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Assessment of a Metabolomics Automated Sample Prep Platform for Low Volume Plasma Samples

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Introduction

In basic and translational research settings, sample preparation prior to 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 inter-day reproducibility. Additionally, in some research settings limited amounts of plasma can be obtained from infants/children or from animal models. Here we evaluate a modification to an existing automated metabolomics sample prep method to accommodate low volume plasma samples (25 µL). This method precipitates plasma proteins to quench enzymatic activity, depletes lipids, and extracts metabolites, providing a clean metabolite sample for LC/MS analysis. With this modified protocol we evaluated metabolite recovery and reproducibility compared to a manual preparation processed by multiple laboratory staff.

Experimental

Samples and Reagents

A single healthy pooled human plasma sample (BioIVT) was used for all experiments. Chemical standards from the MSMLS library (IROA Technologies) were individually acquired with the LC/MS method to obtain retention times and MS/MS spectra. Unlabeled and ^{13}C-labeled yeast metabolite extracts ("ISO1-UNL" and "ISO1", Cambridge Isotopes) were used to aid in metabolite identification. ISO1 was additionally used as a spike-in for recovery estimation and normalization purposes.

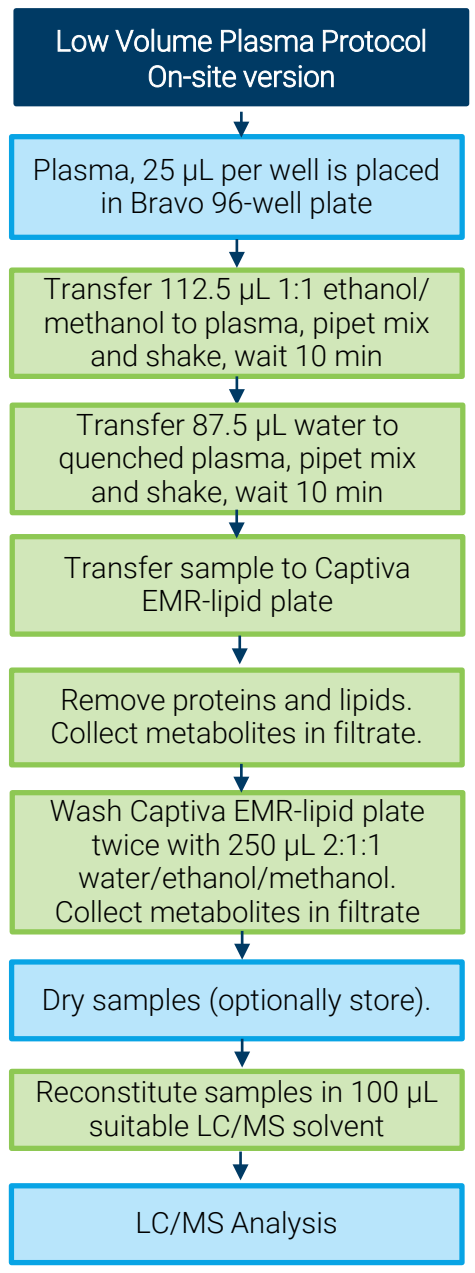
Software

Compounds confidently identified in plasma and yeast samples were used to create a subset Personal Compound Database and Library (PCDL) from the Agilent METLIN PCDL. The custom PCDL with curated retention times was imported by Agilent MassHunter Quantitative Analysis software (Ver 10.1) to easily create a quantitative method.

Method and Workflow Overview

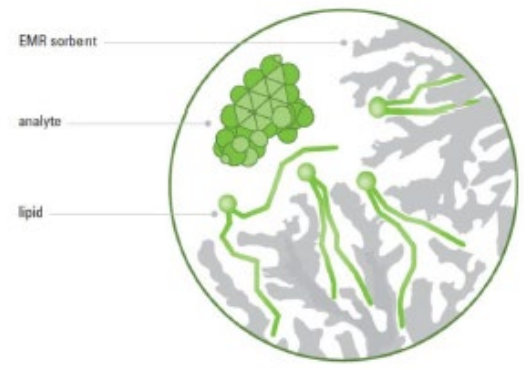
Beginning with the Agilent Bravo Metabolomics Sample Prep Platform[1], modifications were made to the protocol that include reducing the starting plasma volume from 100 µL to 25 µL:

Experimental



Bravo automation steps shown in green

Captiva EMR-Lipid 96-well plate traps lipids efficiently



InfinityLab Poroshell 120 HILIC-Z column

6546 LC/Q-TOF System

A 1260 Infinity II Prime LC system was coupled to a 6546 LC/Q-TOF with a Jet Stream ionization source. Negative-ion mode LC conditions and MS parameters were very similar to those previously described[2].

