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Capillary-Scale Frontal Affinity Chromatography/ MALDI Tandem Mass Spectrometry Using Protein-Doped Monolithic Silica Columns

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Frontal affinity chromatography (FAC) interfaced with electrospray mass spectrometry (ESI-MS) has been reported as a potential method for screening of compound mixtures against immobilized target proteins. However, the interfacing of bioaffinity columns to ESI-MS requires that the eluent that passes through the protein-loaded column have a relatively low ionic strength to produce a stable spray. Such low ionic strength solvents can cause serious problems with protein stability and may also affect binding constants and lead to high nonspecific binding to the column. Herein, we report on the interfacing of bioaffinity columns to matrix-assisted laser desorption/ ionization (MALDI) MS/MS as a new platform for FAC/ MS studies. Capillary columns containing a monolithic silica material with entrapped dihydrofolate reductase were used for frontal affinity chromatography of small-molecule mixtures. The output from the column was combined with a second stream containing α-cyano-hydoxycinnamic acid in methanol and was deposited using a nebulizer-assisted electrospray method onto a conventional MALDI plate that moved relative to the column via a computer-controlled x−y stage, creating a semipermanent record of the FAC run. The use of MALDI MS/MS allowed for buffers with significantly higher ionic strength to be used for FAC studies, which reduced nonspecific binding of ionic compounds and allowed for better retention of protein activity over multiple runs. Following deposition, MALDI analysis required only a fraction of the chromatographic run time, and the deposited track could be rerun multiple times to optimize ionization parameters and allow signal averaging to improve the signal-to-noise ratio. Furthermore, high levels of potential inhibitors could be detected via MALDI with limited ion suppression effects. Both MALDI- and ESI-based analysis showed similar retention of inhibitors present in compound mixtures when using identical ionic strength conditions. The results show that FAC/MALDI-MS should provide advantages over FAC/ESI-MS for high-throughput screening of compound mixtures.

Bioaffinity chromatography has been widely used for sample purification and cleanup,[1] chiral separations,[2] on-line proteolytic digestion of proteins,[3] development of supported biocatalysts,[4] and more recently screening of compound libraries via the frontal affinity chromatography (FAC) method.[5,6] The basic premise of FAC is that continuous infusion of a compound will allow for equilibration of the ligand between the free and bound states, where the precise concentration of free ligand is known. In this case, the breakthrough time of the compound will correspond to the affinity of the ligand for the immobilized biomolecule—ligands with higher affinity will break through later.

The detection of compounds eluting from the column can be accomplished using methods such as fluorescence,[7] radioactivity,[6] or electrospray mass spectrometry.[5] The former two methods usually make use of either a labeled library or use a labeled indicator compound, which competes against known unlabeled compounds, getting displaced earlier if a stronger binding ligand is present. However, in each case, the methods have limited versatility owing to the need to obtain labeled compounds, and the need for prior knowledge of compounds used in the assay,

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since no structural information is provided by the detector. Hence, fluorometric and radiometric methods tend to be useful only for analysis of discrete compounds.

Interfacing of FAC to ESI-MS, on the other hand, has proven to be a very versatile method for screening of compound mixtures.[5] Use of MS, and in particular MS/MS detection, provides the opportunity to obtain structural information on a variety of compounds simultaneously. In cases where the identity of compounds in the mixture is known, the analytes can be detected simultaneously and in a quantitative manner using the multiple reaction monitoring (MRM) mode, improving the throughput of the method. While this unique aspect of the FAC/MS technique has been touted as a major advantage for applications such as high-throughput screening of compound mixtures,[5,8] there are some potential disadvantages that arise as a result of the use of electrospray ionization for introduction of compounds into the mass spectrometer. For example, obtaining a stable electrospray requires the use of a low ionic strength eluent, which in some cases can be incompatible with maintaining the activity of the proteins immobilized in the column.[9] Low ionic strength can also lead to an ineffective double layer, which can cause significant nonselective binding through electrostatic interactions of compounds with the silica column. Furthermore, only one mode of analysis is possible per chromatographic run when using ESI-MS. Finally, high levels of analytes can lead to large ion currents in the electrospray, which can lead to ion suppression.[10]

To overcome these issues, it is advantageous to decouple the chromatography and mass spectrometry by performing the mass spectrometric detection step off-line. This is most easily achieved by coupling liquid chromatography to matrix-assisted laser desorption/ionization (MALDI) MS. The general approach is to deposit the LC effluent onto a MALDI target, followed by MALDI-MS analysis. Deposition can be done either as discrete spots or as a continuous track using a variety of methods, including the following: fraction collection followed by MALDI deposition;[11] direct deposition of spots[12,13,14] or tracks[15,16] from the capillary; electrodynamic charged droplet processing;[17] deposition using a heated droplet interface;[18] piezoelectric flow-through microdispensing;[19,20] vacuum-assisted deposition;[21] electric field driven droplet deposition;[22] electrospray deposition;[23] or capillary nebulizer spraying.[24,25] Relative to ESI, MALDI analysis has the advantages of higher tolerance to buffers, lower sample consumption per analysis and reduced analysis time.[18] Separation of the LC and MS steps also allows independent optimization of the MS detection parameters for each analyte.

In this work, we describe the integration of FAC, using newly developed sol−gel-derived monolithic bioaffinity columns,[9] with MALDI-MS/MS detection, and compare the operation to FAC-ESI-MS/MS by examining the ability of small enzyme inhibitors to interact with entrapped dihydrofolate reductase (DHFR) using elution at different ionic strengths. The interfacing involves mixing the column effluent with a suitable matrix followed by continuous nebulizer-assisted electrospray deposition of the mixture onto a MALDI plate that is present on a computer controlled x−y translation stage. The chromatographic trace is deposited semi-permanently onto the MALDI plate, allowing for subsequent analysis by MALDI-MS/MS. By scanning the laser over the tracks deposited by the column while monitoring the eluted compounds in MRM mode, the frontal chromatogram can be reconstructed directly to obtain breakthrough curves for each analyte. We show that MALDI-MS/MS has a number of benefits relative to ESI-MS/MS as a detection method for FAC, including the following: better tolerance to high ionic strength elution buffers, which helps maintain the activity of the protein in the column and reduce nonspecific binding; the ability to acquire multiple MS scans from a single plate in a matter of minutes following the FAC run; and the ability to detect high levels of potential inhibitors with limited ion suppression effects. The results show that FAC/MALDI-MS should be well suited for high-throughput screening of compound mixtures.

