Analytical Methods

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In Vivo Solid-Phase Microextraction: Capturing the Elusive Portion of Metabolome**

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The main objective of metabolomics is the analysis of all low-molecular-weight compounds present in a particular living system. Metabolomics data is complementary to proteomics, genomics, and transcriptomics data and provides a better understanding of dynamic processes occurring in living systems.[1] The processes of sampling and sample preparation can significantly affect the composition of the measured metabolome, so the analytical results may not adequately reflect the true metabolome composition at the time of sampling.[2–4] This is due primarily to poor efficiency (or even complete omission) of metabolism quenching step and multi-step handling procedures, which contribute to inadvertent metabolite loss and/or degradation.

Herein we introduce in vivo solid-phase microextraction (SPME) as a new sample preparation method for global metabolomics studies of living systems using liquid chromatography–mass spectrometry (LC-MS). SPME is a non-exhaustive sample preparation procedure in which the amount of analyte extracted is governed by the distribution coefficient of the analyte between the SPME coating and sample matrix if the equilibrium is reached or the rate of mass transfer if a short sampling time is used.[5] In vivo SPME allows accurate extraction of the metabolome directly in the tissue or blood of freely moving animals without the need to withdraw a representative biological sample for analysis, under conditions of negligible depletion where the amount of analyte extracted by SPME is independent of the sample volume.[5–7] The blood-draw-free nature of the sampling method facilitates multiple sampling of the same living system and can capture unstable or short-lived metabolites. Large biomolecules are not extracted by the specially selected biocompatible SPME coating, so the need for a metabolism quenching step is eliminated. The amount of metabolites extracted is proportional to the biologically active unbound concentration. For metabolomics studies, in vivo SPME provides the simplest and most rapid sample preparation tool available to date to study living systems in a format directly compatible with LC-MS detection. Although SPME was successfully applied to metabolomics studies using GC-MS primarily in headspace mode,[8–12] its capability to provide instantaneous metabolism quenching directly during the sampling process to capture true metabolome of blood or tissue has not been previously evaluated.

First, we developed a successful in vivo SPME workflow for direct sampling of metabolome, and applied it to mice as a model system (Figure 1). In this approach, a coated SPME fiber is housed inside hypodermic needle,[13] which is used to pierce the sampling interface containing circulating blood. The fiber is exposed to blood for a pre-set short sampling time of 2 min. During the sampling, analytes are extracted directly into the SPME coating. The key aspect of developing SPME device for metabolomics was selection of the chemical nature of the coating to ensure simultaneous extraction of both hydrophilic and hydrophobic species. We found that mixed-mode, phenylboronic acid, and polystyrenedivinylbenzene polymers were particularly suited for sampling of metabolome.[14] The coating does not extract proteins because of the pore size of the sorbent, and the surface of the coating is protected with a thin-layer of biocompatible polymer, which minimizes protein adhesion to the surface of the coating. Therefore, all the steps of sampling, extraction, and metabolism quenching are combined into a single step. As soon as a

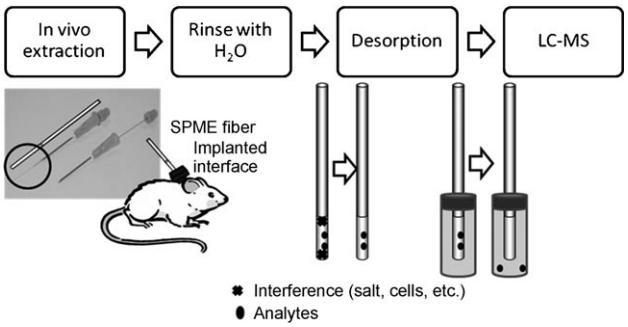

Figure 1. Overview of the main steps of the in vivo SPME procedure. The photograph (lower left) shows the in vivo SPME device (Supelco), which is housed within a hypodermic needle.

