Supporting Information

Bio-Solid Phase Extraction / Tandem Mass Spectrometry for Identification of Bioactive Compounds in Mixtures

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SUPPLEMENTARY EXPERIMENTAL SECTION

Column Leaching. Eluate from 10 cm column segments were collected in 15 µL fractions, mixed with a 10 µL aliquot of 100 µM adenosine and allowed to react for 5 minutes. The reaction was quenched with 75 µL of methanol followed by injection into an AB Sciex QTrap API 2000. The resulting solutions were analyzed in multiple reaction monitoring (MRM) mode for adenosine (268 → 136 m/z) and inosine (269 → 137 m/z) signal ratios and compared to a calibration curve to determine the amount of enzyme leached from the column.

Column Characterization by Michaelis Menten Kinetics. Column activity was assessed on entrapped ADA columns by injecting increasing concentrations of adenosine up to 500 µM via an Eksigent AS-1 autosampler coupled to the ADA column and then connected to the ESI source. The flow rate was 5 µL/min and was teed prior to the source to a makeup flow of 1% acetic acid in LCMS grade methanol at a rate of 5 µL/min. Calibration curves were prepared via a previously described method[1] to correct for the ^{13}C isotope of adenosine interfering with the inosine MRM transition.

Column Characterization by bioSPE. Entrapped ADA columns were used to determine the protein loading by infusing EHNA at increasing concentrations from 10 nM to 2 µM. The columns were washed with 8 bed volumes of 20 mM ammonium acetate prior to elution with 3% acetic acid. The resulting peaks were quantified by use of an EHNA calibration curve prepared in 3% acetic acid. Extracted peaks were plotted versus the infused concentration to determine the maximal loaded concentration and dissociation constants by fitting data to one-site saturation ligand binding using SigmaPlot 10.0.

Mass Spectrometer Settings. Instrument settings for IMER assays were as follow: curtain gas = 45.0, collision gas = medium, ion spray voltage = 5500V, temperature = 200 °C, declustering potential = 45 V,

exit potential = 11 V, collision energy = 26 V, cell exit potential = 3.0 for both the adenosine and inosine MRM transitions. Conditions for compounds used in bioSPE assays are provided in Table S1.

Entrapped ADA Column Optimization. Sol-gel entrapped ADA columns were tested with a simple mixture containing EHNA, fluorescein and huperzine A at concentrations ranging from 10 nM to 2 μM. Mixtures were loaded onto the column using an 85 μL injection loop, washed with 200 mM ammonium acetate pH 7.5, then eluted with either 50% methanol or 3% acetic acid. Competitive displacement of EHNA was also assessed using either 25 μM or 100 μM adenosine.

Column Characterization by FAC. Protein binding sites (B_T in picomoles) were quantified using frontal affinity chromatography-tandem mass spectrometry[2] (FAC-MS/MS) by running increasing concentrations of EHNA, from 1 to 10 μM, through a series of columns (using a fresh column for each ligand concentration) and fitting the data to Equation (1):

$$V - V_o = \frac{B_T}{[A]+K_d} \quad (1)$$

where V_0 is the void volume (μL), V is the retention volume (μL), [A] is the concentration of EHNA (μM), and K_D is the binding constant of the ligand to the protein (μM). The retention volume was determined as the volume where the frontal curve reached 50% of the maximum intensity. Columns with entrapped protein had too small a protein concentration to provide observable shifts in elution volume relative to the void volume, and instead had protein loading calculated by measuring the relative turnover of adenosine to inosine as compared to columns with covalently bound proteins.

The covalently bound ADA columns were assessed for reproducibility by performing replicate extractions of EHNA on a single ADA column. Columns were loaded with 85 μL of a simple ternary

mixture containing 1 μM each of EHNA, fluorescein and trimethoprim, then washed with 200 mM ammonium acetate pH 7.5 for 10 column bed volumes, followed by elution with 3% acetic acid. A calibration curve was generated using EHNA in 3% acetic acid from 50 nM to 10 μM in order to quantify the amount of EHNA extracted from the column. Replicate injections of the mixture were assessed for reproducibility by measuring the EHNA XIC area extracted from the same column.

