**Supplementary Data for:**
**Smithson et al. - Discovery of Potent and Selective Inhibitors of *Trypanosoma brucei* Ornthine Decarboxylase.**

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***Assay Automation and Nitrogen Atmosphere Generation***

All screening data was generated on a High Resolution Engineering (Woburn, MA) integrated screening system using Liconic plate incubators (Woburn, MA) and a Stabuli T60 robotic arm (Stabuli, SC). This system is enclosed in a virtually gas tight Plexiglass enclosure allowing a nitrogen atmosphere to be generated by continual purging with approximately 30 psi nitrogen through twin 8 mm inner diameter tubes. Nitrogen was obtained via an in-house dry-nitrogen system supplied by NexAir (Memphis, TN). Percent oxygen was monitored using an Air Aware oxygen detector (model 6810-0056, Industrial Scientific, Oakdale, PA) and maintained at less than 2.5% throughout all high throughput assays. Assay solutions were dispensed using Matrix Wellmates (Matrix Technologies, NH) equipped with 1µl rated tubes. Plates were centrifuged after all bulk liquid additions using a Vspin plate centrifuge (Velocity11, Menlo Park, CA). Compound transfers were performed using a 384-well pin tool equipped with 10 nl slotted hydrophobic surface coated pins (V&P Scientific, San Diego, CA). This allowed delivery of ~25 nl of DMSO stock solution with CVs of less than 10%. All absorbance data was measured using an EnVision Multilabel Plate Reader equipped with a 340 nm narrow bandwidth filter (Perkin Elmer, 2100-5740).

***ODC-PEPC-MDH Linked Assay***

This assay was performed under nitrogen atmosphere as described.{Smithson, 2010 #173} Assay buffers were prepared under normal atmosphere while flushing with a stream of nitrogen and transferred to the screening enclosure. Assay buffer (66 mM TRIS, 25 mM NaCl, 8 mM $MgSO_4$, 0.01% Triton-X, pH 8.05) was prepared daily in water. The assay reaction was prepared using two master mixes A and B, which were prepared immediately before use. Mix A contained ODC (375 to 750 nM), PLP (150 µM) and DTT (5.7 mM) in assay buffer. Mix B contained Infinity™ Carbon Dioxide Liquid Stable Reagent (Infinity™ $CO_2$) and L-ornithine (0 to 10 mM). For testing 10 µl Mix A was added to appropriate wells in a 384-well clear-bottomed plate followed by compounds transferred by pin. Plates and compounds were allowed to equilibrate in the presence of ODC for 20 minutes. Following this 15 µl Mix B was added to start the reaction. Plates were then incubated at room temperature for 15 minutes to allow the signal to stabilize before being moved to the plate reader. Final primary screening assay conditions were 2.3 mM DTT, 60 µM PLP, 625 µM L-Orn, 150 nM TbODC, 10 µM test compound and 60% Infinity™ $CO_2$ (v/v) in assay buffer with a final volume of 25 µl.

Reaction progress was monitored by decrease in absorbance at 340 nm using an Envision plate reader (Perkin Elmer) equipped with a narrow bandwidth 340 nm filter (Perkin Elmer, 2100-5740). Absorbance was monitored for 6 minutes, with time points taken every minute. These data were fit to a linear model using statistical methods described below. The resulting slope of this fit was taken as the rate of the reaction and used as the endpoint for the assay.

Compounds for screening were placed in 384-well polypropylene plates (Corning Life Sciences, Acton, MA) at 10 mM concentrations in DMSO. Sixteen Positive controls (DFMO, 1 M in DMSO) and 16 negative controls (DMSO) were placed in a single separate 384-well polypropylene plate and pin-transferred to test plates after the addition of variable compounds.

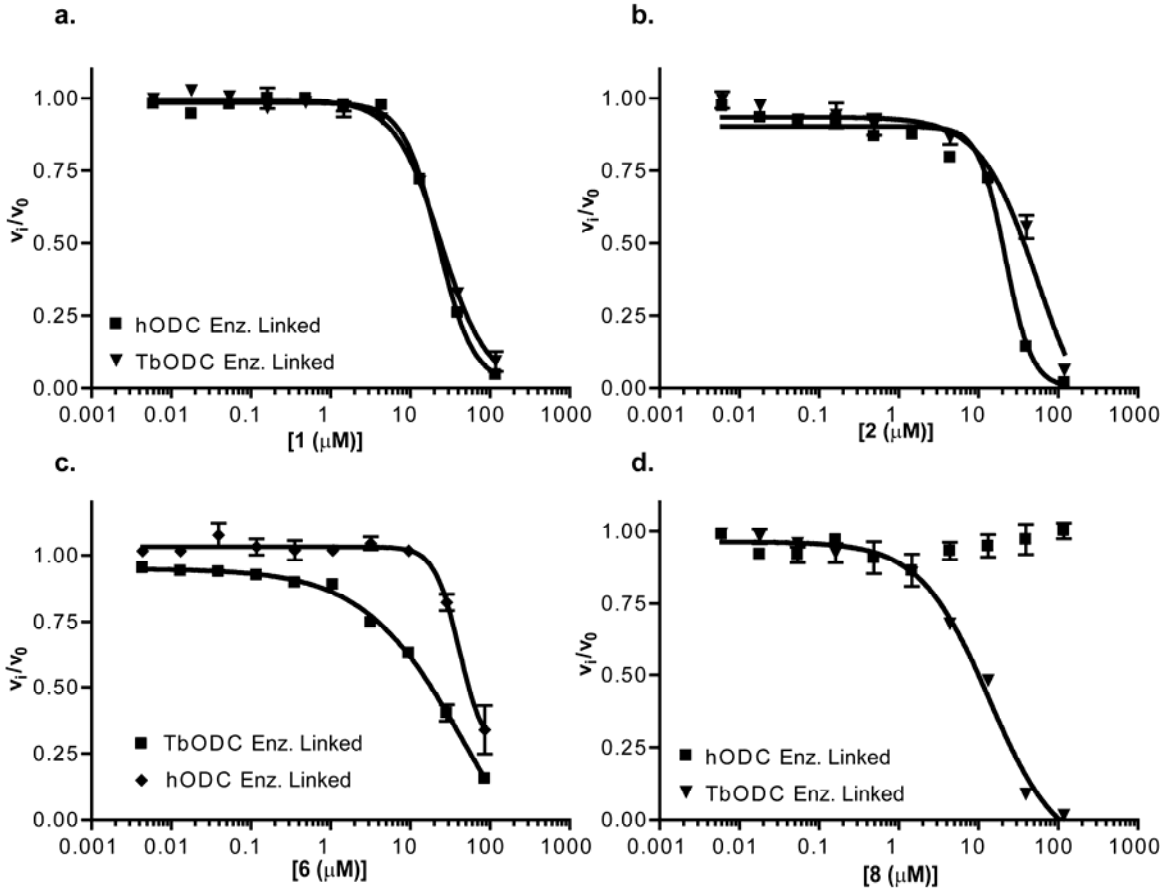
Cuvette assays for low-throughput re-testing were performed as described above with the following minor modifications; the final assay volume in cuvettes was 500 µl at 40% Infinity™ Carbon Dioxide Liquid Stable Reagent, 50 µM PLP, 50 µM DTT, 1% DMSO and varied ornithine concentrations from 10 mM to 100 µM. As with microplate assays, assay buffer (66 mM TRIS, 25 mM NaCl, 8 mM $MgSO_4$, 0.01% Triton-X, pH 8.05) was prepared fresh daily. Cuvette assays were performed under normal atmosphere at 37 °C.

***PEPC-MDH Linked Assay***

For assay of the linking enzymes, assay buffer (66 mM TRIS, 25 mM NaCl, 8 mM $MgSO_4$, 0.01% Triton-X, pH 8.05) was prepared daily using water. The assay reaction was prepared in two master mixes. Mix A contained 1.25 mM sodium bicarbonate (Sigma Aldrich), 100 µM PLP, and 5.7 mM DTT in assay buffer. Mix B was 100% Infinity™ Carbon Dioxide Liquid Stable Reagent. For testing 15 µl Mix B was added to appropriate wells of a 384-well clear-bottom microplate followed by pin-transfer of compound DMSO stocks. Compounds were allowed to equilibrate in the presence of enzymes for 20 minutes before substrate was added. The reaction was started by addition of 10 µl Mix A and reaction progress was monitored by decrease in absorbance at 340 nm.

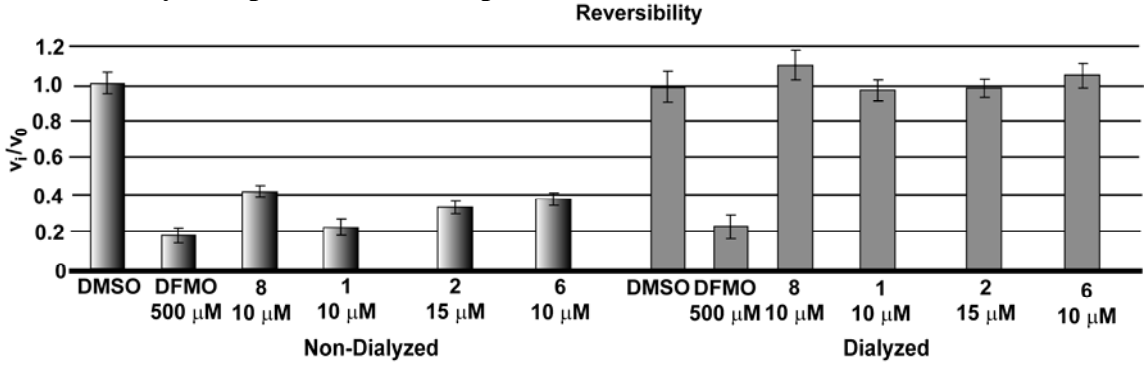
Final assay concentrations were 2.3 mM DTT, 60 µM PLP, 0.75 mM sodium bicarbonate, 60% Infinity™ Carbon Dioxide Liquid Stable Reagent, and 0.01% Triton-X. Reaction progress was monitored by decrease in absorbance at 340 nm using an Envision plate reader (Perkin Elmer) equipped with a narrow bandwidth 340 nm filter (Perkin Elmer, 2100-5740) for 10 minutes with time points taken every minute. Data from minutes two to seven was fit to a linear model using statistical methods as described below.

**Species Selectivity Curves for Compounds 1, 2, 6 and 8**



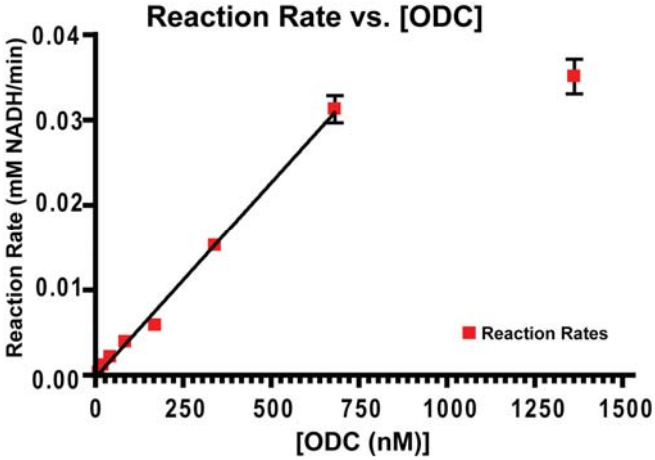

Data was gathered in 384 well plates as described in materials and methods. All curves were determined under isokinetic conditions at [1.5x $K_m$] L-ornithine. (625 μM L-Orn for TbODC, 150 μM L-Orn for hODC) $IC_{50}$ experiments were performed in independent triplicates.

**Reversibility of representative compounds**



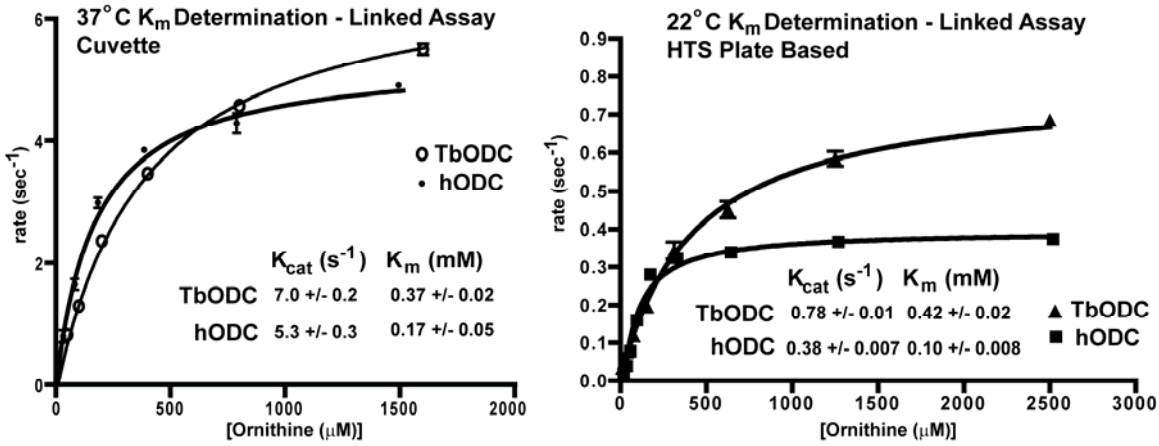

Reversibility was determined as described in materials and methods.

