Application Note

Metabolomics

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Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform

Authors

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Abstract

This application note describes the modification of the Agilent Bravo Metabolomics Sample Prep Platform to lower the required starting plasma volume from 100 to 25 µL. Analyte recovery of the low-volume method was assessed using an Agilent 6546 LC/Q-TOF, and reproducibility was compared with manual sample preparation. The method was shown to enable outstanding metabolite recovery and superior reproducibility compared with manual sample processing methods.

Introduction

In basic and translational research settings, sample preparation before LC/MS-based analysis of plasma metabolites is challenging for several reasons, including the presence of compounds with different physical properties, variability between operators, and interday reproducibility. Additionally, in some research settings, limited amounts of plasma can be obtained from infants/children or from animal models. Here is a description of a modification to the Bravo Metabolomics Sample Prep Platform to accommodate 25 µL of plasma sample volume. Like the original protocol, this modified method precipitates plasma proteins to quench enzymatic activity, depletes lipids, and extracts metabolites, providing a clean metabolite sample for LC/MS analysis. An Agilent 6546 LC/Q-TOF was used to evaluate metabolite recovery of the automated 25 µL method and also compared the reproducibility against manually prepared samples processed by multiple laboratory staff.

Experimental

Materials

Normal human plasma (lithium heparin, pooled, mixed gender) was procured from BioIVT (Hicksville, NY, USA). The Mass Spectrometry Metabolite Library (MSMLS) (IROA Technologies) was purchased through Millipore Sigma (St. Louis, MO, USA). Unlabeled yeast metabolite extract "ISO1-UNL" and U-^{13}C labeled yeast metabolite extract "ISO1" (ISOtopic Solutions) were purchased through Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA).

Agilent LC/MS-grade acetonitrile (part number G2453-85050) was used for LC/MS analysis. Ultrapure water was produced with a Milli-Q Integral system equipped with a LC-Pak Polisher and a 0.22 µm point-of-use membrane filter cartridge (Millipore Sigma). LC/MS grade ammonium acetate was purchased from Millipore Sigma. To minimize the binding of polar ionic metabolites to trace levels of metal in the LC system, Agilent InfinityLab deactivator additive (part number 5191-4506) was added to the mobile phases.

Instrumentation

- Agilent Bravo Metabolomics Sample Prep Platform (part number G5589AA)
- Agilent PlateLoc thermal microplate sealer (part number G5585BA)
- Agilent 1260 Infinity II Prime LC including:
  - Agilent 1260 Infinity II Flexible Pump (G7104C)
  - Agilent 1260 Infinity II Multisampler with thermostat (G7167A)
  - Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1200 Isocratic Pump (G1310A) with a 100:1 splitter (G1607-60000) for reference mass addition
- Agilent 6546 LC/Q-TOF with a Dual Agilent Jet Stream technology ion source

Bravo consumables

- Agilent Captiva EMR—Lipid plates (part number 5190-1001)
- Agilent 250 µL disposable tips (part number 19477-002)
- Agilent PlateLoc peelable aluminum seals (part number 24210-001)
- Agilent Captiva collection plates (part number A696001000)
- Agilent reservoirs, single cavity (part number 201254-100)
- Thermo Scientific Matrix 1.0 mL screw top tubes and rack, 2D V-bottom (Thermo Scientific, part number 3741)

Bravo method

Beginning with the Bravo Metabolomics Sample Prep Platform (p/n G5589AA)[1] (original method), modifications were made to the protocol that include reduction of the starting plasma volume from 100 to 25 µL. Figure 1 summarizes major steps, and Figure 2A is a display of the custom application user interface used for low-volume plasma metabolite extraction.

