

LipidQuan: Quantifying the Lipidome of Transgenic Mice Tissue Extracts, a Rapid and Comprehensive Targeted Approach

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APPLICATION BENEFITS

- Rapid quantification of ~500 lipids in tissue extracts
- A robust and easy to deploy platform reducing method development and training costs, using Quanpedia™
- Improve identification and specificity of phospholipids using MRM transitions from the two fatty acyl chain fragments
- Fast data processing and visualization using TargetLynx™ Software and third-party informatics (i.e., Skyline) for maximum flexibility
- Faster and cheaper than comparable workflow, enabling increased customer productivity

WATERS SOLUTIONS

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[ACQUITY™ UPLC™ I-Class PLUS System](#)

[ACQUITY UPLC BEH Amide Column](#)

[TargetLynx Application Manager](#)

[MassLynx™ Software](#)

KEYWORDS

Lipidomics, targeted, high-throughput, mouse mutant, murine adipose

INTRODUCTION

Leucine-rich- α 2-glycoprotein 1 (LRG1) is a highly conserved member of a leucine-rich repeat grouping of proteins involved in protein-protein interaction, signalling, and cell adhesion.¹ LRG1 is a secreted glycoprotein and was recently described as a novel angiogenic factor with a role in proliferative diabetic retinopathy.¹ However, LRG1 is also expressed by adipose tissue, and has been suggested to be a liver and adipose specific secretory protein.²

Although advances in mass spectrometry (MS) have allowed for more in-depth lipidomic analysis, unambiguous identification and quantification has proven difficult as lipids exhibit a high number of isomeric and isobaric species. Here, we adapt the LipidQuan workflow³ (Figure 1) to provide a comprehensive and quantitative overview of the lipid species from murine adipose tissue extracts.

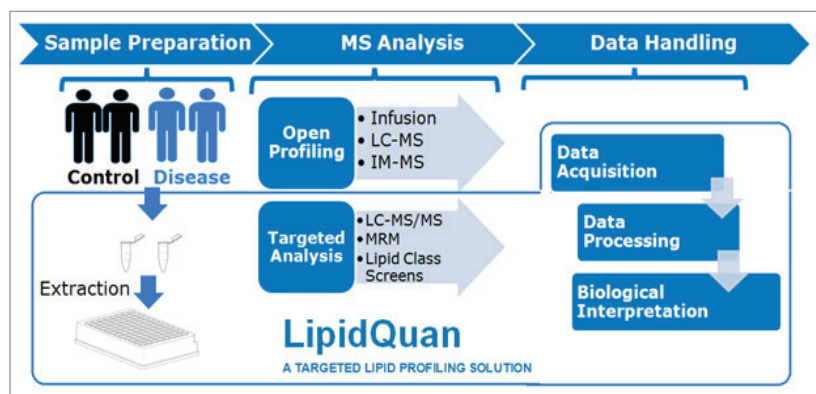


Figure 1. General lipidomic workflow used in most research laboratories, with the LipidQuan workflow highlighted.

EXPERIMENTAL

Samples

Adipose tissue (~30 mg) from wild type and transgenic LRG1 knockout mice (LRG1^{tm1(KOMP)Vlcg}) were extracted using a chloroform:methanol (2:1) Bligh and Dyer method⁴ and subsequently dried down for transportation and analysis.

Samples were reconstituted in 500 µL isopropanol (IPA). Fifty microliters (50 µL) of each sample were pooled in a glass vial (600 µL total volume) and were spiked with stable isotope labelled (SIL) standards (SPLASH® LIPIDOMIX® and deuterated Ceramide LIPIDOMIX®, Avanti Lipids, Alabaster, AL) at seven concentration levels to generate calibration curves for quantification. High, middle, and low QC samples at 80%, 8%, and 1.275% of the highest concentration of the calibration curve were used.

Six biological replicates representing wild type as controls and six mutant mice were analyzed. Samples were injected twice for both positive and negative mode analyses.

Sample preparation

A simple sample preparation procedure was adopted using 40 µL of extract reconstituted in IPA spiked with 5 µL of Odd Chain LIPIDOMIX® (1:100, Std. Mix:IPA) and 5 µL of neat IPA in total recovery Waters™ UPLC vials (p/n: [186005669CV](#)). Samples were vortex mixed prior to LC-MS/MS analysis.