**Reaction Linearity with Respect to [ODC]**

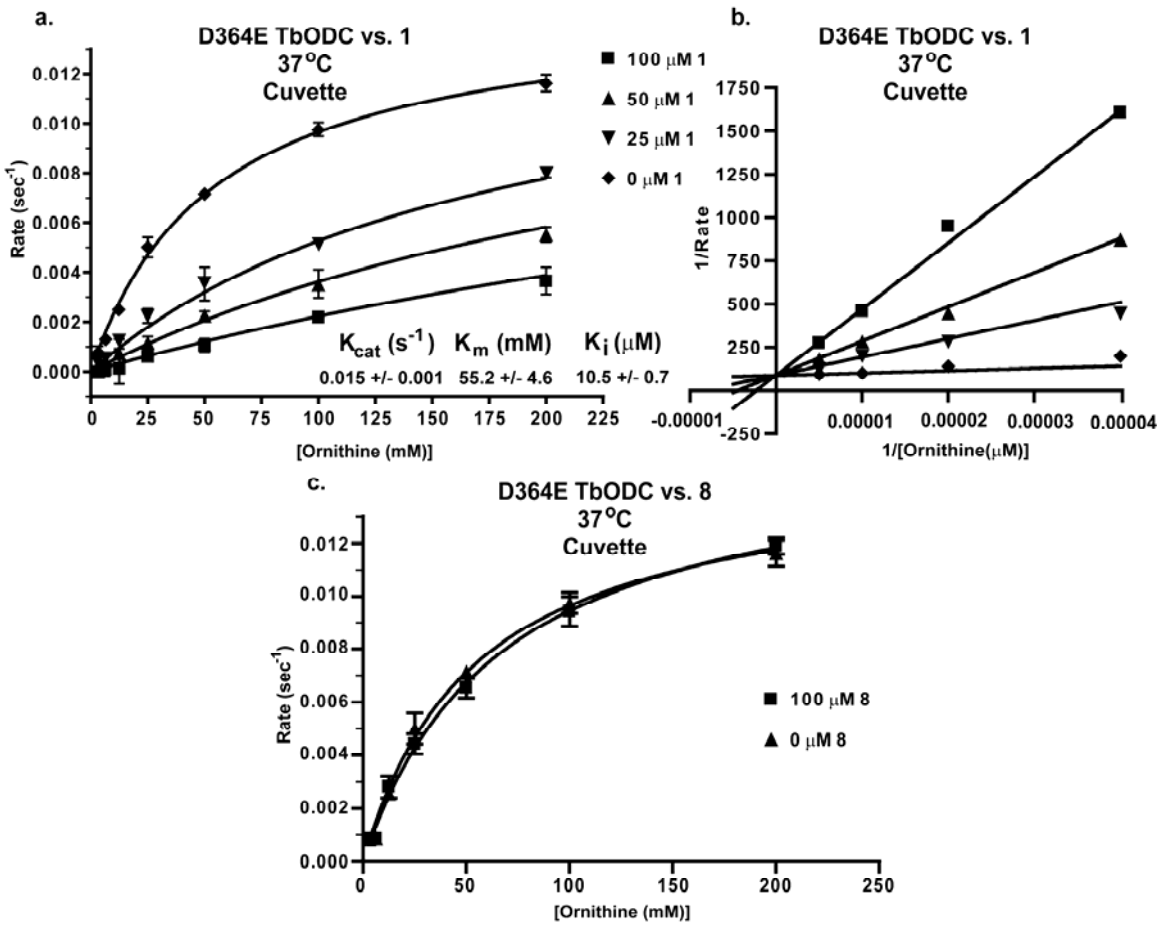



Linearity was determined at 625 μM Orn and 60 μM PLP in 384-well plates using HTS assay protocol under a nitrogen atmosphere.

**$K_m$ Determination for Enzymes used in Study**

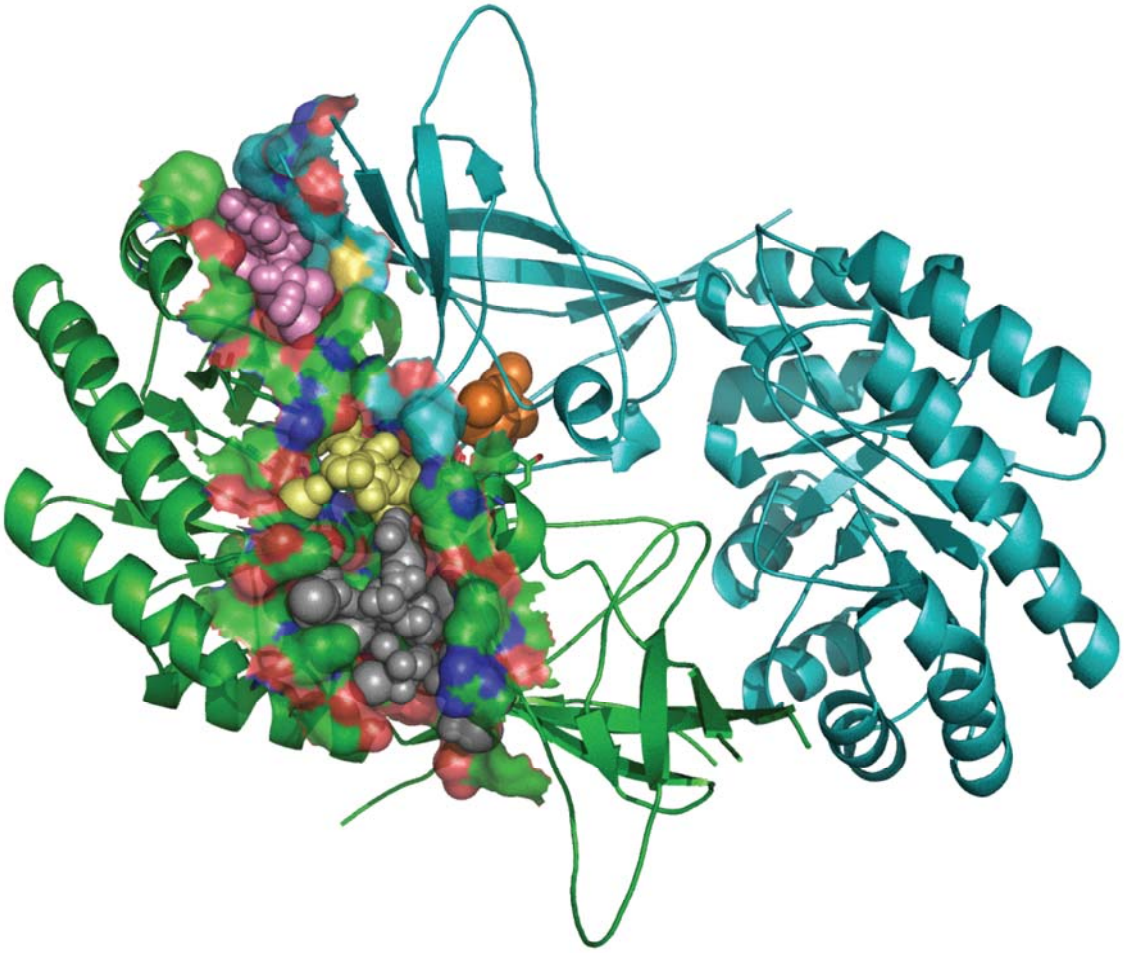



TbODC and hODC in plate based assay were 150nM, TbODC and hODC in cuvette assays were at 100nM.



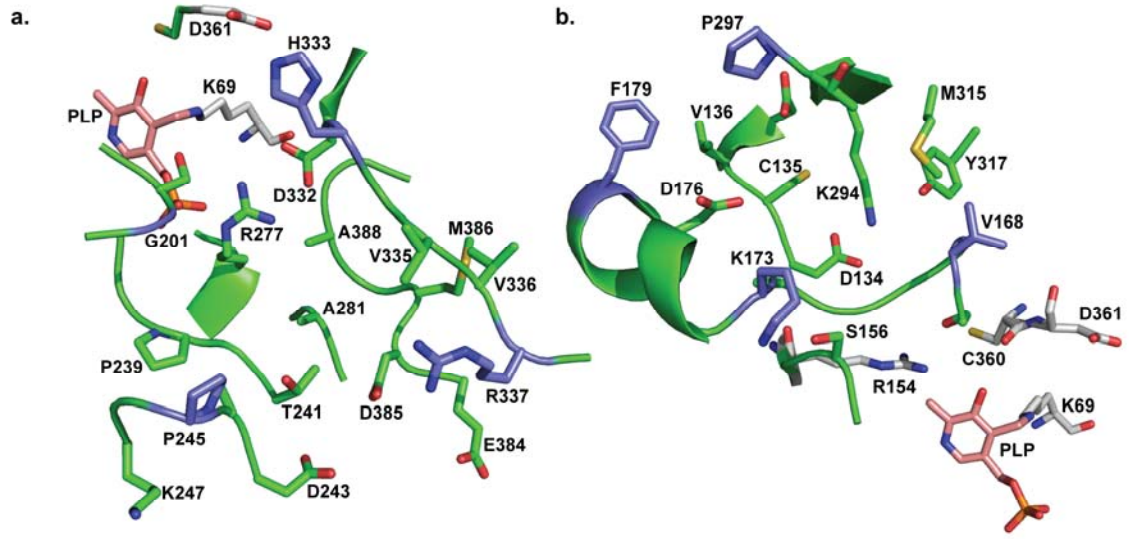

D364E TbODC was tested in cuvettes at 20 μM ODC due to low activity of the enzyme. **a.** Rate data for compound **1** vs. TbD364E fit to the Michaelis-Menton equation for competitive inhibitors showing clear inhibition. **b.** *Lineweaver-Burke* plot of data from **a.** showing that compound **1** is clearly acting as a competitive inhibitor of TbD364E. **c.** Rate data for compound **8** vs. TbD364E showing no effect on enzymatic rates at 100 μM **8** over a wide range of substrate concentrations. Data was fit to the Michaelis-Menton equation.

**Proposed Alternate Binding Sites for Dithioamidine Compounds**



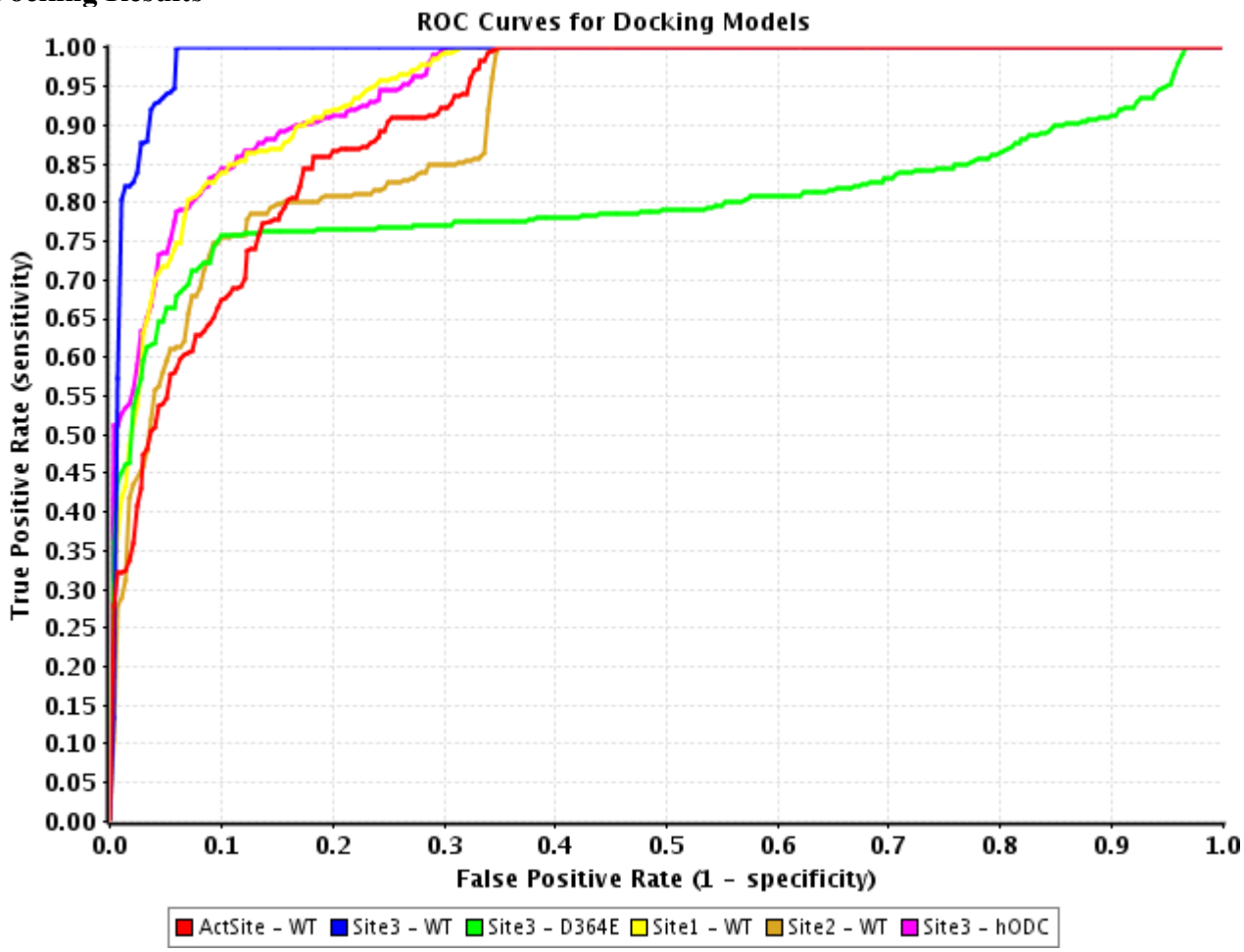
The above structure is based on the TbODC structure 1QU4. Green coloration represents chain C of the dimer and blue coloration represents chain D of the dimer. Yellow spheres represent the enzyme active site where ornithine and PLP bind. Gray spheres represent potential alternate binding site 1, pink spheres represent potential alternate binding site 2 and orange spheres represent potential alternate binding site 3.

