Electronic Supplementary Material (ESI) for Green Chemistry

SUPPORTING INFORMATION

Dual Use of a Chemical Auxiliary: Molecularly Imprinted Polymers for the Selective Recovery of Products from Biocatalytic Reaction Mixtures

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I. General Methods

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, Ontario, Canada). Reagents and solvents were used without further purification except where stated. Flash chromatography and TLC analyses were performed with 60 Å silica gel from Silicycle (Quebec, Canada). Preparative TLC was performed with type 60 F_{254} plates from EMD (Gibbstown, NJ). Purification by reverse-phase HPLC was achieved using an Agilent 1100 modular system equipped with an autosampler, a quaternary pump system, a photodiode array detector, a thermostatted column compartment and a ChemStation (for LC 3D A.09.03) data system. The columns used were an analytical 4.6 × 250 mm, 4 µm SYNERGI 4µ Hydro-RP 80 A (Phenomenex, Torrance, CA), an analytical 2.6 x 100 mm KINETEX 2.6 µ C-18 100 A (Phenomenex, Torrance, CA. Samples were eluted using a combination of mobile phase A (0.1% aqueous formic acid), mobile phase B (acetonitrile containing 0.1% formic acid). The detector was set to 273 nm. The different HPLC elution gradients and flow rates are detailed in Table S1 below. TEMs were performed with a Philips CM200 TEM using toluene as solvent for the dispersion of the solid-phase.

Table S1. Flow rates and linear gradient profiles used for HPLC analyses and purifications. See section VI for the retention times of the various compounds.

Method A: 0.5 ml min^{-1}, with the analytical SYNERGI 4µ Hydro-RP 80A

Time (min)	%A	%B
0	50	50
20	5	95

Method B: 0.5 ml min^{-1}, with the analytical Kinetex 2.6 µ C-18 100 A

Time (min)	%A	%B
0	35	65
8	35	65

II. MIP synthesis

The MIPs were synthesized according to the procedure of Theodoridis and Chromatigr.[1] The desired template ligand (~200 mg, 0.5 mmol) was dissolved in acetonitrile (2 mL). Methacrylic acid (169 μL, 2 mmol), ethylene glycol dimethacrylate (1.2 mL, 6 mmol) and the radical initiator, benzoyl peroxide (16 mg, 61 μmol) were added and the mixture was degassed with nitrogen for 2 minutes before being left to stir at 60°C overnight. The resulting solid was ground into a fine powder and the finest particles were removed by repeatedly suspending the powder in water (3 x 50 mL) followed by decanting the aqueous layer above the precipitate. The powder was then oven-dried, loaded into empty solid-phase extraction cartridges (empty columns) and washed with 5 column volumes of methanol:acetic acid (9:1, v/v). The final wash was analyzed by LC-MS (the detection limit was approximately 1%) to verify that no template ligand was present in the MIP.

III. Expression and purification of enzymes

Purified CYP3A4, was expressed and purified according to previously reported procedures.[2]

A variation of the above procedure was used to prepare CYP3A4-containing membranes. Following the sonication step, the resulting viscous mixture was centrifuged at 10,000 × g for 20 minutes. The DNA-containing pellet was discarded and the supernatant was centrifuged at 53,000 × g for 65 minutes. The new pellet was resuspended in potassium phosphate buffer (15 mL, 0.1M, pH 7.4) per liter of culture, affording a solution of CYP3A4-containing membranes.

The concentration of CYP3A4 was determined as previously described,[3] affording approximately 1 mL of 15 μM per liter of bacterial culture. The solution was frozen at -80^{o}C until use.

IV. Assays and Biocatalytic Studies

Small volume MIP assay

A solution of the desired ligand (~0.7 mg, 2.5 mL, 1.03 mM) was loaded onto the MIPs (2.5 mL) by centrifugation (3, 000 × g for 5 minutes) and the flow through was collected, leaving a dry solid phase. The MIPs were then washed with 2 column volumes (5 mL) of water by centrifugation (3000 × g for 5 minutes each), leaving a dry solid phase. Finally, the MIPs were eluted with 2 column volumes (5 mL) of methanol:acetic acid (9:1) and all fractions were analyzed by LC-UV-MS after dilution to 10 mL in water. Experiments were performed in duplicate except the assay to test for MIP viability, which was performed in quadruplicate.

Large volume MIP assay to separate theobromine-containing compounds from complex mixtures.

Solutions containing the theobromine derivatives (50 mL, 20 column volumes, 8.50 μM) were dissolved in water, Lysogeny Broth, or a solution of CYP3A4-containing membranes, and the resulting 'spiked' mixtures were loaded onto the MIPs (2.5 mL) using a peristaltic pump. The flow-through was collected, leaving a dry solid phase. The MIP was then washed with 2 column volumes (5 mL) of water by centrifugation (3,000 × g for 5 minutes), leaving a dry solid phase. Finally, the MIPs were eluded with 2 column volumes (5 mL) of methanol/acetic acid (9:1) and

all fractions were analyzed by LC-UV-MS after dilution to 100 mL in water. Experiments were performed in duplicate.

Large volume MIP assay to separate theobromine-containing compounds from a reaction mixture resulting from the CYP3A4-catalyzed oxidation of 4.

