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Original Article

Investigating the effects of storage conditions on urinary volatilomes for their reliability in disease diagnosis

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Abstract: Background: Cancer detection presents challenges regarding invasiveness, cost, and reliability. As a result, exploring alternative diagnostic methods holds significant clinical importance. Urinary metabolomic profiling has emerged as a promising avenue; however, its application for cancer diagnosis may be influenced by sample preparation or storage conditions. Objective: This study aimed to assess the impact of sample storage and processing conditions on urinary volatile organic compounds (VOCs) profiles and establish a robust standard operating procedure (SOP) for such diagnostic applications. Methods: Five key variables were investigated: storage temperatures, durations, freeze-thaw cycles, sample collection conditions, and sample amounts. The analysis of VOCs involved stir bar sorptive extraction coupled with thermal desorption-gas chromatography/mass spectrometry (SBSE-TD-GC-MS), with compound identification facilitated by the National Institute of Standards and Technology Library (NIST). Extensive statistical analysis, including combined scatterplot and response surface (CSRS) plots, partial least squares-discriminant analysis (PLS-DA), and probability density function plots (PDFs), were employed to study the effects of the factors. Results: Our findings revealed that urine storage duration, sample amount, temperature, and fasting/non-fasting sample collection did not significantly impact urinary metabolite profiles. This suggests flexibility in urine sample collection conditions, enabling individuals to contribute samples under varying circumstances. However, the influence of freeze-thaw cycles was evident, as VOC profiles exhibited distinct clustering patterns based on the number of cycles. This emphasizes the effect of freeze-thaw cycles on the integrity of urinary profiles. Conclusions: The developed SOP integrating SBSE-TD-GC-MS and statistical analyses can serve as a valuable tool for analyzing urinary organic compounds with minimal preparation and sensitive detection. The findings also support that urinary VOCs for cancer screening and diagnosis could be a feasible alternative offering a robust, non-invasive, and sensitive approach for cancer screening.

Keywords: SBSE-GC-MS, metabolomics, standard operating procedures, volatile organic compounds, urinary biomarkers

Introduction

Cancer diagnoses and detection have drastically evolved over the years; however, many tests and techniques present great invasiveness, are costly, require extensive training and expertise, and often present limitations. These limitations and challenges include a high risk of false-positive interpretation, invasiveness, misdiagnoses, and overdiagnoses [1-3]. Ongoing research has shifted towards adopting "omics" approaches, such as proteomics [4], metabolomics [2], transcriptomics [5], and lipidomics [6] to analyze potential cancer biomarkers. Metabolomics ultimately measures small-molecule byproducts from metabolic processes via non-targeted and targeted approaches, and it is the latest "omics" to capture the researchers' attention in cancer diagnosis. As metabolomics is the most down-streamed profile of the "omics", it most represents the biological system's phenotype. Cancer metabolomics, i.e., determining the metabolite profiles of cancer cells, particularly can provide an accurate readout of tumor cells' physiology and biochemical activity [1].

A large variety of literature reported using serum, tissue, and urinary sample matrices in metabolomics as potential cancer screening techniques [7-9]. Urine is the most common biospecimen collected during routine testing. It comprises urea, water, and waste products filtered through the urinary tract at high void capacity. These components present a significant source of organic compounds representing endogenous and exogenous metabolic activities [1]. Urinary metabolomes could offer valuable insight into an individual's health, are attractive due to their non-invasive collection method, the ability to detect diseases, and monitor pollutant exposures. Moreover, recent studies reported that trained animals could detect cancers by "sniffing" biomatrices (e.g., urine, breath, and tissue) [10-12]. Since the odor of urine perceived by the animals is produced by volatile organic compounds (VOCs), urinary VOCs have been found to offer unique signatures in patients with breast [2], bladder [13], prostate [3, 14, 15], colorectal [16, 17], and lung cancer [18]. Thus, detecting VOCs in urine has drawn interest from the scientific community for cancer detection [3, 19-23].

While urine biomarker research in cancer detection has warranted claims in recent decades, there is no study on the potential covariates that could affect its integrity in such applications. In the case of prostate cancer, prior gas-chromatography/mass spectrometry (GC-MS) studies of urine metabolites have shown the inconsistent utility of specific metabolites [3, 14, 15]. For instance, GC-MS urine metabolites can be unpredictable due to interfering compounds such as creatinine and bilirubin, which can mask the detection of specific metabolites. Reports showed the presence of unexpected chemical properties, the lack of caffeine detection, inconsistencies in metabolite relative concentrations, and the inconsistency of total detected compounds between duplicate GC-MS runs. Researchers have combated the variations in GC-MS urine metabolites using endogenous urinary metabolites (i.e., creatinine, hippuric acid, and trimethylamine oxide) or exogenous metabolites (i.e., glucose, amino acids, and choline) to normalize the results [20-22]. In addition, sample collection and storage conditions could further complicate the use of urine for disease diagnosis. One concern for storing urine at room temperature for an extended period is that it may become a breeding ground for bacteria, leading to the growth of unwanted microorganisms and causing contamination. While some researchers have reported no significant difference between urine samples stored at various temperatures for an extended duration [24-26]. In contradiction, several studies have looked at the effect of storage time and freeze-thaw cycle's effect on urinary biomarker concentrations; findings creatinine, ketone bodies, and albumin as the prominent biomarkers susceptible to significant detection changes [27-30]. Without standard operating procedures (SOPs) and investigation into confounding variables, the accuracy of the results may be compromised due to a lack of uniformity in the methods of sample collection, storage, and analysis.

We reason that differences in sample processing and target selection could have accounted for the discrepant correlation between the VOCs and cancer detection. Since the temperature of storage, collection volumes, and time from collection of urines to an analysis by GC-MS could vary, a reliable method to overcome these variations and detect VOCs in the urine would need to be developed. This study aimed to evaluate the effects of storage temperature, storage duration, urine amount, fasting, and freeze-thaw cycle used in the analysis to determine the integrity of VOC profiles and reproducibility. This is the first study that utilizes a comprehensive approach to study the effect of various factors on urinary profiles. The results of these research experiments are projected to offer critical insight into the importance of sample collection and preparation that can be translated to the adoption in cancer diagnostics.

Materials and methods

All chemicals were of analytical grade. Mirex (99.0%, Dr. Ehrenstorfer GmbH, Germany) was purchased from the National Institute of Standards and Technology (NIST). Methanol was used to prepare the 100 mg/L Mirex solution and was purchased from Burdick & Jackson (Muskegon, MI, USA). Hydrochloric acid (HCl, 37%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure deionized water from the Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare 2 M solutions of HCl.

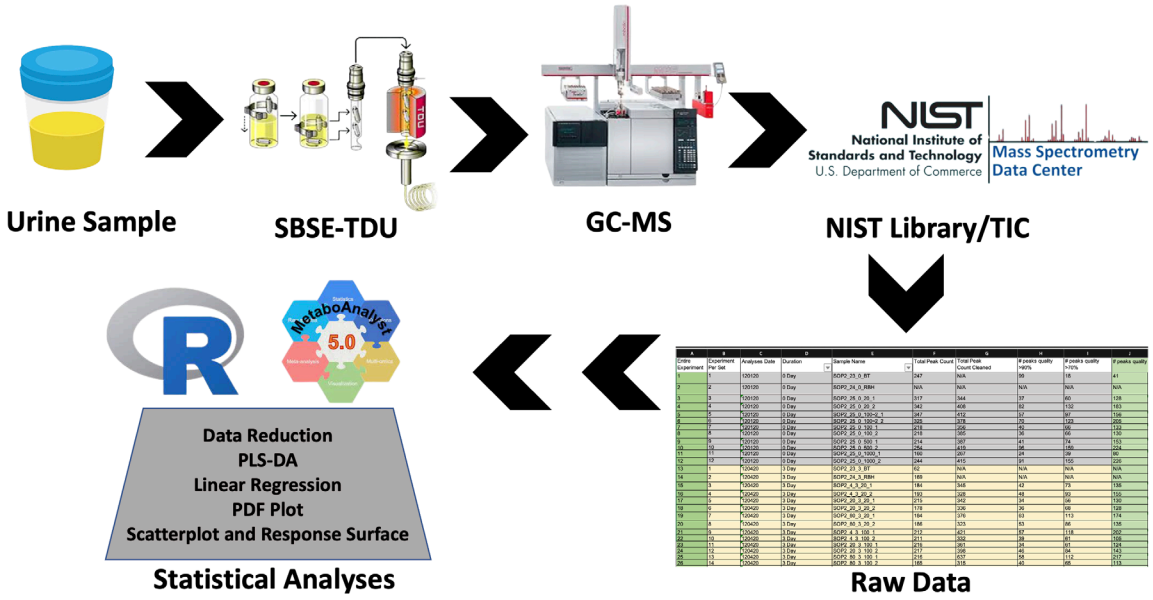


Figure 1. Experimental design flowchart for analyzing variables of interest for all experiments. Abbreviations: SBSE-TDU, stir bar sorptive extraction-thermal desorption unit; GC-MS, gas chromatography-mass spectrometry; NIST, national institute of standards and technology; TIC, total ion chromatogram; VOC, volatile organic compound; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis.

