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Pre-analytic evaluation of volumetric absorptive microsampling and integration in a mass spectrometry-based metabolomics workflow

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Abstract

Volumetric absorptive microsampling (VAMS) is a novel approach that allows single-drop (10 µL) blood collection. Integration of VAMS with mass spectrometry (MS)-based untargeted metabolomics is an attractive solution for both human and animal studies. However, to boost the use of VAMS in metabolomics, key pre-analytical questions need to be addressed. Therefore, in this work, we integrated VAMS in a MS-based untargeted metabolomics workflow and investigated pre-analytical strategies such as sample extraction procedures and metabolome stability at different storage conditions. We first evaluated the best extraction procedure for the polar metabolome and found that the highest number and amount of metabolites were recovered upon extraction with acetonitrile/water (70:30). In contrast, basic conditions (pH 9) resulted in divergent metabolite profiles mainly resulting from the extraction of intracellular metabolites originating from red blood cells. In addition, the prolonged storage of blood samples at room temperature caused significant changes in metabolome composition, but once the VAMS devices were stored at – 80 °C, the metabolome remained stable for up to 6 months. The time used for drying the sample did also affect the metabolome. In fact, some metabolites were rapidly degraded or accumulated in the sample during the first 48 h at room temperature, indicating that a longer drying step will significantly change the concentration in the sample.

Keywords

Metabolomics; Volumetric absorptive microsampling; Mass spectrometry

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Compliance with ethical standards This study was performed in accordance with the ethical standards. The local ethics committee (Comitato etico del comprensorio sanitario di Bolzano) approved the study, and all participants provided written informed consent.

Conflict of interest The authors declare that they have no competing interests.

Introduction

Dried blood spot (DBS) sampling was first introduced in 1963, when whole blood collected on filter paper was successfully applied to the neonatal screening of phenylketonuria [1]. Nowadays, the use of DBS sampling has become a common practice in several clinical applications, for the diagnosis of an inborn error of metabolism [2–4] and for therapeutic drug monitoring and clinical drug development [5, 6], including toxicology [7].

The introduction of DBS sampling in typical analytical workflow simplifies sample collection, transport, storage, and processing. Furthermore, it enables collection of representative samples in a patient's home setting. Despite the numerous advantages of DBS sampling, there are still several issues limiting the use of DBS in routine bioanalysis, such as the influence of hematocrit (HCT), the volume of blood spotted onto the filter paper cards, as well as the spot homogeneity.

Volumetric absorptive microsampling (VAMS) is a novel approach that allows single-drop (10 μ L) blood collection [8]. Besides showing all the recognized advantages of DBS sampling, it overcomes the issues associated with HCT and homogeneity. It has been recently demonstrated that this microsampling device allows to collect an accurate volume of blood for analysis, which is independent of HCT and is representative of the whole blood being sampled [8, 9].

One of the areas of interest for the application of this type of sample is obviously the “omics” field. Metabolomics, which aims to provide a global metabolite profile of a biological sample, can benefit from microsampling in many ways; most notably, it becomes easier to increase a cohort size for biomarker discovery and collect repeated samples in challenge-response experimental designs or in longitudinal studies.

Moreover, the use of VAMS in animal studies supports the principle of the three Rs (3Rs) by reducing the animal sample size and minimizing the potential pain for the animal [10].

Applications of microsampling in metabolomics have been presented using DBS sampling [11–17]. Nevertheless, these studies are still at an early stage and important pre-analytical strategies need to be carefully evaluated. For instance, the effect of different transport and storage conditions on the stability of the metabolome has not been fully investigated. Previous studies on DBS suggested that sample stability is limited at room temperature and that DBS need to be stored at -80°C [14]. Besides that, to date, no applications or studies have been presented integrating VAMS in metabolomics workflows.

In this study, we aim to integrate VAMS technology in a mass spectrometry (MS)-based untargeted metabolomics workflow, as well as investigate pre-analytical strategies including extraction procedures and storage conditions.

Material and methods

Chemicals

All materials were obtained from Sigma-Aldrich (Sigma-Aldrich, Seelze, Germany) unless stated otherwise. Acetonitrile was purchased from VWR International (Radnor, PA, USA). Water was obtained from a Milli-Q water purification system equipped with a LC-Pak polisher (Merck, Darmstadt, Germany). All chemicals and solvents were of analytical grade or higher purity. Metabolomic amino acid mix standard containing isotopically labeled amino acids was purchased from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Tewksbury, MA, USA).

Blood sampling

A pool of human blood was obtained by pooling surplus EDTA blood samples from healthy subjects and was obtained from the Transfusion Center of the Hospital of Bolzano. EDTA blood was selected to avoid coagulation of native blood during VAMS collection.

MITRA VAMS devices were purchased from Neoteryx (Neoteryx LLC, Torrance, CA, USA). For each experimental condition, one drop of the EDTA blood pool was absorbed onto VAMS devices and processed as described in the appropriate section.

