Project: Lengyel 18 hour time point WCMC Pilot & Feasibility Study

Summary Text for OXYLIPIN SPE EXTRACTION & ANALYSIS PROTOCOL

Conducted in the laboratory of Dr. John W. Newman

Oxylipin Extraction

Oxylipins were isolated by solid phase extraction on 10 mg Waters Oasis-HLB cartridges (Milford, MA), as previously described by Luria et al (1). Prior to extraction, cartridges were washed with 1 column volume ethyl acetate followed by 2 column volumes methanol and conditioned with 2 mL of 95:5 v/v water/methanol (MeOH) with 0.1% acetic acid. The column reservoir was spiked with 5 µL anti-oxidant solution, (0.2 mg/ml solution BHT/EDTA in 1:1 MeOH:water), and 10 µL 1000nM analytical surrogates (See Table 2 below for specific compounds). Sample aliquots (250 µL media) were then introduced to the column reservoir and diluted with 1 column volume wash solution (5% MeOH, 0.1% acetic acid). Sample was allowed to gravity extract and the sorbent bed was then washed with 1 column volumes wash solution (20% methanol, 0.1% acetic acid). SPE cartridges were dried by vacuum @ -7.5in Hg for 20 min. Analytes were then eluted by gravity with 0.2 mL MeOH, followed by 0.5 mL Acetonitrile, followed by 0.5 mL Ethyl Acetate, into 2 mL autosampler vials containing 10 µL 20% glycerol solution in MeOH. Eluent was dried by vacuum evaporation for 35 min, and residues were re-constituted with 100uL of 100 nM internal standard solution containing 1-cyclohexyl ureido,3-dodecanoic acid (CUDA) and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU), in 50:50 MeOH:ACN. Vials were vortexed for 1 min to dissolve residues chilled 15 min on wet ice, and extracts were transferred to a centrifugal filter (0.1 µm Durapore, Millipore, Billerica, MA), centrifuged for 3 min at 6ºC at <4500g (rcf) and transferred to 150 uL glass inserts and into the 2 mL amber vials, and cap. Extracts were stored at -20ºC until analysis by UPLC-MS/MS. The internal standard was used to quantify the recovery of the deuterated extraction surrogates by ratio response.

Oxylipin Analysis

Analytes in a 10 µL injection of extract were separated with an Aquity C_{18} BEH 1.7µm 150mm x 2.1mm column utilizing a Waters Acquity UPLC (Waters, Milford, MA) with the solvent gradient described in Table 1, slightly modified from a previously published protocol (2). The autosampler was maintained at 10ºC. Resolved analytes were detected by negative mode electrospray ionization and multiple reaction monitoring on a API 4000 QTrap (AB Sciex, Framingham, MA, USA) using the following operating parameters: Curtain gas = 35.0 psi, temperature = 525 °C, ion source gas 1 = 60.0 psi, ion source gas 2 = 50.0 psi, IonSpray voltage = -4500.00, collision gas = medium, collision cell exit potential = -10.0 V, and entrance potential = -10.0 V. Analyte retention times, mass transitions, declustering potentials, dwell times, and analytical surrogate associations for each analyte are shown in Table 2. Analytes were quantified using isotope dilution and internal standard methodology with 5 to 7 point calibration curves ($r2 \geq 0.997$). Calibrants and internal standards were either synthesized [PHAU and CUDA] or purchased from Cayman Chemical (Ann Arbor, MI) unless otherwise indicated. Larodan Fine Lipids (Malmo, Sweden) provided the linoleate derived triols 9,12,13-TriHOME and 9,10,13- TriHOME. Data was processed utilizing AB Sciex Analyst version 1.6.2. Surrogate recoveries can be viewed in Table 3.

(1) Luria A et al (2007). Compensatory mechanism for homeostatic blood pressure regulation in Ephx2 gene-disrupted mice. *J Biol Chem*. 282:2891-8

(2) Strassburg K et al (2012). Qualitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery. *Anal Bioanal Chem*. 404:1413-26.

