

Evaluation of FASP for Mass Spectrometry-Based Untargeted Metabolomics Analysis of Urine Samples

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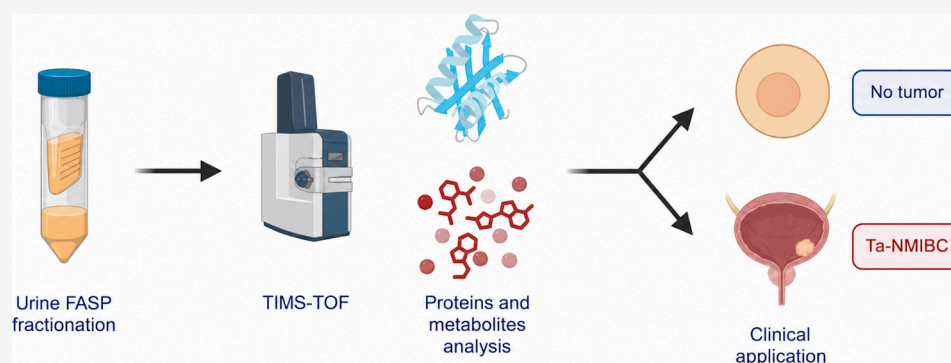
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ABSTRACT: Filter-Aided Sample Preparation (FASP) is a well-established method in proteomics, yet its potential for the parallel recovery of metabolites remains largely unexplored. Herein, we evaluate the performance of FASP as a straightforward workflow for the simultaneous isolation of protein and corresponding metabolite fractions from a single urine sample. The FASP-based LC-MS/MS approach for both proteomics and metabolomics analysis identified 3,163 nonredundant peptides corresponding to 957 unique protein groups. The metabolomic profile comparison of three urine fractions, specifically FASP-concentrated, FASP flow-through, and raw samples, resulted in the identification of 176 common metabolites. Next, as a proof-of-concept, the FASP protocol was applied to compare the metabolomic profiles of clinical urine samples from healthy individuals ($n = 13$) and patients with Ta bladder cancer ($n = 12$). The metabolomic modulation was consistent with previously reported findings, highlighting perturbations in phenylacetate, purine, and tryptophan metabolism, as reflected by changes in metabolites such as adenosine monophosphate (AMP), phenylacetic acid, glutamine, cytosine, and L-tryptophan. FASP protocol can be effectively adapted for the concurrent profiling of both proteomic and metabolomic fractions from urine samples. Thus, FASP-based workflow represents a viable alternative for single-step sample preparation, facilitating subsequent quantitative multiomics data integration.

KEYWORDS: FASP, Proteomics, Metabolomics, Multiomics, Bladder cancer, Urine, Sample preparation

1. INTRODUCTION

Multiomics is a rapidly emerging field that combines multiple methods and data sets from diverse high-throughput technologies, including genomics, proteomics, lipidomics, metabolomics, and other omics.¹ Multiomics integrates quantitative data sets from thousands of analytes and is increasingly recognized as an essential tool for assessing and pinpointing the fundamental causative molecular alterations that may lead to disease and, ultimately, for identifying novel disease biomarkers.^{2,3} Therefore, more recently, the medical bioinformatics section has made great efforts to extract meaningful biological interpretation from layers of “omics” data sets.⁴ This would certainly open new avenues for disease diagnosis and for personalized and/or precise therapies aiming at more effective pharmacological treatments. The major

challenge now lies in integrating multiple data types from multiomics data using data mining and predictive algorithms to support a more comprehensive interpretation of phenotype-genotype relationships than analysis using a single data type.^{4,5}

The majority of existing omics data sets were acquired over the past two decades, obtained from a single, standalone omics approach using specific sample preparation protocols, and were

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frequently generated from cells, tissues, or body fluids exposed to conditions that differed significantly from those in the comparison such as cardiovascular health and disease, where integrating multiomics studies across plasma patient cohorts offers a more complete interpretation of the complex biology than individual-level analysis.⁶ Indeed, a great deal of the current metabolomic data sets were acquired through nuclear magnetic resonance (NMR) spectroscopy-based analysis such as a metabolomics biomarker study for metastatic colorectal cancer using blood serum samples,⁷ which uses sample preparation protocols that are, in many aspects, distant from those employed in proteomics workflows, specifically liquid chromatography mass spectrometry (LC-MS/MS)-based analysis, the proteomics' gold standard technology.^{8,9} Therefore, even when subjected to the most sophisticated bioinformatics methodologies, downstream multiomics statistics and/or integrative bioinformatic analysis must account for the inherent bias and inconsistent interplay among data sets.^{4,10} As such, there is a need to generate multiomics data sets from a single reference sample as a starting point. The field is still searching for a reliable, robust extraction and sample preparation protocol that enables the extraction of primary cellular components (proteins, metabolites, lipids, and nucleic acids) from a single sample aliquot. Recently, in our attempt to build a unified multiomics sample preparation methodology (Zenati et al., 11), we compared the coverage and reproducibility of two extraction methods frequently used in plasma: single-phase methanol precipitation (MeOH ppt) and biphasic chloroform/methanol (MeOH:CHCl₃) extraction.¹¹ The study indicates that both extraction methods are suitable for integrative multiomics analysis. Along the same lines, Muehlbauer et al.¹ presented an efficient and unified in-solution approach for preparing lipids, metabolites, and proteins. Termed Bead-enabled Accelerated Monophasic multiomics.¹ While, the field of multiomics welcomes these and other solid-phase sample preparation methodologies, Filter-aided sample preparation (FASP)¹² is still the method of choice for many in proteomics studies,^{13–15} mainly due to its outstanding effectiveness in eliminating sample contaminants, in protein conversion to peptide, and in peptide cleavages,^{16–18} particularly for body fluids like plasma,^{19–22} urine^{23–26} saliva²⁷ and others. However, to the best of our knowledge, FASP has not been widely tested or used for metabolite isolation during protein sample preparation. In this study, as a complementary approach and proof of concept, we evaluated the use of FASP to compare the metabolomes of urine samples from healthy individuals (no tumor) and Ta bladder cancer patients.