**Proposed Alternate Binding Sites – Residue View**




**a.** Site 1. Apo TbODC structure. PLP is salmon, active site residues are white and residues that form the boundaries of the entrance to the proposed binding site are purple. **b.** Site 2. Apo TbODC structure. PLP is salmon, active site residues are white and residues that form the boundaries of the entrance to the proposed binding site are purple.

**Docking Results**

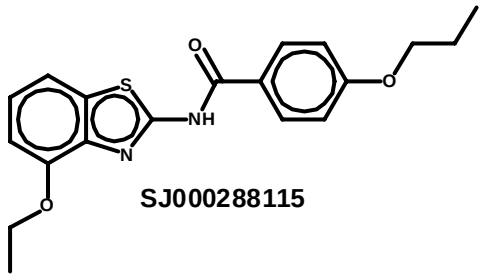



**ROC Curves for Docking Models** – Enrichment plots for each alternate binding site tested using molecular docking approaches. Note that Site 3 outperforms other models, while the remaining sites perform similarly. Site 3 in the D364E mutant model performs much worse than the same site in the wild-type TbODC enzyme. The top 100 poses of each compound were used for calculating enrichment, and true positives were defined as those compounds with $K_i$ values less than 50 μM. AUC scores are as follows: ActSite – WT (Active Site, wild-type TbODC): 0.91, Site3-WT (Site 3, wild-type TbODC): 0.99, Site3-D364E (Site3, D364E TbODC model): 0.80, Site1-WT (Site 1, wild-type TbODC): 0.93, Site2-WT (Site 2, wild-type TbODC): 0.91, Site3-hODC (Site 3, wild-type hODC): 0.93.

**Additional Benzthiazoles Screened**

Note that these compounds did not have $K_i$ values determined, and while identities were confirmed by mass spec, purities were not able to be accurately assessed due to the limited amount of available compound. $IC_{50}$ values were obtained at 625 μM L-ornithine, 60 μM PLP and 150 nM TbODC, fit as described in materials and methods, and are expressed as 95% confidence intervals.

| Compound ID | $IC_{50}$ (μM) |
|---|---|
| SJ000288115 | 4.6 to 7.8 |
| SJ000024884 | 3.7 to 10.1 |
| SJ000002905 | Inactive (>100 μM) |
| SJ000019249 | Inactive (>100 μM) |
| SJ000151009 | Inactive (>100 μM) |
| SJ000246794 | Inactive (>100 μM) |
| SJ000121058 | Inactive (>100 μM) |
| SJ000150783 | Inactive (>100 μM) |



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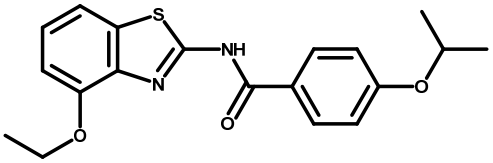
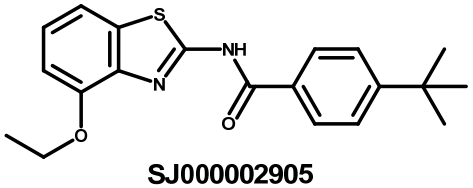


SJ000024884



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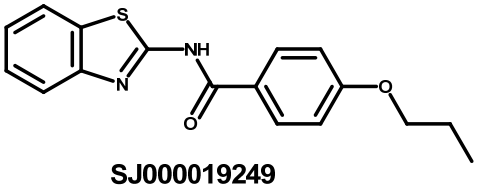

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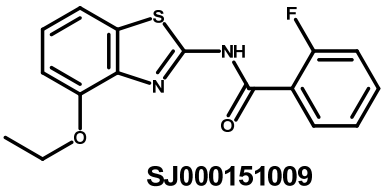

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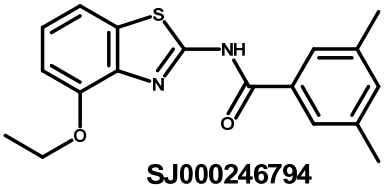
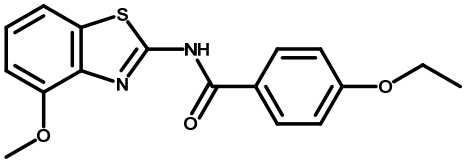
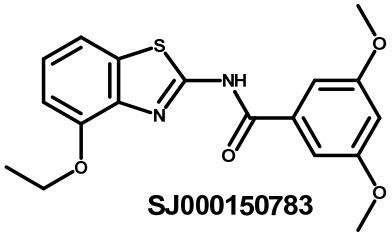


SJ000246794



SJ000121058



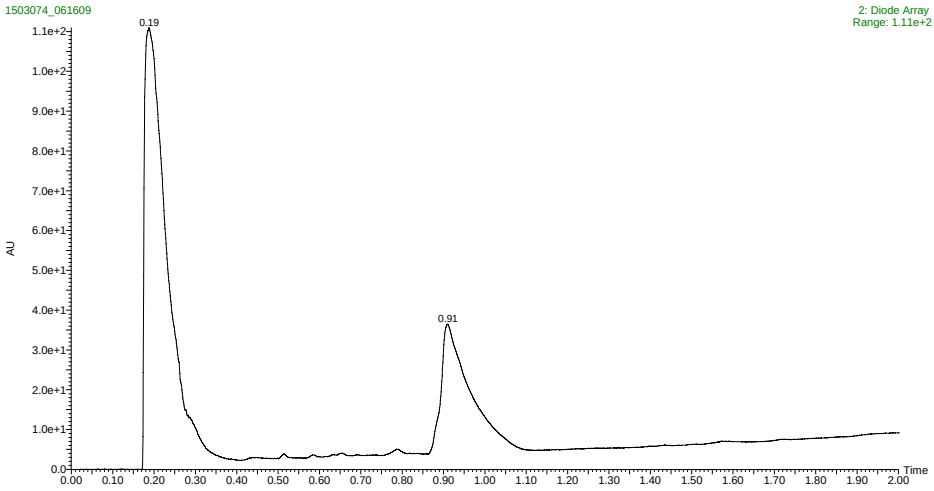
SJ000150783

**Paper numbering to St. Jude Registry Number Key** – please use the below designations when requesting additional information regarding compounds included in this report.

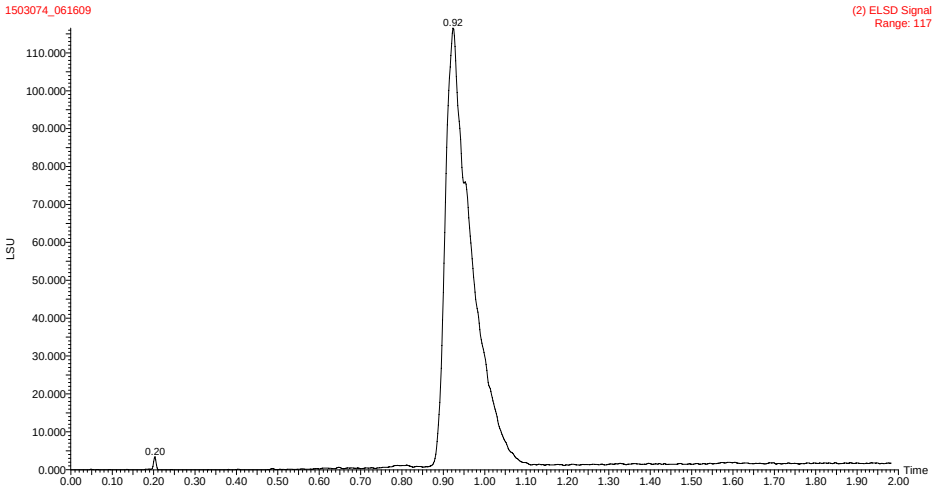
| SJREGNO | Internal Manuscript Numbering |
|---|---|
| SJ000285319 | 1 |
| SJ000026125 | 2 |
| SJ000360936 | 3 |
| SJ000122887 | 4 |
| SJ000298934 | 5 |
| SJ000126684 | 6 |
| SJ000360927 | 7 |
| SJ000288115 | 8 |
| SJ000359132 | 9 |
| SJ000359141 | 10 |
| SJ000285200 | 11 |
| SJ000359413 | 12 |
| SJ000359140 | 13 |
| SJ000359137 | 14 |
| SJ000354134 | 15 |

**Compound UPLC-MS Data**
**(Peaks at 0.2 min are DMSO solvent peaks)**

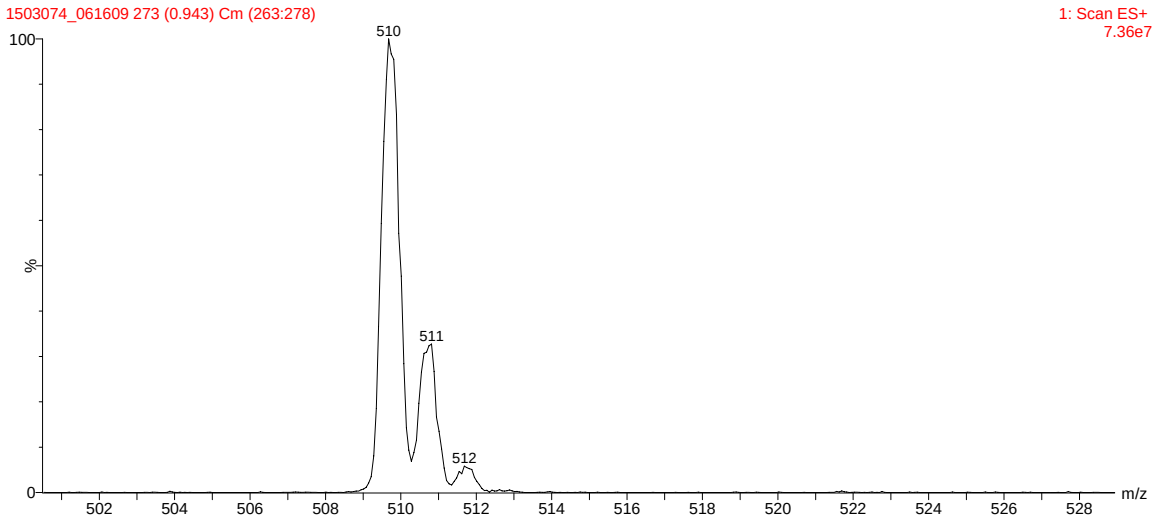
**1** (SJ000285319) UV-TIC – Purity = >95% - Product Mass at 0.91 min



