Newman Lab Lipid Mediator Analysis

Materials & Methods

For plasma analysis, oxylipins, endocannabinoids, and ceramides 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.

For tissue analysis (liver, muscle, adipose, and hypothalamus), samples were pulverized, added to 2mL polypropylene tubes, 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. A total of 50 µL of methanol was added and the tube was placed in a Geno/Grinder for 30 sec. An additional 550µL isopropanol w/ 10mM ammonium formate & 1% formic acid and 100 uL water was added and the tube was placed in a Geno/Grinder for 30 sec before being centrifuged at 10,000g for 5 min at room temp. The supernate was then transferred into Water Ostro Sample Preparation Plate wells. From this point forward, the samples were treated similarly to plasma (detailed previously above), as they were eluted by vacuum, dried, reconstituted, and analyzed.

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 (1), endocannabinoids (2), and ceramides (3)(See pages 3-11 for detailed instrument parameters for each assay). Samples were held at 10ºC. Separated residues were detected by negative mode electrospray ionization for oxylipins and positive mode electrospray ionization for endocannabinoids and ceramides 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), Avanti Polar Lipids Inc. (Alabaster, AL), and Sigma-Aldrich (St. Louis, MO), 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) 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.

(2) 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.
(3) Bielawski J et al (2009). Comprehensive quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. Methods Mol Biol. 579:443-67.

Table 1a. 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 1b. Endocannabinoid Assay UPLC parameters

Time (min)	A%	B%
0	75	25
0.25	75	25
0.5	60	40
1.5	50	50
3	45	55
3.5	20	80
8	15	85
9	5	95
9.25	5	95
9.5	75	25
11	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 1c. Ceramide 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 Ia. 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.

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

Analyte	Common Abbreviation	tR (min)	Transition (Da)	Dwell (msec)	Declustering (V)	Collision (V)	ISTD†
d4-PGF2α Ethanolamide	d4-PGF2α EA	3.24	384.3 > 62.1	25	55	40	CUDA
PGF2αEthanolamide	PGF2α EA	3.25	380.3 > 62.1	25	55	41	d4-PGF2a EA
PGE2 Ethanolamide	PGE2 EA	3.26	378.3 > 62.1	25	58	38	d4-PGF2a EA
PGD2 Ethanolamide	PGD2 EA	2.9	378.3 > 62.1	25	58	38	d4-PGF2a EA
PGF2α 1-glyceryl ester	PGF2a 1G	3.04	411.3 > 301	25	55	19	d4-PGF2a EA
PGE2 1-glyceryl ester	PGE2 1G	3.07	409.3 > 317	25	55	19	d4-PGF2a EA
1-Cyclohexyluriedo-3-dodecanoic acid	CUDA	4.98	341.3 > 216	25	58	25	---
15(S)-HETE Ethanolamide	15-HETE EA	5.19	346.3 > 62.1	25	58	39	d8-AEA
11(12)-EET Ethanolamide	11(12)-EpETre EA	5.58	364.3 > 62.1	25	58	40	d8-AEA
α-Linolenoyl Ethanolamide	αLEA	6.03	322.2 > 62.1	25	72	32	d8-AEA
Docosahexaenoyl Ethanolamide	DHEA	6.42	372.3 > 62.1	25	61	36	d8-AEA
d8-Arachidonoyl Ethanolamide	d8-AEA	6.54	356.3 > 63.1	25	60	30	CUDA
Arachidonoyl Ethanolamide	AEA	6.59	348.3 > 62.1	25	65	33	d8-AEA
Linoleoyl Ethanolamide	LEA	6.66	324.2 > 62.1	25	72	31	d8-AEA
d5-2-Arachidonoyl Glycerol	d5-2-AG	6.92	384.3 > 287	20	63	19	CUDA
2-Arachidonoyl Glycerol	2-AG	6.94	379.3 > 287	20	53	19	d5-2-AG
d8-Arachidonoyl Glycine	d8-NA-Gly	6.95	370.3 > 76.1	25	79	35	CUDA
Arachidonoyl Glycine	NA-Gly	6.98	362.3 > 76.1	25	79	35	d8-NA-Gly
Dihomo-□-Linolenoyl Ethanolamide	Dihomo GLA EA	7.02	350.3 > 62.1	20	65	36	d8-AEA
1-Arachidonoyl Glycerol	1-AG	7.1	379.3 > 287	20	53	19	d5-2-AG
2-Linoleoyl Glycerol	2-LG	7.11	355.3 > 263	20	52	18	d5-2-AG
d4-Palmitoyl Ethanolamide	d4-PEA	7.31	304.2 > 62.1	20	80	35	CUDA
1-Linoleoyl Glycerol	1-LG	7.32	355.3 > 263	20	52	18	d5-2-AG
Palmitoyl Ethanolamide	PEA	7.33	300.2 > 62.1	20	80	31	d8-AEA
Docosatetraenoyl Ethanolamide	DEA	7.45	376.3 > 62.1	20	66	36	d8-AEA
Oleoyl Ethanolamide	OEA	7.6	326.2 > 62.1	20	80	32	d8-AEA