EXPERIMENTAL SECTION

Chemicals. Tetraethyl orthosilicate (TEOS, 99.999%) and (3-aminopropyl)triethoxysilane (APTES) were obtained from Aldrich (Oakville, ON, Canada). Diglycerylsilane precursors were prepared from TEOS as described elsewhere.[26] Trimethoprim, pyrimethamine, folic acid, poly(ethylene glycol) (PEG/PEO, MW 10 000), and fluorescein were obtained from Sigma (Oakville, ON, Canada). MALDI matrix solution (6.2 mg/mL α-cyanohydoxycinnamic acid, CHCA, in methanol) was obtained from Agilent (Part No. G2037A). Recombinant dihydrofolate reductase (from *Escherichia coli*), which was affinity purified on a methotrexate column, was provided by Professor Eric Brown (McMaster University).[27] Fused-silica capillary tubing (250-μm i.d., 360-μm o.d., polyimide coated) was obtained from Polymicro Technologies (Phoenix, AZ). All water was distilled and deionized using a Milli-Q synthesis A10 water purification system. All other reagents were of analytical grade and were used as received.

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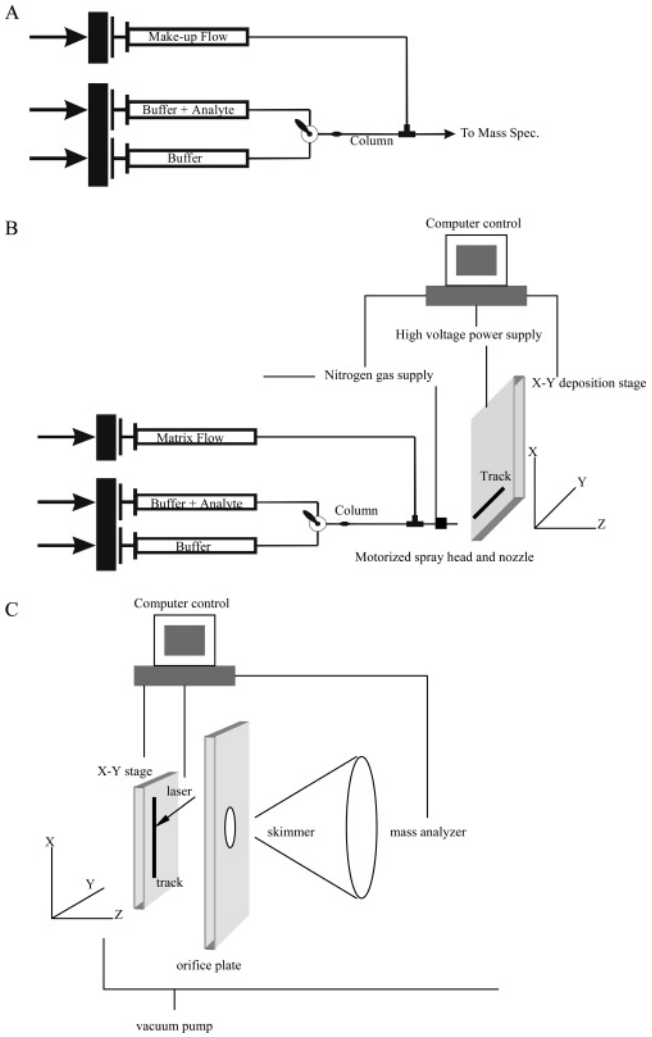

Figure 1. Schematic of the apparatus used for FAC/MS. (A) Apparatus used for FAC/ESI-MS/MS: A switch valve is used to switch from buffer to buffer + analyte, allowing continuous infusion of analytes onto the column. The column outlet is connected to a mixing tee for addition of makeup buffer that flows directly into the PE/Sciex API 3000 triple-quadrupole mass spectrometer. (B) Apparatus used for FAC/MALDI-MS/MS: The column outlet is connected to a mixing tee for addition of MALDI matrix solution that flows directly into the nebulizer to allow spraying of the mixture onto a MALDI plate that is moved under the column outlet on a computer controlled X−Y translation stage. (C) The vacuum-based oMALDI ion source assembly of the API 4000, which places the MALDI sample plate within the region evacuated by the interface vacuum pump in an orientation orthogonal to the analyzer axis.

Instrumentation. FAC/MS System. The system used for FAC/ESI-MS studies is shown in Figure 1a. Syringe pumps (Harvard Instruments model 22) were used to deliver solutions, and a flow-switching valve was used to toggle between the assay buffer and the solution containing the compound mixture. This solution was then pumped through the column to achieve equilibrium. Effluent was combined with a suitable organic modifier to assist in the generation of a stable electrospray and detectability of the sprayed components using a triple-quadrupole MS system (PE/Sciex API 3000). A Rheodyne 8125 injector valve was used to switch from buffer to buffer + analyte streams during operation. Columns were interfaced to the FAC system using Luer capillary adapters (Luer Adapter, Ferrule and Green Microtight Sleeve from Upchurch (P-659, M-100, F-185X)). All other connections between components were achieved using fused-silica tubing.

Instrumentation for FAC/MALDI-MS/MS is shown in Figure 1b. For deposition onto the MALDI plate, column effluent was mixed in a 1:1 volume ratio with CHCA MALDI matrix in methanol flowing at 5 μL/min. The resulting total flow was then deposited onto MALDI plate(s) using a continuous deposition process. In the present experiment, a custom-built nebulizer-assisted electrospray system was used to deposit a track onto an Applied Biosystems MALDI sample plate (Opti-TOF system) mounted on a computer controlled X−Y translation stage. The translation stage is a part of a three-axis positioning system consisting of a 404 series axis, Aries controllers, and ACR PCI control card from Parker Hanifin and Compumotors, respectively, that controls the deposition motion in the $X-Y$ plane and sprayer separation from the MALDI plate along the Z axis. All three axes as well as application of high voltage (custom-built digitally controlled high-voltage power supply, 4 kV) and nebulizer gas flow (Clippard minimatics valve ET-2M) are controlled from a single Dell Precision 340 computer through the ACR control card. The column flow is combined with CHCA makeup flow in a stainless steel Tee junction from Valco. The combined flow is carried by fused-silica tubing (200 μm/100 μm o.d./i.d.) passing through a stainless steel electrode, which itself is inside a nebulizer. Both the fused-silica and stainless steel electrode protrude slightly (1 mm) from the nozzle (0.6-mm i.d.). A mixing Tee is used to mount the nebulizer and introduce the N_2 gas into it. Both the electrospray voltage and nebulizer gas flow are manually adjusted and digitally actuated.

Deposition parameters, including distance of the sprayer above the plate, nebulizer gas flow, and electric field, were optimized to obtain maximum track homogeneity and minimum track width. The translation speed with which the plate was moved under the deposition tip was also optimized to provide optimum track thickness while maintaining the necessary chromatographic resolution. The optimal height of the electrospray tip was 8 mm above the sample plate, while a combination of gas flow (nitrogen at 1.5 L/min) and electric field (3 kV between the electrospray tip and MALDI plate) was used to deposit the traces. For this work, the MALDI plate was moved at 0.2 mm/s relative to the stationary deposition tip.

