Application Note

Metabolomics/Clinical Research

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An Interlaboratory Evaluation of a Targeted Lipidomics Method in Plasma

Authors

Mark Sartain,
Sheher Banu Mohsin,
Carrie Adler, Alex Apffel,
John Sausen,
Pietro Morlacchi, and
Dan Cuthbertson
Agilent Technologies, Inc.

Kristal Maner-Smith and
Eric A. Ortlund
Emory University,
Dept. of Biochemistry,
Atlanta, GA, USA
Emory Integrated
Metabolomics and Lipidomics
Core, Emory University School
of Medicine,
Atlanta, GA, USA

Abstract

A major challenge facing translational metabolomics is the successful implementation of targeted methodologies to measure large population cohorts and to improve interlaboratory precision enough to enable cross-laboratory comparisons of measurements. This application note further documents the performance of a previously developed, highly curated, and proven robust plasma lipidomics LC/TQ method that balances depth of coverage with sample throughput across large population cohorts. The performance was evaluated using results from an interlaboratory multiday study to assess variance. Low intraday retention time (RT) and peak area RSDs were observed, with only minor RT shifts across sites, demonstrating the method's portability to multiple different lab environments. Transferability in method implementation is also demonstrated.

Introduction

Lipidomics seeks to comprehensively profile a diverse range of biomolecules that play critical roles in a variety of essential biological processes, including membrane structure, cell signaling, energy storage, and metabolism. Lipids play important roles in mediating the biological and cellular responses to environmental stimuli, disease states, and pharmaceutical interventions, among many other roles. Crucially, lipidomic measurements are essential to understanding the metabolic underpinnings of disease, either by discovering metabolic dysregulation or production of pro-inflammatory or inflammatory markers. Thus, in this context, it is essential to have a lipidomic methodology that not only covers a diverse range of lipids, provides accurate results, and provides robustness for large cohort studies, but is highly transferrable across multiple laboratories as well. While lipids have diverse properties, they generally have relatively hydrophobic structures, making them amenable to very reproducible reversed-phase chromatography separations, aiding in ease of method implementation. The robust methodology documented here and in another application note (publication number 5994-3747EN)[1] is an ideal choice for researchers evaluating lipid markers for a variety of purposes, including disease research.

There are two different approaches to lipidomics analysis—untargeted and targeted. Untargeted discovery-focused lipidomics workflows are Q-TOF-based methods, using Agilent MassHunter Lipid Annotator tool as a key innovation for in silico library searching of high-resolution MS2 spectra and lipid feature annotation.[2,3] This methodology fits into a complete workflow, including lipidomics support for differential analysis in Agilent Mass Profiler Professional (MPP) software.

In contrast, this application note focuses on targeted lipidomics to achieve fast biological insights for well-characterized matrices. This workflow, using Agilent MassHunter acquisition software for LC/MS systems and Agilent MassHunter Quantitative Analysis software with MPP for differential analysis was already established by Dr. Peter Meikle's group at the Baker Heart and Diabetes Institute in Australia. This is a high-throughput, comprehensive lipidomics profiling method for large population cohorts, all the way from sample extraction to data analysis. Dr. Meikle's lab at the Baker Institute has published their targeted methodology, which uses Agilent instrumentation.[4] The method includes a high level of curation with orthogonal experiments to confirm the annotation level of many MRM transitions, allowing for more accurate lipid specificity. The Meikle Lab method for plasma lipidomics consists of a 16-minute LC/TQ method that covers 44 lipid classes for a total of 763 lipids in 0.1 µL of plasma, and was published as an Agilent application note (publication number 5994-3747EN) in October 2022.[1]

This application note tackles the practical challenge of setting up the methodology in a new lab, for those interested in building a highly specific, comprehensive LC/TQ method covering hundreds of lipids with robust chromatography. To gain a better understanding of method transferability, implementation, and troubleshooting of the method across laboratories and end users, the method was implemented at four sites across the United States. The study design and results of the interlaboratory evaluation are detailed in the Experimental section.

