國立臺灣大學醫學院藥學研究所

碩士論文

Graduate Institute of Pharmacy

College of Medicine

National Taiwan University

Master Thesis

第一部分：建立氣相層析質譜儀方法定量短鏈脂肪酸之濃度並研究糞便檢體樣品製備方法產生之誤差
第二部分：以液相層析質譜儀結合柱後注入內標法分析尿液中的生物胺並應用於研究乳癌化療藥物之療效反應

Part I: Development of a quantification method for short-chain fatty acids by gas chromatography-mass spectrometry and using human fecal sample to study the errors caused by sample handling
Part II: Using liquid chromatography-mass spectrometry in combination with PCI-IS strategy to analyze biogenic amines in urine and its application to investigating chemotherapeutic response to breast cancer patients

許雅琳

Ya-Lin Hsu

指導教授：郭錦樺 博士

Advisor：Ching-Hua Kuo, Ph.D.

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中文摘要

代謝體學為近年新興之研究領域，主要目標為廣泛地分析生物系統中的代謝物，已展現出具有應用於個人化醫療的潛力。標的代謝體學探討特定標的代謝物與臨床疾病之間的相關性，而針對這些標的代謝物開發分析方法可以獲得更準確的結果。本論文運用質譜技術於標的代謝體學，分別測定兩種不同類型的小分子代謝物：短鏈脂肪酸與生物胺。

在本論文的第一部分，我們開發並確效一利用氣相層析飛行式質譜儀定量人體糞便中之六種短鏈脂肪酸濃度，包含：乙酸、丙酸、丁酸、異丁酸、異戊酸與戊酸。為達到去除基質干擾物的目的，我們使用丁醇從經酸化處理的糞便中萃取短鏈脂肪酸。在分析條件的最適化下，利用 VF-WAXms 毛細管層析管柱進行分離，能於十六分鐘內完成此六個短鏈脂肪酸之定量分析。此方法之準確度介於 87.9 至 110.9% (n = 9)，同日 (n = 3) 與異日間 (n = 9) 精密度之相對標準偏差皆小於 10%。我們進一步應用此方法研究糞便檢體製備方式對於分析短鏈脂肪酸可能造成之誤差，包括凍乾與取樣位置的影響。研究發現配對樣品中 (n = 6) ，經凍乾處理的糞便檢體與未經處理的檢體之間的短鏈脂肪酸濃度比值介於 0.6 至 1.4。同時，我們也觀察到從同一糞便樣品 (n = 5) 的六個不同位置取樣所測定的短鏈脂肪酸濃度具有顯著的差異，六個短鏈脂肪酸濃度的相對標準偏差介於 6.0%至 66.3%。我們的結果指出，若僅使用 60 毫克的糞便樣品進行分析，將可能因為不同的取樣位置造成顯著的測量偏差。

第二部分我們利用液相層析質譜儀並結合柱後注入內標校正法建立分析人體尿液中 14 個生物胺的分析方法，包括兩種不同化學結構的多胺與兒茶酚胺。我們以丹磺醯氯作為衍生化試劑並使用 Gemini C18 作為層析管柱，此方法能在十二分鐘內完成 14 個標的生物胺的分離。我們利用經衍生的腐胺同位素 (putrescine-d6) 做為單一的柱後注入內標，能夠有效地校正不同尿液檢體中的基質效應而引起的測量誤差。我們進一步將此方法應用於研究尿液生物胺濃度與乳癌病人化療反應

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的關係。60 個接受化學治療之乳癌病人分別在第一次化療治療前、第二次化療前、第三次化療前三個不同時間點採集尿液檢體，並利用此分析方法分析其尿液中生物胺含量。實驗結果顯示，14 個被選為標的的生物胺中，N^1,N^{12}-diacetylspermine (N1N12) 與 N^1,N^8-diacetylspermine (N1N8) 在化學治療前之尿液含量，在化療有效組 (n = 43) 中顯著高於 (P < 0.05) 化療無效組 (n = 17)。除此之外，N-acetylputrescine (NAP) 與 N1N12 的含量在化療有效組中之化學治療前、後有顯著改變。我們的結果顯示尿液中的 N1N12 含量具有潛力作為評估乳癌化療反應的生物指標，但未來仍需要更多的研究來驗證。

本研究成功建立分別用於測定短鏈脂肪酸與 14 個生物胺的分析平台，未來可更廣泛將這兩個分析方法應用於不同之臨床研究以探討這些代謝物在疾病機轉的角色與協助臨床診斷。

關鍵字：短鏈脂肪酸、生物胺、柱後注入內標、乳癌、尿液、糞便檢體

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Abstract

Metabolomics, an emerging field which aims at comprehensive analysis of metabolites in the living system, has shown its potential in personalized medicine. Targeted metabolomics evaluates the relevance of predefined analytes to a specific clinical problem, and the analytical method could be designed for those target analytes to obtain more accurate results. In this study, a mass spectrometry-based targeted metabolomics approach was employed to determine two different classes of small-molecule metabolites (short-chain fatty acids (SCFAs) and biogenic amines)

In the first part of this thesis, we developed and validated a quantitative method to analyze six SCFAs, including acetic, propionic, butyric, isobutyric, isovaleric, and valeric acids, in human fecal samples by gas chromatography-time of flight mass spectrometry. To remove interfering species, we used butanol to extract SCFAs from acidified fecal suspensions. Six SCFAs could be quantified within 16 mins under optimal conditions by using a capillary VF-WAXms column for separation. The accuracy of the method was ranged from 87.9% to 110.9% (n = 9), and both the intra-day (n = 3) and inter-day precision (n = 9) were below 10% relative standard deviation (RSD). We further applied the method to study sample preparation errors including lyophilization and sampling position. Our findings revealed that the concentration ratios between the paired samples (n = 6), freeze-dried feces and wet feces (without lyophilization) were ranged between 0.6 and 1.4. Also, we observed different concentrations of SCFAs among samples collected from different positions of the same feces (n = 5). The RSD of six sampling from different positions for six SCFA was range from 6.0% to 66.3%. Our results indicated significant bias may be introduced by different sampling position if 60 mg of fecal sample was used for the analysis.

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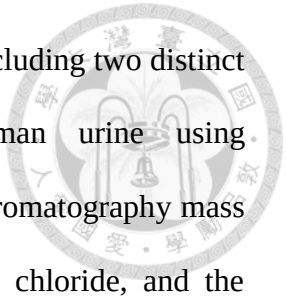

In the second part, we analyzed 14 selected biogenic amines, including two distinct chemical structures—polyamines and catecholamines—in human urine using post-column infused internal standard (PCI-IS) strategy in liquid-chromatography mass spectrometry. The 14 target amines were derivatized with dansyl chloride, and the separation was achieved by a Gemini C18 column within 12 mins. The dansylated putrescine-d6 was utilized as a universal PCI-IS for the 14 target amines, and it effectively calibrated the errors caused by matrix effect in urine samples. The developed method was applied to investigate the relationship between the urinary biogenic amines and the chemotherapeutic response in breast cancer patients. Urine samples were collected from 60 patients at three time points: before chemotherapy was initiated (pre-C1), on the day before the second cycle (pre-C2), and the third cycle (pre-C3) of chemotherapy and they were analyzed by the optimal LC-MS method to investigate the level of biogenic amines. Among the 14 selected biogenic amines, N^1,N^{12}-diacetylspermine (N1N12) and N^1,N^8-diacetylspermine (N1N8) in the responder group (n = 43) were significantly higher ($P < 0.05$) than the nonresponder group (n = 17) prior to the chemotherapy. *N*-acetylputrescine (NAP) and N1N12 showed significantly change in response to chemotherapy in the responder group. Our results revealed urinary N1N12 showed high potential to serve as an indicator for assessment of the response to chemotherapy in breast cancer, though further studies are required to verify this finding.

This study successfully established two analytical platforms for the SCFAs and the biogenic amines which could be used for various clinical studies to explore their potential roles for interpretation of disease mechanism and clinical diagnosis.

Keywords: short-chain fatty acids (SCFAs), biogenic amines, post-column infused internal standard (PCI-IS), breast cancer, urine, fecal samples

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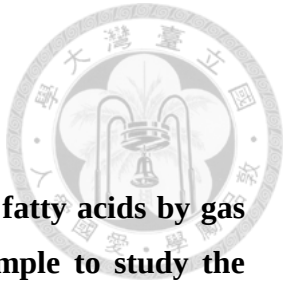

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Table contents

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Part I:

Development of a quantification method for short-chain fatty acids by gas chromatography-mass spectrometry and using human fecal sample to study the errors caused by sample handling

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1. Introduction

1.1 Short-chain fatty acids

Recently, there has been a growing interest in the influence of the gut microbiota on human health. Considering as a forgotten organ [1], gut microbiota exerts beneficial effects on the intestinal mucosa, ranging from protection against infection to the immune system development, and also plays an important role in the regulation of multiple host metabolic pathways [2, 3]. The host and its gut microbiota coproduce diverse range of metabolites essential for the host, such as vitamin K, vitamin B12, folate, and bile acids during the metabolism of nutrients and xenobiotics [2]. The main substrates for fermentation are dietary carbohydrates and proteins that escape digestion in the small intestine [4, 5]. The major end-products of the non-digestible carbohydrates, including resistant starches and dietary fiber, are the short-chain fatty acids (SCFAs), CO_2, and H_2. Other intermediates, such as lactic acid, ethanol, succinic acid, and formate, are also important metabolites during the formation of SCFAs [4, 5]. The bacterial degradation of fermentable proteins results in the formation of branched-chain fatty acids and other potentially toxic metabolites such as amines, phenols, indoles, and volatile sulfur compounds [4-6]. As the major group of these metabolites, the biological relevance of SCFAs to human health has attracted much attention.

SCFAs are monocarboxylic acids that consist of one to six carbons. Effects on SCFAs production in the cecum and proximal colon include the type and amount of carbohydrates in diet, the gut microbiota composition, and also the gut transit time [7, 8]. The most abundant SCFAs in the colon are acetate (C2), propionate (C3) and butyrate (C4), whereas isobutyrate (i-C4), valerate (C5), isovalerate (i-C5), and hexanoate (C6) constitute only 5–10% of total SCFAs [8]. The molar ratio of acetate, propionate, and butyrate in fecal samples has been found to be 60:20:20 [9], which was similar in the

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samples from colonic regions [10]. The produced SCFAs are rapidly absorbed by colonocytes and further transported from the intestinal lumen to the blood compartment, and consequently can be found in the portal, hepatic and peripheral venous blood [10]. Previous studies indicated that SCFAs acted as substrates or signaling molecules in various tissues and were involved in regulation of host energy metabolisms, including lipid, glucose, and cholesterol [6]. It has been estimated that SCFAs can provide approximately 10% of daily caloric requirements in humans [11]. Roediger *et al.* [12] showed that more than 70% of the oxygen was consumed by colonocytes due to butyrate oxidation, indicating that butyrate is a major respiratory fuel in colonocytes. The remaining propionate and butyrate can be taken up by the liver and used for gluconeogenesis [13], whereas acetate can be used as a substrate for cholesterol synthesis and metabolized by peripheral tissues [7]. In addition to the roles of SCFAs in metabolic functions, there is growing evidence that SCFAs contribute to reducing colonic inflammation, preventing colon carcinogenesis, and promoting mucosal healing [14]. Moreover, many human intervention studies revealed that SCFAs were involved in the prevention and treatment of some inflammatory bowel disease, including Crohn's disease [15], ulcerative colitis [16], obesity [17], and colorectal cancer [18, 19].

Although there are only 5% of the produced SCFAs excreted in the feces due to the absorption in colonocytes [20], fecal samples are the most accessible specimens for studying host-gut microbial interactions [21] since the changes in fecal SCFA concentrations could reflect the balance between the production and absorption of SCFA in colon [22]. In addition, differentially represented fecal metabolites in patients with colorectal cancer [23] and inflammatory bowel disease [24, 25] indicated the importance of fecal metabolic analysis to study the correlations between fecal metabolites and human health.

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Therefore, qualitative and quantitative analysis of SCFAs in human feces is crucial for elucidating the role of SCFAs in human health.

1.2 Current methods for analysis of SCFAs in human feces

Several analytical techniques have been used for determination of SCFAs over the past decade, such as gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR) spectroscopy, and capillary electrophoresis (CE). Among all, GC is the most widely used method for SCFAs analysis due to the volatility of these compounds. Moreover, the combination of mass spectrometer (MS) provides better sensitivity and selectivity as compared to the flame ionization detector [26].

To isolate SCFAs from a complex matrix, different sample pretreatment procedures have been reported in GC/MS-based metabolomic analyses. For example, the method from Weir *et al.* was to simply acidify the fecal samples, followed by centrifugation and filtration prior to injection [23]. The acidification step of fecal samples to pH 2–3 enhances extraction efficiency and improves the peak shape of SCFAs [27]. However, direct injection of complex samples into the GC apparatus may lead to a shorter lifespan of column due to the contamination of nonvolatile compounds [28]. As a result, liquid-liquid extraction using organic solvent, such as diethyl ether [29-31], was most frequently employed after the acidification step of fecal samples [26]. On the other hand, Garcia-Villalba *et al.* tested three different organic solvents, including diethyl ether, dichloromethane, and ethyl acetate, to extract SCFAs from acidified fecal suspensions. Although ethyl acetate and diethyl ether showed similar extraction efficiencies, ethyl acetate is preferred due to its connivance and ease of use [28]. After that, solid phase microextraction (SPME), a solvent-less extraction technique, has been applied to extract volatile SCFAs from different matrices such as waste water [32], wines [33], and also fecal samples [34-36]. The technique is based on the use of a

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polymer-coated fiber to concentrate and extract the volatile analytes in a single step, which provides high selectivity and sensitivity due to a better clean-up of the matrix, thus resulting in a longer lifetime of the chromatographic system [26, 37]. However, fibers are relatively expensive and fragile, and it also requires additional devices for automated analysis [28].