2. MATERIAL AND METHODS

2.1. Study Design

The study was carried out in two phases: a technical assessment followed by a clinical application. In the first phase, a single urine sample from a healthy male volunteer was processed using the multiomics FASP workflow to evaluate metabolite recovery across the raw urine, flow-through (filtrate), and concentrate (retentate) fractions, and to confirm that the retentate fraction was suitable for LC-MS/MS-based proteomic analysis. In the second phase, the same protocol was applied as a proof-of-concept to compare the metabolomic profiles of urine samples collected from healthy individuals (no tumor; G1) and patients with nonmuscle-invasive bladder cancer at stage Ta (G2).

2.2. Sample Assessment

The FASP-based multiomics protocol was evaluated using a single urine sample from a healthy male volunteer, with informed consent. After collection, the urine was centrifuged at 5000g to remove precipitated cells. The supernatant was transferred to a new centrifuge tube and stored at -80°C until further use. Raw urine samples were thawed and then centrifuged in a 15 mL/10 kDa Millipore centrifugal filter device at 10,000g and 4°C for 20 min to obtain filtrate and retentate fractions. This sample served as the technical reference for assessing metabolite recovery and protein extraction efficiency across the three fractions produced by the unified FASP protocol (raw, flow-through (filtrate), and concentrate (retentate)) before clinical application.

2.3. Clinical Cohort- Healthy control vs nonmuscle-invasive bladder cancer

Midstream second-morning urine samples were collected from participants in the no-tumor control group (cohort G1) and from patients diagnosed with nonmuscle-invasive bladder cancer at stage Ta (Ta-NMIBC), assigned to cohort G2. Bladder cancer diagnoses were confirmed by histopathological examination. The median age of patients in the Ta-NMIBC group was 76 years (interquartile range [IQR] 68–83), whereas participants in the no-tumor control group had a median age of 67 years (IQR 62–78). All participants were informed about the study procedures and provided written informed consent. The study protocol was approved by the Ethics Committee of the Central Lisbon Hospital Center (approval number: 669/2018).

2.4. Proteomics Sample Preparation, Analysis, and Data Processing

2.4.1. Protein Extraction and Digestion for LC-TOF/MS Analysis. The Bradford assay was performed on the raw, flow-through, and concentrate fractions of the assessment sample to determine total protein content. However, the raw and flow-through fractions had larger volumes (approximately 15 mL) but protein concentrations below the Bradford assay detection limit, so they were not detected. In contrast, the 10 kDa MWCO membrane retained and concentrated proteins, yielding a detectable protein concentration in the concentrate fraction (approximately 1 mL). As a result, protein extraction was performed only on the concentrate fraction.

Ultrasonic-based filter-aided sample preparation (US-FASP) was used on the concentrated portion of urine samples. Briefly, about 300 μL (100 μg protein) was loaded onto the VivaSpin 500 (10 kDa MWCO, Sartorius) and centrifuged for 20 min at 10,000g. The loaded proteins in the membrane were washed with 200 μL of 8 M urea 25 mM ammonium bicarbonate (AmBic) solution, then centrifuged for 20 min. The reduction process for protein disulfide bonds was done by adding 200 μL of 50 mM dithiothreitol (DTT) in 8 M urea and 25 mM AmBic and sonicated using Q500 Sonicator (20 kHz frequency and 500 W maximum power) equipped with cup-horn 431C2 accessory (Qsonica, USA) for 5.25 min (30 s sonication period and 15 s intervals) at 25% amplitude. The solution was then removed by centrifuging at 10,000g for 20 min. The sample was washed with 200 μL of 8 M urea, 25 mM AmBic. The alkylation was performed by adding 100 μL 50 mM iodoacetamide in 8 M urea and 25 mM AmBic solution, then sonicated and centrifuged as reported for the reduction step. The sample was washed twice with 200 μL 25 mM AmBic. The protein extract was finally digested by adding 100 μL of trypsin solution (1:30 trypsin-to-protein ratio) prepared in 12.5 mM AmBic. The trypsin digestion was performed using the same sonication parameters as before. The peptides were collected by centrifugation at 10,000g for 10 min, followed by two additional membrane washing steps with 100 μL of 3% (v/v) acetonitrile that contained 0.1% (v/v) aqueous formic acid and centrifugation for 10 min at 10,000g. Finally, peptides were transferred to a 500 μL microtube and stored at -80°C until further analysis by Nano-LC-MS/MS.