SUPPLEMENTARY RESULTS & DISCUSSION

Column Leaching and Activity. Leaching of the enzyme from the column was observed for the first 8 bed volumes when flushing at 5 μL/min (Figure S1A). The amount of enzyme lost on each column is negligible after this volume and was therefore used as a minimum conditioning procedure prior to using. Without adequate conditioning, PEG and glycerol appear to suppress enzyme function, which is partially due to the microviscosity they impart in the silica matrix that interferes with the pressure driven diffusion of analytes into the mesopores and hence contact with the enzyme, as well as interfering with MS detection by causing ion suppression of analytes of interest. Once conditioned, the Michealis-Menten constant was determined to be 20 ± 7 μM (Figure S1B). This is within error of the solution value of 24 μM[3] and a higher affinity compared to our previous report on ADA activity on column of 100 μM.[1] Enzyme reactor mode is a beneficial way of running initial kinetic studies of the target biomolecule since it does not subject it to a harsh wash and can be used repeatedly for hundreds of injections without inhibiting function.[4]

Entrapped ADA Column Optimization. ADA columns produced via sol-gel entrapment of the enzyme were tested for their ability to extract EHNA from a simple mixture containing EHNA, fluorescein and huperzine A. Figure S2A shows that both 50% methanol and 3% acetic acid were capable of effectively separating EHNA from fluorescein and huperzine A. A competitive extraction assay was also performed using adenosine as the elution solvent to confirm the presence of an active site

inhibitor versus an allosteric inhibitor (Figure S2B). An increase in substrate concentration during elution shows an increase in EHNA signal. However, adenosine does not provide a signal enhancement of EHNA as with 50% methanol or 3% acetic acid. Ion suppression from the high substrate concentrations used coupled with the slow off-rate of EHNA contributes to reduced signal enhancement and elongated peak widths. Successive extractions could not be performed on the entrapped ADA columns without a significant loss of activity, eventually leading to a complete loss of activity after 4 repeated extractions. Once mixture complexity was increased on the columns, the separation efficiency of the columns decreased, leading to incomplete extraction of EHNA versus non-specific binders. Initial bioSPE proof of concept and optimization studies using columns with entrapped ADA did not provide a reproducible elution peak during the harsh washing step, which was determined to be the result of inadequate protein loading. All subsequent bioSPE assays used columns with covalently bound ADA, which produced a much higher number of binding sites (see Results section). Unfortunately, when the entrapped ADA columns were tested with a 20-component EHNA spiked mixture, an extremely low amount of EHNA was extracted. The reason for this was found to be that the amount of protein in the column was only 1.42 ± 0.04 pmol, based on peak areas for EHNA extracted from entrapped ADA columns using 3% acetic acid as compared to the peak area obtained from a column with covalently bound ADA (see Figure S2 and compare to Figure 5). In addition, protein leaching from the macroporous silica matrix resulted in a continually decreasing and irreproducible protein concentration on the columns. This effect, coupled with interfering signals from PEG and glycerol byproducts remaining from column fabrication, caused a significant decrease in inhibitor retention and increased detection limits, making both frontal affinity chromatography (FAC) and bioSPE unfeasible using entrapped ADA columns.

Covalent Column Characterization by Frontal Affinity Chromatography (FAC). The protein loading (B_T) of covalently-bound ADA columns was assessed by FAC using EHNA infused as

concentrations ranging from 1 to 10 μM. In cases where the signal could not reach a 100% signal intensity compared to infusion on a heat denatured ADA column, the maximum signal intensity on the heat denatured column was used as 100% signal intensity to calculate the percent infusion for the signal on the functional ADA column. The retention volume at 50% infusion of EHNA on the ADA column was plotted versus EHNA concentration and fitted to equation (1) using Sigma Plot 10.0 software. The column protein loading was characterized via FAC since specific activity was extremely high and the maximum turnover velocity (V_{max}) could not be reached prior to running into ESI-MS ion suppression effects, thus leading to difficulties in determining K_M. An observed 25 minute frontal retention for 10 μM EHNA was used as a starting point for determining B_T and K_D by FAC. Figure S3A shows increased retention volumes with decreasing EHNA concentration and when fitting the data to equation 1 in Figure S3B, the protein loading (B_T) was determined to be 712 ± 17 pmol. The inhibitor dissociation constant (K_D) was not reliably determined by the FAC method (25 ± 27 nM), as the amount of functional protein was very high compared to the actual K_D, leading to relatively high error upon curve fitting. Covalent columns were therefore chosen to perform all further bioSPE experiments since they were better at producing higher signal with less interferences with a higher reproducibility.