Although various sample preparations are successful in extracting the SCFAs from fecal samples, a standard protocol for fecal sample handling is not yet fully established. Since feces contain numerous microbes, the proper methods for collection, transportation, and storage of the samples after defecation are necessary to prevent the ongoing biological processes [21]. A human fecal sample handling protocol for metabolic profiling using ^{1}H NMR spectroscopy has been proposed by Gratton *et al.* [38]. The fecal samples were recommended to transport on ice (4℃), homogenize, and extract within 24 hours to avoid sample degradation. Moreover, the concentration of SCFAs in fecal water stored at <

-20℃ was more stable compared to crude fecal samples. Other studies also indicated the samples should be kept at -20℃ or -80℃ after collection when analyzing SCFAs in human feces [26]. Compared to storage conditions, the influence of lyophilization to SCFA concentration has less been studied. While the removal of water minimizes analytical bias due to different water content among fecal samples, there was a study indicated the lyophization could result in a decrease in the concentration of acetate, propionate, and butyrate [39].

1.3 Specific aims

The aim of this study was to develop a simple and accurate gas chromatography-mass spectrometry (GC-MS)-based method for quantification of SCFAs (C2–C5) in human feces. The constructed method was further applied to

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investigate the analytical biases during the processes of human fecal sample handling, which include impacts on lyophilization and the sampling location of the original fecal specimen on the measurement of SCFAs.

2. Material and methods

2.1 Chemicals

Short-chain fatty acids standards, including propionic acid, butyric acid, isobutyric acid, isovaleric acid, and valeric acid, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and ortho-phosphoric acid were obtained from Merck (Darmstadt, Germany). 1-butanol (ACS reagent grade, ≥ 99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate-d3 (99% D) and sodium propionate-d5 (98% D) were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada).

2.2 Fecal samples collection

The collections of human fecal samples were approved by the Research Ethics Committee of National Taiwan University Hospital (NTUH REC No: 201703101RIND). Briefly, fecal samples were collected in a plastic box and transported to the laboratory on ice, and divided into 15 ml centrifuge tubes within 48 h. After lyophilization, samples were stored at -80℃ before processing.

2.3 Sample preparation

For the extraction of short-chain fatty acids, 20 mg of lyophilized human fecal sample was suspended in 500 μl of 0.5% phosphoric acid aqueous solution and homogenized thoroughly with Geno/Grinder 2010 (SPEX, Metuchen, NJ, US) at 1,000 rpm for 2 mins. After centrifugation at 18000 rcf for 10 mins, 100 μl of the supernatant was transferred into a 1.5 ml eppendorf tube and diluted with 200 μl of 0.5% phosphoric acid aqueous solution. In the preliminary test, 1, 3, and 5-fold dilutions of the fecal

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aqueous supernatant were evaluated, and finally the 3-fold dilution was found to be optimal for the MS sensitivity. An aliquot of 300 μl butanol was subsequently added to the solution for the liquid-liquid extraction of SCFAs, and the mixture was homogenized for 2 mins and centrifuged for 10 mins as mentioned above. Then, 180 μl of the upper organic layer was transferred into a new tube and 20 μl of butanol containing sodium acetate-d3 and sodium propionate-d5 was added as internal standards (ISs) at a final concentration of 50 μg ml^{-1}. The resulting mixture was filtered with a 0.2-μm PP membrane filters (RC-4, Sartorius, Göttingen, Germany) and transferred into a glass insert prior to analysis. All samples were frozen at -80℃ until analysis.

2.4 GC-TOF analysis

All analyses were carried out on an Agilent 7890A gas chromatograph equipped with a MultiPurpose Sampler MPS (GERSTEL, Mülheim an der Ruhr, Germany) and coupled to a Pegasus 4D GC x GC-TOFMS system (Leco Corporation, St. Joseph, MI, USA). A polar VF-WAXms capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness) (Agilent Technologies) was utilized for the separation and the helium was used as a carrier gas at a constant flow rate of 1 ml/min. One microliter of the sample was injected in a split mode with a ratio 1:10. The oven temperature was initially held at 70℃ for 1 min, and then increased to 170℃ at a rate of 10℃ min^{-1}, to 240℃ at a rate of 25℃ min^{-1}, and finally maintained at 240℃ for 2 mins (total run time 15.8 mins). The temperature of the front inlet, transfer line, and ion source were set at 250℃, 250℃, and 240℃, respectively. The mass spectrometer was operated in the electron impact mode at -70 eV and the data were acquired in full scan mode in the scan range m/z 40–550.

2.5 Data analysis

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Data acquisition and data processing were carried out using LECO's ChromaTOF® software. The identification of each SCFA was confirmed by comparing mass spectra and retention times with those of the in-house library. The peak area of each SCFA was calculated using the unique mass defined by ChromaTOF® .

2.6 Method validation

2.6.1 Recovery

For the extraction recovery, the standard mixture of six SCFAs was added into the pooled human fecal suspension at three concentration levels and followed by extraction with butanol (Table 1, p.21). Three independent samples were prepared at each concentration and the recoveries of two subsequent extractions were also tested by the same procedure. Recoveries were calculated using the following formula:

$$\text{Recovery } \% = \frac{\text{peak area of SCFA in the preextraction spiked fecal sample}}{\text{peak area of SCFA in the postextraction spiked fecal sample}} \times 100\%$$

2.6.2 Linearity

Method validation was evaluated by LLE with butanol from the standard mixture of SCFAs dissolved in 0.5% H_3PO_4. Stock solutions of the six SCFAs were prepared in Methanol/0.5% H_3PO_4 (1:1) at a concentration of 10000 μg ml^{-1}. The stock solutions were further diluted to a standard mixture containing a concentration of 1000 μg ml^{-1} for each SCFA with 0.5% H_3PO_4. The IS was first dissolved in methanol, diluted in butanol, and then added to the butanol layer at a final concentration of 50 μg ml^{-1} for correcting the injection errors, especially for the variability of MS response and the injection volume. Sodium acetate-d3 was used as an IS for acetate, and sodium propionate-d5 was used for the rest of five SCFAs. The calibration curves of six SCFAs were constructed by plotting the peak area ratio of each SCFA to IS versus

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concentration, and the range was defined with reference to the concentration of SCFAs in human feces (Table 2, p. 22). All concentrations were analyzed in triplicate.

2.6.3 Precision and accuracy

The repeatability, intermediate precision, and accuracy for the six SCFAs were evaluated by analyzing three independent replicates at low, medium, and high concentrations on three different days (Table 2, p. 22). The precision was expressed as % relative standard deviation and the accuracy of the intra- and inter-day measurements were determined by the constructed calibration curves.

2.6.4 Stability

The 24 h stability of fecal extractions at room temperature in the autosampler was evaluated in order to assess whether the concentration of SCFAs changes during the time period before injection. For this purpose, we prepared fecal extractions from a pooled human fecal supernatant using the procedure shown in Section 2.3. These samples were kept in the autosampler and analyzed at equal time intervals up to 24 h.

To ensure the long-term stability, the lyophilized feces and the fecal extractions were stored at 4℃ and -20℃ for up to 30 days, respectively. For this purpose, a homogenized fecal sample was first divided into individual 1.5 ml eppendorf. The two groups of fecal extractions were immediately extracted and stored under each condition while the extraction of lyophilized feces was carried out before analysis on day 7 and day 30. The sample for each storage condition was prepared in triplicate.

The standard mixture of six SCFAs which extracted under same condition were used as quality control (QC) samples.

3. Results and Discussion

3.1 Selection of the extraction solvent

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To develop a simple method for extraction of the SCFAs from human fecal samples, six organic solvents including methanol, acetonitrile, acetone, chloroform, ethanol, and hexane were tested. Among all, methanol showed the best extraction efficiency and minimal interferences with acetic acid comparing with the rest of solvents. However, the direct injection of methanol extracts may cause the damage to inlet liner due to the accumulation of non-volatile compounds from fecal matrix. Thus, we modified a liquid-liquid extraction protocol based on the study of Garcia-Villalba *et al.* [28]. Liquid-liquid extraction (LLE), one of the common sample pretreatment methods for the extraction of SCFAs from human fecal samples, involves a process of transferring analytes from an aqueous to an organic layer, thus resulting in good purification. We firstly tested LLE with three different solvents: ethyl acetate, chloroform, and butanol, which are immiscible with water, to extract SCFAs from acidified fecal aqueous solution. As ethyl acetate was used, residual acetic acid could be observed in the solvent blank chromatogram. Although there was no residual acetate found in butanol and chloroform, LLE with butanol showed better extraction efficiency for acetic acid and propionic acid as compared to chloroform. As a result, LLE with butanol was selected in this study.

3.2 Optimization of extraction procedure

The extraction recoveries of the six SCFAs from fecal aqueous supernatants spiked with three concentrations of SCFAs are summarized in Table 1 (p. 21). The recoveries of isobutyric (i-C4), butyric (C4), isovaleric (i-C5), and valeric acid (C5), obtained by single extraction were ranged from 82 to 97%. However, the recoveries of acetic (C2) and propionic (C3) acid were comparatively low (63% and 73%, respectively) when spiked at high concentration (300 μg ml^{-1}). Although slight improvement of extraction recovery for C2 was observed in double extraction, the recoveries for the other SCFAs

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remained unchanged (Table 1, p. 21). Besides, the extraction recoveries were reproducible for all the six SCFAs in single extraction (n = 3, RSD < 5%) while higher variability was observed in double extraction (n = 3, RSD ranging from 0.8 to 14.1%) which probably due to more sample processing steps introduced more analytical errors. The extraction recoveries in this study were similar to a former study which showed the recoveries ranged from 82 to 105% for C3 to C5, and around 65% for C2 using LLE with ethyl acetate [28]. Besides, our results were consistent with the published observations that single extraction using LLE with diethyl ether was incomplete particularly for C2 and C3 due to the high water solubility of these compounds [40]. Since C2 and C3 present in high concentration in human fecal sample, their relatively poor recoveries may not cause the detection problem. Because using single extraction could simplify the sample preparation procedures and also provide better reproducibility, single extraction was used in our study.

Figure 1 (p. 16) shows the chromatograms obtained from a standard mixture of six SCFAs and human fecal samples using the optimal extraction procedure.

3.3 Method validation

3.3.1 Linearity, precision and accuracy

The developed method was validated in terms of linearity, precision, and accuracy using standard mixture of SCFAs and these standards were extracted with butanol. The results of linearity, precision, accuracy are summarized in Table 2 (p.22). All the calibration curves showed a good linearity and a high correlation coefficient greater than 0.99. The RSD values of repeatability and intermediate precision were below 10% and the accuracy of six SCFAs under three concentrations were in the range of 87.9–110.9%. These results suggested that our method can provide accurate and reliable measurements of SCFAs in human feces within the test range.

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3.3.2 SCFAs stability

Since the SCFAs are volatile compounds, particular attention should be paid to the storage of the fecal samples. No significant changes were found in the concentration of six SCFAs while keeping fecal extracts in an autosampler tray at room temperature for a 24-h period (Table 3, p. 23), indicating that using an autosampler was proper for sequential analysis of samples. The stability of SCFAs were further evaluated using lyophilized feces and fecal extracts under two storage temperatures, 4℃ and -20℃, in order to determine whether there is a need to extract the samples immediately after lyophilization. Our results showed that the concentration of fecal samples stored at -20℃ were stable and had no significant changes within 30 days compared to control samples, whereas the concentration of SCFAs tended to decrease in the sample stored at 4℃ (Table 4, p. 24). The variation of SCFAs concentration in fecal extracts stored at 4℃ (RSD 3.6–6.8%) was higher than lyophilized feces (RSD 1.8–4.6%), suggesting that the lyophilized feces were more stable when stored at 4℃ (Table 4, p. 24). The changes in concentrations under the different storage conditions were further illustrated in Figure 2 (p. 17). Although the RSD of concentration is relative higher in fecal extracts stored at 4℃, we found that the decrease in the concentration of SCFAs were still less than 15% of the control samples (Figure 2, p. 17). On the basis of these results, we suggest that the lyophilized feces should be stored at -20℃ without extraction and the extraction procedure should be performed prior to the GC analysis.

3.4 The effect of lyophilization on the measurement of SCFAs

Lyophilization is a common sample pretreatment procedure for fecal samples to minimize comparison bias caused by different water content. The validated method was applied to investigate the impacts of lyophilization procedure on each SCFA concentration in fecal samples. To evaluate whether there was a loss of SCFAs during

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lyophilization, two 60 mg aliquots obtained from the same fecal sample (n = 6) were weighted and placed into two 1.5 ml eppendorfs. One of the paired samples was freeze-dried before extraction. The six pairs of wet and freeze-dried feces from six healthy volunteers were subsequently prepared by the procedure shown in Section 2.3.

