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On-chip solid-phase extraction pre-concentration/focusing substrates coupled to atmospheric pressure matrix-assisted laser desorption/ionization ion trap mass spectrometry for high sensitivity biomolecule analysis

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Atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) has proven a convenient and rapid method for ion production in the mass spectrometric (MS) analysis of biomolecules. AP-MALDI and electrospray ionization (ESI) sources are easily interchangeable in most mass spectrometers. However, AP-MALDI suffers from less-than-optimal sensitivity due to ion losses during transport from the atmosphere into the vacuum of the mass spectrometer. Here, we study the signal-to-noise ratio (S/N) gains observed when an on-chip dynamic pre-concentration/focusing approach is coupled to AP-MALDI for the MS analysis of neuropeptides and protein digests. It was found that, in comparison with conventional AP-MALDI targets, focusing targets showed (1) a sensitivity enhancement of approximately two orders of magnitude with S/N gains of 200–900 for hydrophobic substrates, and 150–400 for weak cation-exchange (WCX) substrates; (2) improved detection limits as low as 5 fmol/μL for standard peptides; (3) significantly reduced matrix background; and (4) higher inter-day reproducibility. The improved sensitivity allowed successful tandem mass spectrometric (MS/MS) sequencing of dilute solutions of a derivatized tryptic digest of a protein standard, and enabled the first reported AP-MALDI MS detection of neuropeptides from *Aedes aegypti* mosquito heads.

Atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry (AP-MALDI MS), first reported by Laiko and co-workers,[1,2] has emerged as a powerful extension of conventional vacuum MALDI (vMALDI). AP MALDI offers the advantages typically associated with MALDI such as reduced sample cleanup needs, simplicity of sample preparation, use of a pulsed laser to generate gaseous ions, and the ability to re-analyze samples from a previously investigated spot. However, in AP-MALDI liquid matrices are more easily implemented,[3] and measurements tend to have higher reproducibility than in vMALDI.[4]

Because ion production in AP-MALDI is decoupled from mass analysis, this ion generation technique has been interfaced to a variety of instruments, including orthogonal acceleration time-of-flight (oaTOF),[2,5,6] ion traps,[7–9] and Fourier Transform ion cyclotron resonance (FT-ICR) mass spectrometers.[10] In most of these instrumental configurations, the AP-MALDI ion source is interchangeable with electrospray ionization (ESI) and other atmospheric pressure ion sources, providing researchers with substantial analytical versatility and making this technique a cost-effective alternative for laboratories with limited budgets.

An important characteristic of the AP-MALDI process is that, following ionization, ions are rapidly thermalized by collisional cooling with atmospheric gases,[11] resulting in lower effective ion temperatures than in vMALDI.[12] Gaskell and co-workers showed that, depending on the differences in gas-phase basicities between analyte and matrix, the internal energy of AP-MALDI-generated ions can be lower than that those generated by ESI.[11] The rapid thermalization obtained by carrying out the MALDI process under atmospheric pressure conditions has proven useful for studying labile biomolecules such as conotoxins[6] and phosphopeptides with minimum unwanted fragmentation,[13] but has the undesirable side effect of forming matrix-matrix and analyte-matrix clusters, resulting in decreased sensitivity when compared to vMALDI.[14] Instrumental advances such as the incorporation

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of an extended stainless steel heated capillary interface,[15] and a capillary inlet incorporating a concentric flow of heated nitrogen directed at the sample[16] have been proposed as a means to inducing ion declustering. Despite these efforts, the limited sensitivity of AP-MALDI continues to be a limitation of this technique.

Another factor influencing sensitivity in AP-MALDI is the limited ion transport efficiency into the first differentially pumped chamber of the mass spectrometer, a problem common to any ion source operating under atmospheric pressure conditions.[17] Laiko *et al*. first used dry nitrogen as a carrier gas to enhance ion transfer from the target plate to the mass analyzer inlet orifice.[2] Sensitivity improvements of 5–10× were later reported using a technique termed 'pulsed dynamic focusing' (PDF), where the AP-MALDI extraction high voltage is switched off several microseconds after the laser is fired, allowing ions to be more easily entrained by gas stream lines converging towards the atmospheric pressure capillary inlet.[18,19]

AP-MALDI, when coupled to trapping instruments such as linear or quadrupole ion traps (IT), is in principle appealing for proteomic and peptidomic experiments, as it could enable multi-stage mass spectrometric (MS^n) analysis in a compact and rather inexpensive platform.[20] However, the difficulty in obtaining good sequence coverage from singly charged peptide ions by low-energy collision-induced dissociation (CID) somewhat limits the applicability of this approach for *de novo* peptide sequencing and protein identification via database searches.[21] In an effort to increase fragment ion yields of singly protonated MALDI peptides, Keough *et al*. introduced a derivatization technique based on C-terminal sulfonation followed by lysine guanidination.[22,23] Addition of a negatively charged group at the C-terminus of tryptic peptides counterbalances the charge of the protonated N-terminal basic amino acid, facilitating charge-directed cleavage of backbone amide bonds by a second, more mobile proton.[24] In AP-MALDI ion trap experiments, peptide derivatives prepared by C-terminal sulfonation have been shown to fragment more extensively than the corresponding native peptides, generating contiguous y-ion series.[25] However, C-terminal sulfonation further decreases AP-MALDI sensitivity, as two protons are to be transferred from the UV-absorbing matrix to the analytes in order to produce positively charged ions.[22]

Here we present an evaluation of the analytical performance (i.e. sensitivity gain, reproducibility) afforded by two types (hydrophobic, WCX) of on-chip solid-phase extraction/focusing substrates for AP-MALDI ITMS. We test these focusing targets for two AP-MALDI applications requiring high sensitivity: (a) the profiling of neuropeptides extracted from *Aedes aegypti* mosquito heads and (b) tandem mass spectrometric (MS/MS) sequencing of dilute sulfonated tryptic digests of a model protein. Previous efforts in the area of on-chip sample preparation include the use of surface-enhanced LDI,[26] anchorchips,[27] silicon microchips,[28] and integrated digital microfluidic chips,[29] focusing mostly on vMALDI applications. To the best of our knowledge, this is the first report of the successful coupling of pre-concentration/focusing substrates to AP-MALDI, resulting in a sensitivity improvement of more than two orders of magnitude.