Compound **4** (0.7 mg, 2.6 mmol) was added to a solution of CYP3A4-containing membranes (5 mL, 15 μM enzyme) and the mixture (15 mL) was pre-incubated at 27°C for 5 minutes before the addition of the cofactor surrogate CHP (481 μL, 2.6 mmol). The mixture was shaken at 250 RPM and 27°C for 2 hours before dilution to 50 mL in water (to lessen the back pressure on the pump). The diluted solution was loaded onto the MIPs (2.5 mL) using a peristaltic pump with minimal back-pressure. The flow-through was collected, leaving a dry solid phase. The MIPs were then washed with 20 column volumes (50 mL) of water by centrifugation (3,000 × g for 5 minutes), leaving a dry solid phase. Finally, the MIPs were eluded with 20 column volumes (50 mL) of methanol:acetic acid (9:1) and all fractions were analyzed by LC-UV-MS after dilution to 100 mL in water. Experiments were performed in duplicate.

V. Determination of MIP Binding Constants

The affinity of **4** for MIPs prepared using either **4** or **6** was determined using Scatchard analysis.[4] The Scatchard equation is:

$$B/U = (B_{max} - B) / K_d$$

Where: *B* is the amount of ligand bound per gram of MIP used. This value was measured by the HPLC absorbance (using the previously determined value ε = 8.81 E+03 M^{-1} cm^{-1})[3] of the elution fraction, indicating the amount of ligand released from the MIP during elution. *U* is the equilibrium concentration of unbound ligand, measured by subtracting *B* from the total amount of ligand initially loaded onto the MIP. B_{max} is the maximum binding capacity and K_d is the equilibrium dissociation constant.

Figure S1 is a plot of these parameters where K_a is the slope and B_{max} is the intercept.

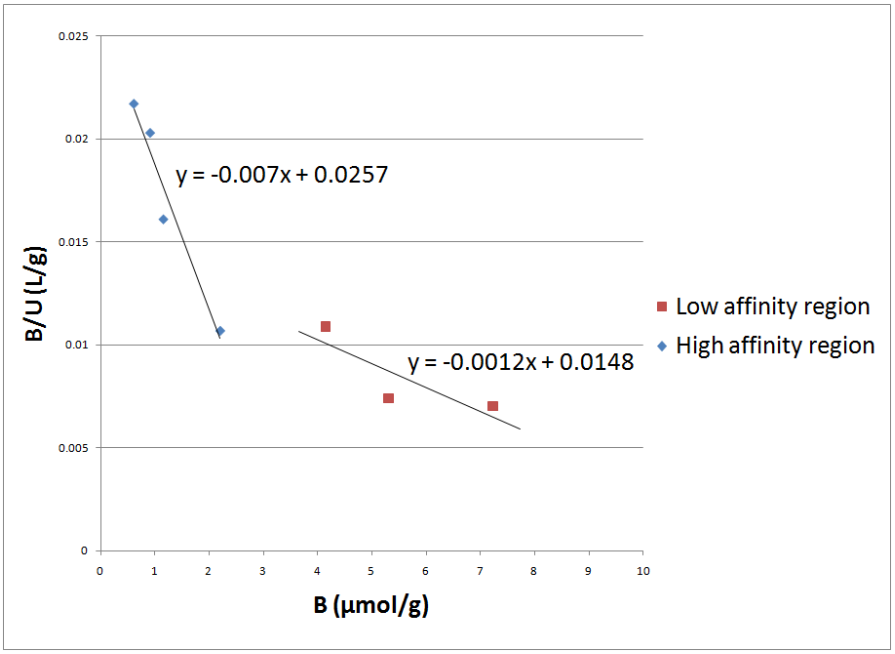


Figure S1: Scatchard plot for compound **4** and the MIP designed for **4**.

K_d is equal to the negative reciprocal of the K_a, the association constant, thus:

$$K_d = -1/K_a$$

This plot features two distinct linear regions, as is expected when using Scatchard analysis to evaluate a MIPs.[5] It is known that MIPs often contain two two types of binding sites

on the polymer surface, high affinity sites and low affinity sites. The steepest linear portion describes the binding parameters of the high affinity sites, and the second linear portion describes the binding parameters of the low affinity sites. The relevant binding parameters used for analysis are determined by the amount of ligand loaded onto the MIP. When using amounts of ligand below the B_{max} of the high affinity region, the K_d of the high affinity region may be considered the relevant equilibrium dissociation constant. From the slope and intercept of the above plot, the equilibrium dissociation constant and maximum binding capacity of **4** on the MIP designed for **4** were $K_d = 1.42\times10^{-4}$ M^{-1}, B_{max} = 3.7 μmol/g for the high affinity binding sites and $K_d = 8.33\times10^{-4}$ M^{-1}, B_{max} = 12.3 μmol/g for the low affinity binding sites. These values are in good agreement with previous evaluations of similar MIPs for similar ligands.[4-5] The equilibrium dissociation constant and maximum binding capacity of **4** on the MIP designed for **6** were $K_d = 6.66\times10^{-4}$ M^{-1}, B_{max} = 1.2 μmol/g (Figure S2).

