# A novel UHPLC-Q-TOF method for the detection and quantification of plasma lipids

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## Gradient

| Time | %A | %B |
|---|---|---|
| 0 | 85 | 15 |
| 2 | 70 | 30 |
| 2.5 | 52 | 48 |
| 11 | 18 | 82 |
| 11.5 | 1 | 99 |
| 12 | 1 | 99 |
| 12.1 | 85 | 15 |
| 15 | 85 | 15 |

## Introduction

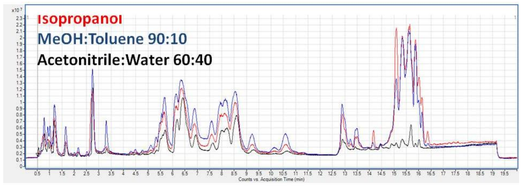
Lipids are dysregulated in the onset and progression of many diseases, and are functionally involved in mechanisms of disease etiologies. This requires the development of high throughput analyses capable of dealing with large numbers of samples which are necessary to reach statistical significance in clinical studies. While a large variety of methods exist, the majority of which employ direct infusion MS or reverse phase LC-MS, there is need for improvement in order to efficiently identify and quantify large arrays of lipids in the context of high throughput studies.

## Lipid Extraction

The extraction protocol was slightly modified from one found in the literature [1]. Different plasma extraction volumes were studied. A 20 µL aliquot plasma was selected in terms of an optimum ratio between the number of extracted features and the number of saturated peaks Also three resuspension solvents were analyzed for a wide lipid class coverage.

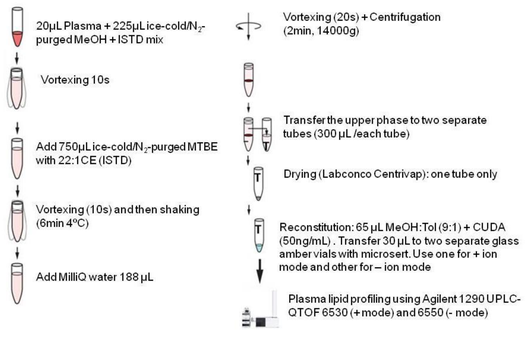
Number of detected molecular features vs number of saturated peaks for different plasma extraction volumes.

| Sample | Molecular features | Saturated peaks |
|---|---|---|
| Blank | 442 | 0 |
| 5 µL plasma | 661 | 5 |
| 10 µL plasma | 941 | 15 |
| 20 µL plasma | 993 | 17 |
| 30 µL plasma | 1166 | 27 |
| 60 µL plasma | 1840 | 48 |




TIC of three plasma samples resuspended in isopropanol (red), methanol:toluene 90:10 (blue) and acetonitrile:water 60:40 (black). Methanol:toluene 90:10 was selected because it provided a good coverage for the most abundant lipid classes in plasma studied (phospholipids, lysophospholipids, triglycerides and cholesterol esters).

### Final Lipid Extraction Protocol




## LC-MS Lipid Method

**Column:** Waters Acquity UPLC CSH C18 1.7 µm 2.1x100mm

**Temperature:** 65ºC **Injection volume:** 3 µL

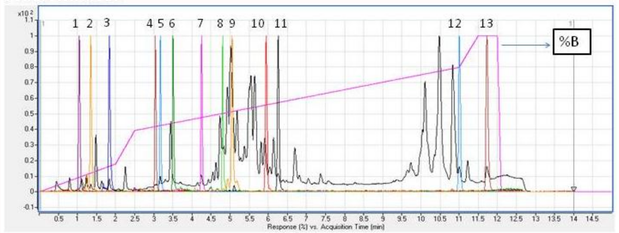
**Flow rate:** 0.6 mL/min

**Solvent A:** Acetonitrile:Water 60:40 + 10mM Ammonium Formiate + 0.1% Formic Acid

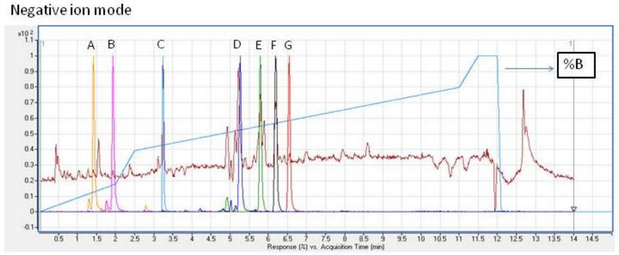
**Solvent B:** Isopropanol:ACN 90:10 + 10mM Ammonium Formiate + 0.1% Formic Acid

