Semiautomated Device for Batch Extraction of Metabolites from Tissue Samples

James J. Ellinger,[†,‡] Dan C. Miller,[†] Ian A. Lewis,[§] and John L. Markley*,[†,‡]

[†]National Magnetic Resonance Facility at Madison, Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, Wisconsin 53706-1544, United States

[‡]DOE Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, Wisconsin 53706-1544, United States

[§]Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey 08540, United States

Ⓢ Supporting Information

ABSTRACT: Metabolomics has become a mainstream analytical strategy for investigating metabolism. The quality of data derived from these studies is proportional to the consistency of the sample preparation. Although considerable research has been devoted to finding optimal extraction protocols, most of the established methods require extensive sample handling. Manual sample preparation can be highly effective in the hands of skilled technicians, but an automated tool for purifying metabolites from complex biological tissues would be of obvious utility to the field. Here, we introduce the semiautomated metabolite batch extraction device (SAMBED), a new tool designed to simplify metabolomics sample preparation. We discuss SAMBED's design and show that SAMBED-based extractions are of comparable quality to extracts produced through traditional methods (13% mean coefficient of variation from SAMBED versus 16% from manual extractions). Moreover, we show that aqueous SAMBED-based methods can be completed in less than a quarter of the time required for manual extractions.

Metabolomics sample preparation methods can be divided into three main phases, (1) homogenization, (2) metabolite extraction, and (3) sample filtration. Each of these steps plays a direct role in the overall yields and error rates associated with metabolite isolation from biological tissues. Given the paramount importance of consistent sample preparation to metabolomics,[1−4] surprisingly few tools are available for automating sample preparation and ensuring consistent metabolite extraction. This is particularly problematic for nuclear magnetic resonance (NMR) spectroscopic studies, which require large sample sizes and substantial volumes of solvents.

Several commercial devices are currently available to automate sample preparation: the Precellys 24 (Bertin Technologies), gentleMACS Dissociator (Miltenyi Biotec), and Tissuelyser (Qiagen). Furthermore, recent studies have shown that these devices are effective for medium to high throughput preparation of metabolite extracts.[5−8] However, these devices focus on automating the homogenization process, whereas the addition of extraction solvents and sample filtration have not been fully streamlined.

In this report, we introduce the semiautomated metabolite batch extraction device (SAMBED). SAMBED is a new tool that supports the parallel extraction of metabolites from NMR-scale samples. Our goal in designing SAMBED was to integrate all of the requisite steps of sample preparation into a single platform while maintaining flexibility with respect to a range of extraction solvents. Consequently, SAMBED was constructed from autoclavable materials that are tolerant of both water and organic solvents and operates effectively at temperatures ranging from 4 to 100 °C. Our prototype accommodates six parallel extractions and is designed for processing large samples (0.05−1 g of tissue). Although SAMBED's scale makes it most appropriate for NMR-based metabolomics, the design could be rescaled for smaller samples, such as those used in mass spectrometry.

SAMBED is composed of six integrated components: (1) milling chamber, (2) vibrational shaker, (3) solvent reservoir, (4) homogenization platform, (5) filtration chamber, and (6) filtration platform (Figures 1 and S1−5, Supporting Information). The system is powered by compressed air supplied from a conventional air compressor. Biological tissues are placed in the milling chamber and are homogenized by ball milling in the vibrational shaker. Our custom milling chamber has a pneumatically controlled plunger in its base that allows extraction solvents to be injected directly into the chamber and raw extracts to be transferred to the downstream filter chambers (Figure S1, Supporting Information). A preallocated
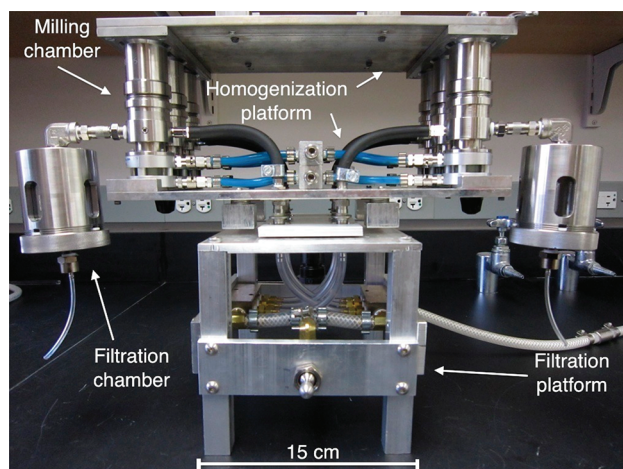
Figure 1. Photograph of the major components of the assembled SAMBED. The air compressor and vibrational shaker are not shown. For more detailed photographs of the components, see Figures S1−5 in the Supporting Information.