Experimental

Curated method

The LC/TQ method referred to in this note is described in Agilent application note 5994-3747EN.[1] Briefly, the method uses an Agilent 1290 Infinity II LC and a robust, reversed-phase Agilent ZORBAX Eclipse Plus C18, 100 × 2.1 mm, 1.8 µm column resulting in chromatography that provides the ability to separate many interesting isomers with the Agilent 6495 triple quadrupole LC/MS. This method is designed to cover all the major lipid classes in plasma from the small lysophospholipids to the larger, very nonpolar triacylglycerols, thus ensuring suitability for biological research by measuring a wide range of biologically important lipid species.

Lipids, in general, are a challenging group of compounds to analyze in a single method due to their modular structure, which facilitates incredible diversity. Lipids may contain a huge number of combinations of acyl chain length, number of double bonds, double bond location, and more. This diversity can cause systematic problems in correctly measuring lipid species within and across classes. One of the benefits of the reversed-phase approach is the improved ability to separate both isobaric and isomeric compounds, which is critical to identifying species and classes, in comparison to other chromatographic approaches. Hence, robust and reproducible chromatography is crucial for maintaining critical separations, not only in a single lab but at multiple sites as well.

Overall, the most critical part of the method is curation, due to the large number of isobaric and isomeric compounds. Simply adding more transitions as qualifier ions is possible in some cases, but is overall not a complete solution. During the development of the core methodology at the Baker Institute,

a deep characterization of representative pooled plasma samples was accomplished using several orthogonal experiments to add specificity and confidence to the developed MRM transitions. The Baker Institute hosts a website to facilitate lipid data review: https://metabolomics.baker.edu.au/method/lipids.

LC/MS configuration and parameters

See application note 5994-3747EN[1] for specific method details.

Interlaboratory study experimental design

An interlaboratory study was designed to evaluate and better understand method transferability, implementation, and troubleshooting across different laboratories and end users. Plasma from two different sources was used for assessment. Sample 1 consisted of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1950 – Metabolites in Frozen Human Plasma, and was obtained from NIST (Gaithersburg, MD, USA). NIST-SRM 1950 has often been implemented as a quality control sample for LC/MS-based lipidomics.[1,5,6] However, note that NIST-SRM 1950 was used in this study as a reference material to assess method variability across laboratories, rather than reporting its lipid composition. Sample 2, "BIO", was Normal Human Plasma, Mixed Gender from BioIVT (Westbury, NY, USA).

The primary goal of this study was to assess variance in LC/MS measurements across labs with the methodology, and not to assess variance due to sample preparation. To minimize variance due to inconsistent lipid extraction, plasma samples were prepared by a single user using a large-scale single-phase butanol-methanol lipid extraction method described previously.[1] The SPLASH II LIPIDOMIX Mass Spec Standard (Avanti Polar Lipids, Alabaster, AL, USA), consisting of a mixture of 12 deuterated class-specific lipid standards, was added to the extraction solvent. Identical aliquots of the lipid extract were shipped to four locations across the United States. These included three independent Agilent laboratories with different end users: one lab in Wood Dale, Illinois, and two separate labs in Santa Clara, California. The fourth lab was a collaborator lab at the Emory Integrated Metabolomics and Lipidomics Core at Emory University in Atlanta, Georgia. Each site used completely independent LC/MS systems and columns of different lots. All users were provided with the mobile phase preparation procedure, description of the LC configuration and plumbing, and a column conditioning procedure, as well as worklist (.wkl) and acquisition method (.m) files.