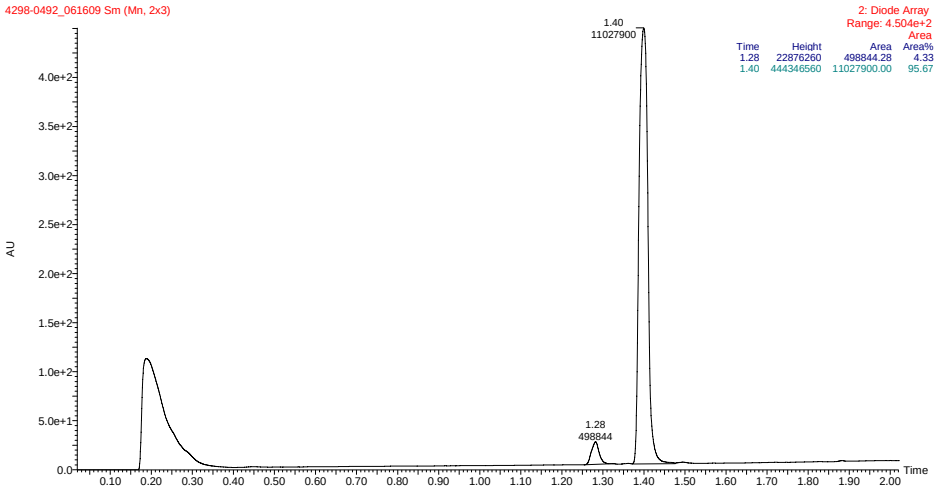

**1** (SJ000285319) ELSD Signal – Purity = >95% - Product Mass at 0.92 min



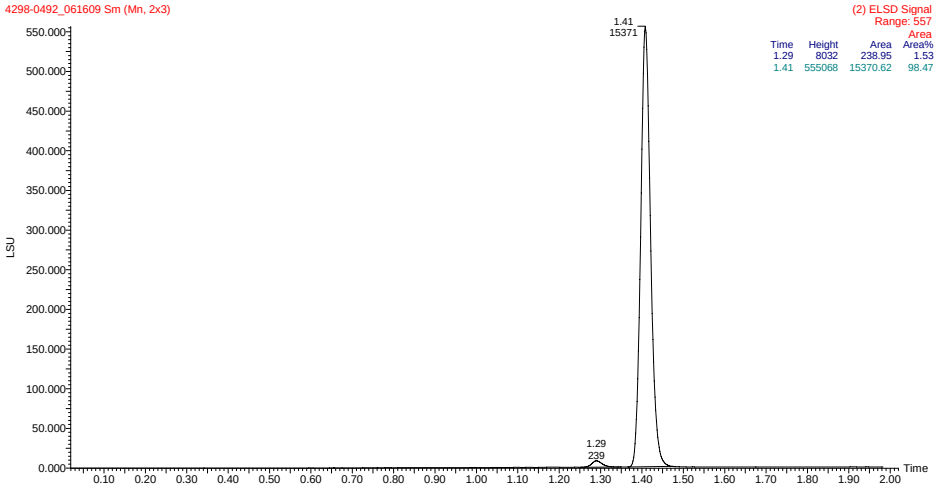

**1** (SJ000285319) Parent Ion – Expected Mass+1: 509.8



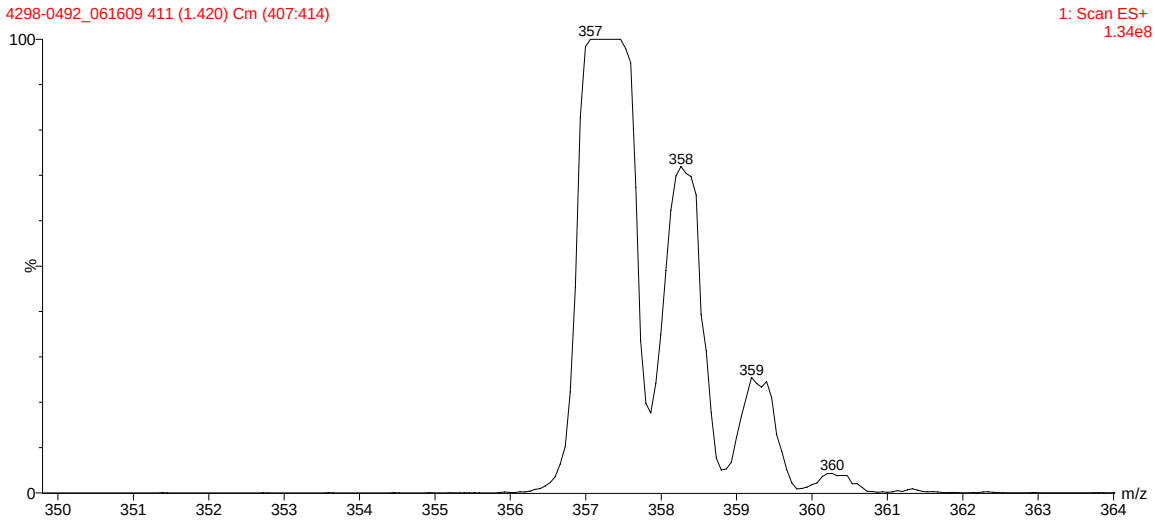
**2** (SJ000026125) UV-TIC – Purity = 95% - product mass at 1.40 min



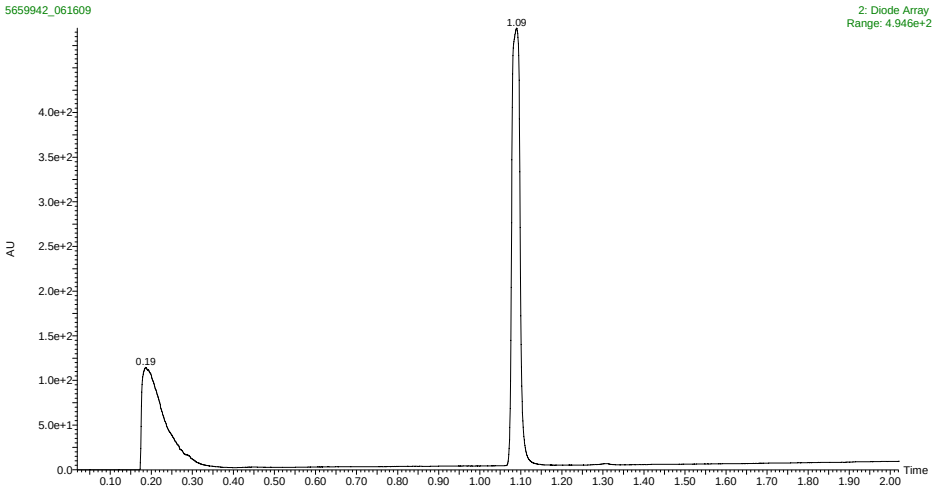

**2** (SJ000026125) ELSD Signal – Purity = 98 % - product mass at 1.41 min



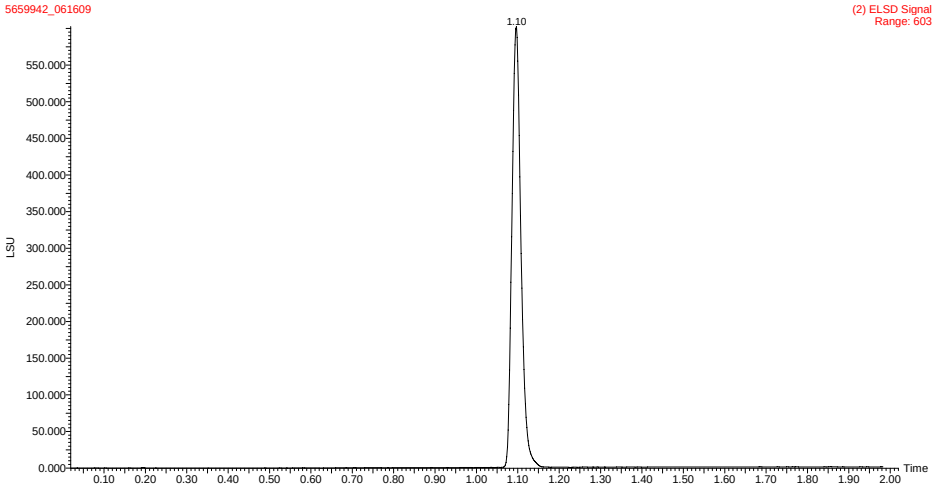

**2** (SJ000026125) Parent Ion – Expected Mass +1: 357.7



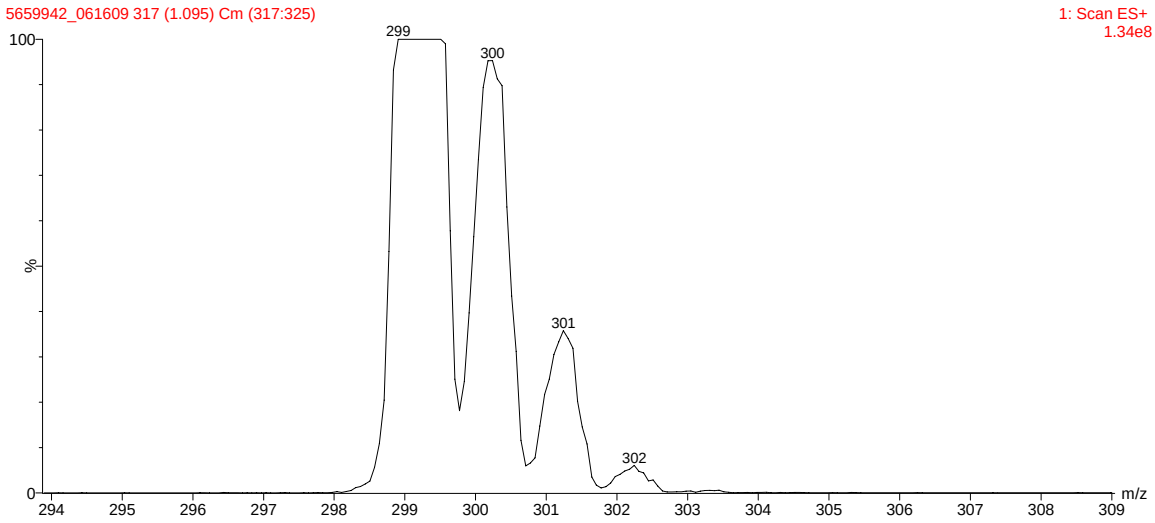
**3** (SJ000360936) – UV-TIC – Purity = >95% - product mass at 1.09 min



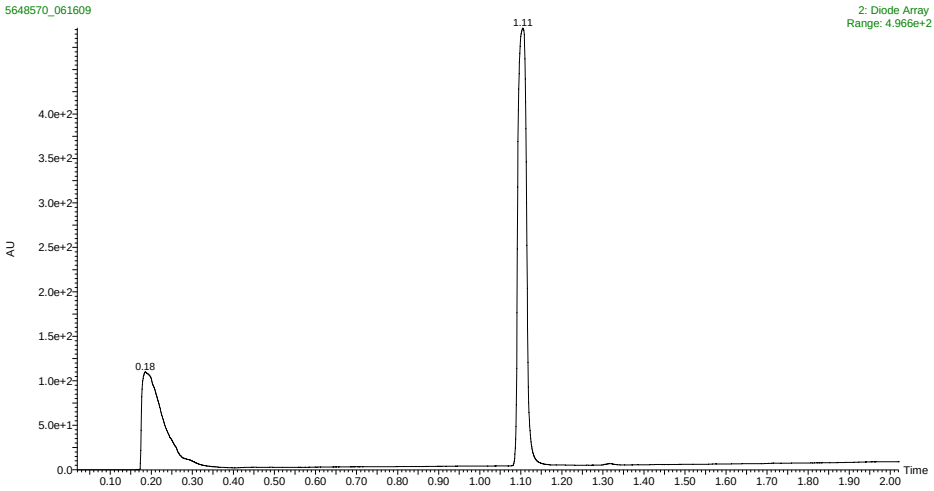

**3** (SJ000360936) – ELSD Signal – Purity = >95% - product mass at 1.10 min



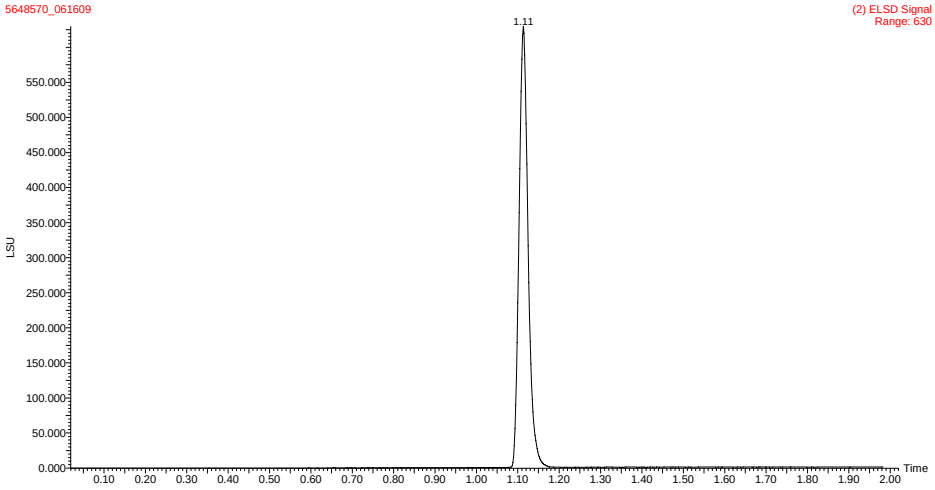

**3** (SJ000360936) Parent Ion – Expected Mass +1: 299.1



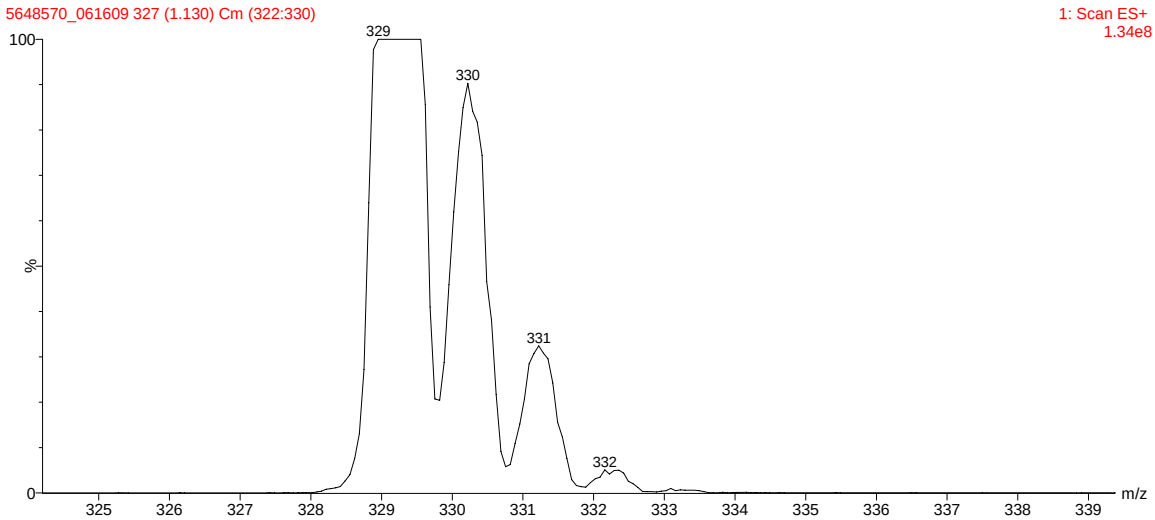
**4** (SJ000122887) UV-TIC – Purity = >95% - product mass at 1.11 min