volume of extraction solvent flows from the solvent reservoir into the milling chamber by gravity. Homogenate is then transferred via the fluid delivery system to a filtration chamber, where metabolites are separated from cellular debris and macromolecules by ultrafiltration.

■ EXPERIMENTAL SECTION

Manual Sample Processing. Bovine liver was obtained from a local grocery store. The liver was frozen, lyophilized, and aliquoted into 500−600 mg (large samples) or 100−150 mg (small samples) portions. Metabolites were extracted by following established aqueous[9−11] or organic protocols.[3,12] Briefly, dry liver samples (large samples for aqueous extractions and small samples for organic extractions) were homogenized with a rounded glass rod and then suspended in either 16 mL of 95 °C deionized water (aqueous extraction) or 3 mL of −20 °C 40:40:20 Acn/MeOH/H$_2$O (organic extraction). Aqueous extractions were vortexed and incubated in a 95 °C water bath for 7.5 min and then placed on ice for 10 min to cool. Organic extractions were vortexed and stored at 4 °C for 15 min. Following extraction, all samples were vortexed and centrifuged at 10 000 rpm for 10 min (4 °C). Supernatants from aqueous extracts were transferred to prewashed centrifugal microfilters (3000 Da cutoff, Sartorius Biolab Products) and centrifuged at 4100 rpm for 10.5 h. The long centrifuge time was necessary for passing the entire sample through the filter membrane (excluding the 200 μL dead volume). Supernatants from organic extracts were decanted into a fresh tube, and the pellet was re-extracted twice with 2 mL of Acn/MeOH/H$_2$O, incubated for 5 min at 4 °C, and centrifuged. Supernatants from the two organic wash steps were combined with the original extract to yield a single 7 mL extract from each sample. All metabolite extracts were frozen, lyophilized, and stored dry at −80 °C until NMR analysis.

Sample Processing by SAMBED. SAMBED was kept at room temperature for aqueous extractions and was conducted in a 4 °C cold room for organic extractions. Lyophilized liver samples (large and small samples for aqueous extractions and small samples for organic extractions) were placed in each of the six milling chambers along with a 1.8 mm diameter grinding ball. Dry samples were milled on the shaker platform for 30 s,

and either 17 mL of 95 °C ddH$_2$O (aqueous extractions) or 8 mL of −20 °C 40:40:20 Acn/MeOH/H$_2$O (organic extractions) were injected from the solvent reservoir into each chamber. Samples were wet-milled for an additional 30 s. For aqueous extractions, the homogenization platform was submerged in a 95 °C water bath for 7.5 min. The homogenization platform was coupled to the filtration platform, and six filtration chambers (prechilled for 1 h to 4 °C) containing prewashed ultrafiltration membranes (3000 Da cutoff, Millipore) were attached. The contents of each milling chamber were transferred to the downstream filter chamber under compressed air at 35 psi. The pressure was increased to 70 psi, and filtration was allowed to progress until most of each sample had passed through the filtration membrane. Each milling chamber was flushed with 5 mL of ddH$_2$O, and filtration was allowed to progress until outflow from the filter chambers ceased. Aqueous extracts were collected in tubes placed on ice. All metabolite extracts were frozen, lyophilized, and stored at −80 °C until NMR analysis.

