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BIOTRANSFORMATION SCALE-UP REPORT

Production of metabolites using biotransformation: PolyCYPs® Scale-up.

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Contributing & Reviewing Scientists' Statements

We, the undersigned, hereby declare that the work described herein was performed by employees of Hypha Discovery Ltd in accordance within Hypha Discovery's Laboratory Recording policy and with compliance to local authority Health & Safety regulations and Risk Assessment out-comes.

Responsibility	Person	Signature
Contributing scientists	Emily Hopkins, MSci Chris Drake, BA	n/a
Report compiled by (Project manager)	Richard Phipps, PhD	
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10th March 2021

Data Reference

Data pertaining to the work described herein is located at Hypha Discovery Ltd., 154B Brook Drive, Milton Park, Abingdon, OX14 4SD, UK.

Table of Contents

Summary

The Open Source Antibiotics Team wish to obtain oxidised metabolites of OSA821 found to be produced by Hypha's PolyCYPs® enzymes. Metabolites of this compound were detected in a previous screen using PolyCYP483. Initially a dose escalation step was performed, which permitted reduction of scale-up volume using the ~50mg of substrate. The reaction was repeated at 153 mL volume at 300mg/L and extracted for returned to UCL for purification.

1. Methods

1.1. Dose escalation

A previous screening study highlighted many PolyCYP enzymes as capable of producing OSA821 metabolites and that the best of these was PolyCYP483. OSA821 was in limited supply, therefore a dose escalation step was completed prior to the scale up reaction to try and increase the parent: metabolite ratio.

The reactions were performed in triplicate in a V-well 96-well polypropylene microtitre plate at 100 µL reaction volume. The reaction comprised 10 µL cofactor reagent stock solution (50 mM G6P, 10 mM NADP+, 10 UN/mL G6PDH, 5 mM $MgCl_2$ and 100 mM potassium phosphate buffer at pH 8 dissolved in cold H_2O), 89.2 µL (for 200 and 300 mg/L doses) or 89.6 µL (for 50 and 100 mg/L doses) PolyCYP enzymes (from 500 µL stock prepared in cold H_2O to give a final buffer concentration of 100 mM potassium phosphate and 5 mM $MgCl_2$), 0.4 or 0.8 µL OSA821 (from 12.5, 25, 37.5 mg/mL stock in DMSO) to give final concentrations of 50, 100, 200 or 300 mg/L respectively. Reactions were shaken at 200 rpm on a Kuhner (AG Switzerland) 5 cm orbital shaker at 27°C for 18 hours and stopped by addition of an equal volume of MeCN.

1.2. Scaled-up reaction

A previously prepared fed-batch-derived cell pellet of recombinant *E.coli* strain expressing PolyCYP483 was thawed and re-suspended with phosphate buffer (100 mM potassium phosphate at pH8 plus 5 mM $MgCl_2$). The cellular suspension was homogenised using a cell disruptor (1.1Kw system, Constant Systems Ltd, UK) set at 20 KPsi, then again at 24 KPsi and finally 30 KPsi. The homogenate was centrifuged at 47,500 x g and the crude extract (supernatant) used for the reaction. A stock solution of OSA821 was prepared by dissolving in DMSO at 37.5 mg/mL. The final reaction comprised 137 mL crude extract, 1.23 mL OSA821 and 15.36 mL co-factor solution

(50 mM G6P, 10 mM $NADP^+$ and 10 UN/mL G6PDH, all dissolved in 100 mM KPi buffer at pH8 + 5 mM $MgCl_2$) to provide a total volume of 153 mL. The bulk reaction was transferred in equal volumes to 3 x 250 mL Erlenmeyer flasks incubated overnight at 27°C and 180 rpm (5 cm diameter orbit). The reaction was checked by LC-MS and the whole reaction harvested and frozen at -80°C until ready for extraction.

1.3. Product extraction

The reaction (170 mL) was defrosted, mixed well with an equal volume of MeCN and centrifuged. Ammonium sulphate (50 g) was added to the supernatant and mixed well. The pellet was extracted with 50% $MeCN_{(aq)}$ centrifuged, added to the supernatant and all stored at -20ºC overnight. The MeCN was decanted off and the aqueous re-extracted with another aliquot of MeCN (100 mL). Both MeCN layers were combined, dried to aqueous under vacuum and lyophilised to provide between 100 and 200 mg of an orange solid (Batch ID: CD88/99x/1). It is likely the majority of the weight is derived from residual ammonium sulphate.