LC conditions

LC system:	ACQUITY UPLC I-Class PLUS or H-Class PLUS System (Fixed Loop (FL) or Flow Through Needle (FTN))
Column(s):	ACQUITY UPLC BEH Amide 2.1 × 100 mm, 1.7 µm
Column temp.:	45 °C
Flow rate:	0.6 mL/min
Mobile phase:	95:5 Acetonitrile/water + 10 mM Ammonium acetate (A) and 50:50 Acetonitrile/water + 10 mM Ammonium acetate (B)
Gradient:	0.1% to 20.0% B for two minutes, then 20% to 80% B for three minutes, followed by three minutes re-equilibration
Run time:	Eight minutes
Injection volume:	2 µL

MS conditions

MS systems:	Xevo TQ-XS, TQ-S, or 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
Nebulizer gas:	7 bar
Ion guide offset 1:	3 V
Ion guide offset 2:	0.3 V

Informatics

The LipidQuan Comprehensive Method Package containing the LC conditions, MS method, and associated TargetLynx processing method (including retention times and MRM transitions) was downloaded from the [Waters Targeted Omics Method Library \(TOML\)](#) and used to generate these methods. The resulting data were processed using either TargetLynx or [Skyline](#) (MacCoss Lab Software, University of Washington). Statistical analysis was performed using [SIMCA P+](#) (Umetrics, Umeå, Sweden) and further data interrogation conducted using [Metaboanalyst](#).⁵

RESULTS AND DISCUSSION

Lipid analysis of murine adipose tissue from wild type and transgenic LRG1 knock-out subjects were conducted using the LipidQuan platform. The samples were prepared for LC-MS/MS by re-constituting total extracts in isopropanol. Samples were randomized and two technical replicates per sample acquired. Targeted LC-MS/MS data were acquired in positive and negative ion electrospray modes using the LipidQuan method package that was downloaded from the TOML website. Deployment of LipidQuan method files using Quanpedia allowed for the fast and easy importing of all MRM transitions and chromatographic conditions, representing a positive and negative mode screen for lipid species. This simple workflow eliminated the manual input of LC-MS methods and eliminated possible transcription errors. The MRMs contain highly specific fatty acyl transitions, in addition to head group fragments for increased identification confidence (Figure 2). Example chromatograms of samples in both positive and negative mode are shown in Figure 3. Using this methodology, more than 400 lipids were identified in the samples.

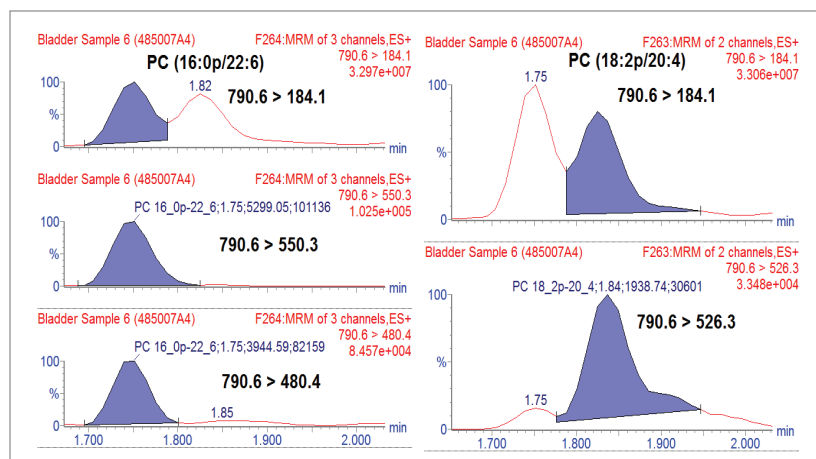


Figure 2. LipidQuan improves isobaric lipids identification by using fatty acyl fragment transitions and retention times for confirmation. For example, PC (16:0p/22:6) and PC (18:2p/20:4) have precursor m/z 790.6 and cannot be distinguished using only the head group transition (m/z 184.1).

