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Ultrasensitive and High Reproducible Detection of Urinary Metabolites Using the Tip−Contact Extraction Method Coupled with Negative LDI-MS

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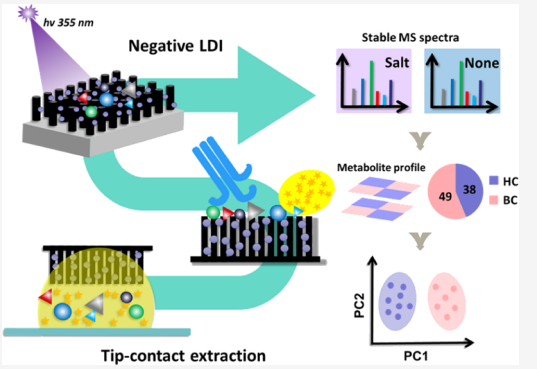
ABSTRACT: More and more evidence has proved that urinary metabolites can instantly reflect disease state. Therefore, ultra-sensitive and reproducible detection of urinary metabolites in a high-throughput way is urgently desirable for clinical diagnosis. Matrix-free laser desorption/ionization mass spectrometry (LDI-MS) is a high-throughput platform for metabolites detection, but it is encountered by severe interference from numerous salts in urine samples, because the crystallized urine salt on dried samples could result in poor reproducibility in LDI-MS detection. The present work proposed a tip−contact extraction (TCE) technique to eliminate interference from the urine salt. Vertical silicon nanowire arrays decorated with the fluorinated ethylene propylene film (FEP@VSiNWs) could effectively extract metabolites from the urine sample dropping on its surface. High salt tolerance was observed in the subsequent LDI-MS detection of the metabolites extracted on the tip of FEP@VSiNWs even in the presence of 1 M urea. Stable and reproducible mass spectra for non-target metabolic analysis were obtained in real urine samples with different dilution folds. Urinary metabolites collected from bladder cancer (BC) patients were reliably profiled by the TCE method coupled with negative LDI-MS. Based on this platform, potential metabolic biomarkers that can distinguish BC patients and normal controls were uncovered.



KEYWORDS: *tip−contact extraction, laser desorption ionization, silicon nanowires, salt tolerance, metabolite fingerprinting, urine metabolomics, non-invasive bladder cancer diagnosis*

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1. INTRODUCTION

Urine is an ideal biological matrix for screening disease biomarkers since the collection procedure is non-invasive and the risk of acquired infections is minimal.[1,2] Metabolic fingerprinting provides the possibility to reflect systematic changes in the whole metabolome of a living organism and identify disease-related differential endogenous metabolites for clinical diagnosis.[3−5] The rise of urinary metabolic fingerprinting provides a new insight for screening of non-invasive biomarkers, which is expected to instantly mirror metabolic perturbations in disease state.[1,6,7]

With the increasing demand on non-invasive diagnosis based on the omics approach, reliable and high-throughput mass spectrometric technologies are urgently needed. Matrix-free laser desorption/ionization mass spectrometry (LDI-MS) such as surface-assisted laser desorption ionization MS has been regarded as a high-throughput platform for detection of metabolites with acceptable sensitivity.[8,9] Usually, the "Drop-dry" method was commonly applied for sample loading before LDI-MS analysis owing to its simplicity and applicability.[10] However, for urine sample analysis, the presence of high concentration of salt is not favorable, as numerous salts in the urine sample could form crystals that unevenly distributed on the dried sample, resulting in laser energy scattering and poor reproducibility.[11,12] To solve the problem, several approaches have been developed for sample desalting. For less polar and higher mass analytes, there are two main strategies to overcome the salt interference before LDI-MS detection. One is to perform extraction and desalting procedures in solutions followed with the drop-dry method using nanomaterials such as graphene nanosheets,[13,14] metal oxide nanospheres,[15,16] and metal nanoparticles.[17] The other is to extract, desalt, and elute the analyte on a target,[18] which was pre-coated with hydrophobic materials such as paraffin,[19] nylon,[20,21] poly(methylmethacrylate),[13] perfluorosilane,[18,22,23] and hydrophobin.[24] However, for low-mass ionic metabolites, extra derivatization steps of analytes are required to enhance extraction and retention onto the pre-coated target, thereby

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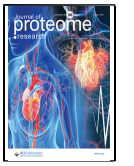

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reducing the speed of LDI-MS detection for biofluid analysis.[25,26] To avoid extra derivatization steps of analytes, recent works tried to directly segregate the crystallization of salt in biofluids by adjusting the contact angle of perfluoro-surface coatings and controlling the rate of droplet evaporation, but the drying process for salty biofluids required a long time and the salt crystallization remained on the sample spot may reduce the shot-to-shot reproducibility.[12]

To meet the needs of rapid, sensitive, and reproducible detection, our group has reported a tip-enhanced laser desorption ionization mass spectrometry based on vertical silicon nanowires (VSiNWs), which can significantly enhance the electric field and electron transfer at the tip of VSiNWs.[27,28] In addition, ultra-low background noise in low-molecular weight region (<400 Da) could be achieved after coating fluorinated ethylene propylene on the VSiNW array (FEP@VSiNWs).[29] With the assistance of the tip-enhanced LDI-MS platform, metabolic analysis of saliva samples has been successfully achieved.[29] However, reproducible detection of urinary metabolites without severe interference from salt on the LDI-MS platform remains a challenge.