2. Results & Discussion

2.1. Reaction optimisation and purification

The dose escalation experiments for PolyCYP483 (Table 1) showed that increasing the dose of OSA821 provided an increase in the production of metabolites. The PolyCYP reaction proceeded as expected. Figures 1 to 7 provide chromatograms and spectra of the extract and the metabolites detected therein.

Table 1: Results for the dose escalation experiment of CYP483 dosed with OSA821 (values are peak areas UV_{298nm}). The focus of metabolism was on the primary +16Da product, with the other tentative 16Da product not changing in yield and therefore suspected as potentially endogenous-derived isobaric component.

Dose (mg/L)	Parent (1.89 mins) 302m/z	+16Da (1.59 mins) 318m/z
50	312	144
100	1311	329
200	4170	401
300	6555	438

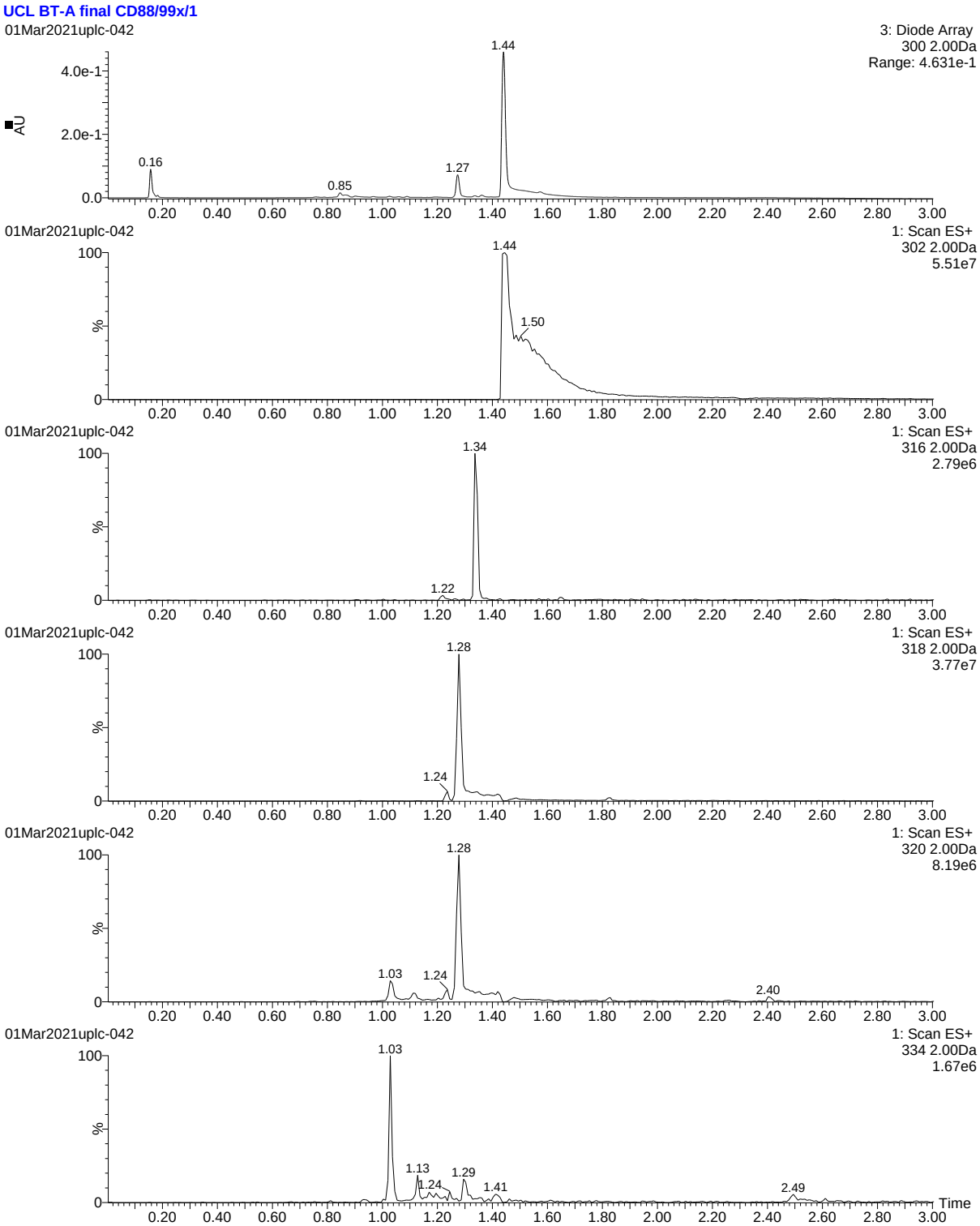

Figure 1: Chromatograms of a reaction extract from PolyCYP483 vs UCL-BT-A (821). From top to bottom the chromatograms are UV_{300nm}, $EIC_{302m/z}$ (UCL-BT-A), $EIC_{316m/z}$ (+14Da), $EIC_{318m/z}$ (+16Da), $EIC_{320m/z}$ (+18Da) and $EIC_{334m/z}$ (+32Da). UCL-BT-A elutes at 1.44 minutes. An expansion of the region between 0.95 and 1.40 minutes is provided in Figure 2 below.

