A Semi-Automated Lipid Extraction Protocol Using the Agilent Bravo Automated Liquid Handling Platform

Application Note

Authors

Magendran Muniandy,
Pradeep Narayanaswamy,
Federico Torta, and Markus R. Wenk
SLING, National University of Singapore, Singapore

Matthew Foong
Agilent Technologies
Singapore

Abstract

Automated protocols to facilitate sample preparation in lipidomics have been previously developed. The aim of these procedures is to minimize human errors resulting from handling large quantities of samples, and to decrease the amount of time spent in sample preparation. This application note describes the semi-automated extraction of phospholipids and sphingolipids from human plasma using the Agilent Bravo Automated Liquid Handling Platform. This procedure includes the transfer of the starting material (plasma is usually delivered in 1.5-mL tubes) to 96-well plates, which can be either stored or used for the lipid extraction. In the latter case, a butanol/methanol (BuMe) mixture is added to the sample. Although the samples are still transferred manually to the sonicator and the centrifuge during the lipid extraction procedure, the reported protocol allows a high number of samples to be processed uniformly, decreasing the sample preparation time and yielding high reproducibility according to the results obtained after analysis of the extracted lipids with an Agilent UHPLC/MS/MS system.

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Introduction

Lipidomics is the branch of metabolomics that seeks to identify all the lipid species present in living organisms and understand their synthesis, regulation, and effects.

Mass spectrometry-based lipidomics is increasingly popular due to the constant development in liquid chromatography-mass spectrometry systems. A large number of methods have been developed, and many biologically important lipids can now be routinely analyzed and quantified[1]. However, the sample preparation techniques used for lipid extraction from biological samples are often long and tedious, and still rely on those techniques established in the late 1950s[2].

Usually, lipidomics labs have to prepare large numbers of samples (for example, in large epidemiological studies), making the use of standard manual preparation impractical. This application note describes use of the Agilent Bravo Automated Liquid Handling Platform to generate a semi-automated, high-throughput method for extracting lipids from human plasma. The results show that this procedure can give precision comparable to, or better than, that achieved using traditional manual methods, while reducing experiment time and costs. Moreover, lipid extraction protocols involve the use of organic solvents that are highly volatile and toxic, and the use of a robotic station can improve safety in the lab.

Instruments and materials

- Agilent Bravo Automated Liquid Handling Platform (G5523A)
- 96-channels LT Disposable Tip Head (G5498B#042)
- CPAC Ultraflat heating and cooling plate (Bravo deck)
- VarioMag Teleshake Plate Shaker (Bravo deck)
- 96-well polypropylene conical bottom (V-bottom) plate, (250 µL well capacity)
- 24-well 1.5 mL Eppendorf tube plate (G5498B#572)
- Agilent 96LT 250 µL sterile, filtered pipette tips (19477-022)
- Agilent VWorks Automation Control software
- Butanol:methanol (1:1) with ammonium formate 10 mM (extraction solvent)
- Empty tip box (tip waste)
- Corning Axygen, Multi-Well 12 Channel Reservoir (plate volume 21 mL)
- Branson Ultrasonic Bath and Sorvall RT legend centrifuge
- Thermo Scientific PCR tube caps, skirted
- Seralab Human Plasma EDTA K2, not filtered

Lipid standards

Purchased from Avanti Polar Lipids:

- 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)
- 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE)
- N-heptadecanoyl-D-*erythro*-sphingosine (Cer)
- N-lauroyl-D-*erythro*-sphingosylphosphorylcholine (SM)
- 1-arachidoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC)

LC/MS/MS analysis

- Data were acquired in both positive and negative dynamic MRM modes using an Agilent 1290 Infinity Series LC interfaced to an Agilent 6460 Triple Quadrupole Mass Spectrometer.
- Agilent ZORBAX Eclipse Plus C18 RRHD 2.1 × 50 mm, 1.8 µm
- Mobile phase A: 60/40 water/acetonitrile in 10 mM ammonium formate
- Mobile phase B: 90/10 isopropanol/acetonitrile in 10 mM ammonium formate

LC/MS/MS method

LC conditions	
Column temperature	40 °C
Injection volume	2 µL
Gradient	0 minutes (A:B = 80:20), 2 minutes (A:B = 40:60), 7 minutes (A:B = 0:100), 9 minutes (A:B = 0:100), 9.01 minutes (A:B = 80:20), 10.50 minutes (A:B = 80:20)
MS conditions	
Ionization mode	ESI
Ionization polarity	positive and negative
Drying gas flow	5 L/min
Drying gas temperature	300 °C
Nebulizer pressure	45 psi
Sheath gas temperature	250 °C
Sheath gas flow	1 L/min
Nozzle voltage	500 V
Capillary voltage	1,000 V

Protocol workflow

Aliquoting samples

As standard procedure in most labs, original samples are initially split into smaller aliquots for long-term storage or analyte extraction. This protocol starts with the transfer of 10-µL aliquots of the original human plasma samples from 1.5-mL tubes into a 96-well plate for sample storage or lipid extraction.