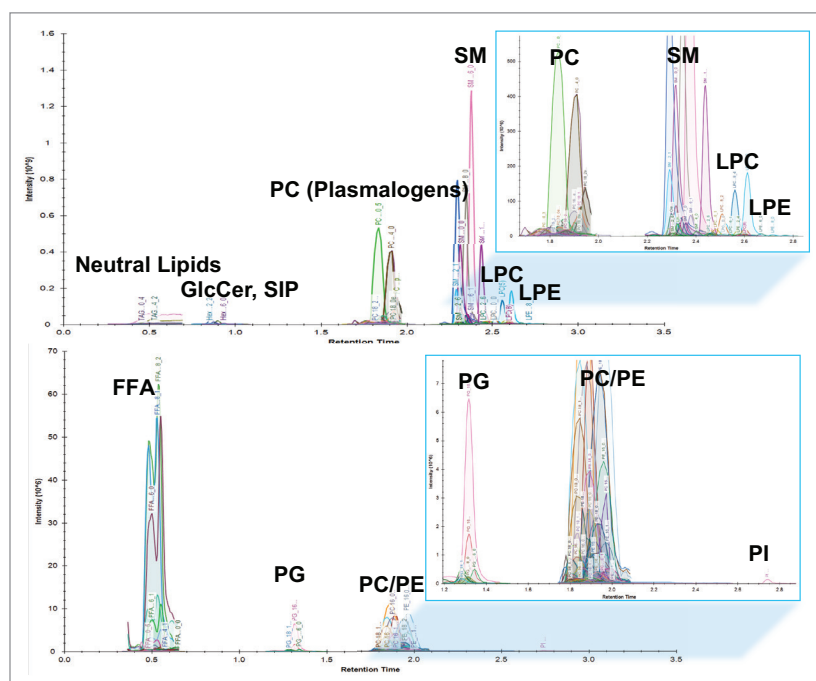


Figure 3. Overlaid chromatograms representing the tissue screen for positive mode (A) and negative mode (B).

Statistical analysis of the data revealed clear separation between the two sample cohorts. A PLSDA model created using SIMCA P+ and MetaboAnalyst resulted in clustering of wild types and mutant mice samples (Figure 4). The Q² values were much greater than 40% (~90%), showing the model created is predictive. Permutation tests were also used to validate these models.