Tiered Selection of Targets Provide Confident Metabolite IDs

An approach was taken to select only the most confident metabolite identifications for the following studies (Fig 1).

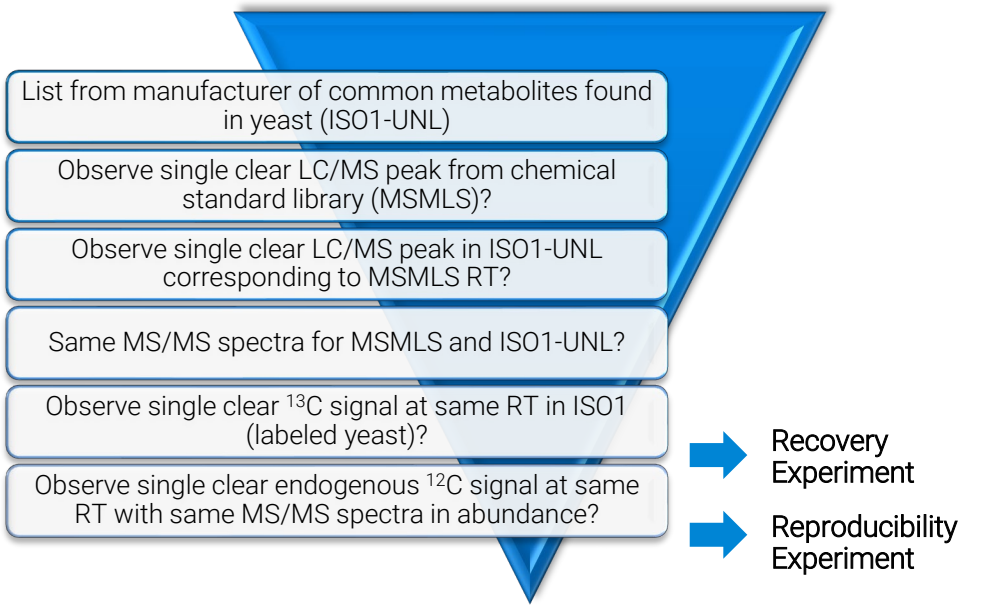


Figure 1. Metabolite selection strategy

Method Provides Overall Excellent Metabolite Recoveries

The ISO1 ^{13}C-labeled yeast extract was spiked into plasma before and after low volume Bravo metabolite extraction. The ^{13}C-compound peak area ratios from six pairs of pre- and post-spiked samples were used to calculate recovery.

Fig 2 shows example chromatograms for two metabolites. Fig 3 shows a histogram summarizing the recoveries, and Table 1 lists individual results. Excellent recoveries (>80%) were observed for 28 of the 32 compounds covering diverse chemical classes. One compound showed poor recovery (D-fructose 1,6-bisphosphate, 38.7%). However, this compound was considered nonendogenous as it was not found at detectable levels in plasma.

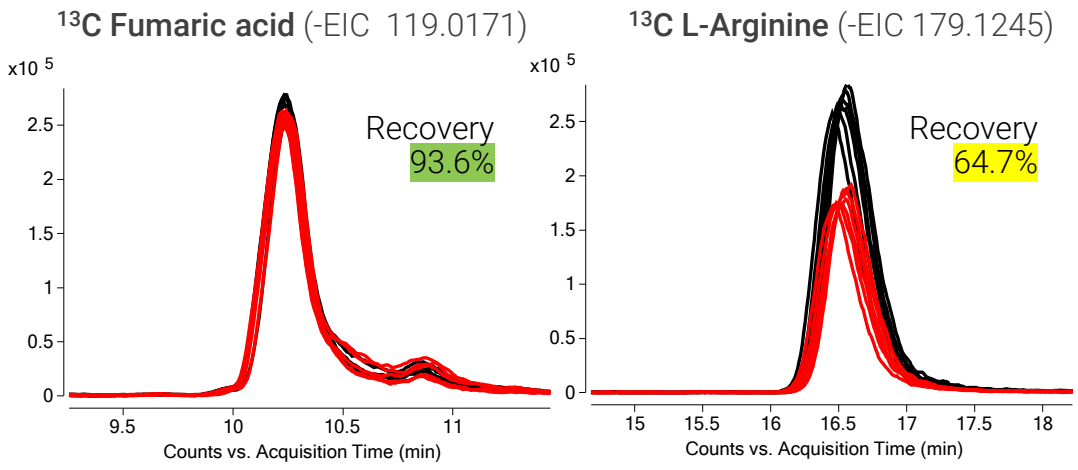


Figure 2. Example EICs for two selected metabolites across 6 pre-spike samples (red) and 6 post-spike samples (black)

