# A ROBUST AND REPRODUCIBLE LIPID DIA UPLC/MS USING WATERS CONNECT™ WITH THE XEVO™ Q-TOF G3

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## INTRODUCTION

**The waters_connect™ platform features comprehensive software tools for the analysis and processing of LC-MS/MS based lipidomic data. Lipidomic workflows can incorporate multiple MS acquisition types such as data-dependent (DDA) and data-independent (DIA) acquisition datasets. Here we provide an evaluation of DIA UPLC/MS in combination with workflow-driven informatics. We demonstrate how the waters_connect™ platform, which leverages Ultra Performance LC (UPLC™) technology coupled with the Xevo G3 QTof mass spectrometer for lipidomics analysis. Using waters_connect applications, samples can be submitted for analysis, processed and reported in the same workflow offering a streamlined, robust, reproducible and accurate means of identification, quantification and reporting (Figure 1).**

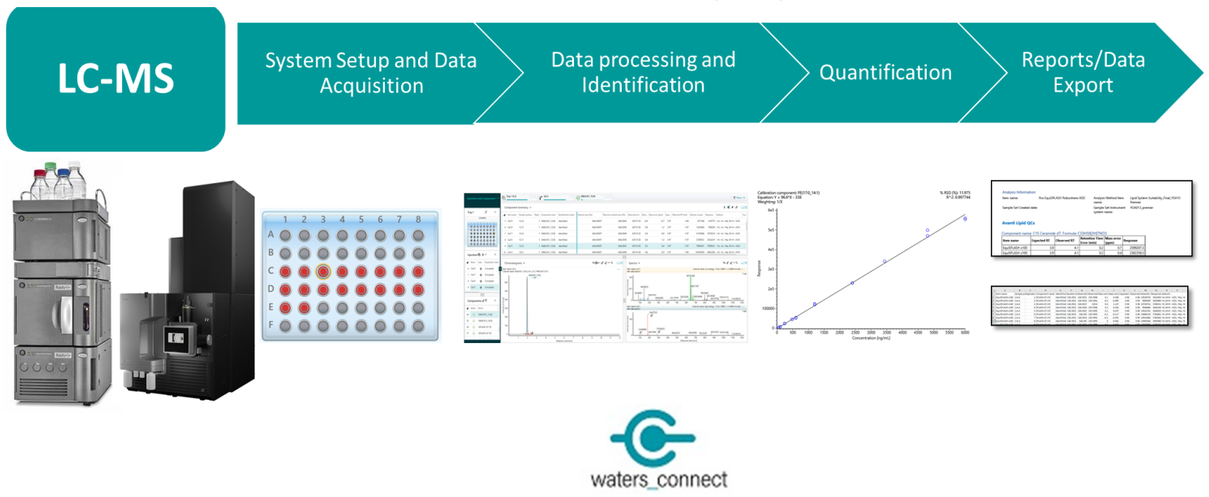


*Figure 1. Overview of the integrated waters_connect workflow from system setup through to data reporting .*

## METHODS

- Test samples for mass accuracy stability and reproducibility consisted of a 100 times dilution of AVANTI EquiSPLASH™ (Avanti, Birmingham, Al, USA) in IPA and plasma extracts using a simple protein precipitation method with 100x EquiSPLASH in IPA [1].
- The calibration curves were generated using AVANTI Odd-Chained LIPIDOMIX™ at three concentrations (10×, 20×, and 50×) using IPA. The neat standard mixture or spiking solutions were added at less than 5% v/v to commercially available pooled “normal plasma” (Peary Court, Novi, MI, USA) to generate a 10-point calibration curve [1]
- High throughput Reversed-Phase Lipid profiling method for large samples sets gradient and MS conditions (12 min run time). Mobile Phase A (600:390:10 Acetonitrile:Water:1M Ammonium Formate, 0.1% Formic Acid) Mobile Phase B (900:90:10 IPA: Acetonitrile:1M Ammonium Formate, 0.1% Formic Acid) starts from 50% to 99% over 12 minutes.
- ACQUITY™ Premier UPLC™ I-Class system and ACQUITY Premier UPLC™ CSH™ C18 (2.1 x 100 mm, 1.7 µm) column.
- Mass Spectrometers used: Xevo G3 QToF instrument
- Data acquisition and processing by waters_connect informatics
- The Waters Lipidomics Profiling CCS Library was used to create the scientific libraries required for processing and compound identification in waters_connect