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metabolite partitions into the coating, it is isolated from the components of the matrix, which can cause its degradation or conversion. This ensures metabolite levels are representative of those in the living system at the time of sampling and are unaffected by enzymatic conversions post-sampling, which can occur owing to inefficient metabolism quenching when employing traditional methods based on blood withdrawal. After the removal of the SPME device from the living system, the SPME probe is briefly rinsed with purified water to eliminate any remaining loosely attached droplets, and the extracted small molecules are desorbed from the coating using an appropriate organic solvent. The desorption step of the proposed SPME workflow is the most time-consuming (60 min), but overall sample throughput can be further enhanced by performing the desorption for all samples in parallel in a 96-well plate format with automation.[15] This reduces the total sample preparation time per sample to less than 5 min, making it one of the most simple and rapid workflows in metabolomics reported to date. Figure 2 A compares the repeatability of in vivo SPME procedure for repeated sampling of the same animal versus the sampling of 8 individual animals. In general, the results show that interanimal variability in unbound (free) metabolite concentrations is much higher than the variability in the concentrations for the repeated samplings of the same animal. The results also show that the technique can be successfully used to study short-lived and/or unstable metabolites, such as glutathione, retinol, and adenosine. Interestingly, adenosine concentrations appear to be highly variable for the same

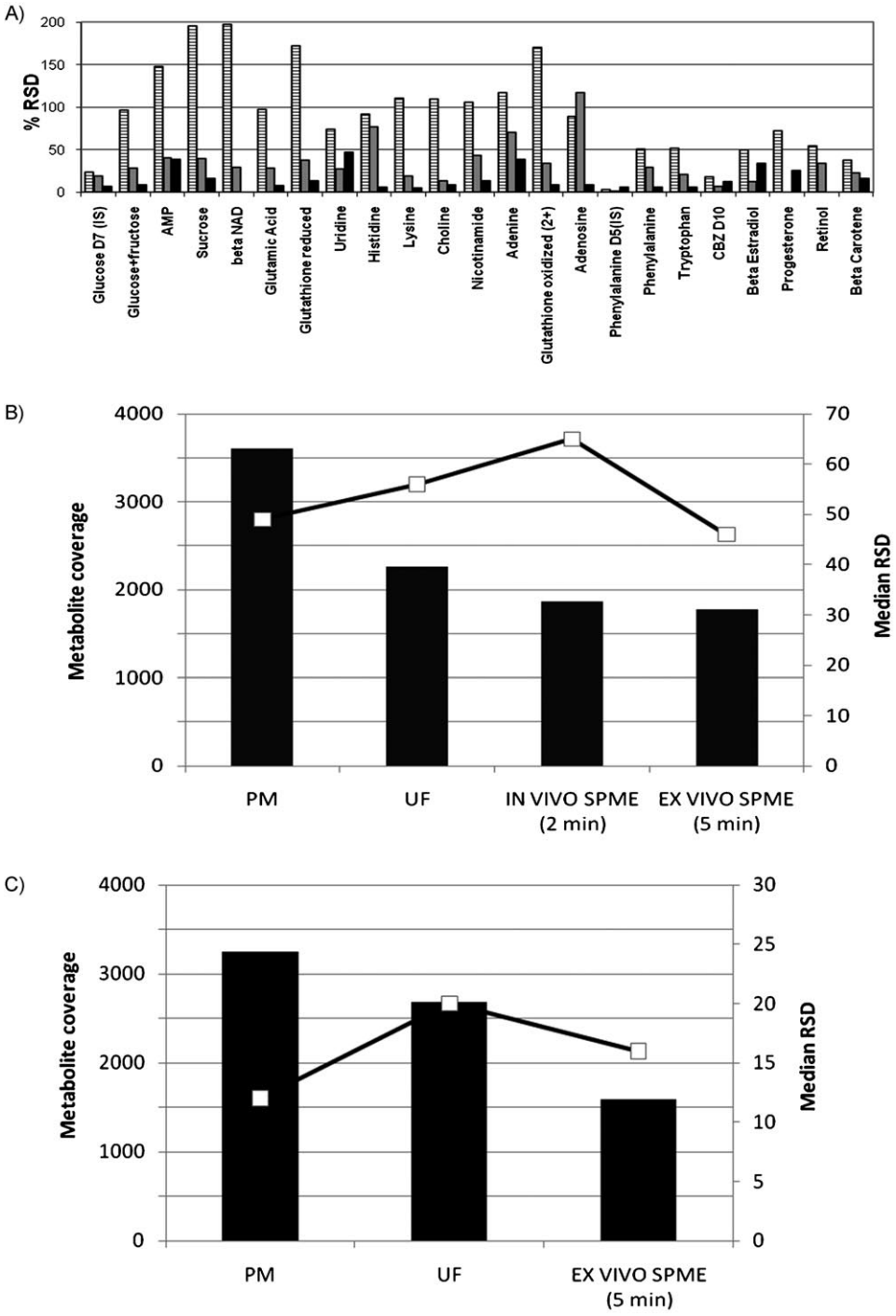

Figure 2. Results of in vivo SPME study for sampling of circulating mouse blood. A) Comparison of intra-animal (gray bars; $n=5$ samplings of the same animal) and inter-animal variability (striped; $n=8$ animals) in free concentrations of identified metabolites. The results for pooled QC sample (black; see Supporting Information) are included for comparison as an indication of LC-MS system performance throughout the run. B) Comparison of in vivo SPME performance versus ultrafiltration (UF), solvent precipitation (PM), and ex vivo SPME expressed as metabolite coverage (number of features detected; bars) and method precision (median RSD of signal intensity of all features detected by each method for $n=4$ mice; data points, secondary *y*-axis). C) Comparison of ex vivo SPME performance versus ultrafiltration (UF) and solvent precipitation (PM) for repeated extraction ($n=7$) of pooled human plasma sample expressed as metabolite coverage (number of features detected; bars) and method precision (median RSD of signal intensity of all features detected by each method; data points, secondary *y*-axis).