Urine samples

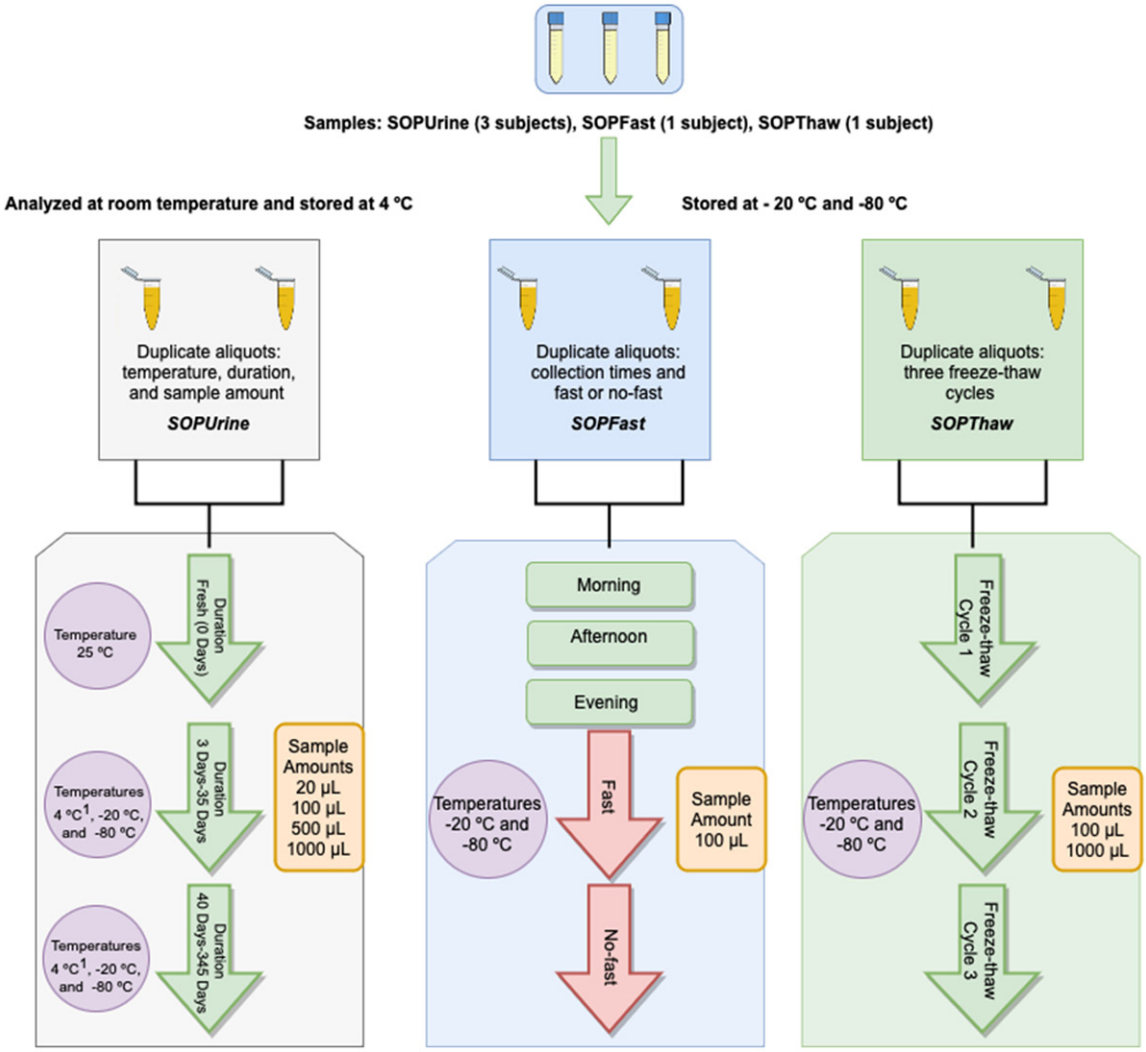
Three healthy volunteer urine samples (one female and two males) were used for the experiment conducted between December 2020 and July 2022. The mean age of the volunteers was 33 ± 10.92 years. The University of Texas approved the study at El Paso Institutional Review Board (IRB), conducted in adherence with the IRB guidelines, and written informed consent was obtained from all volunteers. Urinary samples were collected for different sample preparation conditions for VOC analyses. In total, 11 separate urine samples with 258 total aliquoted samples for all experimental designs.

Study design

The main task for metabolomic and statistical analysis approaches for urine biomarker detection is identifying and quantifying urinary metabolites. **Figure 1** shows the overall experimental workflow. The experimental design was to detect metabolite changes caused by five main factors: storage temperature, storage duration, sample amount, direct influence of fasting/no-fasting, and freeze-thaw cycles on urinary profiles. Urine samples were collected, stored, and analyzed within three primary cohorts (SOPUrine, SOPFast, and SOPThaw).

Urine storage conditions

All the urine samples were subjected to one of the three primary cohorts, "SOPUrine" (Storage Variables), "SOPFast" (Fast/No-fast), "SOPThaw" (Freeze-thaw) (**Scheme 1**; **Table 1**). **Scheme 1** shows the study design and experimental workflow. Experimental procedures were developed to investigate the targeted variables of storage temperature, storage duration, and sample volume for their effects on VOC profiles. All the samples were analyzed within a year of collection, and duplicates were performed for each sample. Five factors were studied on the collection day: storage temperature, storage duration, sample amounts, fasting, and freeze-thaw cycles. Roughly 5 mL of urine were aliquoted into 15 mL centrifuge tubes and held at 4°C, -20°C, -80°C until analyses, attributing to the targeted temperature factor. For SOPUrine, approximately 80 mL and two 35 mL of void morning urine were collected and pooled from the female and two male volunteers, respectively. SOPFast consisted of six subaliquots of urine samples collected from the female volunteers' morning, afternoon, and evening urine with and without fasting (where



Scheme 1. Experimental flowchart indicating storage variables of interest for all three experiments (Samples at 4 °C were not stored longer than two weeks).

Table 1. Summary of experimental sampling cohorts

Experimental Design	Total Samples*	Variables
SOPUrine	204	Temperature (°C): 4; 25; -20; -80 Duration (Days): 0; 3; 7; 13; 21; 35; 40; 77; 171; 345 Amount of urine used (µL): 20; 100; 500; 1000
SOPFast	42	Temperature (°C): -20; -80 Collection Times (Fast/No-fast): Morning; Afternoon; Evening Amount of urine used (µL): 100
SOPThaw	24	Temperature (°C): -20; -80 Duration (Thaw Cycles): Cycle 1; Cycle 2; Cycle 3 Amount of urine used (µL): 100; 1000

*Samples were run in duplicates.

fasting was greater than 5-hr post-meal). Samples were collected within two days of each other according to the specific time frame. Approximately 50 mL of urine was collected, then aliquoted into 10 mL samples and stored in 15 mL centrifuge tubes for three days at

-20°C and -80°C before analyses. SOPThaw consisted of 20 mL of urine collected from the female volunteer, prepared in two centrifuge tubes of 10 mL for three freeze-thaw cycles, and frozen at both -20°C and -80°C for 24 hours before analyses.

Urine variable experimentation

SOPUrine experimentation, samples were removed from their respective storage and thawed on ice for 90 min according to the specific duration, which ranged from 3 days to 345 days, for an investigation into the target duration factor. Samples were centrifuged at 300 × g at 4°C for 10 min for debris removal. Before extraction of VOCs, urine supernatant was subjected to various aliquoted amounts of 20 µL, 100 µL, 500 µL, or 1 mL. The aliquots were mixed with DI water (2 mL or 19 mL), 300 µL of 1-ppm Mirex internal standard solution, and 2 M HCl (300 µL or 600 µL) in a 20 mL amber vial. Samples were prepared in duplicates to analyze sample amounts as a targeted factor on urinary VOC profiles.

SOPFast experimentation samples were centrifuged at 300 × g at 4°C for 10 min. Before extraction of VOCs, samples were then subjected to duplicated aliquots of urine supernatant of 100 µL and the aliquots were mixed with 2 mL of DI water, 300 µL of 1-ppm Mirex internal standard solution, and 300 µL of 2 M HCl in a 20 mL amber vial.

SOPThaw experimentation, samples were thawed on ice for 90 min and centrifuged at 300 × g at 4°C for 10 min. Samples were then subjected to duplicated aliquots of urine supernatant amounts (optimized from SOPUrine); 100 µL and 1 mL were mixed with DI water (2 mL and 19 mL, respectively), 300 µL of 1-ppm Mirex internal standard solution, and 300 µL or 600 µL of 2 M HCl, respectively, in a 20 mL amber vial.