## SYSTEM SETUP

Automated detector set-up and instrument calibration are performed prior to the analysis of samples within waters_connect. This ensures the mass spectrometer is optimal maximizing mass accuracy and response stability. Sample lists of calibrants, quality control and test samples can be submitted with the required LC and MS methods.

A neat standard mixture, diluted 100 times with EquiSPLASH in IPA, was used to verify the reproducibility of retention time and peak shape. As depicted in Figure 2, the resulting chromatograms of the deuterated lipid standards in positive ESI and negative ESI modes provide an overview of the system's performance for lipid analysis.

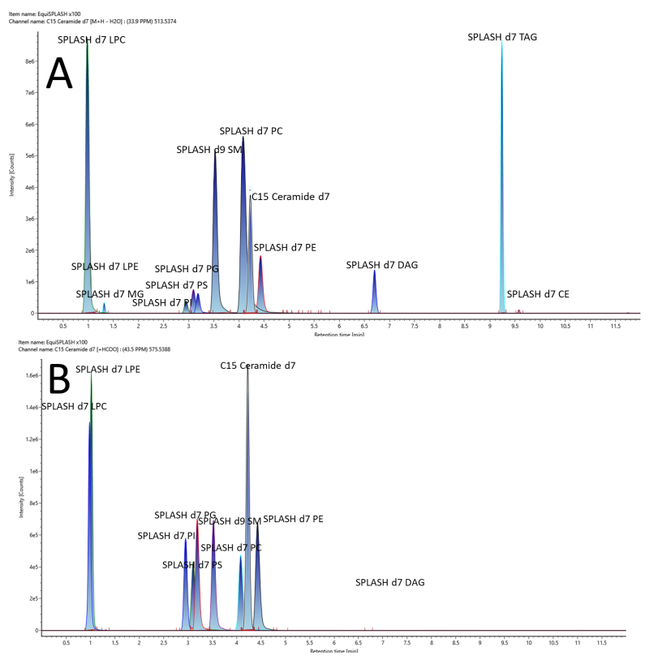


*Figure 2. Chromatograms representing Avanti EquiSPLASH in IPA; (A) Positive mode ESI (2 µL injection) and (B) Negative mode ESI (2 µL injection).*

## REPORTING

Results can be reported using the various templates which can be modified to accommodate the user’s requirements. Furthermore, data generated within waters_connect can be accessed directly and processed with third-party software such as Lipostar [2] using the unique application program interface (API) as shown in Figure 4.

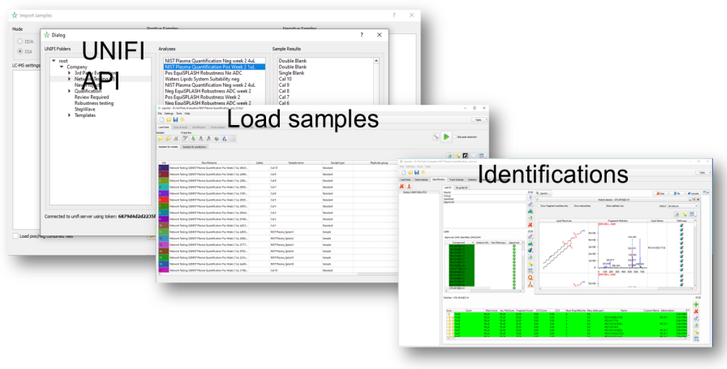


*Figure 4. The application program interface (API) can be used to transfer and process data to third-party software packages such as Lipostar.*