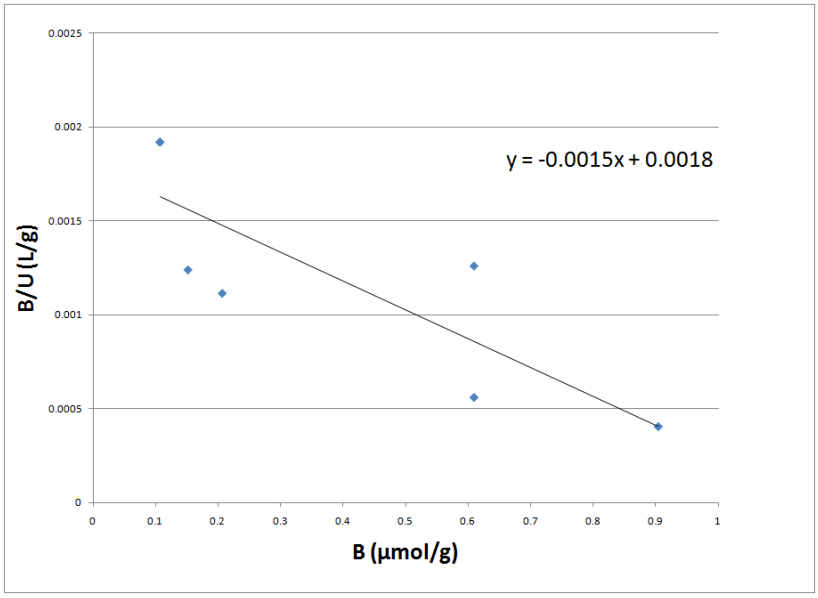
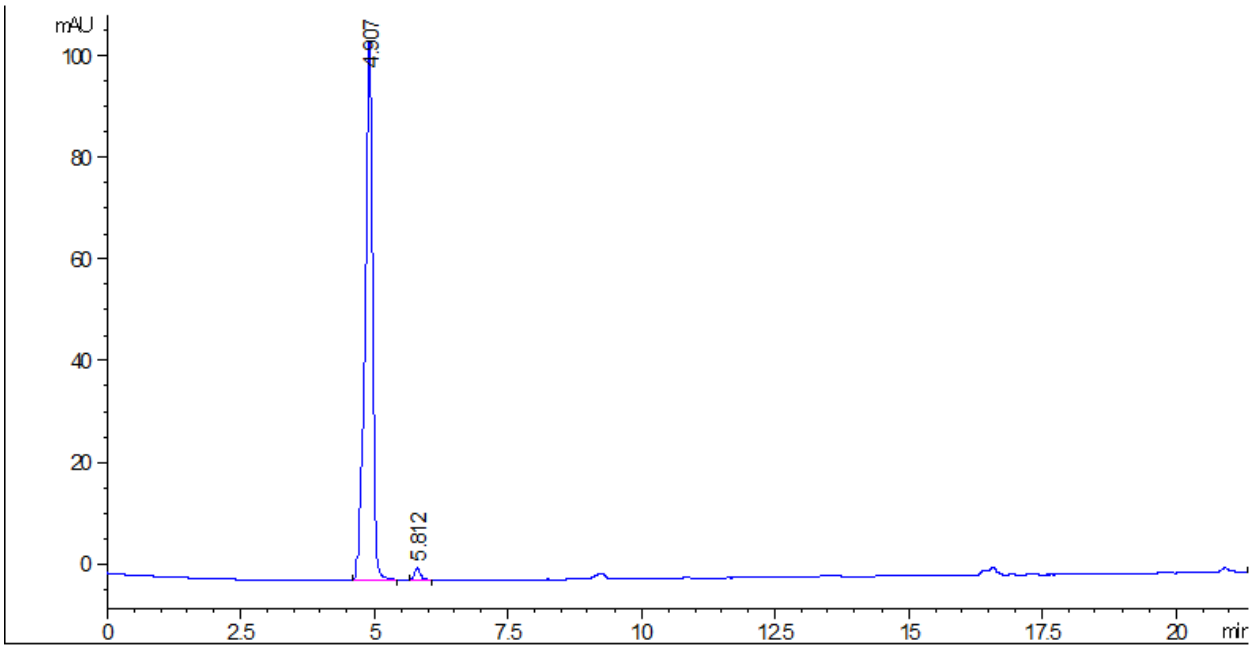


Figure S2: Scatchard plot for compound **4** and the MIP designed for **6**.

