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Harmonizing Routine Oxylipin Profiling: A Multisite Evaluation of Inter-Laboratory Reproducibility

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Introduction

Oxylipins, potent lipid mediators derived from the oxidation of polyunsaturated fatty acids (PUFAs), play a critical role in regulating inflammatory pathways and signaling cascades associated with cardiovascular disease, neurodegeneration, and metabolic disorders.

Despite their biological importance, the structural diversity of oxylipin isomers and their low endogenous concentrations necessitate highly sensitive and robust analytical methods. While individual laboratories have successfully optimized targeted LC-MS/MS assays, the lack of inter-laboratory standardization remains a significant barrier to routine testing, large-scale clinical cohorts, cross-study meta-analyses.

This study evaluates the reproducibility and robustness of a standardized oxylipin profiling method across three independent sites. By utilizing a standardized configuration of the Infinity III Bio LC coupled with the 6495D Triple Quadrupole LC/MS System (Figure 1), we assessed the stability of retention times (RT), ionization efficiency, and quantitative precision for a comprehensive panel of oxylipin targets.

Our findings demonstrate that a standardized instrumental setup significantly mitigates technical variance, providing a framework for routine, high-throughput, and highly reproducible oxylipin analysis. We show that the methodology also serves as an excellent way for new labs to incorporate this measurement into their research as it is proven to be robust and transferable.

Figure 1. Agilent Infinity III 1290 Bio LC (left), and detection with the Agilent 6495D LC/TQ (right). The Bio LC uses the new Altura ZORBAX Eclipse Plus C18 column for Oxylipin analysis, which features Ultra Inert technology to block active metal sites ensuring stable peak shapes and increased sensitivity. The 6495D is equipped with the 4th Gen dual-stage iFunnel to enable high reproducibility at low dwell times.

Experimental

Sample Preparation and LC/MS Analysis of Plasma

Pooled plasma was diluted using a cold 20:80 methanol:water solution (v/v) spiked with heavy-labeled internal standards. Oxylipin enrichment was performed using Agilent Bond Elut C18 SPE cartridges. Samples were aliquoted and distributed to three laboratories, each equipped with an Agilent 1290 Infinity III Bio LC in identical configurations.

Following reconstitution in 50:50 methanol:water (v/v), samples were analyzed via LC/MS using the Altura ZORBAX Eclipse Plus C18. MS source conditions were optimized using Cayman Chemical standards and the built-in Compound and Source Optimizer in MassHunter 12.3.

Table 1. Bio LC and LC/TQ conditions

Infinity III Bio LC Conditions				
Column	Altura ZORBAX Eclipse Plus C18 1.8µm, 2.1x150mm (204215-308)			
Column Temp	40 °C (Autosampler = 4°C)			
Injection	10 µL			
Mobile phase	A = Water + 0.1 % Acetic Acid B = 9/1 ACN/IPA			
Flow rate	0.350 mL/min			
Gradient program	Time	%B	Time	%B
	0	15	17.0	75
	3.5	33	18.5	85
	5.5	38	19.5	95
	7.0	42	21.0	95
	9.0	48	23	15
	15.0	65		

Optimized 6495D MS Conditions	
Sheath Gas Temperature	320 °C
Sheath Gas Flow	12.0 L/min
Gas Temperature	290 °C
Gas Flow	18.0 L/min
Nebulizer	20.0 psi
Capillary	3000 V (-)
Nozzle Voltage	1600 V (-)
iFunnel Mode	Standard

Results and Discussion

Robust Gradient and Maintaining Oxylipin Selectivity

This targeted approach included 191 analytes, 17 of which were heavy-labeled analytes, and 174 endogenous analytes. The TQ dynamic MRM (dMRM) method had a minimum dwell time of 10.1 ms. Transitions for each oxylipin analyte were chosen to enable the best selectivity for a particular isomer. A robust chromatographic separation was achieved for many oxylipins that have isomers using the Altura ZORBAX Eclipse Plus C18 column. The heavy-labeled internal standards included span the chromatographic gradient (Figure 2).