Many of the steps and fundamental principles between the original method and this modified protocol remained the same. Importantly, these included maintaining a 225:225:100 ratio of methanol:ethanol:plasma for effective protein precipitation and use of Captiva EMR—Lipid plates for protein filtering and lipid depletion, as described previously.[1] In addition, this protocol included a 10-minute incubation following quenching, subsequent water addition to the quenched sample to achieve 50% final water content, and washing the Captiva EMR—Lipid plate twice with 1:1:2 methanol:ethanol:water. These combined measures improved recovery for a small percentage of metabolites (data not shown). With the original method, ~55% of the metabolite extract is transferred to a final plate for dry down. In contrast, the entire combined volume of Captiva EMR—Lipid eluant including washes is collected and dried in the modified low-volume plasma protocol, thereby minimizing the overall losses otherwise expected when moving from 100 to 25 µL plasma input.

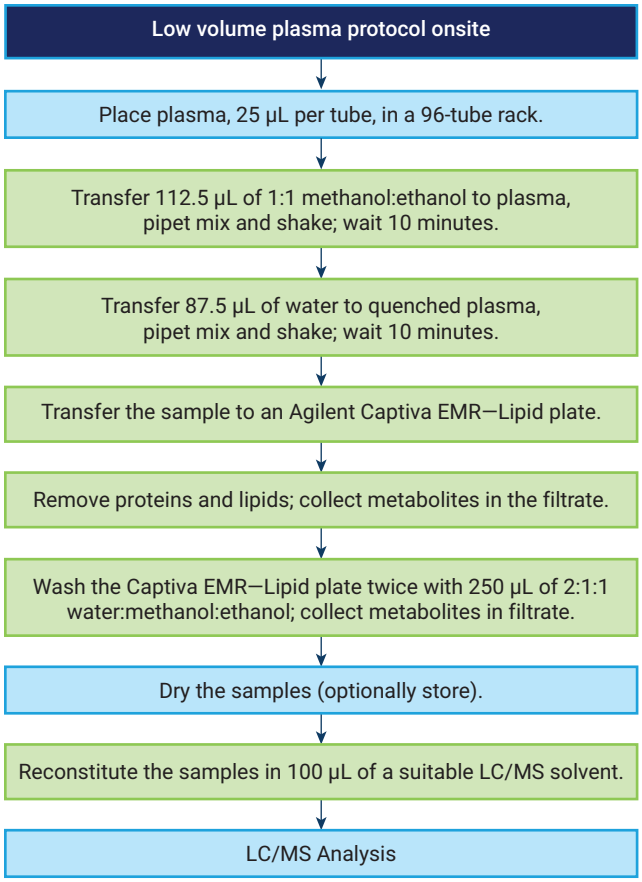

Figure 1. Workflow for onsite, automated, low-volume metabolite extraction from plasma. Green steps are performed with the Agilent Bravo platform.

A

Application Settings

Total Number of Sample Columns (1-12): 4 — **Starting well column (1-12)**

Methanol/Ethanol Plate: 1 — **Plasma Plate**: 1 — **Captiva EMR-Lipid Plate**: 1

Settings	Steps			
	1. MeOH/EtOH Addition	☑ 2. Water Addition	3. Lipid Removal	☑ 4. Wash Buffer ☑ 2x washes
Transfer Volume (2-1,000)	112.5 µL	87.5 µL	240 µL	250 µL
Dispense Distance From Well Bottom (0.5-100)	39 mm	39 mm	2 mm	19 mm
Liquid Class	96 disposable tip 51 - 2	96 disposable tip 51 - 2	96 disposable tip 2 - 50ul	96 disposable tip 51 - 2
Vortex Offline	☐	☐		
On Deck Shaking & Mixing				
Shaker Speed (0-2,000)	1000 RPM	500 RPM		
Mix Cycles (0-100)	10 cycles	5 cycles		
Incubation Time	600 sec	600 sec		
Filtration Time			180 sec	300 sec
Filtration Target Pressure			200 mbar	200 mbar
Filtration Vent Delay			0 sec	0 sec
Filtration Time (blowout)			60 sec	60 sec
Filtration Target Pressure (blowout)			400 mbar	400 mbar
Filtration Vent Delay (blowout)			15 sec	15 sec

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 Matrix 3741, V-bottom, 1mL ScrewTop Storag
5	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
6	empty
7	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
8	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
9	96 V11 LT 250 Tip Box (p/n 19477.002)