In the present work, a sensitive and reproducible detection platform based on the "tip−contact extraction" (TCE) technique coupled with negative LDI-MS was established. Through a simple TCE process, urinary metabolites can be effectively extracted onto the surface of FEP@VSiNWs for subsequent LDI-MS analysis. Mixed standard samples and real urine samples were applied to validate the performance of the TCE methods, respectively. The ability of the FEP coating layer for extracting and enriching urinary metabolites is remarkable. Compared with the "drop-dry" method, the sensitivity and signal-to-noise ratio (S/N) obtained by the TCE approach greatly improved, leading to significant increase in the number of metabolite peaks (∼5 folds). Furthermore, the reproducible metabolite pattern could be obtained even if a same urine sample was diluted into different concentrations. These results substantially proved that urinary salt interference encountered in the "drop-dry" method was eliminated by the TCE approach. Taking into consideration that diseases of the urinary system directly affect the levels of metabolites in urine, application of the TCE method in the urinary metabolic analysis was investigated to uncover the metabolic disturbance in bladder cancer (BC) patients for non-invasive diagnosis. Furthermore, detection of exogenous substance such as melamine, cyromazine, and imazalil in urine samples was also demonstrated. The present work offers an attractive matrix-free and simplified protocol for sensitive and reliable analysis of low-mass molecules in salty samples.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents

Single-crystal silicon wafers (p type, ⟨100⟩, 5−10 Ω cm) were purchased from Lijing Silicon Materials Co. (China). Hydrofluoric acid (HF, 40%) and ethanol (EtOH) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Silver nitrate ($AgNO_3$), urea, malonic acid, carnitine, pipecolic acid, glutamic acid, creatinine, malic acid, aspartic acid, histidine, cysteic acid, γ-aminobutyric acid, proline, taurine, allysine, valine, serine, nicotinic acid, cysteine, lauric acid, citraconic acid, *N*-acetylvaline, and *N*-acetylthreonine standards were purchased from Aladdin Co. (Shanghai, China). Fluorinated ethylene propylene preparation (FEP preparation) was purchased from Jinhua Yonghe Fluorochemical Co. (Jinhua, China).

2.2. FEP@VSiNWs Substrate Fabrication

The detailed fabrication procedure of FEP@VSiNWs has been described in our previous work.[29] In brief, the vertical SiNW array was fabricated via the one-step MACE method. Silicon wafer (p type, 5−10 Ω cm) was cut into 2 cm × 2 cm chips and then immersed in a mixed etching solution of 0.02 M $AgNO_3$ and 4.8 M HF for 15 min. After etching, the Ag catalyst that remained on the surface of the freshly etched chip was dissolved with dilute nitric acid (HNO_3, 1:1 v/v). After washing for 1 h, the Ag catalyst was totally dissolved and the as-prepared materials were washed repeatedly with water and then dried for FEP modification. 200 μL of FEP preparation was dropped onto the surface of the VSiNW chip. The modification time cannot be less than 30 min. Finally, the excess initiator was removed on a spin coater to obtain a uniform surface.

2.3. Preparation of Standard Sample Solution

Mixtures of malonic acid, creatinine, malic acid, proline, carnitine, aspartic acid, pipecolic acid, histidine, glutamic acid, and cysteic acid were dissolved in ultra-pure water with each component's concentration equaling to 100, 200, 500, and 1000 μM. In addition, mixed standard solution containing above metabolites (100 μM) was prepared and then spiked with different amounts of salt (0.05, 0.1, 0.5, and 1 M) to evaluate whether TCE technology can guarantee stable mass spectra under the interference of urine salts. Urea, sodium chloride, potassium chloride, sodium carbonate, potassium carbonate, and sodium urate were employed as urine salt, respectively.

2.4. Urine Sample Collection and Preparation

Urine samples (n = 87) were collected at the Sir Run Run Shaw Hospital of Zhejiang University. Totally, 38 healthy volunteers and 49 patients diagnosed with BC were selected in order to create two groups matched in terms of gender, age, body mass index (BMI), and smoking status. The demographic information of participants was provided in Table S1. Before collection, all individuals were refrained from eating, drinking, or smoking for at least 8 h. Urine specimens were collected at the midstream of first morning urine on an empty stomach at around 7:00 to 9:00 a.m. Next, the collected samples were centrifuged at 8000*g* for 10 min at 4 °C to remove insoluble residues and the final supernatant was stored in a refrigerator at −80 °C until use. The Ethical Committee of Sir Run Run Shaw Hospital of Zhejiang University approved the protocol (no. 20210405-34), and the methods were carried out in accordance with the approved guidelines.

2.5. TCE Process for Sample Pretreatment

Prior to mass spectrometry measurements, urine samples were thawed at 4 °C for a subsequent tip−contact sampling process. 20 μL of urine samples was dropped onto ITO glass, then a 4 mm × 4 mm FEP@VSiNWs chip was attached to the surface of the solution and retained for 20 min. After absorbing metabolites in urine samples, the excess urine droplet was further removed using N_2. For the mixed standard solution, extraction of metabolites from analyte solution or salt-added analyte solution was achieved by contacting a 4 mm × 4 mm FEP@VSiNWs chip with the sample for 20 min. After extraction, the residual droplets were blowing away from

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chips by N_2 stream. Finally, all obtained chips were preserved in a desiccator until LDI-MS detection.