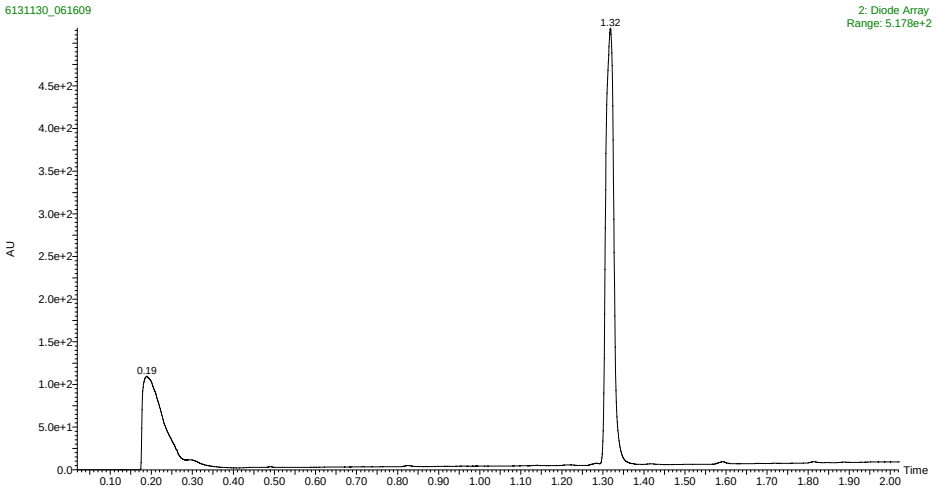

**4** (SJ000122887) ELSD Signal – Purity = >95% - product mass at 1.11 min



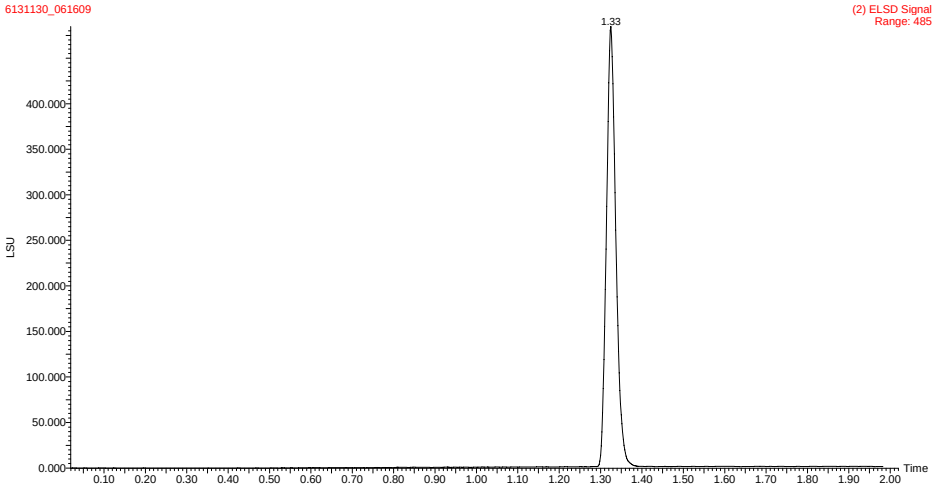

**4** (SJ000122887) Parent Ion – Expected Mass +1: 329.1



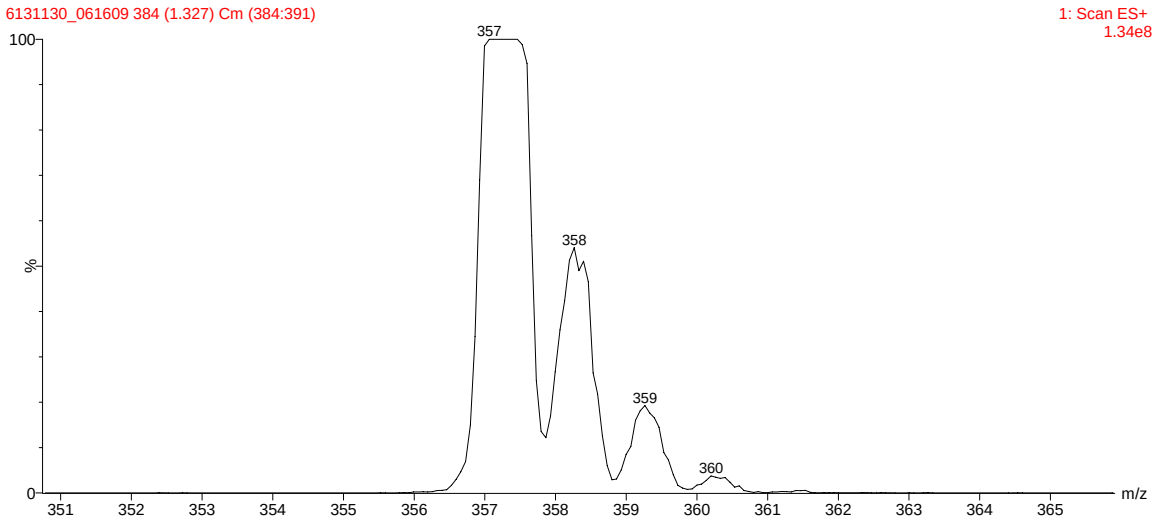
**5** (SJ000298934) UV-TIC – Purity = 100 % - product mass at 1.32 min



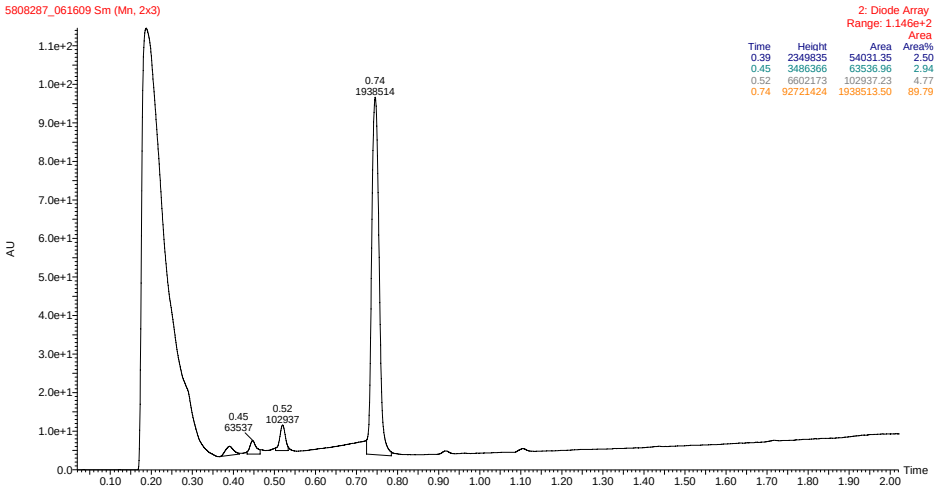

**5** (SJ000298934) ELSD – Purity = 100 % - product mass at 1.33 min



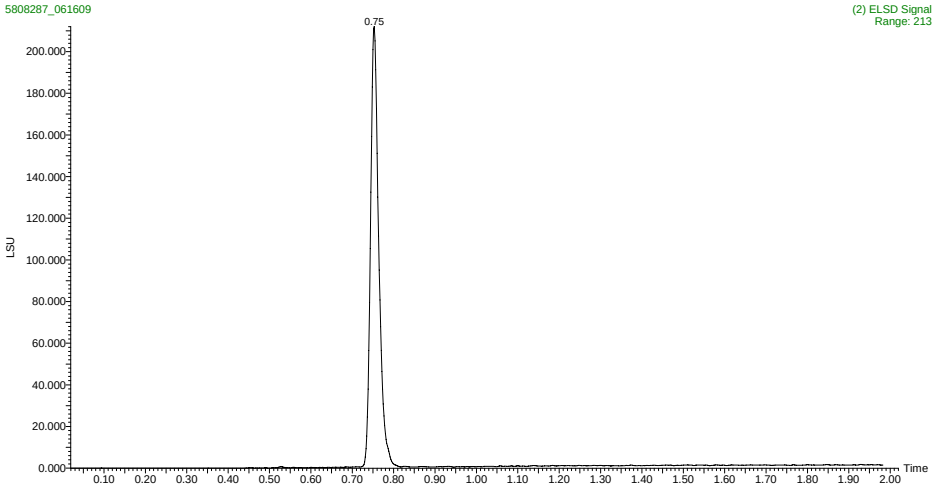

**5** (SJ000298934) Parent Ion – Expected Mass +1: 357.1



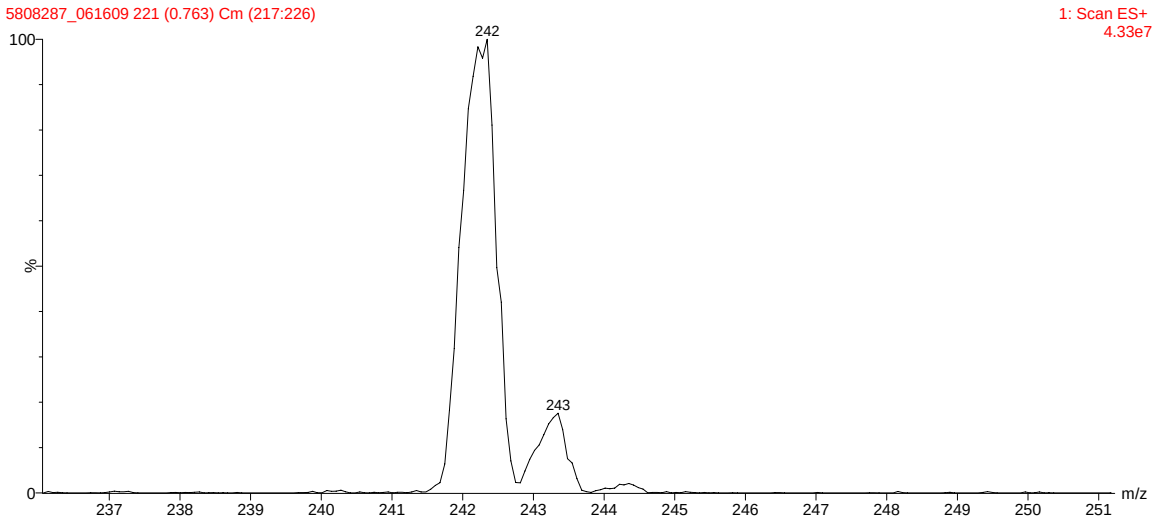
**6** (SJ000126684) – UV-TIC – Purity = 90% - product mass at 0.74 min



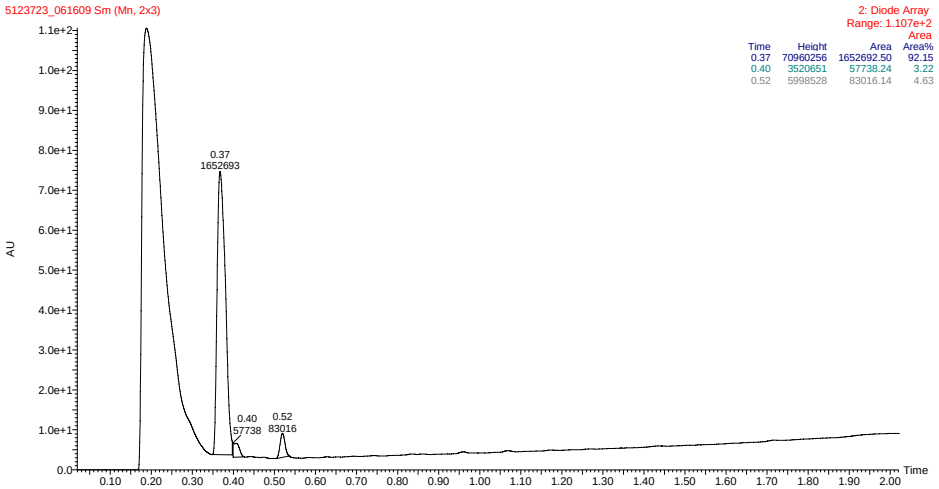

**6** (SJ000126684) – ELSD Signal – Purity = >95% - product mass at 0.75 min



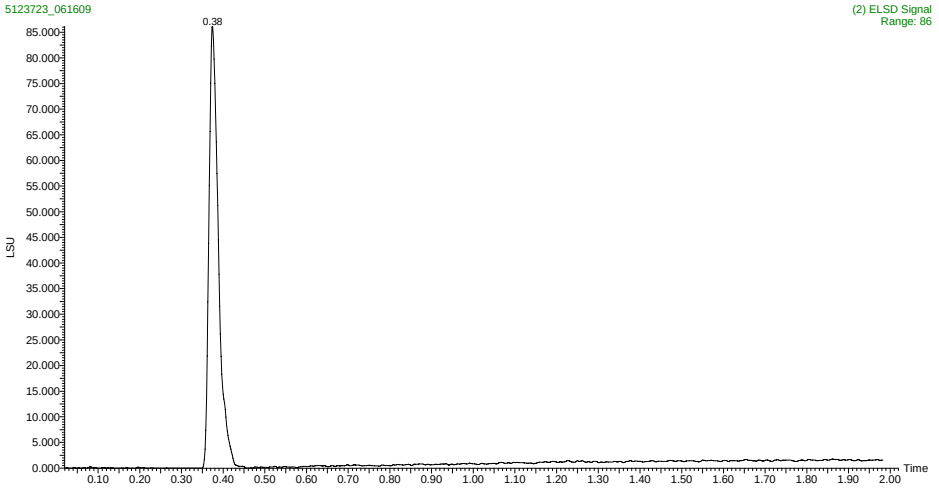

**6** (SJ000126684) Parent Ion – Expected Mass +1: 242.1



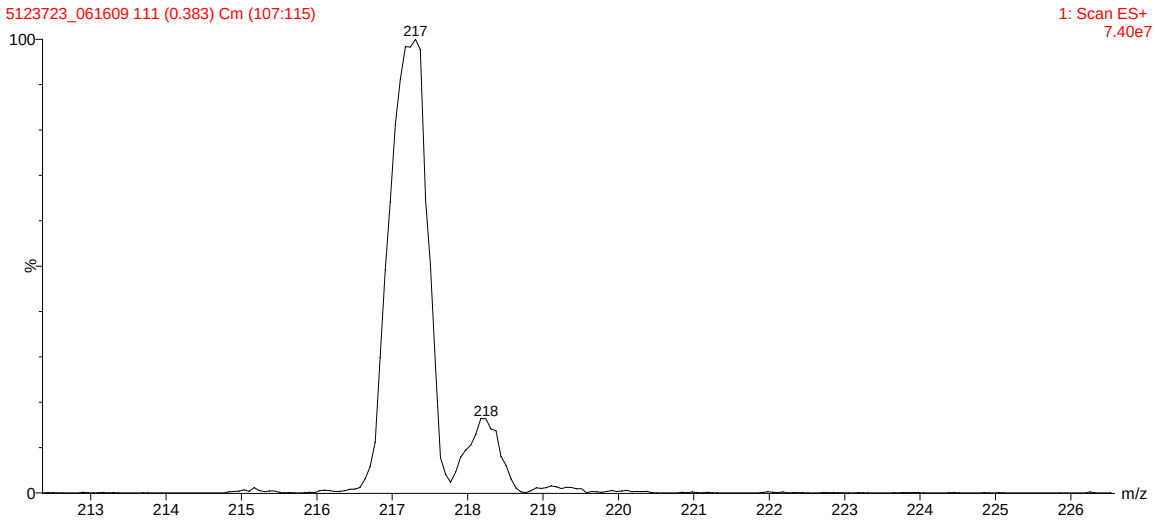
**7** (SJ000360927) – UV-TIC – Purity = 92 % - product mass at 0.37 min