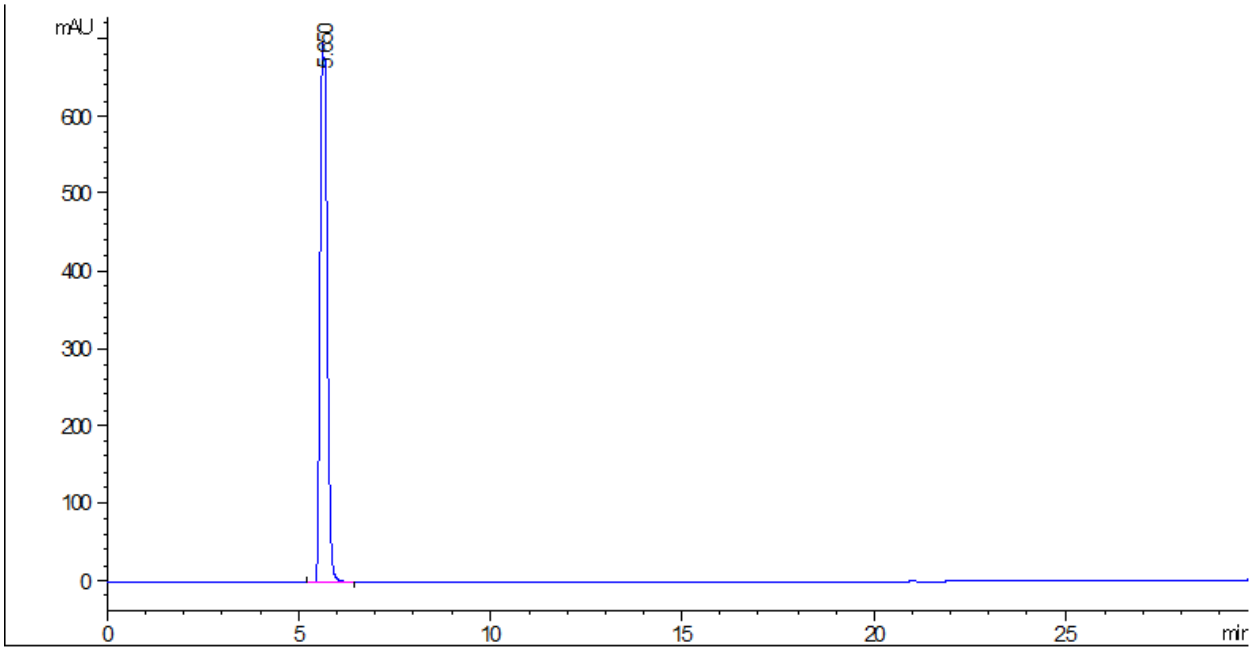
The non-specific binding of **4** on this MIP designed for **6** did not result in heterogeneous binding constants.