The comparison of the concentration of six SCFAs from wet samples and lyophilized samples are shown in Figure 3 (p. 18). The concentration obtained after lyophilization was compared with that obtained from wet sample (without lyophilization) and expressed as ratio to evaluate the effect of lyophilization on the SCFA concentrations in fecal sample. The results showed differential amounts of SCFAs between these two groups with average ratio ranging from 0.6 to 1.4. Acetic acid concentration fluctuations were observed in feces 03, and 04, whereas butyric acid concentration fluctuations were observed in feces 05, and 06. We additionally evaluated the composition of each SCFA in the six pairs of fecal samples (Figure 4, p. 19). We found the composition pattern of SCFA in each pair of sample was similar despite the difference in concentrations between the two groups.

This study uses relatively small amounts of sample (60 mg) to improve the lyophilization efficiency, and thus the different concentrations observed in the freeze-dried and wet sample (Figure 3, p. 18) may be caused by the variation in sampling, given that human feces composed of bacterial biomass and other undigested plant matter [41]. Therefore, the sampling errors may exert a greater impact on the amounts of SCFAs than that of lyophilization procedure. Moreover, the lack of sample replicates is also the limitation in our data and which may increase the variation from sampling. Based on the above results, the lyophilization procedure was incorporated in the sample preparation procedure to avoid potential biases caused by water content.

3.5 The effect of different sampling locations on the measurement of SCFAs

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Unlike other specimens such as urine and plasma, fecal samples are heterogeneous and thus spot sampling may result in high metabolic variations [38]. However, it is inconvenient to homogenize the whole fecal evacuation when the sample size is large. Therefore, we compared the contents of SCFAs from different positions of feces to evaluate the variations within the same fecal sample. We collected 60 mg of feces from the inner layer and outer layer of both ends, and the middle of the original fecal sample (n = 5). All the samples were lyophilized before further extraction.

The concentrations of six SCFAs from different locations in each fecal sample are summarized in Table 5 (p. 25–25). Our results showed that the concentration of six SCFAs in different locations of the original feces were similar in subject 1 and 2 with standard deviation (RSD%) ranging between 6.0 to 14.3% and 10.9 to 17.1 %, respectively. However, the RSD values of each SCFA among these locations were higher in subject 3, 4, and 5, which indicated the variable concentrations in different locations of the original feces. For subject 3, the concentration of C3 and C4 in samples collected from the inner layer of the region C were almost twice than that in the outer layer.

We also compared the composition of six SCFAs in each sampling position (Figure 5, p.20). The distribution patterns were consistent among the different sampling positions in subject 1 and 2. Although the concentrations of six SCFAs were different in subject 4, the composition patterns between the inner and outer layer of the same position were similar. Both subject 3 and 5 showed different concentration of six SCFAs, however, the similar or closer patterns from the outer layer of section A and B in subject 3 and the inner layer of section A and B in subject 5 were found in Figure 5 (p. 20). As a result, it is necessary to homogenize each fecal sample to obtain a representative profile of SCFAs from an individual.

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4. Conclusions

In this study, we developed a GC-TOF method to quantify the concentration of six SCFAs in human fecal samples within 16 mins. The results showed good accuracy, intraday and intermediate precision, indicating that the method was valid. For the human fecal sample handling protocol, we recommend the fecal samples should be homogenized after collection, followed by lyophilization, and extracted prior to the GC analysis. The lyophilized feces were stable at -20℃ within 30 days. This established protocol can be used to investigate the casual effects between SCFAs, microbiota, and various diseases.

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5. Figures

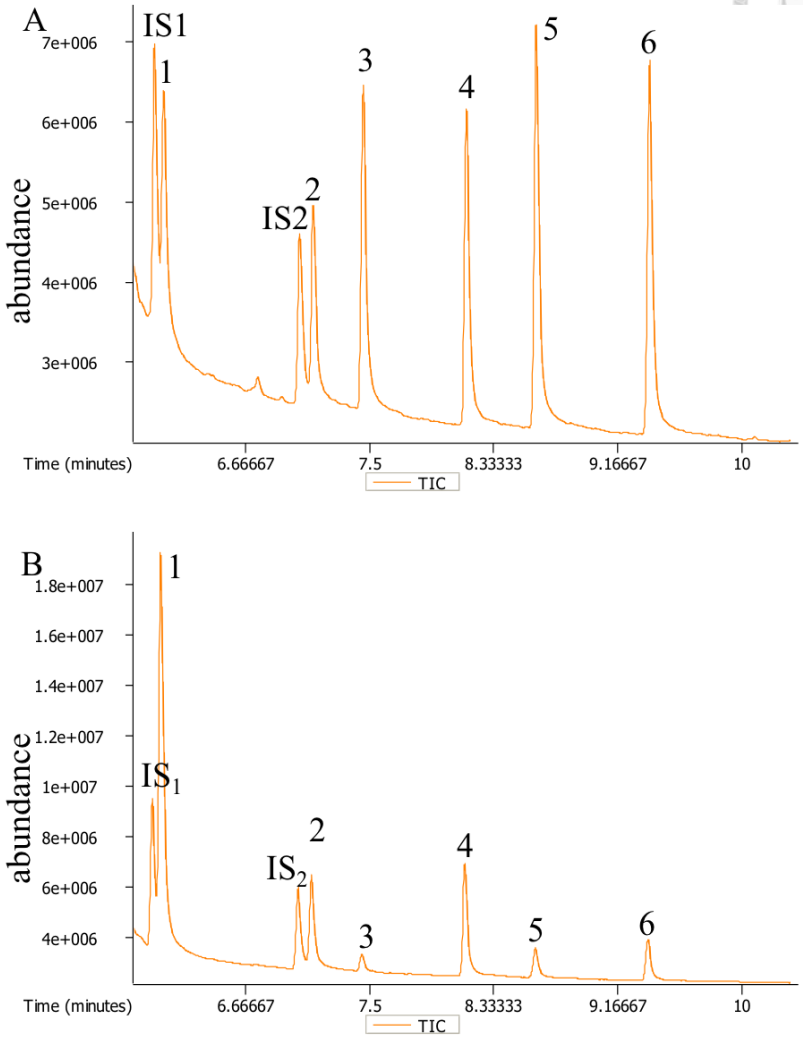

Figure 1. Total ion chromatogram of standard mixture of six SCFAs (A) and a human fecal extract (B). Concentration of standards and ISs are 50 μg ml^{-1}. Peak IS_1, sodium acetate-d3; peak 1, acetic acid; peak IS_2, sodium propionate-d5 ; peak 2, propionic acid; peak 3, isobutyric acid; peak 4, butyric acid; peak 5, isovaleric acid; peak 6, valeric acid.

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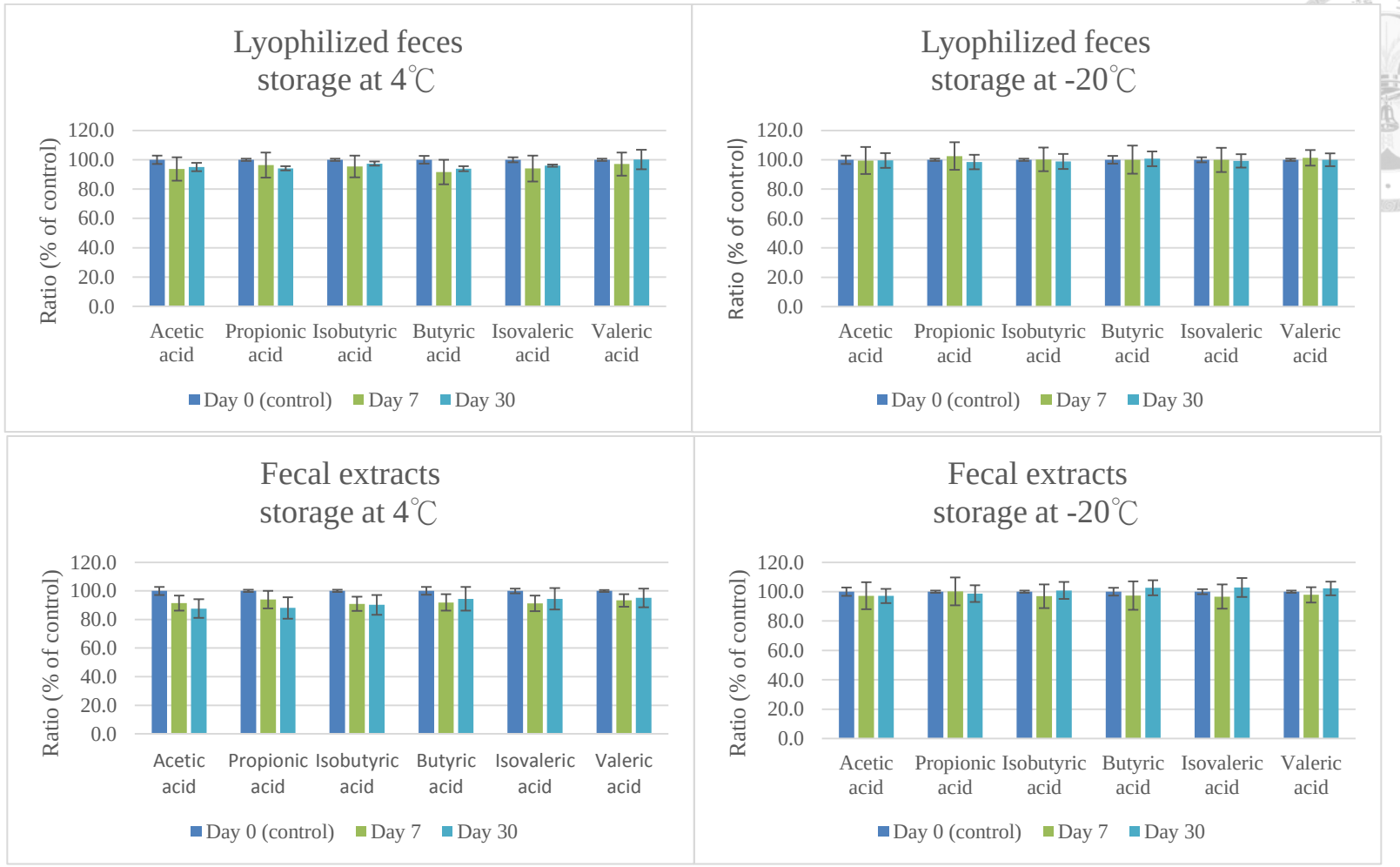

Figure 2. The stability of SCFAs in lyophilized feces *(top)* and fecal extracts *(bottom)* under 4℃ *(left)* and -20℃ *(right)* storage within 30 days. Data were normalized to the concentration of control samples and expressed as percentage relative to the control samples. The error bars represent the standard deviation of independent triplicates.

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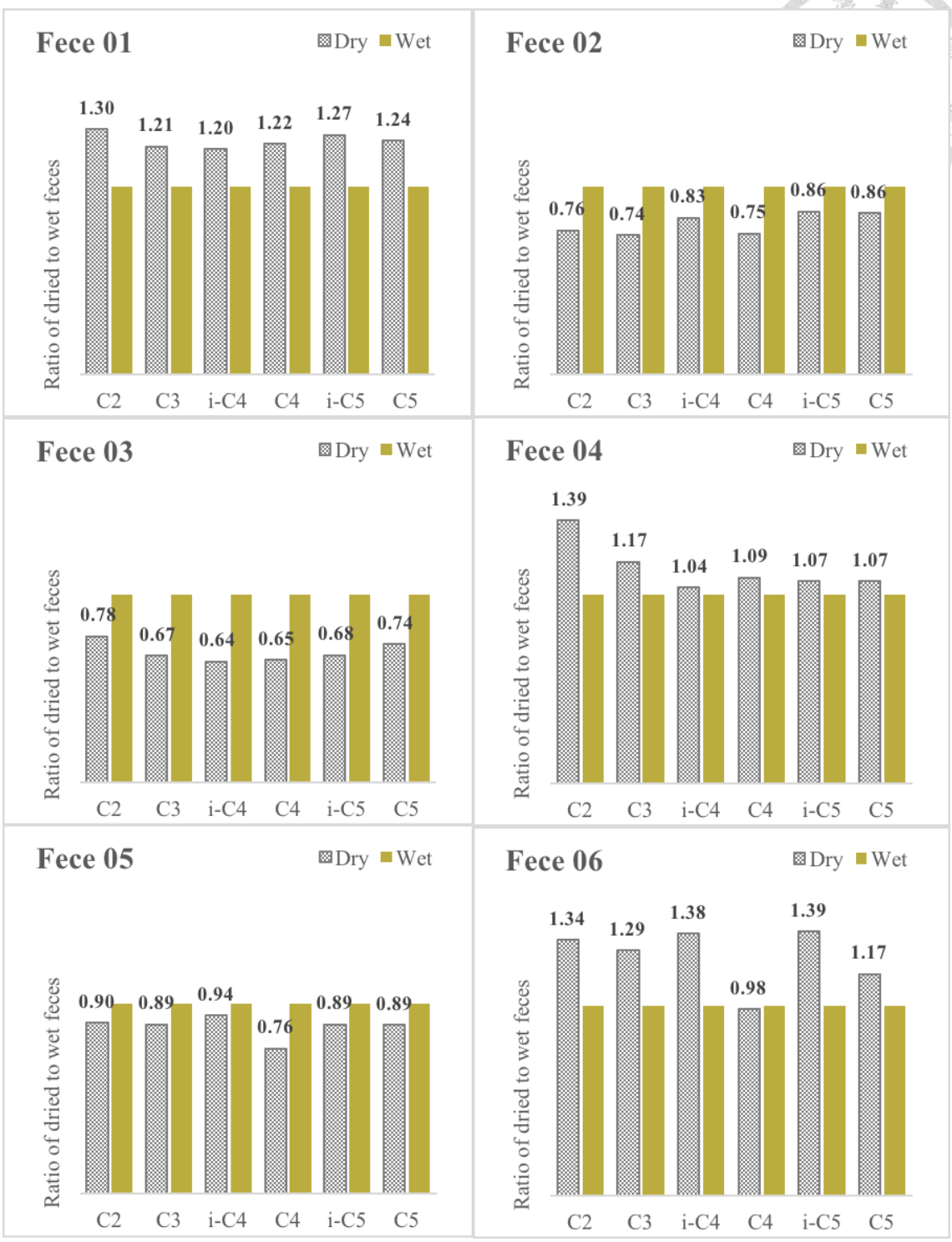

Figure 3. The normalized abundance of six SCFAs in wet and lyophilized fecal samples, n = 6. C2 = acetic acid, C3 = propionic acid, C4 = butyric acid, i-C4 = isobutyric acid, i-C5 = isovaleric acid, C5 = valeric acid.