2.4.2. Proteomics NanoLC-MS/MS Analysis. The frozen samples stored in -80°C freezers were defrosted at room temperature. Afterward, they were filtered once more using a syringe filter with a PTFE membrane with a pore size of 0.2 μm . Finally, a 4

μL portion of each sample was injected into a nanoflow LC system (nanoElute, Bruker Daltonik GmbH) coupled to timsTOF with CaptiveSpray ion source (Bruker Daltonics). The sample were desalted using Acclaim PepMap C18 ($5\ \mu\text{m}$, $100\ \text{\AA}$, $300\ \mu\text{m}$, i.d. $5\ \text{mm}$) trap cartridge (Thermo Fisher Scientific) and the peptides were separated using Bruker FIFTEEN separation column (C18, $75\ \mu\text{m}$ i.d. $\times 150\ \text{mm}$, $1.9\ \mu\text{m}$) and mobile phase of solvent A (0.1% formic acid in deionized Water) and solvent B (0.1% formic acid in acetonitrile) in 140 min gradient mode at $300\ \text{nL/min}$ flow rate as the following: initially 5% B for 5 min increased to 35% B over a period of 115 min; Subsequently increased to 95% B in 5 min and maintained for 10 min then a rapid decreased to initial condition of 5% B occurred within 0.2 min, the final condition was held constant until the end of the entire run which spanned a total duration of 140 min. The Captive Spray ion source ionized the samples at a gas flow of $3.0\ \text{L/min}$ at a temperature of $150\ ^\circ\text{C}$ and a capillary voltage of $1600\ \text{V}$. The acquisition was performed in positive mode at $2\ \text{Hz}$ over the mass scan range $150\text{--}2200\ m/z$, using the auto MS/MS function. The MS2 acquisition was a data-dependent acquisition (DDA), and the collision energy was adjusted between 23 and $65\ \text{eV}$.

2.4.3. Proteomics Data Processing Using MaxQuant. For protein analysis, label-free quantification (LFQ) was performed using MaxQuant V2.2.0.0 with standard parameters, integrated with the Andromeda search engine, against the human UniProt reference proteome (UP5640).

2.5. Metabolomics Sample Preparation, Analysis, and Data Processing

2.5.1. Metabolomics Sample Preparation. From each fraction of the assessment sample, $100\ \mu\text{L}$ was then transferred to an HPLC vial for injection, while the raw fraction was filtered through a $13\ \text{mm}$ $0.2\ \mu\text{m}$ PTFE membrane syringe filter before injection. Additionally, based on the assessment sample results, the flow-through fraction from the clinical urine samples was used for metabolomics analysis.

2.5.2. Metabolomics LC-MS/MS Analysis. The metabolites were separated and profiled using an Elute UHPLC (Bruker Daltonik GmbH, Bremen, Germany) coupled to an electrospray ionization (ESI) timsTOF Q-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The metabolites were eluted by injecting $10\ \mu\text{L}$ of the sample twice into an Intensity Solo 2 C18 column ($100\ \text{mm} \times 2.1\ \text{mm}$, $1.8\ \mu\text{m}$ beads) that maintained at $35\ ^\circ\text{C}$ (Bruker Daltonik), using 30 min gradient method of solvent A: 0.1% FA in LC-MS grade water and solvent B: 0.1% FA in LC-MS grade Acetonitrile (ACN).

The gradient elution protocol employed in this study involved the following steps: initially, a 1% B solution was used for the first 2 min, after which it gradually increased to 99% B between 2 and 17 min. The system was then held at 99% B for 3 min, maintaining a flow rate of $0.25\ \text{mL/min}$. Subsequently, the flow rate increased to $0.35\ \text{mL/min}$ while maintaining 1% B from 20.1 to 28.3 min, thereby re-equilibrating. Finally, from 28.5 to 30 min, the flow rate was reduced back to $0.25\ \text{mL/min}$.

Urine metabolic fingerprinting was investigated using positive polarity, as the most common metabolites were detected in that polarity. The capillary voltage was $4500\ \text{V}$, $500\ \text{V}$ end plate offset, $10\ \text{L/min}$ drying gas flow, while the drying temperature was $220\ ^\circ\text{C}$ and the nebulizer pressure was $2.2\ \text{bar}$. The time-of-flight scan range was $20\text{--}1300\ m/z$. Auto-MS/MS mode was operated with a collision energy of $20\ \text{eV}$; the cycle time was $0.5\ \text{s}$, with a relative minimum intensity threshold of 400 counts per thousand and a target intensity of 20,000.

2.5.3. Metabolomics Data Processing and Statistical Analysis. Data acquisition for metabolites was controlled by Bruker Compass HyStar 5.0 for elute UHPLC, while otControl 6.2 was used for tims TOF-MS. Human metabolite identification and annotation were performed using MetaboScape 4.0 software (Bruker Daltonics). In the T-ReX 2D/3D workflow, the parameters for molecular feature detection were as follows: a minimum intensity threshold of 1,000 counts, a minimum peak length of 7 spectra for peak detection, and peak area for feature quantitation. The mass recalibration was performed over a retention time range of $0\text{--}0.3\ \text{min}$. On the other

hand, the MS/MS import method was set to average. The parameters for data bucketing were assigned as follows: retention time range from 0.3 to $25\ \text{min}$, and mass range from 50 to $1000\ m/z$.

First, the assessment sample fractions were identified to compare results and determine which fraction best suited the clinical samples. For these clinical samples, differences in metabolites between G1 (no tumor) and G2 (Ta-NMIBC) patients were shown using PCA and orthogonal partial least-squares discriminant analysis (OPLS-DA). The variable importance in projection (VIP) scores from the OPLS-DA model were used to identify metabolites that most effectively distinguished the two groups. Additionally, p-values for each variable in the data matrix were calculated using the unpaired Wilcoxon rank-sum test in MetaboAnalyst 6.0 (www.MetaboAnalyst.ca). The false discovery rate (FDR) was also calculated to account for multiple comparisons. Variables with $\text{VIP} > 1.5$ and $\text{FDR} < 0.05$ were considered metabolites with statistically significant differences between the groups. Next, receiver operating characteristic (ROC) curve analysis assessed the diagnostic potential of this metabolite set for bladder cancer. Finally, the Pearson correlation coefficient (r) between age, BMI, gender, and this significant metabolite group was computed in MetaboAnalyst 6.0 to evaluate the possible influence of these confounding factors.