2.6. LDI Mass Spectrometry Measurement

After the TCE process, FEP@VSiNW chips were stuck onto the custom-made aluminum plate with carbon conductive adhesive tape and inserted into an ultrafleXtreme MALDI-TOF/TOF instrument (Bruker Daltonics Co.) equipped with a 355 nm Nd:YAG laser beam for subsequent analysis. Each aluminum plate can be installed with 48 chips at a time, and two plates can be sent into the instrument at the same time to complete the detection of 96 samples. For the detection of real urine samples or analytes, the relative laser pulse energy was set at 57% of the total energy under reflecting negative-ion mode. The voltage of ion source 1 and ion source 2 were set at 20.00 and 17.75 kV, respectively. The lens was set at 8.50 kV, the reflector 1 and reflector 2 were set at 21.10 and 10.70 kV, respectively. The pulsed ion extraction was set at 120 ns, and the laser parameter was set at 4_large. For each sample, MS spectra were obtained at the m/z range of 20−350 Da with 2000 laser shots. All measurements were repeatedly performed three times. For 96 samples, the total detection process can be completed in 5 min, which reveals the potentials in high-throughput detection of biological samples.

To evaluate the MS performance of the TCE technique and traditional drop-dry method, the average S/N ratio and total S/N ratio of metabolites peaks in MS detection were calculated by eqs 1 and 2, where n represents the number of detected metabolite peaks (S/N > 3).

$$\text{average S/N ratio} = \frac{\sum_{i=1}^{n} (\text{S/N})_i}{n} \quad (1)$$

$$\text{total S/N ratio} = \sum_{i=1}^{n} (\text{S/N})_i \quad (2)$$

2.7. Data Normalization Methods Applied to Urine Samples

Prior to urinary metabolic biomarker discovery, six kinds of normalization techniques were screened to confirm the presupposition that extraneous factors-to-sample variation has been eliminated for disease biomarker mining. These statistical techniques aimed to eliminate batch-to-batch variation caused by extraneous factors and thereby reduce the RSD value of relative peak intensity for each metabolite. Four aspects were taken into consideration to unbiasedly evaluate the performance of normalization methods, including variation of replicated measurements, correction effect on urine dilution, variation of metabolites in urine samples from the same group, as well as the discrimination results. In detail, the normalization algorithm used here were normalization to intensity of the highest peak signal, normalization to MS "total useful signal", linear baseline, cyclic loss, PQN, and cubic spline. The last four normalization methods were implemented in the statistical computing language R using R 3.5.2 software. The detailed R code was provided in Supporting Information.

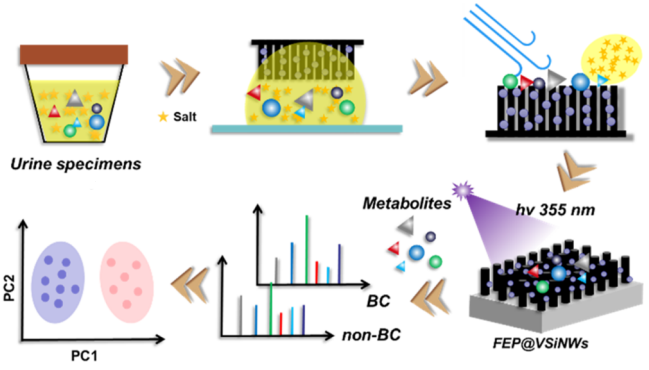
2.8. Identification of Metabolites in Urine

Metabolic biomarker candidates detected in urine were further identified using UPLC-MS/MS analysis combined with MALDI-TOF/TOF tandem mass spectrometry. Briefly, exact molecular weights and main fragment peaks of metabolites in urine samples were obtained from UPLC-MS/MS to identify the differential peaks through searching Human Metabolome Database (http://www.hmdb.ca/). Furthermore, standard reagents were employed to verify the identified metabolites. Exact mass and fragment profile of metabolites detected in standard samples and urine specimens were acquired by MALDI-TOF/TOF tandem mass spectrometry and further compared to eventually confirm the identification. The detailed experimental parameters of UPLC-MS analysis were provided in Supporting Information.

2.9. Data Handling and Statistical Analysis

After TCE and matrix-free LDI-MS detection, raw mass spectra of mixed standard solutions or urine samples were obtained and analyzed to confirm whether the TCE approach can guarantee stable mass spectra under the interference of background salt or during the dilution process. Mass spectra acquisition and processing were performed by FlexAnalysis (Bruker Daltonics Co.). ClinproTool (Bruker Daltonics Co.) was utilized for peak finding (S/N > 3). To statistically describe the similarity of mass spectra, box plots were completed by calculating the ratios of peak after normalization to the intensity of creatinine. The procedure of the metabolic analysis of urine samples from the BC group and controls is presented in Scheme 1. With the assistance of multivariate analysis, potential biomarker candidates that can discriminate between patients diagnosed with BC and healthy volunteers were sorted out. After normalizing the obtained mass spectra with the selected method, student's t-test was performed using MATLAB software to find differential metabolites correlated to BC ($p < 0.05$). Then, unsupervised principal component analysis (PCA) and cluster analysis were conducted using MATLAB and supervised orthogonal partial least squares discrimination analysis (OPLS-DA) was performed by SIMCA software to present the metabolic distinction between two groups. The disturbed metabolic pathway of BC-related metabolites was obtained by importing the statistically significant metabolites into MetaboAnalyst.