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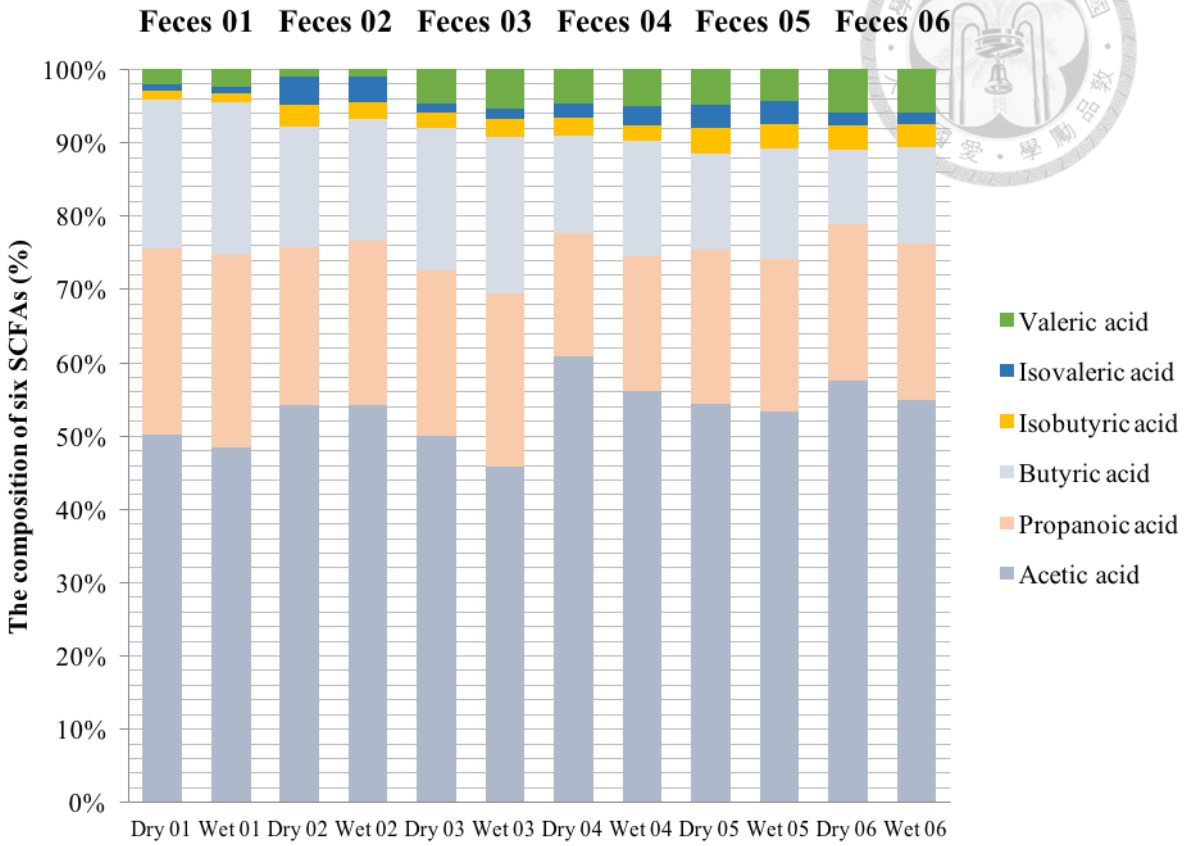

Figure 4. Composition analysis for fecal samples processed with or without lyophilization.

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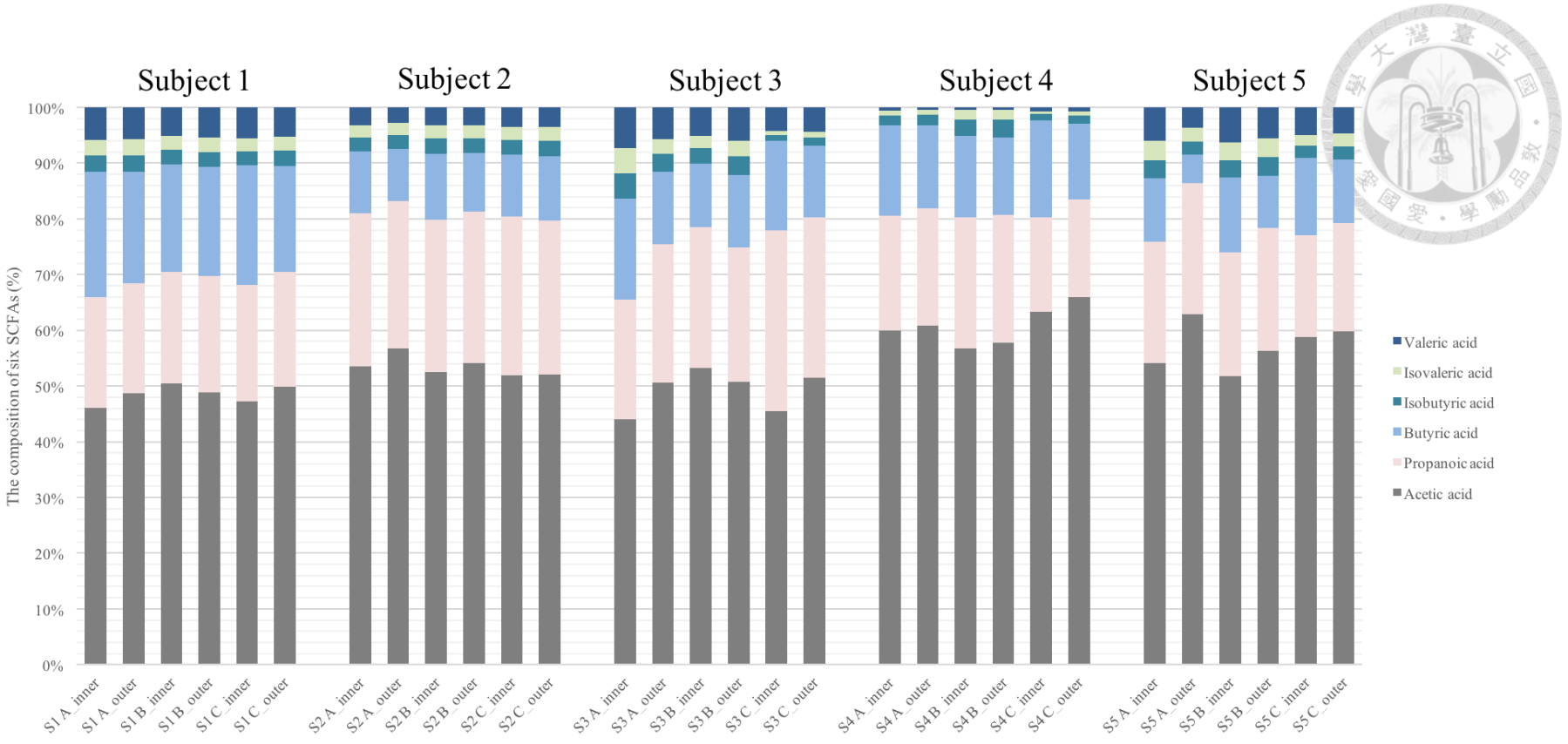

Figure 5. The composition of six SCFAs in human fecal samples obtained from inner and outer layers of three different locations (n = 5). The bottom axis, S1, refers to the subject 1, while A, C, and B refer to the samples collected from both ends and the middle of the original fecal sample, respectively.

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6. Table

Table 1. The extraction recoveries of the six SCFAs (n = 3). Recoveries were evaluated by pooled human fecal extracts spiked with three different amounts of standard mixture of SCFAs.

Compound	Single extraction		Double extraction	
	Concentration ($\mu g\ ml^{-1}$)	Recovery (%)*	Concentration ($\mu g\ ml^{-1}$)	Recovery (%)*
Acetic acid (C2)	20 (4 μg)	86.7 ± 0.9	20 (5 μg)	91.6 ± 0.8
	100 (20 μg)	73.9 ± 1.4	100 (25 μg)	83.1 ± 1.8
	300 (60 μg)	63.2 ± 0.9	300 (75 μg)	73.2 ± 1.1
Propionic acid (C3)	20 (4 μg)	91.8 ± 0.9	20 (5 μg)	88.0 ± 0.3
	100 (20 μg)	88.5 ± 1.0	100 (25 μg)	81.8 ± 2.9
	300 (60 μg)	73.1 ± 0.8	300 (75 μg)	75.1 ± 2.2
Isobutyric acid (i-C4)	2 (0.4 μg)	95.5 ± 0.9	2 (0.5 μg)	96.6 ± 1.7
	10 (2 μg)	90.8 ± 0.7	10 (2.5 μg)	90.4 ± 2.4
	30 (6 μg)	87.9 ± 1.0	30 (7.5 μg)	77.0 ± 2.6
Butyric acid (C4)	20 (4 μg)	93.9 ± 0.4	20 (5 μg)	94.1 ± 0.3
	100 (20 μg)	93.1 ± 2.0	100 (25 μg)	90.7 ± 3.4
	300 (60 μg)	82.1 ± 1.5	300 (75 μg)	75.6 ± 2.5
Isovaleric acid (i-C5)	2 (0.4 μg)	94.2 ± 1.0	2 (0.5 μg)	97.9 ± 0.6
	10 (2 μg)	89.5 ± 1.6	10 (2.5 μg)	93.2 ± 3.4
	30 (6 μg)	83.5 ± 1.7	30 (7.5 μg)	78.2 ± 2.6
Valeric acid (C5)	2 (0.4 μg)	97.4 ± 0.2	2 (0.5 μg)	98.6 ± 1.1
	10 (2 μg)	91.6 ± 3.1	10 (2.5 μg)	92.6 ± 2.7
	30 (6 μg)	85.8 ± 1.2	30 (7.5 μg)	79.7 ± 3.4

*Mean ± SD

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Table 2. The calibration curve, repeatability (n = 3), intermediate precision (n = 9), and accuracy of six SCFAs.

Compound	Target ion	Linearity			Concentration ($\mu g\ ml^{-1}$)	Repeatability (n = 3, RSD %)	Intermediate Precision (n = 9, RSD %)	Accuracy* (%)
		Range ($\mu g\ ml^{-1}$)	Calibration curve	R^2				
Acetic acid	60	10–300	y = 0.0158x - 0.0565	0.9998	20	2.8	4.9	110.0 ± 4.1
					100	3.6	4.7	92.9 ± 4.0
					300	3.2	2.7	96.7 ± 2.7
Propionic acid	74	10–300	y = 0.0231x - 0.0976	0.9992	20	2.9	2.9	103.2 ± 2.4
					100	6.1	6.1	88.0 ± 5.1
					300	2.4	2.2	100.9 ± 2.3
Isobutyric acid	73	1–50	y = 0.0244x - 0.0029	0.9986	2	3.0	8.5	100.2 ± 7.1
					10	7.0	8.3	93.3 ± 7.3
					30	2.5	6.0	102.9 ± 5.4
Butyric acid	60	20–300	y = 0.0752x - 0.3467	0.9997	20	2.8	4.7	110.5 ± 3.8
					100	7.8	6.4	93.8 ± 6.1
					300	2.9	3.0	110.9 ± 3.3
Isovaleric acid	60	1–50	y = 0.0741x - 0.0161	0.9993	2	2.5	8.5	102.3 ± 6.8
					10	7.2	8.9	96.2 ± 8.0
					30	3.1	6.8	109.4 ± 6.6
Valeric acid	60	1–50	y = 0.0748x - 0.0296	0.9979	2	4.0	9.7	101.9 ± 7.1
					10	7.1	8.1	87.9 ± 6.6
					30	3.1	6.5	98.1 ± 5.6

*Mean ± SD

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Table 3. The 24 h stability of six SCFAs in pooled human fecal extracts at room temperature. Seven independent samples were left in an autosampler tray and analyzed sequentially within 24 h. C2 = acetic acid, C3 = propionic acid, i-C4 = isobutyric acid, C4 = butyric acid, i-C5 = isovaleric acid, C5 = valeric acid. Results are expressed as mean concentration ± standard deviation (SD) (μg ml^{-1}).

