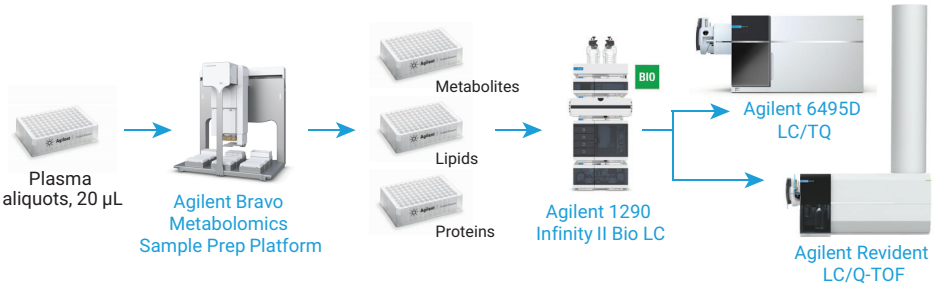
Technical Overview

Automated Fractionation of Low-Volume Plasma Samples for LC/MS Multi-Omics

Bravo enabled end-to-end LC/MS metabolomics, lipidomics, and proteomics workflows



Authors

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Abstract

LC/MS multi-omics analyses dramatically benefit from automated sample preparation, which reduces variability introduced during manual handling of multiple sample fractions. This technical overview demonstrates a comprehensive, automated method for the fractionation of polar metabolites, lipids, and proteins from single 20 µL plasma samples. The method comprises multiple configurations, which enables the user to selectively collect any combination of sample fractions, from metabolites alone to metabolites, lipids, and proteins. Automated sample fractionation is achieved using an Agilent Bravo Metabolomics Sample Prep Platform with an Agilent Captiva EMR–Lipid plate, which separates polar metabolite and lipid fractions. Using the Agilent AssayMAP Bravo Protein Sample Prep Platform, the optionally collected protein precipitate is further prepared with standard methods. Performance of method configurations for metabolite, lipid, and protein fractionation was assessed with LC/MS using the Agilent 1290 Inifinity II Bio LC System or Agilent 1290 Infinity II LC System with an Agilent 6495 triple quadrupole LC/MS (LC/TQ) or Agilent Revident quadrupole time-of-flight (LC/Q-TOF). The assessment detected broad compound coverage with highly reproducible recovery, making this configurable and automated sample preparation platform an integral component of an Agilent end-to-end LC/MS multi-omics workflow.

Introduction

LC/MS omics analyses, which comprise metabolomics, lipidomics, and proteomics, offer a way to measure the molecules and enzymes involved in metabolism—the life-sustaining chemical reactions in organisms.[1] These measurements of metabolic molecules and the enzymes that interconvert them provide a snapshot of the metabolic state present in a biological sample. Comparing relative or absolute metabolite, lipid, and protein abundances across different sample groups can identify alterations in the metabolic state, which can be used to answer essential biological questions, including clarifying the impact of a disease state on metabolism and elucidating the mechanisms of action and off-target effects of a therapeutic candidate.

Comprehensive sample preparation for LC/MS multi-omics analysis requires extraction and fractionation of polar metabolites, lipids, and proteins from single biological samples. For each collected fraction, the goal is to obtain superior sample preparation reproducibility, broad compound coverage, and excellent compound recovery. Sample preparation reproducibility in particular can be improved significantly through automation, which can reduce variability by ~ 50%.[2,3] Improved reproducibility improves statistical power and enables detection of smaller changes in relative abundance of low-abundant compounds, which are more prevalent when starting with small (e.g., 20 µL) plasma sample volumes. Although automation is highly desirable, many methods for extraction and fractionation of polar metabolites, lipids, and proteins rely on liquid-solid-liquid phase separations that are difficult to automate, especially while maintaining maximal compound recovery. As with reproducibility, maximal compound recovery is important for sensitive detection of low-abundance compounds from small sample volumes.

To meet the goals of obtaining a highly reproducible, automated multi-omics sample preparation method that provides maximal compound recovery, adaptations of previous work extracting and fractionating polar metabolites and lipids from cell samples were investigated.[4] This study used a Captiva EMR–Lipid plate, which specifically binds compounds with long hydrocarbon chains, for fractionation of polar metabolites and lipids. To add a protein fractionation option, multiple method configurations for removing the proteins while maintaining optimal polar metabolite and lipid recovery were investigated. This involved identifying the ideal step to complete protein pelleting, optimizing centrifugation parameters for protein pelleting, and adding a protein pellet wash for improved lipid recovery. Ultimately, the collection of the protein fraction has a trade-off. It improves recovery of more hydrophobic lipid classes, such as triglycerides and cholesterol esters, while moderately reducing recoveries of polar metabolites. To address this trade-off, the sample preparation platform is configurable, enabling the user to select which combination of metabolite, lipid, and protein fractions to collect and optimize to meet the requirements of a particular LC/MS omics study.

Experimental

Materials

- Ultrapure water produced with a Milli-Q Integral System equipped with an LC-Pak Polisher and a 0.22 µm point-of-use membrane filter cartridge (MilliporeSigma)
- LC/MS-grade methanol
- High purity > 99.5% ethanol
- GC/MS-grade dichloromethane

Instrumentation, software, and consumables

Metabolite, lipid, and protein fractionation: The following instrumentation, software, and consumables were used for metabolite, lipid, and protein fractionation:

- Agilent Bravo Metabolomics Sample Prep Platform (part number G5589AA)
- Agilent VWorks automation control software supplementary protocols. For supplementary protocol installation, please contact your Agilent Automation Workflow Specialist.
- Agilent Captiva EMR–Lipid plates (part number 5190-1001)
- Agilent 250 µL disposable tips (part number 19477-002)
- Agilent PlateLoc thermal microplate sealer (part number G5585BA)
- Agilent PlateLoc microplate heat seal, thin, clear pierceable (part number 17318-001)
- Agilent adhesive sealing film for temporary plate sealing (part number 410186)
- Agilent Captiva collection plates (part number A696001000)
- Agilent reservoirs, single cavity (part number 201244-100)
- Thermo Fisher Scientific SureSTART WebSeal Plate+ 9-well deep well plates (part number 60180-P338)

Protein digestion: The following instrumentation, software, and consumables were used for protein digestion:

- Agilent AssayMAP Bravo Platform with Protein Sample Prep Workbench software (part number G5571AA)
- Agilent AssayMAP reversed-phase (RP-S) cartridges, 5 µL (part number G5496-60033)
- Greiner Bio-One 96-well plate, U-bottom, white polypropylene (part number 650207)
- Agilent reservoirs, 12-column, low-profile (part number 201280-100)
- Eppendorf 96-well PCR plate, full skirt, polypropylene (part number 30129512)

Method configuration for metabolite, lipid, and protein fractionation

The configurable plasma sample preparation platform provides the opportunity to extract and fractionate any combination of metabolites, lipids, and proteins needed for a particular study. As shown in Figure 1, two decisions are made to choose the optimal method configuration: 1) whether proteins will be collected, and 2) whether it is preferred to maximize recovery for metabolites or hydrophobic lipids. Based on these decisions, a method configuration with or without protein fractionation is selected. In an additional point of configuration, the user can stop the method after collecting all required fractions. For instance, if only polar metabolites are required, a method without protein fractionation is recommended, and this method can be stopped after the metabolites are collected and before lipid fractionation. In total, there are seven metabolite, lipid, and protein combinations that can be collected using eight method configurations (Figure 1).

