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 C18 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 ≥ 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.

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| **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 |  |  |  |

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

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| **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. |  |  |  |  |  |  |
| † - 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. |  |  |  |  |  |  |

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| **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. |  |  |  |  |  |  |
| † - 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. |  |  |  |  |  |  |

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| **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. |  |  |  |  |  |  |
| † - 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. |  |  |  |  |  |  |

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| **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. |  |  |  |  |  |  |

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| **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 |  |  |  |