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Comprehensive Lipidomics Workflow Using Automated Flow Injection Analysis for Data Independent Acquisition of Lipids Generated After Activation of Human Platelets

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Introduction

The advantages of shotgun lipidomics are the speed of analysis and the reliable quantification1. It provides an efficient way to test the integrity of samples, the efficiency of extraction and the more detailed composition of lipidomes from extracts of biological samples with no chromatographic separation. Coupled with data independent acquisition, it is a powerful tool for sequentially fragmenting all the masses in the spectrum with no constraints of cycle time.

We present here a comprehensive lipidomics workflow using an automated large volume loop injection (FIA) coupled to a Q-TOF LC/MS mass spectrometer for Data Independent Acquisition (DIA) of lipids, optimised using human plasma extracts. The lipid species in the sample were identified and quantified using SimLipid software.

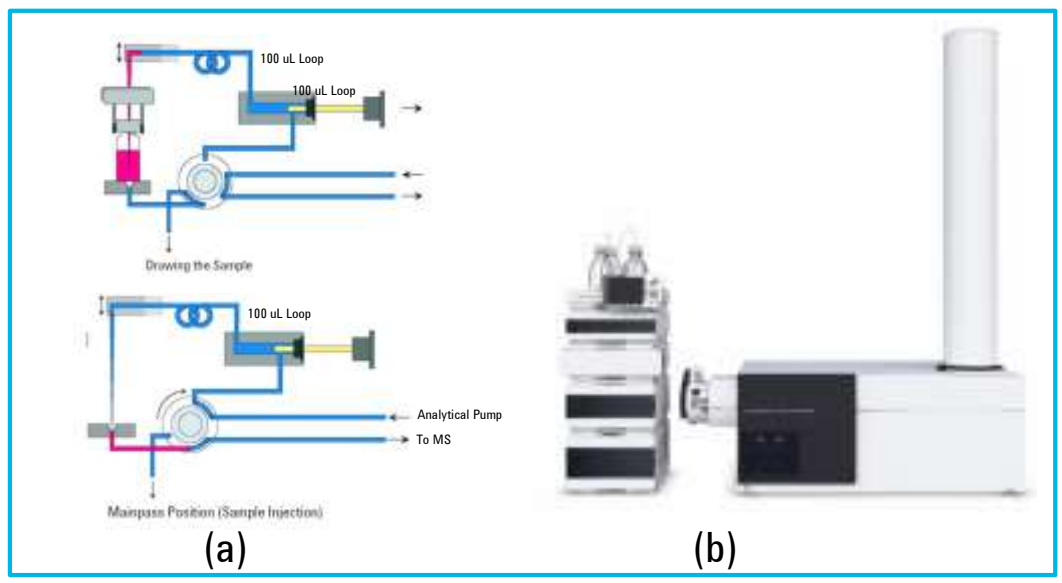

Figure 1. Autosampler with a 100 uL loop for injecting a slug of sample into the peek tubing to the MS. The slug is pushed at a rate of 12 µL/min by the HPLC pump. (b) Schematic of the LC/QTOF system.

Platelets are blood cells with essential functions in hemostasis, wound healing and immune system. Platelet lipid metabolism is highly regulated and central for platelet function. Platelets activation leads to formation and release of lipids [2,3]. In this work we used FIA-DIA to identify and quantify lipids in non-activated and activated platelets.

Experimental

Human Platelets were isolated from whole blood in presence of anticoagulants using a differential centrifugation approach (unpublished protocol). Platelets (150 millions) were activated with 20 µM thrombin receptor-activating peptide-6 (TRAP-6) at 25°C for 10 and 30 min. Platelet isolate purity and activation were confirmed by analyzing CD61 and CD62P surface expression.

Experimental

Lipid extraction. Platelet suspensions were first extracted with butanol:methanol (1:1, v:v)[4]. Dried extracts were then reconstituted in 180 µL of 150 mM ammonium bicarbonate and re-extracted in 810 µL methyl tert-butyl ether:methanol (7:2, v:v) containing 10 µL of the Avanti SPLASH mix and 10 µL of the AvantiCer/Sph mix[5] (shaken for 15 min at 20°C) and then centrifuged (3000 g, 5 min). The organic phase was dried, re-suspended in 240 µL of mobile phase and transferred to vials for injection.