Compound	Concentration (μg ml^{-1})							Average	RSD (%)
	Time 0 h	4 h	8 h	12 h	16 h	20 h	22 h		
C2	157.1 ± 1.6	164.7 ± 0.9	173.7 ± 2.0	169.2 ± 1.5	172.2 ± 1.1	167.5 ± 0.7	168.2 ± 1.1	167.5 ± 5.5	3.3
C3	65.6 ± 0.1	68.5 ± 0.2	70.5 ± 0.1	69.9 ± 0.5	71 ± 0.5	69.4 ± 0.1	69.8 ± 0.6	69.2 ± 1.8	2.6
i-C4	7.1 ± 0.1	7.5 ± 0.0	7.6 ± 0.1	7.5 ± 0.1	7.8 ± 0.1	7.6 ± 0.1	7.6 ± 0.1	7.5 ± 0.2	2.7
C4	70 ± 0.2	73.9 ± 0.2	77.3 ± 0.5	76.4 ± 0.5	76.6 ± 1.0	74.7 ± 0.2	74.5 ± 0.7	74.8 ± 2.4	3.3
i-C5	6.1 ± 0.0	6.5 ± 0.0	6.7 ± 0.0	6.5 ± 0.0	6.7 ± 0.0	6.5 ± 0.0	6.5 ± 0.1	6.5 ± 0.2	3.0
C5	2.4 ± 0.0	2.5 ± 0.0	2.5 ± 0.1	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	2.5 ± 0.0	1.8

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Table 4. The stability of six SCFAs in lyophilized feces and fecal extracts stored at 4℃ and -20℃. The concentrations of SCFAs were evaluated at day 0 (control samples), day 7, and day 30. The data are expressed as mean concentration ± standard deviation (SD) (μg ml^{-1}) (n = 3).

Lyophilized feces at 4℃ storage				
Compound	Control	Day 7	Day 30	RSD (%)
Acetic acid	183.0 ± 5.1	171.6 ± 14.6	174.0 ± 5.2	3.4
Propionic acid	77.7 ± 0.6	75.0 ± 6.6	73.2 ± 1.1	3.0
Isobutyric acid	8.1 ± 0.1	7.7 ± 0.6	7.9 ± 0.1	2.4
Butyric acid	88.1 ± 2.4	80.7 ± 7.4	82.7 ± 1.5	4.6
Isovaleric acid	5.7 ± 0.1	5.4 ± 0.5	5.5 ± 0.0	3.1
Valeric acid	2.5 ± 0.0	2.5 ± 0.2	2.6 ± 0.2	1.8
Fecal extracts at 4℃ storage				
Compound	Control	Day 7	Day 30	RSD (%)
Acetic acid	183.0 ± 5.1	167.5 ± 9.6	160.4 ± 11.9	6.8
Propionic acid	77.7 ± 0.6	73.0 ± 4.8	68.5 ± 5.8	6.4
Isobutyric acid	8.1 ± 0.1	7.4 ± 0.4	7.3 ± 0.6	5.8
Butyric acid	88.1 ± 2.4	80.9 ± 5.1	83.2 ± 7.3	4.4
Isovaleric acid	5.7 ± 0.1	5.2 ± 0.3	5.4 ± 0.4	4.6
Valeric acid	2.5 ± 0.0	2.4 ± 0.1	2.4 ± 0.2	3.6
Lyophilized feces at -20℃ storage				
Compound	Control	Day 7	Day 30	RSD (%)
Acetic acid	183.0 ± 5.1	182.0 ± 16.8	182.3 ± 9.2	0.3
Propionic acid	77.7 ± 0.6	79.7 ± 7.4	76.6 ± 3.8	2.0
Isobutyric acid	8.1 ± 0.1	8.1 ± 0.7	8.0 ± 0.4	0.7
Butyric acid	88.1 ± 2.4	88.1 ± 8.5	88.7 ± 4.4	0.4
Isovaleric acid	5.7 ± 0.1	5.7 ± 0.5	5.6 ± 0.3	0.4
Valeric acid	2.5 ± 0.0	2.6 ± 0.1	2.5 ± 0.1	0.8
Fecal extracts at -20℃ storage				
Compound	Control	Day 7	Day 30	RSD (%)
Acetic acid	183.0 ± 5.1	177.8 ± 2.9	177.8 ± 8.9	1.7
Propionic acid	77.7 ± 0.6	77.9 ± 1.3	76.7 ± 4.4	0.8
Isobutyric acid	8.1 ± 0.1	7.9 ± 0.1	8.2 ± 0.5	2.1
Butyric acid	88.1 ± 2.4	85.7 ± 1.8	90.4 ± 4.4	2.7
Isovaleric acid	5.7 ± 0.1	5.5 ± 0.1	5.9 ± 0.4	3.1
Valeric acid	2.5 ± 0.0	2.5 ± 0.1	2.6 ± 0.1	2.2

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Table 5. The concentration (μg/ml) of six SCFAs in human fecal samples collected from the inner and outer layers of three different locations (n = 5).

Feces S1								
Compound	A inner	A outer	B inner	B outer	C inner	C outer	Mean ± SD	RSD (%)
Acetic acid	155.5	178.1	176.7	143.6	146.3	154.3	159.1 ± 14.9	9.4
Propanoic acid	67.3	71.9	70.1	61.5	65.0	63.3	66.5 ± 4.0	6.0
Isobutyric acid	9.9	11.1	9.4	8.1	7.9	8.4	9.1 ± 1.3	13.8
Butyric acid	76.0	73.1	67.3	57.2	66.7	58.9	66.5 ± 7.5	11.2
Isovaleric acid	9.4	10.3	8.5	7.4	7.3	7.8	8.4 ± 1.2	14.3
Valeric acid	19.8	21.0	18.0	16.0	17.0	16.3	18.0 ± 2.0	11.2
Feces S2								
Compound	A inner	A outer	B inner	B outer	C inner	C outer	Mean ± SD	RSD (%)
Acetic acid	130.9	127.0	148.1	137.4	105.1	122.5	128.5 ± 14.5	11.3
Propanoic acid	67.5	59.2	77.4	69.0	57.7	64.9	65.9 ± 7.2	10.9
Isobutyric acid	6.2	5.8	7.5	6.7	5.3	6.7	64.0 ± 0.8	12.2
Butyric acid	26.9	20.7	33.7	26.6	22.5	27.3	26.3 ± 4.5	17.1
Isovaleric acid	5.5	4.9	6.6	6.1	4.8	5.9	5.6 ± 0.7	12.4
Valeric acid	7.7	6.1	9.3	8.1	7.1	8.1	7.7 ± 1.1	14.0
Feces S3								
Compound	A inner	A outer	B inner	B outer	C inner	C outer	Mean ± SD	RSD (%)
Acetic acid	148.9	117.9	131.8	129.0	215.3	144.4	147.9 ± 34.9	23.6
Propanoic acid	72.8	57.9	62.7	61.5	153.3	81.0	81.5 ± 36.2	44.4
Isobutyric acid	15.5	7.3	7.0	8.6	5.3	4.1	8.0 ± 4.0	50.4
Butyric acid	61.0	30.4	28.3	32.9	75.7	35.9	44.0 ± 19.6	44.4
Isovaleric acid	14.9	6.1	5.4	7.3	3.2	2.9	6.6 ± 4.4	66.3
Valeric acid	24.8	13.4	12.6	15.1	19.9	12.3	16.4 ± 5.0	30.7

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Feces S4

Compound	A inner	A outer	B inner	B outer	C inner	C outer	Mean ± SD	RSD (%)
Acetic acid	208.5	172.2	151.8	167.6	267.7	210	196.3 ± 42.0	21.4
Propanoic acid	71.7	59.3	63	66.4	71.7	55.8	64.7 ± 6.5	10.1
Isobutyric acid	6.1	5.5	7.8	9.1	5.1	4.5	6.3 ± 1.8	27.9
Butyric acid	56.8	41.9	39.2	40.3	73.8	43.4	49.2 ± 13.6	27.6
Isovaleric acid	2.9	2.5	4.8	5.2	2	2.1	3.3 ± 1.4	42.7
Valeric acid	2	1.3	1	1.2	2.8	2.5	1.8 ± 0.7	41.1

Feces S5

Compound	A inner	A outer	B inner	B outer	C inner	C outer	Mean ± SD	RSD (%)
Acetic acid	185.2	127.3	129.6	167.9	181.6	177.8	161.6 ± 26.3	16.3
Propanoic acid	74.5	47.8	55.5	65.8	56.4	57.7	59.6 ± 9.3	15.5
Isobutyric acid	11.2	4.8	7.7	9.7	6.4	6.9	7.8 ± 2.3	29.7
Butyric acid	38.9	10.4	33.5	28	43.2	34	31.3 ± 11.5	36.6
Isovaleric acid	11.6	4.9	8	10.3	6.2	6.7	8.0 ± 2.6	32.3
Valeric acid	20.7	7.4	15.8	16.4	15.1	14	14.9 ± 4.3	28.9

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Part II:

Using liquid chromatography-mass spectrometry in combination with PCI-IS strategy to analyze biogenic amine in urine and its application to investigating chemotherapeutic response to breast cancer patient

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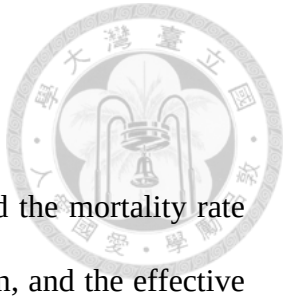

1. Introduction

1.1 Breast cancer and neoadjuvant chemotherapy

In Taiwan, breast cancer is one of the top 10 fatal cancers, and the mortality rate was 12.0 per 100,000 people in 2015 [42]. Screening, early detection, and the effective therapies are crucial to predict outcome and improve prognosis of breast cancer. According to American Cancer Society (ACS) guideline, women in their 40s were recommended for having regular mammography screening, and which led to a reduction in breast cancer mortality [43]. On the other hand, neoadjuvant chemotherapy is usually used for reducing the tumor size prior to surgery such as breast-conserving surgery or mastectomy [44]. Different combinations of chemotherapy drugs, including anthracyclines, taxanes, 5-fluorouracil, cyclophosphamide, and carboplatin, were given in cycles, and the length of treatment depended on the choice of regimen. For example, a sequential chemotherapy consisted of four cycles (every two weeks) of doxorubicin and cyclophosphamide, followed by weekly paclitaxel for 12 weeks was given to HER2-negative patients [45]. For example, the reported regimen for HER2-negative patients was four cycles (every two weeks) of doxorubicin and cyclophosphamide, then paclitaxel given weekly for 12 weeks [45]. Since a complete chemotherapy can be 3 to 6 months long, a routine assessment of tumor response and indicators for monitoring chemotherapeutic efficacy are necessary during the treatment [46]. In addition, early recognition of poor response or tumor progression contribute to find out each patient's effective treatment more efficiently, and avoid wasting time in ineffective and long processes for treatment.

The change in tumor burden has been used as the standard criteria for evaluating the response to treatment, especially in solid tumors such as breast cancer [47]. Imaging exams including mammograms, breast ultrasound, and breast magnetic resonance

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imaging (MRI) are common methods to monitor the response to chemotherapy [48]. Although it has been demonstrated the superiority of MRI in tumor size measurement as compared to the mammography and sonography [49], the MRI are relatively expensive [50]. Therefore, other effective methods to assess chemotherapeutic response are still required to be developed. Among the potential strategies, monitoring biomarkers in biofluids is a promising one.

1.2 Using Metabolomics approach for breast cancer research

Metabolomics refers to the comprehensive and quantitative analysis of the metabolic products of a biological system at a specific time [51]. As the end products that are produced from multiple cellular processes, the levels of metabolites can reflect the system status and the corresponding genetic or environmental changes [52, 53]. By measuring the altered metabolic profiles, the metabolomics approach has been applied in many aspects, including drug development, toxicity assessment, and exploring biomarker for diagnosis and therapeutic evaluation of various diseases [54-56]. Moreover, analyzing different metabolites such as amino acid and polyamines has revealed their potential as predictive markers in early detection of breast cancer and chemotherapeutic response [57, 58].

1.3 The function of biogenic amines

Biogenic amines, a group of amine-containing biogenic compounds, include polyamines, tryptamine, and neurotransmitters such as catecholamines, serotonin, and histamine [59, 60]. The three catecholamines, dopamine, norepinephrine, and epinephrine are composed of a catechol moiety and a side-chain amine (Figure 6, p. 49), and act as neurotransmitters and hormones for regulating a wide range of physiological functions [61]. Measuring the catecholamines and their metabolites in urine and plasma

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has been applied in diagnosis of pheochromocytoma, a tumor characterized by overproduction of catecholamines and symptom of hypertension [62, 63].