B

Application Settings

Total Number of Sample Columns (1-12): 4 — **Starting Well Column (1-12)**

Sample Plate: 1

Settings	Steps		
	1. Organic Solvent Addition	2. Water Addition	☐ 3. Class Specific Standard Addition
Transfer Volume (2-1,000)	80 µL	20 µL	10 µL
Dispense Distance From Well Bottom (0.5-100)	2 mm	2 mm	2 mm
Liquid Class	96 disposable tip 2 - 5(	96 disposable tip 51 - 200u	96 disposable tip 2 - 50ul
Shaker Speed (0-2,000)	1000 RPM	1000 RPM	1000 RPM
Shake Time	25 sec	120 sec	25 sec

Labware Table

Deck Location	Labware Type
1	V11 LT 250 Tip Box (p/n 19477.002)
2	Vacuum Manifold (Base)
3	Vacuum Manifold (Collar - Deep)
4	96 Agilent 203426-100 PP, 1 mL Rnd Btm
5	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
6	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
7	96 V11 LT 250 Tip Box (p/n 19477.002)
8	Reservoir, Seahorse 201254-100, PP, no walls, pyramid b
9	96 V11 LT 250 Tip Box (p/n 19477.002)

Figure 2. Custom Agilent Bravo settings used for low-volume plasma metabolite extraction (A) and reconstitution (B).

The original method offers both an "offsite" and "onsite" version, where metabolite quenching is either done at a separate location or automated on the Bravo platform, respectively. The only evaluation was an onsite version of this modified low-volume protocol, though an offsite version that simply includes a subset of automation steps from the onsite version could easily be accommodated.

For sample reconstitution, the same application settings were used as the original method, except modifications were made to specifically optimize for downstream sample injection and analysis with HILIC chromatography (Figure 2B). Eighty microliters of acetonitrile (in lieu of 20 µL of methanol) was added to the dried samples, shaken, and followed by addition of 20 µL (in lieu of 80 µL) of water and shaken, still resulting in 100 µL final volume for the reconstituted samples. Plates were sealed immediately with PlateLoc to prevent evaporation.

LC/MS method

Experimental LC/MS methods were largely followed as previously described,[2] except the LC gradient was adjusted to compensate for the larger delay volume of the quaternary pump used in this study. Detailed chromatography and MS parameters are provided in Tables 1 and 2.

Chromatographic conditions and MS parameters

Table 1. Agilent 1260 Infinity II Prime LC conditions.

Parameter	Value
Analytical Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 mm × 150 mm, 2.7 µm, PEEK-lined (p/n 673775 924)
Column Temperature	25 °C
Injection Volume	10 µL
Autosampler Temperature	4 °C
Needle Wash	6 seconds in wash port (50:50 water/methanol)
Mobile Phase	A) 10 mM ammonium acetate in water with 2.5 µM Agilent InfinityLab deactivator additive, pH = 9 B) 10 mM ammonium acetate in water/acetonitrile 15:85 (v:v) with 2.5 µM InfinityLab deactivator additive, pH = 9
Flow Rate	0.25 mL/min
Gradient Program	Time %B 0.00 96 1.00 96 4.50 88 7.50 88 8.00 86 13.00 86 16.00 82 22.00 65 23.00 65 23.50 96 25.00 96
Stop Time	25.00 min
Post Time	3.00 min
Observed Column Pressure	170 to 330 bar

Table 2. Agilent 6546 LC/Q-TOF conditions.

Parameter	Value
Ionization Polarity	Negative
Ionization Source	Dual AJS
Gas Temperature	225 °C
Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
VCap	3,500 V
Nozzle Voltage	0 V
Fragmentor	125 V
Skimmer	45 V
OctopoleRF Vpp	750 V
Reference Mass	*m/z* 59.01385, *m/z* 980.016375 (–)
MS Range	*m/z* 50 to 1,600
MS Acquisition Rate	1.5 spectra/s

Software

Agilent VWorks (Ver 13.1.4) automation control software was used to control the Bravo system. Agilent MassHunter Q-TOF Data Acquisition version 10.1 was used to operate the 6546 LC/Q-TOF. Agilent MassHunter PCDL Manager version B.08 SP1 was used to manage and edit the subset libraries, created from a subset of the Agilent MassHunter METLIN PCDL version B.08. MS data were quantitated using Agilent MassHunter Quantitative Analysis software (version 10.1), and the quantitative method was created from the custom PCD (with curated RT information) containing the metabolite targets. All quantifiers were based on the $[M-H]^-$ ion.