Evaluation of extraction procedure

To test the best extraction protocol, the human EDTA blood pool was extracted using six different conditions:

1. pH 2 solution consisting of a methanol/water (7:3, v/v) mixture using water containing 1% of formic acid (pH 2)
2. pH 7 solution consisting of a methanol/water (7:3, v/v) mixture (un-buffered)
3. pH 9 solution consisting of a methanol/water (7:3, v/v) mixture using water containing 2% of sodium hydroxide (pH 9)
4. ACN solution consisting of 100% acetonitrile
5. MeOH solution consisting of 100% methanol
6. ACN-H₂O solution consisting of a acetonitrile/water (7:3) mixture (un-buffered)

We used 18 VAMS tips to collect the sample from the pooled blood. VAMS tips were dried for 24 h and subsequently inserted into Eppendorf tubes. For each of the six abovelisted conditions, three VAMS devices were extracted by adding 200 μ L of the respective extraction solution to the sample.

Samples were sonicated for 15 min and then vortexed at 1200 RPM for 60 min, both at 20 °C. VAMS tips were removed, and the sample was centrifuged at 1800g for 10 min at 20 °C to remove protein-like material. The supernatant was then filtered by using a Waters positive pressure-96 processor applying 12 psi pressure (Waters Corporation, Milford, MA, USA), through a Sirocco protein removal plate (Waters Corporation, Milford, MA, USA), and the extract was evaporated to dryness under vacuum at 35 °C for 120 min in a EZ-2

vacuum evaporator (Genevac, Ipswich, UK). Finally, samples were reconstituted with 150 μ L of acetonitrile/water (50:50, v/v) solution, containing the labeled amino acid standards at the following concentrations: alanine 13C315N (0.9 μ g/mL), arginine 13C615N4 (1.8 μ g/mL), aspartic acid 13C415N (1.3 μ g/mL), cystine 13C615N2 (1.2 μ g/mL), glutamic acid 13C515N (1.5 μ g/mL), glycine 13C215N (0.8 μ g/mL), histidine 13C615N3 (1.6 μ g/mL), isoleucine 13C615N (1.3 μ g/mL), leucine 13C615N (1.3 μ g/mL), lysine 13C615N2 (1.5 μ g/mL), methionine 13C515N (1.5 μ g/mL), phenylalanine 13C915N (1.7 μ g/mL), proline 13C515N (1.2 μ g/mL), serine 13C315N (1.1 μ g/mL), threonine 13C415N (1.2 μ g/mL), tyrosine 13C915N (1.8 μ g/mL), and valine 13C515N (1.2 μ g/mL).

Evaluation of sample stability

VAMS tips were used for sampling 69 samples from the same human pool of blood. Thirty samples were stored at room temperature, 15 samples were stored at -80°C after drying for 2 h, 12 samples were stored at -80°C after drying for 24 h, and finally, 12 samples were stored at -80°C after drying for 48 h. Samples stored at room temperature were extracted after 2 h, 24 h, 48 h, 4 days, 1 week, 2 weeks, 3 weeks, 1 month, 3 months, and 6 months. Samples stored at -80°C after drying for 2 h were extracted after 24 h, 1 week, 1 month, 3 months, and 6 months. Samples stored at -80°C after drying for 24 h were extracted after 1 week, 1 month, 3 months, and 6 months. Samples stored at -80°C after drying for 48 h were extracted after 1 week, 1 month, 3 months, and 6 months.

All samples were extracted using the ACN-H₂O solution and the procedure described above, and then they were subsequently dried for 120 min at 35°C in the EZ-2 vacuum evaporator and finally stored at -80°C until the analysis.

Finally, samples were reconstituted with 150 μ L of acetonitrile/water (50:50, v/v) solution, containing the labeled amino acid standards at the following concentrations: alanine 13C315N (0.9 μ g/mL), arginine 13C615N4 (1.8 μ g/mL), aspartic acid 13C415N (1.3 μ g/mL), cystine 13C615N2 (1.2 μ g/mL), glutamic acid 13C515N (1.5 μ g/mL), glycine 13C215N (0.8 μ g/mL), histidine 13C615N3 (1.6 μ g/mL), isoleucine 13C615N (1.3 μ g/mL), leucine 13C615N (1.3 μ g/mL), lysine 13C615N2 (1.5 μ g/mL), methionine 13C515N (1.5 μ g/mL), phenylalanine 13C915N (1.7 μ g/mL), proline 13C515N (1.2 μ g/mL), serine 13C315N (1.1 μ g/mL), threonine 13C415N (1.2 μ g/mL), tyrosine 13C915N (1.8 μ g/mL), and valine 13C515N (1.2 μ g/mL).

Pooled QC samples were prepared by pooling together an equal amount (30 μ L) from each extracted sample.