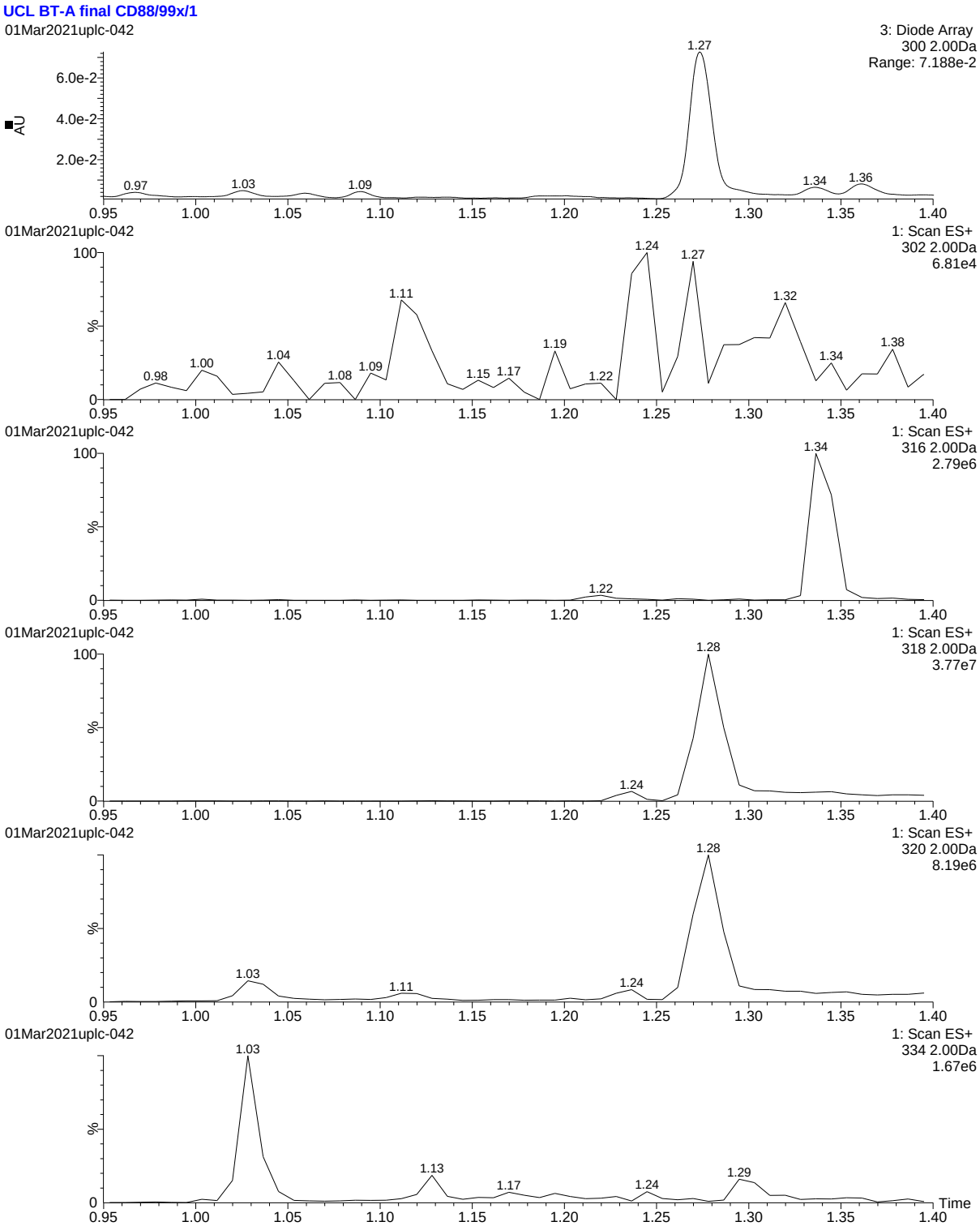

Figure 2: Expansion of the chromatograms above between 0.95 and 1.40 minutes. The UV and ESI MS spectra of the peaks at 1.03, 1.06, 1.27, 1.34 and 1.36 minutes are provided in Figures 3 to 7 below.