**MS:** Agilent Q-TOF 6530 and 6550 operating at full scan range m/z 65-1700

Positive ion mode



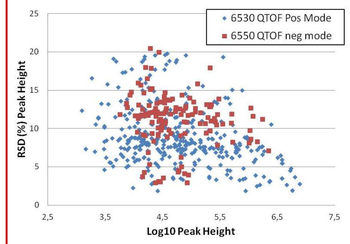

Negative ion mode

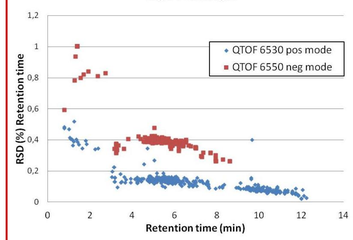



| Mode | ID | ISTD | Formula | RT | m/z |
|---|---|---|---|---|---|
| Positive Ion Mode | 1 | C17 Sphingosine | $C_{17}H_{35}NO_2$ | 1.102 | 286.2740 |
| | 2 | LPE(17:1/0:0) | $C_{22}H_{44}NO_7P$ | 1.438 | 466.2928 |
| | 3 | LPC(17:0/0:0) | $C_{25}H_{52}NO_7P$ | 1.981 | 510.3554 |
| | 4 | MG(17:0/0:0/0:0) | $C_{20}H_{40}O_4$ | 3.137 | 345.2999 |
| | 5 | DG(18:1/2:0/0:0) | $C_{23}H_{42}O_5$ | 3.257 | 416.3370 |
| | 6 | PC(12:0/13:0) | $C_{33}H_{66}NO_8P$ | 3.621 | 636.4599 |
| | 7 | DG(12:0/12:0/0:0) | $C_{27}H_{52}O_5$ | 4.371 | 474.4153 |
| | 8 | Cholesterol-d7 | $C_{27}H_{39}D_7O$ | 4.968 | 376.3953 |
| | 9 | SM(d18:1/17:0) | $C_{40}H_{81}N_2O_6P$ | 5.250 | 717.5905 |
| | 10 | Cer(d18:1/17:0) | $C_{35}H_{69}NO_3$ | 6.137 | 552.5350 |
| | 11 | PE(17:0/17:0) | $C_{39}H_{78}NO_8P$ | 6.476 | 720.5538 |
| | 12 | TG(17:0/17:1/17:0)-d5 | $C_{54}H_{97}D_5O_6$ | 11.155 | 869.8323 |
| | 13 | 22:1 Cholesteryl ester | $C_{49}H_{86}O_2$ | 11.834 | 724.6966 |
| Negative Ion Mode | A | LPE(17:1/0:0) | $C_{22}H_{44}NO_7P$ | 1.438 | 464.2783 |
| | B | LPC(17:0/0:0) | $C_{25}H_{52}NO_7P$ | 1.981 | 554.3463 |
| | C | Palmitic Acid-d3 | $C_{16}H_{29}D_3O_2$ | 3.197 | 258.2515 |
| | D | SM(d18:1/17:0) | $C_{40}H_{81}N_2O_6P$ | 5.250 | 761.5814 |
| | E | PG(17:0/17:0) | $C_{40}H_{79}O_{10}P$ | 5.615 | 749.5338 |
| | F | Cer(d18:1/17:0) | $C_{35}H_{69}NO_3$ | 6.137 | 596.5259 |
| | G | PE(17:0/17:0) | $C_{39}H_{78}NO_8P$ | 6.476 | 718.5392 |

Odd chain and deuterated lipid ISTD used in the method were spiked in the methanol used for extraction. These ISTD lipids cover the main lipid classes and are used for both QC of the injections and for quantification of the identified lipids.