Mass spectrometry-based metabolomics

All samples were analyzed by ultra high-performance liquid chromatography (UHPLC) (Agilent 1290; Agilent Technologies, Santa Clara, CA, USA) coupled to a Q-TOF mass spectrometer (TripleTOF 5600+; AB Sciex, Foster City, CA, USA). The chromatographic separation was based on hydrophilic interaction liquid chromatography (HILIC) and performed using an Acquity BEH amide, 100×2.1 mm column (Waters Corporation, Milford, MA, USA).

Separation was achieved using acetonitrile + 0.1% formic acid as mobile phase A and water + 0.1% formic acid as mobile phase B. The injection volume was 5 μ L, and the flow rate was 0.6 mL/min. The following linear gradients were used: 0 min 95% A and 1 min 95% A, 4 min 30% A and 5 min 30% A, and 5.1 min 95% A and 8 min 95% A.

The mass spectrometer was operated in full scan mode in the mass range from 50 to 1000 m/z and with an accumulation time of 250 ms. In ESI+ mode, the source temperature was set at 700 °C, the declustering potential at 30 V, the collision energy at 6 V, the ion spray voltage at 5120 V, the curtain gas at 25 psi, and the ion source gases 1 and 2 at 60 psi. In ESI – mode, the source temperature was set at 650 °C, the declustering potential at – 45 V, the collision energy at – 6 V, the ion spray voltage at – 3800 V, the curtain gas at 25 psi, and the ion source gases 1 and 2 at 30 psi. The instrument was mass calibrated by automatic calibration infusing the Sciex Positive Calibration Solution (part no. 4460131, AB Sciex, Foster City, CA, USA) for positive mode and Sciex Negative Calibration Solution (part no. 4460134, AB Sciex, Foster City, CA, USA) for negative mode after every two sample injections.

To prevent acquisition of low quality data, at the beginning of each batch, we performed maintenance of the LC-MS system (curtain plate cleaning, mass calibration, and chromatographic column equilibration). We then injected a system suitability solution in acetonitrile/water (50:50, v/v) containing labeled amino acid standards at the following concentrations: alanine 13C315N (0.9 μ g/mL), arginine 13C615N4 (1.8 μ g/mL), glutamic acid 13C515N (1.5 μ g/mL), methionine 13C515N (1.5 μ g/mL), phenylalanine 13C915N (1.7 μ g/mL), serine 13C315N (1.1 μ g/mL), and valine 13C515N (1.2 μ g/mL). We used the system suitability mix to monitor retention time, mass accuracy, and signal intensity to ensure that the LC-MS system was performing properly.

Several studies have shown that the LC-MS system might not be completely stable at the beginning of the analytical run [18, 19]. To ensure stability of the LC-MS system, we inject 15 times pooled QC samples to equilibrate the LC-MS system before running the samples in both positive and negative modes. At the end of the equilibration injection sequence, we injected blanks to check for carryover. Samples were then analyzed in randomized order, and pooled QC samples were injected between every eight samples.

We used pooled QCs and total ion current (TIC) to check the intrabatch and interbatch variability, as applicable. The FDA guidance for biomarker analysis suggests the acceptance of up to 30% coefficient of variation for targeted analysis [20]. In our untargeted approach, we apply an acceptance of 30%. Therefore, features/metabolites with RSD higher than 30% in pooled QCs were removed and not considered further in data analysis. Failed injections were checked by monitoring the intensity of the spiked labeled amino acid standards.

Data analysis

Data were converted to .mzML, using ProteoWizard MS Convert [21] employing their wavelet-based algorithm for m/z peak picking (signal-to-noise threshold = 0.9 and minimum peak spacing = 0.1). The data was processed in R (v 3.3.3) using the XCMS package [22]. The centWave method [23] was used for chromatographic peak detection with parameters:

ppm = 40, snthresh = 5, prefilter = c(2, 200), peak width = c(0.8, 15), and noise = 100. The “peak group” method was used for alignment of the samples (span = 1 and requiring peaks used for alignment to be present in at least 85% of samples) after an initial peak grouping (“density” method, bw = 1.5, minfrac = 0.5, mzwid = 0.02). Final correspondence of retention time-corrected peaks was performed using the “peak density” method (bw = 2) requiring chromatographic peaks to be present in at least 50% of replicates per sample group. Missing peak values for the identified features were filled in using the “chrom” method.

Intrabatch variability, usually due to drift in instrument performance during the analytical run, was corrected in an approach similar to Wehrens et al. [24]. In detail, we applied robust linear regression [25] to estimate the injection orderdependent signal drift for each feature based on the intensities measured in all samples (since the sample order in the run was randomized) and subsequently adjusted the intensities based on the fitted model. This was performed on features with valid measurements in all samples (1016 and 1072 for positive and negative modes, respectively). To evaluate its performance, we calculated the RSD for each feature within each sample group before and after adjustment and averaged these across sample groups. These averaged RSD values across all features improved from 0.212 to 0.198 in the positive mode and from 0.199 to 0.191 in the negative mode data set. This moderate effect of the adjustment suggests the presence of only a small injection order-dependent signal drift in the data set.

