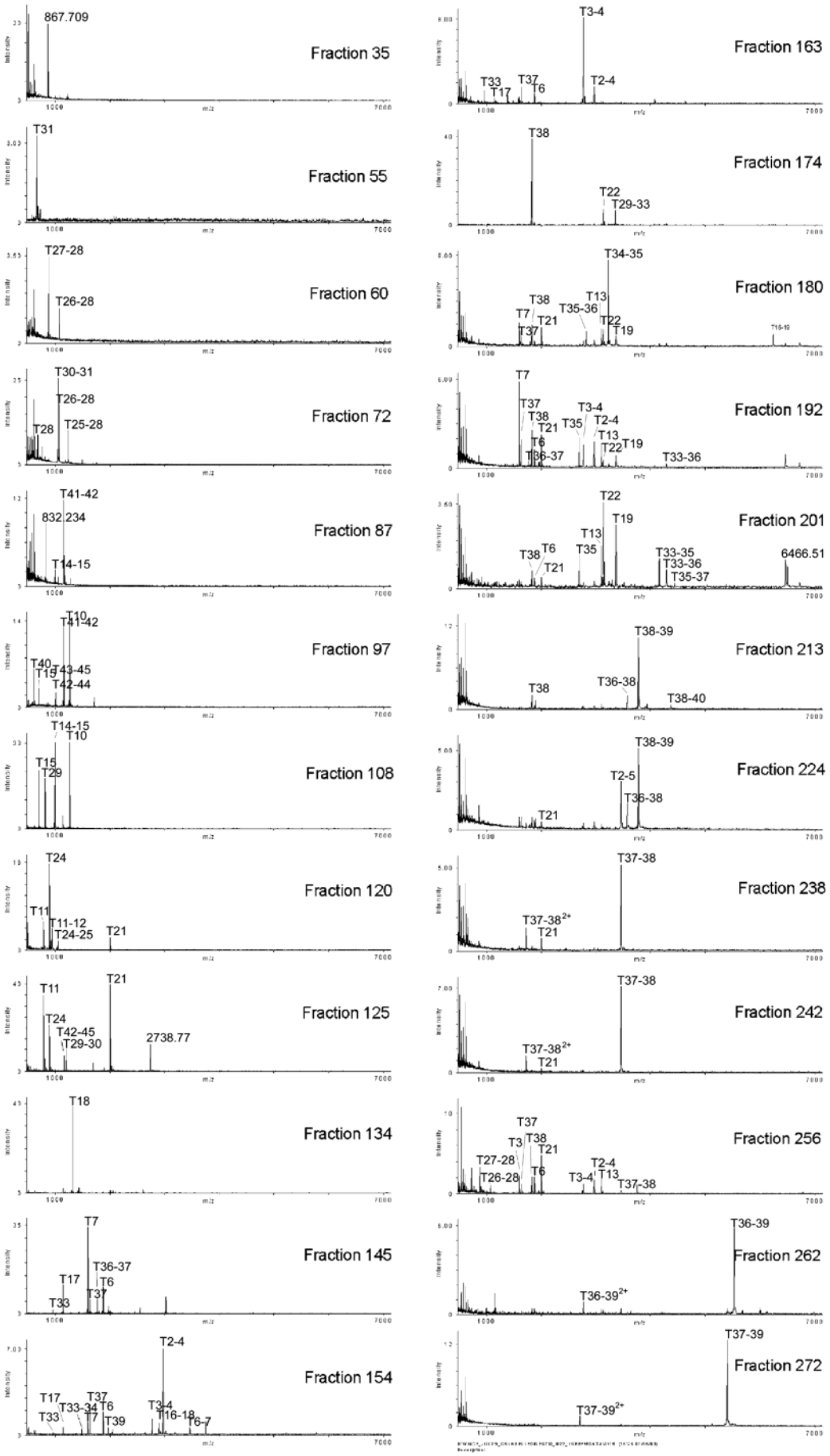

Figure 4. Representative spectra of CS tryptic peptides eluted using the Sep-Dep device.

power. Data were processed using M over Z software[23] with peak m/z values autolabeled and saved to an Excel spread sheet for submission to Profound for peptide-mass fingerprinting.[24]

Matrix-Assisted Laser Desorption/Ionization Tandem Mass Spectrometry. Once MALDI-TOF-MS spectra were analyzed, ions of interest were subjected to MS/MS sequencing. Spots on the 384 target (in DHB matrix) were dissolved in 1 μL of 50% MeCN in water. Solutions were mixed several times by aspiration into the pipet tip to ensure adequate transfer to a gold-plated Intrinsic Bioprobes QqTOF target (Tempe, AZ). Peptides were then sequenced on an in-house-built Manitoba/Sciex prototype MALDI quadrupole/time-of-flight mass spectrometer (QqTOF) located at the University of Manitoba, Department of Physics and Astronomy.[25]

RESULTS AND DISCUSSION

Design and Performance of the Separation/Deposition Device. The overall performance of the Sep-Dep device (Figure 1) demonstrated that efficient peptide separations can be obtained using basic materials and a house vacuum line. The open column allows the application of a narrow-step gradient and the elution of peptides directly onto a prespotted MALDI target. This method improves MS peptide coverage by reducing or eliminating ion suppression effects.