3. RESULTS

3.1. Experimental Design to Evaluate FASP Performance for Metabolomics Analysis

This work explores the use of FASP for a side-by-side proteomic and metabolomic analysis of the same starting sample. For this, we used a single urine sample, which was split into three fractions: urine raw, FASP-concentrated, and FASP-flow-through (Figure 1).

The FASP-concentrated protein fraction was analyzed using the standard nanoLC-MS/MS proteomics methodology (see Section 2.4). On the other side, all three urine metabolite fractions were subjected to LC-MS/MS metabolomics-based analysis as illustrated in Figure 1.

FASP is a well-established sample preparation method for proteomics analysis, including body fluids such as urine, serum, plasma, among others.^{23–26} As a proof-of-concept, we have performed urine proteomics analysis of the FASP-concentrated fraction, as shown in Figure 1. The proteomics analysis of the FASP-concentrated fraction yielded 10,696 spectra that resulted in the identification of 3163 nonredundant peptides, 957 of which were unique to protein groups supported by 48,922 peptide-spectrum matches (PSMs), indicating substantial proteome coverage. Comparable to previous studies employing FASP that found over 970 urine proteins in newborns²⁶ and 1063 proteins identified in Yu et al.²⁴ To further investigate the possible applicability of FASP to the most used MS-based metabolomic workflow(s), we next performed LC-MS/MS to profile all three urine fractions as indicated above (Figure 1). The LC-MS/MS analysis identified 178 metabolites across all analyzed urine sample fractions. Considering metabolite detection in at least two of the three technical replicates, 176, 177, and 177 metabolites were detected in FASP-concentrated, FASP filtrate, and raw samples, respectively. See Table 1 below, and the complete list of metabolites detected across all FASP portions is available in Supplementary Table S1.1.

An additional LC-MS/MS data set showed no difference in the average metabolite area under the curve (AUC) across the three analyzed fractions (Figure 2A), and the median RSD% across all urine fractions was less than 8% (Figure 2B). When compared to raw samples, the AUC values of raw and FASP-

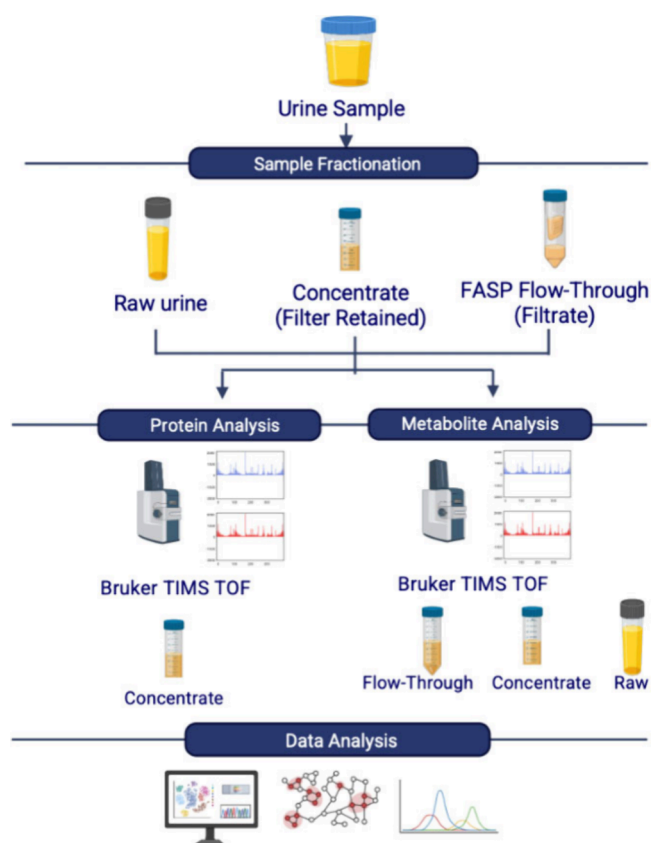


Figure 1. Experiment workflow describes the steps taken to assess the performance of multiomics Filter Aided Sample Preparation (FASP) for proteomics and metabolomics analysis from a single urine sample. The urine sample was divided into three fractions: raw, concentrate (the fraction retained by the filter), and FASP flow-through (the filtrate fraction pushed through the 10 kDa filter after centrifugation). The protein fraction and all three metabolite fractions were analyzed by LC-MS/MS.

flow-through were strongly positive (Supplementary Figure S1A). This confirmed the minimal variance in the metabolomics analysis of the three fractions.

Next, to assess the overall efficacy of FASP for metabolite extraction in subsequent LC-MS/MS analysis, we compared the metabolomics data set obtained from three urine fractions

(Figure 1) using principal component analysis (PCA) followed by ANOVA with Tukey's HSD post hoc test. The PCA clearly indicates a separation between the three fractions (Figure 2C), and ANOVA confirmed a difference between the fractions; specifically, 46 metabolites were found to be differentially enriched ($p < 0.05$) among the three fractions (Figure 2D and 3).