Data sets were log transformed and scaled by using Pareto scaling before principal component analysis (PCA). Heatmaps were obtained using MetaboAnalyst [26].

Metabolites were identified by verifying retention time, accurate mass, and tandem mass spectrometry against our in-house and/or online databases, including HMDB [27] and METLIN [28]. Only metabolites identified in all technical replicates were considered. Tentatively identified metabolites were integrated using MultiQuant 3.0 (Sciex, Foster City, CA, USA). Extracted ion chromatograms (EICs) were extracted using a 0.02-mDa window centered on the expected m/z for each targeted compound.

Results and discussion

The primary aim of this study was to develop a protocol suitable for integrating VAMS technology into a typical MS-based metabolomics workflow designed for untargeted analysis of the polar blood metabolome.

To address this, we first tested different procedures for the extraction of the metabolome sampled by VAMS. Subsequently, we investigated the stability of the blood absorbed onto the microsampling devices.

Evaluation of extraction procedures

Ideally, an extraction method suitable for untargeted metabolomics should be able to quantitatively extract the broadest possible range of metabolites, and at the same time, it should be simple and fast to prevent metabolite loss and/or degradation. However, this is a

very challenging task to achieve considering differences in chemical mass, physicochemical properties, and a wide range of expected concentrations.

Moreover, extraction protocols are sample dependent [29, 30], and specific extraction procedures are recommended for cells [31], serum [18], urine [32], and dried blood spots [15, 33], respectively.

To evaluate the best extraction protocol for combining VAMS with metabolomics applications focusing on polar metabolites, we therefore selected six different extraction solutions as described in the “Material and methods” and compared the metabolic profiles obtained. We have previously demonstrated that HILIC-MS methods are suitable for separating polar metabolites [31, 19]. Since we are interested in polar metabolites, in this study, we analyzed the metabolomes extracted from VAMS by using a HILIC-MS method designed for the large-scale studies of plasma and serum samples [34]. All extracting solutions, except for pH 9 solution, gave clear (uncolored) extracts. On the contrary, pH 9 extracts resulted in a brownish solution, indicating the presence of hemoglobin.

In positive mode, we detected the highest number of features when the blood metabolome was extracted using ACN-H₂O (Fig. 1a). In contrast, the extraction using only ACN proved to be the least effective in terms of the number of features detected (Fig. 1a). Similar results were obtained in negative mode, with ACN-H₂O extracting the highest number of features (Fig. 1b). The mass chromatograms obtained were very similar except for the samples extracted using the pH 9 solution (Fig. 1c, d). The obvious difference of the pH 9 mass chromatogram suggests that for this condition, we extracted a quite different metabolic profile (Fig. 1c, d) with 1919 unique features in positive mode, which were not extracted with the other conditions (see Electronic Supplementary Material (ESM) Fig. S1a). The same was true for negative mode, with 1446 unique features extracted (ESM Fig. S1b).

The PCA confirmed these findings, showing, in both positive and negative modes, that samples extracted with either ACN or pH 9 clustered separately from the other samples (Fig. 1e, f). The first principal component (PC1) accounted for 47% of the total variance, and it clearly separated the extraction at pH 9 and extraction using only ACN from the other samples (Fig. 1a). The second principal component (PC2) accounted for 36% of the total variance and could separate the ACN and the pH 9 extraction (Fig. 1a). All other extractions clustered very closely, suggesting that they recovered a similar metabolome.

We then focused on annotated metabolites. We tentatively identified 133 metabolites, and once again, the metabolomes recovered from pH 9 and ACN extractions were very different compared with the others (Fig. 2, ESM Fig. S2). Next, we evaluated the efficiency of each extraction method depending on the specific classes of metabolites (Fig. 2). As expected, the majority of carboxylic acids was better extracted using basic conditions with the exception of citric acid. Citric acid is a weak carboxylic acid and was better extracted at pH 7 (Fig. 2a). In addition, carboxylic acids were also well extracted using pH 7 and ACN-H₂O (Fig. 2a).

The main components of blood are plasma, red blood cells (RBCs), platelets, and leucocytes. Plasma and RBCs account for more than 99% of the blood content, so it appears reasonable to detect RBC intracellular metabolites in the whole blood metabolome. Indeed,

in the extracted metabolome, we found several intracellular metabolites that are typically present in high amounts in the RBC metabolome, such as heme, AMP, IMP, glucose-6-phosphate, and fructose 6-phosphate [35–37]. Although these metabolites were extracted in all conditions (Fig. 2b), they were detected in particularly high amounts under basic conditions (Fig. 2b). Alkaline conditions seem most effective in extracting RBC's metabolites. This is further supported by the observation that heme and metabolites involved in the glycolysis and purine metabolism were also found in higher amounts at high pH compared with the other protocols. Moreover, as mentioned before, pH 9 extracts resulted in a brownish solution, indicating the presence of hemoglobin.