Extraction of VOCs by SBSE-GC-MS

All urine sample solutions contained in a 20 mL vial were analyzed by stir bar sorptive extraction (SBSE) coupled with thermal desorption (TD) and gas chromatography/mass spectrometry (GC-MS) to extract the organic metabolites (i.e., VOCs). A commercially available stir bar coated with polydimethylsiloxane (Twister, 10 mm × 1 mm, Gerstel, Gerstel, Mülheim an der Ruhr, Germany) was inserted into each vial, and the solution was stirred for 2 hr at 1000 rpm (Gerstel 15 Position Twister Stir Plate, Mülheim an der Ruhr, Germany). Once stirring was completed, stir bars were removed from the vial, rinsed with DI water, dried with lint-free paper, and then placed into a thermal desorption tube for chemical analysis. Procedures for calibrations/conditioning of stir bars, cleaning thermal desorption tubes (TDTs), 20 mL vials, and stir bar storage vials followed our published protocol [14].

A thermal desorption unit, TD3.5 (Gerstel), coupled with an 8890 GC system and a 5977B Mass Selective Detector (Agilent Technologies, Wilmington, DE) was used to analyze VOCs in urine samples. The initial TDU temperature was set at 45°C. After holding for 0.5 min, the temperature of TDU was increased to 300°C at 60°C/min and held for 5 min. The desorption gas flow was set at 1.0 mL/min. During desorption, all the desorbed compounds were concentrated in a cryo injection system, CIS-4 (Gerstel), at -40°C before GC injection. Once the desorption process was completed, the CIS temperature was ramped to 300°C at 12°C/sec and held for 5 min in a splitless mode. The GC analysis was conducted through an HP-5MS UI capillary column (30 m × 0.25 mm × 0.25 µm with 5% phenyl-95% dimethylpolysiloxane, Agilent, USA) under solvent vent mode. The oven temperature was programmed as follows: held for 5 min at 35°C, ramped at 10°C/min to 300°C, and held for 10 min at 300°C. A Mass Selective detector detected the VOCs in urine samples in scan mode (40-500 m/z).

Specific urinary VOCs were identified using ChemStation software (Agilent Technologies, U.S.A.) with NIST (National Institute of Standards and Technology) Mass Spectral Library. Mirex has been previously chosen as the internal standard (IS) due to its exogenous nature in the human urine [14]. The protocol for normalizing the data to the response of Mirex was as follows: the relative area determined by the NIST Library of each VOC peak was normalized against Mirex (VOC Area divided by Mirex Area = Area Ratio), allowing semi-quantitative statistical analysis of VOCs and calculation of specific VOC area ratios.

An average of 150 compounds were detected per NIST library entry file, accounting for over 16,000 (SOPUrine), 12,700 (SOPFast), and 4,900 (SOPThaw) different VOCs within the three experimental designs. The NIST library utilizes a probability-based matching (PBM) algorithm that can reversely verify a spectrum to the reference spectrum. The pre-filtered search assigns significance to each peak, where the accuracy is depicted by the quality percentage value, where a higher quality value represents greater reliability of the unknown peak matches with the referenced compound spectrum. A streamlined analyses approach was adopted to remove all VOCs with less than 50% quality, where the remaining VOCs were used for the SOP experimental investigation. The remaining VOCs were screened, with various statistical analyses performed in R version 4.2.1 software and MetaboAnalyst 5.0 program to conduct qualitative and semi-quantitative studies of VOCs detected in the urine samples. We set the condition at Temperature: 25°C, Duration: 0 Days, and Sample Amount: 1 mL urine in 20 mL of water as the "gold standard reference condition" while other conditions were denoted as "alternative testing conditions".

All VOCs' responses were normalized to the response of internal standard Mirex as described in the Materials and Methods section. In addition, several quality control/reference analyses were performed throughout the experiment to validate and extend the results described in the following subsections.

Statistical analysis

Urinary VOCs were identified by the NIST libraries. The instrument response (i.e., peak area) of each compound was divided by the area of internal standard Mirex to get an area ratio representing its concentration. The complexity of the dataset presented fit-modeling issues; therefore, all variables (i.e., area ratios of VOCs) within each dataset were log-transformed and followed a light-tailed distribution similar to LaPlace or Gaussian distributions [31, 32]. Urinary-specific profiles of duplicated samples were evaluated by a two-sided t-test, where targeted independent variable comparison was used to determine the difference between sample duplicates. This was performed by linear regression fit-models with storage temperature as the covariate; the covariates were replaced with storage duration and sample amount respectively. All statistical analyses were performed in R version 4.2.1 software and MetaboAnalyst 5.0 program libraries as specified below [33, 34].

Exploratory PDF plots generation: Probability Density Function (PDF) plots illustrate the probabilities associated with reference and alternative conditions, displaying differences in probabilities (y-axis) against variations in area ratios (x-axis) with a 95% confidence level. These plots were individually generated for each cohort dataset to compare how different conditions impact urinary profiles directly. They enable the calculation of the likelihood of a random variable falling within specific value ranges, describing the distribution of values for that variable. Additionally, PDFs help identify the most probable outcome given specific conditions [35-37]. In this context, PDFs were employed to explore various combinations of target variables and assess their effects on the response factor and area ratio. This analysis provides valuable insights into the relationships between variables and their impact on the observed urinary profiles [38].

Exploratory combined scatterplot and response surface generation: Combined Scatterplot and Response Surface (CSRS) plots offer a visual representation of the interplay between two independent variables, depicted along the scatterplot axis, and a dependent variable, showcased as a surface plot. In constructing these plots, the independent variables chosen were temperature and urine amount (duration), while the log10 of the area ratio remained the constant dependent variable. These CSRS plots were generated separately for each cohort dataset to discern whether the interaction between the two dependent variables, temperature, and urine amount, significantly impacted the measured area ratios. By examining these plots, we gain insights into how temperature and urine amount changes collectively affect the area ratios' behavior, providing a comprehensive understanding of the underlying relationships within the data.

PLS-DA analyses: In PLS-DA (Partial Least Squares Discriminant Analysis), the VIP (Variable Importance in the Projection) score are computed to quantify the significance of a

variable in effectively distinguishing between the two classes under consideration. Post hoc diagnostic plots were created to assess heteroskedasticity and distribution of model predictions and residuals. Nonparametric and bootstrap mean comparisons were performed to determine the model prediction effect of the raw dataset compared to the transformed; the Wilcoxon rank-sum test presented zero inflation among many VOCs. A final logistic regression model was evaluated via PLS-DA and scatterplot/surface response [38]. The outcomes of this analysis were visualized through graphs that depicted the complex interactions among three key variables: storage temperature, storage duration, and sample amount. These visual representations provided insights into how these variables correlate with the area ratio, enhancing our understanding of their collective impact on the studied phenomenon.

Results

To investigate the influence of storage temperature, storage duration, and sample amount on human urinary biomarker profiles, analytical and computational screening were performed using stir-bar sorptive extraction coupled with gas chromatography-mass spectrometry (SBSE-GC-MS) and machine learning (ML) approaches. The VOCs in the urine collected from three healthy volunteers (one female, two males) were analyzed to study the effects of sample collection conditions on urine metabolome. The investigated variables included storage temperature, storage duration, sample volume, sample collection condition, and freeze-thaw cycles. The urine samples were assigned to three sub-cohorts: "SOPUrine" (Storage Variables), "SOPFast" (Fast/No-fast), "SOPThaw" (Freeze-thaw) (shown in **Scheme 1** and **Table 1** in the Materials and Methods section).

For the three experiments, freshly collected urine samples at a temperature of 25°C, duration at 0 Days, and sample amount of 1 mL urine were determined as the "gold standard reference condition"; additional conditions were denoted as "alternative testing conditions". Supervised PLS-DA plots and variable importance in projection (VIP) scores in MetaboAnalyst were produced throughout the experimental analyses to investigate the effects of conditions on urinary profiles. VIP scores are calculated by the PLS weighted sums, which is beneficial in explaining the y-variance (i.e., VOC quantities in area ratio responses) within each x variable (i.e., targeted sample variables). These VIP scores are proper measurements determining the most explained y-variance contributing to and accounting for the overall changes in the urinary VOC profiles and specific VOCs. Detailed observation in comparing the targeted variables of sample temperature, sample amount, and sample duration within the three experimental designs is described in the following sections. Collectively, the overall trend supported that flexibility within storage conditions is acceptable in sample preparation and analyses.