EXPERIMENTAL

Materials and samples

Proteomic grade trypsin, angiotensin I, equine heart myoglobin, and ammonium dihydrogen phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium bicarbonate, 1,4-dithio-DL-threitol, and 2-sulfobenzoic acid cyclic anhydride were purchased from Fluka (St. Louis, MO, USA) and ammonium hydroxide from Fisher Scientific (Pittsburgh, PA, USA). *O*-Methylisourea hemisulfate was purchased from Acros Organics (Morris Plains, NJ, USA). HPLC grade acetonitrile (ACN), ethanol (EtOH), ammonium citrate dibasic and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Qiagen Inc. (Valencia, CA, USA). Allatostatin-C (AS-C) standards were synthesized by Alpha Diagnostic International Inc. (San Antonio, TX, USA) and allatotropin(AT) standards at the Center for Biotechnology Research, Kansas State University (Manhattan, KS, USA). These standards were purified chromatographically and assessed to be ≥100% (AS-C) and ≥97% (AT) pure by reversed-phase liquid chromatography, mass spectrometry and amino acid analysis. Mosquito heads were collected by decapitation. Head samples were homogenized in Bennett's solution (1% NaCl, 5% formic acid and 1% trifluoroacetic acid (TFA) in 1 M HCl).[30] After centrifugation (14 000 *g*, 10 min) the supernatant was lyophilized and stored at −80°C. Desalting/focusing hydrophobic MALDI targets (64 wells, MassSpecFocus Desalting Chip, type 4), weak cation-exchange (WCX) targets (25 wells, prototype chip), and gold-coated stainless steel 'tuning targets' without surface functionality, but otherwise identical to the hydrophobic/WCX targets, were obtained from Qiagen Inc. C18 particle-embedded pipette microtips (NuTips, 1–10 μL) were obtained from Glygen Corp. (Columbia, MD, USA). All aqueous solutions were prepared with nanopure water (dH_2O) from a Nanopure Diamond laboratory water system (Barnstead International, Dubuque, IA, USA).

Procedures for signal-to-noise ratio (S/N) gain and reproducibility studies

Serial dilutions of angiotensin I and AS-C containing 0.1–5 pmol/0.5 μL of peptide were prepared in 50% ACN/0.1% TFA. Triplicates of each solution were pre-mixed with 10 mg/mL matrix and spotted on conventional targets (Fig. 1(a)) using the dried-droplet method. A second set of serial dilutions containing 0.075–1 pmol in 5 μL of angiotensin I were desalted and concentrated in triplicate or duplicate on hydrophobic and WCX focusing targets, respectively (Fig. 1(b)). The optimized focusing procedures are described below. S/N gain studies at fixed peptide concentration were performed by spotting 1 μL of 2×10^{-7} M angiotensin I solution on a conventional target and 5–20 μL of the same solution on hydrophobic and WCX focusing targets. S/N gain studies at fixed amounts of peptide were performed by spotting 1 μL of 1×10^{-6} M angiotensin I solution on a conventional target and 5 μL of 2×10^{-7} M of the peptide solution on hydrophobic and WCX targets, resulting in equal amounts of angiotensin I (1 pmol) per spot on each investigated target.

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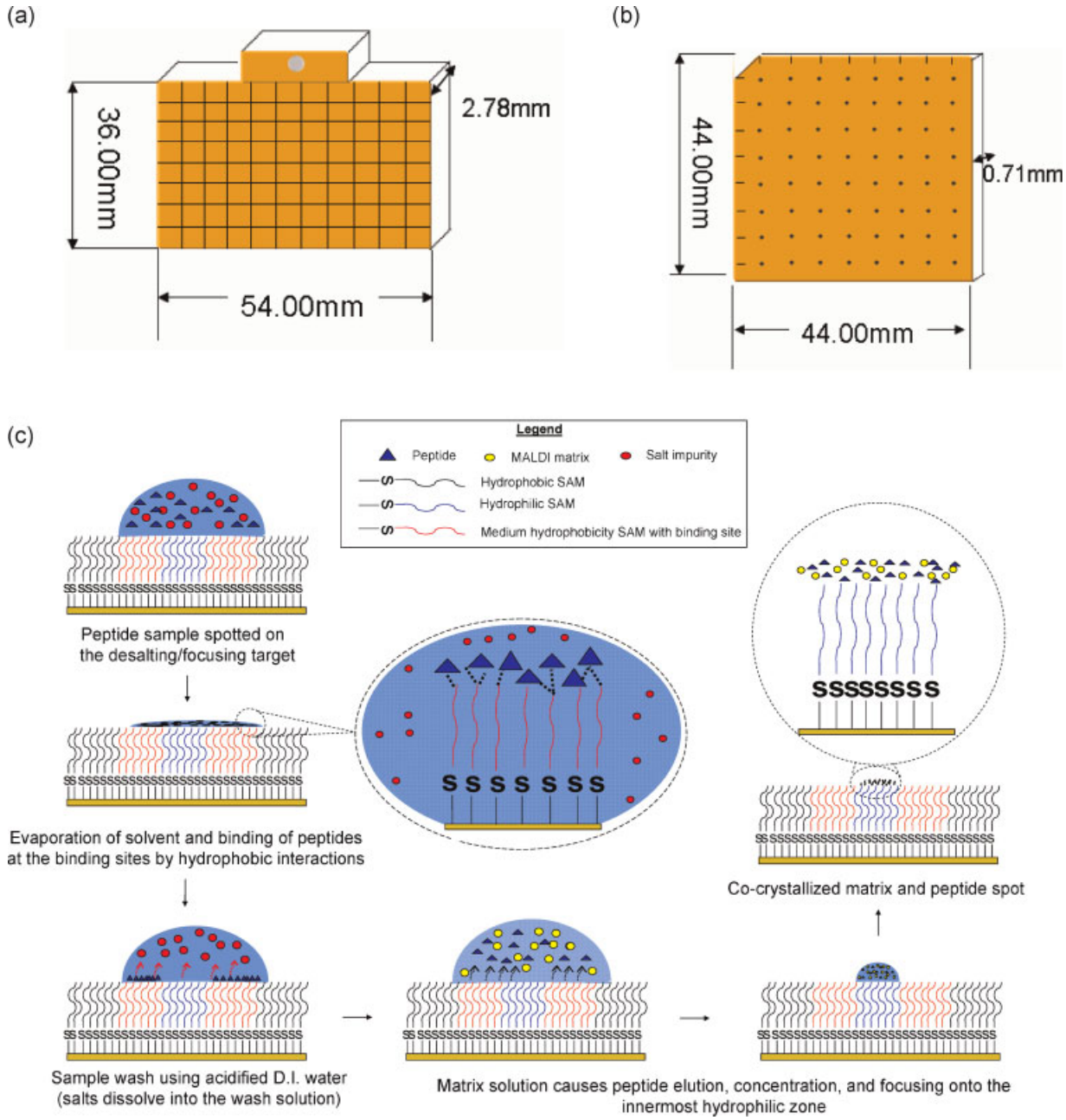