As for design, some features of the device required consideration for adequate performance. For example, the thumb-controlled release valve is positioned near a corner of the top panel to prevent disruption of the newly deposited sample spots by the returning atmosphere. This valve allows for the control of the column flow rate. Closed-cell weather stripping was used rather than a rigid rubber seal because of its compression characteristics that aided sample deposition. Chamber pressures between 0.45 and 0.6 atm generated a drop of eluent (1.0−1.5 μL) in approximately 5−10 s, and higher vacuum (0.2−0.4 atm) resulted in full compression of the weather stripping. Compression along the z-axis was effective in bringing the tip of the column (and eluent drop) to the sample surface without the Geloader tip physically contacting the target. This feature helped to reduce sample carry-over and clogging of the column by matrix that had been already deposited.

Polybutadiene Barrier. The polybutadiene hydrophobic barrier can be applied to any target surface in any desired geometry using the pen-type applicator. To date, no compatibility issues were encountered with the use of the polymer as described. The polymer is extremely hydrophobic in nature and very insoluble in water, methanol, and acetonitrile solutions. As the target surface directly under the samples is not modified, target conductivity and charging properties are maintained. As a result, instrument settings such as acceleration voltage and ion optics require minimal reoptimization.

It should be noted that new Pap Pen markers are "runny". Sufficient care should thus be taken when applying a thin (4 mm wide) layer onto the target surface. Wiping excess material from the pen tip with a lint-free tissue prior to application and using single strokes to partition each sample row and column leads to adequate results. It is also important to allow sufficient time to cure newly treated targets, as Pap Pen solvents produce a significant amount of vapor. The barrier is reusable after washes or rinses with 30% acetic acid, water, and methanol. Removal of the hydrophobic barrier can be achieved using toluene and as described by product literature.

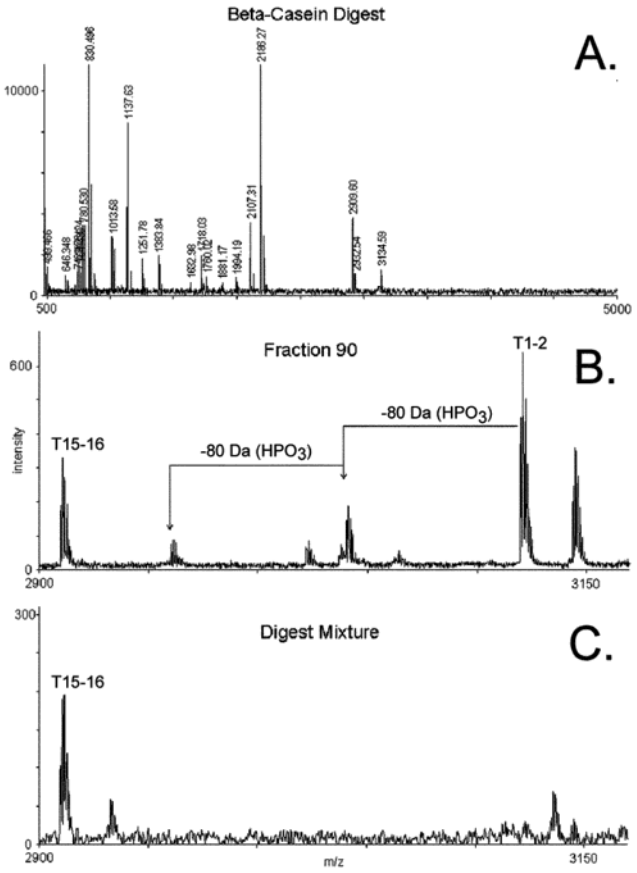

Figure 5. (A) MALDI-TOF-MS of peptides derived from 2 pmol of phosphorylated β-casein. No m/z values corresponding to predicted phosphorylated peptides were observed by direct spotting. (B) Identification of tetraphosphorylated T1−2 (m/z 3123.98) and consecutive losses of HPO_3 (−80 Da) observed in fraction 90. (C) No m/z values corresponding to phosphorylated or dephosphorylated T1−2 were observed in the peptide digest mixture.