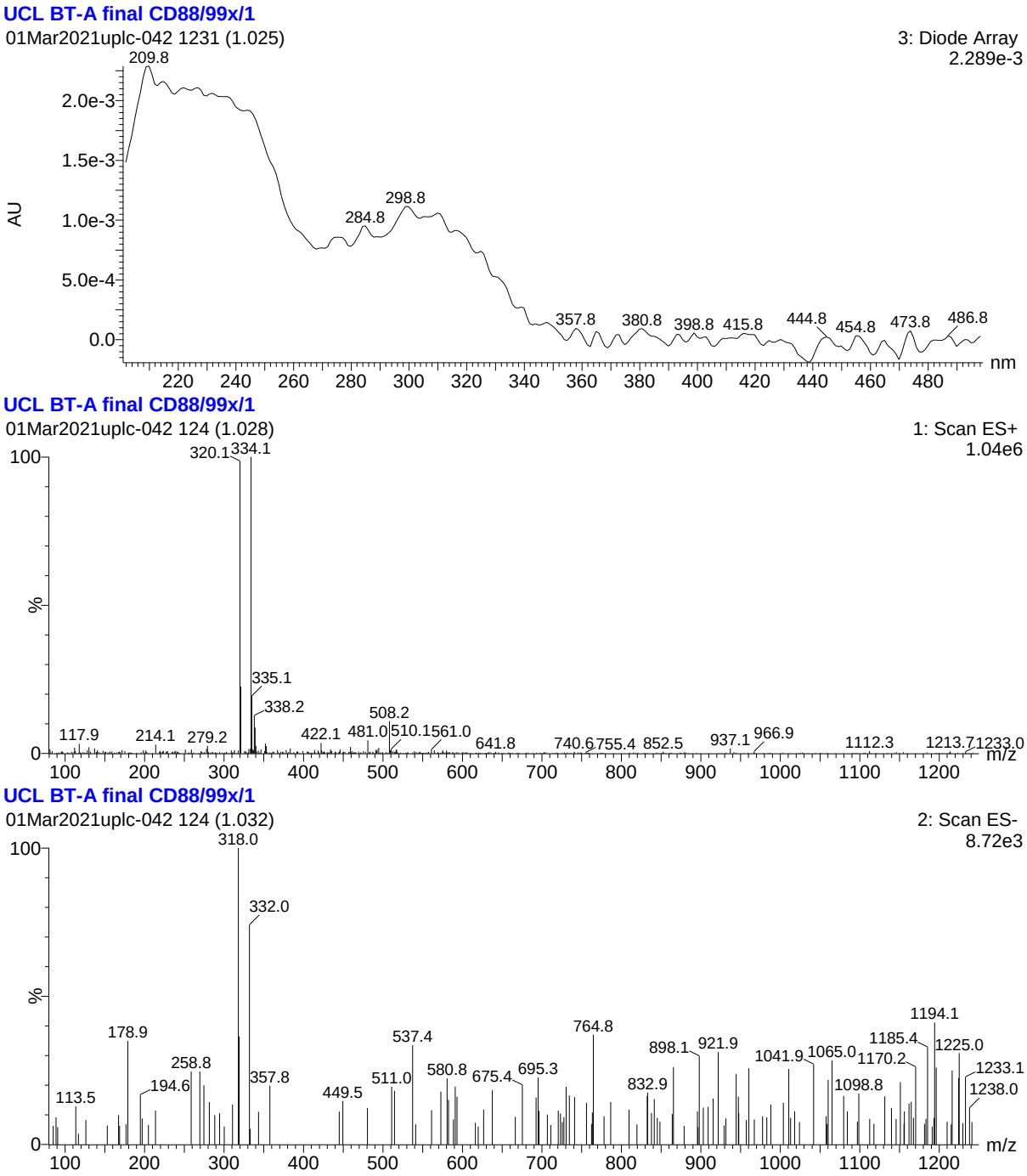

Figure 3: UV, positive ion and negative ion ESI MS spectra of the metabolite eluting at 1.03 minutes from a PolyCYP483 vs UCL-BT-A extract.