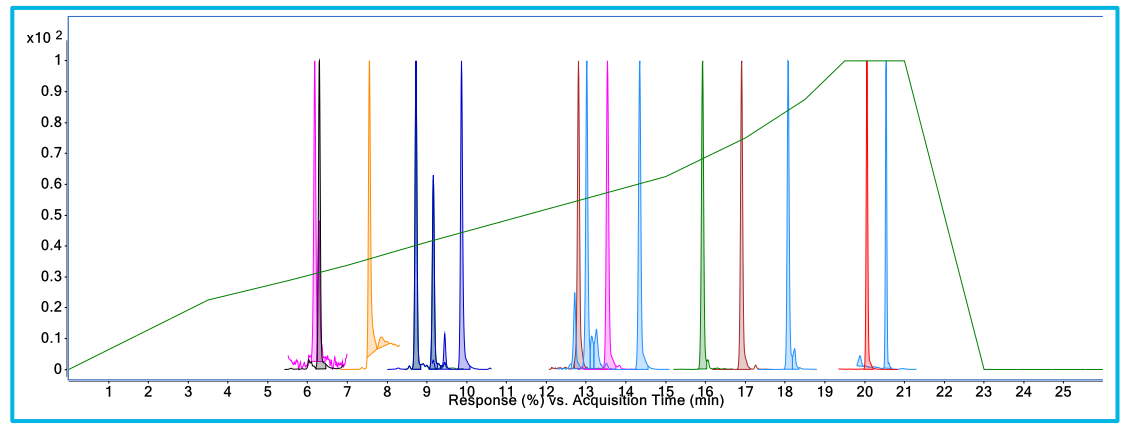

Figure 2. Elution order of heavy-labeled internal standards overlaid with the %B gradient of the method.

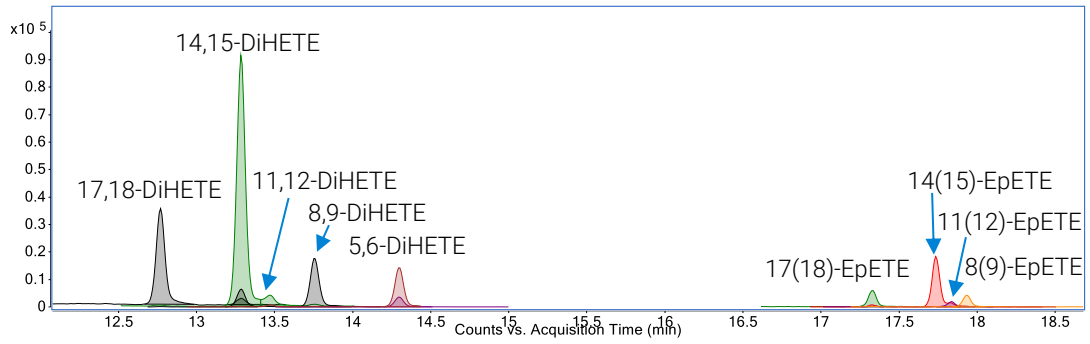

Figure 3. Regioisomers of epoxides and dihydroxy fatty acids from the metabolism of eicosapentanoic acid (EPA) via CYP450.

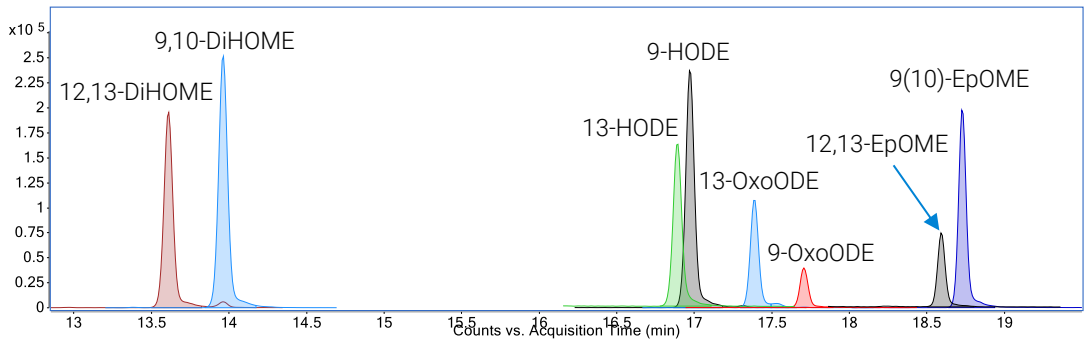

Figure 4. Oxylipins derived from linoleic acid (LA).