With the hydrophobic barrier, more collected fractions produced useful MALDI spectra than with the traditional dried drop method. The barrier reduced edge effects and reduced the time that the automated acquisition routine took to find a "sweet spot". The effectiveness of the barrier was especially noticeable with fractions eluting at higher MeCN concentrations, i.e., with lower surface tension and usually spreading over a wider area on the target. The polybutadiene barrier was sufficiently hydrophobic to contain 60−75% MeCN with volumes greater than 10 μL/spot.

The hydrophobic barrier also helps in the rapid application of matrix using the tilt-and-tap technique. All 384 target positions were easily prespotted with DHB solution and allowed to crystallize over a short time (Figure 2). The solubility characteristics of DHB in water and MeCN resulted in the 1.0−1.5 μL of Sep-Dep eluent nearly fully redissolving the matrix. Peptides were uniformly dispersed through the matrix when predeposited DHB was used, compared to less soluble matrixes such as α-cyano-4-hydroxycinnamic acid.

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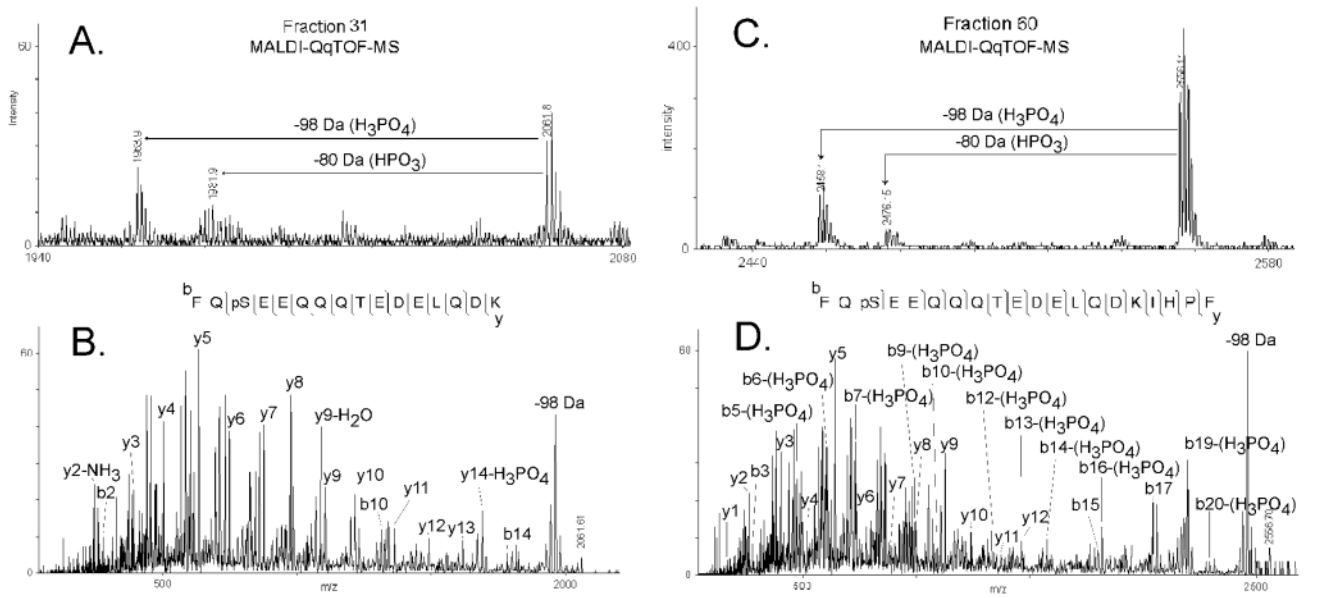

Figure 6. (A) Phosphorylated β-casein peptide T6 identified after partitioning using the Sep-Dep device with MS/MS sequencing (B). (C) A peptide from nonspecific cleavages shows characteristic losses of phosphate moieties. (D) MS/MS sequence analysis identified the phosphorylated peptides as a nontryptic cleavage corresponding to FQpSEEQQQTEDELQDKIHPF.

The Sep-Dep apparatus can be seen as a low-performance LC device. The combination of Fluoropore column frit, column-packing material having a larger particle and pore size (than most HPLC media), bed volume, and house vacuum resulted in suitable flow rates for proper separation and deposition.

Reduction of Ion Suppression Effects. Cx43 peptides mixed with a greater than 19-fold molar concentration of angiotensin I (and sodium ions) underwent significant ion suppression. Only two of the theoretical four Cx43 peptides were detected as low-abundance ions (Figure 3A). Similar suppression effects were observed by Preisler et al.[17] using an equimolar mixture of peptides with 50-fold excess of angiotensin II. After separation using the Sep-Dep device, a significant reduction of suppression was achieved and all four theoretical Cx43 fragments were detected as abundant parent ions (Figure 3B). Tandem MS experiments were performed on ions of interest to certify the identity of Cx43-derived peptides as shown in Figure 3C.