Column Reproducibility. Figure S4 shows the reproducibility of EHNA extraction from covalently bound ADA columns. The RSD was 8.8% for 8 replicate extractions with no significant loss in extracted EHNA area over the day tested. This indicates that columns can be reused multiple times for extraction without adversely affecting ADA activity.

Supplemental Tables & Figures

Table S1. MRM transitions for bioactive compounds in screening mixture

Compound ID	Q1	Q3	Time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
N5-butyl-1,2,4-thiadiazole-3,5-diamine	173.2	117.1	500	30	10	30	3
N'-(2,6-dimethoxybenzoyl) nicotinohydrazide	179.2	90	500	68	10	41	2.5
3,8-dithia-1,6-diazaspiro[4.4]nona-1,6-diene-2,7-diamine	189.1	113.1	500	39	10	21	3
1-[(3-pyridylamino)methyl] pyrrolidine-2,5-dione	206.2	107	500	30	10	30	3
epibatidine	208.9	126.1	500	62	11	32	3
1-{[(6-methyl-2-pyridyl)amino]methyl} pyrrolidine-2,5-dione	220.1	121.2	500	31	8	23	3
2-[2-(2-propyn-1-ylsulfanyl)phenyl]-1,4,5,6-tetrahydropyrimidine	231.2	192.2	500	45	10	27	3
huperzine A	243.2	226.2	500	62	11	30	4
N2-[3-(1H-imidazol-1-yl)propyl]-3-nitropyridin-2-amine	248.2	180.2	500	35	9	20	3
pyrimethamine	249.2	177.1	500	83	11	39	4
N-{2-[[(acetoxy)imino](amino) methyl]-3-fluorophenyl}acetamide	254.2	152.2	500	32	9	16	3
vidarabine	268.2	136.1	500	65	10	30	3
methyl-N-(4-methoxyphenyl)-4-morpholinecarimidothioate	267.2	180.1	500	45	8	27	3
tubercidin	267.2	135.1	500	50	11	27	3
2-(4-chlorophenyl)-2-oxoethyl-N,N-dimethylcarbamodithioate	274.1	88.1	500	27	8	28	3
sanguinine	274.3	199	500	65	11	32	3
(erythro-9-(2-hydroxy-3-nonyl)adenine)	278.2	136.1	500	68	10	30	3
galanthamine	288	213.1	500	65	11	31	3
trimethoprim	291.2	230	500	80	10	33	3
N'-(2,6-dimethoxybenzoyl)nicotinohydrazide	302.2	165.2	500	30	10	30	3
N-[2-(diethylamino)ethyl]-2,3,4,5,6-pentamethylbenzenesulfonamide	327.3	100.2	500	60	11	31	2
6-{[3-(dimethylamino)propyl]amino}-2-morpholino-3-nitrobenzonitrile	334.3	230.2	500	42	10	27	3.5