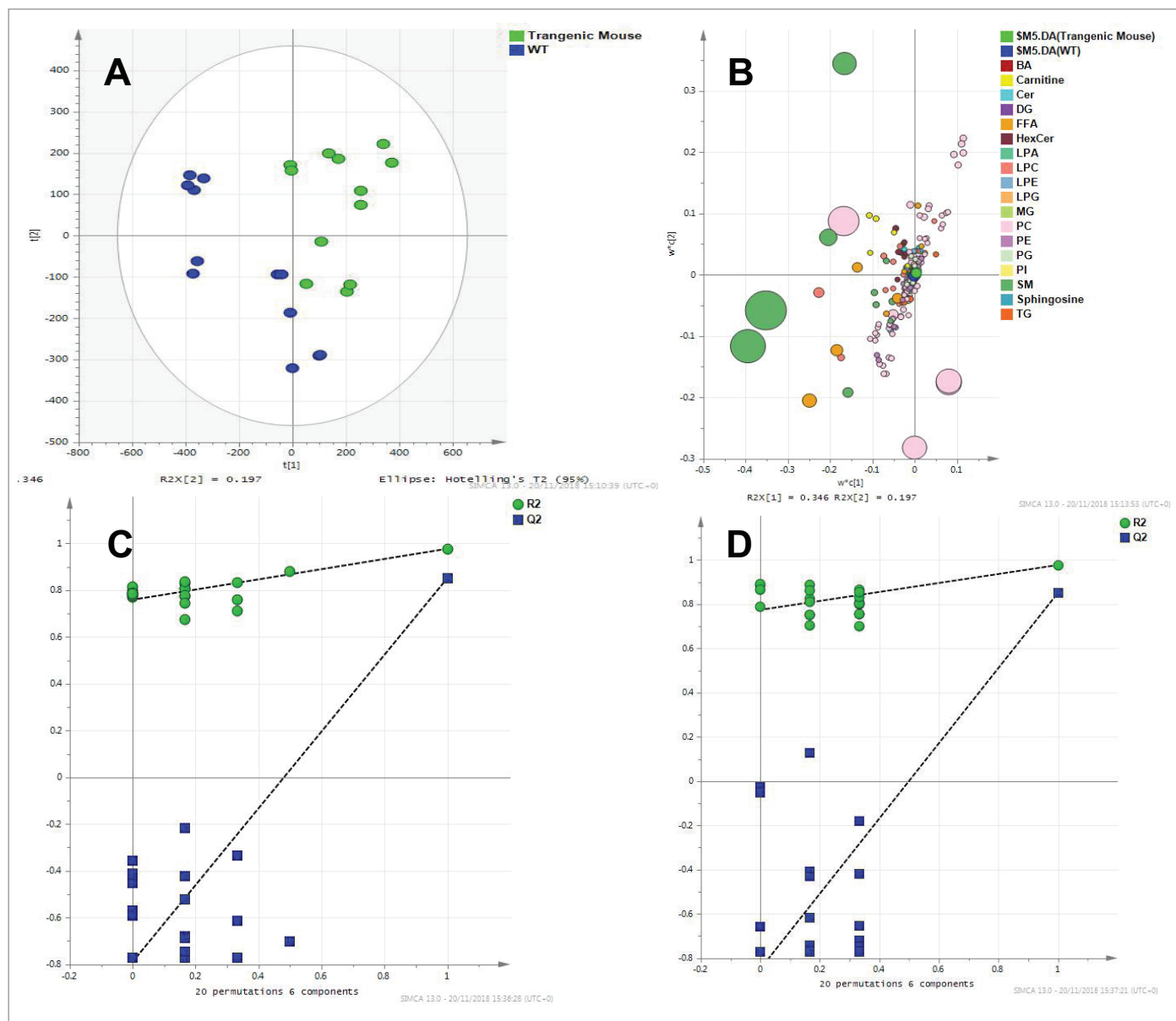


Figure 4. Multivariate statistical packages such as SIMCA P+ can be employed to assess findings. SIMCA P+ Score (A) and loadings plot (B) for an unsupervised PCA, pare to scaled model ($R^2=0.93$, $Q^2=0.911$). (Note: the size of the plot markers corresponds to the magnitude of the specific observation.) Model was validated using permutation tests C and D. The higher R^2 (fit) and Q^2 (prediction) values of the original model and of the lower permuted models R^2 and Q^2 indicates that the original model is valid since it could not have been generated using random data.

A VIP scores plot and heatmap based on VIP scores from the PLS-DA models (Figure 5) was generated using MetaboAnalyst. The top 25 VIP scores revealed LPC, PC, and SM classes to be the main contributors to sample type clustering. The majority of lipids have elevated levels in wild type mouse samples. However, some plasmalogens levels are elevated in transgenic mouse samples, for example PC (18:1e/16:0), PC (16e/18/1), and PC (18:0e/16:1). The ANOVA/t-test, with %1 FDR, highlights lipid species with p-values greater than 0.05 and example chromatograms of these statistically significant peaks are overlaid to show potential for quantification (Figure 7).

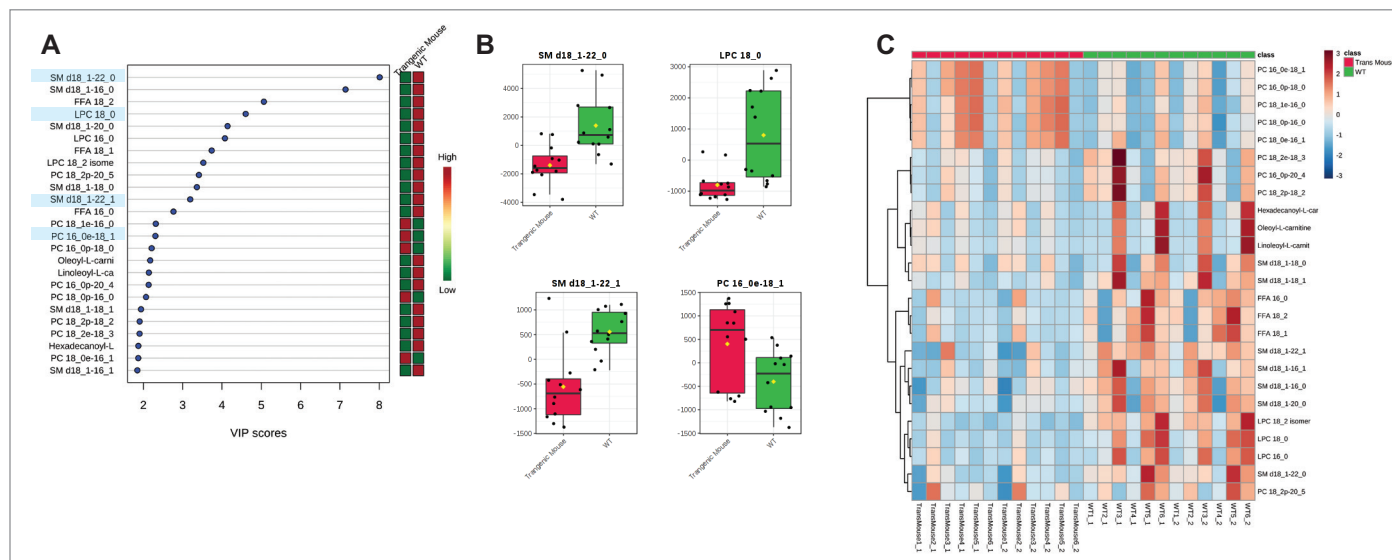


Figure 5. Multivariate statistical packages such as SIMCA P+ or Metaboanalyst can be employed to assess findings. Metaboanalyst VIP scores (A) showing the top 25 most significant variables (lipids); selected box plots of the most significant variables (B); and hierarchical clustering of the top 25 lipids (C) highlights the averaged differential expression across the three groups.