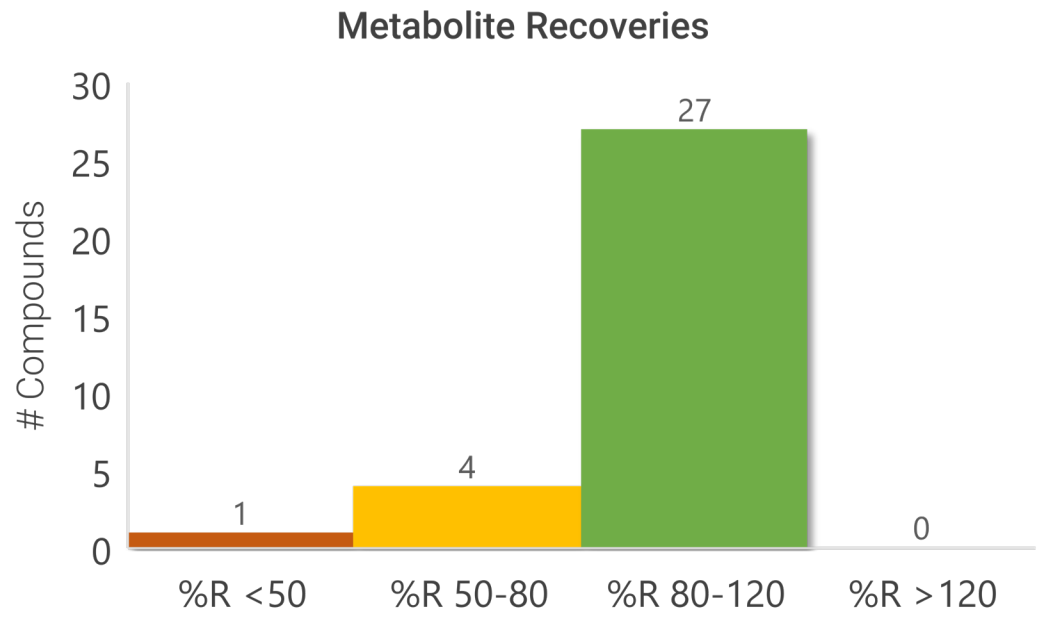


Figure 3. Summary of recoveries

Spiked ^{13}C Metabolite	Endogenous to Plasma	% Recovery	% RSD
Amino Acids and Derivatives			
Glycine	✓	91.6%	4.6%
L-Alanine	✓	89.2%	1.6%
L-Arginine	✓	64.7%	2.4%
L-Asparagine	✓	87.2%	8.4%
L-Aspartic Acid	✓	91.6%	5.6%
L-Citrulline	✓	88.1%	3.0%
L-Glutamic acid	✓	92.5%	5.1%
L-Glutamine	✓	91.0%	3.3%
L-Histidine	✓	90.0%	6.3%
L-Isoleucine	✓	84.4%	9.2%
L-Leucine	✓	84.8%	6.2%
L-Proline	✓	90.1%	4.8%
L-Serine	✓	96.1%	7.8%
L-Threonine	✓	91.1%	1.8%
L-Tryptophan	✓	94.6%	8.1%
L-Tyrosine	✓	84.5%	6.1%
L-Valine	✓	78.6%	8.9%
SAH / S-Adenosyl-L-homocysteine	✓	90.3%	8.6%
Nucelobases, Nucleosides, and Nucleotides			
Adenine	✓	77.1%	13.2%
5'-AMP / Adenosine 5'-monophosphate	✓	89.9%	18.1%
IMP / Inosine 5'-monophosphate		84.3%	16.4%
Uridine	✓	101.2%	10.3%
Organic Acids			
alpha-Ketoglutaric acid	✓	95.5%	10.2%
Fumaric acid	✓	93.6%	6.9%
D-Gluconic acid	✓	92.7%	6.8%
Malic acid	✓	84.7%	9.1%
Sugars, Sugar Alcohols, and Sugar Phosphates			
D-Arabitol		92.3%	2.5%
D-Fructose 1,6-bisphosphate		38.7%	11.9%
D-Mannose 6-phosphate		72.8%	12.7%
Trehalose	✓	84.1%	9.4%
Vitamins and Coenzymes			
Nicotinamide adenine dinucleotide (NAD)		81.3%	7.3%
	Average	86.2%	8.2%