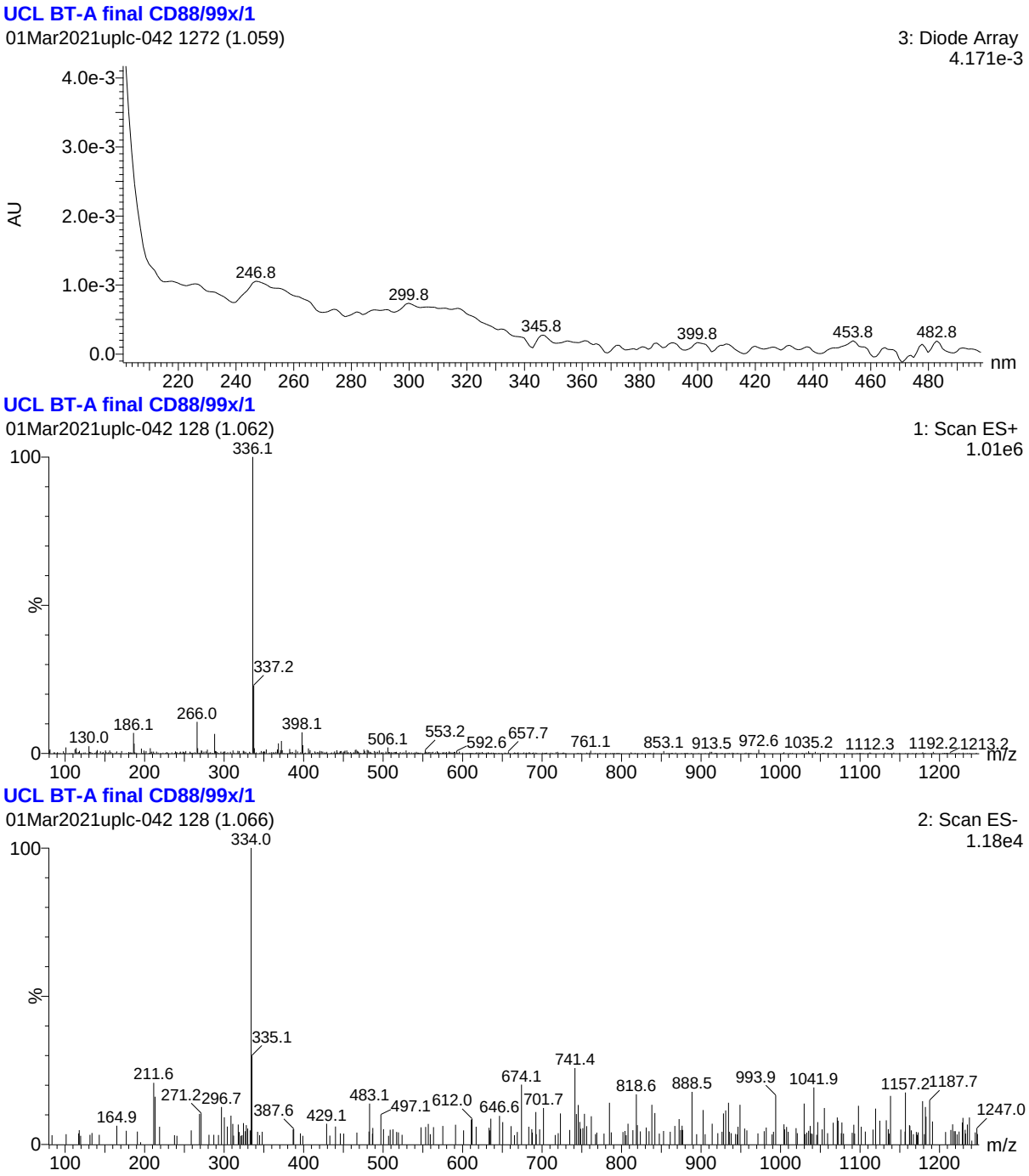

Figure 4: UV, positive ion and negative ion ESI MS spectra of the metabolite eluting at 1.06 minutes from a PolyCYP483 vs UCL-BT-A extract.

