Newman Lab Free & Total Oxylipin Analysis

Materials & Methods

For plasma analysis, free (non-esterified) oxylipins were isolated using a Waters Ostro Sample Preparation Plate (Milford, MA). Sample aliquots (50 µL plasma) were introduced into the plate wells and spiked with a 5 µL anti-oxidant solution (0.2 mg/ml solution BHT/EDTA in 1:1 MeOH:water) and 10 µL 1000nM analytical deuterated surrogates. Acetonitrile (150 µL) with 1% formic acid was forcefully added to the sample and aspirated three times to mix. Samples were eluted into glass inserts containing 10 µL 20% glycerol by applying a vacuum at 15 Hg for 10 min. Eluent was dried by speed vacuum for 35 min at the medium BP setting, before switching to an aqueous setting for an additional 35 min. Once dry, samples were re-constituted with the internal standard 1-cyclohexyl ureido, 3-dodecanoic acid (CUDA) and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) at 100 nM (50:50 MeOH:CAN), vortexed 1 min, transferred to a spin filter (0.1 µm, Millipore, Billerica, MA), centrifuged for 3 min at 6ºC at <4500g (rcf), before being transferred to 2 mL LC-MS amber vials. Extracts were stored at -20ºC until analysis by UPLC-MS/MS. The internal standard was used to quantify the recovery of surrogate standards.

The total rat lung lipid extract was isolated as previously reported (1,2). Specifically, rat lung samples (~25 mg) were enriched with 5 mL 0.2 mg/ml butylated hydroxytoluene/EDTA in 1:1 methanol/water (v/v), and enriched with the extraction surrogate dodecatrienoic acid (22:3n3; NuChek Prep). Lipids were then extracted with 10:8:11 cylcohexane/2-propanol/ammonium acetate (v/v/v). Briefly, enriched samples were mixed with cyclopropane/2-propanol, phases were split with ammonium acetate, the organic phase was isolated and the aqueous phase was re-extracted with cyclohexane. The combined organic total lipid extract was reduced to dryness and reconstituted in 100 µL of 1:1 methanol/toluene (v/v). To liberate esterified oxylipins, aliquots of total lipid extract (40 µL) spiked with deuterated oxylipin surrogates were incubated with 100 µL 0.5M sodium methoxide for 1hr at 60 ºC, mixed with 100 µL water, and returned to 60 ºC for 1hr. Samples were then diluted with 0.5 mL 0.1%AA/5%MeOH, neutralized with 10 µL 20% glacial acetic acid.

Total (free and esterified) oxylipins were isolated from the total lipid extract by solid phase extraction on 10 mg Waters Oasis-HLB cartridges (Milford, MA), as previously reported (3). 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. Sample aliquots (40 µL total lipid extract) were then introduced to the column reservoir and diluted with 1 column volume wash solution (5% MeOH, 0.1% acetic acid), followed by 1 column volumes wash solution (20% methanol, 0.1% acetic acid). SPE cartridges were dried with vacuum @ -25psi for 20 min. Analytes were then extracted into 2 mL autosampler vials (pre-prepared with 10 µL 20% glycerol solution in MeOH) with 0.2 mL MeOH, followed by 0.5 mL Ethyl Acetate, by gravity. Eluent was dried by speed vacuum for 35 min, before they were re-constituted with the internal standard 1-cyclohexyl ureido,3-dodecanoic acid (CUDA) and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) at 100 nM (50:50 MeOH:ACN), vortexed 1 min, transferred to a spin filter (0.1 µm, Millipore, Billerica, MA), centrifuged for 3 min at 6ºC at <4500g (rcf), before being transferred to 2 mL

LC-MS amber vials. Extracts were stored at -20ºC until analysis by UPLC-MS/MS. The internal standard was used to quantify the recovery of surrogate standards.

Analytes in a 100 µL extract aliquots were separated utilizing a Waters Acquity UPLC (Waters, Milford, MA) with a solvent gradient using modifications of a previously published protocols for oxylipins (4,5) (See Table 1 & 2 for detailed instrument parameters). Samples were held at 10ºC. Separated residues were detected by negative mode electrospray ionization using multiple reaction monitoring on an API 4000 QTrap (AB Sciex, Framingham, MA, USA). Analytes were quantified using internal standard methods and 5 to 10 point calibration curves ($r2 \geq 0.997$). Calibrants and internal standards were either synthesized [10,11-DHN, 10,11-DHHep, 10(11)-EpHep and CUDA] or purchased from Cayman Chemical (Ann Arbor, MI), unless otherwise indicated. Loradan Fine Lipids (Malmo, Sweden) provided the linoleate derived triols 9,12,13-TriHOME and 9,10,13- TriHOME. Data was processed utilizing AB Sciex MultiQuant version 3.0.1.

(1) Smedes F (1999). Determination of total lipid using non-chlorinated solvents. Analyst. 124:1711-1718.