For other classes of metabolites, such as amino acids and carnitines, we found a very similar trend (Fig. 2c–e), with ACN-H₂O, MeOH, pH 2, and pH 7 performing well and ACN and pH 9 showing a lower recovery. As expected, basic conditions did not extract basic amino acids, such as arginine and lysine, but only acidic amino acids, such as aspartic acid and glutamic acid.

The extraction protocol using 100% of ACN was found to be the least effective in recovering the blood metabolome. Only lipids were extracted in a reasonable amount (Fig. 2f). As expected, MeOH was, however, the most effective in the recovery of lipids (Fig. 2f).

Overall, ACN-H₂O and MeOH-H₂O at pH 7 were the most efficient procedures, both in terms of coverage of the metabolome and the amount of metabolites recovered (Figs. 1 and 2). Given that the LC-MS method employed in this study uses a HILIC approach, starting with a high organic gradient (mobile phase A—ACN), it is reasonable to inject the sample extracted with ACN-H₂O directly into the LC-MS system. This would avoid additional sample preparation steps, including lyophilization and reconstitution. For these reasons, we selected ACN-H₂O as an extraction protocol, which was used for subsequent analyses.

Next, we evaluated whether the extraction of the blood metabolome required multiple extraction steps. To address this question, the blood was sampled onto the VAMS devices and extracted using up to three consecutive steps. The resulting extracts were then analyzed separately.

The results obtained are reported in Fig. 3. Most of the polar metabolites (carnitines, amino acids, carboxylic acids, and phosphorylated compounds) were already extracted during the first step. In fact, around 80% of the total extractable content from all three extraction steps came from the first step. For polar metabolites, the second step extracted no more than 17% of the total extractable content and the third extraction step resulted in the recovery of the remaining 3% of the total extractable polar metabolome. Lipids, on the other hand, required more than one-step extraction. During the first step only, 50% of total extractable lipid content was recovered, and second and third extractions were necessary to allow the additional recovery of 35 and 15% of the total extractable content, respectively (Fig. 3).

Considering that this study is focused on the analysis of blood polar metabolites and that the first step extracted around 80% of the total polar metabolome, we think that the 20% loss of polar metabolites is acceptable and compensated by the fact that the one-step procedure is much faster and simpler to execute.

We are aware that other combinations of solvents and extraction steps might yield similar or better results and that based on the scope of the study, other researchers might be interested in different sets of metabolites. Therefore, we recommend to validate internally the best extraction protocol, according to the scope of the study and the experimental design.

Evaluation of the metabolome stability

To expand the use of VAMS in metabolomics applications, it is important to evaluate the stability of the samples at different storage conditions. Notably, understanding how the storage conditions affect the stability of the metabolome is of great importance.

VAMS devices collect a single drop (10 μ L) of blood, but a variable time frame (from 2 h to several days) might pass between the collection and the extraction of the metabolome especially if the sampling is done remotely, at home or at the convenience of the participant.

To address this open question, we designed an experiment where multiple VAMS samples were collected from a pooled human blood sample and stored at different conditions (Fig. 4a). The first set of samples was stored at room temperature for up to 6 months, and samples were extracted at different time points to evaluate the stability of the metabolome (Fig. 4a). Another three sets of samples were stored at -80°C after drying for 2, 24, and 48 h, respectively (Fig. 4a).

We tentatively annotated 103 metabolites and performed PCA as the first step for data visualization (Fig. 4b). The annotated metabolites are presented in the heatmap (Fig. 5). PCA defines a clear trend for the samples stored at room temperature that are clustering based on the time of storage. In fact, the metabolic profile changed over the time when VAMS devices were left at room temperature. On the other hand, samples stored at -80°C did not show any trend related with the time of storage. Nevertheless, while the samples stored at -80°C after drying for 24 or 48 h clustered together, samples stored at -80°C after drying for 2 h clustered separately, suggesting a difference in the observed metabolome (Fig. 4b).

To confirm this observation, we performed PCA on all ion features extracted in both positive and negative modes, and in both cases, this analysis shows a similar trend clustering samples stored at room temperature based on the time of storage (ESM Fig. S3).