Recovery estimation

One vial of ISO1 U-^{13}C yeast extract (~2 × 10^9 *Pichia pastoris* cells) was dissolved in 400 µL of 1:1 methanol:ethanol (M:E), and insoluble particulate was removed by centrifugation. For the set of six prespike samples, 10 µL of ISO1 was added to 25 µL of thawed plasma (hand-pipetted). For the set of six postspike samples, 10 µL of 1:1 M:E was hand-pipetted to 25 µL of thawed plasma. The remainder of the low-volume automated protocol was the same, except 102.5 µL instead of 112.5 µL of 1:1 M:E was used for quenching, thereby ensuring a consistent ratio of methanol:ethanol:plasma for all experiments. Following Captiva Lipid—EMR collection, 10 µL of 1:1 M:E and 10 µL of ISO1 were added to the pre- and postspike sample wells, respectively. The plate was dried by vacuum concentration and reconstituted as described. The resulting ^{13}C-compound peak area ratios from six pairs of pre- and postspiked samples were used to calculate metabolite recoveries and % RSD values.

Assessment of automated versus manual protocol performance

Sixty 25-µL plasma samples were processed with the low-volume plasma protocol using the Bravo instrument. A manual version of the protocol was provided to three experienced technicians and each processed 20 samples. The manual protocol was designed to emulate the experimental steps of the Bravo protocol as closely as possible. Figure 3 illustrates the experimental workflow. Except for pipette tips, the same consumables were used. Hand pipettors, a benchtop vacuum filtration station, and a thermal mixer were used in lieu of the Bravo instrument and accessories. The same starting pooled plasma aliquot and the same reagent bottles were used (same lot numbers) for both the Bravo and manual experiments, and all experiments were conducted on the same day.

