QUANTIFYING THE LIPIDOME FOR RESPIRATORY DISEASE: A RAPID AND COMPREHENSIVE HILIC-BASED TARGETED APPROACH

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HIGHLIGHTS

- **Comprehensive and robust high-throughput HILIC-based LC-MS/MS method with over 2000 MRMs.**
- **Highly specific MRM transitions based on the fatty acyl chain and headgroup fragment ions.**
- **Lipid class based separation reduces the number of stable isotope lipid standards (SILS) which results in significant cost saving.**

INTRODUCTION

Respiratory linked conditions associated with chronic obstructive pulmonary disease (COPD), asthma, and infection are increasing with significant associated socio -economic costs.

Although advances in mass spectrometry (MS) have allowed for more in-depth lipidomics analysis, unambiguous identification and quantification has proven difficult as lipids exhibit a high number of isomeric and isobaric species.

Furthermore, MS spectra often contain peaks and fragments from multiple compounds making confident identification and relative quantification of specific molecular species difficult and time consuming.

A hydrophilic interaction chromatography (HILIC) based approach for the separation of lipids by class prior to MS analysis is a proven method of reducing identification ambiguity. An additional benefit of separating lipid species by class is that fewer stable isotope labelled (SIL) standards are required for quantification, conferring a cost saving.

Here we describe a comprehensive and high-throughput HILIC-based LC-MS/MS method for the separation and quantitation of both polar and non-polar lipid classes (Figure 1); (www.waters.com/targetedomics).

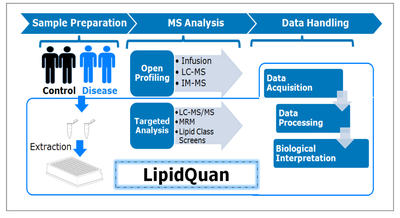

Figure 1. *General lipidomics workflow used in most research laboratories, with the LipidQuan workflow highlighted.*

METHODS

SAMPLE PREPARATION

A simple sample preparation procedure was adopted using protein precipitation with a pre-cooled isopropanol (IPA) at 4 ºC (1:5, plasma:IPA). Samples were vortex mixed for 1 minute and placed at -20 ºC for 10 minutes. Samples were vortex mixed again for 1 minute and placed at 4 ºC for 2 hours to ensure complete protein precipitation. The extracted samples were centrifuged at a maximum of 10,300 g for 10 minutes at 4 ºC before transferring the supernatant to glass vials for LC-MS analysis.

INSTRUMENT CONDITIONS

LC Conditions:

LC system:	ACQUITY UPLC I-Class with FTN or Fixed Loop
Column:	ACQUITY UPLC BEH Amide (2.1x100mm, 1.7 µm)
Column temp:	45°C
Injection volume:	2 µL
MP A:	95/5 ACN/Water (10 mM ammonium acetate)
MP B:	50/50 ACN/Water (10 mM ammonium acetate)
Gradient:	0.1% to 20.0% B for 2 minutes, then 20% to 80% B for 3 minutes followed by 3 minutes re-equilibration

MS Conditions:

MS systems:	Xevo TQ-XS or Xevo TQ-S micro
Ionization mode:	ESI (+/-)
Capillary voltage:	2.8 kV (+)/1.9 kV (-)
Acquisition mode:	MRM
Source temp.:	120 °C
Desolvation temp.:	500 °C
Cone gas flow:	150 L/hr
Desolvation flow:	1000 L/hr
Nebuliser gas:	7 bar
Ion guide offset 1:	3 V
Ion Guide offset 2:	0.3 V

INFORMATICS

A LipidQuan Quanpedia method file that contains the LC conditions, MS method (with over 2000 MRM transition), and associated TargetLynx processing method (including retention times) was generated.

The Avanti SPLASH LIPIDOMIX™ was used as stable isotope labelled (SIL) standards for lipid quantitation.