Table 1. UPLC parameters

Time (min)	A%	B%
0	75	25
1	60	40
2.5	58	42
4.5	50	50
10.5	35	65
12.5	25	75
14	15	85
14.5	5	95
15	75	25
16	75	25

Solvent A = 0.1% Acetic Acid;
Solvent B = 90% Acetonitrile / 10% isopropanol flow rate = 0.25 mL/min, column 2.1 X 150mm, 1.7 µm BEH C18 (Waters, Milford, MA), column temp = 60 °C

Table 2. UPLC/Electrospray ionization QTRAP analyte and instrument specific parameters*

Analyte	tR (min)	Transition (Da)	Dwell (msec)	DP (V)	CE (V)	ISTD†
PHAU	2.7	249.2 > 130.1	10	50	18	---
20-carboxy-LTB4	3.0	365.3 > 347.3	10	70	27	d4-6-keto PGF1α
Resolvin E1	3.1	349.3 > 195.2	50	65	21	d4-6-keto PGF1α
6-keto PGF1α	3.1	369.3 > 163.1	10	70	40	d4-6-keto PGF1α
d4-6-keto PGF1α	3.1	373.3 > 167.1	10	70	40	PHAU
20-hydroxy-LTB4	3.1	351.3 > 195.2	10	60	24	d4-6-keto PGF1α
PGF4 screen	3.7	377.3 > 113.1	20	60	21	d4-PGF2α
PGF3	4.8	351.3 > 115.1	20	40	21	d4-PGF2α
PGE3	3.8	349.3 > 269.2	20	50	21	d4-PGF2α
d4-TXB2	3.9	373.3 > 173.1	20	40	27	PHAU
TXB2	3.9	369.3 > 169.1	20	50	27	d4-TXB2
9,12,13-TriHOME	4.3	329.2 > 211.2	20	50	33	d4-PGF2α
d4-PGF2α	4.4	357.3 > 197.2	20	80	36	PHAU
PGF2α	4.4	353.3 > 193.2	20	60	33	d4-PGF2α
9,10-13-TriHOME	4.4	329.2 > 171.1	20	70	27	d4-PGF2α
PGE2	4.5	351.3 > 271.2	20	50	27	d4-PGD2α
PGE1	4.7	353.3 > 317.2	20	50	21	d4-PGD2α
d4-PGD2	4.8	355.3 > 275.2	20	40	27	PHAU
PGD2	4.8	351.3 > 271.2	20	40	24	d4-PGD2α

* - See Table I for UPLC conditions. Collision-induced dissociation was performed with 2.3 mTorr nitrogen. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

† - Internal Standards (ISTD) - Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA) and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) were introduced immediately prior to analysis and used to quantify surrogate recoveries.

†† - Compounds labeled as "screen" are compounds for which we did not have calibration standards. These compounds were identified based on retention time and transition (Da) and produced qualitative data.

Table 2. UPLC/Electrospray ionization QTRAP analyte and instrument specific parameters (continued)*

Analyte	tR (min)	Transition (Da)	Dwell (msec)	DP (V)	CE (V)	ISTD†
Resolvin D1	5.3	375.3 > 121.1	50	60	40	d4-PGF2α
11,12,15-THET	5.3	353.3 > 167.1	50	70	30	d4-PGF2α
Lipoxin A4	5.4	351.3 > 217.2	50	50	27	d4-PGF2α
PGJ2/ δ 12-PGJ2	6.3	333.2 > 233.2	5	40	15	d4-PGF2α
LTB5	6.5	333.3 > 195.2	5	70	21	d4-LTB4
d3-LTE4	6.6	441.4 > 336.3	50	50	27	CUDA
LTE4	6.6	438.4 > 333.3	50	70	27	d3-LTE4
15,16-DiHODE	6.9	311.2 > 235.2	10	60	24	d11-14,15-DiHETrE
12,13-DiHODE	7.0	311.2 > 183.2	10	50	27	d11-14,15-DiHETrE
8,15-DiHETE	7.0	335.3 > 235.2	10	80	21	d11-14,15-DiHETrE
Hepoxilin A3	7.0	335.2 > 171.1	50	40	24	d11-14,15-DiHETrE
9,10-DiHODE	7.0	311.2 > 201.2	10	50	27	d11-14,15-DiHETrE
17,18-DiHETE	7.3	335.3 > 247.2	10	60	24	d11-14,15-DiHETrE
5,15-DiHETE	7.3	335.3 > 173.1	10	50	21	d11-14,15-DiHETrE
6-trans-LTB4	7.4	335.3 > 195.2	20	60	21	d4-LTB4
14,15-DiHETE	7.6	335.3 > 207.2	10	60	24	d11-14,15-DiHETrE
CUDA	7.6	339.4 > 214.2	10	60	36	---
d4-LTB4	7.7	339.3 > 163.1	20	50	33	CUDA
LTB4	7.7	335.3 > 195.2	10	50	24	d4-LTB4

* - See Table I for UPLC conditions. Collision-induced dissociation was performed with 2.3 mTorr nitrogen. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

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†† - Compounds labeled as "screen" are compounds for which we did not have calibration standards. These compounds were identified based on retention time and transition (Da) and produced qualitative data.