1. The positions of the plates for the sample transfer step are shown in Figure 2A. The 24-well holder for 1.5-mL Eppendorf tubes and the 96-well destination plate are kept on the CPAC Ultra-flat cooling stations (Station 4) to maintain the sample temperature at 4 °C.

2. A method generated with VW software can aliquot 24 samples from Station 4 to the 96-well plates in Station 6. The dispensing destination, head mode, and tips filled at Station 1 have to be changed in the method according to Table 1.

3. Once all the wells needed for the sample extraction procedure have been filled with sample, the lipid extraction protocol can be started separately. We chose to extract the lipids in a butanol/methanol (BuMe) mixture to be able to work with a single organic phase, avoiding the need for phase separation, as reported in previous manual methods. Since the presence of two phases would make sample recovery with the Bravo platform problematic, we optimized this method for single-phase extractions.

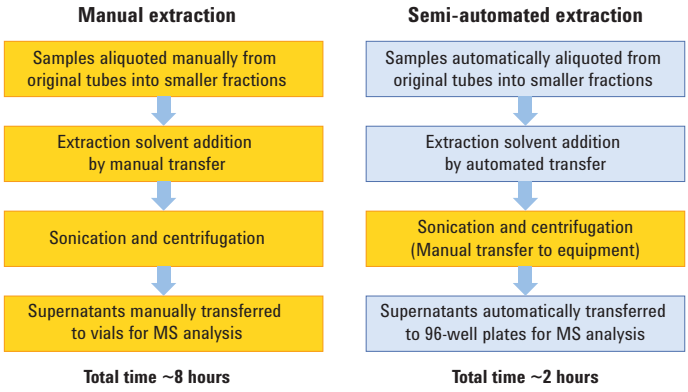

Figure 1. Workflow for manual and semi-automated procedures.

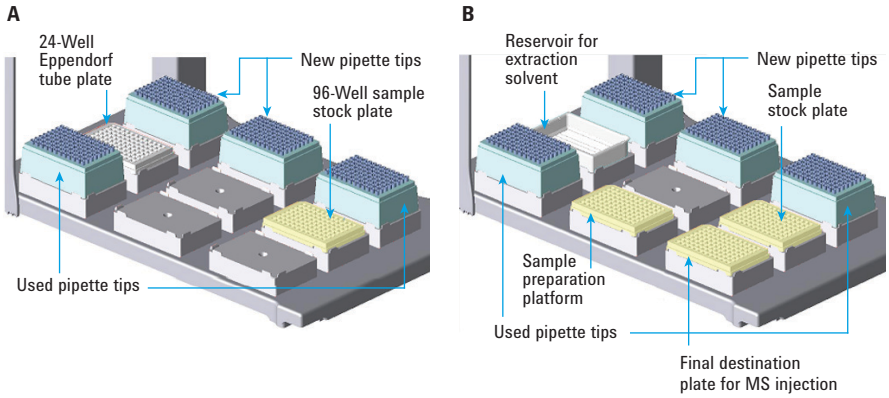

Figure 2. A) Sample transfer configuration. B) Lipid extraction configuration.

Table 1. Head mode, dispensing destination, and pipette tips to be filled at Station 1. Parameters used for initial sample transfer.

Head mode		Tips to be filled at Station 1		
Subset mode	Subset orientation	Column	Row	Dispense location
Full column	Front left	2, 4, 6, 8, 10	A, C, E, G	Dispense: 1 Selection column: 1
Full row	Back left	2, 4, 6, 8, 10	A, C, E, G	Dispense: 1 Selection row: 1
Full column	Front left	2, 4, 6, 8, 10	A, C, E, G	Dispense: 1 Selection column: 2
Single barrel	Back left	2, 4, 6, 8, 10	A, C, E, G	Dispense: 1 Selection: B2

Lipid extraction

1. The plate orientation for the lipid extraction is shown in Figure 2B. The 96-well plate containing the samples and the multiwell 12-channel reservoir containing BuMe + internal standard (IS) mix for subsequent lipid quantification, are kept on the CPAC Ultra-flat cooling plates (Station 4 and Station 6) to maintain a constant temperature of 4 °C.

2. Samples are extracted at Station 8 on the VarioMag Teleshake Plate Shaker as the protocol requires uniformly mixing the plasma and BuMe before the sonication step.

3. The tips are taken from Station 1 and primed with the extraction solvent in Station 6. Then, 100 µL of the extraction solvent with a post-aspiration air-volume of 10 µL (to prevent the solvent from leaking while transferring) are delivered to the plate containing the samples. Due to differences in liquid vapor pressure and surface tension, the BuMe mixture needs to be characterized as a separate liquid class, based on solvent-specifi c aspiration and dispensing volumes. We empirically optimized these values following the

procedure reported in a dedicated Application Note[3]. By aspirating and dispensing different volumes of the specific solvent, a volume correction factor table is generated to ensure accuracy. This procedure is essential, and its effect on the reduction of the error associated with dispensing small volumes of organic solvents was significant.