To define the number of metabolites that were significantly changing with the time of storage we used, univariate analysis (one-way ANOVA, $p < 0.005$). Thin analysis suggested that at room temperature, around 75% of the identified metabolites (77 metabolites) changed significantly over time (one-way ANOVA, $p < 0.005$; Tables 1, 2, and 3). Three different time-dependent trends were observable. The largest group of metabolites (36 or 35%) showed a decrease in concentration over time of storage (Table 1). Among them, histidine, glutamine, and asparagine underwent a degradation process overtime (Fig. 6, Table 1). However, the degradation process was not the same for all metabolites. For instance, the degradation of histidine started after 2 h of storage and continued until 3 weeks of storage, whereas the degradation of asparagine started to be important with a variation higher than 30%, after 1 week of storage

Of note, a total number of 32 metabolites (31%) showed an opposite trend, and their levels increased with the duration of storage (Table 2). An example is shown in Fig. 6 where the concentration of glutamic acid, glyceric acid, and methionine sulfoxide increased overtime. Several mechanisms might be responsible for this increase. For instance, it has been shown that glutamine is converted into glutamic acid in DBS due to the presence of HCl and butanol used for the derivatization process employed [38]. In our experiments, since no HCl and butanol were used, we suggest that this conversion might be due by some residual activity of glutaminase, which converts glutamine to glutamic acid. The increase of glycerol instead is likely due to the breakdown of glycolysis intermediates present in RBCs [35, 37] such as 2- and 3-phosphoglycerate and 1,3-bisphosphoglycerate and 2,3-bisphosphoglycerate. Finally, the storage at room temperature might also cause oxidation of some metabolites. We observed that the levels of methionine sulfoxide increased with the time of storage (Fig. 6). In fact, it has been shown that oxidation of methionine occurs also in samples stored at -25°C , causing accumulation of methionine sulfoxide in the sample [39].

A small portion of these metabolites (9 metabolites or 9%) had a mixed trend instead, where it was possible to record an increase in their concentration in the first 48 h of storage followed by a gradual degradation overtime (Table 3). An example of this behavior is shown in Fig. 6, where the profile of phosphorylethanolamine is presented.

Finally, some metabolites such as leucine and isoleucine were stable at room temperature (Fig. 6).

While the storage at room temperature causes significant changes in the composition of the metabolome, once the VAMS devices are stored at -80°C , the metabolome was shown to be stable for up to 6 months. However, it is worth to stress that even the time of drying after the microsampling collection can affect the final metabolome. In fact, some metabolites are rapidly degraded or generated over the first 48 h at room temperature (Fig. 6). For these metabolites such as methionine sulfoxide, glutamic acid, and histidine, a longer drying step will significantly change their concentrations in the sample (Fig. 6).

Our results are in agreement with a previous study in DBS, where Wilson and colleagues reported experiments to assess the stability of dried blood spots at different storage conditions, suggesting that general sample stability for VAMS is limited at room temperature and that for metabolomics purposes, VAMS devices need to be stored at -80°C [14] in order to ensure stability.

Nevertheless, another study integrating targeted metabolomics with DBS collection shows that some metabolites were stable for 4 weeks, even at room temperature, stored in sealed bags with desiccant packages [33].

The stability seems to be both chemical class and metabolite dependent; for instance, Zukunft et al. showed that glutamine and histidine stored at room temperature in sealed bags with desiccants are degraded after 2 weeks, while other amino acids such as asparagine were stable for weeks [33]. On the other hand, the same paper showed that lipids such as

phosphatidylcholines and sphingomyelins are unstable at room temperature stored in sealed bags (in all samples or after hours/days/weeks) [33].

Several solutions might be employed for stabilizing blood samples [40, 41]. Lowering the temperature is the most common approach used for stabilizing small molecules. However, pH adjustment and the addition of inhibitors and/or antioxidants are also commonly employed in therapeutic drug monitoring [40–42].

Nevertheless, in metabolomics studies, it is challenging to find a solution able to stabilize the whole metabolome and lowering the storage temperature is the most used and the most successful approach [14, 33].

Due to the fact that VAMS is a recent technology, only few data on stability of small molecules have been presented, and they were produced investigating the stability of single drugs/chemicals, such as paracetamol [43] and caffeine [9]. Paracetamol was shown to be stable for 7 days at room temperature [43], while caffeine was stable for up to 48 days at room temperature [9].

According to the guideline from the vendor, VAMS sampling requires a drying step that can vary from 2 to 24 h. This step needs to be performed at room temperature. Moreover, in order to enable remote sampling, it is of paramount importance to improve the short-term stability of VAMS samples to ensure minimal degradation during the drying step and the following first hours of storage at room temperature. Limited stability is likely due to a combination of several causes, including oxidation, residual enzymatic degradation, as well as degradation under ambient humidity conditions. Therefore, further studies are necessary to investigate the possibility of using different conditions to stabilize the storage of VAMS samples for short-term periods.

Based on the presented results, we suggest to dry the VAMS device for 2 h and then immediately freeze and store the sample at -80°C for untargeted metabolomics. Specific metabolites might still show adequate stability over longer times; therefore, we suggest that for targeted metabolomics, the stability of the specific metabolites in question should be verified before deciding the final sample collection strategy. In this paper, we showed that pre-analytical conditions are critical for the use of VAMS device.