Figure 1. Schematic diagrams of (a) a conventional AP-MALDI target; (b) a focusing target, with location of the innermost sample focusing zone shown by dots; and (c) illustration of the on-chip purification and pre-concentration steps on a hydrophobic focusing plate. This figure is available in color online at www.interscience.wiley.com/journal/rcm.

Protein digestion and tryptic peptide derivatization

A myoglobin solution (1×10^{-4} M) was prepared in 50 mM ammonium bicarbonate/40% ACN, and 100 μL of this solution were incubated sequentially with (a) 5 μL of 200 mM 1,4-dithio-DL-threitol in 100 mM NH_4HCO_3 for 1 h and (b) 4 μL of 1 M iodoacetamide in 100 mM NH_4CO_3 for 20 min at room temperature. Trypsin working solution (20 μg/mL, prepared as per the manufacturer's protocol) was then added to the mixture. After 18 h incubation at 37°C, the digest was dried in a Speed-Vac (Savant Instruments Inc., Holbrook, NY, USA) and reconstituted in 60 μL of dH_2O. Guanidination of 20 μL of reconstituted digest was performed using 7 M NH_4OH and 8 M *O*-methylisourea hemisulfate solution for 10 min at 65°C.[31] A 26 μL aliquot of this solution was dried, reconstituted in 10 μL of 50:50 ACN/0.1% TFA and purified using a C18 NuTip.[32] The eluate was subsequently dried, reconstituted in 40 μL of 0.1 M ammonium bicarbonate, and N-terminal sulfonation was carried out following the procedure described by Keough *et al.*,[22] with the exception that ammonium bicarbonate instead of trimethylamine was used to maintain a pH value of 8. The derivatized digest was serially diluted and each dilution was spotted in duplicate on conventional and tuning targets using the dried-droplet method, and on hydrophobic focusing targets using the on-chip sample focusing pro-

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cedure described below. A CHCA matrix stock solution (10 mg/mL) was prepared in 84:13:3 v/v/v ACN/EtOH/5 mM ammonium citrate.

Pre-purification of mosquito head extract samples

Frozen lyophilized mosquito head extract obtained from 10 mosquito heads was dissolved in 30 μL of 50:50 ACN/0.1% TFA in dH_2O. A 10 μL sample aliquot was subjected to on-tip purification on a hydrophobic focusing target according to the following procedure: C18 NuTips were first washed with 5×10 μL 50% ACN and equilibrated with 3×10 μL 0.1% TFA. Then 30 μL of sample were aspirated and expelled through the micro-column 10 times, followed by a washing step using 2×10 μL of 0.1% TFA. Finally, elution of bound components was carried out with 5 μL of 50:50 ACN/0.1% TFA. The eluate, containing an extract equivalent to four mosquito heads, was directly deposited on a hydrophobic focusing target for on-chip pre-concentration and focusing. A second frozen lyophilized extract obtained from 10 mosquito heads was dissolved in 30 μL of 20 mM ammonium hydrogen phosphate (pH 6.4) and a 10 μL aliquot was directly spotted on a WCX focusing target for on-chip pre-concentration and focusing.

On-chip sample focusing

Variable volumes (5–20 μL) of sample solution prepared in 50:50 ACN/0.1% TFA were deposited on hydrophobic target loading sites marked by gridlines (Fig. 1(b)) and allowed to evaporate completely. Controlled evaporation was performed in a humidity chamber, with the relative humidity maintained between 45–65%. Under these conditions, a 10 μL sample required approximately 45 min to dry completely. On-chip desalting was achieved by twice depositing and subsequently removing after 2 min 10 μL of 0.1% TFA wash solution. In the last step, elution and focusing were performed simultaneously by depositing 2 μL of 0.063 mg mL^{-1} CHCA solution on the spots. The optimum matrix concentration was determined in exploratory experiments by focusing 0.1 pmol angiotensin I with different concentrations of CHCA matrix (data not shown). The average diameter of the focused sample spots measured with an optical microscope (2.5×, Olympus, USA) was 569 ± 48 μm. For WCX targets, variable volumes of samples prepared in 20 mM ammonium phosphate buffer (pH 6.6) were deposited on the sample loading sites for 30 min and rinsed prior to complete dryness. Elution and focusing were performed separately. Bound peptides were first eluted by depositing and air drying 5 μL of 50:50 ACN/0.1% TFA on the desalted spot, followed by focusing with 2 μL of CHCA matrix solution. In all cases, signal-to-noise ratio (S/N) gains were calculated by dividing the S/N obtained for the experiment performed on the focusing target by the S/N obtained with the conventional target.

AP-MALDI MS

An AP-MALDI ion source (model 611, MassTech Inc., Columbia, MD, USA) equipped with a nitrogen laser (337 nm, max. repetition rate of 10 Hz, pulse width 4 ns) and a pulsed dynamic focusing (PDF) module was mounted on an LCQ DECA XP+ quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). A detailed description of this ion source and the PDF module can be found elsewere.[15,18,33] In order to adapt the focusing targets to the AP-MALDI ion source, the original detachable plate holder was replaced by an Opti-TOF plate holder (Applied Biosystems, Foster City, CA, USA). The target layout stored in the ion source control software (MassTech) was custom-modified by adjusting the settings for sample plate geometry and target position offset parameters. For 64-well hydrophobic and 25-well WCX targets, the (X,Y) position offsets were set to (10 mm, 1 mm) and (4.7 mm, −3.50 mm), respectively. Sample spacing parameters were set to 4.5 mm (X) and 9.0 mm (Y) for both types of targets. A spiral motion velocity of 2 mm/min and a between-turn spacing of 0.08 mm were used throughout all experiments.