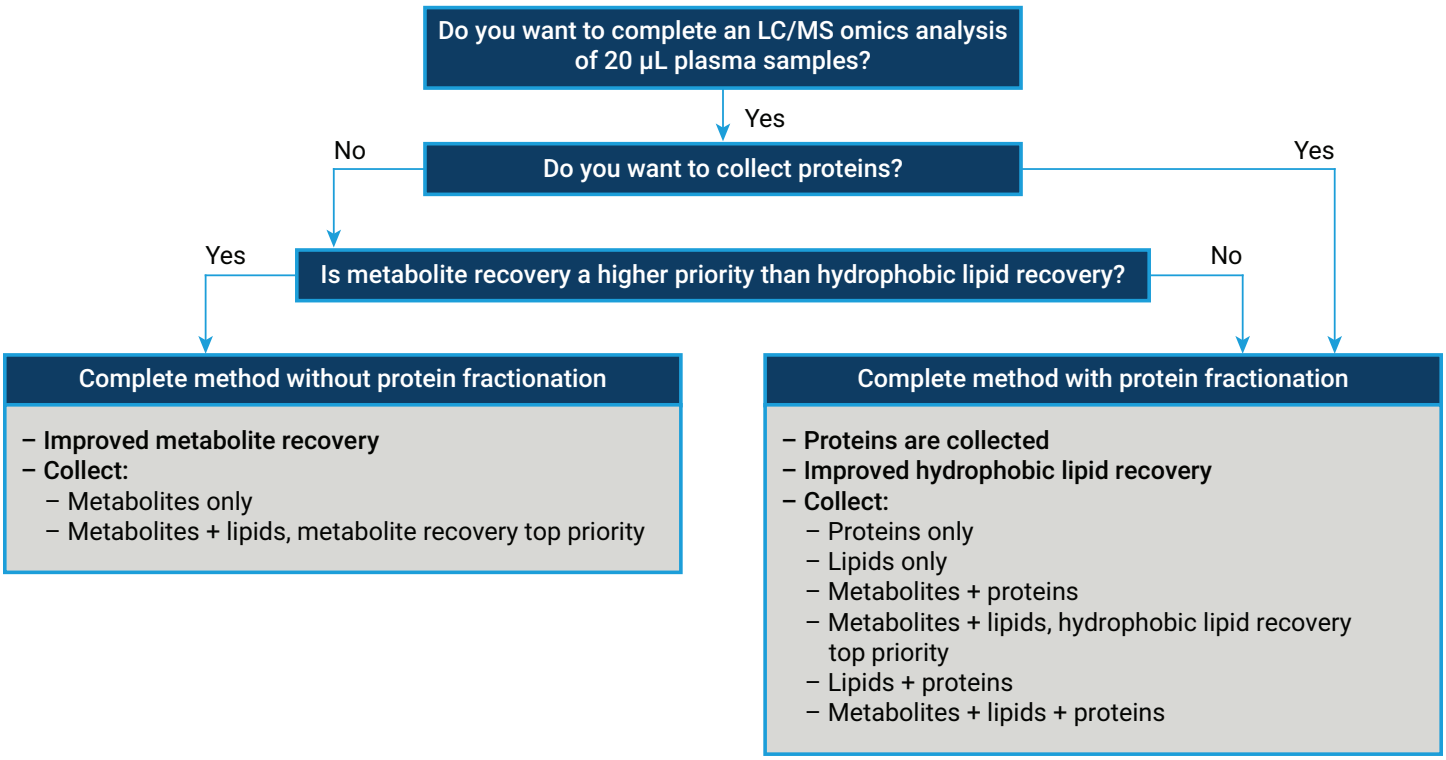


Figure 1. Method configuration decision tree. Study goals are used to select the optimal method configuration.

General method steps

The order of operations for the plasma sample preparation method configurations is outlined in Figure 2. When collected, proteins are fractionated first, followed by polar metabolites and, finally, lipids. After protein precipitation and pelleting, the water-diluted[4] sample supernatant is filtered through a Captiva EMR–Lipid plate, which adsorbs the lipids and provides a polar metabolite containing eluate. The protein pellet, if collected, is washed with a lipid elution solution, and the wash is passed through the Captiva EMR–Lipid plate to elute the adsorbed lipids.

Collected metabolite and lipid fractions are dried using nitrogen or under vacuum, then sealed with a PlateLoc Thermal Microplate Sealer and optionally stored at –80 °C. Dried metabolite and lipid fractions can be reconstituted using the reconstitution application provided with the Bravo Metabolomics Sample Prep Platform.[3] Bath sonication, orbital shaking, or vortex mixing can optionally be used to assist with sample redissolution. Following this, a brief, gentle centrifugation is recommended (30 seconds, 250 × g) to bring all liquid to the bottom of the wells. Collected protein fractions are directly sealed with a PlateLoc Thermal Microplate Sealer and optionally stored at –80 °C before protein sample preparation using an AssayMAP Bravo.