## IDENTIFICATION

Lipid species to be identified or screened can be added to an “Analysis Method” from the scientific library such as the Waters Lipidomic Profiling CCS Library. In Figure 3A, we show the positive ion spectrum, which has a precursor mass error of +2.53 ppm. The high energy spectrum displays the typical PC head group of 184 m/z and various other theoretical fragments, suggesting a sum composition of PC(34:2). Complimentary negative ion mode (Figure 3B) data provides a high energy spectrum with more diagnostic fragment ions relating to the fatty acyl chains. The sn1 RCOO- ion fragment of 255.235 m/z confirms the presence of a 16:0 fatty acyl chain, and the sn2 RCOO- ion fragment at 279.235 m/z indicates the presence of an 18:2 fatty acyl chain. We can therefore assign the identification of PC(16:0_18:2) with a high degree of confidence.

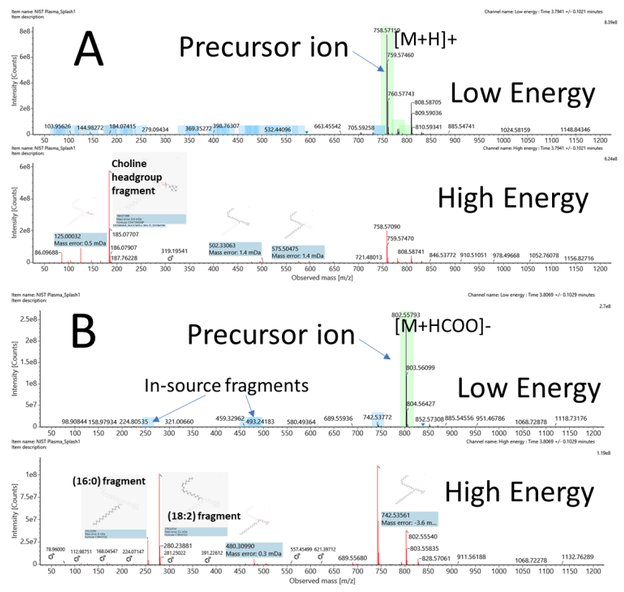


*Figure 3. Low and high collision energy spectra of PC (16:0_18:2); (A) Positive mode ESI and (B) Negative mode ESI.*

## MASS ACCURACY

The mass accuracy stability revealed a mean mass error of less than 0.57 ppm across several lipid classes from Avanti EquiSPLASH in IPA for positive ionisation mode, and less than -1.93 ppm in negative mode over 75 injections (Figure 5). Similar results were found for spiked standards in plasma over 125 injections.

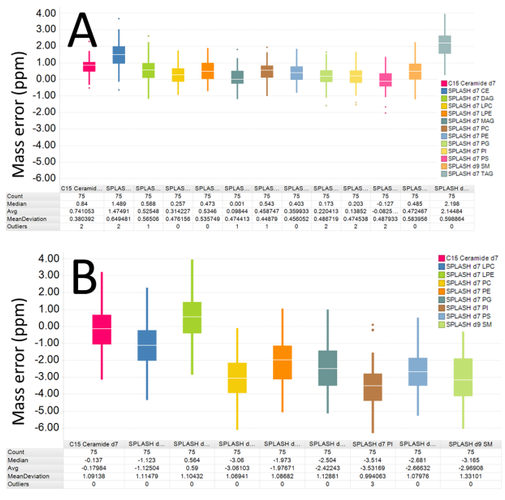


*Figure 5. Mass accuracy stability of lipid standards over 75 injections; Box and whisker pots of mass error values of lipid standards A) Positive mode ESI and (B) Negative mode ESI.*

## REPRODUCIBILITY

By enabling automatic detection correction, we achieved raw responses with coefficients of variation (CVs) of less than 5% across multiple lipid classes (Figure 6 A), even when outliers were included. Our analysis of over 125 plasma injections revealed CVs of less than 5% for endogenous lipids across various lipid classes. Figure 6B shows the retention time stability of less than 0.5% across 125 injections.