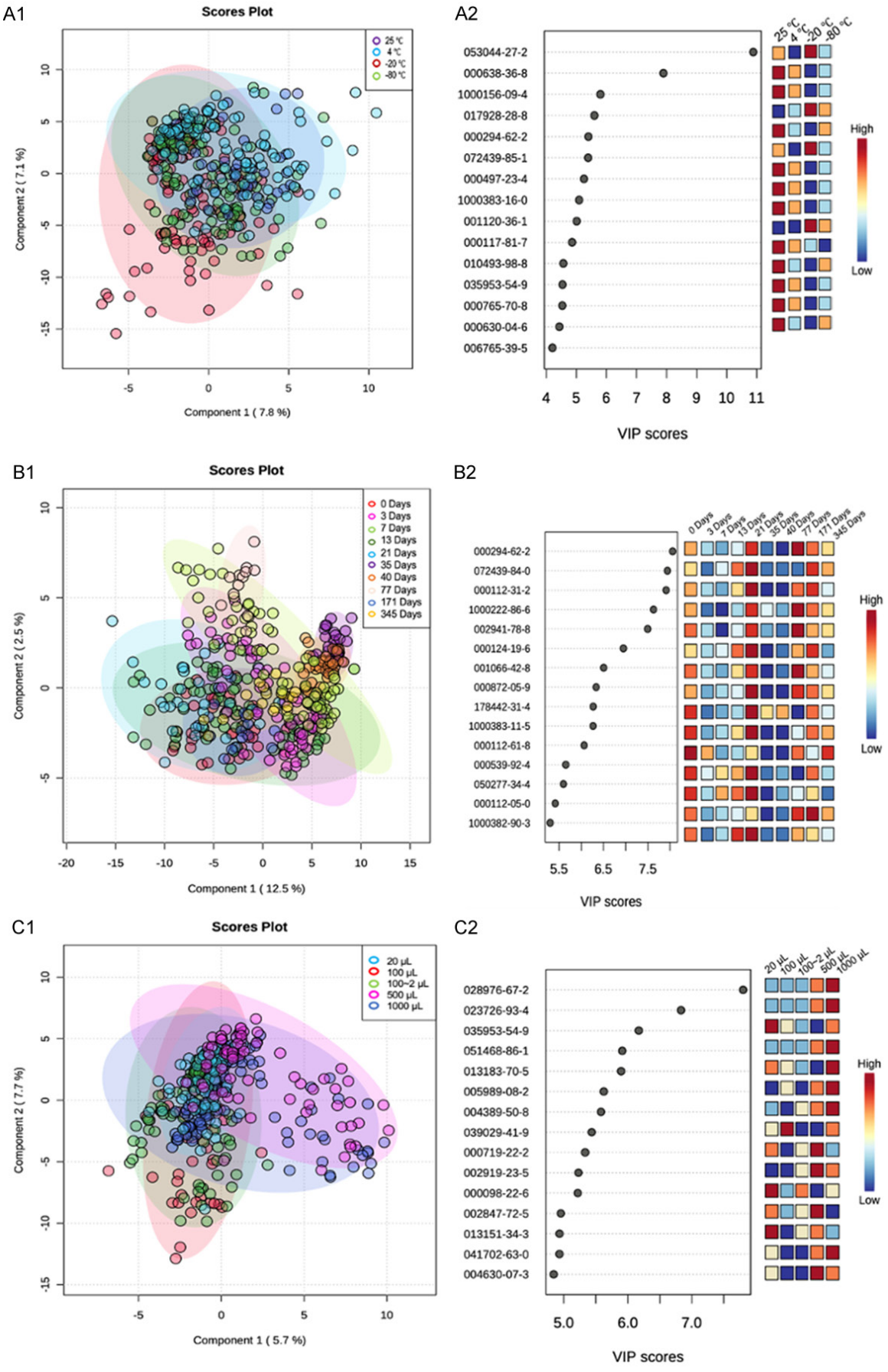
Influence of storage conditions and sampling preparation on variables urine samples dataset

The initial investigation of the targeted variables of storage temperature, storage duration, and sample amount was performed on 204 urine samples collected and stored from December 2020 to July 2022 (**Table 1**). Using PLS-DA, we observed no evidence of significant differentiation according to the variables analyzed. This suggests that the VOC profiles of the entire cohort were not significantly influenced by storage temperature, duration, and amount of urine used for extraction (**Figure 2A1-C1**).

Using PLS-DA, significant VOCs were identified under each targeted variable: temperature (311 VOCs), duration (233 VOCs), and amount (270 VOCs) with a complete list of the 814 VOCs (data are available upon request). The VIP score plots of **Figure 2A2-C2** show the top 15 VOCs for each analysis, a comprehensive list of the 69 overlapping VOCs from the variables temperature, duration, and amount was recorded (data are available upon request). These VOCs were selected from the 814 total VOCs are filters with the VIP scores greater than or equal to one. From these analyses, sample duration contributes to the most significant percentage of explained variability yet is still statistically insignificant.

Influence of collection times and fasting on urinary metabolites

Following the initial investigation of the storage variables in SOPUrine samples, SOPFast uti-


A1
Scores Plot
25 ℃
4 ℃
-20 ℃
-80 ℃
Component 2 (7.1 %)
Component 1 (7.8 %)
A2
053044-27-2
000638-36-8
1000156-09-4
017928-28-8
000294-62-2
072439-85-1
000497-23-4
1000383-16-0
001120-36-1
000117-81-7
010493-98-8
035953-54-9
000765-70-8
000630-04-6
006765-39-5
25 ℃
4 ℃
-20 ℃
-80 ℃
High
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B1
Scores Plot
0 Days
3 Days
7 Days
13 Days
21 Days
35 Days
40 Days
77 Days
171 Days
345 Days
Component 2 (2.5 %)
Component 1 (12.5 %)
B2
000294-62-2
072439-84-0
000112-31-2
1000222-86-6
002941-78-8
000124-19-6
001066-42-8
000872-05-9
178442-31-4
1000383-11-5
000112-61-8
000539-92-4
050277-34-4
000112-05-0
1000382-90-3
0 Days
3 Days
7 Days
13 Days
21 Days
35 Days
40 Days
77 Days
171 Days
345 Days
High
Low
VIP scores
C1
Scores Plot
20 µL
100 µL
100~2 µL
500 µL
1000 µL
Component 2 (7.7 %)
Component 1 (5.7 %)
C2
028976-67-2
023726-93-4
035953-54-9
051468-86-1
013183-70-5
005989-08-2
004389-50-8
039029-41-9
000719-22-2
002919-23-5
000098-22-6
002847-72-5
013151-34-3
041702-63-0
004630-07-3
20 µL
100 µL
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500 µL
1000 µL
High
Low
VIP scores

Figure 2. PLS-DA plots of the investigated storage variables and their effects on VOC profiles, and their corresponding VIP Score Plots (MetaboAnalyst). (A1) Analysis of the impact of storage temperature on VOC profiles; (A2) The top 15 VOCs contributed to generating PLS-DA SOPUrine-Temperatures VIP Score Plot; (B1) Analysis of the effect of storage duration on VOC profiles; (B2) The top 15 VOCs contributed to SOPUrine-Duration VIP Score Plot; (C1) Analysis of the impact of sample amount on VOC profiles; and (C2) The top 15 VOCs contributed to SOPUrine-Sample Amount VIP Score Plot. VIP scores were calculated and filtered by scores greater than and equal to 1, where Red indicates the highest concentration and Blue indicates the lowest concentration.

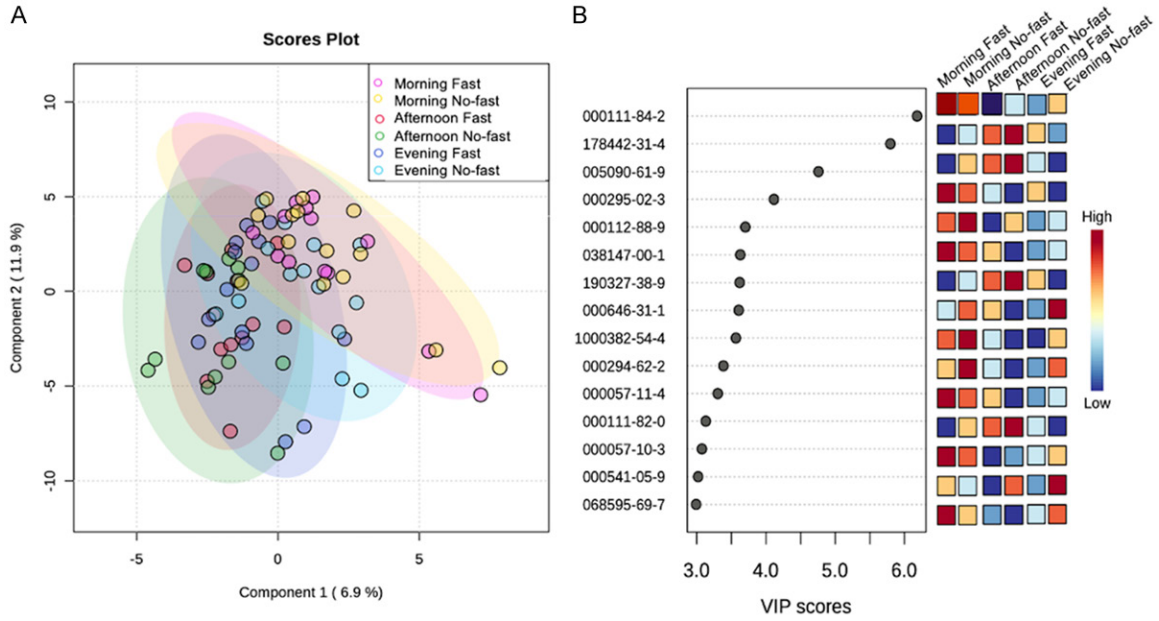

Figure 3. (A) PLS-DA plots of the SOPFast target variables on their effects on VOC profiles and (B) the top 15 VOCs out of 138 contributed to generating PLS-DA plots. VIP scores were calculated and filtered by scores greater than and equal to 1, where red indicates the highest concentration and blue indicates the lowest concentration.