The deposited plates were analyzed using an AB/Sciex API 4000 triple quadrupole mass spectrometer equipped with an AB/Sciex oMALDI ion source and high repetition rate (1.4 kHz) PowerChip NanoLaser (355 nm) from JDS Uniphase. The vacuum-based oMALDI ion source replaces the normal orifice/interface assembly of the API 4000 and its Turbo V source, thus placing the MALDI sample plate within the region evacuated by the interface vacuum pump in an orientation orthogonal to the analyzer axis, as shown in Figure 1c. Normal source parameters were used to setup and control the oMALDI ion source. Within the source, the MALDI plate is held on an X−Y translation stage in front of an orifice and skimmer that separate it from the analyzer. The modified API 4000 retains its full capability of scan modes and scan speeds. During MALDI analysis, the deposited track (plate) was moved relative to the desorbing laser beam at a constant speed of 3.8 mm/s by the MALDI source X−Y stage,

unless otherwise stated. The desorbing laser beam was focused to a 180 × 230 μm spot on the track surface.

Procedures. Preparation of Columns. Macroporous silica columns containing entrapped DHFR were prepared as described in detail elsewhere.[9] Briefly, 250-μm-i.d. capillaries were first coated with a layer of APTES to promote electrostatic binding of the monolithic silica column. Silica sols were prepared by first mixing 1 g of DGS (finely ground solid) with 990 μL of H_2O to yield ~1.5 mL of hydrolyzed DGS, after 15−25 min of sonication. A second aqueous solution of 50 mM HEPES at pH 7.5 was prepared containing 16% (w/v) PEO (MW 10 000) and 0.6% (v/v) APTES. This aqueous solution also contained ~20 μM DHFR. A 100-μL sample of the buffer/PEG/APTES/DHFR solution was mixed with 100 μL of hydrolyzed DGS, and the mixture was immediately loaded via syringe pump into a fused-silica capillary (~2 m long). The final composition of the solution was 8% w/v PEO (10 kDa), 0.3% v/v APTES, and 10 μM DHFR in 25 mM HEPES buffer. The mixture became cloudy due to spinodal decomposition (phase separation) over a period of 1−3 s about 2−3 min prior to silica polymerization (~10 min) to generate a hydrated macroporous monolithic column containing entrapped protein. After loading of the sol−gel mixture, the monolithic columns were aged for 2−5 days at 4 °C and then cut into 5-cm lengths before use. The columns had an initial loading of 25 pmol of active DHFR in 5 cm, of which −6 pmol was active and accessible in the column.[9]

FAC/MS Studies. Typical FAC/MS experiments involved infusion of mixtures of compounds containing 50 nM concentrations of each compound, including *N*-acetylglucosamine, or fluorescein, both as void markers, folic acid (micromolar substrate), and pyrimethamine and trimethoprim (nM inhibitors). Before the first run, the column was flushed with 50 mM NH_4OAc buffer (pH 6.6, 100 mM NaCl) for 30 min at a flow rate of 5 μL·min^{-1} to remove any glycerol and nonentrapped protein and then equilibrated with 0−100 mM NH_4OAc for 30 min at 5 μL·min^{-1}. All compounds tested were present in 0−100 mM NH_4OAc and were delivered at a rate of 5 μL·min^{-1} using the syringe pump. The makeup flow (used to assist in the generation of stable electrospray ionization) consisted of methanol containing 10% (v/v) NH_4OAc buffer (2 mM) and was delivered at 5 μL·min^{-1}, resulting in a total flow rate of 10 μL·min^{-1} entering the ESI mass spectrometer. For MALDI, the makeup flow was replaced with a flow of matrix (CHCA 6.2 mg/mL in methanol) at 5 μL·min^{-1}. The ESI mass spectrometer was operated in MRM mode with simultaneous detection of m/z 222 → 204 (*N*-acetylglucosamine CE 15 eV); m/z 249 → 233 (pyrimethamine CE 42 eV); m/z 291 → 230 (trimethoprim CE 35 eV); m/z 333 → 202 (fluorescein CE 100 eV), and m/z 442 → 295 (folic acid CE 15 eV). MALDI MS/MS analysis was also performed using MRM scan mode but due to fragmentation during the MALDI desorption process the transitions for *N*-acetylglucosamine and folic acid were changed to m/z 204 → 138 (CE 18 eV) and m/z 295 → 176 (CE 30 eV).

The much shorter analysis times achievable with MALDI makes necessary a reduction in signal accumulation bin duration (dwell time) in order to maintain sufficient sampling frequency. The ESI-based MRM analysis used 1000-ms dwell while the MALDI MRM dwell was reduced to 40 ms per transition. Hence, when comparing steady-state MRM signal variation between the two ionization methods, the higher noise levels of the MALDI signal are due to an increase in normal statistical variation of the accumulated counts, a side effect of the reduced dwell, and due to variation in homogeneity of the track (ESI samples a small fraction of the spray that is stable in time while the plate captures all analyte including any temporal variations and variations in drying/crystallization).

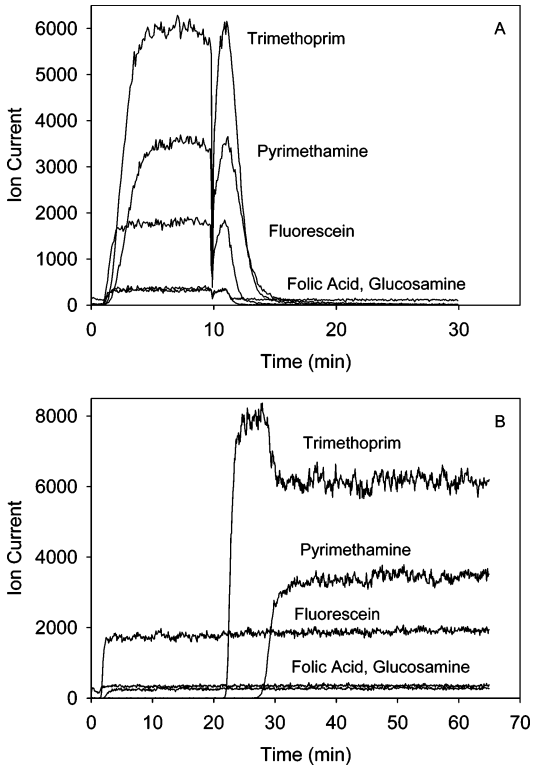

Figure 2. Typical FAC/ESI-MS/MS traces obtained using protein-loaded and blank DGS/PEO/APTES monolithic columns. Panel A, blank column containing no protein; panel B, column containing 25 pmol of DHFR (initial loading). *N*-Acetylglucosamine, fluorescein, folic acid, pyrimethamine, and trimethoprim were infused at 50 nM. Traces show actual ion currents to provide a clearer indication of the ion suppression effect.

DHFR Stability in Ammonium Acetate. DHFR was diluted to 40 nM in 2 or 100 mM ammonium acetate, (which contained 3 μM HEPES and 2 μM NaCl) and was incubated for various periods of time up to 24 h. At specified intervals, 100-μL aliquots were mixed with 100 μL of a solution containing 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 100 μM NADPH, and 100 μM DHF. DHFR activity was measured by monitoring the decrease in absorbance at 340 nm using a Tecan Safire microplate reader. Activity data are reported relative to the activity obtained from a fresh DHFR sample that was diluted in 50 mM Tris-HCl, pH 7.5, containing 2 mM DTT.