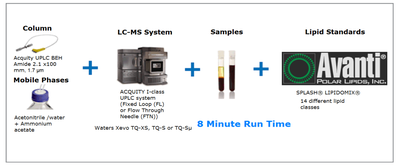

Figure 2. *LipidQuan instrumentation and LC-MS/MS conditions. The LipidQuan Quanpedia method file contains LC conditions, MS method with over 2000 MRM transitions and processing methods.*

RESULTS

A mixture of SIL lipid standards representing different lipid classes from Avanti (SPLASH LIPIDOMIX™) were used to demonstrate the separation of the lipid classes. As shown in Figure 2, lipids are mainly separated into lipid classes according to their polarity within 8 minutes yielding a quantitative method suitable for the lipidomic analysis of large sample sets.

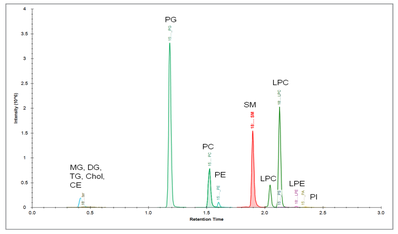

Figure 3. *Positive ion mode chromatogram representing HILIC separation of the SPLASH LIPIDOMIX™ lipid standard mixture.*

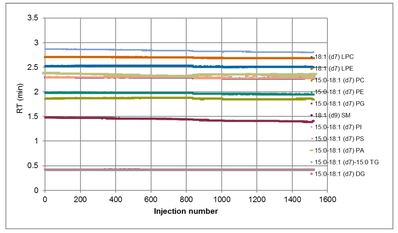

Figure 4. Average retention time (n=1500) *of SPLASH LIPIDOMIX™ lipid standard mixture spiked into NIST 1950 plasma with RSD's <2%.*

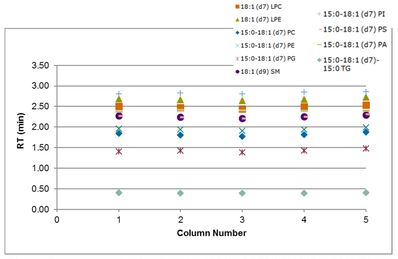

Figure 5. *Average retention time (n=1500) of SPLASH LIPIDOMIX™ lipid standard mixture in IPA using five columns from different batches with RSD's <2%.*

RESULTS

Plasma sample from three biological states of varying phenotypes (healthy control, COPD patients and asthma patients) were analyzed. Samples were randomized and two technical replicates per sample were acquired. Example chromatograms representing endogenous lipids for both positive and negative mode are shown below in Figure 6.

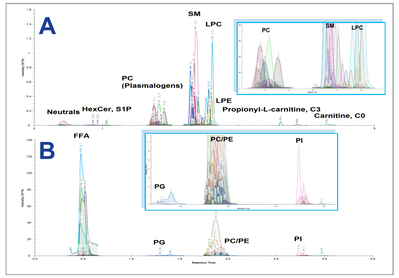

Figure 6. *Example chromatogram of plasma samples analysed using LipidQuan platform.* ***(A)*** *Positive mode screen (with zoomed insert) and* ***(B)*** *Negative mode screen (with zoomed insert) of various lipid classes.*

A large number of lipids were identified from the extensive LipidQuan Quanpedia MRM library that contains highly specific fatty acyl and headgroup MRM transitions for confident lipid identification. Figure 7 shows two PC molecular species with the same precursor mass that were distinguished by the specific fatty acyl chain fragments.

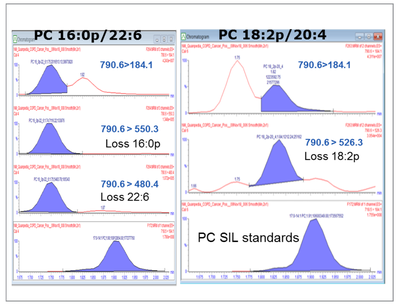

Figure 7. *LipidQuan improves isobaric lipid species identification by using both fatty acyl and headgroup MRM transitions for confirmation. Example, PC (16:0p/22:6) and PC (18:2p/20:4) have precursor m/z 790.6 and can not be distinguished using only the head group transition (m/z 184.1).*

RESULTS

Data processing can be performed using TargetLynx or open source software such as Skyline. Since lipids of the same class elute as discreet bands, stable isotope lipid standards can be used for more accurate quantification of endogenous lipids. Multi-variate statistics can be performed on processed data using packages such as SIMCA-P+ (Umetrics) or MetaboAnalyst through a Symphony data pipeline.