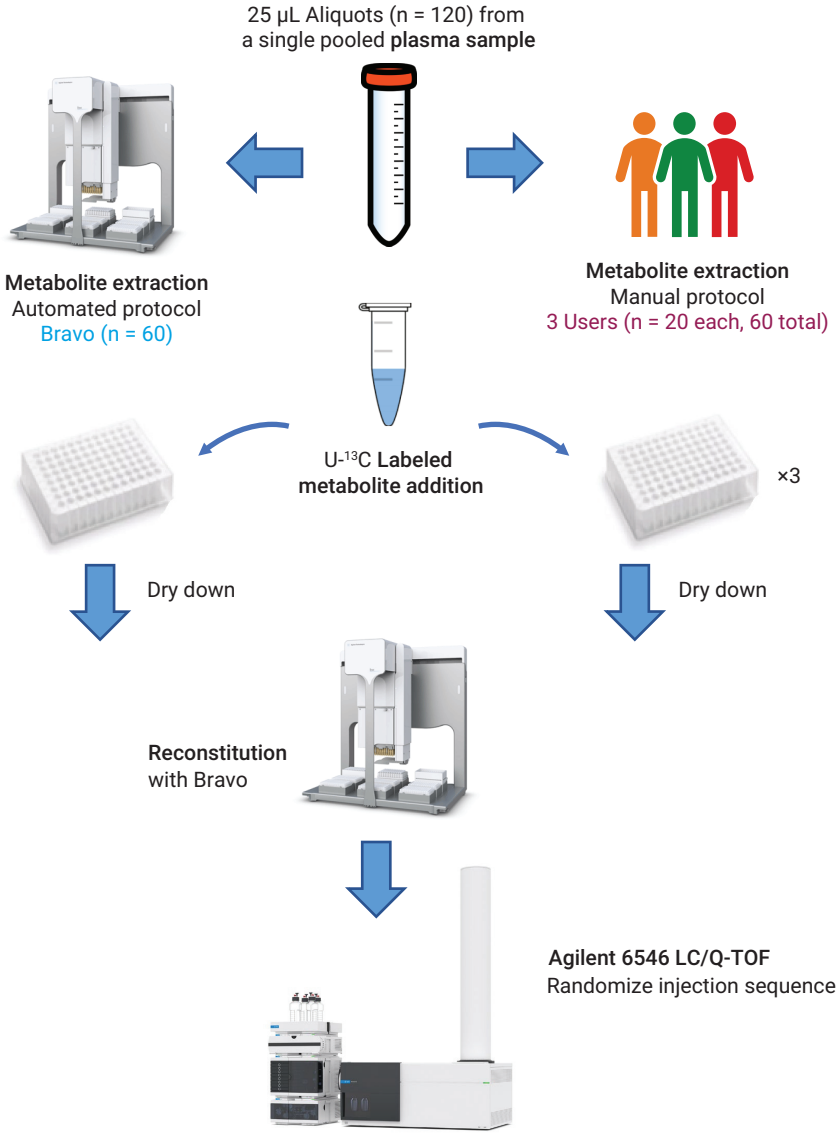

Figure 3. Workflow strategy for automation versus manual metabolite-extraction comparison.

To remove any effects of LC/MS instrument variation during data analysis, ISO1 U-^{13}C yeast extract was added to all plasma extracts following both the Bravo and manual protocols. Briefly, one vial of ISO1 yeast extract (~2 × 10^9 *Pichia pastoris* cells) was dissolved in 2 mL of water, and 8 µL was added to each sample well before drying and reconstitution. All samples were reconstituted with the Bravo protocol in preparation for HILIC-LC/Q-TOF analysis. The sample injection order was randomized.

Normalized peak areas were calculated according to Equation 1.

$$\left(\frac{\text{Peak area of endogenous } ^{12}\text{C plasma metabolite}}{\text{Peak area of corresponding } ^{13}\text{C yeast metabolite}}\right) \times \text{Average of corresponding } ^{13}\text{C metabolite peak areas across 120 samples}$$

Equation 1.

Results and discussion

Tiered selection of targets provides confident metabolite IDs

From the untargeted LC/Q-TOF datasets, lists of confident metabolite identifications were generated, with their selection based on an unbiased selection approach to obtain a representative understanding of the modified Bravo protocol performance across chemical classes (Figure 4). Selecting potential metabolites in plasma limited the experiment to the presence of corresponding metabolites in the ISO1 ^{13}C-labeled yeast extract used for recovery estimation and normalization purposes. It was additionally limited to the most abundant yeast metabolites, because a relatively low amount of ISO1 was intentionally added for spiking purposes to minimize confounding variables from the yeast matrix. Leveraging a chemical standard library (MSMLS), both MS/MS and retention time information were used to build curated subset PCDs, which were used to build methods for the MassHunter Quantitative Analysis software.