Scheme 1. Schematic Illustration of the TCE Process Based on the FEP@VSiNWs Substrate and Metabolic Analysis of Urine Samples from BC Group and Controls



3. RESULTS AND DISCUSSION

3.1. Establishment and Optimization of TCE Method

Herein, the FEP@VSiNWs substrate was fabricated and employed for extracting metabolites from mixed standard solutions or human urine samples followed by negative LDI-MS detection (Scheme 1). The TCE procedure was optimized by adjusting the extraction time. Figure S1 displayed total intensity of metabolites in a mixed standard solution (100 μM)

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at different extraction times which was varied from 0.5 to 30 min. The result indicated that TCE with 20 min was able to provide sufficient intensity for subsequent analysis. Figure 1A

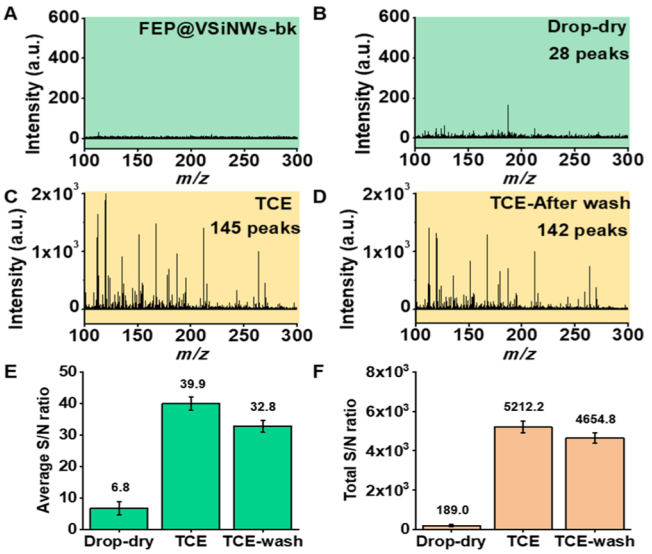

Figure 1. Analytical performances of the TCE method compared with the traditional drop-dry method. (A) Background noise of the FEP@VSiNWs substrate in low-molecular weight region. As a control, the chip was contacted with pure water. Raw mass spectra of urine sample obtained by the (B) traditional drop-dry method, (C) TCE method, and (D) TCE method with an extra washing procedure, respectively. (E) Average S/N ratio and (F) total S/N ratio of metabolite peaks detected in real urine samples using different sample preparation procedures.

displayed the low background noise of the substrate after contacting with the blank ultra-pure water. Besides, contact angle measurements indicated that FEP@VSiNWs substrate displays amphiphilic nature, which may promote the extraction of metabolites onto the substrate (Figures S2 and S3). Furthermore, 145 common peaks (S/N > 3) of salty urine samples were observed with the TCE technique, whereas the traditional drop-dry method displayed only 28 peaks (Figure 1B,C). An extra washing procedure after extraction was performed, and the majority of metabolic peaks was still observed, further proving the effect of the TCE method (Figure 1D). Compared with the drop-dry method, TCE can dramatically improve the average S/N ratio (~6 folds) and the total S/N ratio (~25 folds) of metabolite peaks (Figure 1E,F). The total S/N ratio not only represents the quality of the detected peak but also reveals the number of metabolite peak obtained by different methods. These results demonstrated the feasibility of TCE in revealing more metabolic information.

3.2. Evaluation of TCE Performance

To evaluate whether TCE technology can guarantee stable mass spectra under the interference of background salt, a mixed standard solution containing ten metabolites (100 μM) was prepared and then different amounts of extra urea (0, 0.05, 0.1, 0.5, and 1 M) were added. As shown in Figure S4, thick and uneven salt crystals remained on the sample spot were observed when the traditional drop-dry method was employed, which would significantly interfere the metabolite profiles of analyte solutions. On the contrast, no obvious liquids and aggregates was observed on the surface of the substrate right after the removal of the residual droplet, indicating the potential of the TCE method in providing stable and reproducible metabolite profiles (Figure S4). Therefore, stable mass spectra were obtained under salt interference, which reflects the high salt tolerance of the TCE technique (Figure 2A,B). To statistically describe the similarity of these mass spectra, box plots were presented by calculating the normalized peak ratios between salt-added samples and the original sample. Under the reasonable assumption that there are little biological and physical variations of metabolites during the process of adding salt, the log-normalized peak ratios should be close to 0 for most metabolites. As shown in Figure 2C,D, the TCE technique can largely enhance the stability of mass spectra and improve the S/N ratio of metabolites in the high-salt environment when compared with the drop-dry method (Figure S5). Furthermore, the high salt tolerance of the TCE technique was also confirmed when applying to urea-added urine samples (Figures 2E,F, S6 and S7). Furthermore, the