* - 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) was introduced immediately prior to analysis and used to quantify surrogate recoveries.

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

Analyte	Common Abbreviation	tR (min)	Transition (Da)	Dwell (msec)	Declustering (V)	Collision (V)	ISTD†
N-Oleoyl Glycine	NO-Gly	8.14	340.2 > 76.2	20	80	26	d8-NA-Gly
2-Oleoyl Glycerol	2-OG	8.2	357.3 > 265	50	52	18	d5-2-AG
1-Oleoyl Glycerol	1-OG	8.86	357.3 > 265	50	52	18	d5-2-AG
Stearoyl Ethanolamide	SEA	8.97	328.2 > 62.1	50	80	35	d8-AEA

* - See Table Ib 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 - Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA; and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) was introduced immediately prior to analysis and used to quantify surrogate recoveries.

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

Analyte	tR (min)	Transition (Da)	Declustering (V)	Collision (V)	ISTD†
1-Cyclohexyl Urea 3-Dodecanoic Acid	3.0	341.3 > 216.2	60	24	---
17:1 Sphingosine	3.4	286.4 > 268.3	40	15	CUDA
18:1 Sphingosine	3.8	300.4 > 282.4	40	21	17: 1 Sphingosine
17:1 Sphingosine-1P	3.7	366.4 > 250.3	50	23	CUDA
18:1 Sphingosine-1P	4.0	380.4 > 264.4	50	25	17: 1 Sphingosine-1P
18:0 Sphinganine-1P	4.0	382.4 > 266.4	50	25	17: 1 Sphingosine-1P
C14 Ceramide	8.8	510.7 > 492.6	50	21	C17 Ceramide
C16 Ceramide	9.5	538.8 > 264.4	55	37	C17 Ceramide
C18:1 Ceramide	9.8	564.5 > 546.4	60	24	C17 Ceramide
C17 Ceramide	9.9	552.8 > 534.5	55	24	CUDA
C18 Ceramide	10.3	566.7 > 264.4	55	37	C17 Ceramide
C18 dihydroceramide	10.6	568.7 > 266.4	85	33	C17 Ceramide
C20 Ceramide	11.3	594.4 > 576.5	55	21	C17 Ceramide
C24 Ceramide	13.3	650.9 > 264.4	55	42	C17 Ceramide
C24 dihydroceramide	13.6	652.9 > 266.4	55	42	C17 Ceramide
C25 Ceramide	11.3	664.9 > 264.4	55	45	C17 Ceramide

* - Analytes were separated under conditions described in Table Ic. Collision-induced dissociation was performed with nitrogen at a pressure of 2.3 mTorr.

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) was introduced immediately prior to analysis and used to quantify surrogate recoveries.