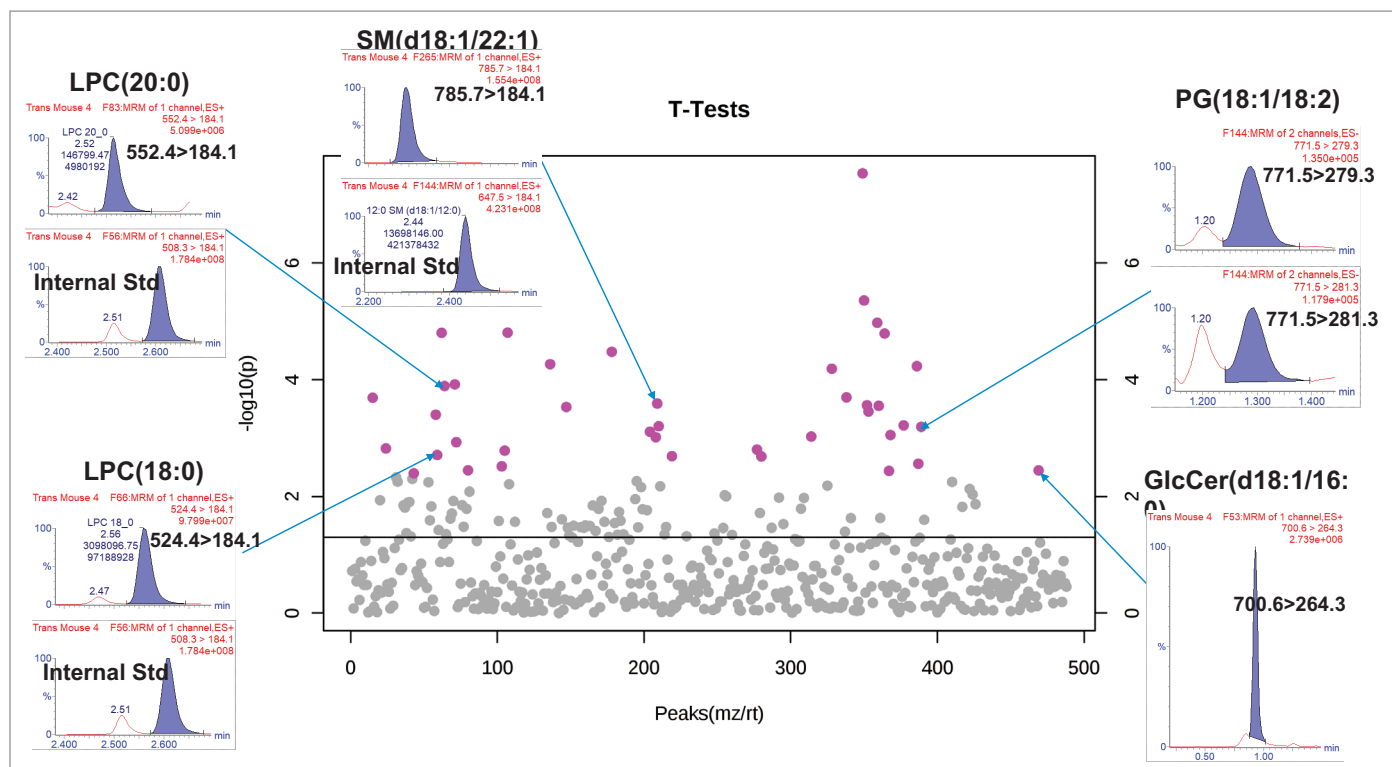


Figure 6. ANOVA/t-test with %1 FDR. Lipid species with p-values greater than 0.05 highlighted in pink. Some example chromatograms of highlighted peaks included to illustrate potential for quantification. (Note: Most phospholipids use the fatty acyl MRM transitions to improve specificity.)