RESULTS

FAC/ESI-MS/MS. Figure 2 shows FAC/ESI-MS/MS traces obtained for elution of mixtures of DHFR inhibitors and control compounds through DGS/PEO/APTES columns containing no protein (panel A) or an initial loading of 25 pmol of active DHFR (panel B). The blank column shows the expected breakthrough of all compounds in the first few minutes (between 1 and 4 min), although both pyrimethamine and trimethoprim, which are cationic, are retained slightly longer than the anionic compounds

fluorescein and folic acid. The retention, which is present when 2 mM ammonium acetate buffer is used, is indicative of nonselective interactions between the cationic compounds and the anionic silica column, showing that normal-phase silica chromatography is not fully suppressed at low ionic strength. Panel B shows significant retention of the two DHFR inhibitors, trimethoprim ($K_d = 4$ nM, elution time of 22 min) and pyrimethamine ($K_d = 45$ nM, retention time 28.5 min), less retention of a weak substrate (folic acid, $K_d = 11$ μM, retention time 3 min), and no retention of nonselective ligands (fluorescein, *N*-acetylgluconamide, retention time 1.5 min) on DHFR-loaded columns. This result indicates that DHFR is active when entrapped in the column, in agreement with recent results from our group showing good activity of DHFR when entrapped in DGS derived materials.[9,28] An interesting aspect of the ESI-MS/MS derived chromatogram is the large reduction in ion current for trimethoprim upon elution of pyrimethamine. Such effects have previously been associated with a "roll-up" phenomenon, wherein stronger binding compounds bump off weaker binders, causing a transient overconcentration of the weaker binding ligand.[5] However, in the present case, the loss in ion current is not due to a roll-up effect, but rather is due to suppression of the trimethoprim ion current, which is prevalent at the concentration of inhibitor used in this study (50 nM). Previous FAC/ESI-MS/MS studies using these compounds at lower levels (20 nM) did not show such an effect.[9] The ion suppression effect is further confirmed by FAC/MALDI-MS/MS data that are presented below.

The reversal in the expected elution times for trimethoprim and pyrimethamine (based on their respective K_d values) has been reported previously[9] but is not fully understood at this time. We suspect that this phenomenon may be related to differences in on and off rates, which are likely to play a significant role in determining the overall retention time of compounds on the column. This is being examined in further detail and will be discussed in a future article.

FAC/MALDI-MS/MS. Optimization of MALDI MRM Transitions. A useful feature of offline MS analysis by MALDI is the ability to rerun sample tracks multiple times to allow different MS data to be acquired, which allows for optimization of MRM parameters. Figure 3 shows a MALDI Q1 spectrum of a mixture of the four target analytes (folic acid, pyrimethamine, trimethoprim, fluorescein) after appropriate background subtraction to reduce CHCA background signals. Peaks are evident for each of the four compounds; however, the primary peak for folic acid occurs at m/z 295 rather than at m/z 442, indicative of a fragment ion being the primary species present for this compound. Focusing on folic acid, product ion scans obtained from the same track using the m/z 295 parent ion clearly show a maximum peak at m/z 176 with an intensity of 5.5×10^5 counts/s. The most abundant product ion obtained from the m/z 442 parent ion was only 15% as intense as the m/z 295 → 176 ion pair. This is in contrast to the case for ESI, where the m/z 442 → 295 transition showed maximum intensity, and highlights the importance of being able to optimize MRM transitions directly on the MALDI plate.

Optimization of MALDI Parameters. Analysis of the deposited tracks on the MALDI plate show that the typical track width obtained using our deposition parameters was ~2.5 mm. The spot size of the laser was 180 × 230 μm, which generally lead to the burn track being ~10% the width of the deposited track. The utilization of only a small amount of the deposited sample during MALDI acquisition offers an advantage in method setup, where MRM selection and analyzer optimization can be achieved with a fraction of the sample (~10 pg) as compared to ESI (~100 pg), through track/spot rerunning.

A question that arises is the number of times that a particular region of a track can be rerun, as this determines how to best utilize the ability to rerun an already sampled portion of the track and hence increase the efficiency of the detection process. The number of times a track can be rerun depends on the laser fluence and the speed with which the laser is translated over the sample. The laser fluence used for the MALDI process was set to 3 μJ/pulse. This value optimized the signal-to-noise ratio while minimizing thermal degradation of the track surface, thus allowing maximum sample utilization. The effect of sampling speed on the number of possible reruns over the same region of the track is shown in Figure 4. It is clear that sample consumption depends on the speed with which laser traverses the track, with greater speed causing less sample consumption and allowing more reruns. The maximum speed of the MALDI source stage (3.8 mm/s) allows ~30 reruns prior to sample exhaustion occurring in a given region of the track, where the majority of the signal is desorbed during the first 15 passes. However, since only a small portion (~10%) of the total track is sampled, it is likely that up to 7−8 different regions could be sampled per track, and thus in practice a single deposited track could be sampled over 100 times. Varying the laser translation speed through the values allowed by the source (0.5, 1, and 3.8 mm/s) shows that there is a significant increase in the maximum signal intensity at slower speeds but a decrease in the number of reruns that can be done. Thus, the total signal obtained by complete exhaustion of a given sample region remained relatively constant and independent of the speed with which the data were generated. Using the high translation speed offers the fastest acquisition of an interpretable signal, hence maximizing the throughput.

The analyte/matrix ratio was also varied in the range of 3:1−1:3 (v/v) to achieve optimum detection for the four compounds. The results, expressed as signal over background per unit of analyte, are summarized in Table 1. It is clear that the optimum ratio is compound specific. However, use of the 1:1 (v/v) ratio offers the best compromise between overall sensitivity and ability to detect all compounds. Indeed, detection of fluorescein was possible only at a 1:1 analyte/matrix ratio, as the matrix background for the m/z 333 → 202 transition was extremely high and overwhelmed the fluorescein signal at other analyte/matrix ratios. It has also been observed that MALDI performance at higher buffer concentration improves with slightly higher CHCA content, which may improve both crystallization and competition for charge.