lized sample storage temperatures of both -20°C and -80°C and a sample amount of 100 µL of urine in 2 mL of water to observe the VOC profile changes due to the subjection of fasting and the time of collection (morning, afternoon, evening). A total of 42 urine samples were collected and stored from December 2020 to July 2022 (**Table 1**). We observed no evidence of significant clustering according to the fast, no-fast, and time of sample collection, suggesting that there is flexibility within sample collection and condition that does not influence urinary VOC profiles as an entire cohort (**Figure 3A**). SOPFast determined a 6.9% explained variability, supporting the interpretation that sampling conditions are not a determining factor in this cohort (**Figure 3A**). A total of 11,086 VOCs were identified of which 579 VOCs were selected by the PLS-DA, and 119 VOCs were considered significant from the SOPFast analysis. A comparison of the selected conditions indicated that morning fast samples contributed to having greater VOC concentrations compared to the rest of the group (**Figure 3B**). However, these VOCs do not contribute to the significance of SOPFast conditions.

Influence of freeze-thaw cycles on urine metabolites

Lastly, SOPThaw utilized sample storage temperatures of both -20°C and -80°C and sample amounts of 100 µL of urine in 2 mL of water and 1000 µL of urine in 20 mL of water to observe the integrity of the VOC profiles due to repeated sample storage cycling Within SOPThaw; urine samples were collected and stored from December 2020 to July 2022, accounting for 24 analyzed urine samples (**Table 1**). Clear evidence of significant clustering was observed based on freeze-thaw cycles, signifying that increased cycles impact the urinary VOC profiles (**Figure 4A**). **Figure 4B** also denotes that at higher cycles, the top VOC con-

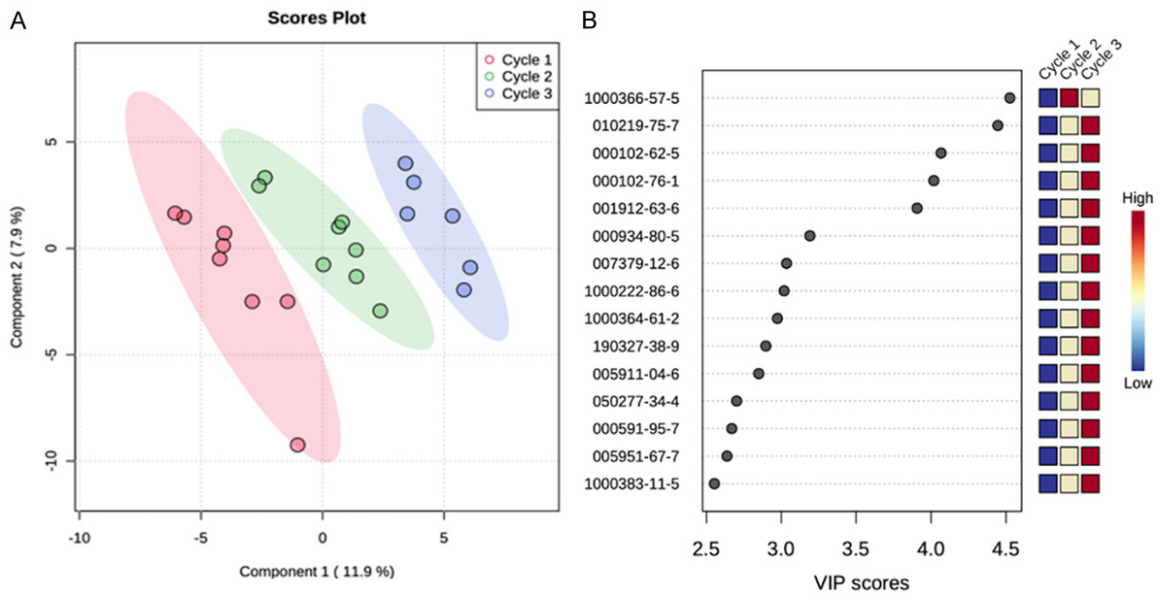

Figure 4. A. PLS-DA plots of SOPThaw target variables on their effects on VOC profiles (MetaboAnalyst). B. The top 15 VOCs out of 103 contributed to generating PLS-DA plots. VIP scores were calculated and filtered by scores greater than and equal to 1; Red indicates the highest concentration and blue indicates the lowest concentration.

tributors (top 15 VOCs from a total amount of 394 SOPThaw VOCs) show an increase in concentration which is a direct correlation of thawing and duration effects on urinary profiles.

Exploratory statistical analyses of experimental datasets

To re-examine the previous findings, we conducted exploratory analyses on the previous experimental datasets (SOPUrine, SOPFast, and SOPThaw). Determination of the extent to of urinary profiles are affected in response to the storage variables is an essential question for understanding the chemical changes and detecting VOC profiles in various storage conditions. Exploratory statistical analyses of the flexibility of optimal conditions and modeling approaches were performed to detect and determine urinary VOC profile differences amongst experimental conditions. We performed unsupervised clustering analyses of all experimental urinary variables, revealing no definitive clustering or influence within the observation of the total SOPUrine and SOPFast datasets, with significant clustering in SOPThaw (**Figures 2**-**4**). The results indicated that storage temperature, storage duration, and sample amount do not significantly contribute to the changes in urinary VOC profiles, further suggesting that sample preparation conditions can be flexible while maintaining the integrity of the VOC analyses.

Exploration of variable responses was further investigated against the "gold standard condition" using probability density function plots (PDF) (**Figures 5**-**7**). PDFs are generated to describe the probability of a specific outcome within a given range of values. This mathematical function assigns probabilities to each dataset value, where the sum of all probabilities equals one. The assigned PDF values are then multiplied by the probability to calculate the given PDF scores, and significant plots are determined by a 95% confidence/prediction level; created by bootstrapping modeling to estimate confidence intervals based on the data used to generate the PDF plot (*data not shown*). In visual representation, PDF plots of the reference and alternative conditions that differ in associated probabilities (y-axis) are considered significant in relationship to the differences in area ratios (x-axis). Variables of temperature and sample amount were held constant for the reduction of other bias and a greater explanation of the targeted variable effects on the urinary VOC profile response for the duration of SOPFast and SOPThaw. **Figure 5** investigates the reference condition of tem-