FIA system. Samples were injected with an automated FIA system utilizing a 1260 Agilent HPLC. The Autosampler was fitted with a 100 µL loop. A Capillary pump was utilized for pushing the slug of sample to the mass spectrometer. The Capillary pump was operated at 12 µL/min flow rate during sample analysis and at 20 µL/min for a 2 minutes final wash with the same mobile phase. The total injection time was 15 min. Connections to/from the autosampler valve and to the source micronebulizer were made using silica/PEEK capillaries having internal diameters of 50 µm and including an in-line filter to avoid clogging of capillaries. The mobile phase was IPA:ACN 90:10 + 10mM ammonium formate.

MS system. The HPLC system was interfaced with a 6550 Agilent Q-TOF with optimized ESI parameters. Samples were analyzed in positive and negative mode during separate acquisitions. Signals were acquired in MS mode until 1 min after injection of the sample. After 1 min the signals were acquired in targeted MS/MS mode. Data Independent Analysis was triggered by a targeted precursor list from 400.3 to 947.7 with a moving scale of 1 m/z intervals and a narrow isolation width of 1.3 amu. MS/MS spectra were acquired at a rate of 2 spectra/sec with a collision energy of 35 V in positive and 45 V in negative mode. The total cycle time to go through approximately 500 precursors was around 6 minutes.

Data analysis. The raw data in mzData format files exported from the MassHunter software were imported into SimLipid software. A custom database containing ten lipid structures of Cer/Sph Avanti mix was created in the SimLipid server database. Structures of SPLASH Avanti mix standards are already present in the main database. MS/MS database search was performed using 0.65 Da tolerance for the parent ions of the MS/MS bins, and 10 ppm tolerance for the product ions.

FIA and QTOF-based lipidomic DIA method optimisation

To evaluate the performance of the FIA configuration, a human plasma lipid extract was injected multiple times to test for signal intensity, stability, carryover and reproducibility of the measurements in both MS and MSMS mode.