Among the differentially enriched metabolites detected by LC-MS/MS analysis, some showed a clear trend of higher levels in the FASP concentrate compared to the other two fractions, as illustrated in Figure 3 (panel A–J). Whereas other metabolites were less abundant in the FASP concentrated fraction than in the other fractions, Figure 3 (panel K–O).

3.2. Clinical samples of no tumor and nonmuscle-invasive bladder cancer at stage Ta (Ta-NMIBC)

Next, as a proof-of-concept, the FASP protocol was then employed to compare the metabolomic profiles of clinical urine samples from healthy individuals ($n = 13$) and patients with Ta bladder cancer ($n = 12$). A total of 290 metabolites were identified in the Human Metabolome Database (HMDB-4.0) for the whole urine samples (Table S1.2). An OPLS-DA model was constructed to explore group separation in the data set. The resulting model demonstrated good performance with one predictive and two orthogonal components ($R^2X_{\text{cum}} = 0.421$, $R^2Y_{\text{cum}} = 0.989$, $Q^2_{\text{cum}} = 0.651$). The 2D score plot showed apparent clustering between groups, supported by 95% confidence ellipses (Figure 4A).

These results suggest that the model provides a reliable overview of class separation. Then, VIP values derived from the OPLS-DA model were used to identify differential metabolites. We defined significance in this case to be those metabolites with $VIP > 1$ and thus identified 128 metabolites as significant (VIP values > 1). Additional significant metabolites were identified using classical statistics (t test). As a result, 105 metabolites were significantly different (P -value < 0.05 and $|\log_2FC| > 1.5$) between the groups, of which 104 increased (marked in red), and one decreased (marked in blue). As visualized in the heat map generated for the top 25 altered metabolites (Figure 4B), there was complete separation between no tumor (G1) and Ta-NMIBC (G2).

Altered metabolites were initially identified using VIP scores ($VIP > 1.0$) from the OPLS-DA model, followed by univariate Student's t tests ($p < 0.05$) and absolute $\log_2$ fold change ($|\log_2FC|$

Table 1. Summary of the Type of Metabolites Identified in FASP Flow-Through (Filtrate) Followed by LC-MS/MS

Number of metabolites	Group	Metabolite
8	Organic Oxygen Compounds	Galactose; N-Acetyl-D-glucosamine; Gluconic acid; Threonic acid; N-Acetylglucosamine; Acetone; Sedoheptulose; scyllo-Inositol;
6	Organic acid and derivatives	Glutamic acid; Aspartic acid; Saccharopine; Creatinine, Leucine; 5-Aminopentanoic acid
5	Organoheterocyclic compounds	2-Hydroxyadenine; Xanthurenic acid; Levofloxacin; 9-Methyluric acid; 4-Aminohippuric acid
5	Benzenoids	Homoveratric acid; Hippuric acid; Salicylic acid; Phenylglyoxylic acid; 4-Aminohippuric acid
3	Nucleosides, nucleotides, and analogues	Adenosine monophosphate; Adenosine; 2'-Deoxyguanosine 5'-monophosphate
2	Organic nitrogen compounds	Sphinganine; Spermine
16	Lipid and lipid-like molecules	Cortisolone; Deoxycorticosterone; Androstenedione; Cortisol; Dehydroepiandrosterone; Glycocholic acid; L-Acetylcarnitine; Pregnenolone; 17-Epiestradiol; 17-Hydroxyprogesterone; Epitiolcholanolone; Dodecanedioic acid; Hexadecanedioic acid; Linoleic acid; Tetrahydrocortisone; all-trans-Retinoic acid
1	Phenylpropanoids and polyketides	Kaempferol