The target plate voltage was set to an optimum value of 3.0 kV. Optimization of laser focusing, laser alignment and ion optics settings was done by spotting 5 pmol of angiotensin I on a 'tuning target' with a geometry identical to focusing targets, but without surface functionalities. The optimum ion transfer capillary and tube lens offset voltages were 43 V and 15 V, respectively, with an inlet capillary temperature of 300°C. The skimmer-multipole 1 voltage difference was set to 60 V for enhancing declustering of matrix-analyte adducts by low-energy collisions. Other ion optic voltages were as follows: multipole 1 offset: 0.25 V, intermultipole lens voltage: −16 V, multipole 2 offset: −7.00 V, and entrance lens: −66 V. The optimum PDF module pulse delays were 23.5 μs for conventional and 17.5 μs for focusing targets. Automatic gain control (AGC) was turned off, and the ion injection time was fixed to 220 ms. Spectral data was acquired for 1 min, and averaged over the entire acquisition time (approximately 30 scans). Averaged spectra were smoothed with a 9-point Boxcar filter and exported to Origin 7.5 (OriginLab Corp., MA, USA) for baseline correction.

For protein identification experiments, ion trap data were acquired for 2 min in two sequential events. The first event was to perform a full scan of the 1300–1805 m/z range, and the second event consisted of successive MS/MS scans on five different precursor ions. Each MS/MS scan was acquired for 0.2 min and the precursor ion m/z added to the exclusion list. Other MS/MS parameters were as follows: isolation width: 5.0, normalized collision energy: 100%, activation Q: 0.25, activation time: 80 ms, minimum signal required for MS and MS^n scans: 2×10^4 and 2×10^3, respectively, exclusion mass width: 2.0, ion injection time: 500 ms, and number of microscans: 20. Raw data was directly imported into PEAKS Studio (version 4.2, Bioinformatics Solutions Inc., Waterloo, ON, Canada) for auto *de novo* followed by database searches against SwissProt. The data refinement feature of the software was used to pre-process the raw data by noise filtering, peak centroiding and merging scans of the same precursor ion. The database search mass error tolerances for precursor and fragment ions were 2.0 and 0.8, respectively. User-defined C-terminal guanidination and N-terminal sulfonation, together with the built-in cysteine carbamidomethylation, were considered as fixed peptide modifications for auto *de novo* and database searches.

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RESULTS AND DISCUSSION

Signal-to-noise ratio (S/N) gains

The focusing targets used in this work consist of gold-coated stainless steel plates with their surfaces modified by arrays of concentric rings of alkanethiol self-assembled monolayers (SAMs) (Fig. 1(b)). Depending on the nature of the terminal groups of these alkanethiols, three wettability zones, namely the outermost hydrophobic, a central moderately hydrophobic and the innermost hydrophilic zone, exist on the target surface. The diameters of the central and the innermost zones are approximately 3000 and 600 μm, respectively. The central zone can be alternatively modified with weak cation-exchange functionalities, so as to provide binding sites for retention of positively charged chemical species. A schematic depicting the typical process of on-chip desalting, pre-concentration, and focusing on a hydrophobic target is shown in Fig. 1(c). In the first step, a volume of up to 20 μL of sample is deposited without spreading due to the containment effect afforded by the large hydrophobicity of the outermost zone. Following deposition, the sample solution is given sufficient time to interact with the surface. This step allows increased retention of peptide components by their hydrophobic interactions with the binding sites on the middle zone (red). Following binding, unbound chemical species and salt impurities are washed off the surface with acidified dH_2O in the second step. Bound peptides are then eluted and co-focused with MALDI matrix onto the innermost zone. For hydrophobic targets, this step is carried out in a dynamic fashion. The liquid droplet simultaneously evaporates and migrates from the medium hydrophobicity surface area to the high wettability innermost zone.

Initial characterization studies were directed at investigating the extent to which focusing targets enhanced AP-MALDI sensitivity. Two phenomena factor into the observed S/N gains. First, the focusing targets enable the deposition of a larger sample volume than in the conventional AP-MALDI experiment, effectively resulting in a pre-concentration effect where more peptide is probed by the AP-MALDI laser. The second effect is related to the focusing of the co-crystallized matrix and analyte into a spot of a smaller diameter than what is obtained in a conventional AP-MALDI plate provided by the manufacturer. Two sets of experiments were designed to investigate these phenomena independently from each other. We first deposited variable volumes of a fixed concentration (2×10^{-7} M) angiotensin I solution to investigate the pre-concentration effect. These experiments were followed by a second set of runs where a fixed amount (1 pmol/spot) of peptide was deposited to investigate the focusing effect, independently of any pre-concentration factors. Both conventional and focusing AP-MALDI targets were used in these experiments, and S/N gains were calculated from the resulting baseline-corrected mass spectra (Figs. 2(a)–2(f)).

For experiments performed with a fixed peptide concentration per spot (Figs. 2(a)–2(c)), the observed S/N values were 0.5, 117 and 56, for conventional, hydrophobic and WCX focusing substrates, respectively. This is equivalent to S/N gains of 234 and 112 for the hydrophobic and WCX focusing targets. The observed higher absolute signal of the protonated peptide on the focusing targets (Figs. 2(b) and 2(c)) compared to that on the conventional target (Fig. 2(a)) was attributed to the fact that the improved spot homogeneity reduced the appearance of 'sweet-spots', yielding on average more ions per laser shot, along with the pre-concentration effect. The focusing targets produced mass spectra with clean baselines, devoid of matrix species such as the protonated CHCA matrix ion at m/z 190, matrix clusters and salt adducts at m/z 270.8 ($[M+Na+K+H_2O+H]^+$), 379 ($[2M+H]^+$), 442 ($[2M+Na+K+H]^+$), and 630 ($[3M+Na+K+H]^+$), commonly observed with conventional targets. This observation was partially attributed to the fact that by following the manufacturer's protocols recommended in each case, the surface matrix density was almost 10 times higher for the conventional (5.76×10^{-3} mg/mm^2) than for the focusing targets (4.95×10^{-4} mg/mm^2). Additionally, on-chip desalting minimizes the abundance of Na^+ and K^+ ions necessary for forming the above-mentioned clusters. The addition of diabasic ammonium citrate to the CHCA matrix solution further suppresses the most common matrix adducts, resulting in reduction of baseline chemical noise.[34–36]

When the CHCA concentration used for the focusing targets (0.063 mg/mL) was spotted on the conventional target, there was no signal observed for 1 pmol of angiotensin I due to insufficient matrix (data not shown). However with the 10 mg/mL CHCA concentration, peptide signal was observed with S/N of 7.8 (Fig. 2(d)). The addition of ammonium citrate did not improve the spectral quality (data not shown). This suggested that, for the conventional target, a higher matrix concentration should be used without the addition of diabasic ammonium citrate salt. Therefore, the matrix concentrations and solvent compositions used for the two types of targets were independently optimized for the subsequent sensitivity and reproducibility experiments.