Before analyzing the samples, each LC/MS system underwent a two-day instrument preparation procedure, which included a standardized LC configuration, LC/TQ tuning, LC flushing and background check, new column flushing and equilibration, and the injection of a test sample to check and adjust the retention times in the acquisition method if needed. On day 1, 80 samples were analyzed: NIST-SRM 1950, N = 30, followed by BIO N = 20, and then 30 additional NIST-SRM 1950 samples were analyzed. On day 2, after freshly preparing the mobile phase, 60 NIST-SRM 1950 samples were analyzed. All data files from the four locations were submitted for review by a single analyst. MassHunter Quantitative Analysis software (version 12.1) was used to analyze the sample batches with a quantification method that implemented retention time referencing integration as described previously.[1]

Results and discussion

Retention time evaluation

All data files from the four laboratory sites were analyzed and compared with the reference sample chromatogram from the method curation database https://metabolomics.baker.edu.au/method/lipids. As shown in Figure 1, using Lipid LPC (18:3) as an example, all four sites achieved reproducible chromatograms for complex isomers, with the same pattern seen in the database (Figure 1A). Using this lipid as a "quality-control" lipid can inform users about the performances of the method chromatography, and be used to evaluate whether adjustments like correct plumbing, delay volumes, etc. are needed to achieve the expected lipid separation. Overall, there is a minimal retention time drift for LPC (18:3) across all sites.

Furthermore, the retention times of the 12 internal standards fell within the dynamic MRM windows in all sites, as shown in Figure 2. The retention time reproducibility was also favorable, with RDS values lower than 0.25% for all internal standards (Table 1). There is remarkable intraday consistency with a slight, but acceptable interday shift observed for two of the four sites. This variation is attributed to new mobile phase preparation on the second day, emphasizing that users should account for this potential source of analytical variability and troubleshoot as needed. Table 1 shows the intraday retention time reproducibility, with all RSD values less than 0.25% for all 12 internal standards.

Additionally, when looking at 664 endogenous lipids at all four sites for two days, the RSDs were all less than 0.20% for intraday retention time reproducibility. Not all 763 lipids from the method were used in the analysis.