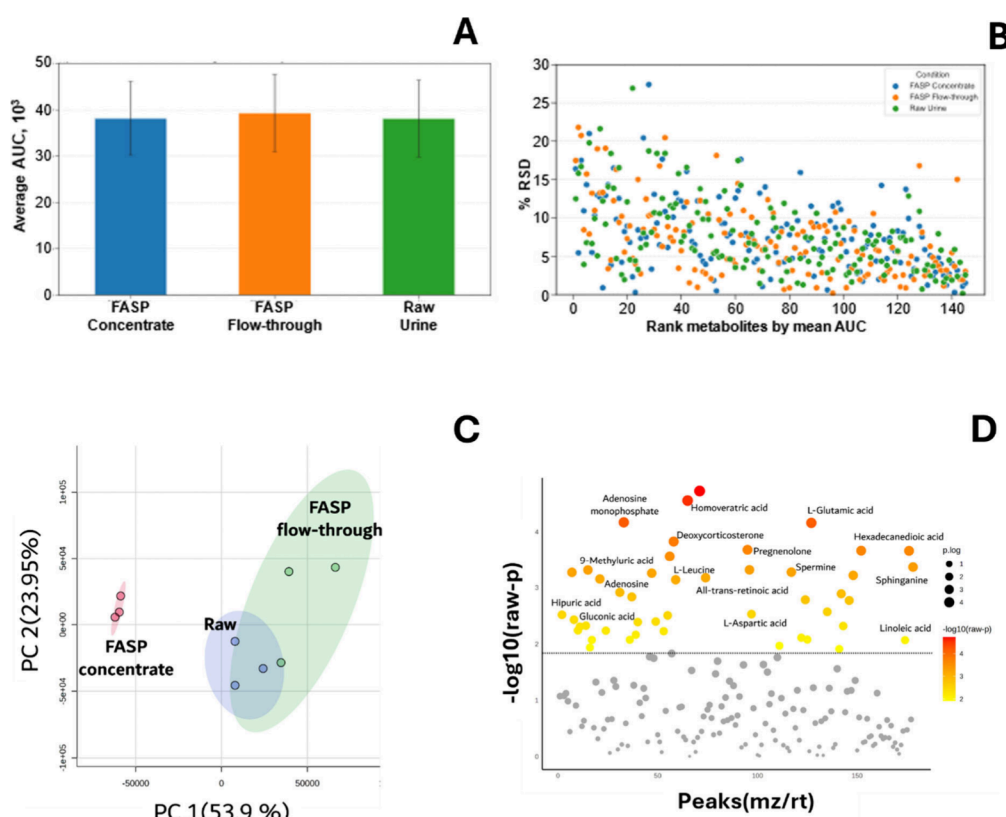


Figure 2. (A) The relation between the metabolites identified by LC-MS/MS for all fractions, average AUC of all identified metabolites, (B) ranking of metabolites by AUC and RSD%. (C) Comparison of the urine PCA clusters obtained by LC-MS/MS, and (D) Manhattan plot showing the metabolites that demonstrate exploratory differences ($p < 0.05$) enriched across the different fractions.

> 1.5) to confirm significance. Metabolites meeting these threshold criteria were further evaluated using ROC analysis, with an AUC ≥ 0.85 yielding a final set of 73 altered metabolites with diagnostic potential (Table S1.2).

Pathway enrichment analysis was performed using MetaboAnalyst 6.0 on the 73 altered metabolites identified using our multistep selection criteria (VIP > 1.0, $p < 0.05$, $|\log_2\text{FC}| > 1.5$, and AUC ≥ 0.85). Pathway enrichment analysis revealed that phenylacetate metabolism exhibited the highest enrichment, followed by purine metabolism and tryptophan metabolism, as shown in Figure 5.

4. DISCUSSION

Simultaneous extraction of metabolites and proteins from a single biological sample sets a significant advance in multiomics workflows, reducing analytical variability and enhancing data integrity for systems biology studies. In this study, we evaluated a new FASP-based multiomics workflow using an assessment urine sample (Figure 1). Subsequently, we validated it clinically in urine samples from patients with bladder cancer, demonstrating its usefulness in a preliminary cohort. The results showed that this approach, in addition to protein extraction and analysis, also offers improved metabolomic profiling by producing a clean, protein-free filtrate that preserves the urinary metabolome without the need for organic solvents or dilution.

4.1. FASP-Based Multiomics: A Methodological Advancement

The traditional metabolomics extraction methods commonly used, such as liquid–liquid extraction (LLE) or protein

precipitation (PPT), are effective but have significant drawbacks. LLE is time-consuming and requires large volumes of organic solvents, potentially leading to selective metabolite loss.²⁸ At the same time, PPT can suffer from incomplete protein removal and coprecipitation of metabolites.²⁹ Our FASP-based approach can address these challenges. This method uses a 10 kDa MWCO membrane to retain high-molecular-weight proteins on the filter while allowing low-molecular-weight metabolites to pass into the filtrate.

This strategy offers two main advantages. First, it eliminates the need for organic solvents in the metabolomics workflow, enabling direct injection of the filtrate into the LC-MS system. In addition to saving processing time and reducing complexity, this approach also prevents selective extraction bias associated with different solvents. The second advantage is that the filtrate provides a cleaner matrix due to membrane filtration. This is crucial for LC-MS analysis, as it plays an essential role in the sensitivity of the mass spectrometer and protects the analytical column from fouling, thereby extending its operational lifespan, a significant practical and economic benefit in high-throughput clinical studies. Therefore, our technical validation demonstrates that this method effectively removes unwanted high-molecular-weight components, such as proteins, while maintaining a metabolite profile with minimal loss relative to the raw fraction at the global level. However, as pointed out (Figure 3) above some metabolites were consistently enriched in the FASP-concentrate this could be due to their hydrophobic physicochemical properties and/or protein-binding interactions, for example 17-Epiestriol, that binds via binding to the estrogen receptor beta (ER β), Tetrahydrocortisone, which circulates largely bound to corticosteroid-binding