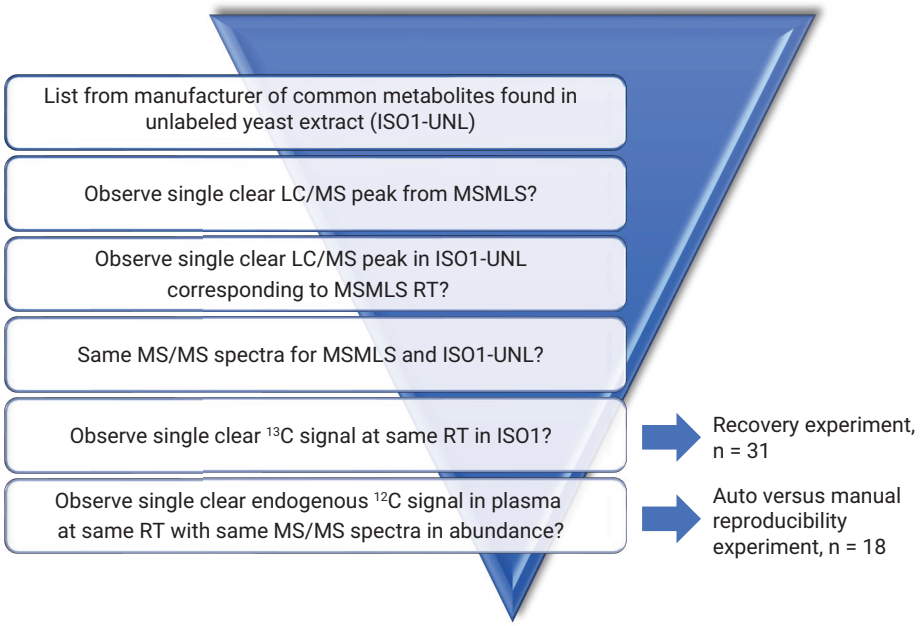

Figure 4. Metabolite selection strategy.

The low-volume method provides overall excellent metabolite recoveries

The ISO1 U-^{13}C labeled yeast extract was spiked into plasma before and after low-volume Bravo metabolite extraction, and the ^{13}C metabolite peak area ratios (prespike/postspike) were used to estimate metabolite recoveries. Figure 5 shows chromatograms for two metabolites. Figure 6 shows a histogram summarizing the recoveries, and Table 3 lists recoveries for each metabolite. Excellent recoveries (>80%) were observed for 26 of the 31 compounds covering several chemical classes. The compound D-fructose 1,6-bisphosphate showed poor recovery (38.7%), however, this compound was considered nonendogenous to plasma as it was not found at detectable levels.

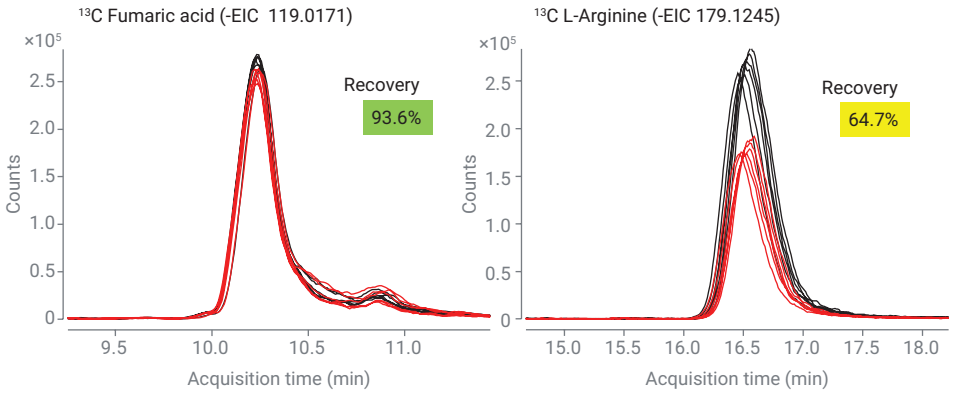

Figure 5. Example EICs for two selected metabolites across six prespike samples (red) and six postspike samples (black).

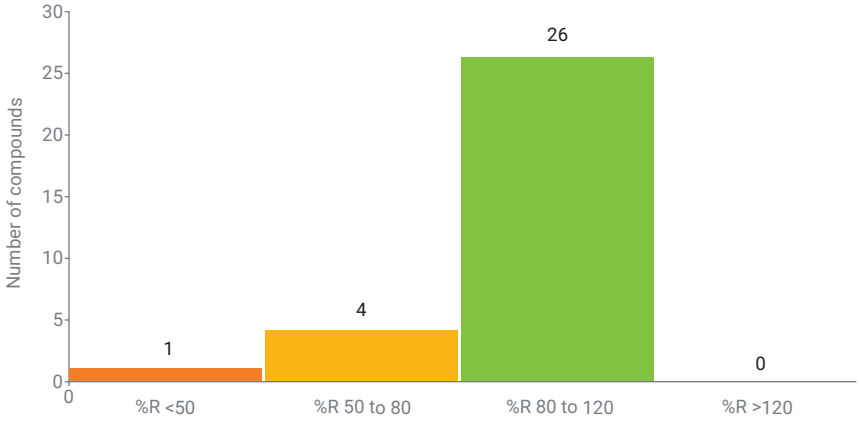

Figure 6. Histogram summary of recoveries.