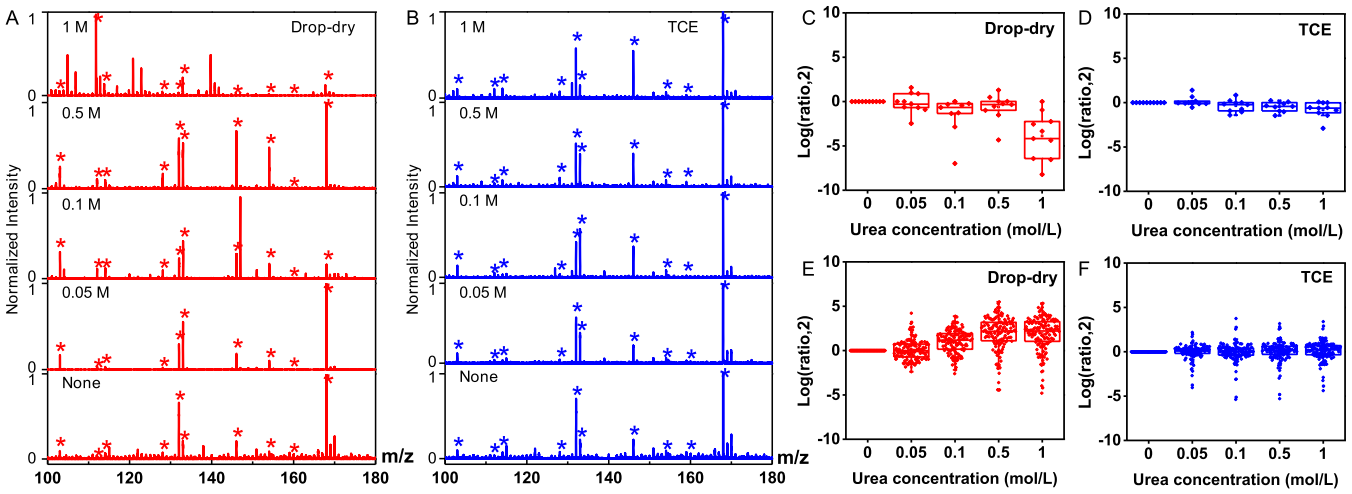

Figure 2. The mass spectra and peak ratio distributions of urea-added samples (0, 0.05, 0.1, 0.5, and 1 M) were presented after normalization. (A,B) Normalized mass spectra of urea-added mixed standard samples obtained by the TCE technique and traditional drop-dry sampling method, respectively. (C,D) Peak ratio distributions of urea-added mixed standard samples using the TCE technique and traditional drop sampling-dry method, respectively. (E,F) Peak ratio distributions of urea-added urine samples.

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effectiveness of TCE technology on reducing the interference from different types of salt in urine samples was also proved (Figure S8). The secretion volume of urine samples is largely affected by water consumption and other external factors, which distinguishes urine from many biofluids used in metabolomics studies. To investigate whether similar mass spectra can be obtained during the dilution process, a series of diluted mixed standard solutions and diluted urine samples were prepared for analysis. As shown in Figure 3A, for urine

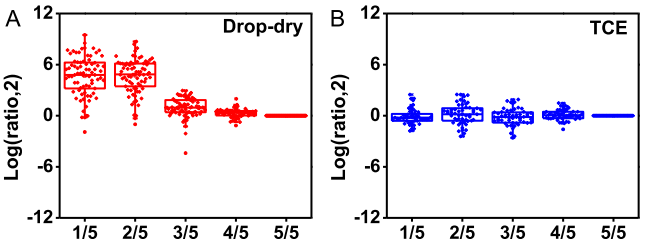

Figure 3. Peak ratio distributions of diluted urine samples (1, 0.5, 0.25, 0.125, and 0.0625 U) were presented using box plots after normalization. (A) Traditional drop-dry method. (B) TCE technique. 1, 2, 3, 4, and 5 represent 1, 0.5, 0.25, 0.125, and 0.0625 U samples, respectively. The intensity of the MS peaks detected in each sample were compared with sample 5 (0.0625U).

samples, salt concentrations varied with the dilution process; therefore, dissimilarity of mass spectra would be observed if the drop-dry method was employed. On the contrast, relatively stable and reliable normalized peak data was obtained with the TCE technique during the dilution process (Figure 3B). Besides, the same phenomenon was also observed in the analysis of diluted mixed standard samples (Figure S9). To explore whether the difference between individuals could be eliminated, urine samples from five healthy volunteers were collected and utilized for subsequent MS analysis. When using the traditional drop-dry method, metabolic spectra varied greatly due to the difference in the salt content between individuals, which severely hinders the application of urine samples in biomarker screening and disease diagnosis (Figure S10A). As shown in Figure S10B, the mass spectra obtained using the TCE technique presented a certain consistency, reflecting an effective role in eliminating interference from salt differences between individuals. Furthermore, the stability of intra-batch and inter-batch detection for urine samples was examined to confirm the repeatability in the MS signal. As shown in Figure S11, acceptable intra-batch (medium RSD = 13.3%) and inter-batch stability (medium RSD = 15.7%) were obtained with the TCE method, which guaranteed the reliability of collective data. However, traditional drop-dry method displayed unsatisfactory intra-batch (medium RSD = 35.4%) and inter-batch stability (medium RSD = 47.4%). These results gave us confidence that the TCE method coupled with the negative LDI-MS system has robust performance for acquiring reproducible metabolic profiles of urine samples.