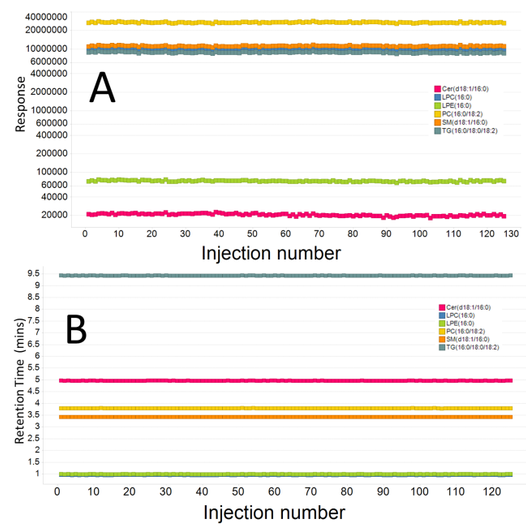


*Figure 6.(A) Robustness of signal responses in NIST Plasma Extracts (n=125) (B) Reproducibility of endogenous lipid RT in NIST Plasma Extracts (n=125)*

## QUANTIFICATION

We established excellent linearity (R2 >0.97) for bioactive phospholipid calibration curves based on spiked Odd Chain lipid standards in plasma, covering typical biological ranges (Table 1).

| Peak No. | Lipid Standard | R2 | |
|---|---|---|---|
| | | ESI + | ESI- |
| 1 | LPC(17:1) | 0.973 | 0.990 |
| 2 | LPE(17:1) | 0.993 | 0.985 |
| 3 | LPG(17:1) | 0.993 | 0.997 |
| 4 | LPI(17:1) | 0.994 | 0.995 |
| 5 | LPS(17:1) | 0.969 | 0.994 |
| 6 | PC(17:0/14:1) | 0.968 | 0.968 |
| 7 | PE(17:0_14:1) | 0.968 | 0.998 |
| 8 | PG(17:0_14:1) | 0.968 | 0.996 |
| 9 | PI (17_0-14_1 | 0.968 | 0.994 |
| 10 | PS (17_0-14_1 | 0.968 | 0.992 |
| 11 | SM(d18:1/12:0) | 0.968 | 0.984 |

*Table 1. : Correlation coefficients (R2) representing the calibration curves for the polar lipids present in the Odd Chain Lipidomix.*

## CONCLUSION

Simple and robust acquisition strategy, providing high-quality, comprehensive data quickly and efficiently.

Highly flexible lipid identification approach, incorporating spectral matching and in-source fragment ion recognition.

Easy and accurate quantitation using calibration curve capabilities.

Compatibility with third-party tools such as Lipostar, MzMine and Skyline, provides users with greater flexibility for data analysis

**References**

1. Munjoma, N.; Isaac, G.; Muazzam, A.; Cexus, O.; Azhar, F.; Pandha, H.; Whetton, A.D.; Townsend, P.A.; Wilson, I.D.; Gethings, L.A.; Plumb, R.S. (2022) High Throughput LC-MS Platform for Large Scale Screening of Bioactive Polar Lipids in Human Plasma and Serum. J Proteome Res. 2022 Nov 4;21(11):2596-2608. doi: 10.1021/acs.jproteome.2c00297. Epub 2022 Oct 20. PMID: 36264332; PMCID: PMC9639203.
2. Laura Goracci, Sara Tortorella, Paolo Tiberi, Roberto Maria Pellegrino, Alessandra Di Veroli, Aurora Valeri, and Gabriele Cruciani (2017) LipoStar, a Comprehensive Platform-Neutral Cheminformatics Tool for Lipidomics, Analytical Chemistry 2017 89 (11), 6257-6264, DOI: 10.1021/acs.analchem.7b01259

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