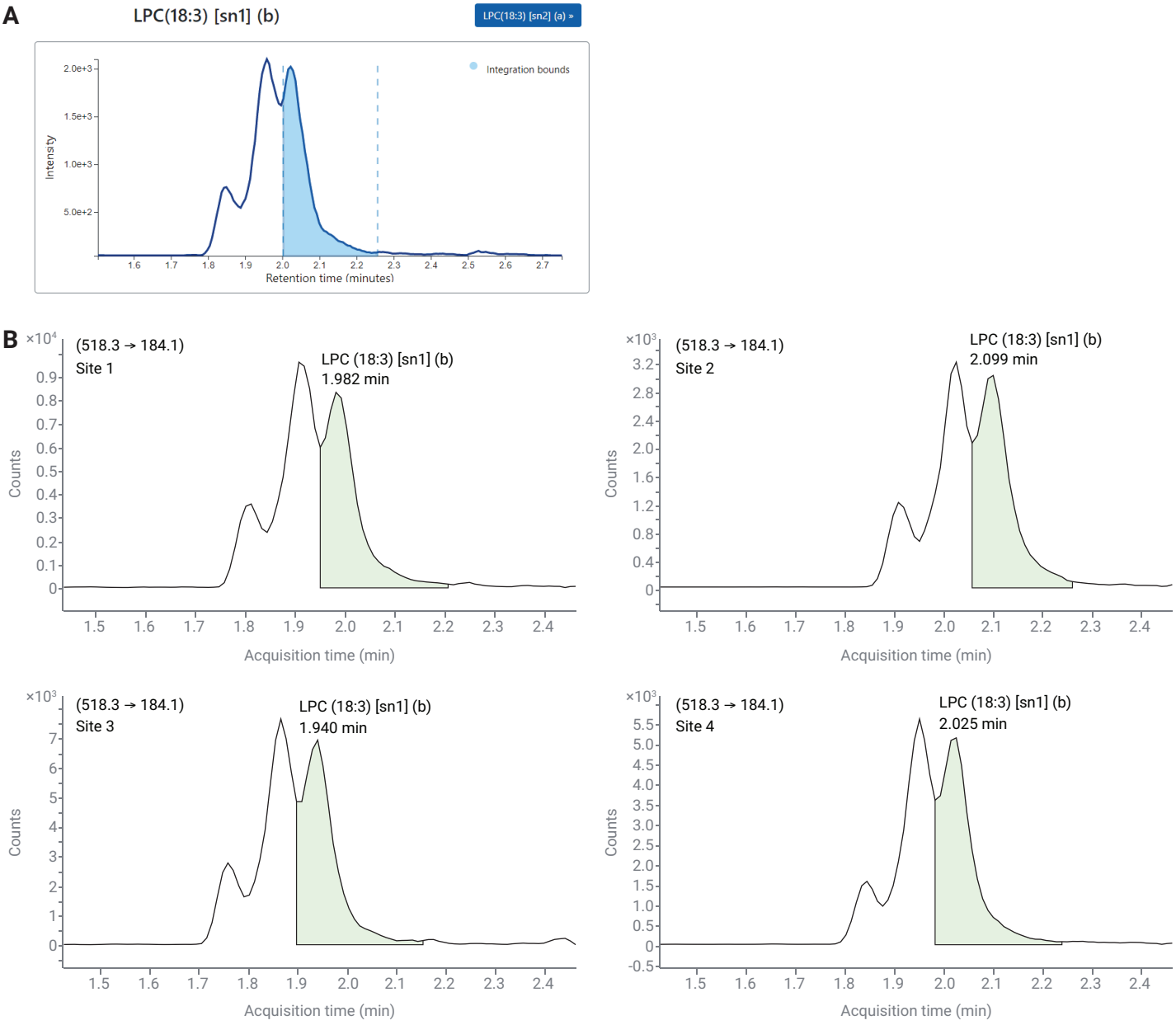

Figure 1. Lipid LPC (18:3). (A) Reference chromatogram from the curated method database: https://metabolomics.baker.edu.au/method/lipids. (B) Chromatograms from the four laboratory sites. Lipid PLC (18:3) was used as a "quality-control" lipid as it is a complex triplet peak. This lipid was monitored to show consistency across sites.

There are two reasons for the removal of some lipids: 1) The NIST plasma used is healthy plasma, and therefore does not contain some lipids included in the method that are only present with certain disease states, and 2) some lipids fell outside of the dMRM RT window, and could not be analyzed. It is important to note that when implementing this method, especially with a new column, an RT adjustment procedure to modify the dMRM RT windows for a particular system may be needed before proceeding. A limitation of this study was that this RT adjustment was not used on all datasets. This RT adjustment is now offered as an automated procedure and available to users to correct this shift—details are outlined in supporting documents for the method implementation.