Table 1. Metabolite Recoveries

Results and Discussion

Automation Improves Reproducibility

Bravo
Automation

versus

Manual Preparation
3 Users

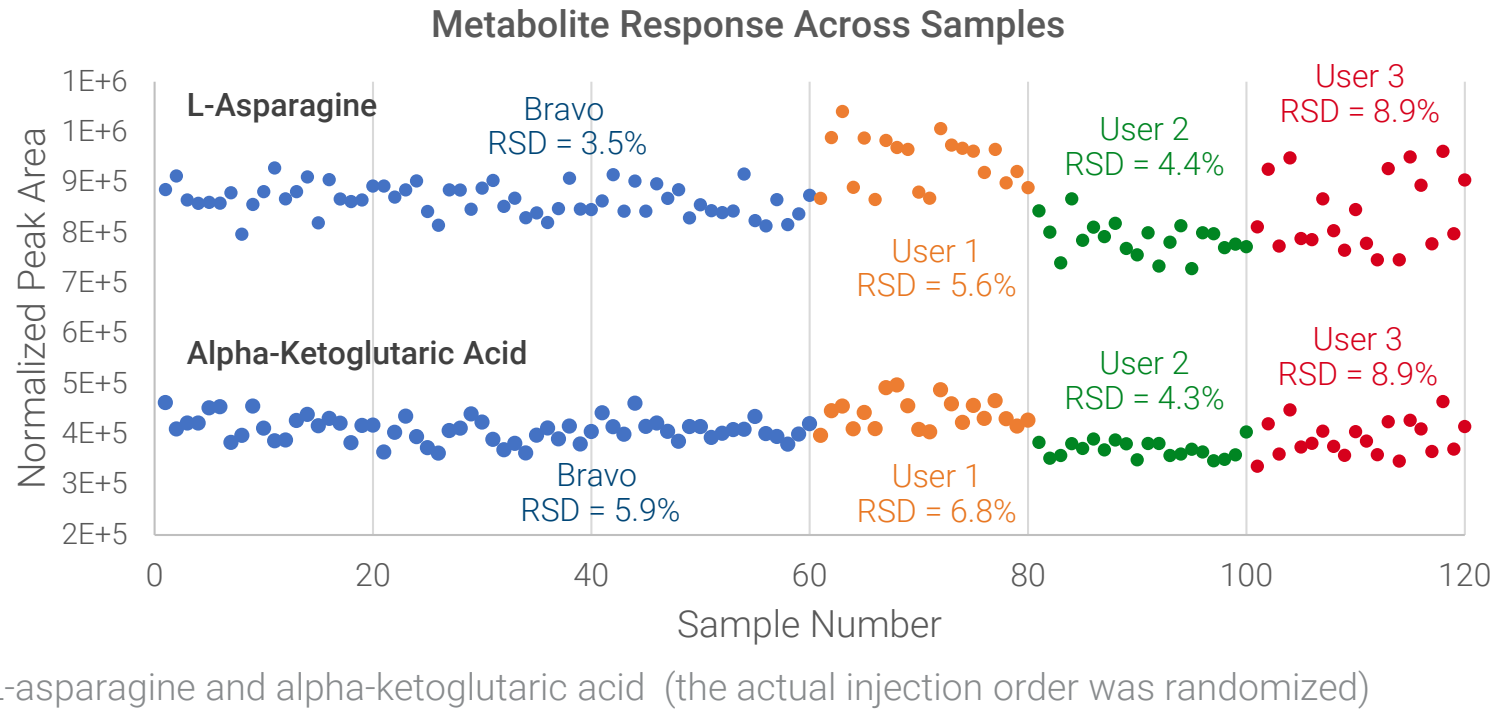


Figure 4. Normalized peak area % RSDs for L-asparagine and alpha-ketoglutaric acid (the actual injection order was randomized)

The performance of the automated method was compared against manual preparation. Sixty 25-µL plasma samples were processed with the low volume plasma protocol using the Bravo instrument. A manual version of the protocol, with the same key steps, was provided to three experienced technicians and each processed twenty samples. Prior to drying and reconstitution, a ^{13}C metabolite extract was added for normalization purposes to remove effects from LC/MS instrument variation. The sample injection order was randomized. Fig 4 shows results for two representative metabolites, and Table 2 summarizes the results for all metabolites. Bravo metabolite extraction reproducibility was comparable to User 2, and outperformed User 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.

	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%**

Table 2. Normalized peak area % RSDs across metabolites

Conclusions

We describe modifications to the Agilent Bravo Metabolomics Sample Prep Platform that reduce the required starting plasma volume from 100 µL to 25 µL. Excellent metabolite recovery with the method was demonstrated across representative chemical classes of compounds. We also showed that 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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