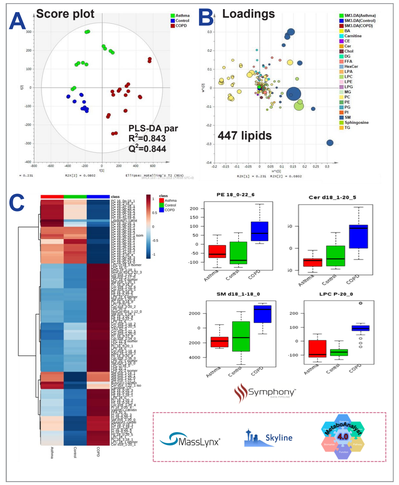

Figure 8. *LipidQuan data from a COPD/Asthma study was statistically analysed using* ***(A)*** *SIMCA-P+ and* ***(B)*** *Metaboanalyst statistical packages via Symphony data pipeline to enable biological interpretations. (C) Hierarcjical clustering of the top 100 lipid species (ANOVA/t-test with FDR < 1%) highliting the average differential expression acroos the three groups. The box plots show example of altered lipid species on the different cohort samples.*

Statistical analysis of the data revealed clear separation between the various cohorts (Figure 8).

Validated PLS-DA models resulted in the clustering of healthy controls, COPD, and asthma patients (Figure 8A). Additional validation, through the use of permutation tests, indicated that the applied PLS-DA models did not over fit the data (R^2=0.843, Q2=0.844).

The loadings plot (Figure 8B), indicated that FFAs, PCs, LPCs, SMs, and Ceramides are the main contributors of statistical separation.

Additional curation of the data using ANOVA/t-test revealed the 100 most statistically significant lipids for hierarchical clustering, showing differential lipid expression trends for the three cohorts (Figure 8C).

RESULTS

Quantification was achieved using calibration curves of plasma spiked with known concentrations of SIL standards prior to extraction. Further details of calibration ranges and acceptance criteria can be found in the LipidQuan Method Reference (www.waters.com/targetedomics). Using surrogate standards prepared and analyzed under identical conditions as the control and disease samples, the quantification of endogenous lipids within the same class was achieved.

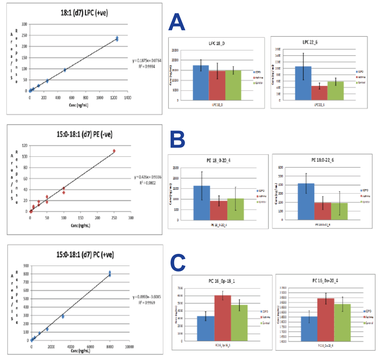

Figure 8. *Calibration curve and quantitative data representing stastically significant lipid species (ANOVA/t-test with an FDR <1%) for over expressed* ***(A)*** *LPC,* ***(B)*** *PE and* ***(C)*** *down regulated PC.*

CONCLUSIONS

- **Streamlined and integrated lipidomics workflow (from sample preparation through to biological interpretations).**
- **Highly specific MRM transitions based on the fatty acyl chain fragments when applicable instead of the typical head group fragments to improve identification and specificity.**
- **Routine targeted quantification of common lipids in plasma and serum.**
- **Lipid class based separation reduces the number of stable isotope lipid standards (SILS) which results in significant cost saving.**
- **Fast data processing using TargetLynx or open source software such as Skyline.**
- **Data visualization using SIMCA-P+ (Umetrics) or MetaboAnalyst.**

Acknowledgements

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References

1. Munjoma, N., Isaac, G., Plumb, R., Gethings, L., (2019) Quantifying the Lipidome for a Respiratory Disease Study Using LipidQuan: A Rapid and Comrehensive Targeted Approach., Application Note (720006542EN).
2. Isaac, G., Munjoma, N., Gethings, L., Plumb, R., (2018) LipidQuan for Comprehensive and High-Throughput HILIC-based LC-MS/MS Targeted Lipid Quantitation., Application Note (720006402EN).

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