Table 3. Metabolite recoveries for identified metabolites.

Spiked ^{13}C Metabolite	Endogenous to Plasma	METLIN	CAS	% Recovery	% RSD
Amino Acids and Derivatives					
Glycine	✓	20	56-40-6	91.6%	4.6%
L-Alanine	✓	11	56-41-7	89.2%	1.6%
L-Arginine	✓	13	74-79-3	64.7%	2.4%
L-Asparagine	✓	14	70-47-3	87.2%	8.4%
L-Aspartic acid	✓	15	56-84-8	91.6%	5.6%
L-Citrulline	✓	16	372-75-8	88.1%	3.0%
L-Glutamic acid	✓	19	56-86-0	92.5%	5.1%
L-Glutamine	✓	18	56-85-9	91.0%	3.3%
L-Histidine	✓	21	71-00-1	90.0%	6.3%
L-Isoleucine	✓	23	73-32-5	84.4%	9.2%
L-Leucine	✓	24	61-90-5	84.8%	6.2%
L-Proline	✓	29	147-85-3	90.1%	4.8%
L-Serine	✓	30	56-45-1	96.1%	7.8%
L-Threonine	✓	32	72-19-5	91.1%	1.8%
L-Tryptophan	✓	33	73-22-3	94.6%	8.1%
L-Tyrosine	✓	34	60-18-4	84.5%	6.1%
L-Valine	✓	35	72-18-4	78.6%	8.9%
SAH/S-adenosyl-L-homocysteine	✓	296	979-92-0	90.3%	8.6%
Nucleobases, Nucleosides, and Nucleotides					
Adenine	✓	85	73-24-5	77.1%	13.2%
5'-AMP / adenosine 5'-monophosphate	✓	34478	61-19-8	89.9%	18.1%
IMP / inosine 5'-monophosphate		3490	131-99-7	84.3%	16.4%
Uridine	✓	90	58-96-8	101.2%	10.3%
Organic Acids					
alpha-Ketoglutaric acid	✓	119	328-50-7	95.5%	10.2%
Fumaric acid	✓	3242	110-17-8	93.6%	6.9%
D-Gluconic acid	✓	345	526-95-4	92.7%	6.8%
Malic acid	✓	118	6915-15-7	84.7%	9.1%
Sugars, Sugar Alcohols, and Sugar Phosphates					
D-Arabitol		63139	488-82-4	92.3%	2.5%
D-Fructose 1,6-bisphosphate		147	488-69-7	38.7%	11.9%
D-Mannose 6-phosphate		5987	3672-15-9	72.8%	12.7%
Trehalose	✓	3479	99-20-7	84.1%	9.4%
Vitamins and Coenzymes					
Nicotinamide adenine dinucleotide (NAD)		101	53-84-9	81.3%	7.3%
			Average	**85.9%**	**7.7%**

Automation improves reproducibility

The interday reproducibility of the automated original method was previously demonstrated to be better compared to manual preparation.[1] A different but complementary approach to assess potential improvements in reproducibility offered by automation in laboratory environments where there are often multiple technicians was taken for this application note. The reproducibility of the low-volume plasma Bravo protocol was compared to a manual protocol with three different users. Figure 7 shows results for two representative metabolites, and Table 4 summarizes the results for all metabolites. Overall, Bravo metabolite extraction reproducibility was comparable to User 2, and outperformed Users 1 and 3. For all metabolites, the Bravo % RSDs were significantly lower than the combined % RSDs for the 60 manually prepared samples across the three users.