(2) Gladine et al (2014). Lipid profiling following intake of the omega 3 fatty acid DHA identifies peroxidized metabolites F4-neuroprostanes as the best predictors of atherosclerosis prevention. PLOS ONE.

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

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

(5) Grapov D et al (2012). Type 2 Diabetes Associated Changes in the Plasma Non-Esterified Fatty Acids, Oxylipins and Endocannabinoids. PLoS ONE. 7(11):e48852.

Table 1. Oxylipin Assay UPLC parameters

Time (min)	A%	B%
0	90	70
2	90	80
5	60	85
5.5	60	90
13.5	20	95
13.75	20	99
14.5	5	99
14.7	5	70
15.2	90	70

Solvent A = 5 mm NH4COO 0.2% formic acid; Solvent B = 5 mm NH4COO 0.2% formic acid in MeOH, flow rate = 0.25 mL/min, column 2.1 X 100mm, 1.7 µm BEH C8 (Waters, Milford, MA), column temp = 60 °C

Table 2a. Oxylipin UPLC/ESI QTRAP Analyte and Instrument-specific Parameters * † ††

	Name	Expected RT	Internal Standard (ISTD) Name	Parent ion	Daughter ion
1	**PHAU**	2.67		249.2	130.1
2	6-keto PGF1a	3.04	IS PGD2	369.3	163.1
3	**d4-6-Keto PGF1a**	3.04	IS PHAU	373.3	167.1
4	PGF3a	3.56	IS d4-PGF2a	351.3	307.4
5	PGE3	3.69	IS d4-PGF2a	349.3	269.2
6	**d4-TXB2**	3.74	IS PHAU	373.3	173.1
7	TXB2	3.75	IS d4-TXB2	369.3	169.1
8	**9_12_13-TriHOME**	4.22	IS d4-PGF2a	329.2	211.2
9	**d4-PGF2a**	4.27	IS PHAU	357.3	197.2
10	PGF2a - Select	4.28	IS d4-PGF2a	353.3	193.2
11	PGE2	4.40	IS d4-PGD2	351.3	271.2
12	PGE1	4.58	IS d4-PGD2	353.3	317.2
13	**d4-PGD2**	4.69	IS PHAU	355.3	275.2
14	PGD2	4.71	IS d4-PGD2	351.3	271.2
15	15-Keto PGE2	4.75	IS d4-PGD2	349.2	331.3
16	Resolvin D1	5.23	IS d4-PGF2a	375.3	121.1
17	Lipoxin A4	5.35	IS d4-PGF2a	351.3	217.2
18	LTB5	6.40	IS d4-LTB4	333.3	195.2
19	**d3-LTE4**	6.51	IS CUDA	441.4	336.3
20	LTE4	6.54	IS d3-LTE4	438.4	333.3
21	15_16-DiHODE	6.85	IS d11-14_15-DiHETrE	311.2	235.2
22	12_13-DiHODE	6.93	IS d11-14_15-DiHETrE	311.2	183.2
23	8_15-DiHETE	6.95	IS d11-14_15-DiHETrE	335.3	235.2
24	9_10-DiHODE	6.98	IS d11-14_15-DiHETrE	311.2	201.2
25	17_18-DiHETE	7.23	IS d11-14_15-DiHETrE	335.3	247.2
26	5_15-DiHETE	7.28	IS d11-14_15-DiHETrE	335.3	173.1
27	6-trans-LTB4	7.40	IS d4-LTB4	335.3	195.2
28	14_15-DiHETE	7.57	IS d11-14_15-DiHETrE	335.3	207.2
29	**CUDA**	7.62		339.4	214.2
30	**d4-LTB4**	7.63	IS CUDA	339.3	163.1
31	LTB4	7.67	IS d4-LTB4	335.3	195.2
32	12_13-DiHOME	7.94	IS d11-14_15-DiHETrE	313.3	183.2
33	**10_11-DHHep**	8.02	IS CUDA	301.2	283.2
34	9_10-DiHOME	8.35	IS d11-14_15-DiHETrE	313.3	201.2
35	**d11-14_15-DiHETrE**	8.51	IS CUDA	348.4	207.2
36	19_20-DiHDoPA	8.57	IS d11-14_15-DiHETrE	361.3	273.20