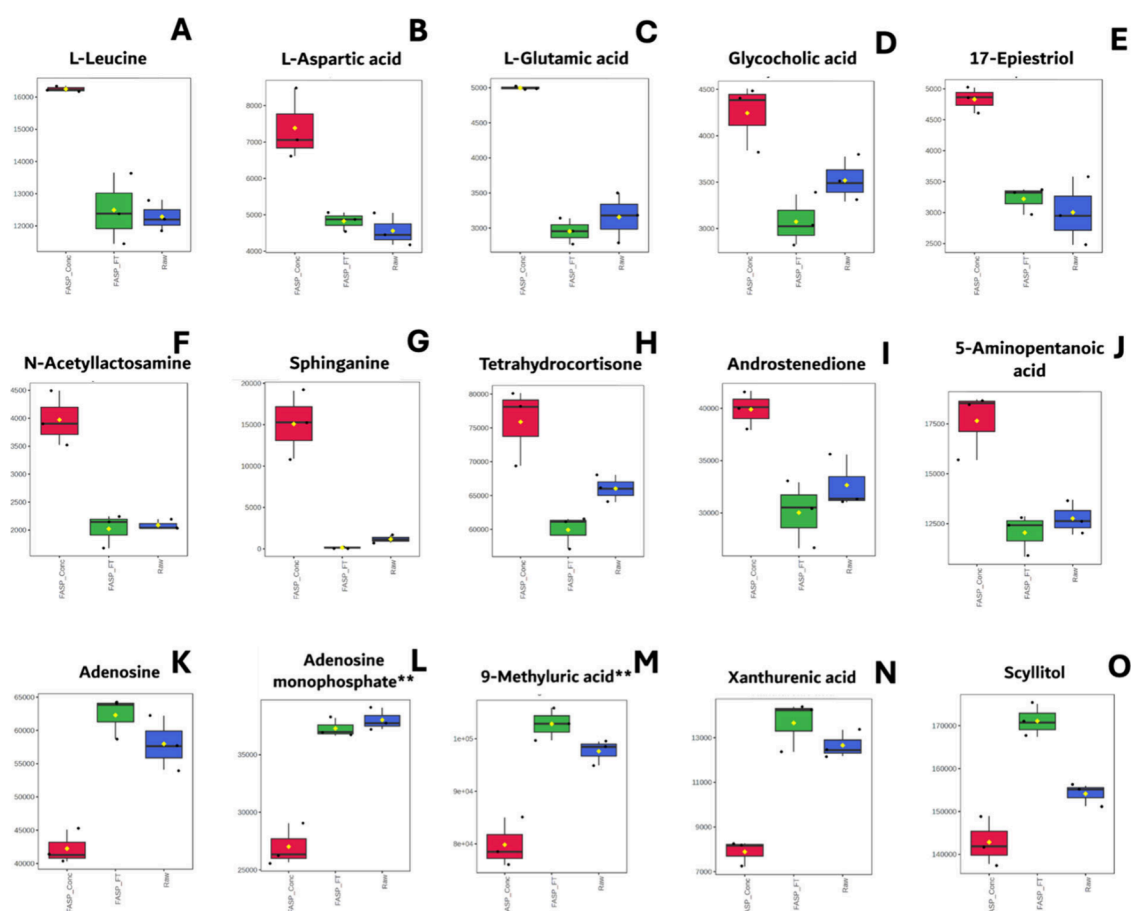


Figure 3. Box plots of the metabolites detected by LC-MS/MS. Panels A–J metabolites that displayed higher levels in the FASP concentrate in comparison to the other two fractions. Panels K–O metabolites showed lower levels in FASP than in the other fractions.

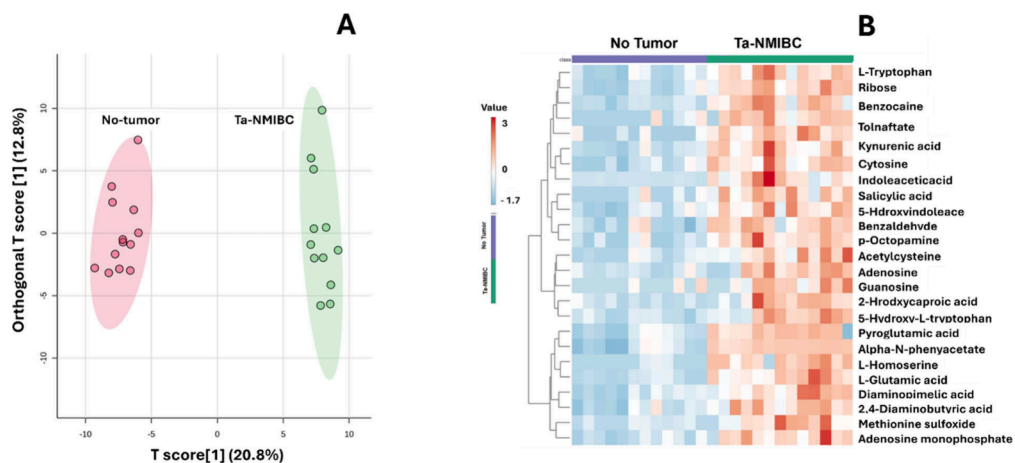


Figure 4. Multivariate analysis distinguishing no tumor (G1) and Ta-NMIBC (G2). A) Two-dimensional score plot of orthogonal partial least-squares–discriminant analysis (OPLS-DA) showed a clear separation between the no tumor (G1) and Ta-NMIBC (G2) cohorts, indicating metabolic profile differences between the groups. B) Heat map of selected significantly altered metabolites between no tumor (G1) and Ta-NMIBC (G2) groups, with hierarchical clustering applied to both samples (columns) and metabolites (rows). The relative abundance was expressed by color intensity, with higher abundance shown in red and lower abundance in blue across the groups.

globulin (CBG) and albumin, and Sphinganine, which interacts with adaptor proteins MyD88 and TIRAP.^{30–32} In contrast, metabolites were significantly depleted in the retentate, likely because they are highly polar and low-molecular-weight metabolites such as adenosine, 9-Methyluric acid, and Scyllitol that efficiently passed through the molecular-weight cutoff membrane into the flow-through fraction.³³

Further experimental work is required to better understand the enrichment trends of metabolites during FASP, specifically identifying which metabolite classes are preferentially retained in the concentrate and which partition into the flow-through (filtrate). The differential distribution of metabolites across the resulting FASP fractions presents an opportunity for further investigation and could potentially be exploited as a strategic