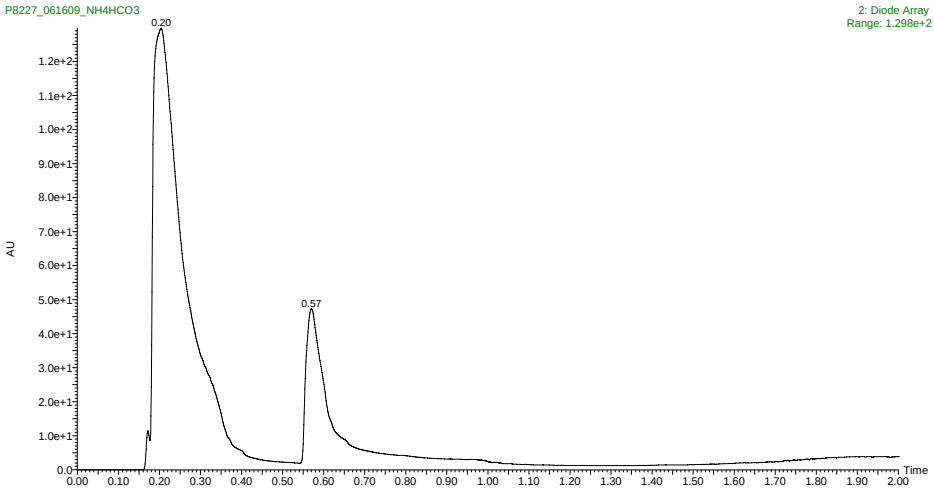

**7** (SJ000360927) – ELSD Signal – Purity = >95% - product mass at 0.38 min



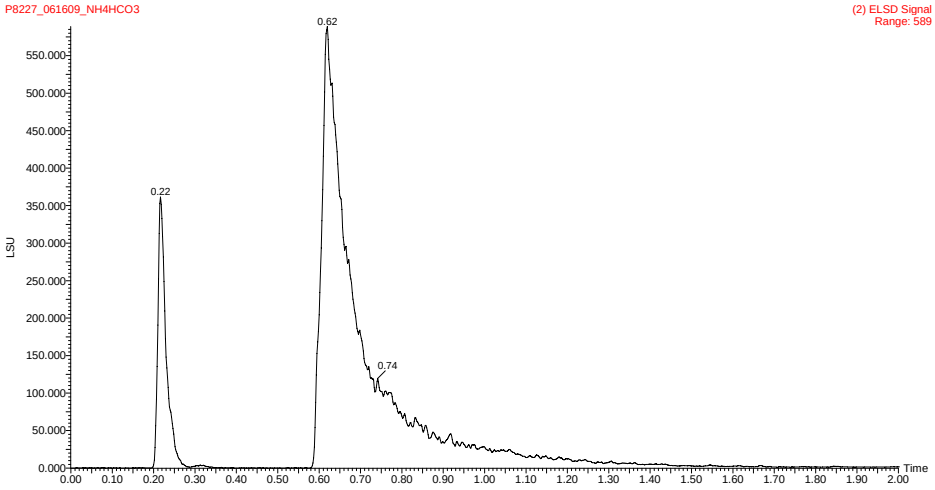

**7** (SJ000360927) Parent Ion – Expected Mass +1: 217.1



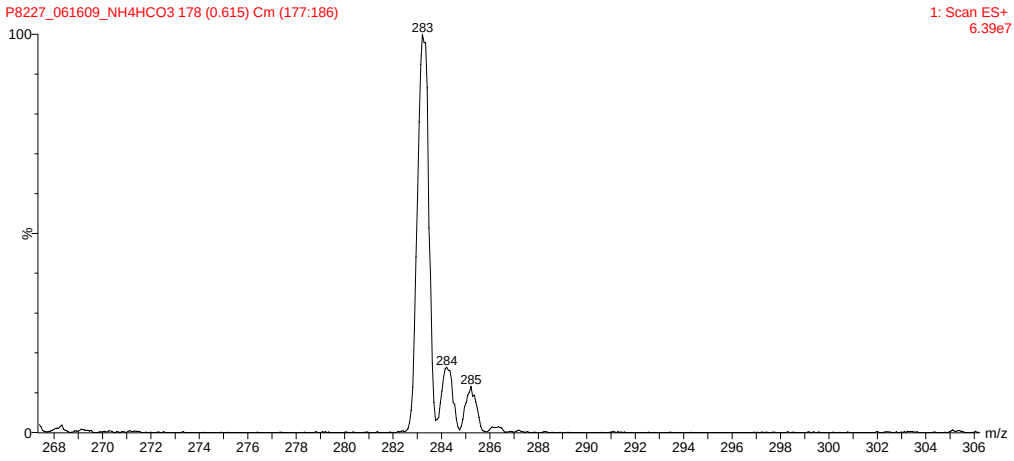
**8** (SJ000288115) UV-TIC – Purity = >95% - Product Mass at 0.57 min



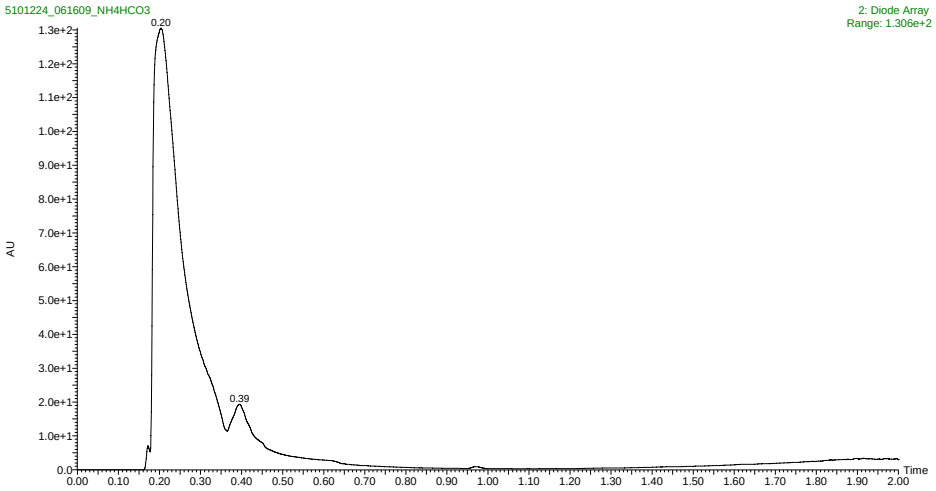

**8** (SJ000288115) ELSD Signal – Purity = >95%



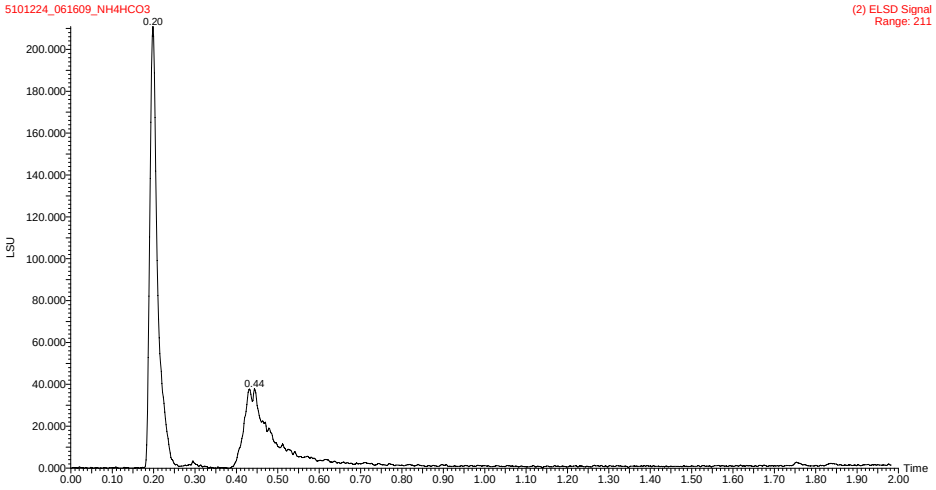

**8** (SJ000288115) Parent Ion – Expected Mass +1: 283.1



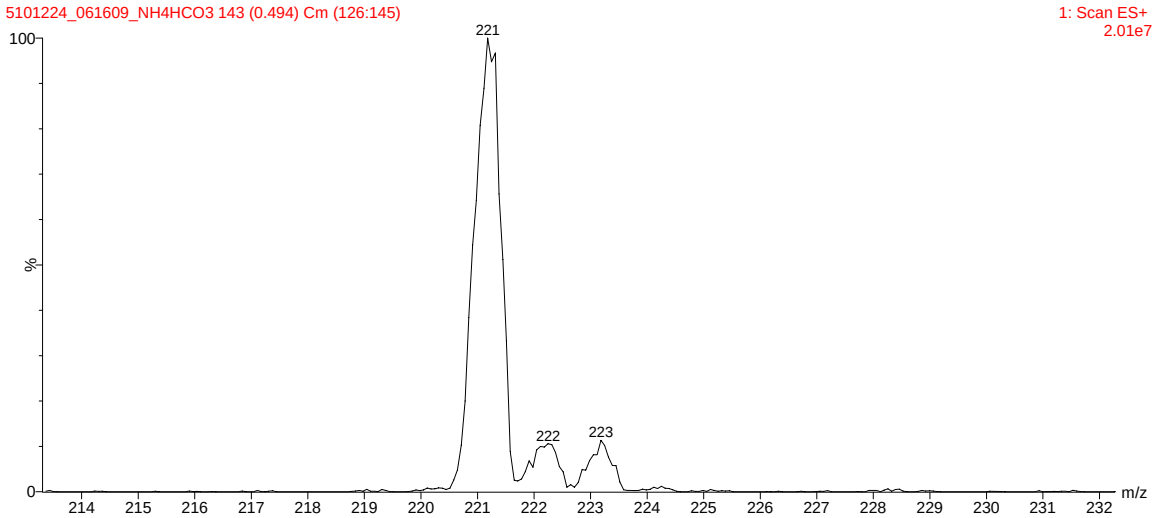
**9** (SJ000359132) UV-TIC – Purity = >95%, product mass at 0.39 min