Separation of Tryptic Digests of Proteins. Following the successful separation of relatively simple peptide mixtures, the Sep-Dep method was tested on tryptic peptides from larger proteins, *E. coli* citrate synthase (CS) and bovine β-casein. CS is a protein with 427 amino acids and a molecular weight of ~48 000. The protein has been well characterized[26,27] and used to evaluate separation techniques used in hyphenation with mass spectrometry.[28] The separation of CS tryptic fragments was achieved using a 0−80% MeCN narrow-step gradient with the collection of 344 sample spots. Figure 4 shows the separation MALDI profile of 24 of the 344 spots that were analyzed by the autoacquisition routine. Submission of m/z values to the Profound (data not shown) mass-fingerprint database led to the unambiguous verification of CS,

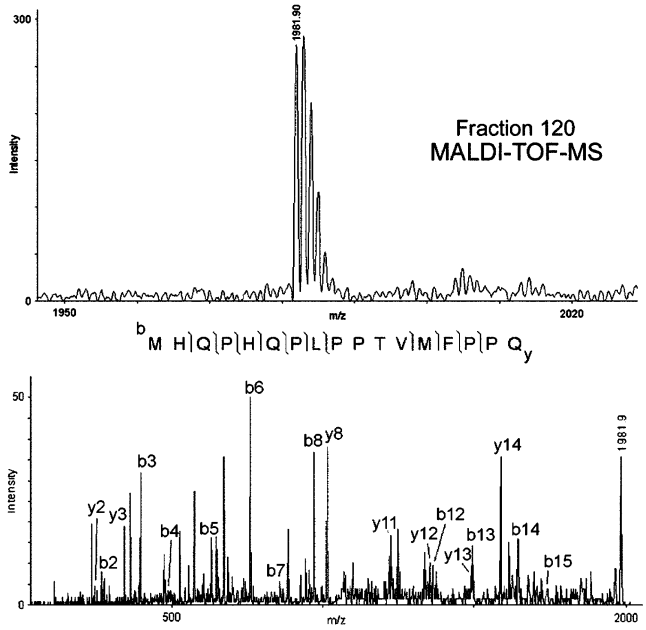

Figure 7. Sep-Dep detection of a β-casein peptide at m/z 1981.9 (top), i.e., the same mass as dephosphorylated T6 observed in fraction 31. MS/MS and sequence analysis identified the peptide MHQPHQPLPPTVMFPPQ, a product from nonspecific cleavage (bottom). Identification of both peptide isobars would have been difficult without separation.

with 52 matching peptides and a minimum sequence coverage of 94%.

Separation of β-casein tryptic peptides was performed under similar conditions and aimed at the analysis of phosphorylated peptides. β-Casein is a pentaphosphorylated protein, with serines modified at positions 15, 17, 18, 19, and 35. Direct spotting of 2 pmol of β-casein digest using DHB yielded no detectable phosphorylated peptides, likely due to ion suppression effects. Phosphopeptides are also known to yield reduced ionization efficien-

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cies.[29] Once separated, both predicted phosphorylated peptides, T6 (fraction 31) and T1−2 (fraction 90), were detected as $[M + H]^+$ ions (Figure 5). Losses of HPO_3 moieties (80 u, by MALDI-TOF-MS) and H_2PO_3 (98 u, MALDI-QqTOF-MS) were also observed (Figure 6). These losses were helpful to identify a nonspecific phosphorylated peptide observed at m/z 2556 in fraction 60. Sequence analysis of m/z 2556 ions yielded FQpSE-EQQQTEDELQDKIHPF, a fragment not produced by trypsin and most likely due to the presence of chymotrypsin in the TCPK treated protease. Separation also resolved a second nonspecific β-casein peptide corresponding to MHQPLPPTVMFPPQ (m/z 1981.9, Figure 7) observed in fraction 120, interestingly an isobar of dephosphorylated T6 observed in fraction 31. Detection of both peptides corresponding to m/z 1981.9 and their respective sequencing and identification by MS/MS would not have been possible without separation of the peptide mixture.

CONCLUSION

The Sep-Dep device produces reasonable separations for MALDI analysis using simple, inexpensive materials and taking advantage of house vacuum. The technique significantly increases peptide coverage and enhances MALDI spectral quality in general. The main drawbacks to this technique are the manual efforts necessary for separation and deposition. The incorporation of features such as an automated X−Y−Z stage and a solvent delivery/gradient system would decrease the need for user time and would likely improve separation efficiency. The addition of such features would surely be an asset, but at the expense of adding layers of complexity and of generating higher costs. In this report, we demonstrated that a minimalist approach to peptide separation, the Sep-Dep device, can be used to reduce ion suppression effects and increase the quality of peptide analysis by MALDI-MS.

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