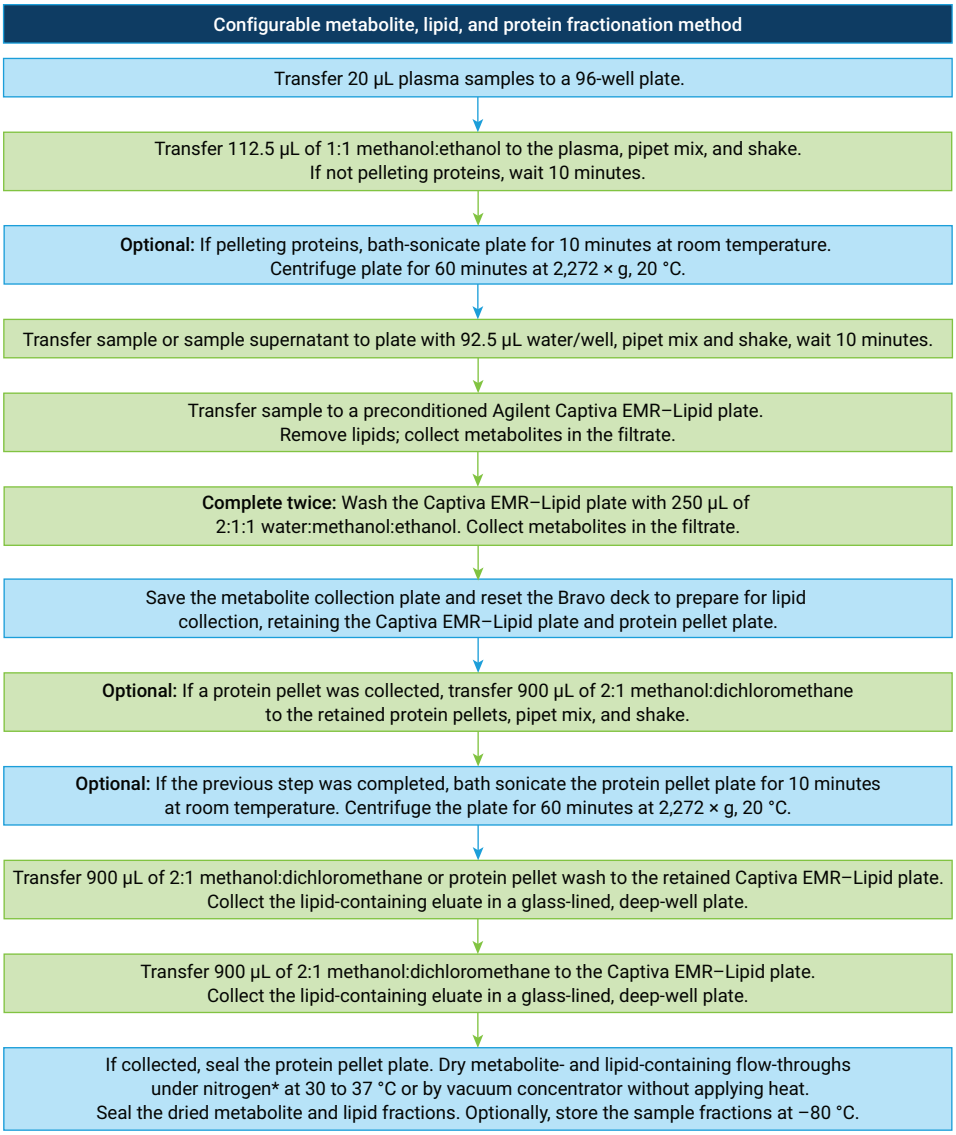


Figure 2. General steps in the configurable metabolite, lipid, and protein fractionation method. Green steps are performed by the Agilent Bravo Metabolomics Sample Prep Platform. *Nitrogen drying note: Use of high nitrogen flow that is continuously at a close distance to the top of the sample reduces drying time. It is recommended that nitrogen flow is decreased when samples are reduced to less than 50 µL to prevent ejection of dried samples from the plate wells. Samples should be removed from the nitrogen flow, sealed, and stored as soon as they are dry.

Method configuration control with VWorks protocol forms

Supplementary protocols and user interface forms have been developed for easy setup of the metabolite, lipid, and protein fractionation method configurations.

Configurable multi-omic fractionation user interface form, part 1

Figure 3 shows the user interface form for the initial steps completed for any of the method configurations. This form and associated protocol are used to complete optional protein pelleting, polar metabolite fractionation, and lipid removal.

The form's default settings include a manual centrifugation step to pellet precipitated proteins. Protein pelleting is completed through plate centrifugation for one hour at a minimum of 2,272 × g to achieve the pelleting equivalent to a typical high-speed microfuge tube centrifugation of 15 minutes at 18,000 × g. Completing a shorter plate centrifugation will reduce protein recovery and may reduce hydrophobic lipid recovery.

The form includes an adjustable pipet tip aspiration height for removing the sample supernatant from the protein pellet. Notably, the volume of sample left behind with the protein pellet after sample supernatant removal is negligible (~ 1 µL), highly consistent well to well, and does not disturb the protein pellet. When proteins are not collected, that is, to maximize metabolite recoveries, **Manual Centrifugation** must be deselected and **Mix sample before transfer to water plate** must be selected.

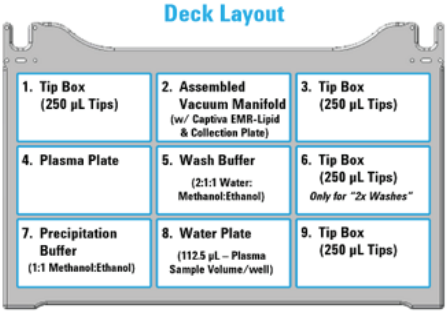
Application Settings

Starting Sample Volume (1-25)	20 µL	**Total Number of Sample Columns (1-12)**	1	
Starting Well Column (1-12)				
Plasma Plate	1	**Captiva EMR-Lipid Plate**	1	
Precipitation Buffer	1	**Water Plate**	1	**Wash Buffer** 1

Settings	Steps			
	1. MeOH/EtOH Addition	2. Addition of Sample to Water	3. Lipid Removal	4. Wash Buffer ☑ 2x Washes
Transfer Volume (2-1,000)	112.5 µL	160 µL	240 µL	250 µL
		Mix sample before transfer to water plate ☐		Transfer to water plate before transfer to filter plate ☑
Aspirate Distance from Well Bottom (0.5-5)		0.8 mm		
Dispense Distance from Well Bottom (0.5-100)	19 mm	19 mm	2 mm	19 mm
Liquid Class	96 disposable tip 51 - 200ul	96 disposable tip 2 - 50ul	96 disposable tip 2 - 50ul	96 disposable tip 51 - 200ul
Vortex Offline	☐	☐		
On Deck Shaking & Mixing				
Shaker Speed (0-2,000)	1000 RPM			
Mix Cycles (0-100)	10 cycles	5 cycles		
Incubation Time	600 sec	600 sec		
Manual Centrifugation	☑			
Filtration Time 1			Auto sec*	Auto sec*
Filtration Target Pressure 1			200 mbar	200 mbar
Filtration Vent Delay 1			0 sec	0 sec
Filtration Time (blowout)			Auto sec*	Auto sec*
Filtration Target Pressure (blowout)			400 mbar	400 mbar
Filtration Vent Delay (blowout)			15 sec	15 sec
Manually confirm Filtration is complete (user intervention required)			☐	☐

* Do not enter values in green boxes if automatic calculation of parameters is desired.

Deck Layout



Labware Table

Deck Location	Labware Type
1	96 V11 LT 250 Tip Box (p/n 19477.002)
2	Assembled Vacuum Manifold (w/ Captiva EMR-Lipid & Collection Plate)
3	96 V11 LT 250 Tip Box (p/n 19477.002)
4	96 Thermo Plate+ 60180-P338 2mL Square Glass coated
5	Reservoir, Seahorse 300mL 201244-100, PP, no walls, py
6	96 V11 LT 250 Tip Box (p/n 19477.002) *Add only if "2x Washes" selected.*
7	96 Agilent A696001000 Captiva collection plate
8	96 Agilent A696001000 Captiva collection plate
9	96 V11 LT 250 Tip Box (p/n 19477.002)

Figure 3. Agilent Bravo Metabolomics Sample Prep Platform low-volume plasma multi-omic fractionation part 1 user interface form, which includes customized settings for the supplementary protocol used for plasma protein pelleting and metabolite fractionation.

After each vacuum filtration sequence, there is an option to manually check whether the filtration has been completed for all samples. If any wells have not been emptied, additional filtration time at a user-defined vacuum pressure can be added sequentially until all wells are emptied. It is recommended that the user selects **Manually confirm Filtration is complete** and that manual filtration checks are completed when proteins are not fractionated.

Configurable multi-omic fractionation user interface form, part 2

Figure 4 shows the user interface form for the latter method steps that are completed when lipid collection is desired. The form's default settings include washing the protein pellet with the lipid elution solution to increase all lipid recoveries and achieve near-quantitative hydrophobic lipid recoveries. This form includes an adjustable aspiration height and aspiration volume for removal of the protein pellet wash from the protein pellets. A default aspiration height of 1.3 mm and aspiration volume of 1,300 µL have been optimized for transfer of the 900 µL protein pellet wash to the Captiva EMR–Lipid plate. These settings provided the most complete and consistent wash removal of the multiple settings combinations tested. When proteins are not collected, that is, to maximize metabolite recoveries, **Perform Wash of Protein Pellet** must be deselected.

During vacuum filtration for lipid elution, the optional manual filtration checks occur before the blowout filtration step. The earlier filtration check ensures that additional low vacuum pressure filtration can be completed, as needed, before higher vacuum blowout filtration. This enables lipid elution to proceed slowly, which is crucial for maximizing lipid recoveries. It is recommended that the user selects **Manually confirm Filtration is complete** and that manual filtration checks are completed when proteins are not fractionated.