FAC/MALDI-MS/MS Analysis. Figure 5 shows the FAC traces obtained upon desorption from MALDI plates onto which the eluent from either blank (Figure 5A) or DHFR columns (Figure 5B) had been deposited using 2 mM ammonium acetate as the running buffer. In Figure 5A, the compounds elute in the first two traces that are deposited onto the MALDI plate (arrows

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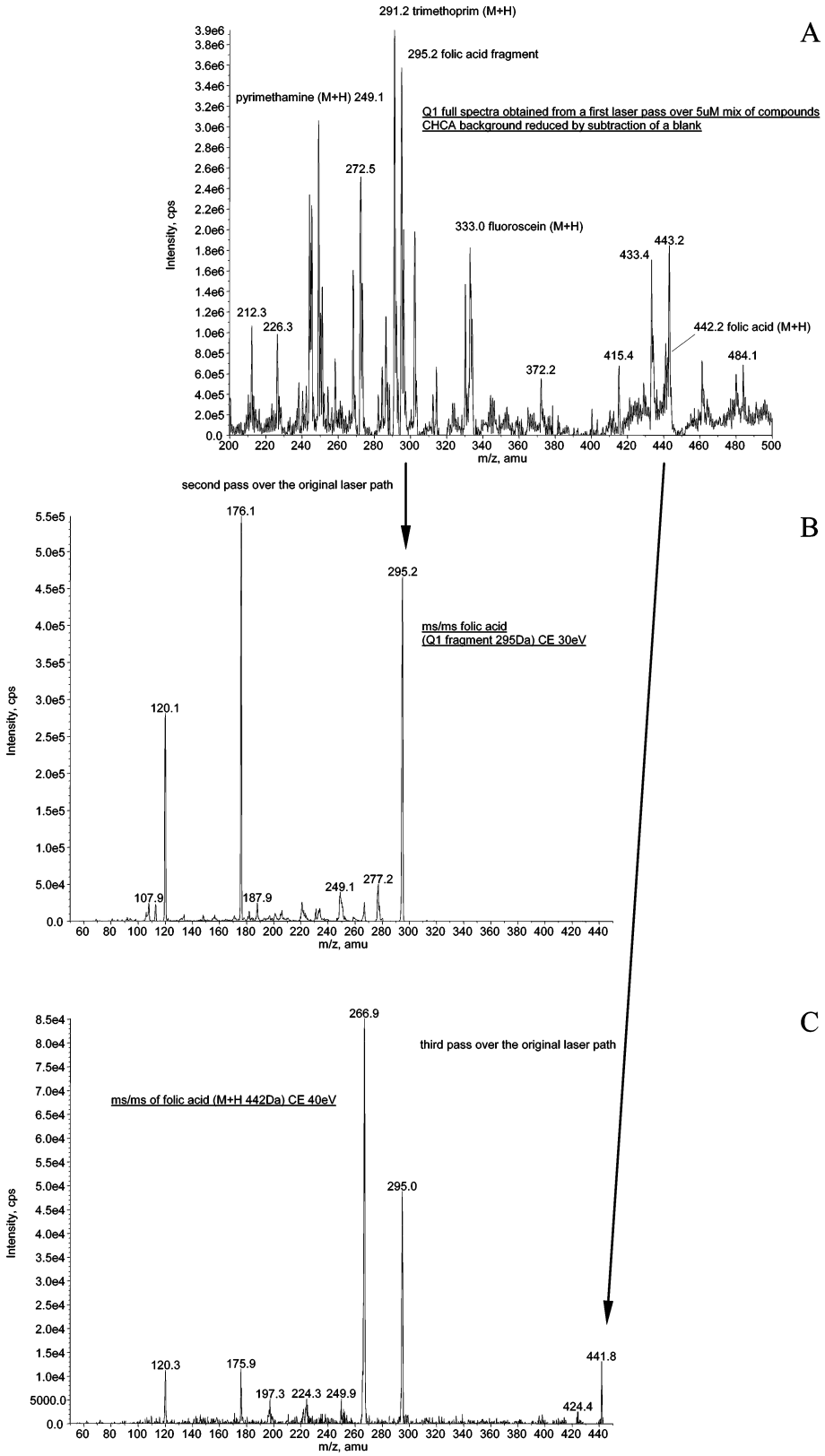

Figure 3. Multipass selection of MRM transition for folic acid. Panel A shows a Q1 full scan of a mixture of folic acid, fluorescein, pyrimethamine, and trimethoprim (50 nM each) mixed 1:1 (v/v) with 6.2 mg/mL CHCA in MeOH and deposited on a MALDI plate. The Q1 spectrum has had background signals originating from the matrix removed by subtraction. Panel B shows the Q3 product ion scan originating from the *m*/*z* 442 parent ion. Panel C shows the Q3 product ion scan originating from the *m*/*z* 295 parent ion. All scans were obtained using medium laser translation speed (1 mm/s) and are the average of 5 reruns over a given sample region.

show the traces that have been analyzed). The bottom scale of Figure 5 shows MALDI analysis time, which can be converted into LC elution time using the ratio of deposition speed to laser readout speed as a multiplication factor, which is 19 in this case.

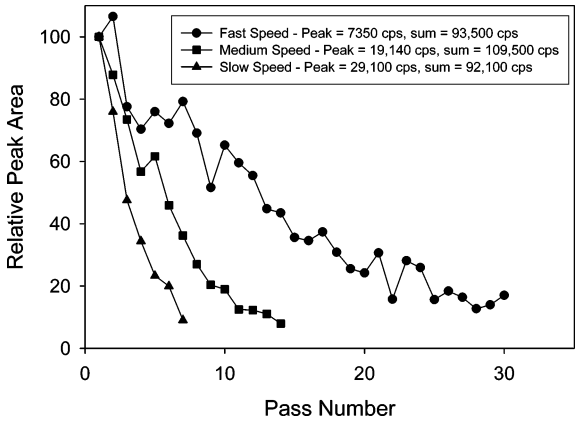

Figure 4. Signal intensity as a function of number of reruns of a given region for slow (0.5 mm/s, ▲), medium (1 mm/s, ■), and fast (3.8 mm/s, ●) translation speeds. The peak value refers to the number of counts obtained for the first pass over the track; the sum value refers to the total number of counts obtained from all runs over a given track at a particular speed.

Table 1. Effect of Analyte/Matrix Ratio on Signal above Background per Unit of Analyte

CHCA vol added to a unit vol of analyte	total signal (counts) above background signal per unit of analyte			
	folic acid	trimethoprim	pyrimethamine	fluorescein
0.333	81 659	38 619	17 422	0
1.0	49 082	39 611	15 100	13 431
3.0	26 827	24 357	15 111	0