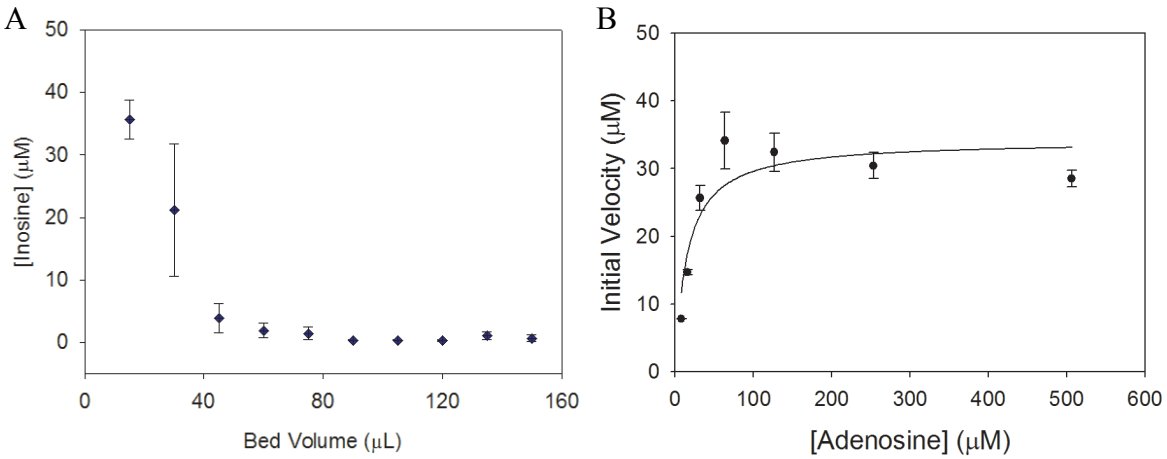

Figure S1. (A) Leaching of adenosine deaminase from a 10 cm sol-gel entrapped ADA column. Eight bed volumes are sufficient for removing leachable protein as a way of pre-conditioning the column prior to use. (B) Enzyme activity versus adenosine concentration on entrapped ADA columns shows a K_M value of 20 ± 7 μM, as determined by fitting the data to a Lineweaver-Burke model.

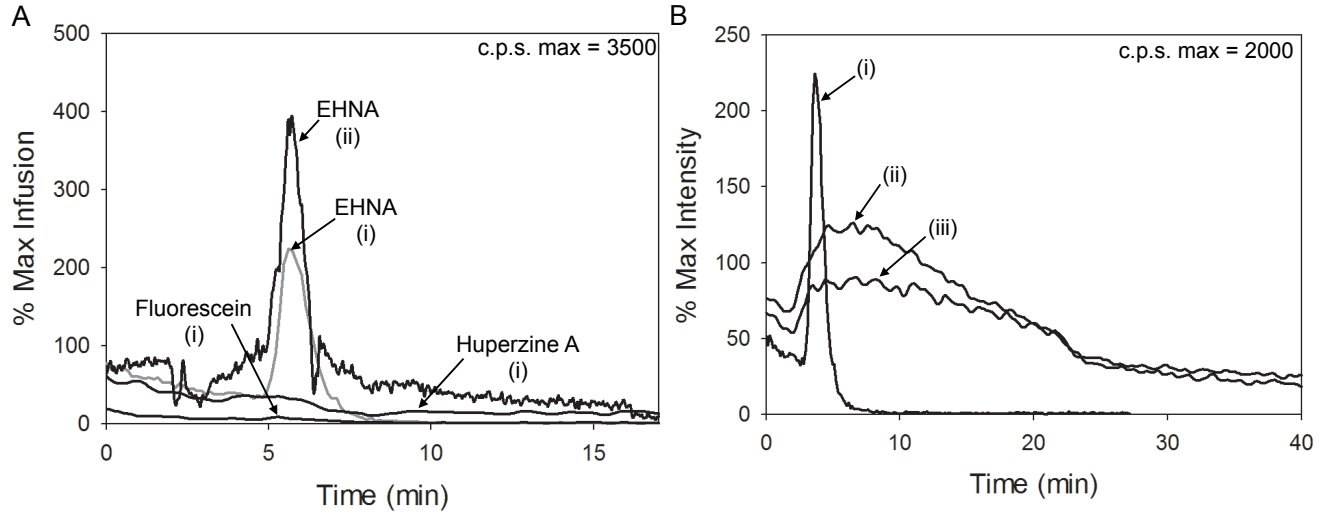

Figure S2. (A) Extraction of simple mixture from sol-gel entrapped ADA columns using (i) 50% methanol, and (ii) 3% acetic acid. (B) Extraction of EHNA from a sol-gel entrapped ADA column with (i) denaturing 50% methanol, or competitively with (ii) 25 μM adenosine, and (iii) 100 μM adenosine.