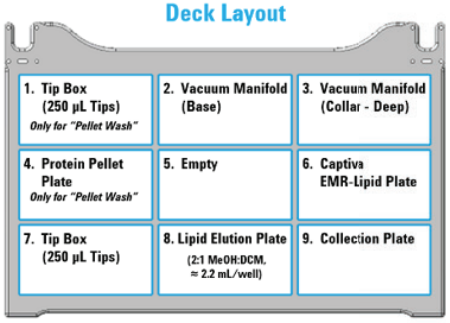
Application Settings

Starting Sample Volume (1-25)	Total Number of Sample Columns (1-12)	Starting Well Column (1-12)			
20 µL	1	Methanol/DCM Plate	1	Protein Pellet Plate	1
		Captiva EMR-Lipid Plate	1		

Settings	Steps 2:1 MeOH:DCM Addition	
Perform Wash of Protein Pellet	☑	
Transfer Volume (2-900)	900	µL
Aspirate Distance from Well Bottom (0.5-5)	1.3	mm
Aspiration Volume from Protein Pellet Plate (2-2000)	1300	µL
Dispense Distance from Well Bottom (0.5-100)	19	mm
Liquid Class	MeOH:DCM dispense	
Incubation Time	0	sec
Filtration Time 1	300	sec
Filtration Target Pressure 1*	Auto	mbar
Filtration Vent Delay 1	0	sec
Manually confirm Filtration 1 is complete (user intervention required)	☐	
Filtration Time (blowout)*	Auto	sec
Filtration Target Pressure (blowout)	400	mbar
Filtration Vent Delay (blowout)	15	sec

* Do not enter values in green boxes to automatically calculate based on *Starting Sample Volume*

Deck Layout



Labware Table

Deck Location	Labware Type
1	96 V11 LT 250 Tip Box (p/n 19477.002)
2	Vacuum Manifold (Base)
3	Vacuum Manifold (Collar - Deep)
4	96 Thermo Plate+ 60180-P338 2mL Square Glass coate
5	Empty
6	Captiva EMR-Lipid Plate
7	96 V11 LT 250 Tip Box (p/n 19477.002)
8	96 Thermo Plate+ 60180-P338 2mL Square Glass coate
9	96 Thermo Plate+ 60180-P338 2mL Square Glass coate

Figure 4. Agilent Bravo Metabolomics Sample Prep Platform low-volume plasma multi-omic fractionation part 2 user interface form, which includes customized settings for the supplementary protocol used for protein pellet washing and lipid elution.

Completion length for plasma fractionation method configurations

Table 1 describes the estimated total and hands-on time to complete each method configuration, with a minimum of approximately 25 minutes hands-on time for protein-only collection, and a maximum of approximately 1 hour and 10 minutes hands-on time for protein, metabolite, and lipid collection. All method configurations begin with Captiva EMR–Lipid plate preconditioning[5,6] and setting up the Bravo Metabolomics Sample Prep Platform with 96-well plates, which accounts for more hands-on time than any other step.

The manual transfer of plasma samples to a 96-well plate will add to the total and hands-on time estimates, with the amount of added time depending on the number of samples transferred. Typically, a full plate of 96 samples will take approximately 1.5 hours to transfer, assuming that each sample is unique. The metabolite and lipid fraction drying time estimates assume nitrogen drying. Vacuum drying is an alternative option and can be completed overnight.

Statistical treatment of LC/MS data

Statistical treatment of the LC/MS data described in the following sections included first completing an F-test to compare variances. All F-test P values were corrected using a false discovery rate (FDR) correction with a 5% threshold. The FDR-corrected F-test results were used to select either a homo- or heteroscedastic Student's *t*-test for comparisons of means. All Student's *t*-test P values were FDR-corrected with a 5% threshold and are indicated as q-values.

Table 1. Completion length estimates for method configurations.

	Without Protein Fractionation Time (Hands-On Time), Hours		With Protein Fractionation Time (Hands-On Time), Hours				
Method Step	Metabolites and Lipids Collected[#]	Metabolites Collected	Metabolites, Lipids, and Proteins Collected[+]	Metabolites and Proteins Collected	Lipids and Proteins Collected	Proteins Collected	Lipids Collected
1. Plate Preconditioning	0.33 (0.083)	0.33 (0.083)	0.33 (0.083)	0.33 (0.083)	0.33 (0.083)	0.33 (0.083)	0.33 (0.083)
2. Plate and Bravo Setup	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)	0.25 (0.25)
3. Metabolite Fractionation	1.2 (0.083)[*]	1.2 (0.083)[*]	1.2 (0.083)	1.2 (0.083)	1.2 (0.083)[‡]	—	1.2 (0.083)[‡]
4. Lipid Fractionation	0.5 (0.083)	—	0.5 (0.083)	—	0.5 (0.083)	—	0.5 (0.083)
5. Protein Fractionation	—	—	2.5 (0.167)	1.25 (0.083)[¤]	2.5 (0.167)	1.25 (0.083)[¤]	2.5 (0.167)
6. Metabolite Fraction Drying	3.0 (0.25)	3.0 (0.25)	3.0 (0.25)	3.0 (0.25)	—	—	—
7. Lipid Fraction Drying	3.0 (0.25)	—	3.0 (0.25)	—	3.0 (0.25)	—	3.0 (0.25)
Total Time (Total Hands-On Time)	5.28 (1.00)[‡]	4.78 (0.67)	7.78 (1.17)[‡]	6.03 (0.75)	7.78 (0.92)	1.83 (0.42)	7.78 (0.92)

[*] Metabolite collection filtration times may vary when protein is not fractionated. Contact an Agilent Automation Workflow Specialist if the filtration time is inconsistent.

[#] Use for collecting metabolites and lipids when maximum metabolite recovery is desired.

[+] Use for collecting metabolites and lipids when maximum hydrophobic (e.g., TG and CE) lipid recovery is desired.

[‡] Assumes metabolites and lipids are dried simultaneously.

[‡] Although metabolites are not collected, the metabolites must be removed from the sample before lipid collection.

[¤] If not collecting lipids, the protein pellet does not need to be washed with the lipid elution solution, which reduces completion length by removing a sonication and centrifugation step.

LC/MS assessment of the polar metabolite fraction

Overall, the metabolite extraction and fractionation performance of the plasma sample preparation method configurations was assessed through metabolite coverage, reproducibility, and recovery analyses. Agilent LC/MS methods and instrumentation and the Agilent MassHunter software suite were used for data acquisition and analysis.[7] New LC columns and 1290 Infinity II LCs were phosphorylated to obtain optimal peak shapes of metal-sensitive analytes.

Metabolite coverage and reproducibility assessment

Metabolite coverage was analyzed using a method that included protein fractionation. Metabolite coverage was assessed using targeted LC/TQ analysis, which detected 314 of 523 targeted metabolites. The detected metabolites covered major metabolite classes and metabolic pathways, including amino acids, organic acids, sugars, and nucleobases and their derivatives.