animal on the timescale of the experiment (five consecutive 2 min samplings), which is in line with previous findings in humans[16] and is likely a consequence of adenosine involvement in multiple pathways, including blood flow regulation, cell energy homeostasis, and neurotransmission. Overall, the results illustrate good repeatability of in vivo SPME technique and its suitability to study interindividual variability and also temporal changes in metabolite concentrations. The repeated sampling of mice over short time periods is usually limited by the requirement to sample $\leq 20\,\%$ of total blood volume, whereas in vivo SPME presents an opportunity to sample same animal repeatedly over short time periods without any limitations in the number of samples that can be taken.

The optimized SPME workflow was subsequently used to perform full metabolomic study on mice ($n = 4$; Supporting Information, Figure S1) after in vivo administration of 2 mg kg^{-1} carbamazepine to investigate any advantages of in vivo SPME sampling versus ex vivo approaches based on blood withdrawal: ex vivo SPME, ultrafiltration, and plasma protein precipitation[17] using methanol/ethanol (PM). Figure 2B shows metabolite coverage and method precision obtained for all four methods studied. The coverage by SPME was significantly lower (3609 versus 1868 features) than by solvent precipitation (PM). This may be attributed to the fact that solvent precipitation methods disrupt any protein binding so that the amount extracted is proportional to the total metabolite concentration and/or lower analytical sensitivity of SPME method owing to the non-exhaustive nature of the extraction (Supporting Information, Figure S2). Ultrafiltration detected a similar number of features to SPME (2262 versus 1868), but the examination of ion maps (retention times versus m/z ratio) for each method (Figure 3) showed that the use of ultrafiltration results in a significant loss of hydrophobic metabolites by adsorption to the membrane, as indicated by the very few metabolites observed with retention times of more than 10 minutes in both positive and negative ESI modes. This data is in agreement with NMR metabolomic studies, where an additional method for extraction of hydrophobic species from the ultrafiltration membrane was proposed to overcome this difficulty.[18] However, this significantly reduces sample throughput and introduces new sample preparation steps which can cause inadvertent analyte loss or degradation. In contrast, SPME is able to provide a balanced coverage of both polar and hydrophobic species, as indicated by the numerous metabolites present with retention times of more than 10 minutes in Figure 3.