Polyamines are polycationic alkylamines that are derived from the decarboxylation of the relevant amino acid (Figure 6, p. 49). The three polyamines—putrescine, spermidine, and spermine are ubiquitous and essential components for cell growth and development. Under normal physiological conditions, these polycations could bind with negatively charged macromolecules such as DNA, RNA, proteins, and the acidic sites on cell membranes, indicating their multiple functions in the living organism. These functions include the maintenance of membrane stability, protection from oxidative stress, regulation of ion-channels, as well as regulation of specific gene expression [64-67]. Owing to their important physiological roles, their levels are highly regulated during biosynthesis, catabolism, and transport [68]. The polyamine metabolic pathway is shown in Figure 7 (p. 50), putrescine produces spermidine and then converts to spermine by a series of enzymes, further, the acetylation sequentially occurs to reduce the positive charges on these polyamines. After that, the acetylated derivatives are eliminated by excretion [65]. It was reported the monoacetylated form of polyamines accounted for more than 90% of the total polyamines excreted in human urine, followed by the free polyamines, and the diacetylated form in the decreasing order [69]. Since Russell [70] *et al.* reported in 1971 that the elevated levels of polyamines, including putrescine, spermidine, and spermine, were found in urine samples from cancer patients compared to healthy individuals. The levels of urinary polyamines have been proposed as potential diagnostic biomarkers in many cancers, including prostate, breast, and colon cancer [71-73].

Considering the roles of these biogenic amines in biological functions and the association with cancer cell proliferation, it is important to develop an analytical

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platform for simultaneous determination of these amines. We selected 14 biogenic amines as target analytes in our study based on the published analytical methods [74-76] and their relevance to breast cancer. These biogenic amines included the three catecholamines—dopamine (DA), norepinephrine (NE), and epinephrine (EPI); histamine (HIS); tryptamine (TRP); five free form polyamines—1,3-diaminopropane (DAP), cadaverine (CAD), putrescine (PUT), spermidine (SPD), and spermine (SPM); two monoacetylated polyamines—*N*-acetylputrescine (NAP) and N^1-acetylspermine (N1-SPM) as representative by considering their clinical roles; and two diacetylated polyamines—N^1,N^8-diacetylspermidine (N1N8) and N^1,N^{12}-diacetylspermine (N1N12).

1.4 Analytical method for biogenic amines

Recently, liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) has become an important tool for determining endogenous metabolites in biological samples due to its higher sensitivity and specificity. Because catecholamines and polyamines are small and polar molecules that are poorly retained on reverse-phase (RP) column, different approaches such as hydrophilic interaction (HILIC) chromatography [77] and ion-pairing reagents [74, 75] have been applied for chromatographic separation and retention of these biogenic amines. However, these approaches have some disadvantages, for example, distortion of peak shape was often shown in HILIC compared to RPLC [78]. Also, continuously introducing the ion-pair reagents into mass spectrometry may cause contamination in system, and require additional cleaning procedures [79]. In addition, these biogenic amines are usually present in low concentration in biological fluids [80], and therefore proper sample preparation such as derivatization [81-83], solid-phase extraction (SPE) [75, 84], and microextraction in packed syringe [85] are incorporated to improve the detection sensitivity and pre-concentrate the trace amounts of analytes, respectively. Dansyl

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chloride (DnsCl), a chemical derivatization reagent, is one of commonly used methods for labeling amine- and phenol-containing metabolites [86]. The reaction scheme for dansylation is shown in Figure 8 (p. 51). To enhance the detection sensitivity while analyzing by ESI MS, the dansylation was based on the introduction of a more easily protonated dimethylamino group and a hydrophobic naphthalene moiety into an analyte and thus resulting in a higher ionization efficiency. It has been reported that the dansylation could enhance 10- to 1000-fold of the ESI signal of metabolites including amino acids [87]. Moreover, the improved separation and retention of these polar biogenic amines on RPLC column was achieved after derivatization with dansyl chloride.

When using LC-MS to analyze biogenic amines in biological samples, matrix effects (MEs) may affect quantification accuracy. [88]. Matrix effects result from co-eluting matrix components, and they can affect the efficiency of the ionization process in the mass spectrometer, causing ion suppression or enhancement of the analyte of interest, thus result in quantification errors [89]. The use of stable isotope labeled internal standard (SIL-IS) is effective to adjust MEs since they are structurally and chemically similar to the target analyte [90]. Unfortunately, SIL-IS is generally expensive, and only a limited number of commercial target analytes are available. To overcome MEs, several strategies have been developed, for example, Guo and Li *et al.* described a differential isotope labeling method using ^{13}C and ^{12}C-dansylation to introduce differential isotope-tagged analytes for quantitative urinary metabolome analysis [87]. The peak pairs of $^{13}C/^{12}C$ labeled analytes are co-eluted and thus encountered similar MEs, providing higher accuracy and precision. Instead of adding an IS during sample preparation, the postcolumn infused-internal standard (PCI-IS) method could be used for measuring the MEs at each time point by continuous

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postcolumn infusion of an IS, and the MEs were calibrated by dividing the signal intensity of target analyte by that of PCI-IS [88, 91]. Besides, using a structural analog or a single SIL-IS to calibrated multiple analytes in the PCI-IS method can provide a more economical way to improve the quantification accuracy [88]. The scheme of PCI-IS system is shown in Figure 9 (p. 52).

1.5 Specific aims

In this study, we developed a LC-MS method based on the PCI-IS strategy to analyze fourteen biogenic amines simultaneously in urine samples. We additionally applied this platform to samples collected from breast cancer patients to investigate the association between the changes of these biogenic amines and the therapeutic response in the early course of chemotherapy.

2. Material and Methods

2.1 Chemicals

Amine standards, including spermine, spermidine, dopamine hydrochloride, (±)-epinephrine, (-)-norepinephrine, histamine, *N*-acetylputrescine hydrochloride, tryptamine, 1,3-diaminopropane and cadaverine were purchased from Sigma-Aldrich (St. Louis, MO, USA). N^1-acetylspermine n-hydrochloride was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N^1, N^{12}-diacetylspermine dihydrochloride, N^1, N^8- diacetylspermidine and N^1, N^8-diacetylspermidine-d6 were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Putrescine and the derivatization agent, dansyl chloride (DnsCl) (98%), were purchased from Acros Organics (Fair Lawn, NJ, USA). The isotope-labeled internal standards of tryptamine-α, α, β, β-d4 HCl (97% D) and putrescine-d8 (98% D) were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Sodium hydrogen carbonate and sodium carbonate anhydrous were purchased from Showa Chemical Industry Co., Ltd. (Tokyo, Japan) and Thermo

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Fisher Scientific (Waltham, MA, USA), respectively. MS-grade formic acid solution (99%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide and LC-MS- grade acetonitrile (ACN) were obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2 UHPLC-MS/MS system

An Agilent 1290 UHPLC system coupled to an Agilent 6460 triple quadrupole system with a Jet Stream electrospray as the ion source was used for the analysis. PCI-IS was diluted into 0.1% FA in ACN at concentration of 2 ng ml^{-1} and introduced into the ESI interface at a flow rate of 0.1 ml/min using an Agilent 1260 quaternary solvent pump (Agilent Technologies, Waldbronn, Germany). The separation was carried out on a Phenomenex Gemini C18 column (2.0 mm x 10 cm, 3 μm particle size, 110 Å pore size) at a flow rate of 0.3 ml/min. The mobile phase was composed of 0.1% formic acid in 5% ACN (solvent A) and 0.1% formic acid in ACN (solvent B). The linear elution gradient was as follows: 0–1 min, 45% B; 1–2 min, 45–60% B; 2–8 min, 60–90% B; 8–9.5 min, held at 90% B; 9.5–9.6 min, 90–45% B;9.6–11.6 min, held at 45% B for column re-equilibration. Due to high salt concentration after dansylation, the eluent was diverted to waste in first minute to avoid contamination of MS ionization source. The sample injection volume was 10 μl. The sample reservoir and column oven were maintained at 4℃ and 40℃, respectively. The MS system was operated in positive electrospray ionization mode and parameters were set as follows: drying gas temperature 325℃, drying gas flow rate 10 L/min, nebulizer pressure 35 psi, sheath gas temperature 350℃, sheath gas flow rate 10 L/min, capillary voltage 4000 V, and nozzle voltage 1000 V. MS acquisition was executed in multiple reaction monitoring (MRM) mode and the optimized conditions are summarized in Table 6 (p. 61).

2.3 HPLC-UV system

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The HPLC system consisted of L-2130 quaternary solvent pump, L-2200 auto-sampler, L-2420 UV-VIS detector, and data processing system (HITACHI, Tokyo, Japan) was used to evaluate the stability of PCI-IS after derivatization. The chromatographic separation was achieved on a Phenomenex Luna C18 column (4.6 mm x 250 mm, 5 μm particle size, 110 Å pore size) with a C18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase was composed of 0.1% formic acid in DI (solvent A) and 0.1% formic acid in ACN (solvent B) at a flow rate of 0.5 ml/min. The gradient program was as follows: 0–5 min, 60–70% B; 5–8 min, 70–80% B; 8–24 min, 80–90% B; 24–25 min, 90–95% B; 25–28 min, held at 95% B for complete elution; 28–29 min, 95–60% B; 29–30 min, held at 60% B for column re-equilibration. The sample injection volume was 20 μl. The wavelength of detection was 338 nm [92].

2.4 Dansyl chloride derivatization

The derivatization reaction of amines with DnsCl was carried out according to the method previously described by Zhou and Li *et al.* with some modifications [93]. Briefly, the frozen urine sample was thawed and centrifuged at 15,000 rcf for 10 mins. Fifty μl of the urine sample was mixed with 25 μl of 0.5 M $NaHCO_3/Na_2CO_3$ buffer solution and 75 μl of 50 mM DnsCl solution (13.5 mg ml^{-1} in ACN). After vortexing, the mixture was incubated at 60℃ for 60 mins. Then 10 μl of 250 mM NaOH aqueous solution was added to the derivatized sample and incubated at 60℃ for another 10 mins to quench the excess DnsCl. The resulting solution was acidified with 50 μl of 425 mM formic acid solution (in ACN/H2O, 1:1) and filtered using a 0.2-μm PP membrane filters (RC-4, Sartorius, Göttingen, Germany) for LC-MS analysis.

2.5 Preparation of the postcolumn infused-internal standard (PCI-IS)

For preparation of the PCI-IS, 50 μl of the internal standard solution containing N^1, N^8-diacetylspermidine-d6, tryptamine-d4 and putrescine-d8 at a concentration of 100 μg

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ml^{-1} in deionized (DI) water was derivatized with DnsCl without the addition of formic acid solution and subsequently diluted to a final volume of 1 ml with DI. Following derivatization, the mixture was applied to the Oasis HLB cartridge (Waters, MA, USA), which was preconditioned with 1 ml of methanol and 1 ml of DI. The $NaHCO_3/Na_2CO_3$ buffer salts and other interfering substances from the dansylation reaction may affect the MS signal sensitivity, so we used the solid-phase extraction (SPE) with HLB cartridge to remove these interferences. After washing with 1 ml of 10% ACN in DI, the extracts were eluted with 1ml of ACN and then added into 200 ml of 0.1% formic acid in ACN.

2.6 Matrix effect evaluation

Matrix effect is usually assessed by comparing the peak area of standards and standards spiked into biological samples after extraction [94]. Since the dansylation procedure was incorporated into the sample preparation, an alternative strategy was used to determine the degree of matrix effect under the chromatographic condition. A blank urine, a standard solution containing 14 target amines (1μg ml^{-1}), and a reagent blank sample (DI) were labeled by DnsCl as mentioned in section 2.4. After derivatization, these samples were mixed with each other at equal amounts (e.g., the post-spiked sample is composed of 50 μl of the dansylated urine blank and 50 μl of the dansylated standard mixture). The matrix effect was calculated as follows:

$$\text{Matrix effect (\%)} = \frac{(\text{peak area of analytes in post} - \text{spiked sample} - \text{peak area of analytes in urine blank})}{\text{peak area of analytes in standard solution}} \times 100\%$$

2.7 Strategy for selection of PCI-IS

The diversity of urine concentration among individuals contributes to the different degrees of MEs and thus results in signal changes when analyzing different urine samples. To investigate the correction performance of using different compounds as PCI-IS, urine samples obtained from three healthy volunteers were serially diluted at 1,

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2, 5, 10-fold to mimic the diversity of urine concentration. Following dansylation, these urine samples were post-spiked with a high concentration of dansylated standards containing 100 μg ml^{-1} of DA, 10 μg ml^{-1} of NAP and NE, and 1μg ml^{-1} for the remaining 11 target amines in order to neglect the different amounts of these endogenous amines and reach the equal concentration among these samples. To perform correction by using PCI-IS, the peak intensity of the target analyte was divided by the peak intensity of PCI-IS. The ratios and the peak intensity without correction between the 4 different concentrations urine samples were calculated for their relative standard deviations (RSD).

2.8 Urine sample collection and preparation

Urine samples of breast cancer patients were collected from National Taiwan University Hospital during January 2010 to March 2016. The study was approved by the institutional review board of the National Taiwan University Hospital (NTUH REC No: 200809033R), and all the participants have assigned the informed consents. For each subject, urine samples were collected before chemotherapy was initiated and on the day prior to each cycle of chemotherapy. When we received the urine samples, these samples were centrifuged at 2,500 rcf for 15 mins at 4℃, then sodium azide as preservative was added into the supernatant to a final concentration of 10 mM. These samples were separated and stored in 1.5 ml eppendorfs at -80℃ until analysis.