NMR Analysis. All dry metabolite extracts were dissolved in 800 μL (large samples) or 200 μL (small samples) of D$_2$O containing 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS, chemical shift standard) and 500 μM NaN$_3$ (microbial growth inhibitor). The resulting solution was titrated with concentrated DCl or NaOD as needed to achieve a glass electrode pH reading of 7.40 ± 0.01. NMR data were collected at the National Magnetic Resonance Facility at Madison on a 600 MHz Bruker Avance III spectrometer equipped with a triple-resonance (^{1}H, ^{13}C, ^{15}N, ^{2}H lock) 1.7 mm cryogenic probe. The probe was tuned, matched, and locked to deuterium for the first sample. Each sample was shimmed, and the 90° pulse width was determined. A 2D ^{1}H−^{13}C HSQC spectrum (Bruker sequence hsqcetgpsisp2.2) was then collected for each sample. Data were processed using custom NMRPipe scripts written in-house.[13] Metabolites were identified and quantified using the rNMR software package following established methods.[14,15] Briefly, metabolites were identified by submitting peak lists to the Madison-Qingdao Metabolomics Consortium Database (MMCD);[16] assignments were verified by overlaying NMR spectra of standards from the BioMagResBank (BMRB).[17] Metabolite concentrations were calculated on the basis of calibration curves from metabolite standards prepared at 2, 5, and 10 mM. Peak amplitudes used for quantitation were obtained by fast maximum likelihood reconstruction as implemented in the Newton software package.[18]

Statistical Analysis. To measure the relative consistency of manual versus SAMBED-based preparations, we computed the coefficient of variation (CV) observed for each metabolite across the 18 replicates of each sample preparation method. Overall variability was then expressed as the mean CV associated with each method. All calculations were performed using the R statistical software program (www.r-project.org).

■ RESULTS AND DISCUSSION

One of the primary motivations for developing SAMBED was to make sample preparation more efficient by automating and parallelizing the metabolite purification process. Consequently, we measured the time required to prepare 18 samples (three trials of 6 samples each) via established aqueous[9−11] and organic extraction methods.[3,12] Sample preparation times were compared between manual and SAMBED-based extractions (Table 1). As expected, SAMBED greatly decreased the time required to prepare samples. SAMBED-derived extracts were

Table 1. Average Times Required to Prepare Metabolite Extracts by Manual and SAMBED-Based Protocols[a]

protocol	prefiltration time (min)	filtration time (min)	total time (min)
A (aqueous, manual)[b]	51.14 ± 5.62	630 ± 0.00	681 ± 6
B (aqueous, SAMBED)[b]	17.02 ± 0.55	145 ± 3	162 ± 4
C (aqueous, SAMBED)[c]	18.11 ± 0.43	68.0 ± 0.9	86.1 ± 0.5
D (organic, manual)[c]	51.22 ± 3.83	30.0 ± 0.0[d]	81.2 ± 3.8
E (organic, SAMBED)[c]	N/A[e]	N/A[e]	88.7 ± 7.8

[a]Data are reported as the mean of three trials ± the range/2. [b]Large sample (500−600 mg per sample). [c]Small sample (100−150 mg per sample). [d]Time required for high speed centrifugation. [e]Separating the prefiltration and filtration times was not possible.

generated in 21% of the time required for manual preparation of aqueous extracts. Most of the time savings are attributable to the ultrafiltration step, which is 5 times more efficient by SAMBED because of its large surface area filters. For organic extractions, SAMBED-based extractions required slightly more time than manual preparation (81 versus 89 min). However, these times are not directly comparable because the SAMBED-based organic extractions were subject to microfiltration whereas the manual sample processing method omitted this step. In contrast to the centrifugal microfilters, the filter membranes used by SAMBED are resistant to the Acn/MeOH/H$_2$O solvent used in this study. Our data show that the additional microfiltration step only adds ∼10% to the total processing time and comes with the clear benefit of reduced labor.

A second motivation for developing SAMBED was to standardize sample processing by eliminating manual sample manipulation. To measure SAMBED's success in producing consistent metabolite extracts, we measured variations in 29 metabolite levels observed in 18 liver extracts produced via traditional versus our new SAMBED-based protocol. Two established methods, one organic the other aqueous, were evaluated in this study. For each metabolite, we compared the yields per gram of liver and the average variability of metabolite levels associated with the different protocols (Table S1, Supporting Information). As expected, SAMBED-generated extracts were comparable to those prepared by an experienced technician. Metabolite concentrations observed in SAMBED extracts were linearly related to those observed in manual extractions across multiple orders of magnitude (Figure 2). Moreover, the mean CV of metabolites observed in aqueous SAMBED extracts was 12.3% whereas manual sample preparation resulted in a mean CV of 13.6% (N = 522). Manual preparation of organic extracts was the least consistent protocol tested with a mean CV of 18.1%; this variability was reduced to 14.1% when extracts were prepared by SAMBED. We attribute the more consistent performance of the organic SAMBED-based method to the microfiltration step, which is not possible via the traditional method due to membrane/solvent incompatibility.