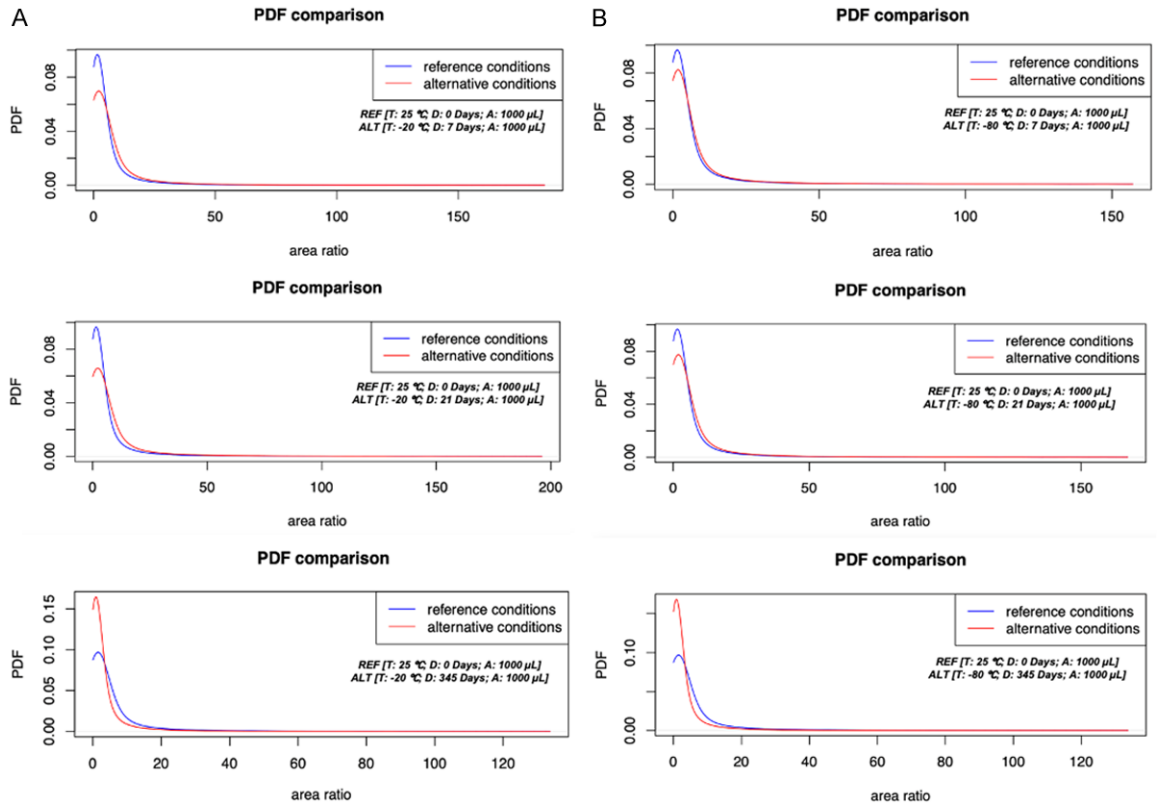

Figure 5. PDF Comparison Plots of SOPUrine to reference condition (i.e., Temperature 25°C, 0 days, and 1000 µL of urine). A. Temperature -20°C; B. Temperature -80°C [Three durations: 0, 7, 21, 345 days; performed using 1000 µL of urine]. Additional PDF comparisons were generated (*data not shown*) [Reference Conditions: REF; Alternative Conditions: ALT; Temperature: T; Duration: D; Sample Amount: A].

perature 25°C, 0 days, and 1000 µL compared to temperature -20°C and -80°C at three durations for cohort SOPUrine. The plots further support the previous results that temperature does not significantly affect the urinary profile, and duration only suggests a noticeable change after 345 days for both temperatures. These results indicate storage condition flexibilities in two variables (temperature and duration) for acceptable urinary profiling reliability. As shown in **Figure 6**, PDFs compare SOPFast variables of fast and no-fast to all three collection times (morning, afternoon, and evening). The results indicate slight differences between collection times and fast/no-fast, signifying that samples can be collected at various times with or without fasting and urinary profiles will not be affected by the sample collection conditions SOPThaw PDFs confirm that freeze-thaw cycles affect urinary profiles where profiles begin to display differences at Cycle 2, indicating that free-thaw cycles should be limited if possible (**Figure 7**); additional PDF comparisons were generated (*data not shown*).

Analyses of combined scatterplot and response surface were implemented to display the relationship between variables and VOC profiles (expressed as area responses in GC/MS analysis). **Figure 8A**, SOPUrine indicates a slight concavity, where area ratio response is significant when all variables (i.e., temperature, duration, and amount) are considered. However, further analyses (PLS-DA/VIP Scores) were needed to determine the significance of the variable and VOC responsible for the area ratio response. Both SOPFast and SOPThaw were observed to have a flat/non-significant effect on the urinary VOC profiles specific to the experimental datasets (**Figure 8B** and **8C**). With the consideration that there were statistical analysis trends and similarities in all three experiments, the scatterplots/surface response plots support the previous findings of the SOPUrine and SOPFast by PLS-DA and VIP Scores, SOPThaw sampling conditions using supervised PLS-DA of duration/cycles results show significant clustering within freeze-thaw cycles indicating a relatively high variable explanation of 11.9%. Scatterplots

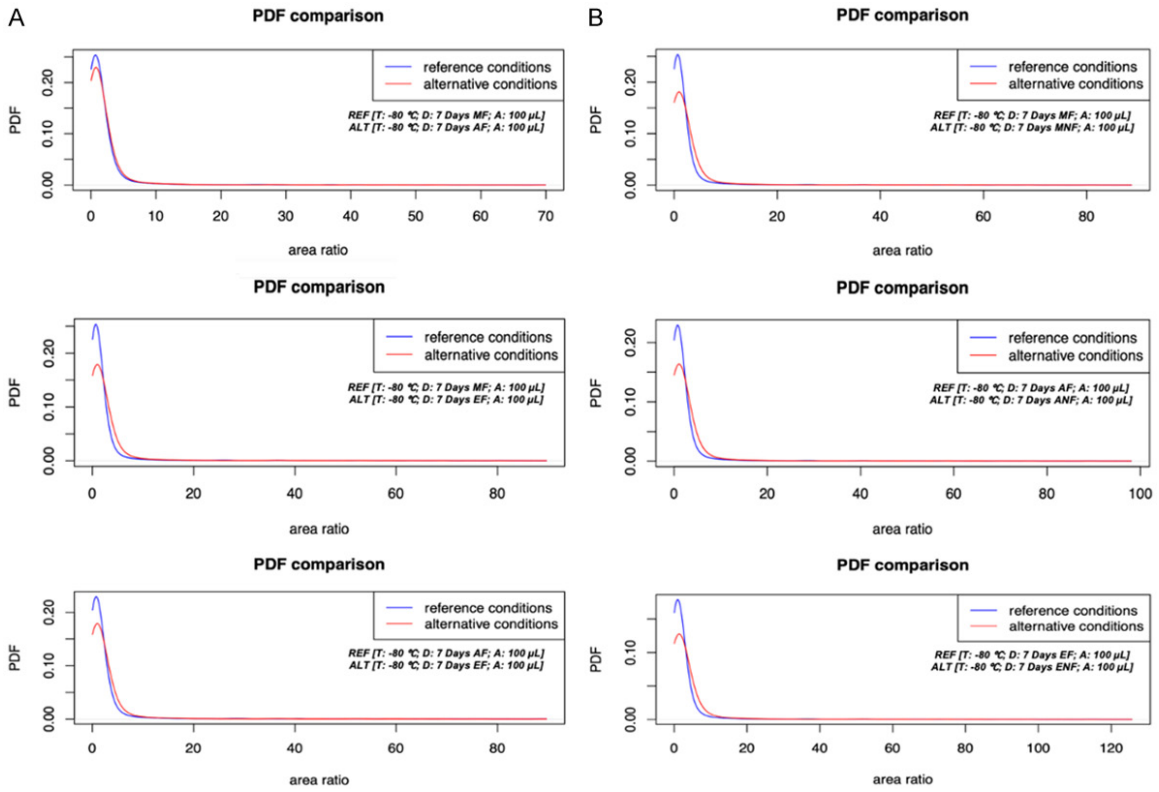

Figure 6. PDF Comparison Plots of SOPFast to reference condition (i.e., Temperature 25°C, 0 days, and 1000 µL of urine). A. Collection Times and Fast; B. Collection Times and No-fast [Three collection times: morning (M), afternoon (A), evening (E), and two Fast: fast (F) and no-fast (NF); performed at 100 µL]. Additional PDF comparisons were generated (*data not shown*) [Reference Conditions: REF; Alternative Conditions: ALT; Temperature: T; Duration: D; Sample Amount: A].

and response surface plots benefit both statistical analyses and visual representation to determine whether there is a correlative relationship between multiple continuous variables.

Discussion

Urinary metabolomic studies are a powerful tool for gaining insights into human health and diseases [2, 9, 37, 39]. Several research teams have utilized mass spectrometry to measure and analyze the metabolites in a urine sample to gain insights into the metabolic processes in the body [4]. These studies can be used to identify biomarkers of disease, to help diagnose and monitor conditions, to measure levels of therapeutic drugs, and to gain insights into the effectiveness of treatments [4, 37]. Statistics can then be incorporated to analyze the metabolite levels and their correlations with different clinical outcomes, such as disease progression or drug efficacy [4, 6, 7, 18, 39].