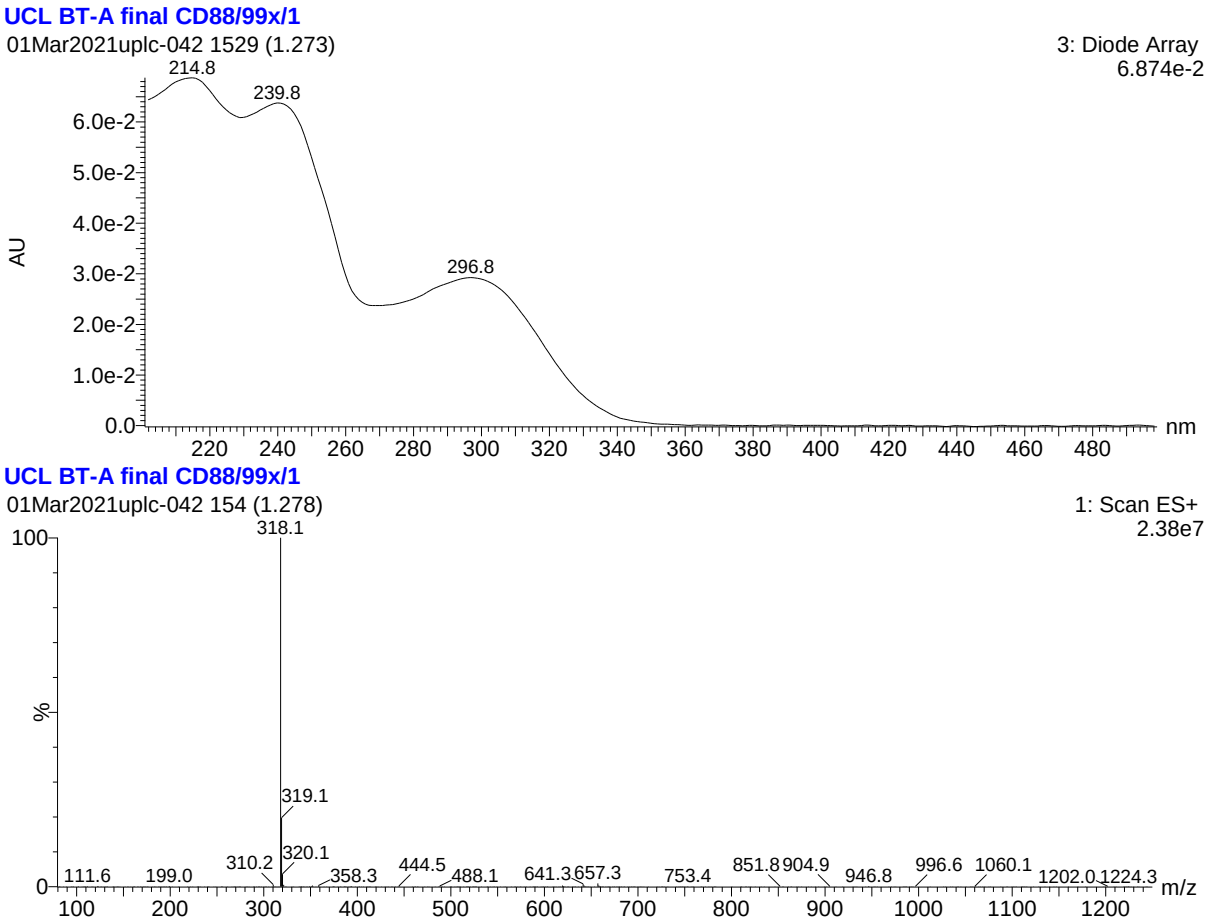

Figure 5: UV, positive ion and negative ion ESI MS spectra of the metabolite eluting at 1.28 minutes from a PolyCYP483 vs UCL-BT-A extract. This metabolite did not ionise under standard negative ion conditions.