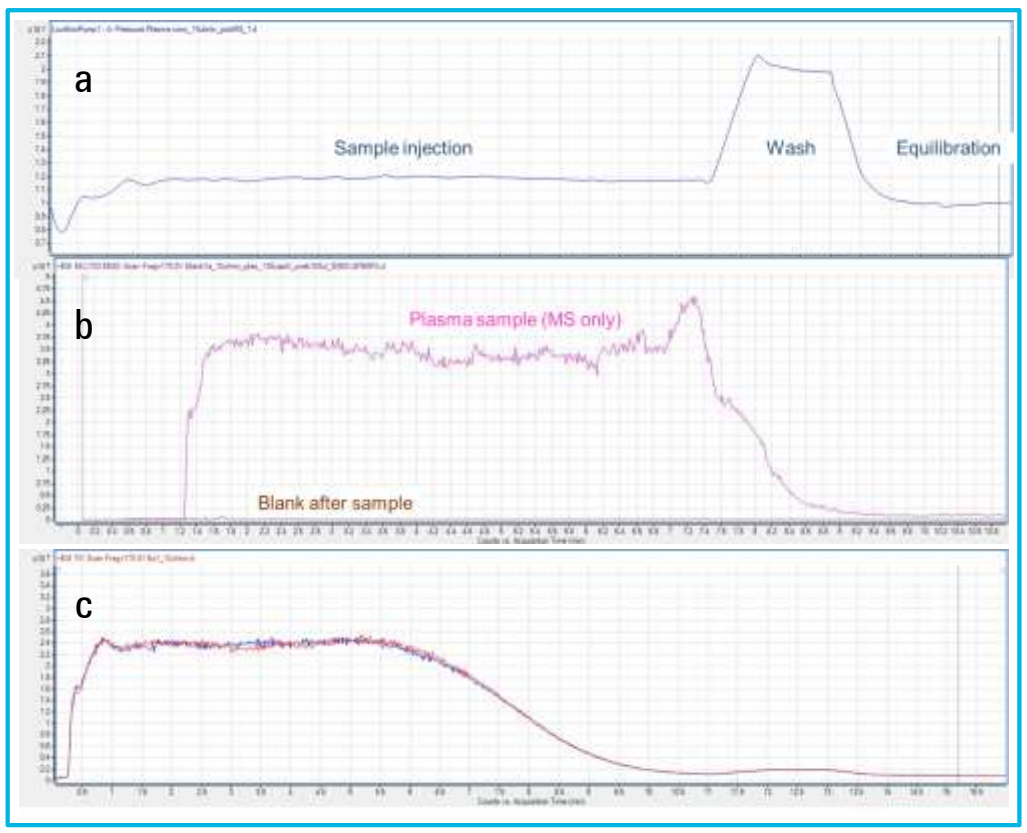

Figure 2. (a) Pressure profile of sample injection (12 µL/min), wash (20 µL /min) and re-equilibration (12 µL /min) with mobile phase IPA/ACN 90/10 + 10 mM Ammonium formate. (b) Measure of carryover using a blank after a human plasma extract injection with the method reported. (c) Reproducibility of consecutive injections with the method reported.

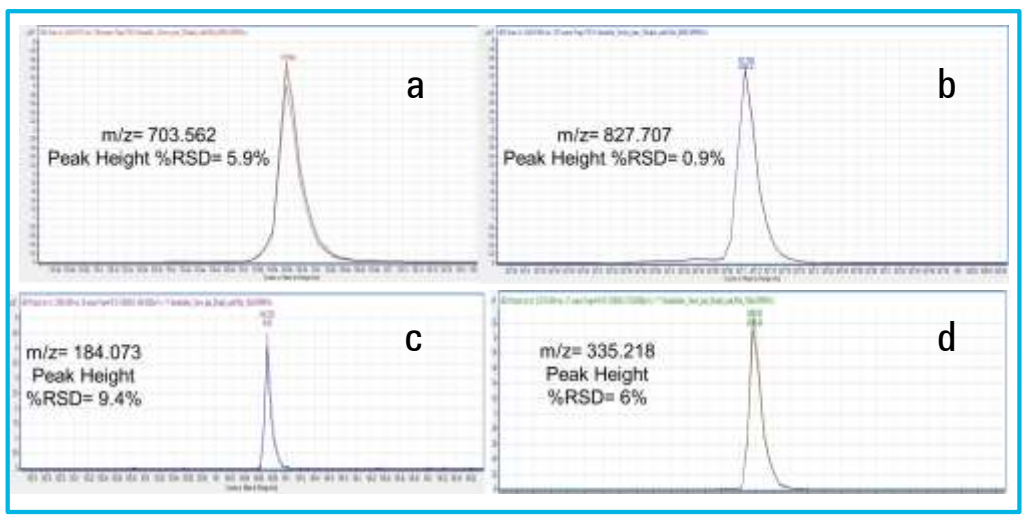

Figure 3. (a-b) Reproducibility of MS intensity signals (n=4). (c-d) Reproducibility of MSMS intensity signals for low and high intensity ion signals (n=4).

Efficient lipid extraction for FIA-DIA lipidomic analysis

The importance of the lipid extraction method for FIA-DIA lipidomic analysis depends on the absence of any cleaning/enrichment device (column) in the FIA setup. In the absence of a liquid chromatography-based separation, this method does not require long time for development but the choice of the lipid extraction method can significantly influence the final results.