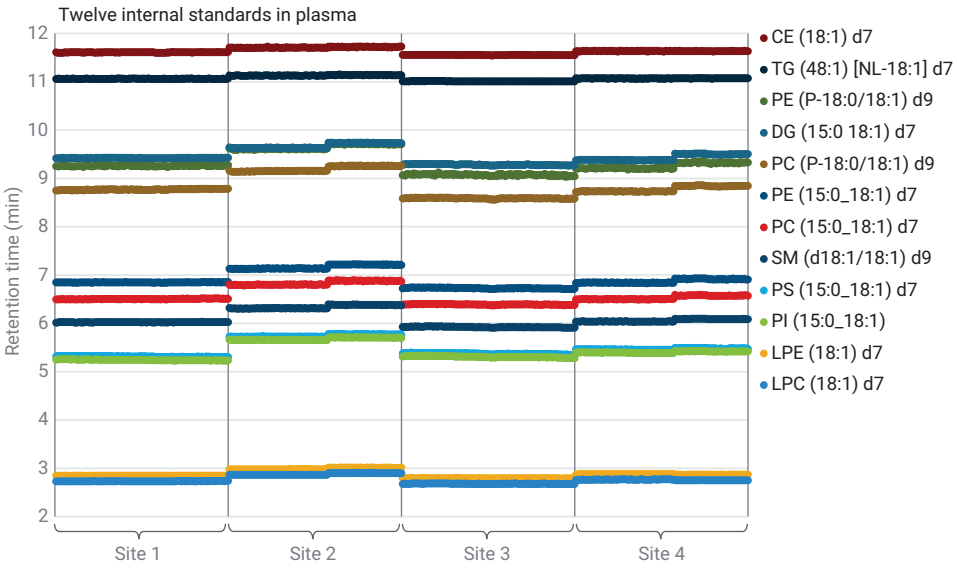

Figure 2. Retention time evaluation of 12 internal standards in plasma. All four sites captured the 12 internal standards within the instrument acquisition window.

Table 1. Intraday retention time reproducibility.

Site 1		Site 2		Site 3		Site 4	
Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
0.04	0.05	0.04	0.06	0.04	0.04	0.05	0.04
0.04	0.04	0.05	0.05	0.04	0.00	0.04	0.02
0.05	0.06	0.06	0.05	0.13	0.07	0.06	0.08
0.10	0.11	0.11	0.09	0.19	0.20	0.13	0.15
0.07	0.08	0.09	0.06	0.14	0.08	0.07	0.08
0.05	0.08	0.09	0.07	0.15	0.10	0.08	0.12
0.09	0.10	0.11	0.10	0.14	0.09	0.09	0.09
0.07	0.05	0.08	0.07	0.16	0.10	0.07	0.07
0.09	0.07	0.09	0.09	0.21	0.14	0.11	0.14
0.11	0.07	0.09	0.07	0.22	0.15	0.12	0.10
0.10	0.05	0.12	0.17	0.14	0.13	0.07	0.14
0.07	0.20	0.00	0.15	0.20	0.17	0.20	0.07

Table 2. Intraday retention time reproducibility of 664 endogenous lipids, with %RSD less than 0.20% for all lipids.

	Site 1		Site 2		Site 3		Site 4	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Mean	0.11	0.11	0.12	0.12	0.19	0.18	0.11	0.15
Median	0.07	0.08	0.09	0.08	0.14	0.10	0.08	0.09

Peak abundance evaluation

To further evaluate robustness and reproducibility, peak abundance was analyzed. For each NIST plasma sample at each of the four sites, a histogram RSD plot was generated (Figure 3) to show the number of lipids that fall within an RSD threshold. Depending on the site, 377 to 516 lipids had less than 10% RSD. In Table 3, the intraday mean and median of peak abundance are also shown, with all median percent RSDs below 10%, except for site 4, day 2, which is explained further.

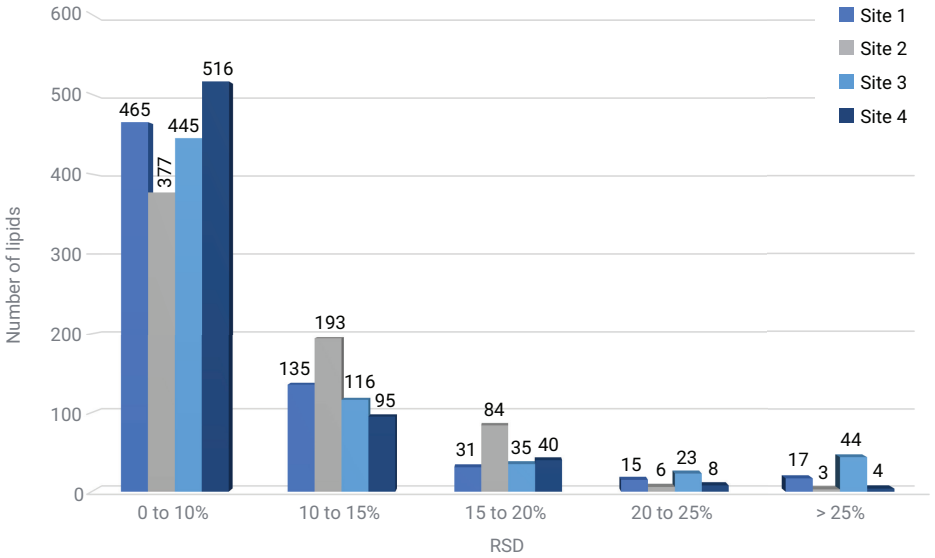

Figure 3. Histogram of intraday peak abundance evaluation, showing the number of lipids at each site that fell within the RSD buckets of 0 to 10%, 10 to 15%, 15 to 20%, 20 to 25%, and greater than 25%.