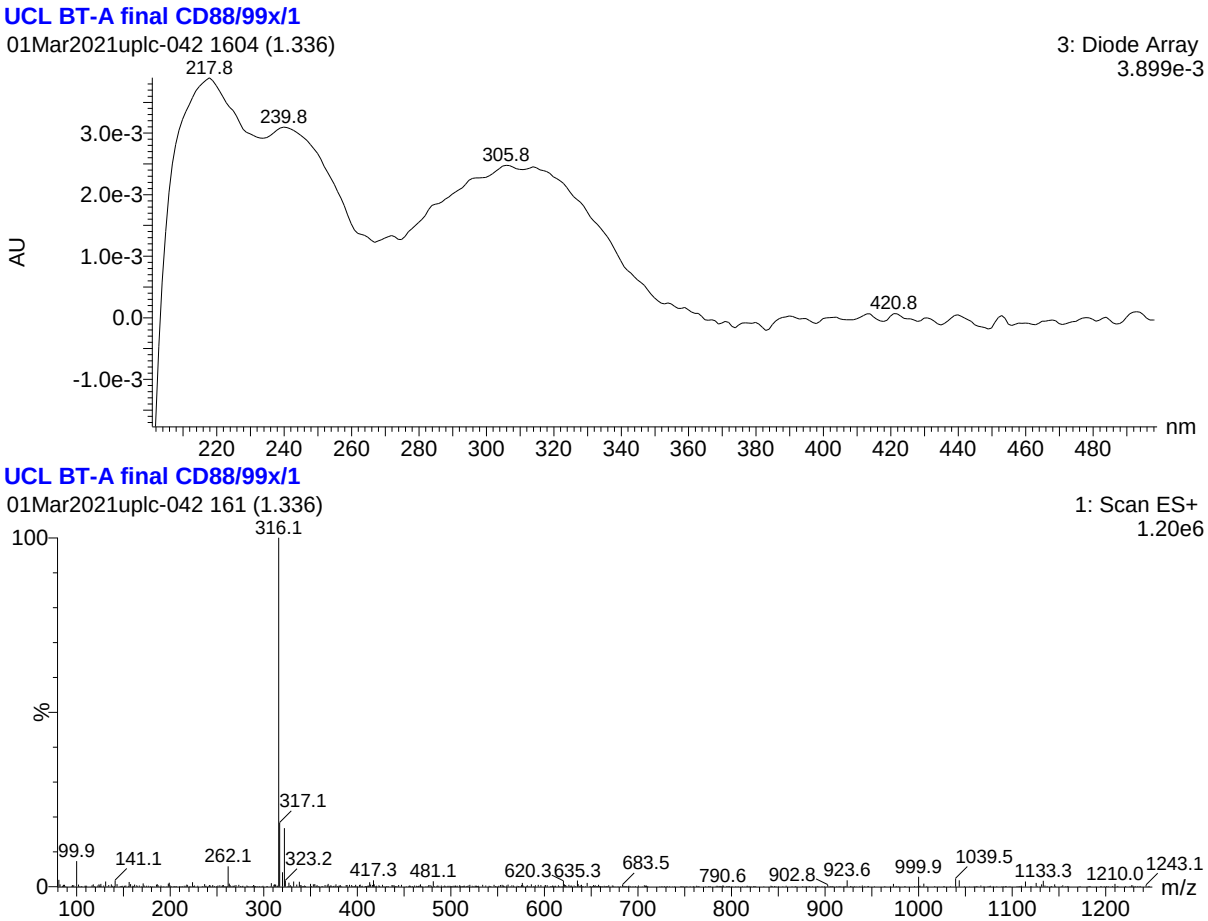

Figure 6: UV, positive ion and negative ion ESI MS spectra of the metabolite eluting at 1.34 minutes from a PolyCYP483 vs UCL-BT-A extract. This metabolite did not ionise under standard negative ion conditions.