We used SBSE-GC-MS and machine learning approaches for urinary VOC detection and profiling as an effect of sample conditions. Stir bar sorptive extraction (SBSE) is a robust, reliable, and limited solvent sample preparation technique that offers several advantages over traditional solvent extraction techniques [40, 41]. These benefits for urinary VOC extraction include a green chemistry approach, reduced sample preparation, increased selectivity due to the coating attraction of hydrophilic analytes, and improved detection sensitivity. SBSE has also produced more reproducible results than other extraction methods and is supremely cost-effective in the repeatable use of up to 100 analyses per stir bar [41]. Chambers et al. investigated using an alternative sorptive extraction method, HiSorb, which allows for a more sorptive phase, thus increasing sorptive capacity and applicability for the automation [42]. When extraction techniques are coupled with robust analytical methods, analyses are sensitive and comprehensive in the character-

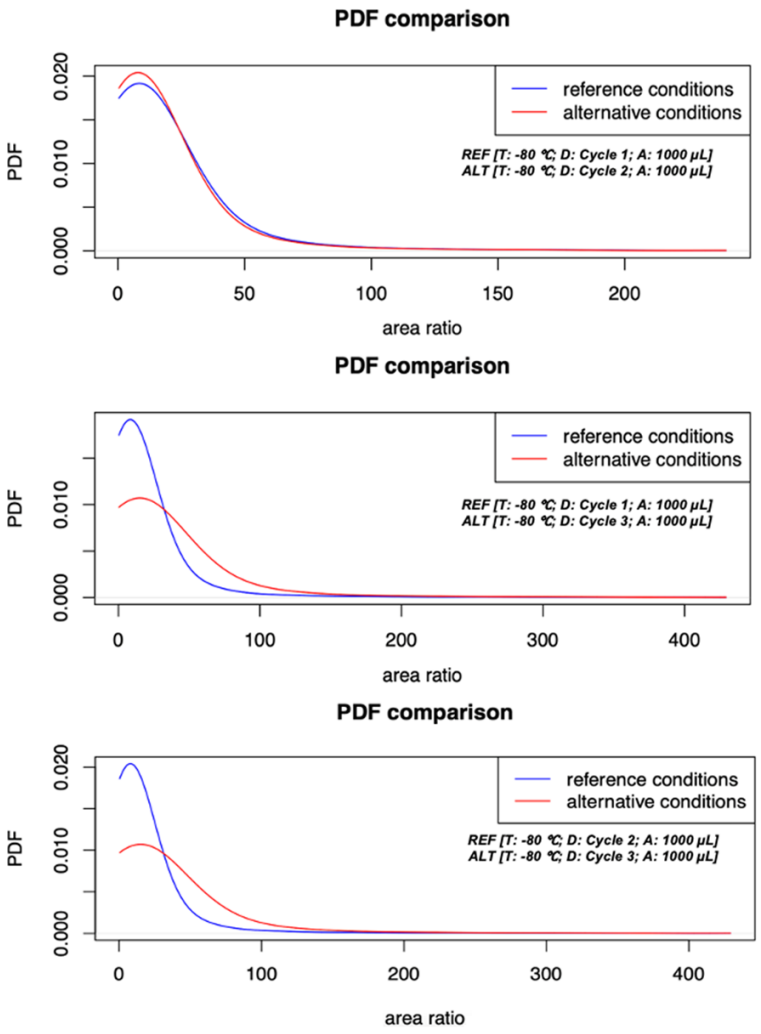

Figure 7. PDF Comparison Plots of SOPThaw Cycles to reference condition (i.e., Temperature 25°C, 0 days, and 1000 µL of urine) [Three freeze-thaw cycles 1-3, performed with 1000 µL urine]. [Reference Conditions: REF; Alternative Conditions: ALT; Temperature: T; Duration: D; Sample Amount: A].

ization of metabolites, therefore increasing the use in clinical screening, diagnosis, and additional detection applications [9, 37, 43, 44].

Standard operating procedures are critical to any analytical analyses for various applications. Having a standard operating procedure in place helps to ensure uniformity of operations and consistency in the quality of sample collection, storage, preparation, and analyses. A universal and incorporated SOP detects subtle differences in metabolite levels that could otherwise be overlooked, making it possible to compare results between different experiments. Ultimately, having a urinary standard operating procedure contributes to overall accuracy and reproducibility within analyses.

It is of great interest to have an SOP that provides reliable VOC profiles that can be applied to diagnose primary cancers, such as prostate and renal cell carcinoma. SOP development and machine learning approaches present excellent outcomes and improvement to sampling conditions to current molecular diagnostics and prognostic methods [3, 4, 6]. To date, research is minimal on pre-sampling and sample preparation for detecting urinary metabolites. Hence this study set out to investigate tandem influences of different conditions of urinary profiles. Literature has shown support and argument that urine metabolome can be influenced by various factors (i.e., sample amount, diet, fasting, freeze-thaw cycling, etc.) [29, 30, 37, 45].

Factors such as improper storage temperature, contamination, and exposure to light or air can all lead to metabolite degradation. Inadequate sample collection procedures and delayed processing can also lead to inaccurate results [42, 46-48]. Additionally, errors in the metabolite analysis can occur if the sample has been stored for an extended period or if the metabolite concentration is too low or too high. To ensure the accuracy and reliability of the analytical techniques and statistical approaches, protocols and reporting for sample collection and storage must be standardized. Wen et al. are one of the first experimental studies to investigate and optimize various sampling conditions (temperature and time, acidification, sample volume, dilution factor) for application in discriminating between healthy and carcinomic cohorts [49]. The most widely accepted storage temperature for biological matrices is -80°C, and storage

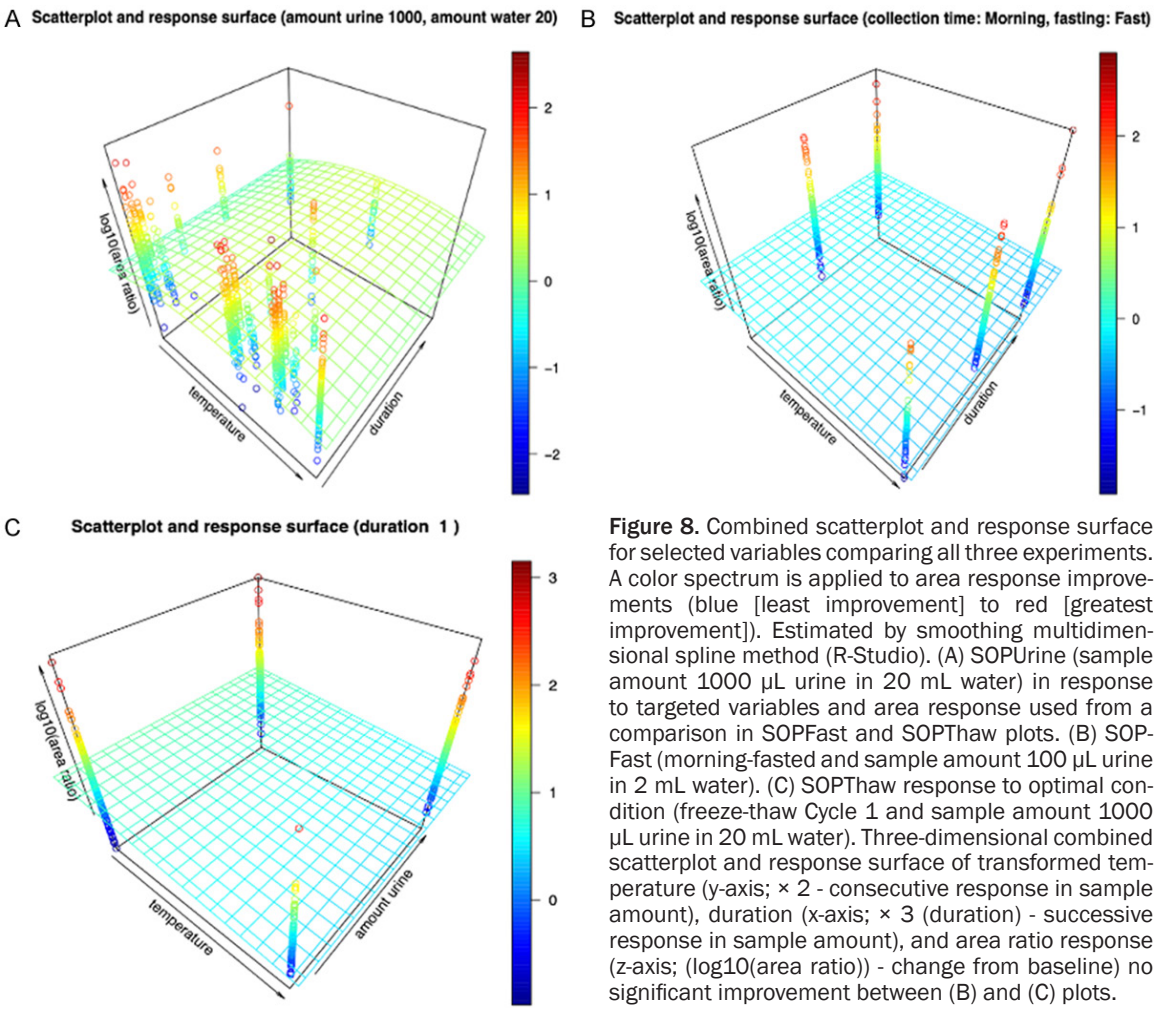

Figure 8. Combined scatterplot and response surface for selected variables comparing all three experiments. A color spectrum is applied to area response improvements (blue [least improvement] to red [greatest improvement]). Estimated by smoothing multidimensional spline method (R-Studio). (A) SOPUrine (sample amount 1000 µL urine in 20 mL water) in response to targeted variables and area response used from a comparison in SOPFast and SOPThaw plots. (B) SOPFast (morning-fasted and sample amount 100 µL urine in 2 mL water). (C) SOPThaw response to optimal condition (freeze-thaw Cycle 1 and sample amount 1000 µL urine in 20 mL water). Three-dimensional combined scatterplot and response surface of transformed temperature (y-axis; × 2 - consecutive response in sample amount), duration (x-axis; × 3 (duration) - successive response in sample amount), and area ratio response (z-axis; (log10(area ratio)) - change from baseline) no significant improvement between (B) and (C) plots.