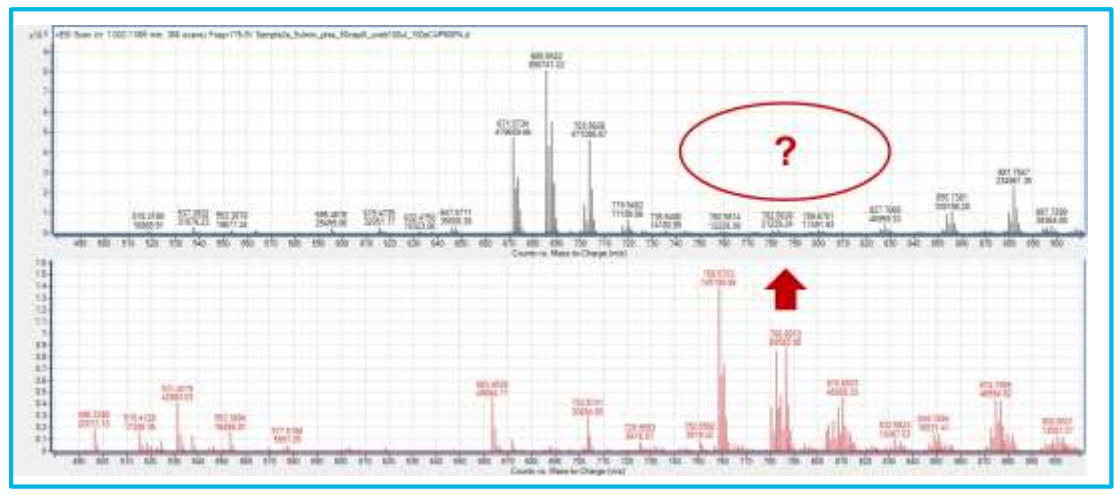

Figure 4. Lipid profile (MS only , positive mode) of human plasma after a single phase Butanol/Methanol extraction (black) and after a two phases MTBE extraction (red). After the single phase extraction the main signals are from Na adducts of some of the most abundant lipids, while lipid peaks usually measured as $M+H^+$ are absent. $M+H^+$ signals instead are abundant after a two phase MTBE extraction.

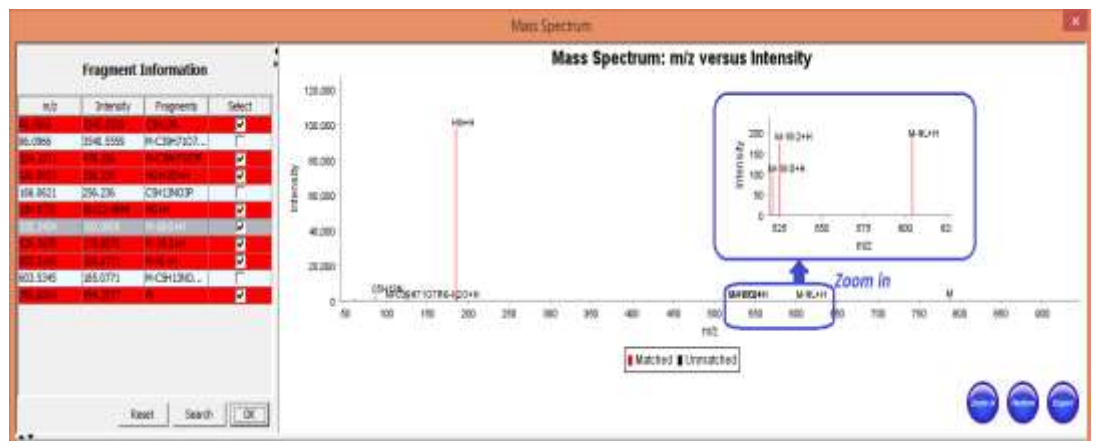

Figure 5. Typical SimLipid software GUI showing annotated MS/MS spectrum (positive mode) at m/z bin 786.6 with characteristic ions of the identified lipid species PC(18:0_18:2) from the human plasma after two-phase MTBE extraction .