Reproducibility of polar metabolite extraction and fractionation was assessed for a method with protein fractionation. Peak area percent relative standard deviations (%RSDs) were calculated for two groups of 20 µL plasma samples either prespiked (n = 8) or postspiked (n = 8) with a ^{13}C-labeled yeast extract. Metabolite abundances for 37 unlabeled compounds originating from the plasma were extracted and background-subtracted, where the background was measured from a processed sample containing 20 µL of PBS and the ^{13}C-labeled spike-in. The %RSDs were calculated for each compound, by group. Each group was treated separately to account for differing matrix effects for the pre versus post spike-in of the ^{13}C-labeled yeast extract. The %RSDs were averaged across the two sample groups and all compounds to provide an average %RSD of 4.28%. The majority (78%) of metabolites had a %RSD < 5%; 95% of metabolites had a %RSD < 11%; and all %RSDs were < 21%.

Metabolite recovery assessment

Polar metabolite recovery values were estimated through the addition of pre and post spike-ins of a ^{13}C-labeled yeast extract to the samples, as previously described.[4] Recovery values were averaged across pairs of pre- and postspiked samples and were calculated after subtracting any background signal detected in no-spike-in plasma samples. Metabolite recoveries were assessed for method configurations with and without protein fractionation, and included assessment of organic acids, amino acids and their derivatives, nucleobases and their derivatives, and sugar derivatives (Figures 5A and 5B).

When proteins were not fractionated, polar metabolite recoveries generally recapitulated those found for previous methods without protein fractionation[2,4] (Figure 5A). Across 41 polar metabolites, 78.0% of compounds had an average recovery between 90 and 110%, and 87.8% of compounds had an average recovery between 80 and 120%. In addition to the outstanding polar metabolite recoveries, the variability of the recoveries was excellent. The average percent standard deviation (SD) across all compounds and spike-in levels was 8.35%. Also, 92.7% of compounds had an average SD < 15% (average from two spike-in volumes), and 100% of compounds had an average SD < 19%.

When proteins were fractionated, polar metabolite recoveries were generally reduced to approximately 80%, with a few metabolites exhibiting lower recoveries (Figure 5B). Ninety percent of the reduced recoveries were statistically significant (Student's *t*-test, 5% FDR correction). Thus, when proteins are not required and metabolite recoveries must be maximized, protein fractionation is not recommended. Although metabolite recoveries were moderately reduced following protein fractionation, metabolite recovery variability remained exceptional, with an average SD of 7.01% across all compounds and spike-in levels.

Metabolite recoveries were consistent between the 10 and 20 µL spike-in volumes, with a few exceptions. When protein was not fractionated, 10 metabolites exhibited an increased percent recovery from an average of 94% recovery for the 10 µL spike-in to an average of 105% recovery for the 20 µL spike-in (Student's *t*-test, 5% FDR correction). For the method with protein fractionation, N-acetyl-serine had a significant increase in recovery, from 103 to 121% between the 10 and 20 µL spike-in volumes (Student's *t*-test, 5% FDR correction). Overall, metabolite extraction and fractionation were stable in the presence of varying absolute quantities of each metabolite, which enables robust metabolite abundance comparisons across samples from different sample groups.

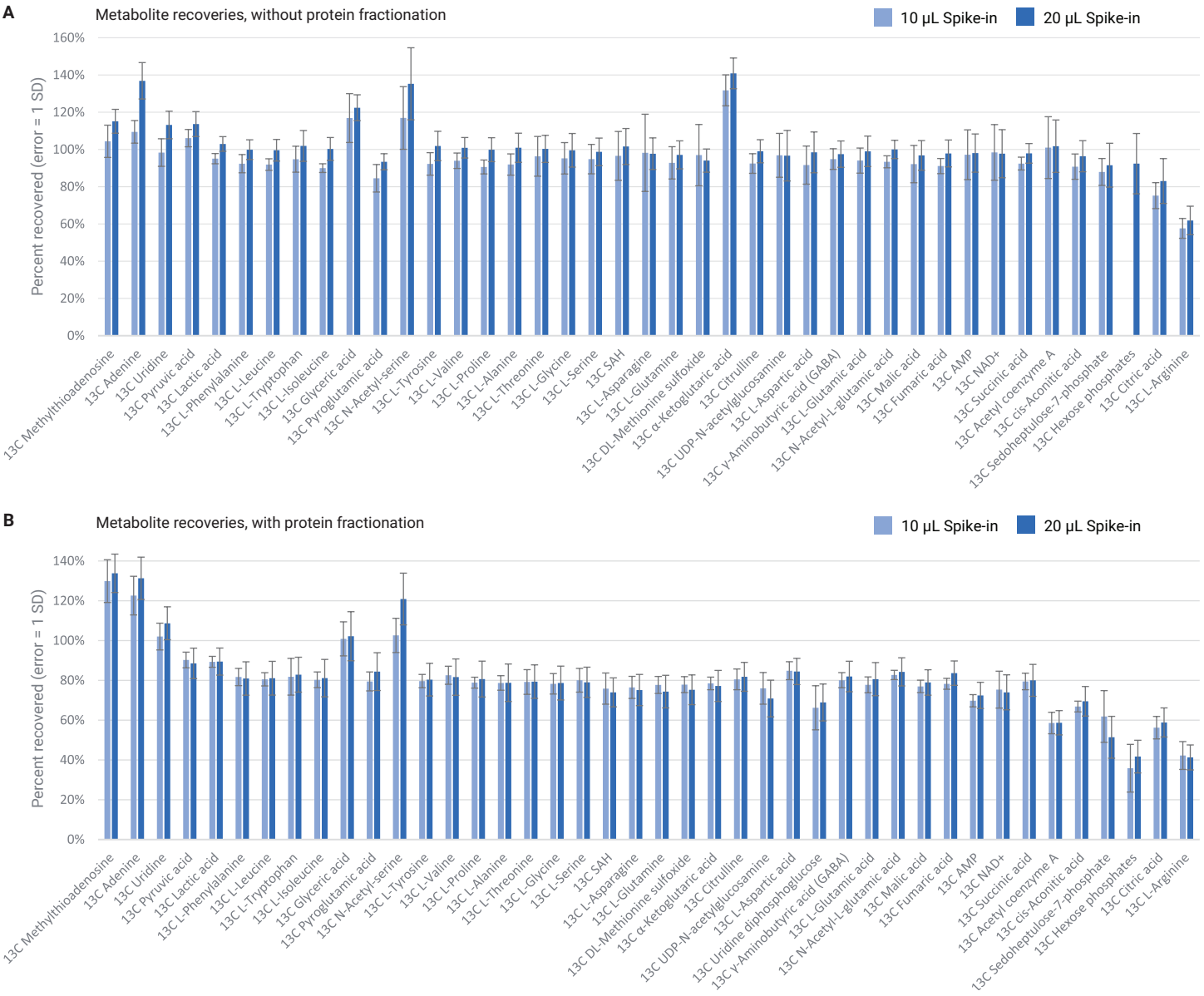


Figure 5. Recoveries (A) without and (B) with protein fractionation for ^{13}C-labeled metabolites across key compound classes and two ^{13}C-labeled metabolite spike-in volumes (n = 7 to 8 pairs of pre- and postspiked samples).

LC/MS assessment of the lipid fraction

Overall lipid extraction and fractionation performance of the plasma sample preparation method configurations was assessed through lipid coverage, reproducibility, and recovery analyses, which used Agilent LC/MS methods and instrumentation and the MassHunter software suite for data acquisition and analysis.[8] Reproducibility assessment also used Skyline software from the MacCoss Lab at the University of Washington.