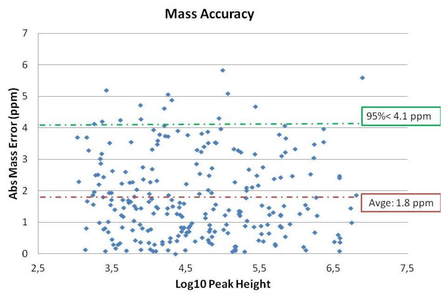
## Method Evaluation



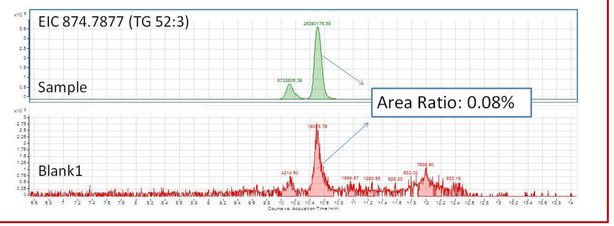




The method was evaluated in terms of reproducibility (%RSD of both the retention time and the peak height) and mass accuracy, displaying an RSD of 0.1% for the retention time and 1.7% for peak area based on replicate analysis of plasma samples (n=10).
For long term reproducibility, 32 quality control citrate plasma samples were analyzed within a batch of ~300 samples (1 QC injected every 10 samples) in the course of 5 days of study. The 243 identified compounds (89 in negative mode and 154 in positive mode) were used for this evaluation.
The results show a %RSD lower than 20% in terms of peak height for all the lipids. The reproducibility in the retention time was more column dependent, being lower than 0.6% for the column used for positive mode, and lower than 1% for the column used in negative mode.

Mass accuracy evaluation showed an average mass error of only 1.8 ppm for the 243 identified lipids. This mass accuracy allows the annotation of lipids based on their retention time and exact mass.



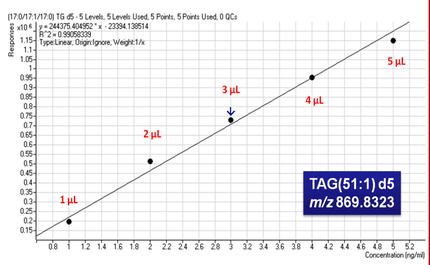

The carryover was analyzed by running a blank sample after 10 injected samples. One of the most abundant triglycerides in plasma (TG52:3) was used for the evaluation. The ratio of the peak area was as low as 0.08%.

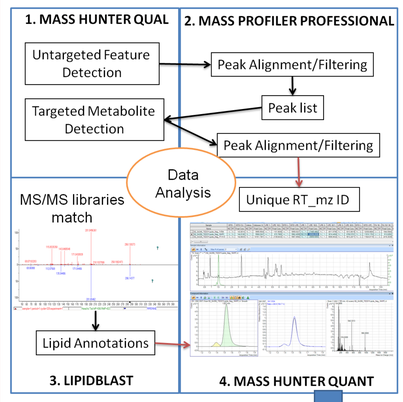



**References**
[1] Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lip Res 2008, 49: 1137-1146
[2] Kind T, Meissen JK, Yang D, Nocito F, Vaniya A, Cheng YS, Vandergheynst JS, Fiehn O. Qualitative analysis of algal secretions with multiple mass spectrometric platforms. J Chromatogr A. 2012, 1244: 139-147
[3] Meissen JK, Yuen BT, Kind T, Riggs JW, Barupal DK, Knoepfler PS, Fiehn O. Induced pluripotent stem cells show metabolomic differences to embryonic stem cells in polyunsaturated phosphatidylcholines and primary metabolism. PLoS One. 2012, 7 (10): e46770.

## Data Analysis

The linearity of the method was evaluated by injecting 1-5 µL of sample and calculating the peak height of the ISTD. The method was linear in the interval 1-4 µL for all ISTD (TG 51:1 is shown as an example, $R^2$=0.99). An injection volume of 3 µL was selected to allow for possible reinjections of more diluted or concentrated samples without modifying the extraction protocol.