Table 2. UPLC/Electrospray ionization QTRAP parameters (continued)*

Analyte	tR (min)	Transition (Da)	Dwell (msec)	DP (V)	CE (V)	ISTD†
12,13-DiHOME	8.0	313.3 > 183.2	5	70	30	d11-14,15-DiHETrE
10,11-DHHep	8.0	301.2 > 283.2	10	70	33	CUDA
9,10-DiHOME	8.4	313.3 > 201.2	5	60	30	d11-14,15-DiHETrE
d11-14,15-DiHETrE	8.5	348.4 > 207.2	10	70		CUDA
19,20-DiHDoPA	8.6	361.3 > 273.2	10	80	24	d11-14,15-DiHETrE
14,15-DiHETrE	8.6	337.3 > 207.2	10	60	27	d11-14,15-DiHETrE
11,12-DiHETrE	9.2	337.3 > 167.1	10	60	27	d11-14,15-DiHETrE
9-HOTE	9.4	293.2 > 171.1	10	60	21	d4-9(S)-HODE
12(13)-Ep-9-KODE	9.4	309.2 > 291.2	10	70	21	d4-9(S)-HODE
13-HOTE	9.5	293.2 > 195.2	10	70	21	d4-9(S)-HODE
8,9-DiHETrE	9.7	337.3 > 127.1	10	55	30	d11-14,15-DiHETrE
15-deoxy PGJ2	9.8	315.2 > 271.2	10	70	21	d11-14,15-DiHETrE
d6-20-HETE	9.9	325.3 > 281.2	10	80	24	CUDA
15-HEPE	10.0	317.2 > 219.2	10	60	18	d8-12(S)-HETE
20-HETE	10.0	319.2 > 275.2	10	80	21	d6-20-HETE
12-HEPE	10.3	317.2 > 179.1	10	60	21	d8-12(S)-HETE
5,6-DiHETrE	10.4	337.3 > 145.1	10	70	24	d11-14,15-DiHETrE
9-HEPE	10.5	317.2 > 167.2	10	60	21	d4-9(S)-HODE

* - See Table I for UPLC conditions. Collision-induced dissociation was performed with 2.3 mTorr nitrogen. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

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†† - Compounds labeled as "screen" are compounds for which we did not have calibration standards. These compounds were identified based on retention time and transition (Da) and produced qualitative data.

Table 2. UPLC/Electrospray ionization QTRAP parameters (continued)*

Analyte	tR (min)	Transition (Da)	Dwell (msec)	DP (V)	CE (V)	ISTD†
13-HODE	10.8	295.2 > 195.2	30	70	24	d4-9(S)-HODE
5-HEPE	10.9	317.2 > 115.1	10	40	21	d4-9(S)-HODE
d4-9(S)-HODE	10.9	299.2 > 172.1	10	70	27	CUDA
9-HODE	11.0	295.2 > 171.1	10	80	24	d4-9(S)-HODE
15(16)-EpODE	11.1	293.2 > 275.2	50	60	18	d4-12(13)-EpOME
17(18)-EpETE	11.2	317.2 > 259.2	30	70	15	d4-12(13)-EpOME
15-HETE	11.2	319.2 > 219.2	50	60	18	d8-12(S)-HETE
13-KODE	11.3	293.2 > 179.1	10	60	24	d4-9(S)-HODE
15-HpETE screen††	11.4	335.2 > 113.1	10	60	20	d8-12(S)-HETE
9(10)-EpODE	11.3	293.2 > 275.2	10	60	18	d4-12(13)-EpOME
17-HDoHE	11.3	343.3 > 281.2	10	50	18	d8-12(S)-HETE
13-HpODE screen	11.6	311.2 > 179.1	10	40	20	d4-9(S)-HODE
12(13)-EpODE	11.5	293.2 > 183.2	10	50	24	d4-12(13)-EpOME
14-HDoHE	11.6	343.3 > 281.2	10	50	18	d8-12(S)-HETE
15-KETE	11.5	317.2 > 273.2	10	50	21	d8-12(S)-HETE
11-HETE	11.6	319.2 > 167.1	30	50	21	d8-12(S)-HETE
14(15)-EpETE	11.6	317.2 > 247.2	30	60	21	d4-12(13)-EpOME
11(12)-EpETE	11.8	317.2 > 167.3		50	21	d4-12(13)-EpOME

* - See Table I for UPLC conditions. Collision-induced dissociation was performed with 2.3 mTorr nitrogen. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

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†† - Compounds labeled as "screen" are compounds for which we did not have calibration standards. These compounds were identified based on retention time and transition (Da) and produced qualitative data.