37	14_15-DiHETrE	8.60	IS d11-14_15-DiHETrE	337.3	207.2
38	11_12-DiHETrE	9.16	IS d11-14_15-DiHETrE	337.3	167.1
39	9_10-e-DiHO	9.31	IS 10_11-DHHep	315.2	297.2
40	9-HOTE	9.42	IS d4-9(S)-HODE	293.2	171.1
41	12(13)-Ep-9-KODE	9.43	IS d4-9(S)-HODE	309.2	291.2
42	13-HOTE	9.55	IS d4-9(S)-HODE	293.2	195.2
43	8_9-DiHETrE	9.70	IS d11-14_15-DiHETrE	337.3	127.1
44	15-deoxy PGJ2	9.77	IS d11-14_15-DiHETrE	315.2	271.2
45	**d6-20-HETE**	9.93	IS CUDA	325.3	281.2
46	15-HEPE	9.97	IS d8-12(S)-HETE	317.2	219.2
47	20-HETE	9.97	IS d6-20-HETE	319.2	275.2
48	12-HEPE	10.33	IS d8-12(S)-HETE	317.2	179.1
49	5_6-DiHETrE	10.46	IS d11-14_15-DiHETrE	337.3	145.1
50	9-HEPE	10.53	IS d4-9(S)-HODE	317.2	167.2
51	13-HODE	10.83	IS d4-9(S)-HODE	295.2	195.2
52	5-HEPE	10.91	IS d4-9(S)-HODE	317.2	115.1
53	**d4-9(S)-HODE**	10.95	IS CUDA	299.2	172.1
54	9-HODE	11.01	IS d4-9(S)-HODE	295.2	171.1
55	15(16)-EpODE	11.09	IS d4-12(13)-EpOME	293.2	275.2
56	17(18)-EpETE	11.19	IS d4-12(13)-EpOME	317.2	259.2
57	15-HETE	11.21	IS d8-12(S)-HETE	319.2	219.2
58	13-KODE	11.27	IS d4-9(S)-HODE	293.2	179.1
59	9(10)-EpODE	11.28	IS d4-12(13)-EpOME	293.2	275.2
60	17-HDoHE	11.32	IS d8-12(S)-HETE	343.3	281.2
61	15-HpETE screen	11.48	IS d8-12(S)-HETE	335.2	113.1
62	13-HpODE screen	11.48	IS d4-9(S)-HODE	311.2	179.1
63	9-HpODE screen	11.88	IS d4-9(S)-HODE	311.2	185.2
64	12(13)-EpODE	11.48	IS d4-12(13)-EpOME	293.2	183.2
65	15-KETE	11.55	IS d8-12(S)-HETE	317.2	273.2
66	14-HDoHE	11.62	IS d8-12(S)-HETE	343.3	281.2
67	11-HETE	11.64	IS d8-12(S)-HETE	319.2	167.1
68	14(15)-EpETE	11.64	IS d4-12(13)-EpOME	317.2	247.2
69	9-KODE	11.71	IS d4-9(S)-HODE	293.2	185.2
70	**d8-12(S)-HETE**	11.78	IS CUDA	327.2	184.2
71	11(12)-EpETE	11.80	IS d4-12(13)-EpOME	317.2	167.3
72	12-HETE	11.88	IS d8-12(S)-HETE	319.2	179.1
73	8-HETE	12.02	IS d8-12(S)-HETE	319.2	155.1
74	9-HETE	12.23	IS d8-12(S)-HETE	319.2	167.1
75	12-HpETE screen	12.00	IS d8-12(S)-HETE	335.2	153.1

76	5-HpETE screen	13.18	IS d8-5(S)-HETE	335.2	155.1
77	**d8-5(S)-HETE**	12.49	IS CUDA	327.2	116.1
78	19(20)-EpDPE	12.57	IS d4-12(13)-EpOME	343.3	281.2
79	5-HETE	12.58	IS d8-5(S)-HETE	319.2	115.1
80	**d4-12(13)-EpOME**	12.65	IS CUDA	299.2	198.1
81	12(13)-EpOME	12.74	IS d4-12(13)-EpOME	295.2	195.2
82	14(15)-EpETrE	12.83	IS d4-12(13)-EpOME	319.2	219.2
83	4-HDoHE	12.88	IS d8-5(S)-HETE	343.3	281.2
84	16(17)-EpDPE	12.96	IS d4-12(13)-EpOME	343.5	273.5
85	9(10)-EpOME	12.97	IS d4-12(13)-EpOME	295.2	171.1
86	5-KETE	13.28	IS d8-5(S)-HETE	317.2	203.2
87	11(12)-EpETrE	13.31	IS d4-12(13)-EpOME	319.2	167.1
88	8(9)-EpETrE	13.51	IS d4-12(13)-EpOME	319.2	155.1
89	10-Nitrolinoleate	13.79	IS d17-10-Nitrooleate	324.3	277.2
90	9_10-EpO	14.37	IS 10_11-DHHep	297.3	279.2
91	**d17-10-Nitrooleate**	14.60	IS CUDA	343.2	307.5
92	10-Nitrooleate	14.7	IS d17-10-Nitrooleate	326.2	279.5
93	9-Nitrooleate	14.80	IS d17-10-Nitrooleate	326.2	308.2
94	9_10-DiHHex	15.21	IS 10_11-DHHep	287.2	227.5
95	**d8-Arachidonic Acid**	15.68	IS CUDA	311.3	267.1

* - Analytes were separated under conditions described in Table I. Collision-induced dissociation was performed with nitrogen at a pressure of 2.3 mTorr. Scheduled mass spectral multiple reaction monitoring was utilized to identify analytes.

† - 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. The compounds were identified based on retention time and transition (Da) and produced qualitative data.