Lipid coverage and reproducibility assessment

Lipid coverage and reproducibility of lipid fractionation were analyzed using a method with protein pelleting and protein pellet washing with the lipid elution solution. Lipid coverage was assessed through a targeted LC/TQ method, which detected 615 of 763 targeted lipids, representing 44 lipid classes. Lipids were detected across commonly analyzed lipid classes, including acylcarnitines, ceramides, cholesterol esters (CEs), diacylglycerols (DGs), triacylglycerols (TGs), phosphatidates (PAs), phosphatidylcholines (PCs), phosphatidylethanolamines

(PEs), phosphatidylglycerols (PGs), phosphatidylinositols (PIs), phosphatidylserines (PSs), and sphingomyelins (SMs).

Reproducibility of lipid preparation was assessed through an individually prepared and pooled sample reproducibility analysis. Twenty-four plasma samples were prespiked with ^{2}H-labeled lipids and prepared individually before combining portions of each sample into a pooled sample. Lipid peak areas for 11 ^{2}H-labeled lipids were extracted, and the peak area %RSDs were calculated for the injections of individually prepared samples (sample preparation and LC/MS %RSD) and repeat injections of the pooled sample (LC/MS %RSD). The average %RSD for the individually prepared sample lipids was 11.3%, and for the pooled sample lipids was 4.10%. Also, 100% of individually prepared sample lipids had a %RSD < 14%.

An F-test was performed for each lipid to assess whether the variance of peak area was different between the individually prepared and pooled samples. After 5% FDR correction, three of 11 lipids—the LPC, the PC, and the TG—had significantly different variances, with the variance being larger for the individually prepared samples compared to the pooled sample.

Lipid recovery assessment

The lipid recovery studies were completed similarly to the metabolite recovery studies with ^{2}H-labeled lipid standards as the pre or post spike-in.[4] Lipid recovery was assessed using method configurations with and without protein fractionation and covered a range of lipids, including a CE, a DG, a TG, a PA, PCs, PEs, a PG, a PI, a PS, and an SM (Figures 6A and 6B).

Lipid recoveries generally recapitulated those found for the dual metabolite + lipid sample preparation method[4], although with marked increases in recoveries of the spiked-in triacylglycerol and cholesterol ester when proteins were fractionated. These improved lipid recoveries were a result of the TGs and CEs being pelleted with the proteins then being dissolved into the lipid elution solution during the protein pellet wash. When the protein pellet was fractionated, seven of 14 lipids had a recovery between 80 and 120%. Although lower recoveries were seen for some lipid classes, the broad lipid coverage described earlier indicates that plasma lipids were easily detected. Moreover,

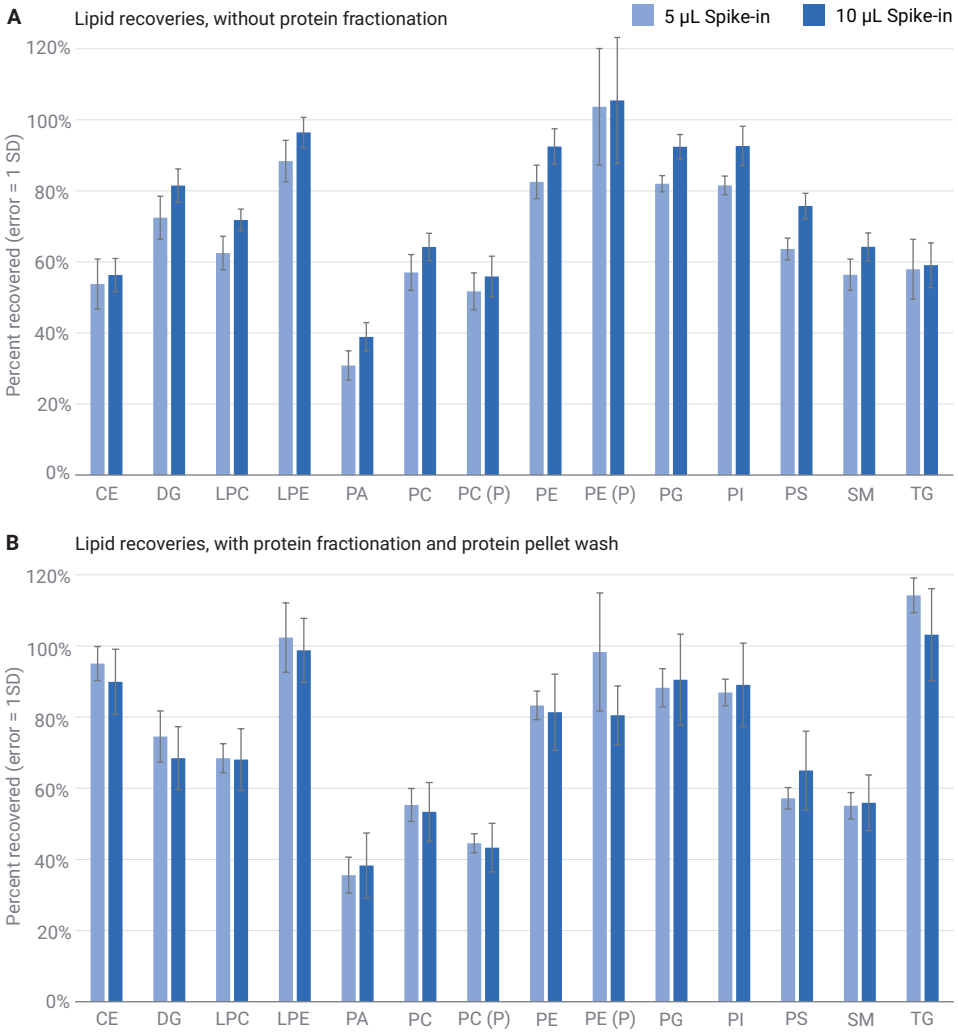


Figure 6. Recoveries (A) without and (B) with protein fractionation for 14 ^{2}H-labeled lipids across two ^{2}H-labeled lipid spike-in volumes. Protein pelleting and protein pellet washing significantly increased recoveries for the TG lipid (97.1% increase, q = 4.52 × 10^{-8} for the 5 µL spike-in; 74.6% increase, q = 2.12 × 10^{-5} for the 10 µL spike-in) and the CE lipid (76.8% increase, q = 1.55 × 10^{-7} for the 5 µL spike-in; 59.7% increase, q = 1.35 × 10^{-5} for the 10 µL spike-in); Student's *t*-test, FDR correction. Protein pelleting and protein pellet washing significantly decreased recovery of the PC (P) lipid (13.9% decrease, q = 0.0105 for the 5 µL spike-in; 22.5% decrease, q = 0.00461 for the 10 µL spike-in) and the PS lipid (10.2% decrease, q = 0.00327 for the 5 µL spike-in; 14.2% decrease, q = 0.0430 for the 10 µL spike-in); Student's *t*-test, FDR correction. A few other lipids had significantly increased or decreased recoveries following protein pelleting and pellet washing, but the changes were not consistent for the 5 and 10 µL spike-ins.

the reproducibility of recovery was exceptional, with an average standard deviation of 7.67% when proteins were fractionated, and 5.56% when proteins were not fractionated. The standard deviations for 86% of measured recoveries were < 10%, and all measured recoveries had SDs < 18%.

When comparing the 5 and 10 µL spike-ins for the method that fractionates proteins, the percent recoveries were consistent across the two spike-in volumes, except for the PE(P) lipid, which exhibited an 18% decrease in the 10 µL spike-in (q = 0.0378; Student's *t*-test, FDR correction). When proteins were not fractionated, recoveries increased by an average of 15% for the DG, LPC, LPE, PA, PC, PE, PG, PI, PS, and SM lipids for the 10 µL spike-in. Thus, percent recovery was more stable across the 5 and 10 µL spike-in volumes when proteins were fractionated. Notably, the percent change of recoveries, when detected, was small relative to the 100% increase in spike-in volume.