VI. HPLC Traces of the Elution Fractions from the Small volume MIP assay

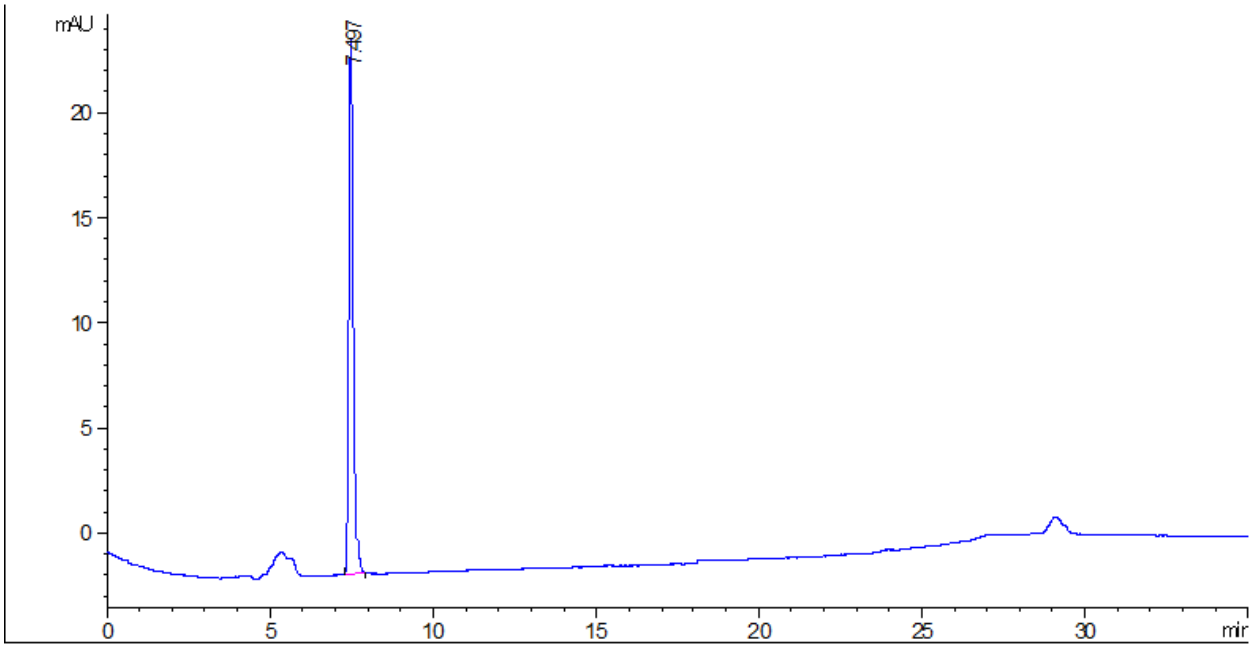
Compound 1



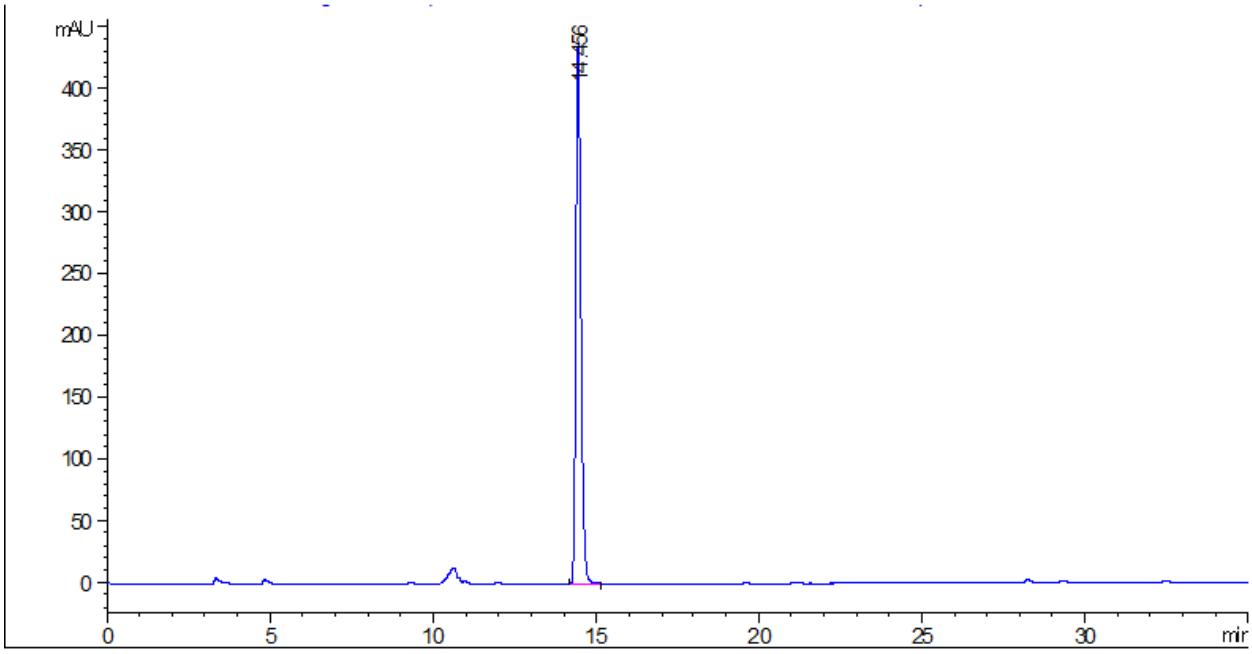