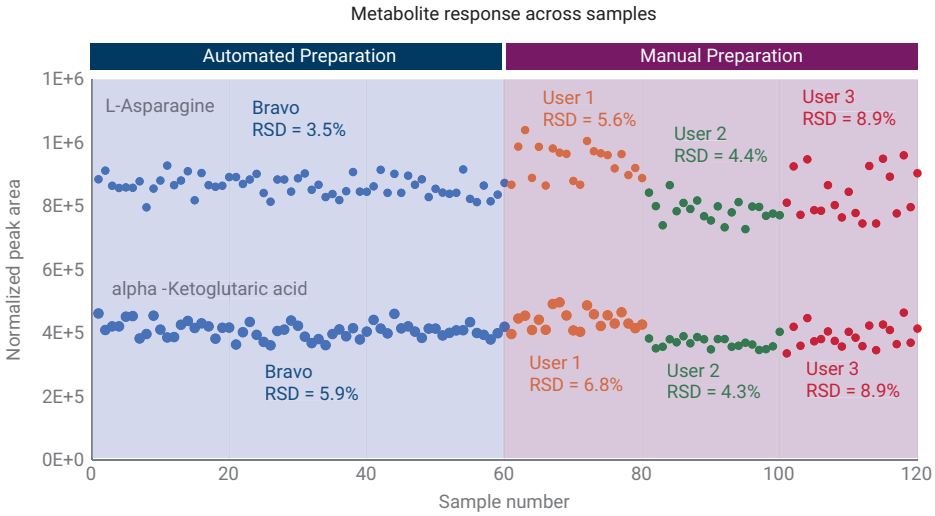

Figure 7. Normalized peak area % RSDs for L-asparagine and alpha-ketoglutaric acid (the actual injection order was randomized).

Table 4. Normalized peak area % RSDs across metabolites.

Metabolite	Bravo n = 60	User 1 n = 20	User 2 n = 20	User 3 n = 20	Users Combined n = 60
α-Ketoglutaric acid	5.9%	6.8%	4.3%	8.9%	10.2%
Fumaric acid	7.5%	5.2%	7.0%	9.8%	10.3%
Glycine	4.1%	6.2%	5.2%	7.3%	9.1%
L-Alanine	5.5%	8.0%	4.9%	9.8%	11.4%
L-Arginine	6.0%	7.6%	5.7%	13.8%	12.6%
L-Asparagine	3.5%	5.6%	4.4%	8.9%	9.9%
L-Aspartic acid	3.9%	6.2%	4.7%	9.4%	9.7%
L-Citrulline	3.1%	5.5%	2.6%	8.5%	9.4%
L-Glutamic acid	3.3%	6.4%	2.8%	9.4%	10.3%
L-Glutamine	3.6%	5.1%	2.8%	9.4%	10.1%
L-Histidine	3.2%	4.6%	2.7%	8.3%	8.5%
L-Isoleucine	7.0%	8.5%	5.2%	10.6%	11.1%
L-Methionine	5.1%	6.6%	3.4%	8.3%	10.6%
L-Ornithine	4.9%	6.8%	6.2%	13.2%	12.3%
L-Proline	6.6%	8.9%	5.3%	11.6%	12.4%
L-Serine	3.6%	5.3%	5.2%	8.5%	9.4%
L-Threonine	4.7%	5.0%	4.2%	10.5%	10.5%
Malic acid	5.0%	4.9%	4.4%	6.8%	7.5%
Average	**4.8%**	**6.3%**	**4.5%**	**9.6%**	**10.3%**

Conclusion

Modifications to the Bravo Metabolomics Sample Prep Platform that reduced the required starting plasma volume from 100 to 25 µL were described in this application note. Excellent metabolite recovery with the method was demonstrated across representative chemical classes of compounds. Additionally, the automated method offers improved reproducibility when compared to a laboratory environment where multiple users manually processed samples.

References

1. Automated Metabolite Extraction for Plasma using the Agilent Bravo Platform. *Agilent Technologies technical overview*, publication number 5994-0685, **2019**.
2. Discovery Metabolomics LC/MS Methods Optimized for Polar Metabolites. *Agilent Technologies application note*, publication number 5994-1492, **2019**.

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RA44175.4137847222

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Printed in the USA, December 8, 2020
5994-2156EN

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