Processing of DIA data

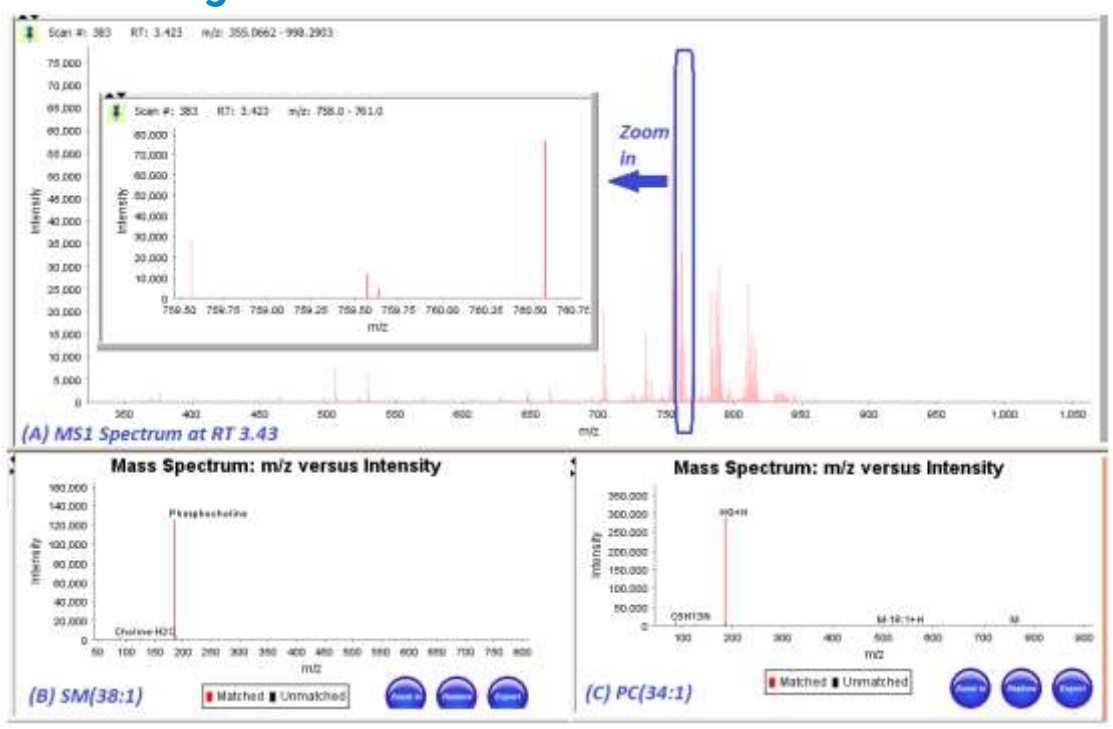

Figure 6. SimLipid software GUIs showing (A) MS1 spectrum (zoom in region shows peaks with overlapping isotopic clusters; MS/MS spectrum (positive mode) at m/z bin (B) 759.6, and (C) 760.6 with characteristic ions of SM(38:1) and PC(16:0_18:1).