3.3. Quantification of Metabolites without Internal Standard

To access the linearity and recovery yield of the TCE method for metabolites, a series of standard samples and standard-added urine samples were prepared. Six putative metabolites in urine samples were identified and selected for the standard addition method including aspartic acid, cysteic acid, pipecolic acid, glutamine, taurine, and proline. Each metabolite was dissolved in ultra-pure water or urine samples to obtain a final concentration of 0, 0.01, 0.1, 0.2, 0.35, 0.5, and 1 mM. As shown in Figures S12 and S13, good linearities were obtained with R^2 > 0.97 for all samples and the recovery yield varied from 95.8 to 105% for standard samples and 92.6 to 105.7% for standard-added urine samples (Figure 4). The results confirmed the reliability of obtained spectra and further revealed the possibility for metabolic analysis of urine samples.

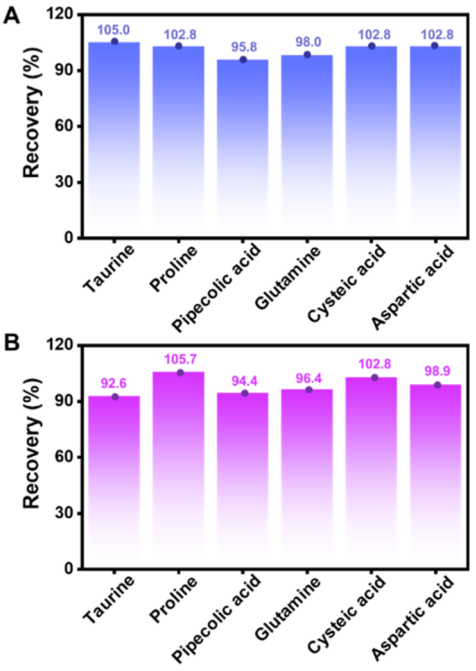

Figure 4. Recovery yield of metabolites in standard samples and standard-added urine samples using the TCE technique. (A) Recovery yield of six identified metabolites in standard samples, including taurine, proline, pipecolic acid, glutamine, cysteic acid, and aspartic acid. (B) Recovery yield of six identified metabolites in urine samples.

3.4. Detection of Exogenous Substance by TCE Method

In addition to endogenous metabolites, exogenous molecules can also be extracted onto the FEP@VSiNWs substrate using TCE technology. In order to demonstrate the feasibility of the TCE technique for exogenous molecule detection in urine samples, three chemical residues were selected and spiked in urine samples for detection, including melamine, cyromazine, and imazalil. Melamine, which is an industrial chemical, used to be added to milk or feed to falsely increase the protein content using the Kjeldahl method. Using the TCE method, the limit of detection (LOD) was 5 μM and a good linearity (R^2 = 0.9996) was obtained for melamine in urine samples, ranging between 12.5 and 2000 μM (Figure S14A,B). Cyromazine, which is a triazine pesticide and insect growth regulator, has been widely used for fly control in animal manure and to protect flowers, fruits, and vegetables in agriculture. In this study, the LOD of cyromazine was 25 μM and a good linearity (R^2 = 0.9930) was obtained, ranging between 50 and 2000 μM (Figure S14C,D). Furthermore, the FEP@VSiNWs chip can also be applied in the extraction of imazalil in urine samples. Imazalil, which is one of the most used bactericides against many fungal diseases that invade