A detailed examination of metabolites present using each method indicated significant changes in metabolome after blood is withdrawn. In vivo SPME detected 70 (positive ESI) and 85 (negative ESI) unique features that could not be detected in blood samples after blood withdrawal, regardless of the sample preparation method used (Supporting Information, Table S1 and S2). One example is β-NAD, which was detected only using in vivo SPME methodology (Figure 4A). High levels of adenosine monophosphate (AMP) were detected using in vivo SPME, and could not be detected using either ultrafiltration or solvent precipitation method (Supporting Information, Tables S3 and S4), indicating significant degradation of this important metabolite. In vivo SPME data indicated the ratio of glutathione to oxidized glutathione of 2.5, which is in excellent agreement with expected values (Supporting Information, Table S5).[19,20]

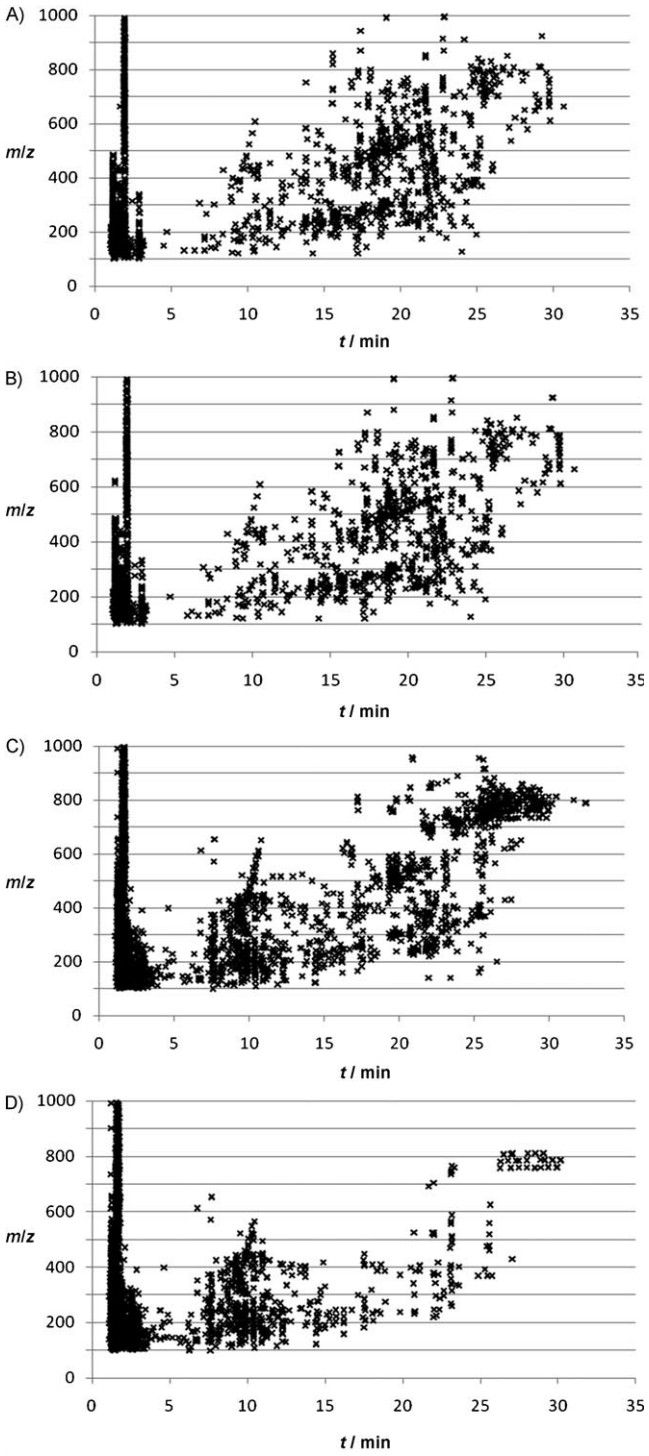

Figure 3. Summary of metabolite coverage obtained in mouse blood/plasma in combination with a pentafluorophenyl column and positive ESI LC-MS. A) in vivo SPME sampling of mouse circulating blood; B) ex vivo SPME sampling of mouse plasma; C) plasma protein precipitation with methanol/ethanol of mouse plasma; and D) ultrafiltration of mouse plasma. t = retention time.