Principal component analysis

Principal component analysis (PCA) is an unsupervised data reduction method that is routinely used in -omics sciences to narrow the number of variables in large data sets, thereby simplifying data visualization and interpretation, while still preserving as much information as possible. MPP software was implemented to perform PCA on the respective datasets. The log_2-transformed MS peak intensities underwent a linear transformation, which allowed the overall variance of the datasets to be expressed in terms of independent components in a tridimensional space.

The 3D-PCA plot in Figure 4 shows proper clustering of the NIST-SRM 1950 and BIO samples analyzed at all four sites on two days. The most important separation of the NIST and BIO sample occurs along the X-axis, contributing to over 40% of the total variability in the study, while the separation associated with the lab sites on the Y-axis has a less important contribution to the study variability. Overall, the grouping and directions of the sample separation shown by the PCA components represents the relationship between the BIO versus NIST samples, and indicates that the primary driver of experimental variability is mainly due to differences in the lipidome composition of the biological samples. Compared to this, the difference within sites is less pronounced, thus documenting that the methodology is fit for purpose and can produce biologically impactful results independent of the site of implementation. Differences between sites could be further minimized by using relative quantitative values instead of raw peak abundances. The process of incorporating class-specific lipid standards and performing relative quantitation is detailed in application note 5994-3747EN.[1] However, this strategy was not pursued for the interlaboratory exercise because not enough internal standards were incorporated to cover most lipid classes.

Table 3. Intraday peak abundance evaluation at all four sites. Median %RSD values were below 10%.

	Site 1		Site 2		Site 3		Site 4	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Mean	8.4	7.9	10.0	9.6	9.4	12.0	7.7	14.4
Median	7.0	6.3	9.1	8.3	6.1	9.1	6.5	13.6

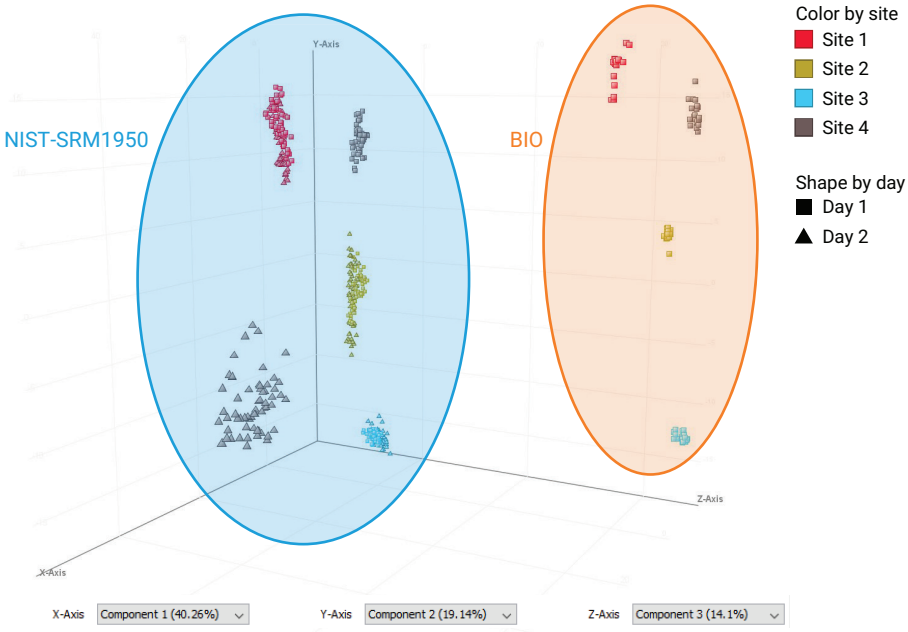

Figure 4. Normalized PCA of four sites, two days, and two samples. The sites are represented by different colors, and days are indicated by different shapes.