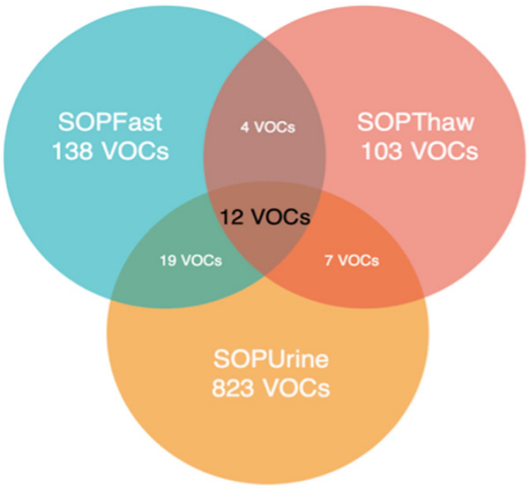

Figure 9. Venn Diagram of Cohorts Overlapping VOCs generated from PLS-DA VIP Scores.

duration can be unlimited. Research shows that even storage at -20°C for six months does not affect the integrity of urinary VOCs, which is a beneficial suggestion when ultra-freezes are unavailable. As shown in **Figures 2** and **3**, VOC profiles showed no significant differences under different storage temperatures, durations, and sample amounts. This implies that within the range of conditions tested, variations in these factors did not substantially impact the detection of VOCs in the urine samples. Based on our findings, it can be inferred that these factors can be manipulated within a reasonable range without significantly altering the urinary profiling results. Overall, this study contributes to the existing knowledge by providing insights into the effects of acidification and storage conditions on the detectability of VOCs in urine samples. The study highlights the potential benefits of acidification for improving the detection of VOCs. It suggests that storage temperature, duration, and sample volume can be optimized based on convenience and practi-

Table 2. Characteristics of the twelve overlapped VOCs from experimental cohorts

CAS Number	Compound Name	Molecular Weight (amu)
000629-73-2	Cetene	224.250
000112-80-1	Oleic Acid	282.256
000334-48-5	n-Decanoic acid	172.146
000101-39-3	2-Propenal, 2-methyl-3-phenyl-	146.073
000556-67-2	Cyclotetrasiloxane, octamethyl-	296.075
002941-78-8	2-Amino-5-methylbenzoic acid	151.063
005090-61-9	(2R,8R,8aS)-8,8a-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,7,8,8a-hexahydronaphthalene	202.172
017928-28-8	Methyltris(trimethylsiloxy)silane	310.127
050277-34-4	4-Isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahydronaphthalene	200.157
055429-29-3	Arsenous acid, tris(trimethylsilyl) ester	342.048
1000364-61-2	1,1,3,3,5,5,7,7-Octamethyl-7-(2-methylpropoxy)tetrasiloxan-1-ol	370.148
190327-38-9	(1S,7S,8aR)-1,8a-Dimethyl-7-(prop-1-en-2-yl)-1,2,3,7,8,8a-hexahydronaphthalene	202.172

A complete list of overlapped VOCs is available upon request.

cal considerations without compromising the accuracy of urinary profiling.

It has been suggested that aliquoting samples should be performed at sample collection for the number of analytical testing and also to minimize freeze-thaw cycles. Our findings agreed with other reports that freeze-thaw cycles could significantly influence urinary VOCs, which can lead to a decrease in the number of urinary VOCs present. Studies have shown that freeze-thaw cycles can alter the concentration of specific metabolites, including creatinine, urea, and uric acid, and current changes in the pH of urine samples; these metabolic perturbations can lead to the reduction in validity and reproductivity in results [30, 50].

Research has shown that fasting can affect urinary VOCs and increase and decrease the concentrations of some urinary VOCs. Thus, fasting is a target condition that should be investigated to determine its potential alterations to urinary profiles. Fasting increases urinary creatinine excretion but decreases the excretion of albumin, uric acid, and a range of electrolytes. Fasting is also related to decreased creatinine to albumin, suggesting that fasting alters the body's balance between catabolic and anabolic processes. As a result, fasting can change the biomarkers of health and disease derived from urinary VOCs [43, 47]. Favé et al. observed that collection times and fast presented distinct profiles [51]. This contradicts our findings that both collection times and fast/no-fast did not significantly alter the cohort urinary profile. Our findings suggest that fasting does not significantly influence urinary VOCs (**Figure 3**), resulting in acceptable flexible sample collection times with and without fasting.

We have studied the effects of storage temperature, storage duration, sample amount, collection times, fast/no-fast, and freeze-thaw cycles on urine metabolomics using SBSE-GC-MS. Within all three datasets, freeze-thaw cycles caused the most significant changes in urinary metabolite concentrations. Using VIP (Variable Importance in the Projection) scores in PLS-DA offers an advantage over *p*-values in that they can assess the importance of individual variables in the data discrimination. Twelve VOCs were selected from the PLS-DA VIP scores with scores greater or equal to 1 in SOPUrine, SOPFast, and SOPThaw (**Figure 9** and **Table 2**). A comprehensive analysis of the VIP scores demonstrated limited effects on biomarker discovery and exogenous value on urinary profiles (*extended data not shown*). However, this application could be further enhanced to determine exogenous urinary biomarkers that can be pinpointed as having statistical effects on urine profiling.

In this study, statistical analysis were performed to highlight the comparison between reference condition (i.e., room temperature, 0 days storage) and other alternative storage conditions. As shown in **Figures 5**-**7**, Probability density function (PDF) plots help visualize the

probability distribution of a continuous variable. They can be used to identify the most likely values for a variable and any outliers or unusual values. PDF plots can also be used to compare the distributions of two variables or to identify any correlations or relationships between variables. Additionally, PDF plots can be used to identify any asymmetries in a distribution, which may indicate a potential underlying cause. Finally, they can also determine the data spread or the range of values a variable can take. These plots can be further utilized to extrapolate data points that have yet to be experimentally conducted, to expand the robustness of variable screening. PDFs can be combined with scatterplot and surface response (**Figure 8**) and benefit from the ability to represent the simultaneous relationship between three or more variables. This analysis can also add to the robustness of identifying data trends and extrapolating areas of higher or lower values.

As shown in **Figures 2**-**4**, some VOCs are found to have some degree of significance. Using PLS-DA, significant VOCs were identified under each targeted variable: temperature (299 VOCs), duration (233 VOCs), and amount (291 VOCs) [823 Total] (data not shown). **Figure 9** is a Venn diagram representing the PLS-DA results of each cohort's total number of VOCs. A comprehensive list of the top forty-two VOCs consistently detected between all three cohorts (data not shown). Furthermore, twelve VOCs were found to be statistically significant in all tested variables (**Table 2**). Three of the twelve compounds found in the literature have biomedical relevance: Cetene, Oleic Acid, and n-Decanoic Acid. In future urinary metabolomic studies, it is crucial to explore the identification of internal biomarkers that can serve as references for storage conditions and potentially replace creatine as an internal standard, all while maintaining the reliability of the urinary profile.

Conclusions

We analyzed various sample conditions in the effect of urinary profiles in healthy cohorts. Results revealed limited association and impact on urinary VOC profiles. These results were obtained by subjecting each experimental dataset to investigate five target variables: storage temperature, storage duration, sample amount, fasting, and freeze-thaw cycles. Utilizing both machine learning and GC-MS analysis in the exploration and identification of urinary "VOCs" response to different conditions. While our finding supported preferred storage conditions of -80°C and a minimal freeze-thaw cycle for the analysis [29, 30, 45, 52], we also demonstrated that no statistical significance within the urine profiles under the three variables of storage temperature, storage duration, and sample amount, permitting a certain degree of flexibility of conditions when these optimal conditions are not or cannot be achieved.

The strength of this study is represented the tandem study design where temperature, duration, fasting, and freeze-thaw cycles were taken into consideration in their effect on urinary profiling. The analytical technique of SBSE-GC-MS presented high throughput and reproducibility of urinary metabolite detection with a lower cost and greener approach to urinary volatome extraction. The quality of the study was also enhanced by the robustness of the exploratory statistical analyses and strategies in detecting comprehensive sets of compounds without compromising the assumptions and sensitivity. One of the significant limitations of our current study was that SBSE-GC-MS is limited to analyze hydrophobic metabolites thus our findings are only applicable to hydrophobic (i.e., not water soluble) metabolites in urine. A second limitation is that we did not look for specific cancer biomarkers. Future study could focus on how the factors in this study could affect the specific urinary VOCs that have the potential for cancer diagnosis. Future work can explore greater experimental designs to study the elicited impact for a longer experimental duration and to focus on a particular panel of biomarkers to determine the interaction effects of each storage and preparation variable.

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Disclosure of conflict of interest

None.

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