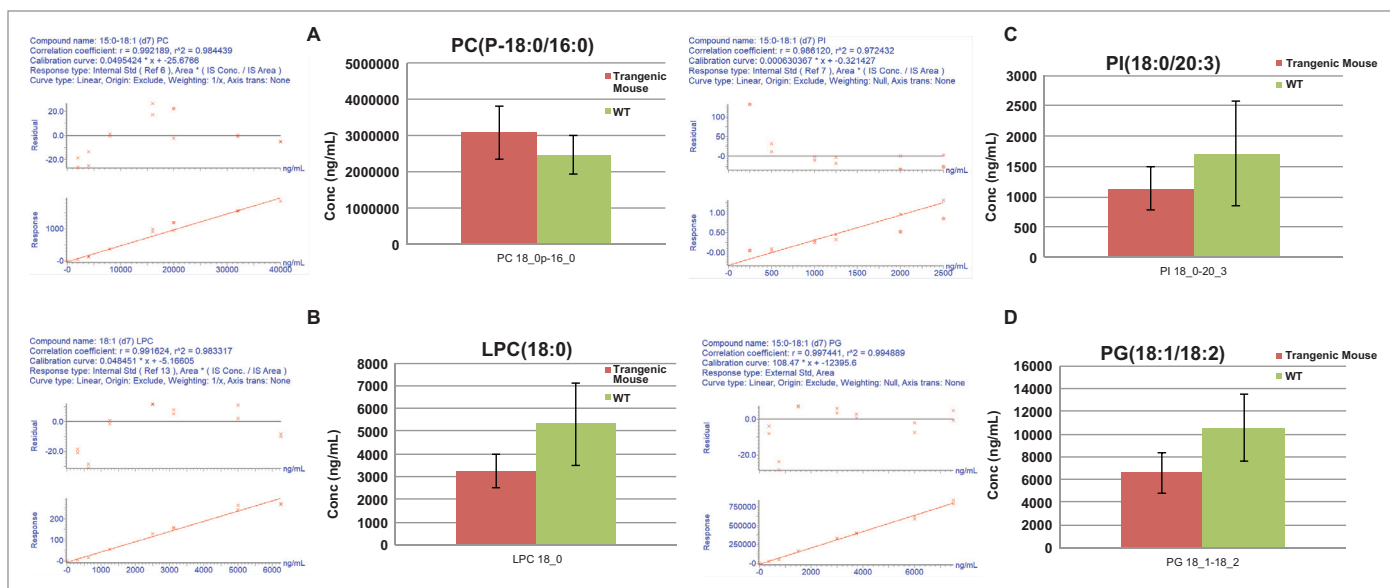


Figure 7. Selected calibration curves from positive mode data PC (A) $R^2 = 0.984$ and LPC (B) $R^2 = 0.983$. In negative mode PI (C) $R^2 = 0.971$, and PG (D) $R^2 = 0.994$. Chromatograms of statistically significant lipid species when wild type samples are compared to transgenic mouse samples also shown. These lipids can be quantified using the calibration curve of the surrogate lipid standards.

Quantification was achieved using calibration curves of spiked extracts with known concentrations of SIL standards prior to extraction. By using surrogate standards prepared and analyzed under identical conditions to those of endogenous lipids, the quantification of endogenous lipids within the same class was achieved. One caveat with regards to this method of quantitation is that the MS detector responses for lipid species from the same class are reported to vary in intensity depending on the FA chain length.⁶ The use of a commercially available, premixed SIL solution, rather than an SIL for each measured lipid, significantly reduces the overall cost of large studies. Deuterated standards were used to assess linear response, with typical R^2 values ranging from 0.97–0.99 for the various lipid classes in both modes of ionization. Figure 7 shows example calibration curves and calculated concentrations for various lipids found to be statistically significant.

The genetic deletion of the putative adipokine, LRG1, appears to remodel membrane associated phospholipids in the brown adipose tissue. LRG1 $-/-$ mice have enriched phosphatidylcholines containing abundant and monounsaturated fatty acyl chains (16:0/18:1) and depleted sphingomyelin species. A decrease in free fatty acid and L-carnitine species reflected alterations to energy metabolism.

CONCLUSIONS

- A rapid quantitative method was developed for the analysis of adipose tissue.
- The methodology allowed for the clustering of samples by disease state when data is analyzed using multivariate statistical packages.
- The method was shown to be linear over four orders of magnitude and had sufficient sensitivity to allow for the analysis of lipids at endogenous levels in human plasma.
- Employing HILIC-based chromatography allowed lipids to elute according to class, thereby reducing the potential for isomeric/isobaric interferences and the number of stable label isotopes required for quantification (i.e., cost reductions).

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