The inserts in Figs. 2(b) and 2(c) show the S/N gains observed for independent experiments where 5–20 μL of sample solution were deposited on the two types of focusing targets investigated. These ranged from 223 to 931 for hydrophobic targets and from 112 to 428 for WCX targets. As compared to the hydrophobic target, lower S/N gains were observed for the WCX focusing target. This could be due to the fact that the recommended pH of the binding solution used for on-chip sample pre-concentration on WCX targets was 6.6, which is close to the pI of this peptide (6.69),[37] leading to a lower proportion of positively charged angiotensin in the investigated solution. Secondly, angiotensin I is hydrophobic (hydrophilicity value of −0.5) and hence it has high binding affinity for hydrophobic sites. This hydrophilicity value was calculated using an online tool,[38] based on the Hopps-Woods scale.[39] Regardless of the substrate type considered, the large increase in observed S/N demonstrates the main advantage of these substrates for AP-MALDI analysis.

Results for experiments with a fixed amount (1pmol/spot) of peptide are shown in Figs. 2(d)–2(f). S/N values of 7.8, 126 and 74 for conventional, hydrophobic, and WCX focusing targets were observed, respectively. This corresponds to S/N gains of 16 and 9.5 due solely to the focusing effect. The limits of detection (LODs) for angiotensin I calculated for these

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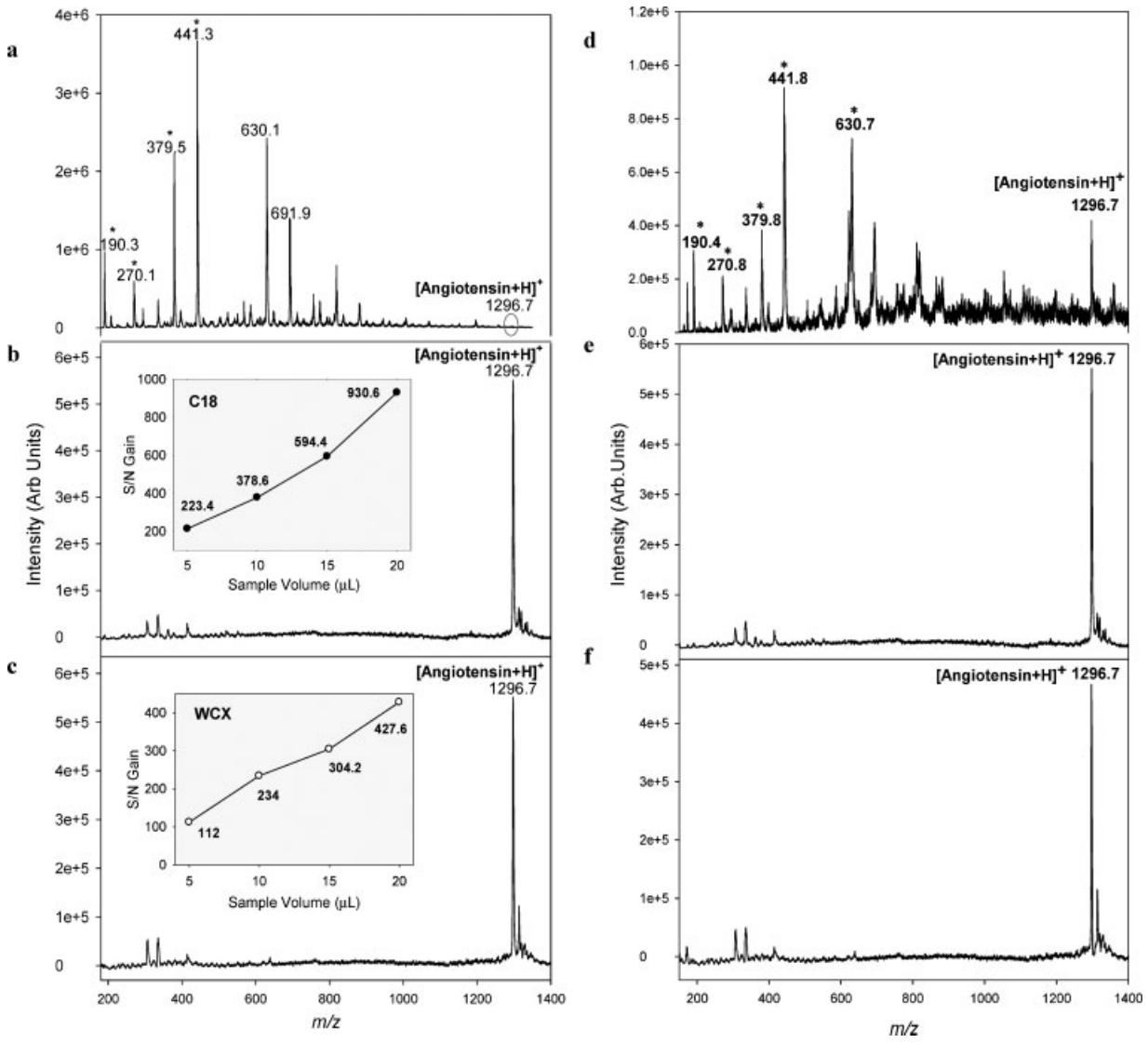

Figure 2. Left panels show spectra for 0.5 μL of 2.10^{-7} M/spot angiotensin I spotted on (a) conventional AP-MALDI target and 5 μL on (b) hydrophobic focusing and (c) WCX focusing targets. The inserts show the S/N gain as a function of sample volume deposited. The panels on the right show mass spectra corresponding to 1 pmol/spot of angiotensin I on (d) conventional AP-MALDI target, (e) hydrophobic, and (f) WCX focusing targets. Peaks corresponding to matrix adducts are denoted by asterisks.

experiments were 384 fmol/μL for conventional targets, and 5 and 8 fmol/μL for hydrophobic and WCX focusing targets, as a direct consequence of the tighter sample spots.