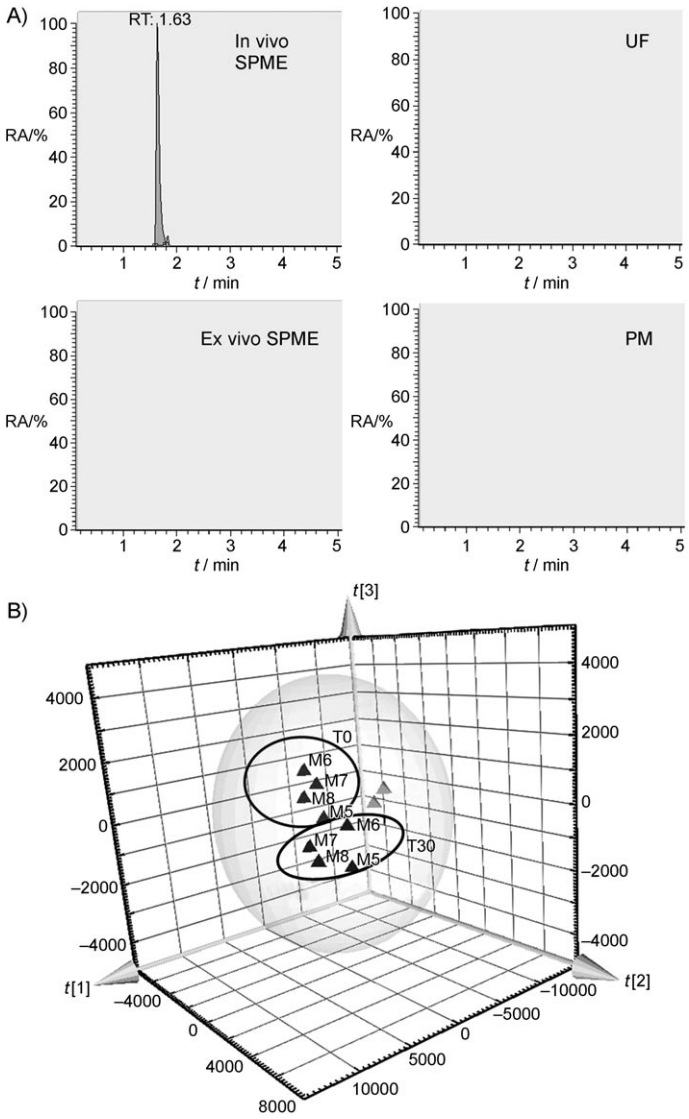

Figure 4. Representative results from a proof-of-concept in vivo SPME metabolomics study. A) Example of metabolite (m/z 662.1007, β-NAD) that was only detected using in vivo SPME and not observed in any of the samples after blood withdrawal, regardless of the sample preparation method employed. RA = relative abundance, t = retention time (RT). B) 3D scores scatter plot, indicating good differentiation between in vivo SPME samples collected prior to dosing, labeled as T0, and samples collected 30 min post-dose, labeled as T30. Two blanks are shown as gray triangles. (2D scores plots and loadings plots of in vivo SPME data are given in the Supporting Information, Figure S5.)

Solvent precipitation, ultrafiltration, and ex vivo SPME yielded ratios of 0.001, 0.005, and 0.2, respectively, indicating significant conversion of glutathione to its oxidized form after blood withdrawal. This data clearly shows the utility of SPME to capture short-lived or unstable species with fast turnover rates, such as energy metabolites, which can be missed or inaccurately depicted by other methods, thus presenting an important advance in the field of metabolomics.