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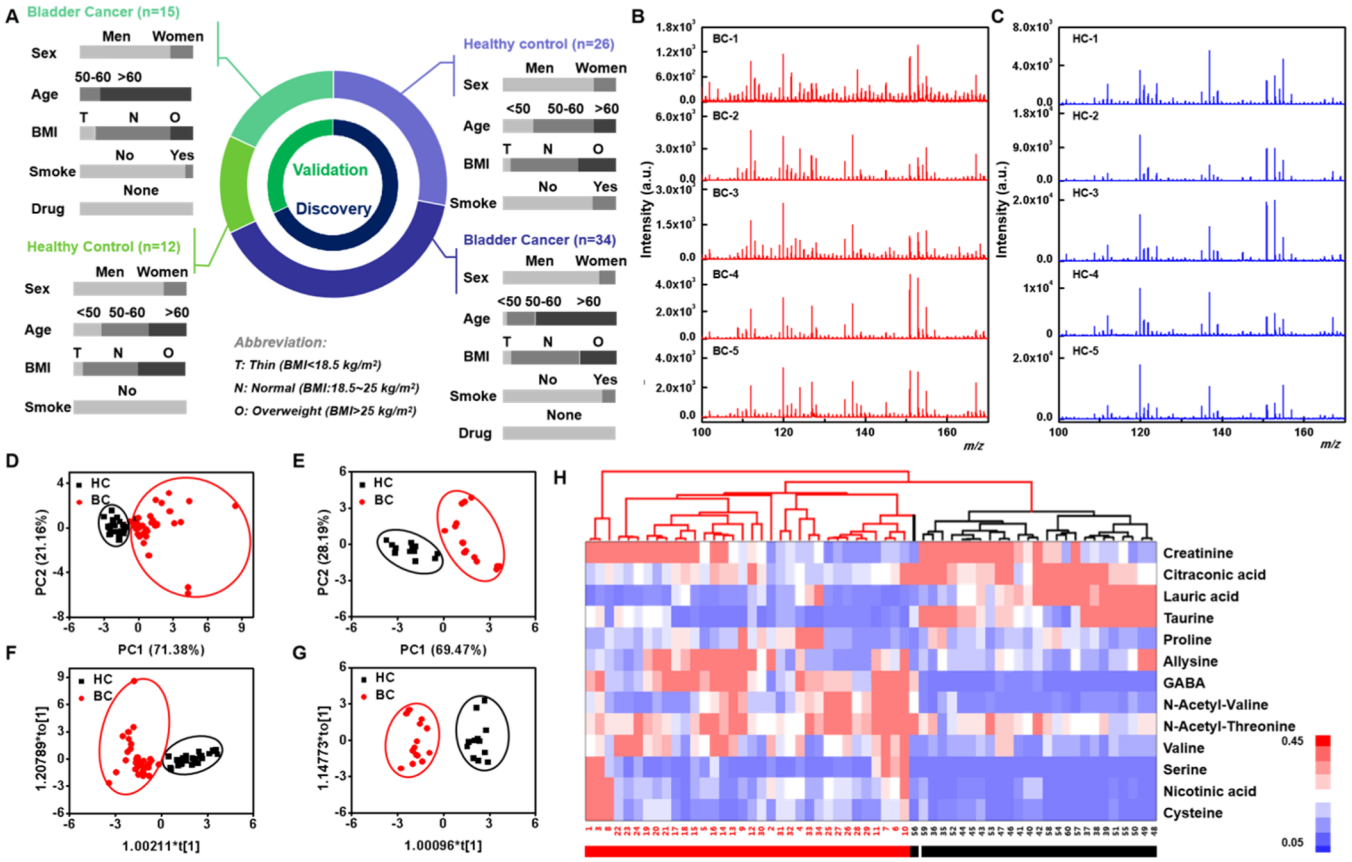

Figure 5. Metabolic analysis of urine samples for discrimination between BC patients and control individuals. (A) Distributions of demographic information from patients with BC and controls. (B,C) Representative LDI mass spectra of urine samples collected from healthy volunteers and BC patients in the metabolic fingerprint region (100−170 Da), respectively. (D,E) Scores plots for the PCA model built with the normalized intensities of 13 selected metabolites in the discovery set and validation set, respectively. (F,G) OPLS-DA results based on the potential biomarkers in the discovery set and validation set, respectively. (H) Cluster analysis results in the discovery set. Intensities of metabolites used here were normalized to [0,1].

fruits, vegetables, and ornamental plants, is attracting more concern over environmental contamination and possibly human health because of its widespread use. The LOD of imazalil was 12.5 μM, and a good linearity (R^2 = 0.9967) was obtained for imazalil spiked in urine samples, ranging between 25 and 500 μM (Figure S14E,F). These results proved that the FEP@VSiNWs array coupled with LDI-MS is an easy-to-use platform for directly detecting food residual agrochemicals or monitoring water body pollution.

3.5. Screening Normalization Methods for Urinary Metabolic Analysis

To guarantee the differences between individual subjects from patients and control groups due to metabolic disorder from disease rather than external interference, a total of six different normalization methods were screened to minimize the impact of external factors such as water consumption on downstream analysis. Data from replicated measurements of the QC sample (a pooled urine sample, represented as 1 U) using the same or different batches of FEP@SiNWs substrates were collected and normalized to calculate the intra-batch and inter-batch stability; box plots were obtained by calculating the peak ratios of replicated measurements after six different normalization methods (Figures S15 and S16). Besides, urine metabolomics studies should consider the variation in solute concentration due to the different secreted volumes. The correction effect of different normalization techniques on urine dilution was expressed as normalized peak ratios between diluted urine samples and the original 1 U sample (Figure S17). Especially, the variation of each metabolite in urine samples from the same group (HC or BC) was also taken into consideration. After calculating the peak ratios of each metabolite to the group average value, the fluctuation of each normalized metabolic peak within the same group was presented in box plots (Figure S18). Furthermore, a discriminant result was also included in the evaluation of the normalization performances. Data acquired from 30 healthy volunteers and 30 BC patients were normalized and then imported into SIMCA software. OPLS-DA is a supervised algorithm based on orthogonal partial least squares model, R^2X and R^2Y represent the interpretation rate of the X and Y matrices, respectively, and Q^2 represents the predictive credibility of the built model. In theory, the closer the R^2Y and Q^2 values are to 1, the more credible the built model is. An unsupervised PCA approach is commonly applied to reduce data dimensionality and to extract relevant information from a given data set. As shown in Figures S19 and S20, cubic spline normalization provided the most satisfactory discrimination result with OPLS-DA (R^2Y = 0.95, Q^2 = 0.93) and unsupervised PCA. Based on all the above descriptions, cubic spline normalization was chosen for subsequent urinary metabolomics analysis.