The data analysis integrates both the Agilent Mass Hunter tools (Qual and Quant) and an in-house written software, LipidBlast [2,3]. A recursive analysis was done to a batch of 300 samples to obtain a list of unique retention time exact mass IDs. In paralel, MSMS files from a pool of samples were subjected to LipidBlast, which gives lipid annotations by matching MSMS spectra to different libraries. These information was gathered into a MH Quant method for quantification of the identified lipids using the ISTD.

| | Data File | | | | | |
|---|---|---|---|---|---|---|
| | | | | | B3_SA602_TEDDYLipids_Pos_112WC.d | B3_SA602_TEDDYLipids_Pos_183W9.d |
| Name | MZ | RT | ID | RT_MZ | Calc. Conc. | Calc. Conc. |
| 1_CE (22:1) [M+NH4]+ IS | 724,6966 | 11,9 | | 11.71_724.70 | 817770,9 | 818007,3 |
| 1_Ceramide C17 [M+H]+ | 552,5350 | 6,11 | | 5.95_552.54 | 5962,4 | 5962,4 |
| 1_Cholesterol d7 [M-H2O | 376,3955 | 4,93 | | 4.81_376.40 | 6760,5 | 6760,5 |
| 1_CUDA ISTD [M+H]+ | 341,2799 | 0,78 | | 0.78_341.28 | 150,0 | 150,0 |
| 1_DG (12:0/12:0/0:0) [M+I | 479,3707 | 4,36 | | 4.26_479.37 | 23728,2 | 23849,6 |
| 1_DG (18:1/2:0/0:0) [M+N | 421,2925 | 3,23 | | 3.17_421.29 | 143097,7 | 143097,7 |
| 1_LPC 17:0 [M+H]+ ISTD | 510,3551 | 1,84 | | 1.82_510.36 | 11924,8 | 11936,8 |
| 1_LPE 17:1 [M+H]+ ISTD | 466,2925 | 1,35 | | 1.34_466.29 | 5962,4 | 5962,4 |
| 1_MG 17:0/0:0/0:0 ISTD | 345,2999 | 3,09 | | 3.03_345.30 | 47699,2 | 47699,2 |
| 1_PC 12:0/13:0 ISTD | 636,4596 | 3,56 | | 3.49_636.46 | 10918,9 | 10918,9 |
| 1_PE 17:0/17:0 ISTD | 720,5561 | 6,41 | | 6.23_720.56 | 17874,3 | 17872,6 |
| 1_SM 17:0 ISTD | 717,5914 | 5,19 | | 5.06_717.59 | 4769,9 | 4765,6 |
| 1_Sphingosine d17:1 ISTI | 286,2752 | 1,05 | | 1.04_286.28 | 2650,0 | 2650,0 |
| 1_TG d5 (17:0/17:1/17:0) | 869,8329 | 11,2 | | 10.98_869.83 | 2981,2 | 2886,7 |
| CE (18:1) [M+NH4]+ | 668,6340 | 11 | | 10.85_668.63 | 40579,7 | 38675,5 |
| CE (18:2) [M+Na]+ no MS | 671,5738 | 10,6 | | 10.37_671.57 | 261812,6 | 208105,3 |
| CE (18:3) [M+NH4]+ | 664,6027 | 10,2 | | 9.96_664.60 | 16117,5 | 20405,2 |
| CE (20:4) [M+NH4]+ | 690,6184 | 10,3 | | 10.13_690.62 | 79028,8 | 205594,9 |
| CE 22:6 M+Na | 719,5738 | 10,1 | | 9.88_719.57 | 10521,9 | 39623,4 |
| Ceramide (d18:1/23:0) [M | 658,6109 | 8,16 | | 7.94_658.61 | 130,3 | 235,1 |
| Ceramide (d40:1) [M+Na] | 644,5937 | 7,83 | | 7.62_644.59 | 183,8 | 299,3 |
| Ceramide (d42:1) [M+Na] | 672,6258 | 8,47 | | 8.26_672.63 | 634,3 | 949,1 |

## Conclusion

The high-throughput UHPLC-QTOF method developed allows the identification and quantification of more than 240 unique lipid species in only 15 minutes analysis time injection to injection. This method shows a good reproducibility, mass accuracy and no significant carryover, allowing the continuous analysis of more than 300 samples per week.

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