Dynamic range and reproducibility

Focusing targets were compared with standard targets to determine spot-to-spot variability, inter-day reproducibility and dynamic range. The observed dynamic range for conventional targets (0.1–5 pmol, Fig. 3(a)) was approximately 5 times larger than for hydrophobic and WCX focusing targets (0.075–0.5 pmol, Fig. 3(b)). The signal for hydrophobic and WCX focusing targets was observed to increase at a lower rate after ~0.7 pmol angiotensin I was deposited (Fig. 3(b)), suggesting partial saturation of the binding surface. Further loading of higher volumes of peptide solution (10–20 μL) did not increase the peptide signal in a linear fashion (data not shown). As a result of the more uniform analyte-matrix co-crystallization, the absolute signal variability measured in terms of % coefficient of variance (CV) was comparatively lower for hydrophobic (2–10%) and WCX (4–17%) focusing targets than for conventional targets (11–37%), as shown by the magnitude of the error bars in Figs. 3(a) and 3(b). Reproducibility studies performed for a binary mixture spotted onto conventional (0.1–5 pmol/spot, Fig. 3(c)) and hydrophobic (0.075–1 pmol/spot, Fig. 3(d)) focusing targets showed % CVs of 25% and 24% for the relative intensity of AS-C with respect to angiotensin I, when averaged over 3 days of measurements and the entire concentration range. Overall, the reproducibility for both absolute and relative signal intensities was found to be acceptable for the focusing targets, indicating that the manual operations involved did not significantly add to the variability of the method.

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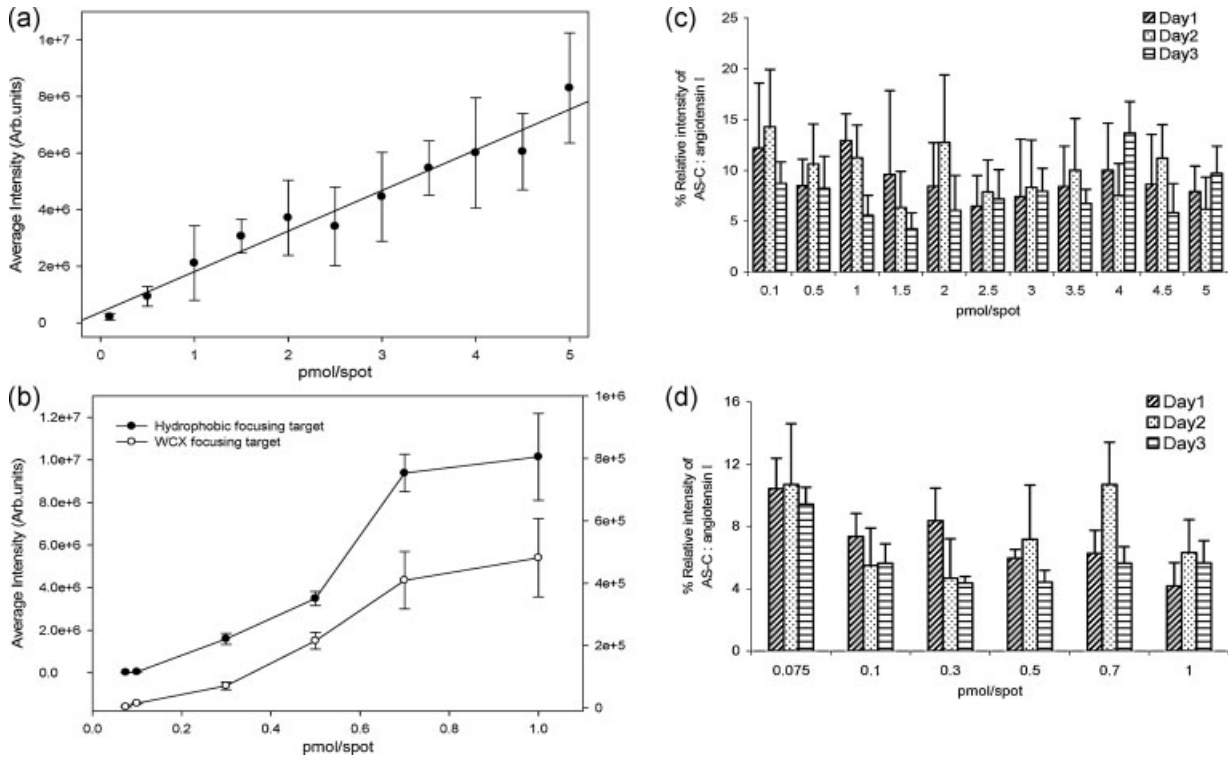

Figure 3. Average signal intensity of the protonated angiotensin I monoisotopic ion as a function of the amount of peptide spotted on (a) a conventional AP-MALDI target and on (b) hydrophobic (y-axis, left) and WCX focusing targets (y-axis, right). Variance in the % relative intensity of allatostatin-C with respect to angiotensin I as a function of the total amount of equimolar peptide mixture deposited per spot on (c) conventional and (d) hydrophobic focusing targets. Experiments were performed on three separate days.

Use of focusing substrates for AP-MALDI ITMS analysis of neuropeptides from *Aedes aegypti* mosquito heads

Allatostatins (AS) and allatotropins (AT) are structurally diverse peptides first described as modulators of juvenile hormone biosynthesis in the corpora allata of a number of insect species.[40–44] It has been recognized that AS and AT have multiple physiological effects, controlling processes such as heart rate and gut motility, control of nutrient absorption, migratory preparedness, and modulation of circadian cycle.[45–48] Profiling AS-C and AT levels in mosquitoes is therefore critical to further understand the physiological roles of these peptides. However, only a few neuroendocrine cells present in mosquito heads[49] are responsible for all of the AS-C and AT production, which is estimated to be in the 30–50 fmol range per insect,[50,51] thus presenting a challenge in terms of analytical sensitivity.