2.9 Study design for predicting response to chemotherapy in BC patients

To predict the response of chemotherapy in the early course of treatment, the levels of the 14 target amines in urine samples obtained from sixty patients with breast cancer were measured. Different regimens of neoadjuvant chemotherapy were given among these patients before surgery, including paclitaxel (Phyxol); docetaxel (Taxotere) plus trastuzumab (Herceptin); Taxotere plus epirubicin (Pharmorubicin); Taxotere plus

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cisplatin; cisplatin plus Pharmorubicin; cisplatin plus vinorelbine (Navelbine); fluorouracil (5-FU); 5-FU plus Navelbine; 5-FU plus Pharmorubicin; cyclophosphamide (Endoxan) plus Pharmorubicin; the combination of Endoxan, Pharmorubicin, and 5-FU; the combination of Endoxan, Pharmorubicin, and Taxotere; the combination of Taxotere, Herceptin, and pertuzumab (Perjeta); the combination of Taxotere, paraplatin (Carboplatin), Herceptin, and Perjeta. Briefly, these patients received approximately four cycles of chemotherapy depending on the regimen, and the urine samples were named based on the timing of collection: before chemotherapy was initiated (pre-C1), on the day before the second cycle (pre-C2), and the third cycle (pre-C3) of chemotherapy. Tumor size measurements were performed by breast ultrasound at baseline, at the end of chemotherapy before surgery, or before changing the regimen. The response to chemotherapy was determined by the diameters of the target lesions and classified into the following categories, "Complete Response (CR)": disappearance of all target lesions, "Partial Response (PR)": at least a 30% reduction in size of the tumor, "Stable Disease (SD)": neither decreasing nor increasing in size of the tumor, and "Progressive Disease (PD)": at least a 20% increase in size of the tumor [47]. For these sixty breast cancer patients, subjects who met the criteria with CR or PR were defined as responder group (n = 43), and those with SD or PD were classified into the nonresponder group (n = 17). The creatinine concentration (mg/dL) of each urine sample was measured in Taipei Institute of Pathology, Taiwan. A total of 180 urine samples were analyzed using the LC-MS method and corrected by PCI-IS.

3. Result and Discussion

3.1 Method development

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3.1.1 Development of the LC-MS/MS method (identification of dansylated amines)

Chemical derivatization with dansyl chloride (DnsCl) was used in this study in order to improve the sensitivity and the separation of the target amines on RP column. As shown in Figure 8 (p. 51), DnsCl can react with compounds containing primary amine, secondary amine, and phenolic hydroxyl group. Labeling a dansyl group resulted in an increase of 233 Da for the corresponding derivative. When the compound contains more than one amine or phenol group, multiple products could be formed after derivatization. Therefore, full MS scan was first performed for each standard amine to find out the predominated derivative of the target amine. Next, we used different collision energies to obtain the two most abundant product ions as quantifier and qualifier ions. The number of dansyl tags and the mass parameters were listed in Table 6 (p. 61). We assumed that the number of dansyl tags should be equal to the sum of the amine and the phenolic groups in a target analyte under the optimal reaction. Take norepinephrine as an example, DnsCl could react with the two phenol groups on the catechol moiety and the primary amine on the side-chain, and thus the tri-dansylated norepinephrine should be the most predominated form. While the numbers of dansyl groups were labeled as expected in most amines, we found that tryptamine and histamine were mono- and di-dansylated, respectively. It has been reported that DnsCl can react with imidazole ring and form *N*,*N*-bis-Dns-histamine at higher pH level [95]. In contrast, the indole ring of tryptamine may not be labeled efficiently [87]. Our observation is consistent with the previous study, where mono-dansylated tryptamine was also selected as the major form [96].

The LC conditions were further optimized and several gradient programs have been tested to shorten the analysis time. Under the optimal conditions, the analysis could be completed within 10 mins. Figure 10 (p. 53) showed the chromatographic

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profiles of the 14 dansylated amines in standard solution and a demonstration of urine sample obtained from a healthy volunteer. Each amine showed good peak shape despite low levels of these urinary endogenous amines under healthy conditions.

3.1.2 Selection of PCI-IS

In this study, we used the PCI-IS strategy to reflect the ME encountered at the retention time of each target analyte and to correct for response variation in MS detection. To evaluate the necessary for using the PCI-IS to minimize analytical bias, we used a mixed urine sample to test the ME for the 14 amines. We observed the MEs occurred over the entire chromatographic run, and the MEs for 14 amines were ranged from 18 to 87 % (details showed in Table 7, p. 62), which reveals it is essential to calibrate the ME caused errors.

A suitable PCI-IS is an analyte that possess a similar ionization ability to the target analytes, in this way, the ME encountered by this PCI-IS can better reflect that of the target analytes. Since the ability of an analyte to generate signals is determined by its physicochemical properties such as the proton affinity, hydrophobicity, and hydrophilicity of the analyte, a structure analog or an SIL-IS is taken into consideration for the selection of potential PCI-IS [88]. Therefore, compounds including DnsCl and three dansylated SIL-IS: putrescine-d8 (PUT-d8), N^1, N^8-diacetylspermidine-d6 (N1N8-d6), and tryptamine-d4 (TRP-d4), were selected to test the correction performance using the PCI-IS strategy for correction the differences in peak intensity resulting from MEs. For the purposes of this experiment, we spiked the same amount of amine standards into four different dilutions (i.e., 1, 2, 5, 10-fold dilutions) of urine samples. The divergence of the target amine signal intensities in four different dilutions of urine was expressed by their relative standard deviation (RSD%) to evaluate the efficiency of using PCI-IS in correcting MEs. Then, we compared the RSD (%) of the

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target amines in the four urine samples before and after correction by PCI-IS. Despite the similar structures between DnsCl and the dansylated amines, poor adjustment was observed after correction by DnsCl. We found that there were unknown interferences from the matrix contribute to the mass transition of DnsCl (270 > 254.9) without introducing the PCI-IS, and their retention times were close to N1N8, NAP, and PUT, which may cause correction error in these target amines. Besides, the retention time of DnsCl was similar to HIS, and thus the residual DnsCl after derivatization may also influence the PCI-IS adjustments.

Because DnsCl showed limited effects on correcting MEs caused signal changes, the dansylated PUT-d8, N1N8-d6, and TRP-d4 were tested for their correction efficiency. These dansylated PCI-IS are not commercially available, and were therefore prepared in house by derivatization with DnsCl. We found that the high level of salts resulting from dansylation caused significant signal suppression of the target amines when introducing these dansylated PCI-IS. Therefore, we additionally used solid-phase extraction (SPE) to remove the salts after dansylation when preparing these PCI-IS. With regard to the correction performance, the peak intensities of the target amines, especially in N1N8, N1N12, TRP, DAP, and PUT exhibited large variations in different dilutions of urine before correction (Table 8, p. 63), and the RSD values decreased after correction, which indicated that using these dansylated SIL-IS as PCI-IS can effectively correct ME caused signal changes in the different concentration of urine samples. Figure 11 (p. 54) demonstrated the use of PCI-IS method for correction the MEs in different dilutions of urine samples. The four urine samples spiked with the same amount of amine standards showed similar peak intensity after correction.

To further evaluate the signal stability of the PCI-IS, we repeatedly analyzed of the same urine sample during an 8-hour period with postcolumn infusion of the three

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dansylated SIL-IS. The divergence of the peak intensities of the 14 amines among the 8 times analyses during an 8-hour period were expressed as their relative standard deviation (RSD%). Before correction, only slight deviations of the peak intensity were observed among a series of analysis (Table 9, p. 64). However, we found that using the dansylated N1N8-d6 as PCI-IS might introduce some bias and thus the RSD values increased after correction (Table 9, p. 64). Moreover, the responses of the dansylated N1N8-d6 remained unstable after continuous infusion of the three PCI-IS for 8 hours compared with the PUT-d6 and TRP-d4 shown in the MRM chromatogram of the three PCI-IS (Figure 12, p. 55).

As dansylated PUT-d6 showed the best signal stability among three dansylated SIL-IS, we tested if it can be used as the single PCI-IS for correcting MEs of 14 target amines. The results indicated that when the dansylated PUT-d6 was used as a single PCI-IS, the RSD values were significantly reduced to less than 15% for most of the target amines (Figure 13, p. 56). Although a slightly higher RSD value was observed in N1N8 after correction, it still showed good performance in adjustment. Therefore, based on the previous correction results and the 8-hr signal stability of the system, the dansylated PUT-d6 was chosen as a single PCI-IS in our study.

Next, the concentration of PCI-IS was optimized. The correction performance was tested at PCI-IS concentrations of 20 ppb, 2 ppb and 200 ppt. Urine sample obtained from a healthy volunteer was repeatedly measured for three times and the signals of 14 amines were adjusted by the PCI-IS prepared at the three different concentrations. The results showed the RSD (%) values between repeated measurements were ranged from 4.9 to 12.1% among the target amines using the PCI-IS at the concentration of 20 ppb, whereas the RSD values were ranged from 0.8 to 6.5% using the PCI-IS at the concentration of 2 ppb. Although the precision obtained by the PCI-IS at the

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concentration of 200 ppt was good, the intensity of the PCI-IS was too low to reflect the MEs. From the MRM chromatograms (Figure 14, p. 57), the signals of the dansylated PUT-d6 among the analyses comparatively fluctuated at the concentration of 20 ppb. Besides, the infusion rate of the PCI-IS was also optimized to provide the most stable chromatogram. Consequently, the flow rate of the post-column infused PCI-IS solution was set at 0.1 ml/min and 2 ppb of the dansylated PUT-d6 was chosen as the final concentration.

3.2 Method validation

3.2.1 Stability of the dansylated PUT-d6 stock solution

Room temperature stability test of the dansylated PUT-d6 was evaluated by LC-UV system. Room temperature stability was evaluated for PCI-IS at the concentration of 5 ppm, and the PCI-IS stored at room temperature was analyzed in triplicate on day 0, 1, 4 and 7. Our results showed the dansylated PUT-d6 was stable for 7 days storage at room temperature (Figure 15, p. 58). This result is consistent with a previous study that the dansylated putrescine in the serum was stable at 4 ℃ for three days [83].

3.2.2 Intra-day precision of the dansylation derivatization

The intra-day precision test for derivatization procedure was evaluated by analyzing urine samples obtained from three BC patients. Each urine sample was derivatized with DnsCl in triplicate. The peak intensities of the 14 target amines were corrected by the peak intensities of the dansylated PUT-d6. The results showed that 79% of the RSD values of the target amines in samples derivatized in the same batch were less than 15% (Table 10, p. 65). We found that the slight fluctuation in the signals of PCI-IS may cause a higher variability in some amines, such as TRP, PUT, and HIS.

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In addition, the dansylation efficiencies between the target amines may also affect the intra-day precision of the urine samples.

3.3 Urinary biogenic amines in breast cancer patients

The developed method was applied to investigate the association between the concentration of urinary biogenic amines and the therapeutic response in breast cancer (BC) patients. Urine samples were collected from sixty BC patients who were undergoing chemotherapy at three time points: before chemotherapy was initiated (pre-C1), on the day before the second cycle (pre-C2), and the third cycle (pre-C3) of chemotherapy. These patients were classified as responders (n = 43) and non-responders (n = 17) based on the tumor size measurements before and after completing a chemotherapy. A total of 180 urine samples were analyzed by the LC-MS/MS to study the concentration changes of the 14 amines after chemotherapy. Since it has been indicated that the presence of high salts in urine samples influenced the chemical labeling, all the urine samples were diluted according to their creatinine concentration prior to LC-MS/MS analysis [97]. The ratios of the target amine intensity to the dansylated PUT-d6 intensity were also normalized by the creatinine concentration to adjust urine concentrations.

Our results revealed that the amounts of N1N12 and N1N8 in the responder group (n = 43) were significantly higher ($P < 0.05$) than the nonresponder group (n = 17) prior to the chemotherapy, while there was no significant difference in the amounts of the remaining target amines between these two groups (Figure 16, p.59–59). The levels of N1N8 were significantly higher ($P < 0.05$) in the responder group compared to the nonresponder group at the three sampling time points before and after treatment. The levels of N1N12 in the responder group significantly decreased ($P < 0.05$) after the chemotherapy at the stages of pre-C2 and pre-C3, on the contrary, only slightly changes

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in levels of N1N12 were observed in the nonresponder group. Our results also showed that there were significant differences ($P < 0.05$) in NAP in the responder group before and after chemotherapy, and the levels of NAP at pre-C2 and pre-C3 were significantly higher than the levels at pre-C1. It was noticed that similar trend that the levels of NAP elevated at pre-C2 and followed by a decrease at pre-C3 was observed in the nonresponder group, but there were no significant differences. Besides, the significant difference ($P < 0.05$) in the levels of NAP between these two groups was observed until these patients received two cycles of chemotherapy (pre-C3).