Platelet lipids identified by FIA-DIA

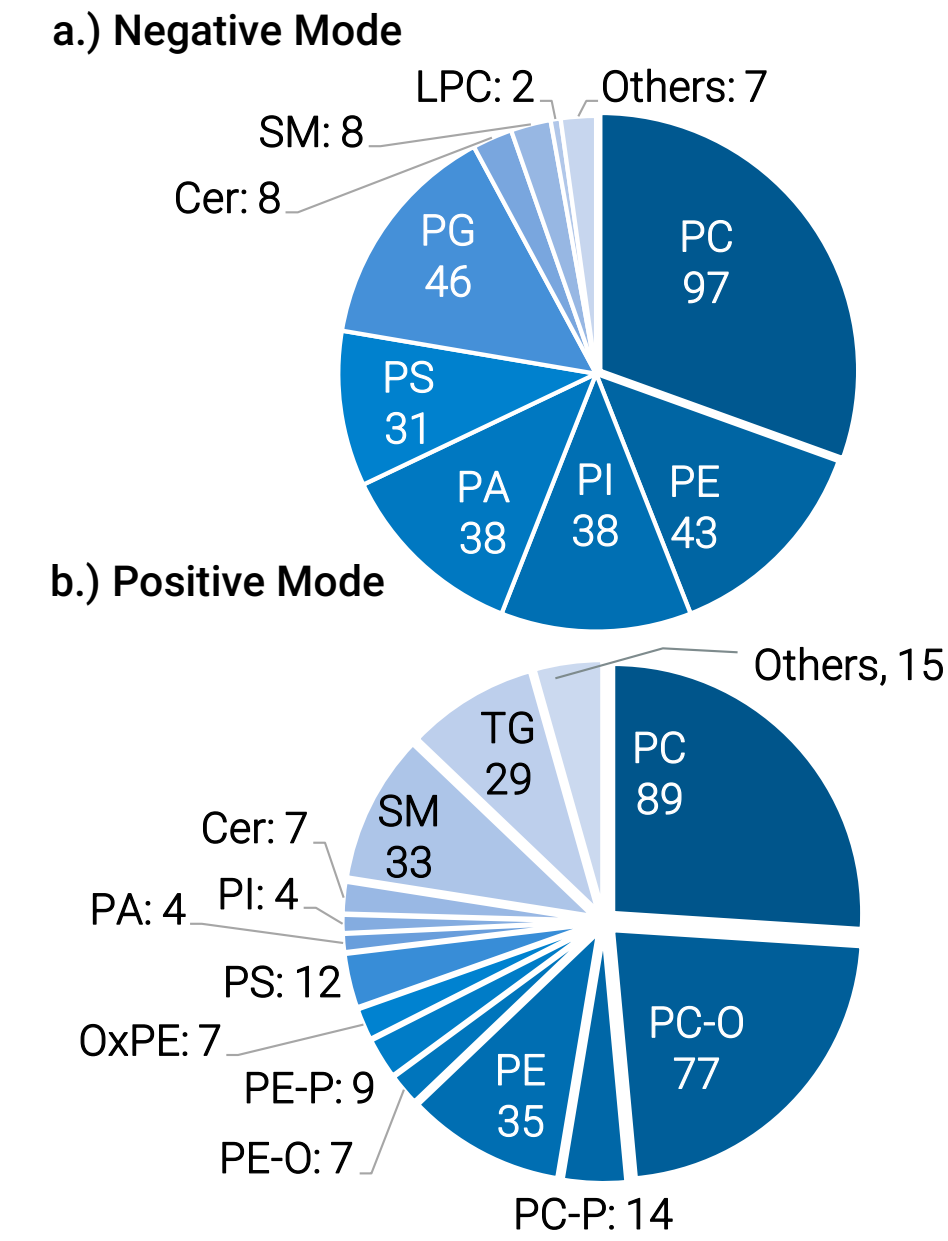

Figure 7. Number of Lipids identified and quantified in both non-activated and activated platelets. A total of 318 were quantified in negative mode (a) and 342 in positive mode (b)

Platelet Phospholipids are Enriched in PUFA

Lipid	Resting	TRAP 10 min	30 min
PS 38:4	24.8	32.1	18.2
PS 36:1	19.5	22.6	17.6
PS 39:4	19.4	24.4	14.5
PE 38:4	20.2	12.0	12.4
PS 37:1	13.7	16.4	12.9
PE P-38:4	16.6	10.0	12.2
PE 39:0	16.8	10.2	9.9
PC O-36:5	19.9	4.2	12.6
PC 38:3	20.0	4.4	9.3
PS 33:4	11.1	13.5	8.2
PE 37:5	13.8	8.0	9.9
PI 38:4	10.4	11.1	9.1
PC 35:5	16.6	3.3	10.2
PC 34:5	16.3	3.3	10.0
PC 36:4	16.2	3.0	7.4
PC 36:0	14.6	3.2	8.2
PC 34:1	15.0	4.1	6.8
PE P-36:4	11.9	7.0	7.1
PC 35:4	15.7	2.9	7.2

Figure 8: Abundances of phopholipids with different numbers of double-bonds in resting platelets.

Table 1: The 20 highest abundant phospholipids detected in platelet samples. Species potentially containing arachidonic acid are indicated in bold.

Conclusions

- A high-throughput workflow for large volume FIA and data independent acquisition (DIA) was used for the first time on an Agilent 6550 QTOF for lipidomics profiling
- This proof-of-concept study shows the feasibility of this FIA-DIA approach to profile the complex lipidome of relevant cells in a robust, high-throughput setup
- We identified 318 and 342 lipids in negative and positive mode, respectively, in both resting and activated platelets. Arachidonic acid (20:4) and other PUFA containing phospholipids constitute a large portion of the platelet lipidome as recently reported[3]
- No apparent differences between resting and activated platelets were found in the identified lipids, in agreement with previous literature[2]. However, the sample size of this study is a limit for our conclusions
- Further work is required to increase coverage, to limit false-positive identifications, improve quantification, and to reduce required sample volumes and analysis time

References

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