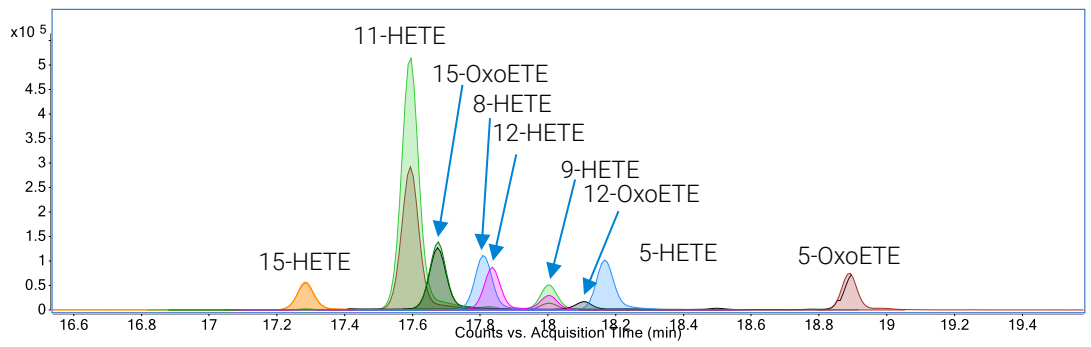

Figure 5. Oxylipins derived from arachidonic acid (AA) via the LOX pathway.

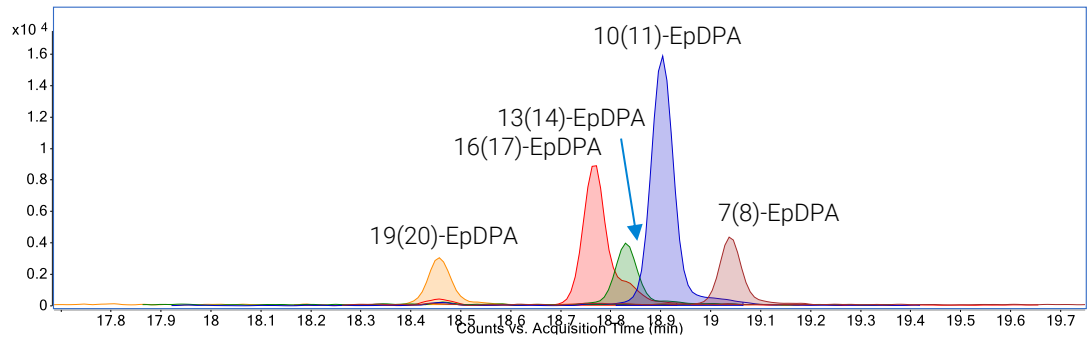

Figure 6. Oxylipins derived from the oxidation of DHA by CYP450.

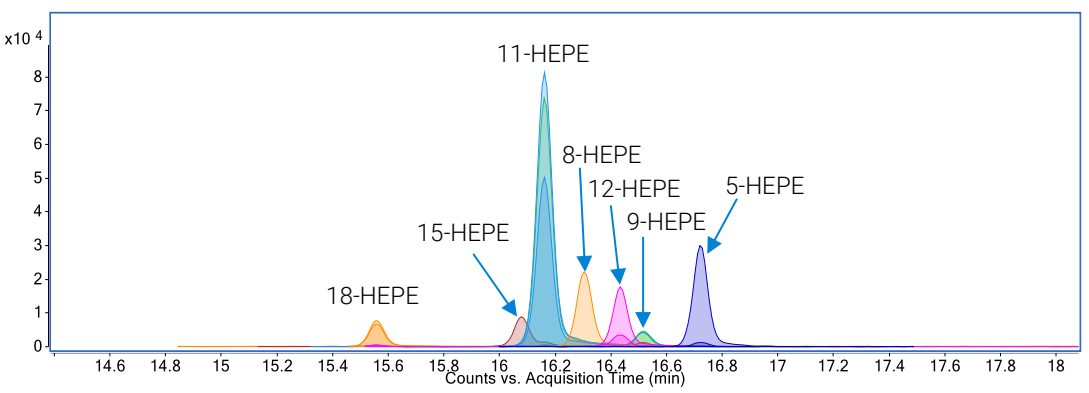

Figure 7. Oxylipins derived from EPA via the LOX pathway.