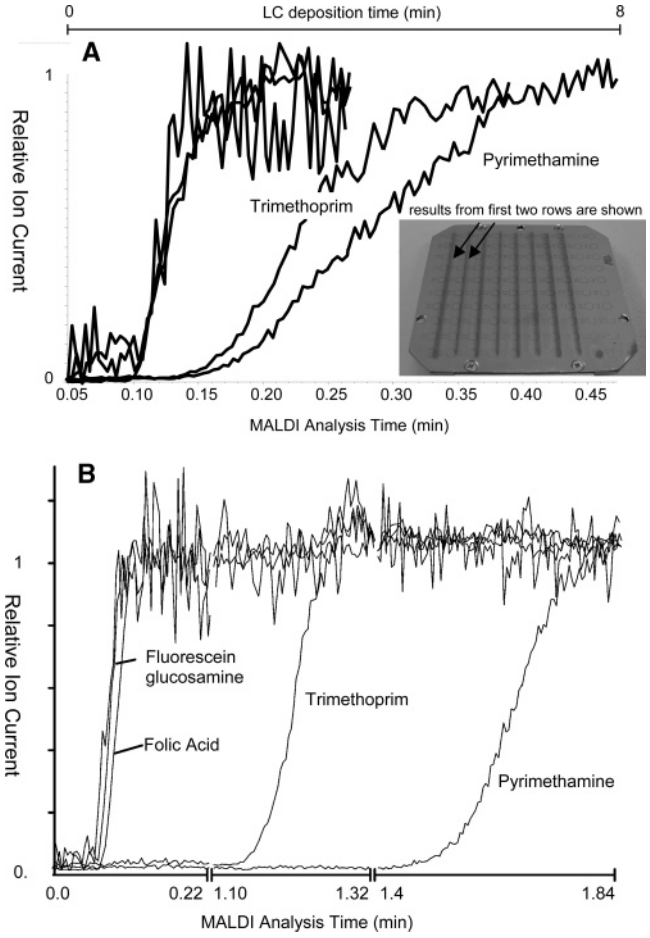

Figure 5. FAC/MALDI-MS/MS traces obtained using protein-loaded and blank DGS/PEO/APTES monolithic columns. Panel A, blank column containing no protein; panel B, column containing 25 pmol of DHFR (initial loading) showing breakthrough of *N*-acetylglucosamine, fluorescein, and folic acid at early times, then trimethoprim, and finally pyrimethamine. All compounds were infused at 50 nM. All traces are normalized to the maximum signal obtained after compound breakthrough. Note that MALDI analysis time is 19-fold faster than LC deposition time. All FAC traces were obtained using a fast laser translation speed (3.8 mm/s) and are the average of five reruns over a given sample region.

As was the case for FAC/ESI-MS/MS, the fluorescein, *N*-acetylglucosamine, and folic acid elute first (1.5-min LC time) followed by trimethoprim (3-min LC time) and pyrimethamine (3.5-min LC time), again showing nonspecific binding of the analytes when using low ionic strength buffers. This is not surprising, as the elution time is dictated by the column rather than the specific type of MS employed for detection. More interestingly, the MS analysis time required for the analysis of the traces on the plate is less than 0.5 min, compared with 8 min of actual LC time. Thus, although the LC deposition time is similar for both methods, it is possible to use multiple modes of MS to interrogate the same sample (see Figure 3) with each mode requiring only a few minutes to run.

Figure 5B shows the data obtained from the DHFR loaded column. Once again, the two nanomolar inhibitors show significant retention, with retention times that are similar to those obtained from FAC/ESI-MS/MS (trimethoprim, $t - t_0 = 20$ min; pyrimethamine, $t - t_0 = 28.5$ min). The slightly longer elution times relative to ESI-MS reflect the fact that the column used for the FAC/MALDI study was slightly longer than the one used for FAC/ESI. An important finding from the FAC/MALDI analysis is the low ion suppression, which shows another important advantage of the MALDI MS/MS method. This may be due to MALDI ionization being closer in nature to chemical ionization (and APCI) than ESI. In the case of MALDI, laser-desorbed species are ionized by interaction with CHCA ions within the plume generated from the surface. In such a case, the results are consistent with the well-established observation of reduced ion suppression in the APCI process.[10,29,30] MALDI signal suppression due to high levels of impurities has been reported by Krause[31] and Gharahdi,[32] but this may be caused more by changes in the crystallization of the sample, where wet spot crystallization under such conditions produces inhomogeneous rimmed spots, or by insufficient CHCA being present in the sample. In such a case, a surface opaque to the laser beam is formed upon drying. In our deposition method, both the crystallization process and CHCA amount were optimized to produce high-density tracks of small crystallites (near-dry spray impinges on the MALDI plate) with a high surface-to-volume ratio, a parameter important to surface-driven processes such as MALDI.

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Table 2. Signal Rate (Counts/s) above a Blank Background for MS/MS Analysis by MALDI and ESI Ionization Methods Using 2 or 100 mM Ammonium Acetate Buffer

	folic acid	trimethoprim	pyrimethamine
MALDI 2 mM AA	7400	42000	20000
MALDI 100 mM AA	1000	6000	3000
ESI (5 μL/min) 2 mM AA	350	6500	3600

Table 3. Total Signal (Counts) above Background Generated by 1 pg of Analyte in 2 mM Buffer

	folic acid	trimethoprim	pyrimethamine
MALDI	120000	180000	85000
ESI (5 μL/min)	350	6500	3700

Table 2 compares the signal-to-background levels obtained from ESI and MALDI MS/MS methods using 2 and 100 mM ammonium acetate (AA) buffer levels for MALDI and 2 mM for ESI and provides a means for conversion of the normalized plots to absolute counts. It should be noted that even though the ESI and MALDI experiments were each made using a different mass analyzer, API 3000 and API 4000, respectively; a general comparison (intended as a guide only) is possible since by converting the API 4000 for MALDI operation by fitting an oMALDI source, its normal orifice/interface and Turbo V source have been removed. It is these components that provide the significant performance enhancement over the API 3000 at flow rates above 50 μL/min. The data show that while MALDI offers approximately the same level of signal with a 100 mM buffer as ESI does with 2 mM buffer, MALDI offers a significant increase in sensitivity with 2 mM AA buffer. This result is further corroborated by comparing total signal generated by the two techniques for a fixed amount of sample, as shown in Table 3. In this case, MALDI analysis generates approximately 20−100× the total signal obtained from ESI. This is because for the ESI process, only a very small portion of sprayed sample actually enters the analyzer and gets detected, while MALDI tracks capture all of the sample and allow repeated analysis of the track and captured sample.

While signal levels in MALDI are higher than those in ESI, MALDI acquisition suffers from more noise owing to a shorter dwell time of 40 versus 1000 ms for ESI and added noise due to inhomogeneity in the track. Even so, the MALDI process offers the ability to reduce its noise by combining signal from numerous reruns of a track. The resulting noise reduction through signal averaging can be applied until a desired level required for data interpretation is reached. The fast laser rerunning of the track and selective application of the summing allows an efficient use of a fixed amount of sample in a time sensitive manner.