Initial attempts to analyze mosquito head extracts by AP-MALDI MS using conventional targets were unsuccessful. Therefore, we evaluated the usefulness of on-chip pre-concentration via focusing targets for analyzing mosquito head extracts by AP-MALDI MS. When crude extracts were directly spotted on focusing plates a film was found to irreversibly form on the target surface where the sample was spotted, probably due to irreversible binding of sample components, such as lipids and proteins. This film caused surface fouling resulting in highly increased wettability which translated in evaporation of the solution droplet at rates too high to enable focusing during the elution step. Previous studies by Kanari *et al*. have shown that the layer of surface-bound water on the hydrophobic SAM stimulates strong protein adsorption.[52] To further prevent surface fouling, a single pre-purification step via on-tip solid-phase extraction (SPE) was used to treat the raw mosquito head extracts prior to on-chip pre-concentration and focusing. With this approach, the pre-purified mosquito head extracts were observed to focus effectively, yielding AP-MALDI mass spectra with detectable signals (Fig. 4). Mass spectra acquired in both wide (Fig. 4(a)) and narrow (Fig. 4(b)) mass ranges using a hydrophobic focusing target showed peaks at *m*/*z* 1614.5, 1920.8, and 1936.1, corresponding to protonated AT, and two forms of AS-C, namely with and without N-terminal glutamine cyclization. These spectra correspond to material extracted from approximately four mosquito heads.

When WCX focusing targets were tested, the sample focused without the additional pre-purification step. Figure 4(c) shows the AP-MALDI mass spectrum corresponding to three mosquito heads. Two forms of protonated AS-C ions were observed in this experiment, along with peaks corresponding to unknown species not seen in the mass spectra obtained from hydrophobic focusing targets. This is most likely due to the different binding specificities of the two targets, which can thus be used to obtain complementary information. The peak at *m*/*z* 1636.8 was assigned to the AT sodium adduct while the peak at *m*/*z* 1654.8 was attributed to a water cluster with the former ionic species. Being both a hydrophobic and basic peptide (pI > 7),

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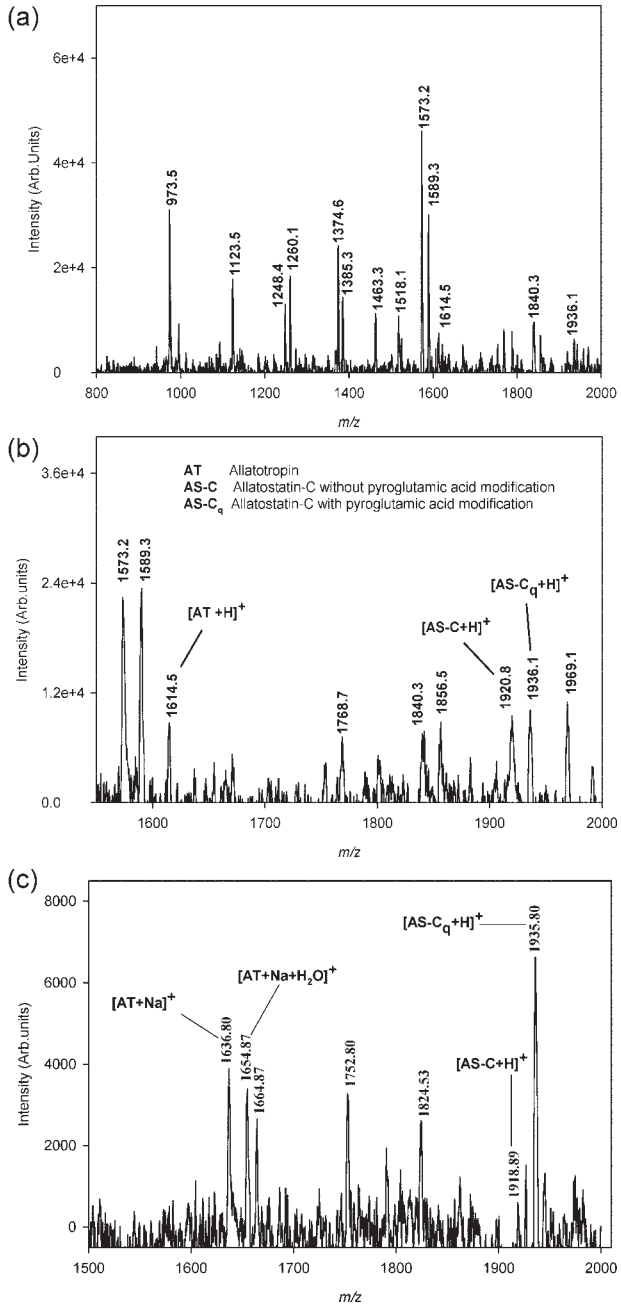

Figure 4. AP-MALDI mass spectra of desalted and focused mosquito head extracts, using hydrophobic and WCX focusing targets. The amount of extract deposited corresponded to 4 and 3 heads/spot, respectively. The mass spectra were acquired in the *m/z* ranges (a) 800–2000 and (b) 1550–2000 for hydrophobic targets and in (c) *m/z* range 1500–2000 for the WCX targets.

AS-C was also detected on the WCX target (Fig. 4(c)). Despite the improvements in sensitivity enabled by the use of focusing targets, the observed gains were not sufficient for performing MS/MS experiments. However, it is expected that by coupling of focusing chips and AP-MALDI to a higher trapping capacity mass analyzer such as a linear ion trap, or by performing AP-MALDI analysis with higher repetition rate lasers now available, sensitivity could be further improved to a point where neuropeptide MS/MS would be possible for these challenging samples.

Use of focusing substrates for enhanced protein identification by AP-MALDI

As a second demonstration of the sensitivity enhancement that focusing chips afford for AP-MALDI MS, we tested their performance for protein identification using dilute tryptic digests of a model protein. Automated data-dependent