Compound 2



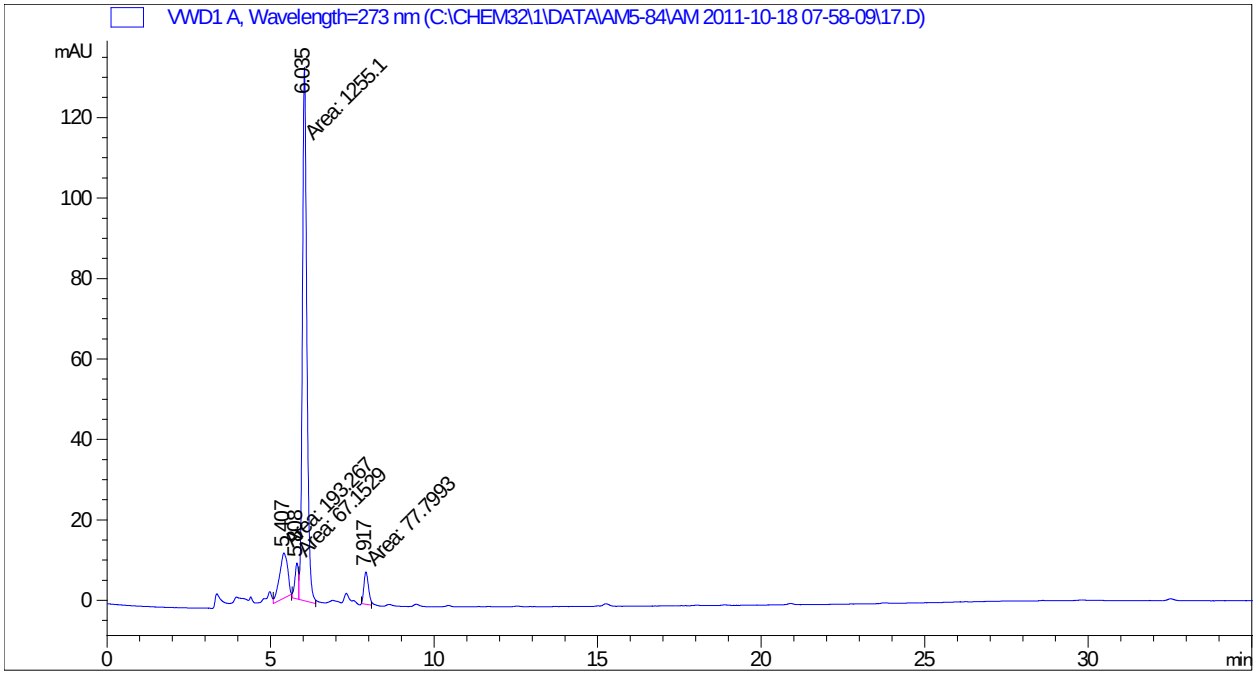
Compound 3



Compound 4



Compound 5



Recovery of 4 and 5 from biocatalytic mixture