4. After dispensing the extraction solvent + lipid standards mixture to the destination plate in Station 8, the plate wells are individually capped with PCR tube caps and shaken for 60 seconds. After this step, the plate has to be removed from the Bravo platform and put in a sonication bath for 60 minutes at room temperature. The plate is then centrifuged in a Sorvall RT centrifuge at 4,200 rpm for 20 minutes to precipitate the protein fraction.

5. The plate is again placed in Station 8 of the Bravo platform, and 70 µL of the supernatant (with a post-aspirate volume of 10 µL) are then transferred to the collection plate at Station 9. This plate can then directly be used in the Agilent 1290 Infinity Autosampler for UHPLC/MS/MS analysis.

Results and Discussion

Experiments were performed to compare the efficiency of the manual extraction of lipids from commercial human plasma with automated extraction using the Agilent Bravo platform. Phospholipids and sphingolipids from different aliquots (n = 12) of the same human plasma were extracted and quantified using an in-house developed LC/MS/MS method, based on reversed-phase separation and multiple reaction monitoring (MRM) detection on a triple quadrupole mass spectrometer. The aim was to evaluate if the manual and automated methods could produce comparable results. One hundred fifteen different lipid molecular species were analyzed, and the mean, standard deviation (SD), and percent relative standard deviation (% RSD) for each species concentration were compared. The mean lipid concentrations measured by manual and automated methods showed high correlation (R = 0.9964), indicating good reproducibility between the two methods (Figure 3).

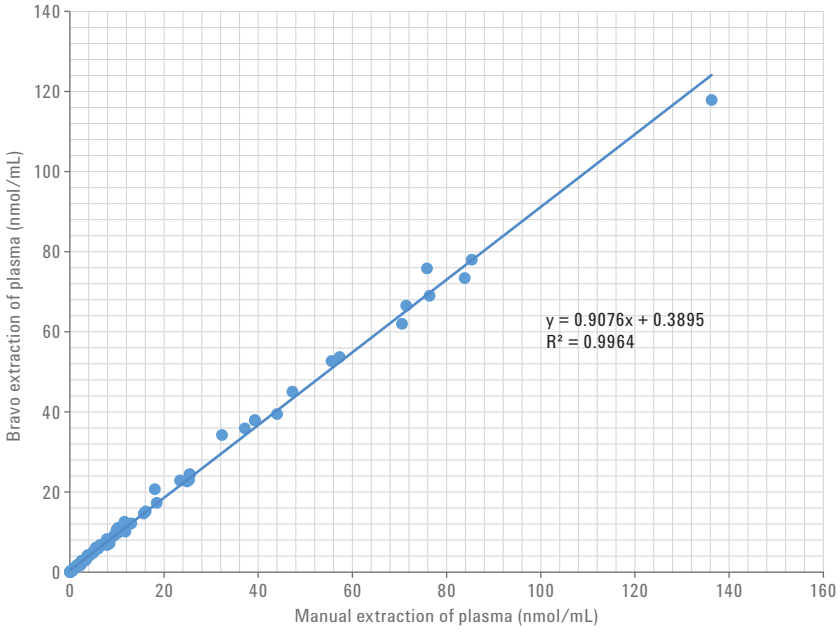

Figure 3. Accuracy and precision of the semi-automated preparation method compared with the manual method. Correlation between the the concentration (nmol/mL of plasma) for the endogenous lipids prepared by the conventional manual method (n = 12) and the semi-automated method (n = 12).

The variation measures for the lipid standards in each class (Table 2) demonstrates that the automated method on the Bravo provides lower %RSDs compared to the manual method.

Table 2. Coefficients of variation of the peak areas of the lipid standards, representative of each class, used in the reported experiments .

Lipid standard	% RSD semi-automated	% RSD manual
PE 14:0/14:0	4.80	7.13
PC 14:0/14:0	5.85	5.72
C17 Ceramide	7.32	11.97
LysoPC 20:0	5.27	5.05
SM 30:1	3.79	4.96
GluCer d18:1/8:0	6.81	6.34

Conclusion

The results demonstrate that the Agilent Bravo Automated Liquid Handling Platform can implement any single phase-based lipid extraction method, generating samples suitable for LC/MS/MS analysis in lipidomics. Automated processing using the Agilent Bravo improved the throughput of the method and showed a consistent analytical precision. While the preparation time for 96 samples with the manual method can take approximately 8 hours, the automated approach requires only 2 hours. Since, by some estimates, 60–80 % of the work activity and operating cost in an analytical lab is spent preparing samples for introduction into an analytical device, this experimental protocol can significantly reduce the cost/sample for lipid analysis.

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