Previous research has shown automated sample preparation is feasible and can produce consistent results.[5−8] Minimizing technical error is a critical design feature of automated sample preparation tools. The mean CV values for SAMBED-based extracts reported here (12−14%) compare favorably to those reported for other automated tools (15−30%).[7,8]
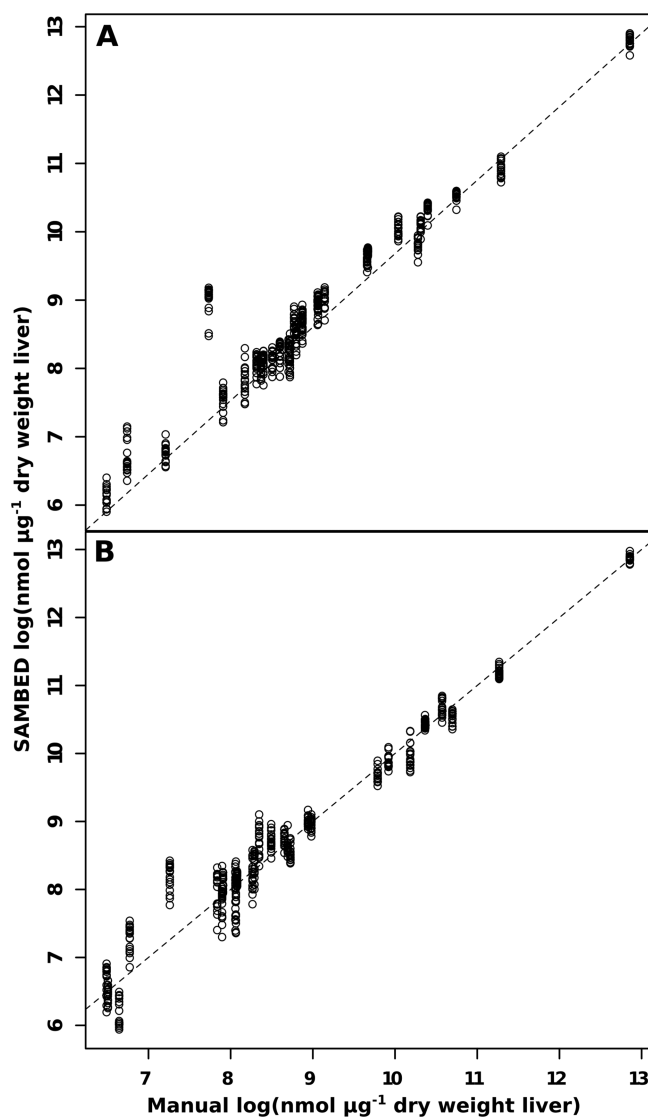
Figure 2. Linear relationship of the metabolite levels measured by manual or SAMBED processing for (A) aqueous and (B) organic extractions. SAMBED-derived metabolite levels (N = 522) are plotted relative to the mean abundance for each metabolite observed in the manual-derived extracts. The dotted black line indicates the theoretical ideal regression (slope = 1). In the case of aqueous extractions, only data from large samples are shown.

■ CONCLUSIONS

We have developed and tested SAMBED, a new device that streamlines and automates the isolation of metabolites from biological tissues. SAMBED consistently generates metabolite extracts that are of comparable, or slightly better, quality than those generated by traditional methods. Our design allows aqueous extractions to be completed in a fraction of the time required for manual sample processing, and the materials used in SAMBED support a wide range of extraction conditions. In summary, SAMBED simplifies one of the most laborious aspects of metabolomics studies without affecting data quality. A complete listing of metabolite levels observed in this study and photographs of SAMBED components are available in the Supporting Information.

ASSOCIATED CONTENT

S Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: (608) 263-9349. E-mail: markley@nmrfam.wisc.edu.

Notes

The authors declare no competing financial interest.

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