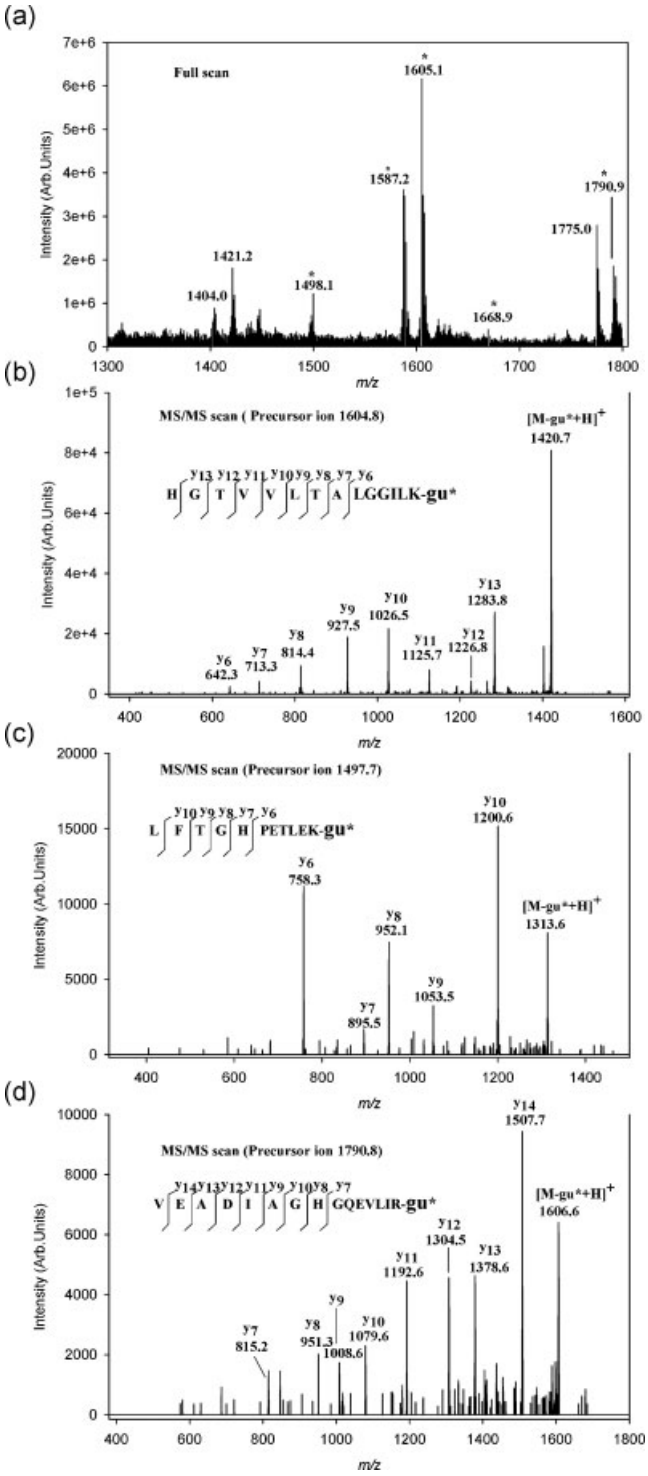

Figure 5. (a) Full scan AP-MALDI mass spectra for 100 fmol derivatized myoglobin digest, spotted on a hydrophobic focusing plate (5 μL) with precursor ions selected for MS/MS experiments marked by asterisks and MS/MS scans for guanidinated and sulfonated peptide precursor ions at (b) *m/z* 1604.8, (c) *m/z* 1497.7, and (d) *m/z* 1790.8.

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acquisition (DDA) was performed on 2×10^{-6} M, 2×10^{-7} M, and 2×10^{-8} M solutions of a derivatized myoglobin digest. A volume of 5 μL of each digest dilution was deposited on the hydrophobic focusing target and 0.5 μL on conventional and tuning targets. Figure 5(a) shows a typical full scan AP-MALDI mass spectrum obtained for a 2×10^{-8} M derivatized myoglobin digest spotted on a hydrophobic focusing target. The five selected guanidinated-sulfonated peptide precursor ions are denoted by asterisks. Subsequent MS/MS spectra for three of the five pre-selected precursors with protonated precursor ions at *m/z* 1604.8, 1497.7, and 1790.8 are shown in Figs. 5(b)–5(d). Fragmentation of precursor ions produced guanidinated (gu*) peptide ions $[M–gu^*+H]^+$ by loss of O_3S-C_6H_5-CO (184 Da) and a series of high intensity y-ions. It was noted that y-ions smaller than y_5 were not observed for any of the three types of targets. We believe that the second, more mobile proton in these derivatized peptide ions tends to populate carbonyl groups further away from the positively charged guanidinated C-terminus due to charge repulsion.[24] This effect, together with the limited energy imparted during ion trap collisional activation, limits the extent of fragmentation. In the case of FTGHPEILEK, fragmentation N-terminal to proline is also favored.[22,53] No b-ions were observed, as, when the charge is retained in the N-terminus, the negatively charged SO_3 group neutralizes the charge of the fragment ion.

Table 1 shows the PEAKS protein identification scores obtained when the AP-MALDI mass spectral data obtained from different substrates and various dilutions was subjected to database searching. All three concentrations deposited on the hydrophobic focusing target, with the lowest (2×10^{-8} M) containing 100 fmol digest, yielded the correct protein identification. For conventional and tuning targets, only the highest concentration (2×10^{-6} M) was positively matched. The quality of the MS/MS spectra obtained from lower concentrations was extremely poor for conventional and tuning targets. The amounts of digest spotted on these two targets were 100 fmol (0.5 μL, 2×10^{-7} M) and 10 fmol (0.5 μL, 2×10^{-8} M) per spot. As a result, most of the fragment peaks were removed during auto noise filtration by the database search software. Again, these results show that focusing targets improved AP-MALDI sensitivity, increasing the quality of MS/MS spectra and protein identification scores. Additionally, focusing targets enabled shorter acquisition times (2 min), which compares favorably to previous reports that used acquisition times of 5–7 min.[54,55]

Table 1. Comparison of PEAKS protein identification scores for derivatized myoglobin tryptic digests spotted on focusing (hydrophobic), conventional, and tuning targets

Concentration of derivatized digest	Hydrophobic focusing target		Conventional AP-MALDI target		Tuning target	
	Spot 1	Spot 2	Spot 1	Spot 2	Spot 1	Spot 2
2×10^{-6} M	61	79	56	59	54	33
2×10^{-7} M	55	51	0	0	0	0
2×10^{-8} M	45	30	0	0	0	0

CONCLUSIONS

Focusing targets with unique surface properties were successfully implemented in an AP-MALDI ion source. Improvements in sensitivity with S/N gains of approximately 200–900 and 100–430 were obtained for peptide standards using hydrophobic and WCX focusing targets, respectively. The use of focusing targets allowed successful detection of neuropeptides in extracts from as low as three mosquito heads, and to identify proteins in nanomolar concentrations. The improved sensitivity was a direct consequence of the on-chip pre-concentration effect, which enabled deposition of larger sample volumes on the MALDI plate, and the subsequent elution onto tightly focused spots.

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