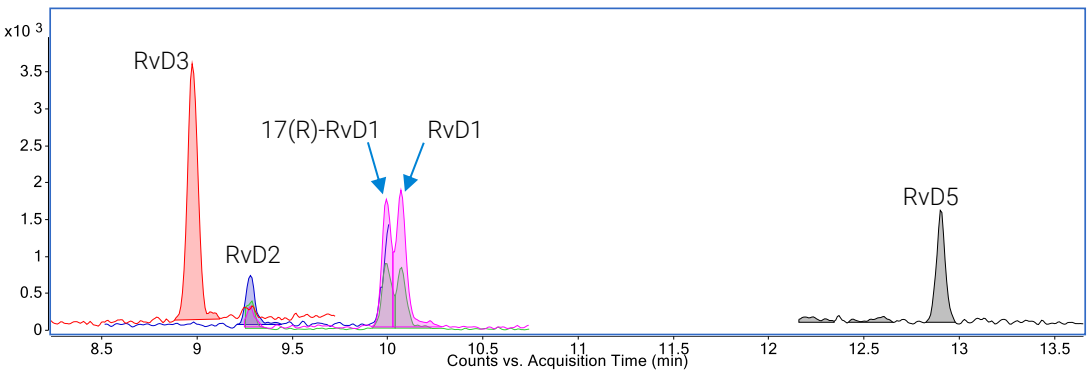

Figure 8. D-series resolvins derived from DHA via the LOX pathway.

Standards from Cayman Chemical were diluted and injected at 0.01 – 1 ng on-column concentration and are shown in Figures 3 – 8 illustrating the retention time order of different oxylipin types and their regioisomers. Some compounds in this method include oxylipins that are derived from the metabolism of eicosapentanoic acid (EPA) via CYP450, such as DiHETEs and EpETEs, compounds derived from linoleic acid (LA) such as DiHOMEs, HODEs, OxoODEs, and EpOMEs. Oxylipins derived from arachidonic acid (AA) via the LOX pathway, such as HETEs and OxoETEs and derived from the oxidation of docosahexaenoic acid (DHA) by CYP450 such as EpDPAs and LOX pathway such as D-series resolvins are included. Endogenous detection varies due to biological phenotype. In this sample, 100 analytes were detected on at least one system and 80 of them were consistently detected across the three sites. The variation can be attributed mostly to sample/analyte stability during sample shipment or slight differences in LC/MS performance for the missing, low responding analytes.