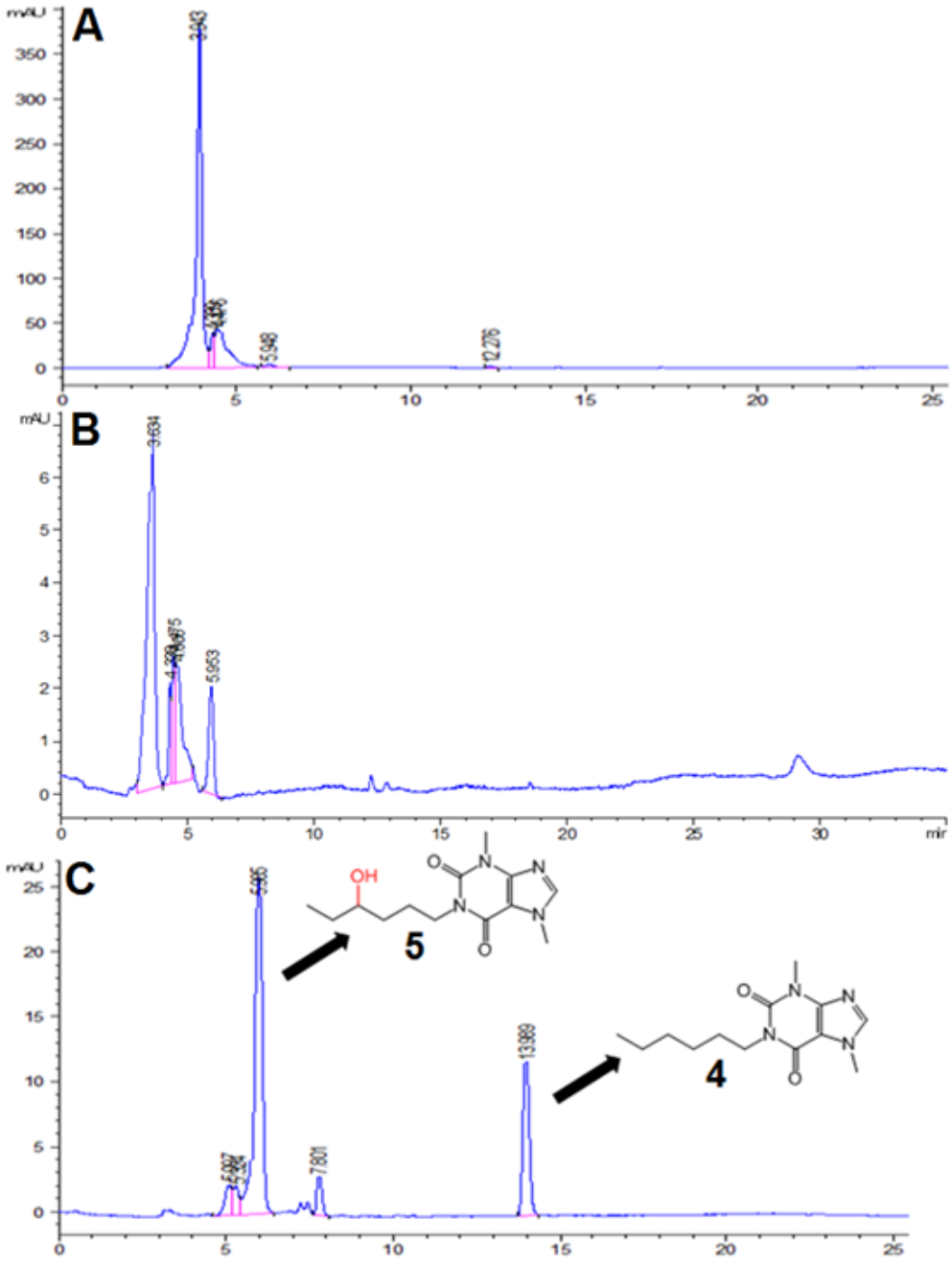
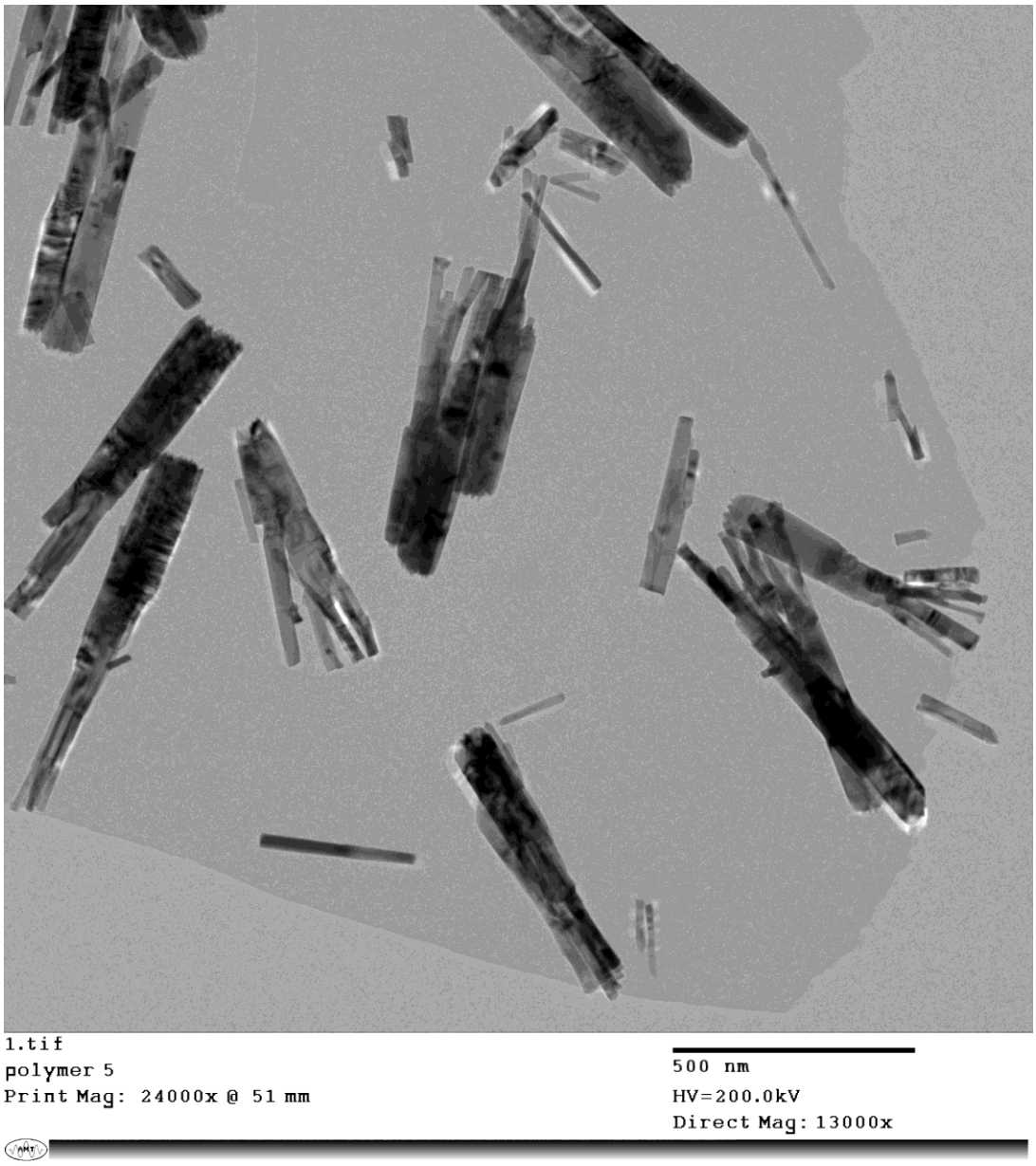
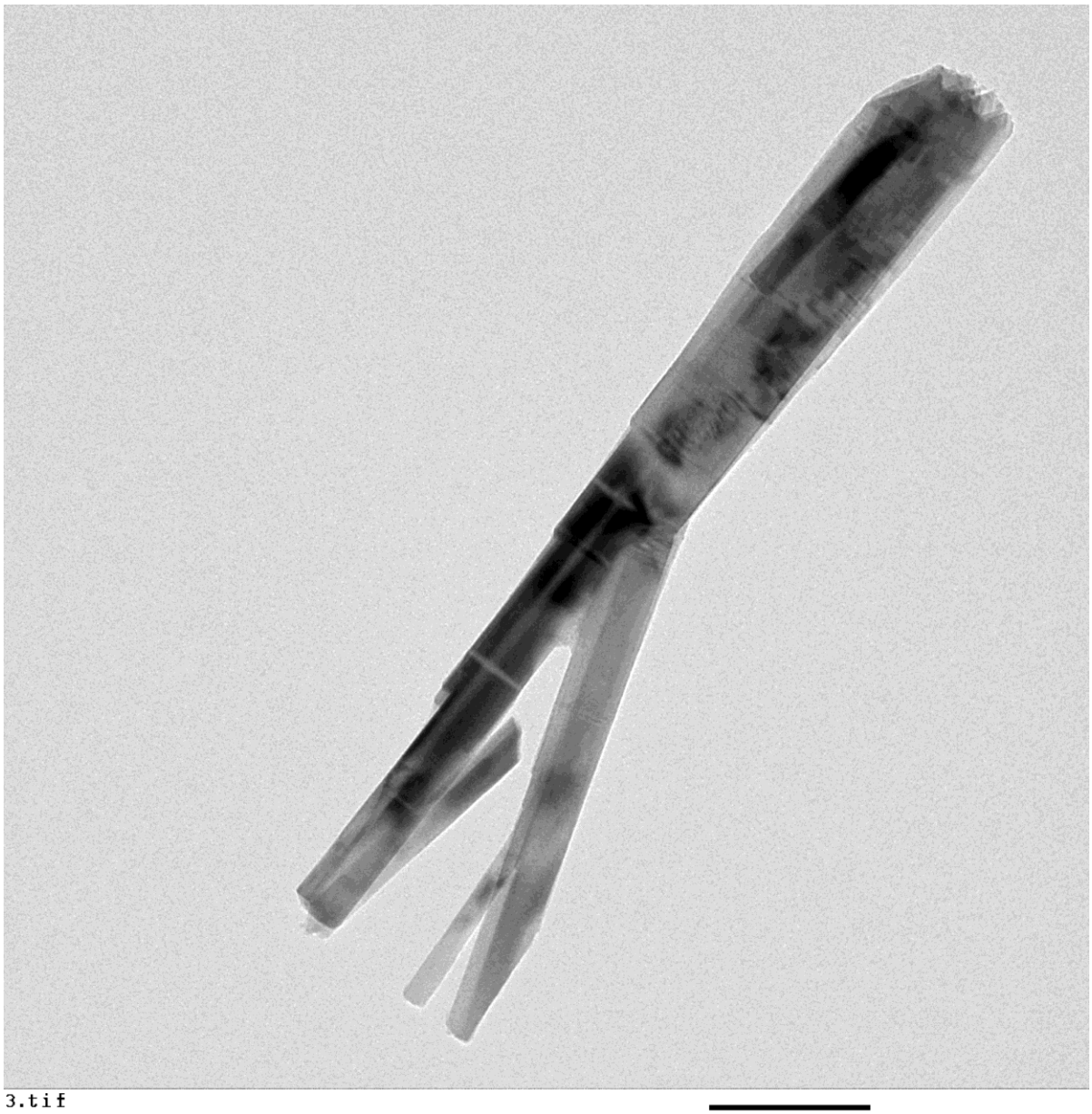
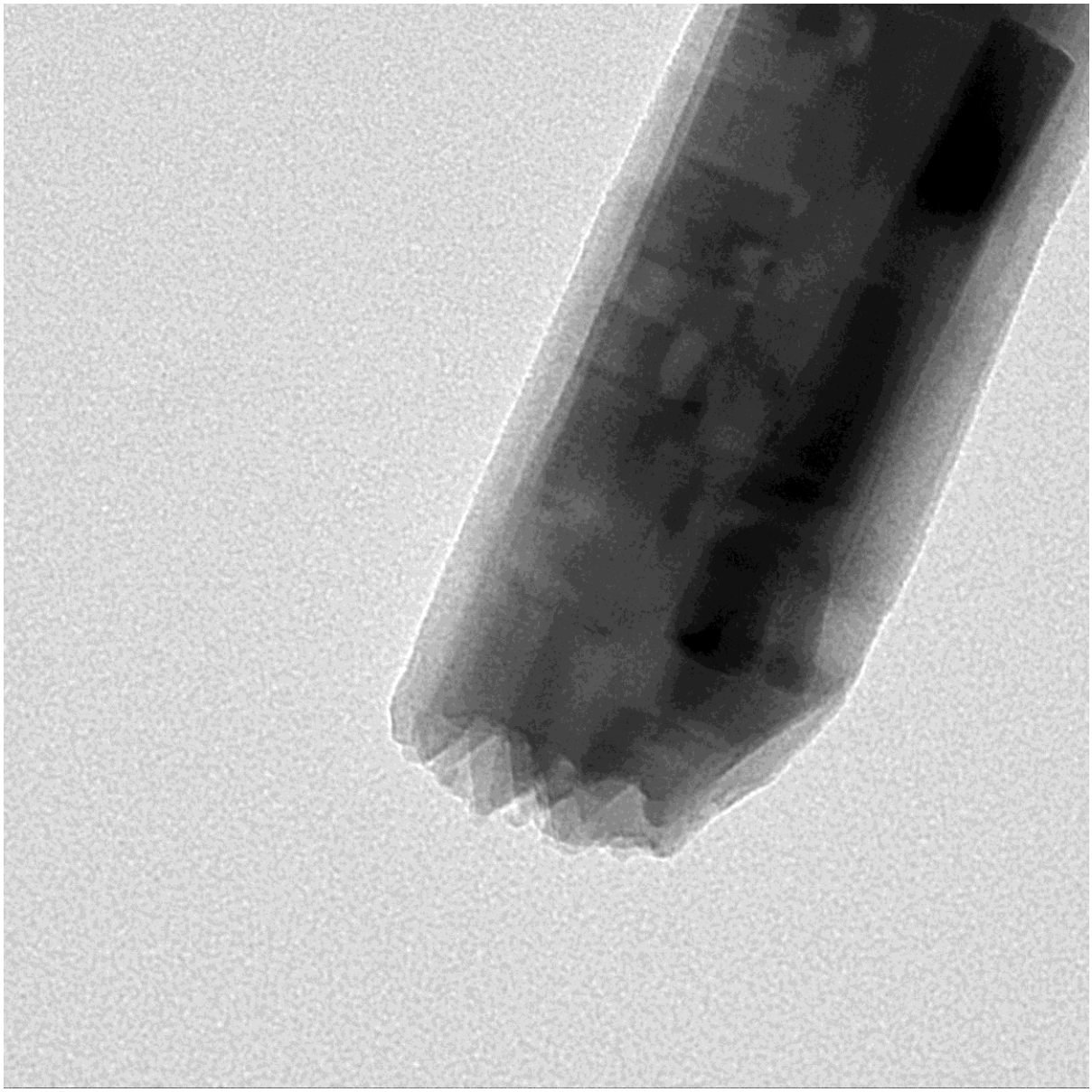