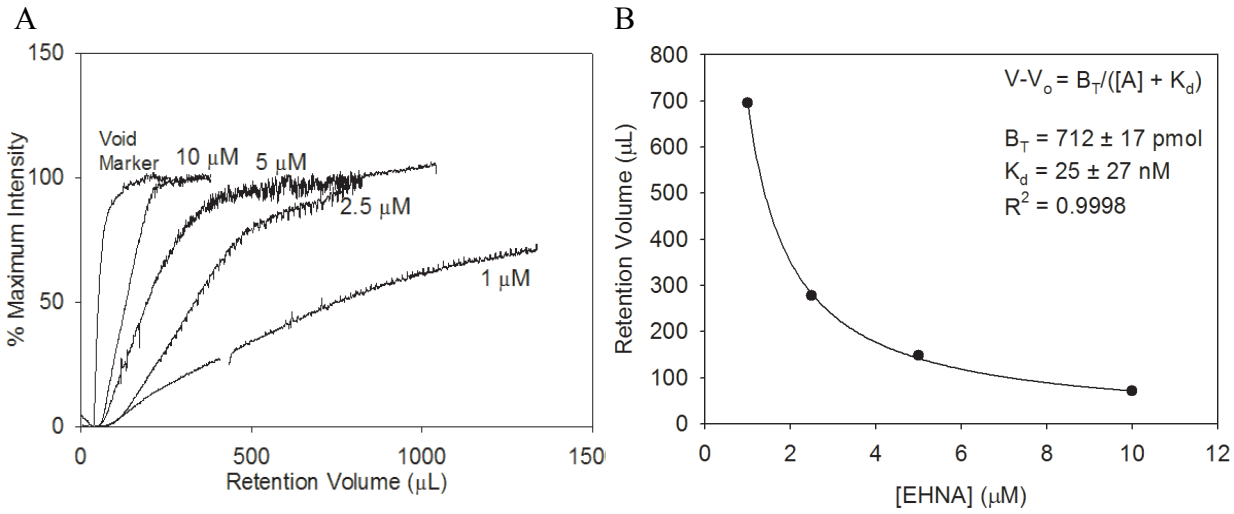

Figure S3. Characterization of covalently bound adenosine deaminase monolithic silica columns using separate 10 cm column segments via frontal affinity analysis. Panel (A) depicts the concentration dependent frontal elution time of EHNA compared to the void marker trimethoprim. Panel (B) shows the fit of the FAC equation to the data showing a protein loading (B_T) of 712 ± 17 pmol and a K_D of 25 ± 27 nM. The high standard error could be reduced by performing replicate runs using smaller column segments.

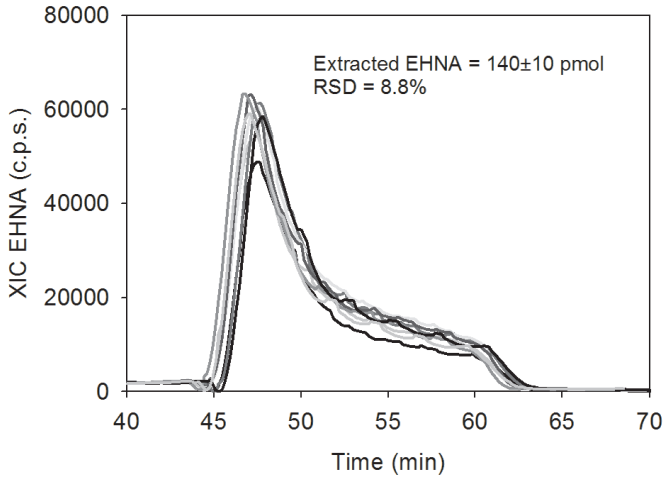

Figure S4. Replicate extractions of EHNA from covalently bound ADA column using 3% acetic acid.

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(3) Murphy, J.; Baker, D. C.; Behling, C.; Turner, R. A. *Anal. Biochem.* **1982**, *122*, 328-337.
(4) Forsberg, E. M.; Green, J. R. A.; Brennan, J. D. *Anal. Chem.* **2011**, *83*, 5230-5236.