Results and Discussion

Multisite Evaluation of Retention Time Reproducibility and Precision

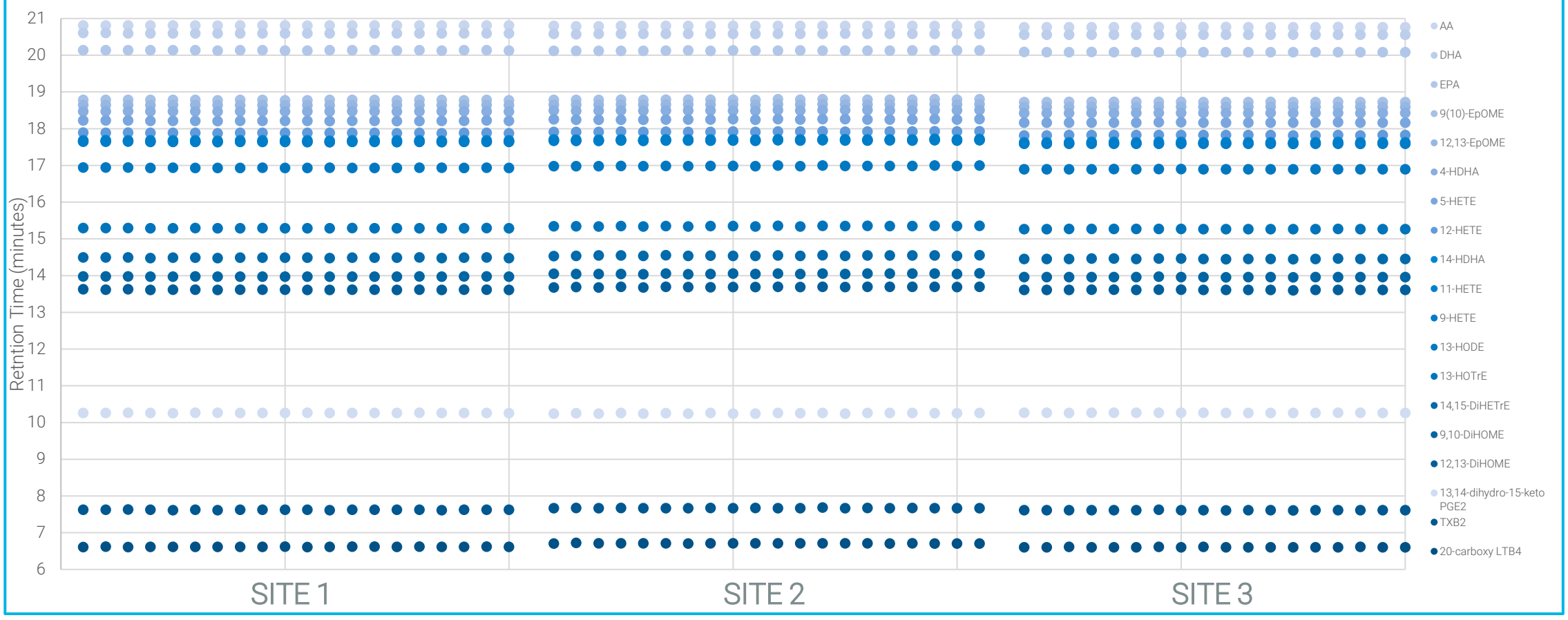

Figure 9. To evaluate method robustness, 19 representative endogenous oxylipins were selected from the 80 species detected across all sites to demonstrate RT stability. These markers were chosen based on their distribution across the full elution profile. The high level of RT precision is maintained across the 191-target method and highlights the standardized instrumental set up of the Infinity III Bio LC and 6495D system.

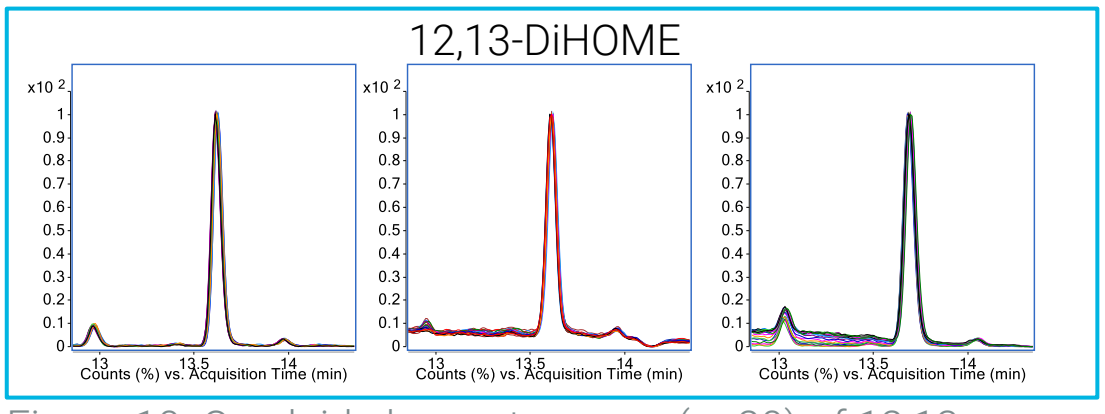

Figure 10. Overlaid chromatograms (n=20) of 12,13-DiHOME from each site.

To assess the reproducibility of the method, endogenous oxylipins were monitored between the three sites. Figure 9 shows the consistent retention times of the representative oxylipins across the chromatographic run. Figure 10 shows overlaid traces from 20 consecutive injections in the same worklist per site of 12,13-DiHOME further showcasing the stable retention time performance.

Conclusions

- **Versatile & Transferable:** Compatible with common SPE-based extraction protocols, facilitating straightforward method adoption and sensitive oxylipin profiling in new laboratories.
- **Comprehensive**: 100 analytes across major biosynthetic pathways were detected in a pooled plasma extract out of the 191 analytes available.
- **Inter-Laboratory Reproducibility**: RT stability was at <0.1 % RSD for 80 analytes found across 3 labs.
- **Flexible:** The standardized LC/MS configuration is also used for established Agilent Omics methods for polar metabolites[3], lipids[4], bile acids[5], and acylcarnitines[6].

https://www.agilent.com/en/promotions/asms

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References

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