Lipid recovery assessment following additional lipid elutions

Whether lipid recoveries could be improved by completing four, instead of two, elutions of the Captiva EMR–Lipid plate with 900 µL of 2:1 MeOH:DCM was also assessed (Figures 7A and 7B). When proteins were not fractionated, additional lipid elutions consistently increased recoveries of the LPC, PC, PC(P), and SM lipids by an average of 28% across both spike-in volumes. For the 5 µL spike-in, the LPE recovery was decreased by 10% when four elutions were completed. When proteins were fractionated, additional lipid elutions again increased recoveries of LPC, PC, PC(P), and SM lipids, in this case by an average of 28% for the 5 µL spike-in, and 42% for the 10 µL spike-in. The PA lipid also exhibited an average increased recovery of 28% when proteins were fractionated. The additional lipid elution steps add approximately 2.5 hours to the overall method length and, in some cases, increase the lipid recovery variance, indicating less reproducible lipid fractionation. For the method without protein fractionation, increased variance with four lipid elutions was detected for the PG, PI, and PS lipids for the 5 µL spike-in and all lipids except for PA and PE (P) for the 10 µL spike-in. For the method with protein fractionation, increased variance with four lipid elutions was detected for the PE, PS, and PC (P) lipids for the 5 µL spike-in and the PE (P) lipid for the 10 µL spike-in (10 µL data not shown). For both spike-in levels and for methods with and without protein fractionation, there were no lipids that exhibited a decreased variance when four lipid elutions were completed instead of two elutions. Thus, completing additional lipid elution steps may not provide enough increase in lipid recovery to outweigh the reduced reproducibility and increased method completion length.

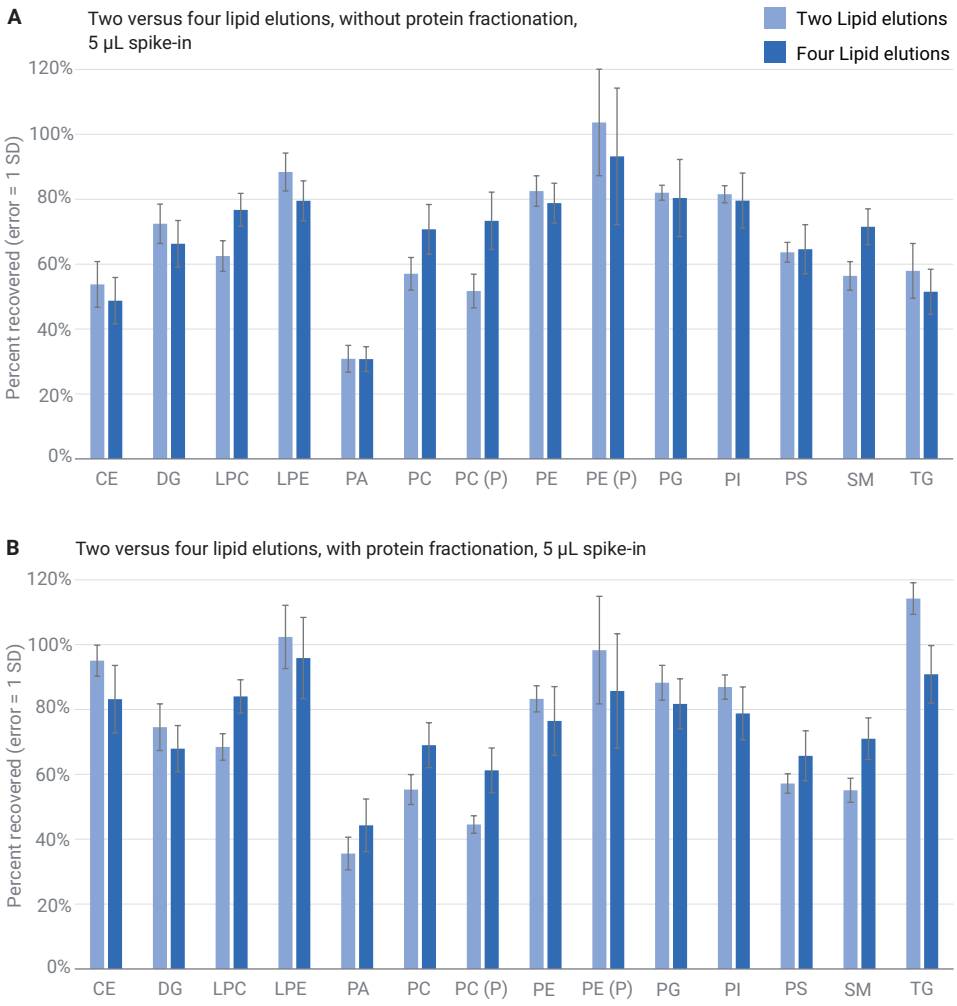


Figure 7. Recoveries following two or four lipid elutions of the Agilent Captiva EMR–Lipid plate for method configurations without (A) and with (B) protein fractionation. Data for the 5 µL ^{2}H-lipid spike-in are shown. Each lipid elution was completed with 900 µL of 2:1 MeOH:DCM, using the first aliquot to wash the protein pellet when proteins were fractionated. Overall, recovery increased for the LPC, PC, PC(P), and SM lipids by 31% when four lipid elutions were completed. For the 5 µL spike-in, a few lipids had decreased recoveries after four lipid elutions, including the LPE lipid for the method without protein fractionation and the TG, CE, and PI lipids for the method with protein fractionation.

Protein pellet preparation with AssayMAP Bravo

The protein pellet fractionated with the Metabolomics Bravo Workbench can be prepared using standard protein preparation methods, many of which are automated on the AssayMAP Bravo. In the present work, protein pellets were dissolved with 9 M urea and the total amount of protein in each well was approximated using a BCA protein assay. An equal amount of protein was taken from each well and transferred to a new plate. The AssayMAP Bravo was used for an in-solution tryptic digest, including denaturation, reduction, and alkylation. Protein digestion was performed overnight at 37 °C using a 1:50 enzyme:protein ratio. The following morning, a second aliquot of the same amount of enzyme was added to each sample, followed by a two-hour incubation at 37 °C. The Peptide Clean-Up Application in the AssayMAP Bravo Sample Prep Workbench software was used to desalt and enrich the peptides after digestion. Samples were dried down using a SpeedVac and sealed with a PlateLoc Thermal Microplate Sealer. Prepared peptide samples can be reconstituted using the AssayMAP Bravo or the Bravo Metabolomics Sample Prep Platform Utility Protocols.

LC/MS assessment of the prepared protein fraction

Assessment of overall performance of the multi-omics sample preparation method for protein fractionation used Agilent LC/MS instrumentation and methods[9], with modifications to ensure elution of hydrophobic peptides. The Agilent MassHunter software suite was used for data acquisition.

Manual versus automated protein fractionation

In the initial analysis, a comparison of peptide identifications was made following manual or automated protein fractionation. Slightly more peptides were identified in protein pellets prepared with automation using the Bravo Metabolomics Sample Prep Platform than in protein pellets prepared manually (Figure 8). This may have resulted from the more controlled and consistent supernatant removal provided by Bravo automation.