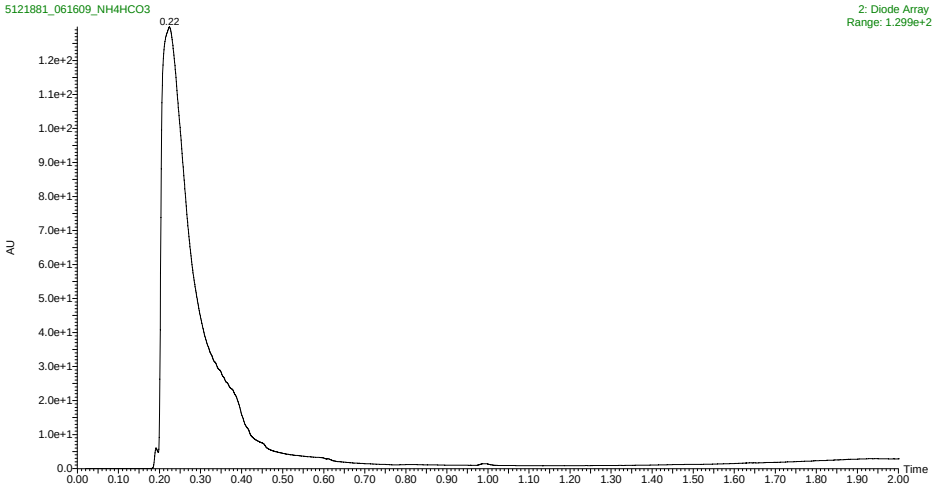

**9** (SJ000359132) ELSD Signal – Purity = >95%, product mass at 0.44 min



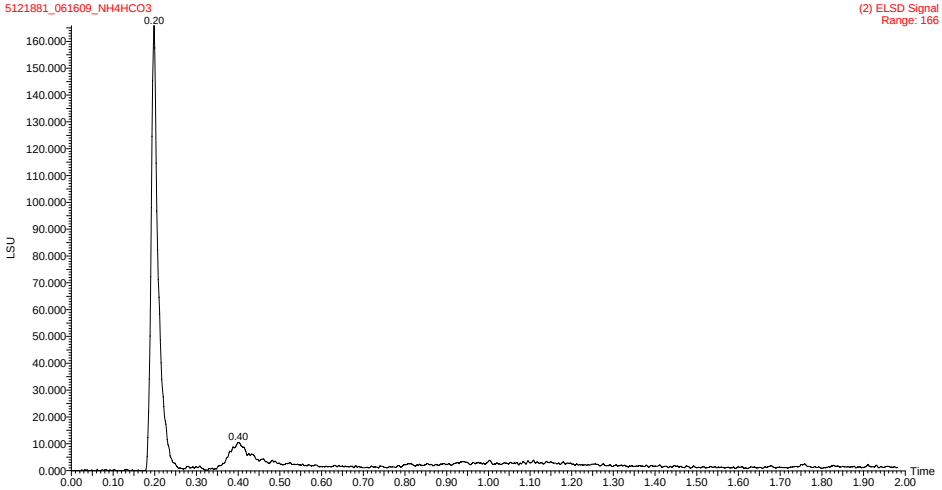

**9** (SJ000359132) Parent Ion – Expected Mass +1: 221.1



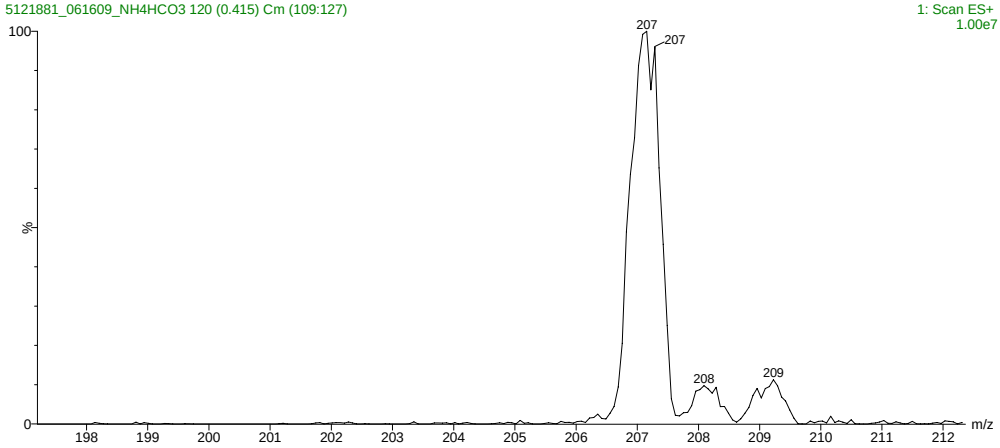
**10** (SJ000359141) UV-TIC – No Detectable UV Signal



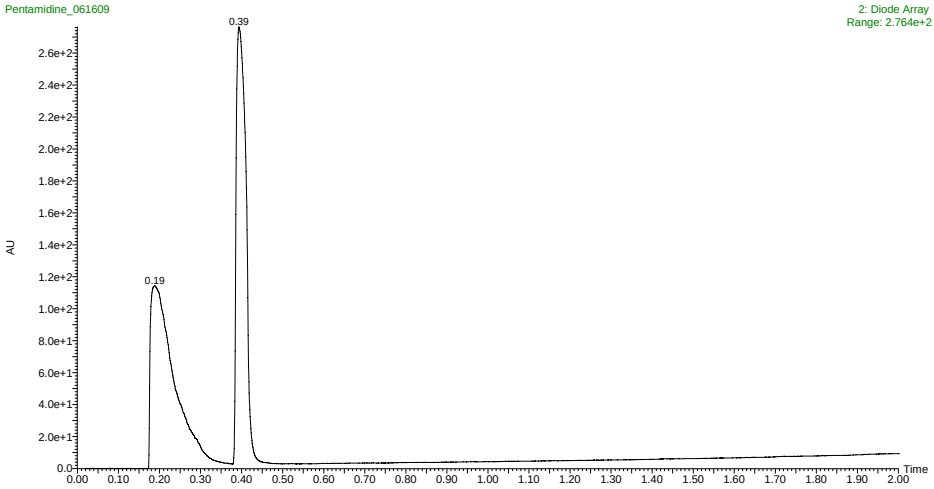

**10** (SJ000359141) ELSD Signal – Purity = >95% - Product mass at 0.4 min



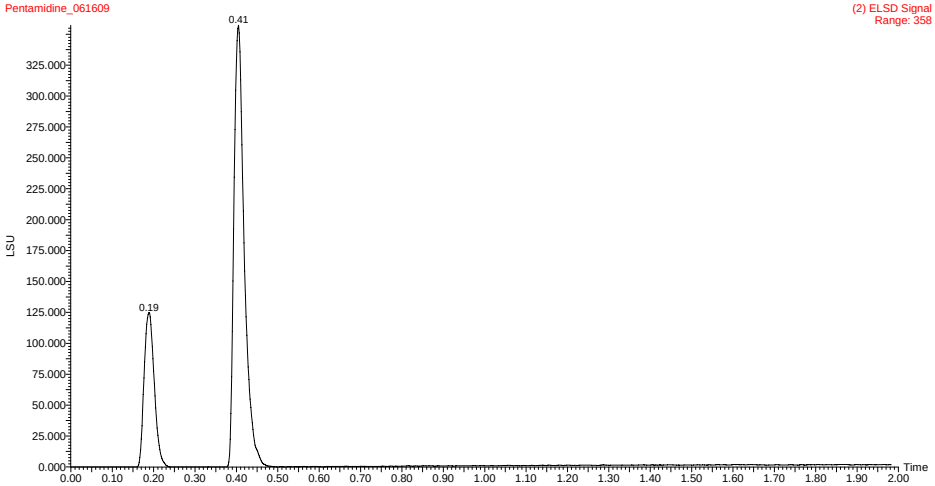

**10** (SJ000359141) Parent Ion – Expected Mass +1: 207.1



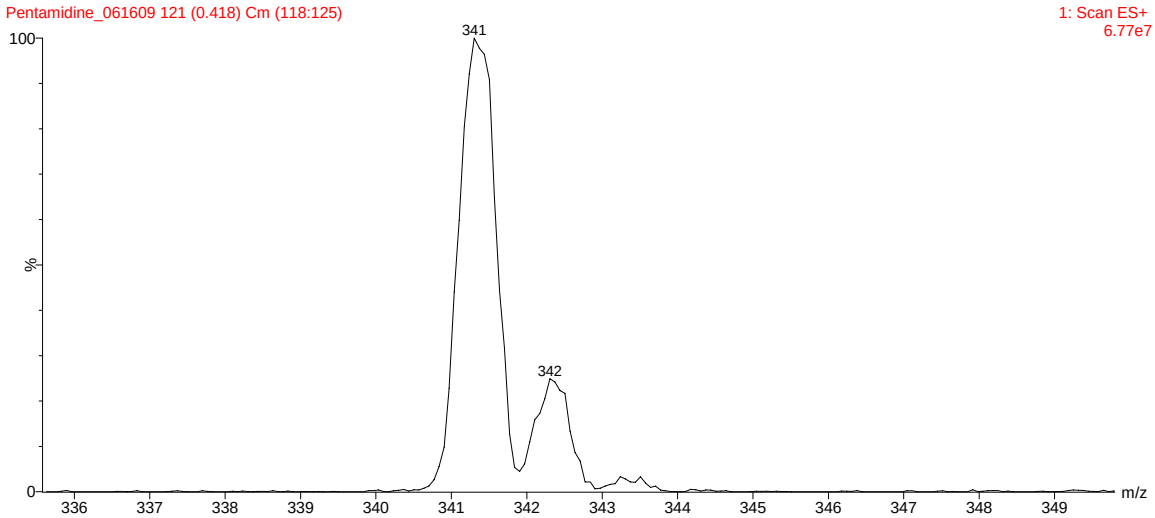
**11** (SJ000285200) UV-TIC – Purity = >95% - product mass at 0.39 min



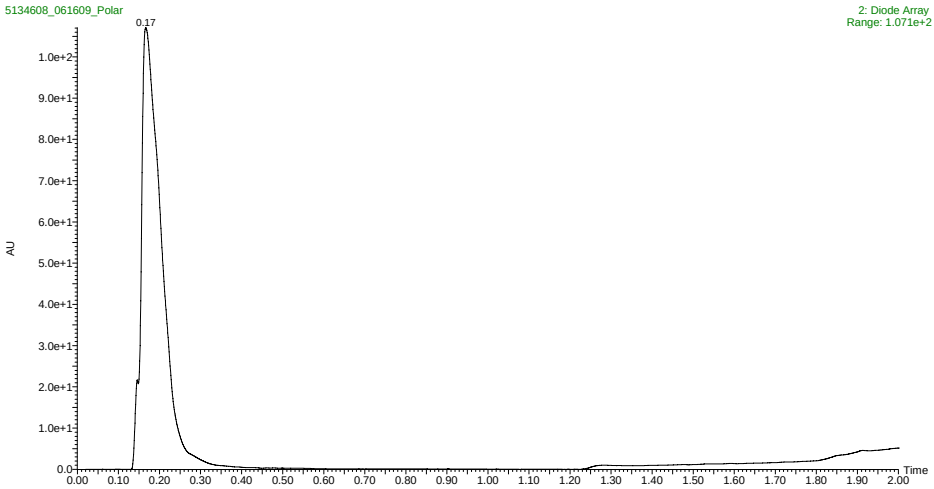

**11** (SJ000285200) ELSD – Purity = 100 % - product mass at 0.41 min




**11** (SJ000285200) Parent Ion – Expected Mass +1: 341.4

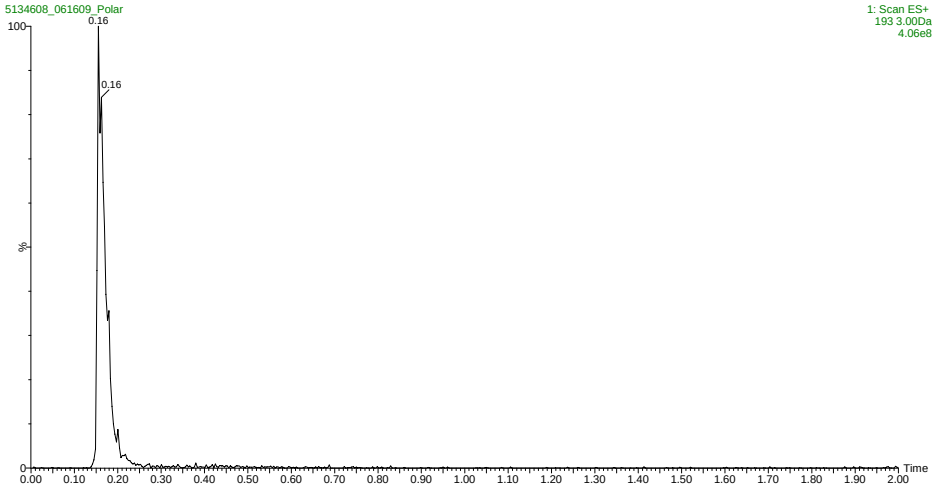


**12** (SJ000359413) UV-TIC – Product in Solvent Peak – attempts at separation unsuccessful – no masses other than product and solvent were detected in solvent peak.