Given that MALDI analysis was possible even with 100 mM AA buffer, we set out to investigate the effect of ionic strength on the FAC process. Figure 6 shows the effects of ionic strength on the degree of nonspecific binding, using blank monolithic columns. In this case, only folic acid, pyrimethamine, and trimethoprim are eluted, with folic acid acting as a void marker. All data were run on the same column, starting at low ionic strength and increasing to 100 mM ionic strength. At 0 mM ionic strength,

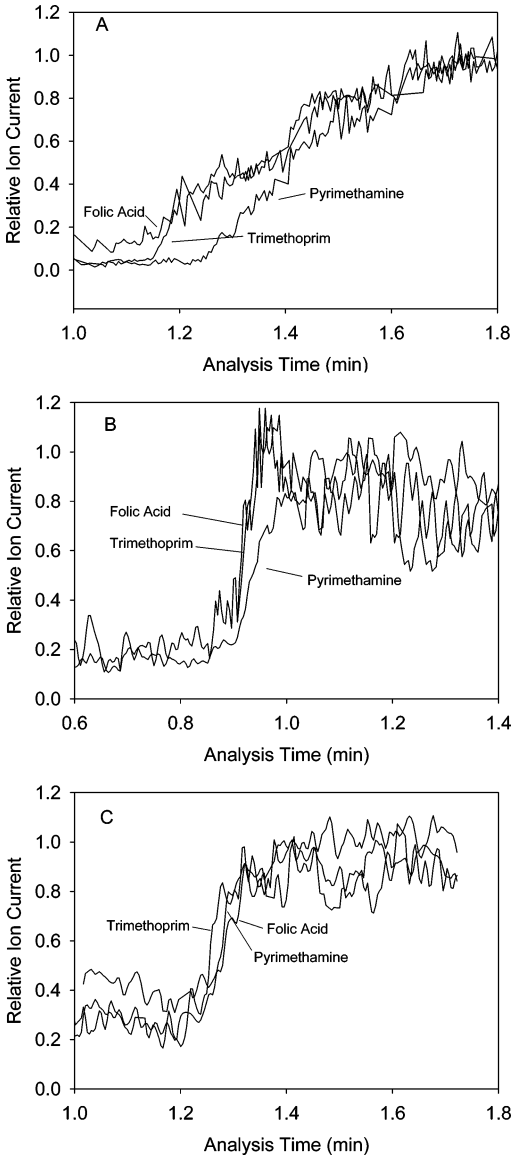

Figure 6. Effect of ionic strength on nonspecific binding of compounds to blank monolithic columns analyzed by FAC/MALDI. MALDI MRM traces are shown for the first run of folic acid, trimethoprim, and pyrimethamine (50 nM each) using (a) 2 mM ammonium acetate buffer, (b) 50 mM buffer, and (c) 100 mM buffer. All data were run on the same column with preincubation of the column in the appropriate buffer prior to introduction of compounds. All FAC traces were obtained using a fast laser translation speed (3.8 mm/s) and are the average of five reruns over a given sample region.

there is both significant retention of all analytes on the column, and more interestingly, the elution is stretched out over a very broad time range, indicative of significant nonspecific binding. Use of 2 mM ionic strength (see above) leads to a sharper elution profile, but causes significant retention of the compounds, including folic acid, by up to several minutes. On the other hand, the compounds elute at earlier times (based on the time to reach 50% of full intensity) and over a much narrower range when using either 50 or 100 mM ionic strength, indicating that the nonspecific binding has been suppressed. An important point from these experiments is that even at 100 mM ionic strength it was possible to deposit the LC eluent satisfactorily using the nebulizer-assisted

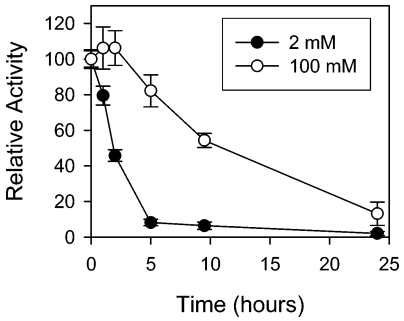

Figure 7. Activity of DHFR as a function of incubation time in 2 and 100 mM ammonium acetate buffer solutions.

electrospray method. Furthermore, the use of ammonium acetate as the buffer did not produce significant adduct ions, allowing identical MRM transitions to be used regardless of the concentration of buffer used. On the other hand, discrete spotting of the 100 mM sample led to most of the matrix/buffer depositing at the edge of the spots (data not shown), leading to significant irreproducibility in the signal and noise levels for MALDI analysis.

Another factor that is dependent on ionic strength is retention of activity of the entrapped protein, which determines whether the bioaffinity column can be reused. Previous studies using FAC/ESI-MS/MS with DHFR columns showed that the use of 2 mM ionic strength resulted in significant decreases in column performance owing to the low stability of DHFR in 2 mM ammonium acetate.[9] Figure 7 shows that the DHFR protein retains full activity after 2-h incubation in 100 mM ammonium acetate but retains less that half of its initial activity after a similar time in the presence of 2 mM ammonium acetate. After 5 h, the protein retains >80% activity in 100 mM buffer but less than 10% activity in 2 mM buffer. These incubation times correspond to the longest run times that would normally be associated with FAC and clearly demonstrate that the use of high ionic strength buffers, which are only compatible with MALDI-MS, should result in retention of activity over the required run time of the FAC experiment.

Figure 8 shows the effects of high ionic strength on the reusability of the monolithic DHFR columns. Panel A shows the FAC/MALDI-MS/MS trace obtained for the initial run of the column using 50 nM folic acid, pyrimethamine, and trimethoprim in 100 mM ammonium acetate, Panel B shows the recovery run obtained using 100 mM ammonium acetate, and panel C shows the second run of the same column under conditions identical to those used in panel A. While there is a small decrease in retention time between the first and second runs, the overall performance of the column when using 100 mM ionic strength is far superior to that obtained when using 2 mM ionic strength. For example, the LC retention time for both trimethoprim and pyrimethamine decreases by only 20% (11.5 to 10 min for trimethoprim, 16.5 to 13.5 min for pyrimethamine) at 100 mM ionic strength, whereas decreases close to 85% in retention time were obtained at 2 mM ionic strength.[9] It is also noteworthy that the retention time for all compounds at 100 mM ionic strength was significantly shorter than was obtained at 2 mM. In part this was due to the use of a shorter column for the latter experiments (5 vs 6 cm), but was likely also due to lower nonspecific binding and perhaps also changes in dissociation constants that may have occurred as a

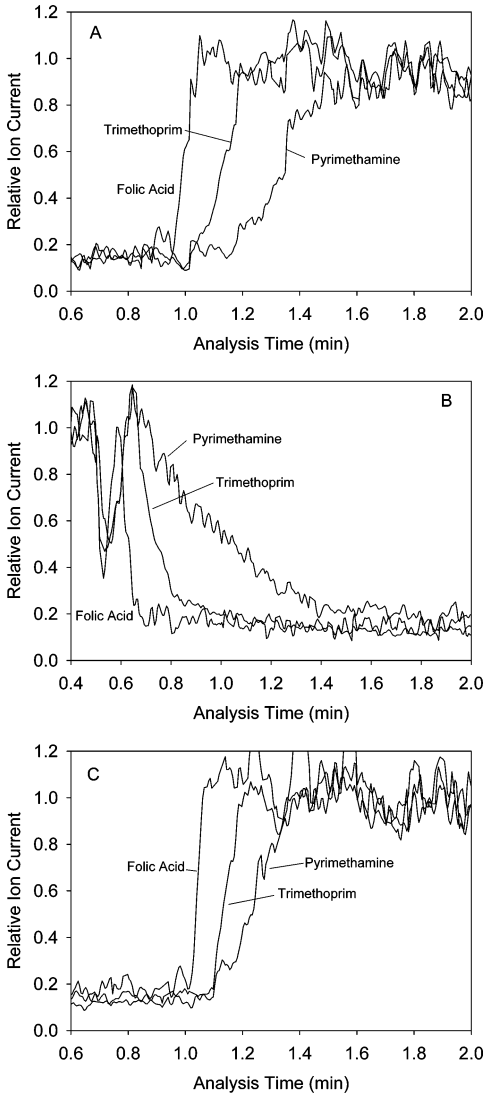

Figure 8. Effect of ionic strength on the reuse of monolithic DHFR columns using FAC/MALDI. Panel A shows a MALDI MRM trace of the first run for folic acid, trimethoprim, and pyrimethamine (50 nM each) using 100 mM ammonium acetate buffer. Panel B shows MALDI MRM traces obtained from plates during deposition of eluent upon the recovery of the column using 100 mM ammonium acetate. Panel C shows the MALDI MRM trace from a plate that was formed by deposition of column effluent during the second run of the compounds listed above through the same monolithic column. Note: total running time was 120 min at 5 μL/min, corresponding to 122 column volumes. All FAC traces were obtained using a fast laser translation speed (3.8 mm/s) and are the average of five reruns over a given sample region.