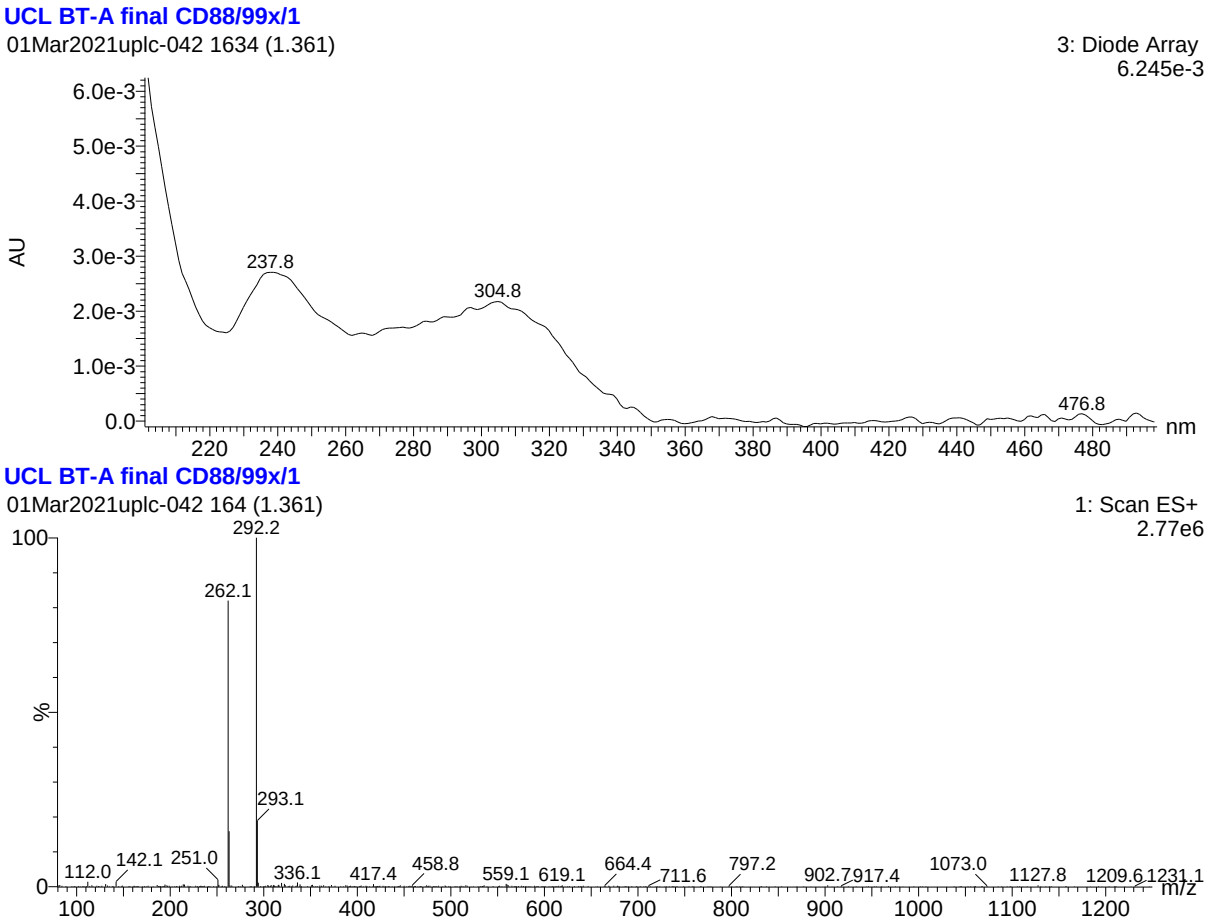

Figure 7: UV, positive ion and negative ion ESI MS spectra of the metabolite eluting at 1.36 minutes from a PolyCYP483 vs UCL-BT-A extract. This metabolite did not ionise under standard negative ion conditions.

3. Appendix: Technical Details

Enzymes:

Hypha's PolyCYPs® are proprietary Cytochrome P450 enzymes derived from microbial origin cloned into an *E.coli* expression system.

Chromatography details

System:

Pumps and autosampler: Waters Acquity UPLC QSM and Waters Acquity UPLC FTN

Detection (UV): Waters Acquity UPLC PDA (UV-Vis detection)

Detection (MS): Waters Acquity UPLC QDA

Basic LC gradient:

Column: Waters BEH C18 1.7 µm, 2.1 mm i.d. x 50 mm length

Column temperature: 45°C

Solvents: A: 10mM ammonium bicarbonate in H_2O/Acetonitrile (95/5), B: Acetonitrile

Gradient (A%/B%): t=0 mins: 98/2 to 2/98 at t=2.4 mins and held for a further 0.4 mins (2.8 mins total), return to 98/2 over 0.05 mins and re-equilibrated for 0.15 mins (t=3 mins) at a flow-rate of 1.0 mL/min.

END OF REPORT