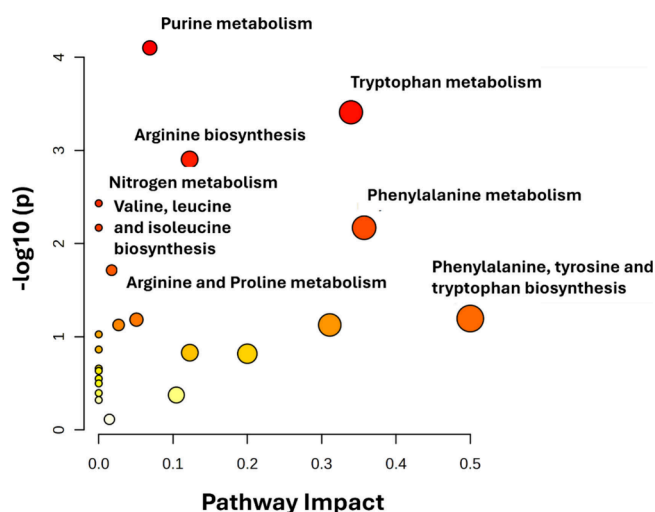


Figure 5. Metabolic pathway analysis of altered metabolites between the no tumor (G1) and Ta-NMIBC (G2) groups. Results obtained from pathway analysis using MetaboAnalyst 6.0 as a bubble plot diagram, where the *x*-axis represents pathways' impact (topological importance), while the *y*-axis represents enrichment significance ($-\log_{10}$ *p*-value). While each bubble indicates a specific pathway, its size and color intensity correspond to pathway impact and the level of statistical significance, respectively.

fractionation approach. Importantly, such an approach could enable the deliberate enrichment of target metabolites by selectively depleting unwanted compounds.

To the best of our knowledge, this study is the first to systematically demonstrate that FASP can be repurposed as an elegant and practical front-end for a unified proteomics-metabolomics workflow from a single urine sample, opening the door for other biological sample studies. Similar to other innovative multiomics sample preparation methods, our approach demonstrates the technical feasibility of multiple omics layers (metabolomics and proteomics) while initially focusing on a single, high-value data type—metabolomics—for urine samples from bladder cancer patients.

4.2. Clinical Validation: Metabolic Signatures of Bladder Cancer

By applying our validated filtrate-based metabolomics workflow to clinical cohort samples, we successfully distinguished Ta-stage NMIBC patients from no-tumor controls. The strong separation of the metabolite results was observed in the OPLS-DA model ($Q^2=0.651$), and the identification of 105 significantly altered metabolites confirms the method's sensitivity in detecting disease-related metabolic changes in real-world clinical samples.

Based on candidate biomarkers with high diagnostic potential in our findings, these metabolites are linked to pathways often involved in bladder cancer development, supporting the biological relevance of our results. However, the most significantly enriched pathway was phenylacetate metabolism; 44% of its metabolites (4 out of 9) were significantly altered, including adenosine monophosphate (AMP), phenylacetic acid, glutamine, and phenylacetylglutamine, which aligns with previous urinary metabolomics studies in bladder cancer.³⁴ Furthermore, we observed significant changes in purine metabolism, driven by alterations in cytosine and tryptophan metabolism due to dysregulation of L-tryptophan and indoleacetic acid, which align with different

studies reported by Zhane et al. and Ming et al.^{35,36} The strong performance of L-glutamic acid (AUC = 1.000) as a biomarker aligns with growing evidence that abnormal glutamine metabolism is a key factor in bladder cancer progression.³⁷ The fact that our simplified, dilution-free method can reliably replicate these known metabolic signatures confirms its analytical robustness and its potential to discover biologically relevant biomarkers.

4.3. Limitations and Future Directions

While this study successfully establishes and validates a novel multiomics workflow, several limitations must be acknowledged. The clinical validation was performed in a pilot-scale cohort (*n* = 25) as a proof-of-concept for the FASP protocol. Therefore, the high AUC values obtained for some metabolites, such as L-Glutamic acid and phenylacetylglutamine, which is 1, will require validation in larger, independent patient cohorts to confirm their diagnostic accuracy.

5. CONCLUSIONS

Here, we introduce a new FASP-based method for isolating proteins and metabolites from a single urine sample before LC-MS/MS analysis. Our findings clearly show that FASP-based workflow proteomics and metabolomics sample preparation is a simple and efficient method; it is a viable alternative for multiomics studies examining both the protein and metabolite fractions of the same sample. As demonstrated by applying this innovative technique to clinical urine samples from patients with bladder cancer. The clinical application of this method successfully identified robust metabolic signatures of bladder cancer, validating its sensitivity and practical utility. Given the advantages FASP offers over existing proteomic sample preparation techniques, we firmly believe the proposed multiomics-FASP approach will be an attractive and viable option for future MS-based integrative omics analysis.

■ ASSOCIATED CONTENT

Data Availability Statement

Metabolomics data are deposited in the Metabolomics Workbench with study ID ST003859. Proteomics mass spectrometry data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository, with the data set submission identifier: PXD079145.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.6c00173>.

Figure S1. Correlation analysis of metabolite abundance between raw urine and FASP flow-through fractions (AUC comparison) (PDF)

Table S1.1. Complete list of metabolites detected across all FASP fractions (raw, flow-through, and concentrate) (XLSX); Table S1.2. List of metabolites identified in the Human Metabolome Database (HMDB-4.0) for whole urine samples from clinical cohorts, including statistical analysis (VIP scores, *p*-values, fold changes, and ROC AUC values) (XLSX)

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Author Contributions

[#]HMS and NCS contributed equally. MKS, HMS, and NCS contributed for the conceptualization. HMS, NCS mentorship; MKS, LBC, ADG, AF, KA, HAH contributed to methodology; MKS, HMS, NCS performed writing original draft preparation; MKS, LBC, ADG, AF, MU, HMS, NCS carried out writing, review, and editing. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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