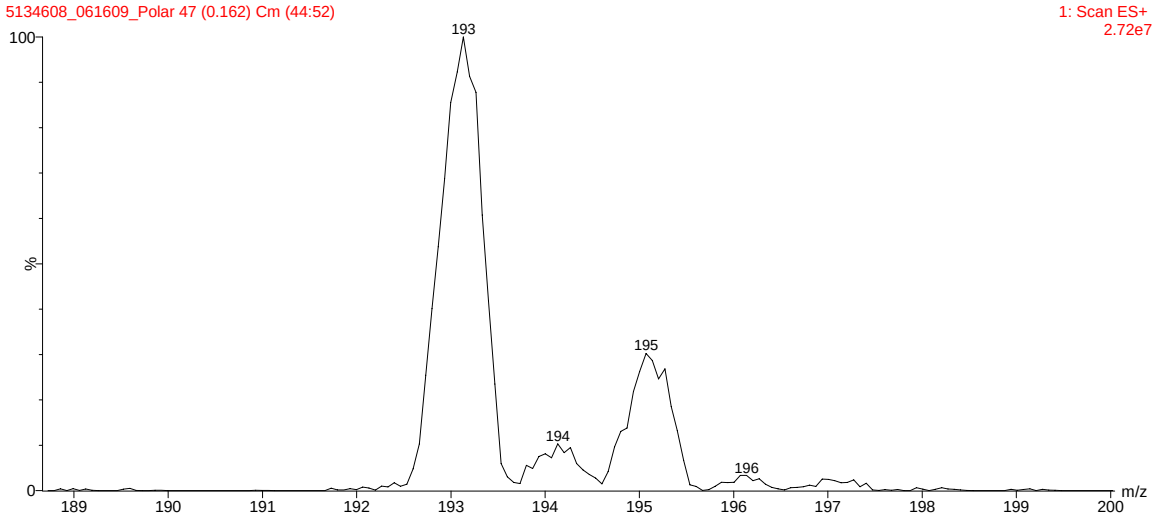



**12** (SJ000359413) ELSD Signal – Product in Solvent Peak – attempts at separation unsuccessful

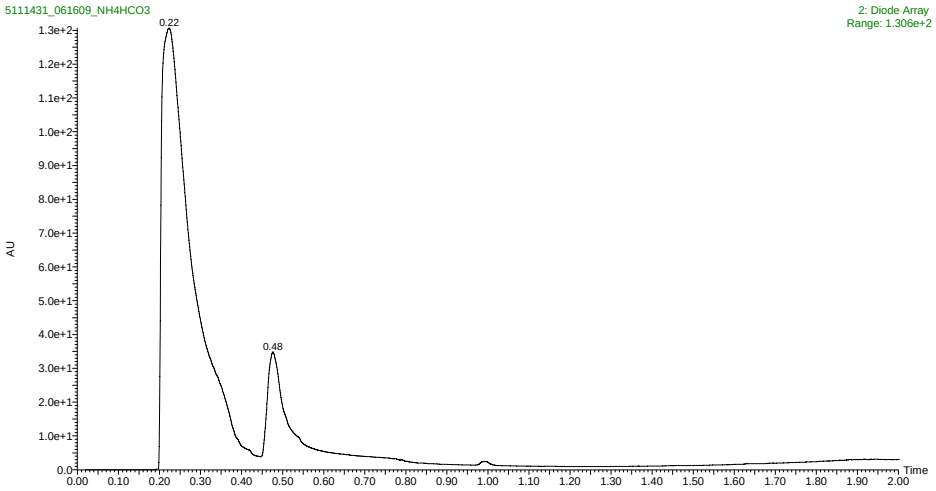

**12** (SJ000359413) Product Parent Ion Chromatogram – Product is in solvent peak



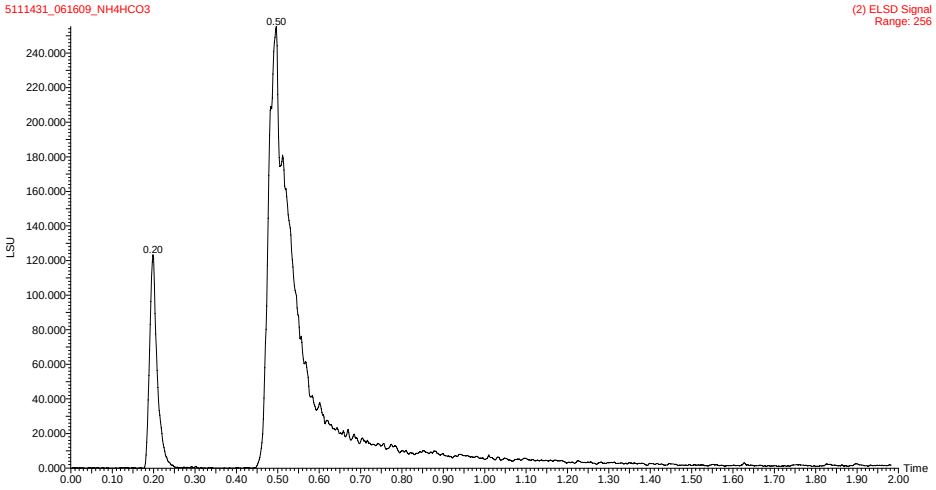
**12** (SJ000359413) Parent Ion – Expected Mass +1: 192.1



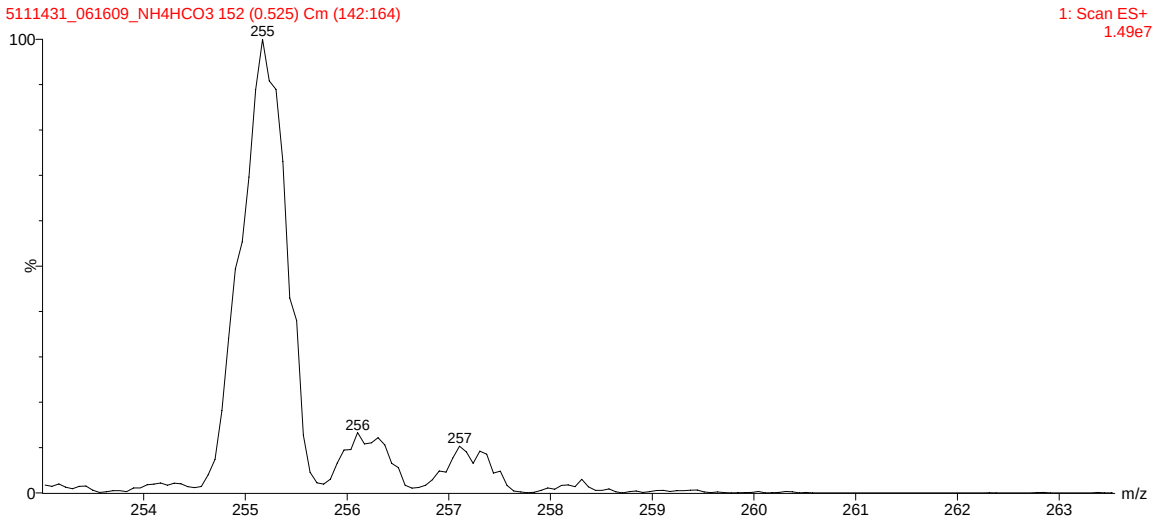
**13** (SJ000359140) UV-TIC – Purity = >95% - product mass at 0.48 min



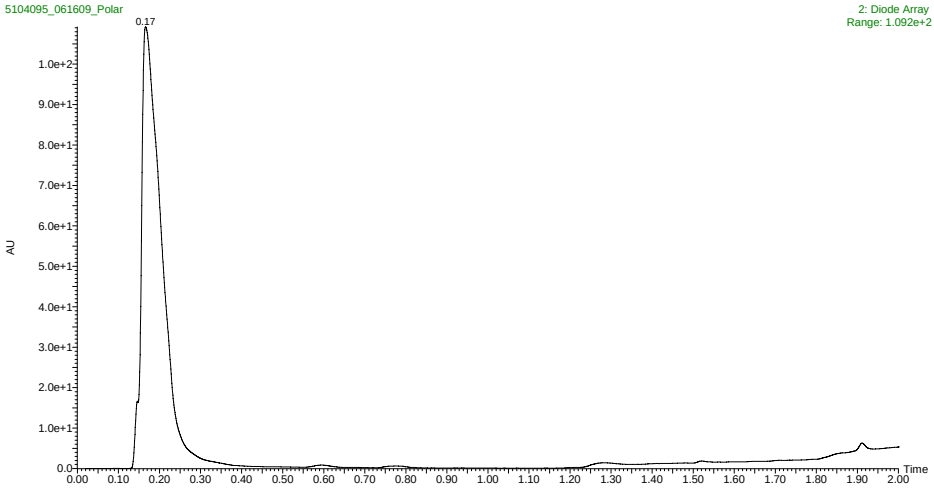

**13** (SJ000359140) ELSD Signal – Purity = >95% - product mass at 0.50 min




**13** (SJ000359140) Parent Ion – Expected Mass +1: 255.1

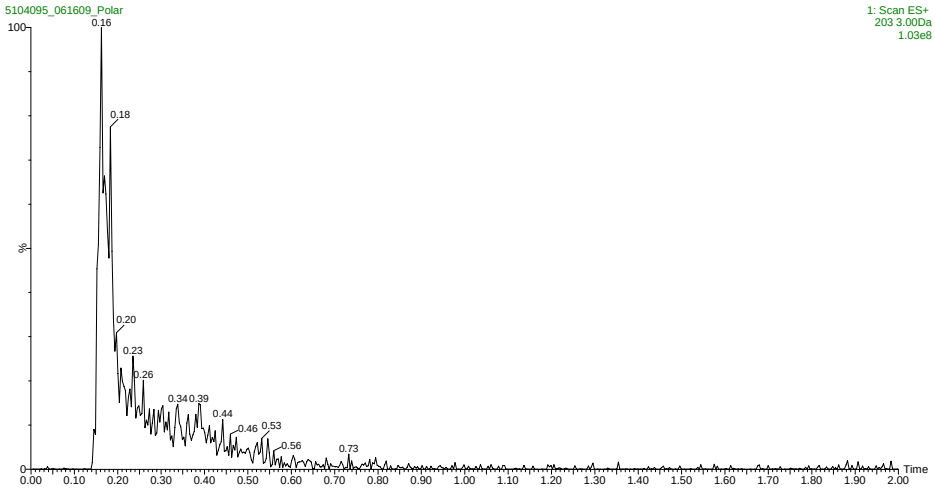


**14** (SJ000359137) – UV-TIC – Product in Solvent Peak – attempts at separation unsuccessful – No other major components detected – Purity = >95%

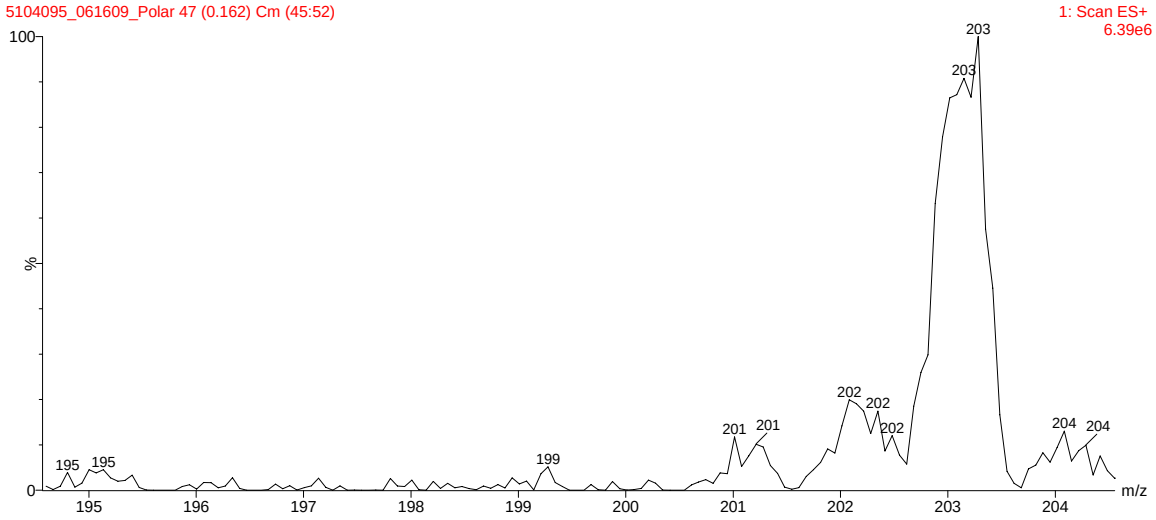



**14** (SJ000359137) – UV-TIC – Product in Solvent Peak – attempts at separation unsuccessful – No other major components detected – Purity = >95%

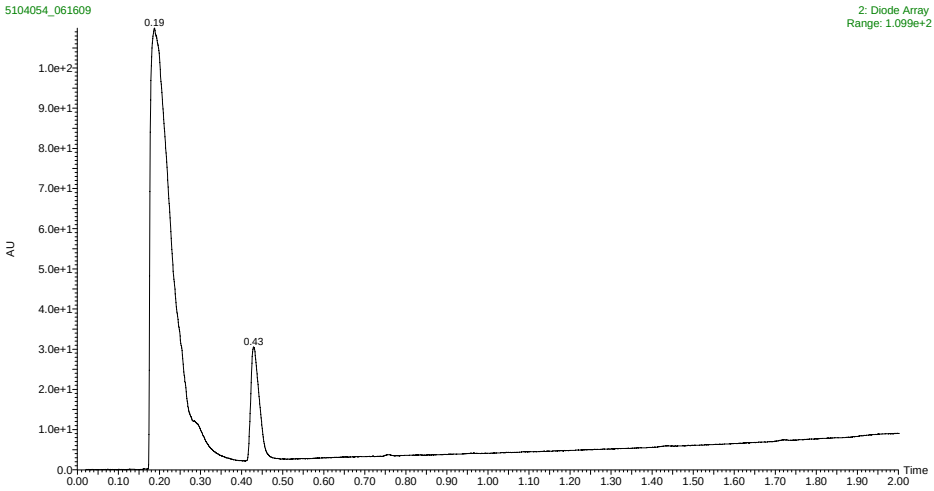

**14** (SJ000359137) - Product Parent Ion Chromatogram – Product is in solvent peak



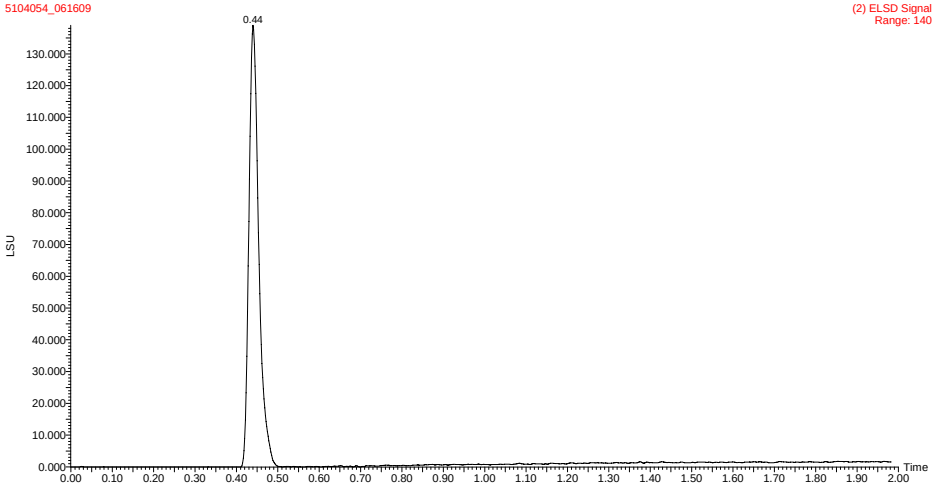
**14** (SJ000359137) Parent Ion – Expected Mass +1: 203.0



**15** (SJ000359134) – UV-TIC – Purity = >95% - product mass at 0.43 min




**15** (SJ000359134) – ELSD Signal – Purity = 100 % - product mass at 0.44 min




**15** (SJ000359134) Parent Ion – Expected Mass +1: 181.1