result of the higher ionic strength. Although a shift of 20% in retention time between runs is still relatively large, such losses may be due to slow leakage of loosely entrapped protein rather than denaturation of protein. Further optimization of the column in terms of pore morphology may allow for further improvements in column reuse, and when coupled with the ability to run the FAC studies at high ionic strength, as demonstrated above, it may be possible to reuse such columns several times.

DISCUSSION

Capillary-scale meso/macroporous sol−gel-based monolithic bioaffinity columns are ideally suited for the screening of com-

pound mixtures using frontal affinity chromatography with mass spectrometric detection for identification of specific compounds in the mixture. A particular advantage of the sol−gel-derived columns is their good compatibility with a variety of different proteins. While the current work focused on entrapment of a soluble enzyme, the sol−gel method employed herein is also amenable to the entrapment of a wide range of important drug targets, including membrane-bound enzymes[28] and receptors,[33] and even whole cells.[34] Furthermore, entrapment into DGS-derived materials allows immobilization of labile enzymes, such as factor Xa and Cox-II,[28] which are difficult to immobilize by other methods. Thus, the monolithic columns may find use in screening of compound mixtures against a wide variety of useful targets.

Another advantage of the low-i.d. monolithic columns is the ability to interface the capillary columns directly to an ESI or a MALDI mass spectrometer, which is likely to make them suitable for HTS of compound mixtures using FAC/MS. In particular, the low i.d. of the present monolithic columns allows them to deposit a relatively thin stream of analyte on a MALDI plate, allowing for high-density deposition (up to 12 traces/plate). The time capacity of a MALDI plate is determined by the width of the deposited track as well as its deposition speed. Reducing the deposition speed will increase the plate capacity but it will also degrade the LC resolution as material eluted at any instant in time is deposited over a finite area, given by the spray diameter, and the overlap of two adjacent events increases. Since the spray diameter directly affects both the capacity of a plate and fidelity of the chromatography record, it is important to keep it as small as possible. In practical terms, the loss of chromatographic resolution that can be tolerated dictates the lowest deposition speed. Since the LC run and analysis are now decoupled into two time-independent events, the ratio between deposition and interrogation speed determines how many reruns and different analysis experiments can be performed over a track at a time, saving significant time over an LC rerun.

While FAC/MALDI-MS/MS has provided good chromatographic results, several issues remain to be explored to optimize performance. For example, new deposition methods should be examined that can produce narrower, less disperse traces to give a higher density of analyte on the plate,[35] which should lead to a higher analyte concentration in laser beam and thus a better LOD. Lower diameter columns may also be beneficial as these could allow faster LC separations with lower flow rates that are compatible with deposition of thin tracks on the MALDI target. At this point, we have been able to load capillaries as thin as 50 μm in diameter; however, such thin capillaries lead to a low loading of protein per unit length of column (25-fold less than would be loaded in a 250-μm column using identical protein concentrations in the sol), which compromises the separation of ligands that bind to the protein from those that do not. In addition to thinner columns, methods to suppress the inherent background from the MALDI matrix would be beneficial, as this would minimize the need for subtraction of matrix background signals from analyte signals. While this is less of a problem when using MRM mode, and indeed was not required in the current study, such effects can become problematic if drug compounds have product ions that are similar in structure to commonly used MALDI matrix species.

A key advantage of MALDI-MS relative to ESI-MS for FAC studies is the ability to use much higher ionic strength buffers during the FAC run. The activity of proteins is known to be highly dependent on factors such as solution pH and ionic strength, and in most cases, maximum activity is obtained using buffers that mimic physiological conditions (i.e., 20−50 mM buffer, 100 mM KCl, pH ~7.4). Furthermore, high ionic strength provides a more effective double layer, which better screens the charge of the anionic silica surface, and thus reduces electrostatic interactions between the charged analytes and the silica surface. In the present study, Na^+ and K^+ were avoided to minimize issues with adduct ion formation. Instead, ammonium acetate, which is a volatile buffer, was chosen to adjust ionic strength. The use of this buffer did not lead to the formation of adduct ions and provided conditions that were amenable to LC deposition even at 100 mM concentrations. It is possible that even higher levels of ammonium acetate could be used for FAC/MALDI, but such levels were not examined in this study. As shown above, the use of high ionic strength led to the expected decreases in nonspecific binding and also produced better retention of protein activity upon repeated use of the column. This clearly shows that use of MALDI/MS has significant advantages over ESI-MS for FAC studies using protein-doped columns.

CONCLUSIONS

The use of MALDI-MS/MS provides significant advantages over ESI-MS/MS for frontal affinity chromatography studies. MALDI-MS/MS provides better tolerance of high ionic strength buffers, less ion suppression, faster MS analysis times, and access to more modes of MS analysis per LC run and potentially offers the ability to acquire data using different mass analyzers (triple-quadrupole, TOF, Q-TOF) from the same sample, which could be beneficial in cases where higher molecular weight species are analyzed. These advantages lead to the ability to perform frontal affinity chromatography under conditions that more closely mimic physiological conditions, leading to better retention of activity for the immobilized proteins and likely providing more reliable binding constant data. The ability to perform multiple MS analyses per LC run can be used advantageously to optimize detection of low concentration analytes or to identify unknown compounds that might be present in a natural product library or similar compound mixture. In ESI-MS, the MRM transitions, and, hence, the identity of compounds must be known prior to the FAC run. Otherwise, unknown compounds must be identified indirectly using an indicator compound in "roll-up" mode, with compound identification done off-line. As shown herein, such roll-up effects can be confused with ion suppression when using ESI-MS/MS, leading to difficulties in identifying true "hits" when using indicator mode. MALDI-MS/MS minimizes these problems, making the indicator mode more reliable, and also allows full MS analysis of deposited analytes, aiding in identification of unknowns. Overall, the results of this study show that MALDI-MS/MS can provide numerous advantages over ESI-MS/MS when used in conjunction with FAC,

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providing an improved method for LC/MS based high-throughput screening.

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