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Table 1. Summary of Feature Peaks That Could be Used to Distinguish BC Patients from Control Subjects

no.	detected m/z	identification	p value (discovery)	p value (validation)	trend
1	102.0549	GABA	2.48×10^{-11}	7.04×10^{-12}	↑
2	104.0303	serine	2.44×10^{-3}	1.35×10^{-4}	↑
3	112.0482	creatinine	8.86×10^{-3}	2.91×10^{-6}	↓
4	114.0512	proline	1.47×10^{-2}	2.11×10^{-2}	↑
5	116.0698	valine	5.05×10^{-4}	1.66×10^{-3}	↑
6	119.0119	cysteine	2.01×10^{-5}	4.42×10^{-6}	↑
7	122.0266	nicotinic acid	3.79×10^{-9}	4.64×10^{-3}	↑
8	123.9969	taurine	6.03×10^{-5}	3.24×10^{-3}	↑
9	129.0071	citraconic acid	1.78×10^{-5}	3.65×10^{-4}	↓
10	144.0621	allysine	5.30×10^{-3}	9.75×10^{-3}	↑
11	158.0702	*N*-acetylvaline	1.12×10^{-8}	2.49×10^{-4}	↑
12	160.0615	*N*-acetylthreonine	1.85×10^{-2}	3.32×10^{-3}	↑
13	199.1744	lauric acid	2.71×10^{-5}	2.89×10^{-2}	↓

3.6. Discovery and Validation of Abnormal Metabolic Signatures in BC

To uncover the metabolic disturbance of BC hidden in urine specimens, a total of 87 urine samples from 38 healthy volunteers and 49 patients diagnosed with BC were collected and utilized for subsequent MS analysis. A schematic procedure for the statistical analysis was provided in Figure S21, and detailed demographic information of participants is shown in Figure 5A and Table S1. To discover the differential metabolites in BC patients, student's *t*-test was performed and thirteen metabolic features were sorted out with p values less than 0.05. These differential features were further verified using the validation cohort, including 12 healthy volunteers and 15 patients diagnosed with BC, and same change trends of these biomarker candidates were observed in the validation set (Table 1). Detailed identification information of the verified metabolic biomarker candidates is described in Tables S2, S3, and Figure S22, representative LDI mass spectra of urine samples from healthy volunteers and BC patients are presented in Figure 5B,C. Meanwhile, box plots were completed to reveal the perturbations of differential metabolites in BC patients (Figures S23 and S24). Compared with the HC group, GABA, serine, proline, cysteine, *N*-acetylvaline, *N*-acetylthreonine, valine, allysine, and nicotinic acid were up-regulated in BC patients, whereas creatinine, taurine, citraconic acid, and lauric acid were down-regulated. Besides, literature supports and relevant metabolic pathways of the potential differential metabolites were provided through searches of HMDB (Table S4). The impact pathway analysis of BC-related metabolites using MetaboAnalyst also confirm the correlation between the selected metabolites and BC disease (Figure S25).

3.7. Multivariate Analysis for Discrimination between BC Patients and Control Individuals

To further investigate the feasibility of this platform in non-invasive BC diagnosis, a panel consisting of 13 differential metabolites was established and applied for discriminant analysis. As shown in Figure 5D−G, on the basis of the established panel, BC patients and healthy controls can be successfully discriminated by unsupervised PCA or supervised OPLS-DA, no matter whether in the discovery and validation cohort. Besides, feature metabolites found in urine samples were normalized and further imported into MATLAB to perform cluster analysis; the cluster tree indicates that urine samples from the BC patients and healthy controls appear in separate clusters with only one exception (BC patient no. 56 was misclassified as a healthy control) in the discovery set and no misclassification in the validation set, respectively (Figures 5H and S26). These results have confirmed the potential of this sensitive and reproducible platform based on the TCE technique coupled with negative LDI-MS in urine diagnosis of BC and further provided a new perspective for rapid non-invasive large-scale screening in the population.

In summary, this work has established a method of TCE coupled with the negative LDI-MS on the basis of the FEP@VSiNWs chip, which has been confirmed to be applicable for reproducible and reliable detection of metabolites in high-salt biofluids. Through the TCE method, stable mass spectra can be generated in the presence of salt with different concentrations or during the dilution process. To minimize the impact of external factors such as water consumption on downstream analysis, six normalization methods were compared and cubic spline normalization method was selected owing to its best performance. Especially, we demonstrated for the first time that urinary metabolic profiling obtained by the TCE method holds potential to diagnose BC. Those potential urine biomarkers uncovered in the present work could be well applied to distinguish BC patients from healthy controls. Furthermore, the results suggest that the exogenous substance can also be conveniently and quickly extracted from the urine sample, and quantitative detection of those exogenous substance was achieved on the TCE coupled LDI platform. The present work reveals the potential wide applications in urine detection for health and environmental monitoring.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00340.

Detailed experimental parameters of UPLC-MS analysis; R-scripts for data normalization; extraction procedure optimization; material characterization; performance of the TCE technique in detection of salt-added samples or real urine samples; linearity and LODs of metabolites using the TCE technique; normalization method comparison; box plots of differential metabolites; metabolic pathway analysis; and discriminant analysis (PDF)

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Notes

The authors declare no competing financial interest.

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