Figure S3: HPLC traces of different fractions following MIP purification of a CYP3A4-containing membrane-catalyzed oxidation of **4**. **A)** The flow-through fraction contains a large amount of material which elutes near the solvent front but contains no starting material (**4**) or product (**5**). **B)** The wash fraction. Note the scale. **C)** The elution fraction contains approximately 10% impurities and 90% of starting material (**4**) and enzymatic reaction product (**5**).

VII. TEM images of MIP made using compound 5 as ligand template







VIII. References

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2. Chefson, A.; Zhao, J.; Auclair, K., Replacement of Natural Cofactors by Selected Hydrogen Peroxide Donors or Organic Peroxides Results in Improved Activity for CYP3A4 and CYP2D6. *ChemBioChem* 2006, *7* (6), 916-919.
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4. Wang, D.; Hong, S.; Yang, G.; Row, K., Caffeine Molecular Imprinted Microgel Spheres by Precipitation Polymerization. *Korean Journal of Chemical Engineering* 2003, *20* (6), 1073-1076.
5. Umpleby Ii, R. J.; Baxter, S. C.; Rampey, A. M.; Rushton, G. T.; Chen, Y.; Shimizu, K. D., Characterization of the heterogeneous binding site affinity distributions in molecularly imprinted polymers. *Journal of Chromatography B* 2004, *804* (1), 141-149.