Figure 2B also compares method precision achievable by all methods on secondary y axis with median RSD (relative standard deviation) values ranging from 46–65% showing that the precision of in vivo SPME is comparable to traditional methods. To show that these relatively high values reflect true interanimal variation rather than problems with the analytical method, we also present the results for method precision for the repeated extractions of pooled human plasma sample using all of the methods (median RSD 12–20%) in Figure 2C. This data further confirms that the precision of SPME is equivalent to traditional methods. Furthermore, SPME method provides cleaner extracts (free from protein and containing smaller amounts of metabolites) than solvent precipitation and ultrafiltration, which minimizes ionization suppression effects.[14]

The results obtained for the effect of carbamazepine dosing using in vivo SPME after principal component analysis show clear clustering of the two sets of samples (Figure 4B, and see the Supporting Information). The first three principal components describe 59.5, 17.4, and 9.2% of variance. The first two principal components show differentiation between animals (regardless of dosing), whereas the third component shows clear differentiation according to dosing. The ability of in vivo SPME to sample same animals before and after treatment removes some of the complexity inherent in data sets where different animals are compared in treatment versus control, thus enabling easier extraction of valuable biological information even when using small cohorts, which may be particularly advantageous for studies dealing with precious or genetically-modified strains.

In summary, this is the first application of SPME in direct extraction mode to global metabolite profiling studies of biological fluids by LC-MS. We show for the first time that it is possible to extract more than 1500 metabolites with a single coated fiber, whereas all existing reports of direct immersion SPME deal with targeted analysis of a few a priori selected analytes. In general, the recoveries of polar compounds, such as small organic acids and sugars (typically less than 2% absolute recovery), are lower than for hydrophobic species, but are still sufficient to be reliably detected within the capability of current analytical instrumentation such as Exactive. It is important to emphasize that the goal of SPME is not exhaustive extraction, so absolute recoveries of 1–2% are acceptable for this type of in vivo sampling. For in vivo SPME using short sampling times, the diffusion coefficient is the only discrimination factor, while for longer sampling times, the distribution constant (and coating chemistry) play an important role in the overall selectivity of the experiment and the achievable metabolite coverage. We show that the mixed-mode coating used in this study exhibits broad metabolite coverage, and additional improvements (for polar compounds in particular) can be achieved with the use of complementary chemistries.[14] Most importantly, we show that in vivo SPME can capture an elusive portion of metabolome, which is not accessible by conventional methods relying on blood withdrawal. In particular, in vivo SPME appears to be particularly suited for metabolites with fast turnover rates and metabolites prone to degradation, such as energy metabolites, glutathione conjugates, carotenes,

thionenes, and glucuronide species, owing to the incorporation of metabolism quenching step directly during the sampling process. The in vivo SPME approach described herein can be extended to other metabolomics applications, including metabolomics of plants, cell cultures, and tissues,[21–23] and permits simultaneous in vivo multicompartmental studies on both biofluids and tissues in awake, freely-moving animals. In vivo SPME also presents a useful alternative to microdialysis, especially in the area of metabolomics, because of advantages associated with improved spatial resolution, improved extraction of hydrophobic species, and better compatibility with LC-MS owing to minimization of ionization suppression effects. In vivo SPME can also be used for the extraction of large biomolecules pending appropriate coating development, thus eliminating molecular weight cut-off restrictions imposed by microdialysis.

Experimental Section

Biocompatible mixed-mode probes (C_{18} with benzenesulfonic acid, 45 μm thickness, Supelco Inc.) were pre-conditioned prior to use for a minimum of 30 min in a methanol/water (1:1 v/v) mixture. In vivo SPME was performed for 2.0 min in the circulating blood of mice with the aid of a sampling interface (see the Supporting Information). Samples were collected prior to and 30 min after 2 mg kg^{-1} intravenous dosing of carbamazepine ($n = 4$ mice). Immediately after sampling/extraction, the probes were rinsed in purified water for 30 s to remove any droplets from the surface. Desorption was performed using 300 μL of acetonitrile/water, 1:1 v/v, and the extract (10 μL) was analyzed directly with the reversed-phase LC-MS method on Exactive Orbitrap instrument (Thermo Scientific) using a pentafluorophenyl stationary phase. Full information on in vivo SPME sampling procedure, LC-MS and statistical data treatment is given in the Supporting Information.

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