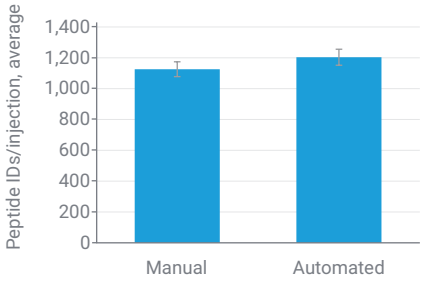


Figure 8. Comparison of peptide identifications for manual and automated protein fractionation. More peptides were identified from protein pellets fractionated using automation, 1,204 (n = 8), than from protein pellets fractionated manually, 1,126 (n = 8), Student's *t*-test (q = 0.01989, FDR corrected). Error bars are SD. In this test, the protein pellet was not washed with the lipid elution solution.

Protein reproducibility assessment

Reproducibility of protein pellet fractionation was assessed using two analyses, measuring reproducibility of total peptide identifications and measuring reproducibility of peptide peak areas. For both analyses, digested protein samples were prepared and injected individually and as a pooled sample. Peptides were filtered in Spectrum Mill based on a peptide FDR of 1.2%, protein FDR of 1%, and a protein score higher than 20.

In the peptide identification analysis, an equivalent number of peptides were identified for the individually prepared samples and the repeatedly injected pooled sample (Figure 9). The %RSD for peptide identifications for the individually prepared samples was 6.63% (sample preparation and LC/MS %RSD) while the %RSD for peptide identifications for the repeatedly injected pooled sample was 6.70% (LC/MS %RSD). An F-test of individually prepared and pooled samples indicated the variances for the number of peptide identifications from the pooled and individually prepared samples were not different, indicating that sample preparation did not add significant variability to the number of peptides identified from a sample.

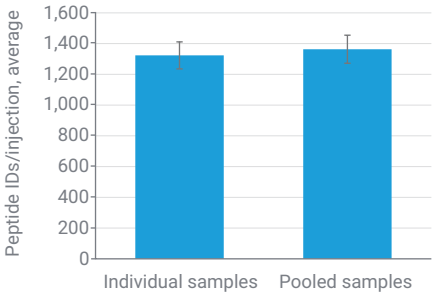


Figure 9. Comparison of peptide identifications for individually prepared and pooled digested protein samples. Peptide identifications from individually prepared samples were 1,323 (n = 24) and peptide identifications from a pooled sample were 1,363 (n = 11 injections), Student's *t*-test (q = 0.3774, FDR corrected). Error bars are SD. The variances for the individually prepared and pooled samples are not significantly different, F-test (q = 0.9201, FDR corrected).

Peptide peak areas of identified peptides from the individually prepared protein samples were extracted from the acquired DDA data using Skyline software from the MacCoss Lab at University of Washington. Using an equalization median normalization, the median peptide peak area %CV was 14.1% (Figure 10). The median peptide peak area %CV from replicate injections (n = 10) of a pooled sample was 9.8%. The reproducibility of both the number of peptides identified and peptide peak areas indicate that this automated plasma preparation method provides robust and reproducible peptide samples.

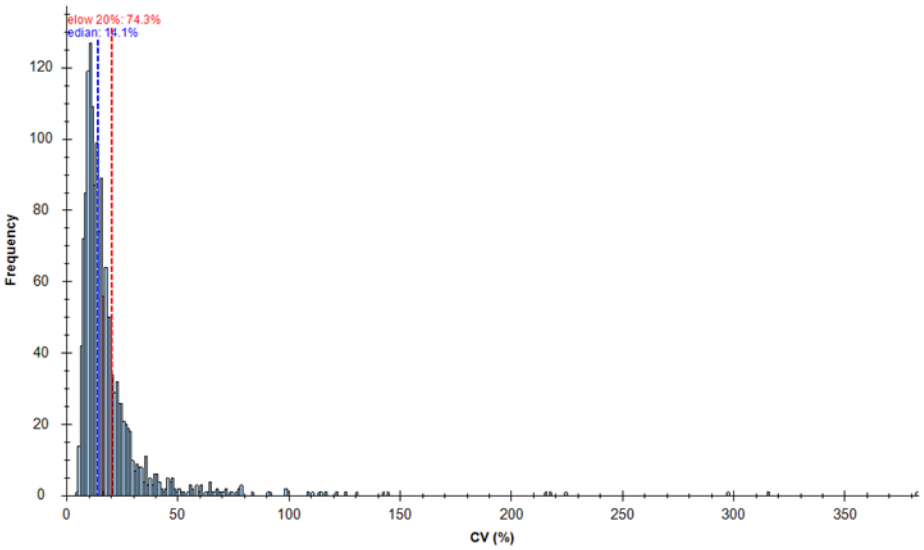


Figure 10. Peptide peak area %CV distribution based on MS1 scan. Using an equalization median normalization, the median peptide peak area %CV was 14.1% across 24 peptide samples prepared from 20 µL plasma samples.

Conclusion

A robust, configurable, automated sample preparation platform for extraction and fractionation of any combination of polar metabolites, lipids, or proteins from 20 µL plasma samples was demonstrated. When combined with Agilent's tested solutions for LC/MS sample analysis and data analysis, an end-to-end LC/MS omics workflow is generated that is easy-to-implement, enabling fast completion of multi-omics studies. Notably, a single LC/TQ or LC/Q-TOF instrument can be adapted to complete an analysis of all three sample fractions, polar metabolite, lipid, and protein.[10] With excellent compound coverage, reproducibility, and recovery, the automated sample preparation platform facilitates routine identification of biological metabolic state perturbations, which enables assessment of how diseases alter the metabolic state, and the mechanism of action and off-target effects of putative therapeutics.

References

1. https://en.wikipedia.org/wiki/Metabolism
2. Sartain, M.; *et al.* Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform. *Agilent Technologies application note*, publication number 5994-2156EN, **2020**.
3. Spivia, W. R.; *et al.* Automated Metabolite Extraction for Plasma using the Agilent Bravo Platform. *Agilent Technologies technical overview*, publication number 5994-0685, **2019**.
4. Van de Bittner, G. C.; *et al.* An Automated Dual Metabolite + Lipid Sample Preparation Workflow for Mammalian Cell Samples. *Agilent Technologies technical overview*, publication number 5994-5065EN, **2022**.
5. Agilent Captiva EMR–Lipid Automated Plate Preconditioning, *Agilent Technologies method guide*, publication number 5994-7479EN, **2024**.
6. Agilent Captiva EMR–Lipid Manual Plate Preconditioning, *Agilent Technologies method guide*, publication number 5994-7481EN, **2024**.
7. Yannell, K. E.; *et al.* An End-to-End Targeted Metabolomics Workflow. *Agilent Technologies application note*, publication number 5994-5628EN, **2023**.
8. Huynh, K.; *et al.* A Comprehensive, Curated, High Throughput Method for the Detailed Analysis of the Plasma Lipidome. *Agilent Technologies application note*, publication number 5994-3747EN, **2021**.
9. Wu, L.; *et al.* In-Depth Peptide Mapping with Iterative MS/MS Acquisition on the Agilent 6545XT AdvanceBio LC/Q-TOF. *Agilent Technologies application note*, publication number 5991-8633EN, **2020**.
10. Simmermaker, C.; *et al.* Enabling Multiomics LC/MS Workflows: Hardware and Methods for Combining Metabolomics, Lipidomics and Proteomics using Plasma Matrix. Poster. Metabolomics, **2023**.

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