Conclusions

In this study, we developed for the first time a workflow integrating VAMS with MS-based metabolomics. We evaluated different extraction protocols to determine the best preanalytical condition for VAMS-based metabolomics to study the polar metabolome of blood and found that a mixture of acetonitrile/water (70:30) yielded the best results for our scope. Nevertheless, other combinations of solvents and extraction steps might be applied, depending on the scope and the experimental design selected.

In addition, our data suggest that blood VAMS samples cannot be stored at room temperature for more than 2 h. This limits the use of VAMS in blood-based metabolomics excluding the possibility to store/transport samples without refrigeration at -80°C .

Improving stability is of paramount importance to fully take the advantage of VAMS technology in human metabolomics studies where remote and longitudinal sampling might be important options.

Limited stability is likely due to several causes, such as oxidation, residual enzymatic degradation, as well as degradation under ambient humidity conditions.

Further investigations to minimize the degradation effect and stabilize the metabolome for short-term storage at room temperatures are recommended to fully exploit the advantages of VAMS devices in untargeted metabolomics.

Nonetheless, we have shown that VAMS sampling can be successfully incorporated in the metabolomics workflow if standardized procedures such as the one described in this study are used.

At the moment, for untargeted metabolomics studies, we suggest to dry the VAMS samples for 2 h and subsequently store them at -80°C . For targeted metabolomics, we suggest to carefully evaluate the stability of the selected metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Evaluation of extraction procedures for the integration of VAMS with untargeted metabolomics. **a** Total count features in positive mode obtained for each specific group after processing the data by using our XCMS. **b** Total count features in negative mode obtained for each specific group after processing the data by using our XCMS. **c** Overlaid base peak chromatograms and feature count along the chromatogram obtained using different extraction procedures in positive mode. **d** Overlaid base peak chromatograms and feature count along the chromatogram obtained using different extraction procedures in negative

mode. **e** Principal component analysis performed on features obtained in positive mode. **f** Principal component analysis performed on features obtained in negative mode. Color code: yellow represents samples extracted using the ACN solution; orange represents samples extracted using the ACN-H₂O solution; violet represents samples extracted using the MeOH solution; red represents samples extracted using the pH 2 solution; green represents samples extracted using the pH 7 solution; blue represents samples extracted using the pH 9 solution

Fig. 2.

Evaluation of the extraction procedures for the recovery of different classes of metabolites. **a** Comparison of extraction procedure for the analysis of carboxylic acid. **b** Comparison of extraction procedure for the analysis of RBC intracellular metabolites. **c** Comparison of extraction procedure for the analysis of other metabolites. **d** Comparison of extraction procedure for the analysis of amino acids. **e** Comparison of extraction procedure for the analysis of carnitines. **f** Comparison of extraction procedure for the analysis of phospholipids

Fig. 3.

Evaluation of the number of consecutive steps necessary for extracting the whole metabolome. **a** Percent of extracted metabolites in each extraction step for selected classes of metabolites. Amino acids, $n = 37$; phosphorylated compounds, $n = 24$; carboxylic acids, $n = 20$; carnitines, $n = 10$; lipids $n = 21$; other compounds, $n = 21$. The percent of extracted metabolites was calculated based on the sum of the signals obtained in each extraction step. **b** Overlaid percent of extracted metabolites in each extraction step for selected classes of metabolites

Fig. 4. Evaluation of sample stability at different storage conditions. **a** Experimental design. **b** Principal component analysis performed on tentatively identified metabolites ($n = 103$)

Fig. 5. Heatmap performed on annotated metabolites extracted from VAMS sample stored at different conditions

Fig. 6.

Time profiles of selected metabolites stored at different storage conditions. The average of three technical replicates is plotted in each chart

Table 1

One-way ANOVA test on samples stored at room temperature. Metabolites that decrease with the time of storage