A thorough analysis of the data in Figure 4 highlights a partial separation between the samples analyzed at site 4 on day 1 and day 2. Although these samples still cluster as expected, the PCA shows a certain degree of variability across them. This difference was due to the lack of appropriate implementation of quality control procedures (QC) at site 4 on day 2 after a restart of the acquisition computer was needed. This is an example of how proper QC application is key before sample analysis, and even when a worklist is resumed after an undesired interruption. Without proper QC, the data is subject to greater experimental variability that can negatively impact the results irrespective of the quality of instrumentation, software, or method implemented. Therefore, users should always ensure adequate QC procedures when using any -omics methodology.

Conclusion

A robust, targeted lipidomics method was implemented at four different sites and showed minimal intralaboratory variations when used to measure lipid levels in plasma samples. The overall intraday RT variability was less than 0.20 %RSD. Modest RT shifts were

noted with the preparation of new mobile phase. While generally small RT shifts were seen between labs, a new RT adjustment procedure is now an automated process in case users need to adjust method RTs at their site. Additionally, the peak abundance evaluation revealed single-digit intraday %RSD values. Lastly, PCA analysis showed distinct lipid profiles for two similar-pooled plasma samples, and the differences in specific lipids were consistent across the four sites, demonstrating suitability for biological studies.

However, the site 4, day 2 data outlier shows that best QC practices are still essential in any -omics laboratory, and particularly with any interlaboratory study. In conclusion, this robust and highly curated targeted lipidomics method produces consistent and high-quality biological results that can be implemented across multiple lab environments.

References

1. A Comprehensive, Curated, High-Throughput Method for the Detailed Analysis of the Plasma Lipidome. *Agilent Technologies application note*, publication number 5994-3747EN, **2021**.
2. Lipidomics Analysis with Lipid Annotator and Mass Profiler Professional. *Agilent Technologies technical overview*, publication number 5994-1111EN, **2020**.
3. Lipid Profiling Workflow Demonstrates Disrupted Lipogenesis Induced with Drug Treatment in Leukemia Cells. *Agilent Technologies application note*, publication number 5994-1356EN, **2020**.
4. Huynh, K.; Barlow, C. K.; Jayawardana, K. S.; Weir, J. M.; Mellett, N. A.; Cinel, M.; Magliano, D. J.; Shaw, J. E.; Drew, B. G.; Meikle, P. J. High-Throughput Plasma Lipidomics: Detailed Mapping of the Associations with Cardiometabolic Risk Factors. *Cell Chem. Biol*. **2019**, *26(1)*, 71–84.e4.
5. Quehenberger, O.; Armando, A. M.; Brown, A. H.; Milne, S. B.; Myers, D. S.; Merrill, A. H.; Bandyopadhyay, S.; Jones, K. N.; Kelly, S.; Shaner, R. L.; *et al.* Lipidomics Reveals a Remarkable Diversity of Lipids in Human Plasma. *JLR* **2010**, *51(11)*, 3299–305. doi:10.1194/jlr.M009449
6. Bowden, J. A.; Heckert, A.; Ulmer, C. Z.; Jones, C. M.; Koelmel, J. P.; Abdullah, L.; Ahonen, L.; Alnouti, Y.; Armando, A. M.; Asara, J. M.; *et al.* Harmonizing Lipidomics: NIST Interlaboratory Comparison Exercise for Lipidomics Using SRM 1950-Metabolites in Frozen Human Plasma. *JLR* **2017**, *58(12)*, 2275–2288. doi:10.1194/jlr.M079012

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