Table 2. UPLC/Electrospray ionization QTRAP parameters (continued)*

Analyte	tR (min)	Transition (Da)	Dwell (msec)	DP (V)	CE (V)	ISTD†
9-KODE	11.7	293.2 > 185.2	10	70	30	d4-9(S)-HODE
d8-12(S)-HETE	11.8	327.2 > 184.2	10	50	21	CUDA
9-HpODE screen	11.9	311.2 > 185.2	10	50	20	d4-9(S)-HODE
12-HETE	11.9	319.2 > 179.1	10	40	21	d8-12(S)-HETE
8-HETE	12.0	319.2 > 155.1	10	50	21	d8-12(S)-HETE
12-HpETE screen	12.0	335.2 > 153.1	10	50	20	d8-12(S)-HETE
15-HETrE	12.1	321.2 > 221.2	10	50	24	d8-12(S)-HETE
9-HETE	12.2	319.2 > 167.1	30	60	21	d8-12(S)-HETE
d8-5(S)-HETE	12.5	327.2 > 116.1	10	40	24	CUDA
19(20)-EpDPE	12.6	343.3 > 281.2	10	70	18	d4-12(13)-EpOME
5-HETE	12.6	319.2 > 115.1	10	50	21	d8-5(S)-HETE
d4-12(13)-EpOME	12.6	299.2 > 198.1	10	60	21	CUDA
12(13)-EpOME	12.7	295.2 > 195.1	10	70	24	d4-12(13)-EpOME
4-HDoHE	13.0	343.3 > 281.2	10	80	18	d8-5(S)-HETE
14(15)-EpETrE	12.8	319.2 > 219.2	10	80	18	d4-12(13)-EpOME
16(17)-EpDPE	12.9	343.5 > 273.5	30	70	18	d4-12(13)-EpOME
9(10)-EpOME	13.0	295.2 > 171.1	10	60	24	d4-12(13)-EpOME
5-HpETE screen	12.8	335.2 > 155.1	10	40	20	d8-5(S)-HETE
5-KETE	13.3	317.2 > 203.2	50	70	27	d8-5(S)-HETE
11(12)-EpETrE	13.3	319.2 > 167.1	30	50	21	d4-12(13)-EpOME
8(9)-EpETrE	13.5	319.2 > 155.1	10	60	21	d4-12(13)-EpOME

* - See Table I for UPLC conditions. Collision-induced dissociation was performed with 2.3 mTorr nitrogen. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

† - Internal Standards (ISTD) - Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA) and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) were introduced immediately prior to analysis and used to quantify surrogate recoveries.

†† - Compounds labeled as "screen" are compounds for which we did not have calibration standards. These compounds were identified based on retention time and transition (Da) and produced qualitative data.

Table 3. Analytical surrogate recoveries

Chemical class	Compound	Mean ± SD	%RSD
prostanoid	d4 6-keto PGF1α	34.1 ± 11	32.3%
thromboid	d4-TXB2	85.7 ± 5.2	6.1%
prostanoid	d4-PGF2α	97.2 ± 6.4	6.6%
prostanoid	d4-PGD2	80 ± 5.7	7.1%
leukotriene	d4-LTB4	59.1 ± 9.3	15.7%
FA diol	d11-14,15-DiHETrE	63.1 ± 6.9	10.9%
FA primary alcohol	d6-20-HETE	53.6 ± 7.9	14.7%
FA secondary alcohol	d4-9(S)-HODE	69.4 ± 7.8	11.2%
FA secondary alcohol	d8-12(S)-HETE	54.8 ± 6.8	12.4%
FA secondary alcohol	d8-5(S)-HETE	42.7 ± 7.6	17.8%
FA epoxide	10,11-DHHep	82.1 ± 5.5	6.8%
FA epoxide	d4-12(13)-EpOME	74.3 ± 6.6	8.9%

† -Relative standard deviation (standard deviation divided by the mean) x 100