	Metabolite	<i>F</i> value	Raw <i>p</i> value	Adjusted <i>p</i> value
Decrease with the time	D-Glucose	543.1	4.83E- 20	4E- 18
	L-Histidine	403.2	6.94E- 19	3E- 17
	Alpha-ketoisovaleric acid	127.8	1.89E- 14	4E- 13
	L-Tryptophan	97.6	2.02E- 13	4E- 12
	L-Asparagine	93.7	2.88E- 13	5E- 12
	1-Methylhistidine	88.9	4.55E- 13	6E- 12
	Glutathione reduced	65.4	6.60E- 12	7E- 11
	Uric acid	54.4	3.23E- 11	3E- 10
	Oxidized glutathione	43.4	2.18E- 10	2E- 09
	Ketoleucine	42.0	2.89E- 10	2E- 09
	UDP	31.3	3.38E- 09	2E- 08
	L-Glutamine	30.2	4.59E- 09	2E- 08
	1-Palmitoylglycerophosphoinositol	28.0	8.51E- 09	4E- 08
	CDP-ethanolamine	27.4	1.00E- 08	5E- 08
	Pyrrolidone carboxylic acid	26.6	1.29E- 08	6E- 08
	L-Arginine	24.9	2.23E- 08	9E- 08
	Dihydrothymine	22.8	4.51E- 08	2E- 07
	L-Lysine	21.9	6.12E- 08	2E- 07
	ADP-ribose	19.8	1.40E- 07	5E- 07
	L-Acetylcarnitine	17.2	4.09E- 07	1E- 06
	Phosphoenolpyruvic acid	17.0	4.45E- 07	1E- 06
	Propionylcarnitine	13.0	3.46E- 06	1E- 05
	Gamma-glutamyl glutamine	12.6	4.41E- 06	1E- 05
	Acetylmethionine	11.4	8.92E- 06	2E- 05
	Pyruvic acid	11.0	1.14E- 05	3E- 05
	D-Fructose 2,6-bisphosphate	10.0	2.25E- 05	6E- 05
	Uridine diphosphate- <i>N</i> -acetylglucosamine	8.2	8.65E- 05	0.0002
	L-Serine	7.7	0.0001	0.0003
	Heme	5.7	0.0009	0.0017
	Carnosine	5.2	0.0015	0.0029
	PC (38:7)	5.1	0.0017	0.0031
	ADP	4.8	0.0023	0.0041
	Phenol sulfate	4.1	0.0053	0.0093
	Ethyl aconitate	3.6	0.0104	0.0176
	L-Phenylalanine	3.1	0.0211	0.0331
	2-Hydroxybutyric acid	3.0	0.0223	0.0347

Table 2

One-way ANOVA test on samples stored at room temperature. Metabolites that increase with the time of storage

Metabolite	<i>F</i> value	Raw <i>p</i> value	Adjusted <i>p</i> value
Glyceric acid	526.6	6.37E- 20	3.76E- 18
Methionine sulfoxide	262.7	3.18E- 17	9.38E- 16
Hypoxanthine	90.4	3.94E- 13	5.82E- 12
PABA	84.7	6.98E- 13	8.23E- 12
L-Beta-aspartyl-L-alanine	57.7	1.95E- 11	1.92E- 10
Hydroxyisocaproic acid	48.0	9.28E- 11	7.30E- 10
PC343	40.6	3.83E- 10	2.51E- 09
L-Glutamic acid	36.1	1.02E- 09	6.36E- 09
Succinic acid	27.6	9.56E- 09	4.51E- 08
L-Cystine	24.0	2.98E- 08	1.21E- 07
Suberic acid	19.2	1.79E- 07	6.38E- 07
PC (38:5)	17.7	3.25E- 07	1.10E- 06
GMP	17.4	3.74E- 07	1.22E- 06
<i>p</i> -Hydroxyphenylacetic acid	13.8	2.26E- 06	6.82E- 06
AMP	13.5	2.65E- 06	7.82E- 06
Glycerophosphocholine	13.2	3.10E- 06	8.91E- 06
Glycerylphosphorylethanolamine	10.9	1.23E- 05	3.16E- 05
Glycolic acid	9.6	3.07E- 05	7.54E- 05
Phosphoric acid	8.9	5.10E- 05	0.0001
Creatinine	8.7	6.09E- 05	0.0001
Glutaric acid	7.9	0.0001	0.0003
Pyroglutamic acid	7.7	0.0001	0.0003
Hydroxyproline	6.9	0.0003	0.0006
Adenosine	5.9	0.0007	0.0015
Glucose 6-phosphate	5.8	0.0008	0.0016
Taurine	5.8	0.0008	0.0017
Gamma-glutamylcysteine	5.7	0.0009	0.0017
Glycerol 3-phosphate	5.4	0.0011	0.0022
PC (36:6)	4.9	0.0021	0.0037
2-Octenedioic acid	3.8	0.0079	0.0137
L-Carnitine	3.6	0.0106	0.0176
IMP	3.1	0.0185	0.0299

Table 3

One-way ANOVA test on samples stored at room temperature. Metabolites that shows a mixed trend

	Metabolite	<i>F</i> value	Raw <i>p</i> value	Adjusted <i>p</i> value
Mixed trend	Ornithine	52.5	4E- 11	4E- 10
	L-Threonine	30.7	4E- 09	2E- 08
	Phosphorylethanolamine	28.7	7E- 09	4E- 08
	Pipecolic acid	19.1	2E- 07	6E- 07
	ADP-glucose	5.1	0.002	0.003
	N6,N6,N6-Trimethyl-L-lysine	3.6	0.010	0.017
	PC363	3.3	0.015	0.024
	Citrulline	3.1	0.021	0.033
	PC342	2.8	0.029	0.045