In this study, we observed that the urine excretion levels of N1N8 and N1N12 were higher in the responder group before the chemotherapy was initiated. Significant changes of N1N12 in response to chemotherapy also only observed in the responder group. Therefore, N1N12 levels in urine shows high potential to serve as a predictive marker for chemotherapeutic response. Patients in our study received diverse chemotherapy regimen, but the changes in N1N12 displayed similar trend regardless of their chemotherapy regimen. It is anticipated that the urinary N1N12 could be served as a universal marker for predicting treatment response. Although the levels of NAP also displayed higher levels compared to the nonresponder group, the difference only being statistically significant after two cycles of chemotherapy (pre-C3). We therefore assumed NAP may not be a sensitive marker compared to N1N12 for predicting treatment response.

Hiramatsu and Sugimoto *et al.* have indiated the diacetylated polyamines, N1N8 and N1N12, in urine may be served as tumor markers [69, 98]. The level of N1N12 and N1N8 were markedly increased in malignant cases such as urogenital cancer patients compared to benign cases [98]. Moreover, it has been reported that the sensitivity of urinary N1N12 (60.2%) for detecting breast cancer was higher than the known tumor

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markers CA15-3 (37.3%) and CEA (37.3%). Its' sensitivity (75.5%) for detecting colon cancers is also more sensitive than CA19-9 (14.1%) and CEA (39.9%) [73]. Little attentions have been paid for its' predicting ability for chemotherapeutic response in previous studies. Our study indicated N1N12 could not only be served as tumor marker, it shows high potential to be used for predicting chemotherapeutic response.

Previous studies have reported that N1N12 seemed to be more related to cancer development than other polyamines [99]. Unlike the high-abundant monoacetylated polyamines such as NAP and N^1-and N^8-acetylspermidine (about 90%) in the urine, the levels of N1N12 and N1N8 were found to be very limited but stable in healthy human urine [69], indicating the amounts of N1N12 and N1N8 were highly regulated. The levels of N1N12 were further found to be elevated in the tumor tissue at early stage of colorectal cancer [100]. Moreover, N1N12 was not reabsorbed in the renal proximal tubules, by contrast, other monoacetylated forms were reabsorbed [101], implied the amount of N1N12 could more directly reflect the elevated amounts in the body. These observations may explain why N1N12 shows higher sensitivity at the early stage in our study.

Compared to diacetylated polyamines, the levels of other polyamines after chemotherapy remained unchanged in both groups. Also, the difference in concentration was not significant between two groups before chemotherapy. Russell [70] *et al.* reported in 1971 that the elevated levels of polyamines, including PUT, SPD, and SPM, were found in urine samples from cancer patients compared to healthy individuals. Researches on the levels of other polyamines, including free polyamines and the monoacetylated form have also been conducted for their association with cancer [69]. These studies have implied that the urinary polyamines could serve as markers for evaluating cancer activity, cancer diagnosis, and prediction of chemotherapeutic

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response [102-105]. However, later studies indicated high levels of urinary polyamines were also observed in benign disorders such as nonmalignant gastrointestinal diseases [106] and benign urogenital diseases [98] raising the doubts of using these free and monoacetylated polyamines as tumor markers. The lacking of significance between responder and nonresponder group for free and monoacetylated polyamines observed in our study additionally revealed the limitation of these polyamines for clinical use.

This study found higher levels of N1N12 and N1N8 in the responder group as compared to the nonresponder group before chemotherapy. It has been indicated that the activity of polyamine's biosynthetic enzymes was increased as growth rate increases and resulted in the increased of polyamine levels [107]. The increased polyamine excretion may be attributed to either the production from the proliferating cancer cells or the release from dead cells due to the replacement of cells in active growing tissues [108]. Although we do not fully understand the underlying mechanism in the higher levels of N1N12 and N1N8 observed in the responder group as compared to the nonresponder group before chemotherapy, we assumed the higher levels of N1N12 and N1N8 excreted in the responder group may be due to the defense mechanism to lower the elevated polyamines by accelerating the acetylation in the cells [109]. Their lower levels in the nonresponder group may imply a dysfunction in the polyamine transport pathway or the interconversion pathway in the nonresponder group. From our results, we also observed there were an increase in the levels of NAP and a decrease in the levels of N1N12 in the responder group after chemotherapy (Figure 7, p. 50). We assumed that the activated polyamine biosynthesis was sensitive to the chemotherapy in the responder group, and results in the accumulation of the upstream metabolites such as NAP that were directly converted from PUT. On the contrary, the downstream metabolites such as N1N12 tended to decrease as a result of depletion in their precursors.

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We concluded that the urinary N1N12 may serve as an indicator for predicting chemotherapeutic response. Further studies are required to confirm our observation and also to set a cutoff value for distinguishing the two groups to increase its clinical values It is also important to elucidate the underlying mechanism of polyamine pathway in cancer development.

4. Conclusions

In this study, we developed an LC-MS/MS method which combined the use of dansyl chloride as the derivatization reagent and the PCI-IS strategy for sensitive and accurate analysis of 14 biogenic amines in human urine. The use of putrescine-d6 (PUT-d6) as a single PCI-IS was economical and effective to calibrate the matrix effects caused errors in urine sample analysis. The established method was applied to investigate the association between the changes of the 14 amines and the chemotherapeutic response in breast cancer patients. Our results revealed the level of N1N12 was significant higher in the responder group prior to chemotherapy, and it was decreased significantly after chemotherapy in the responder group. Although further studies are required to confirm our results, these observations provide a valuable information for future studies in elucidating the roles of polyamines in cancer either for clinical treatment or cancer biology.

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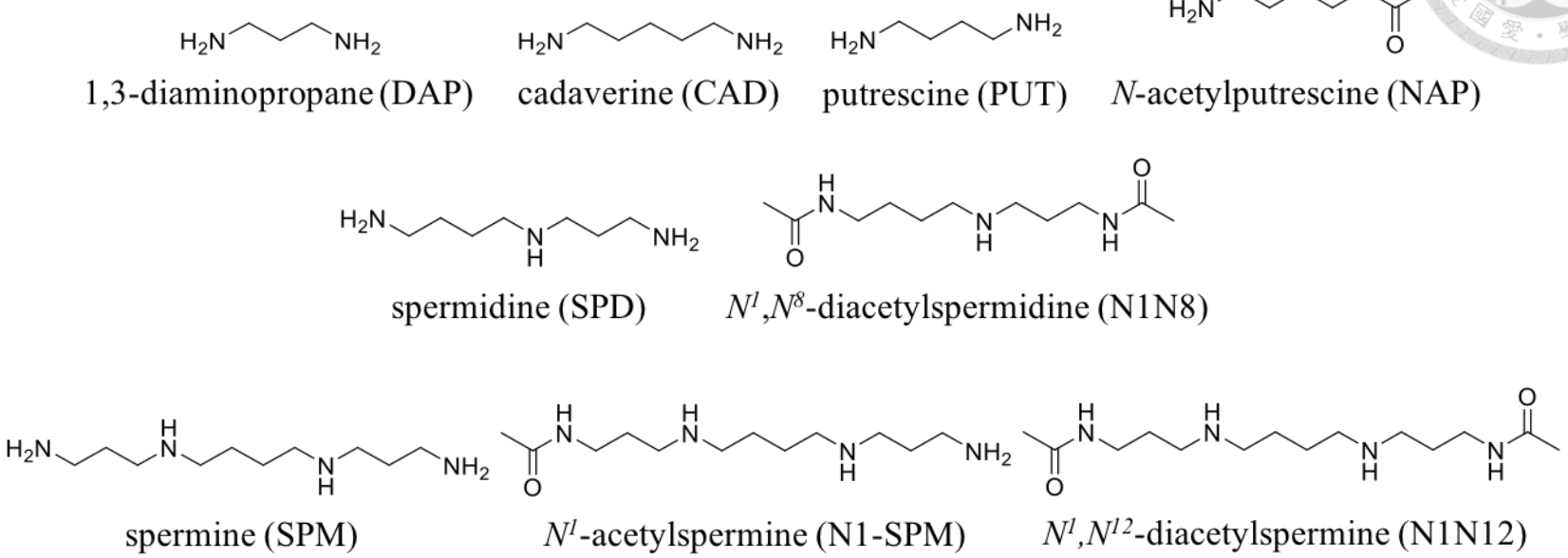
5. Figures

(A) Polyamine



(B) Catecholamine and others

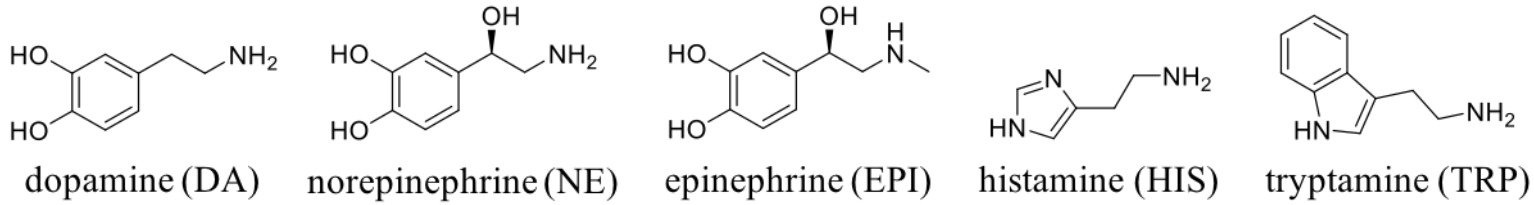


Figure 6. Chemical structures and abbreviations of 14 biogenic amines analyzed in the study.

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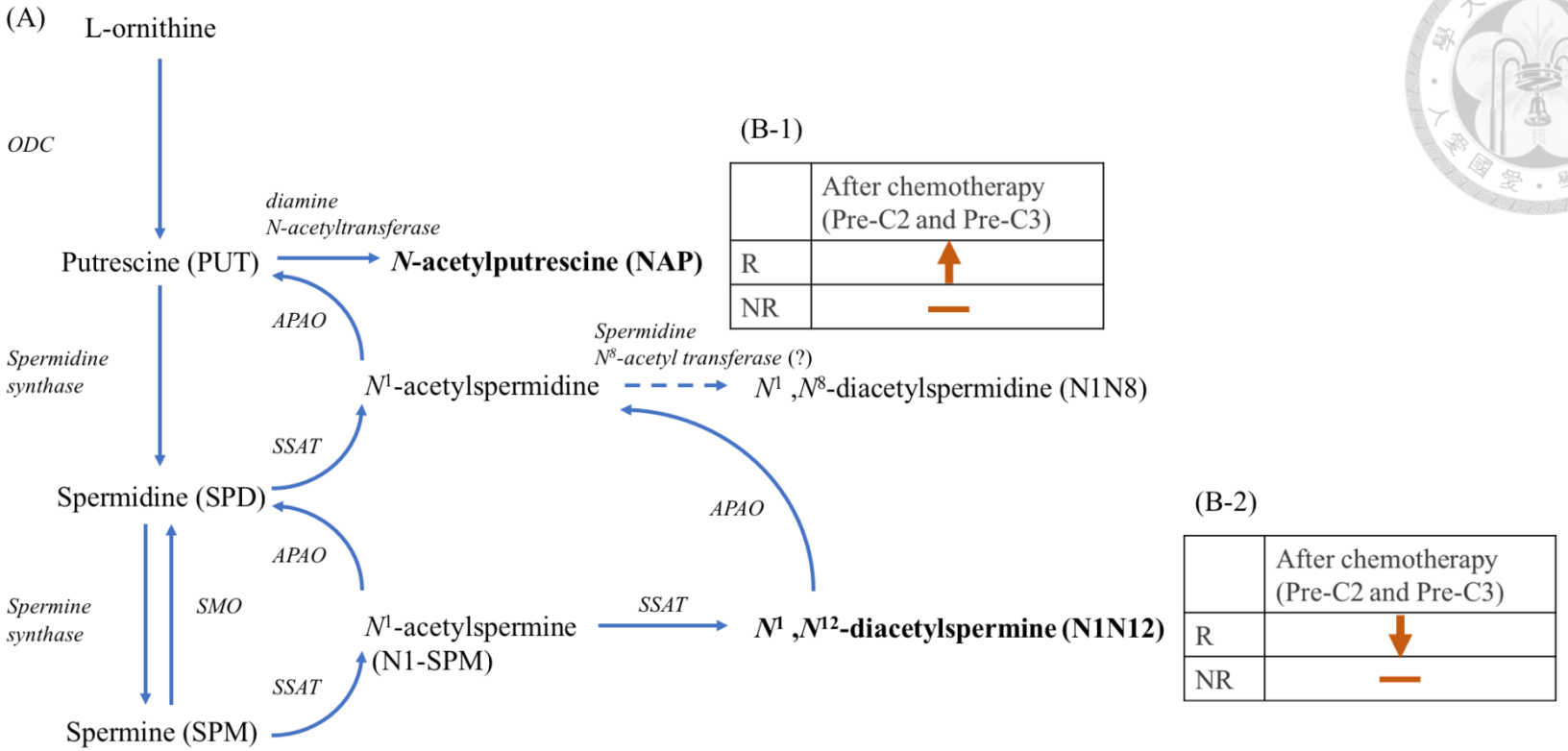

Figure 7. (A) The polyamine biosynthesis and metabolic pathway [64, 65] and (B) the change of *N*-acetylputrescine (NAP) and N^1,N^{12}-diacetylspermine (N1N12) in the responder (R) and nonresponder (NR) group after chemotherapy. ODC, L-ornithine decarboxylase; SMO, spermine oxidase; APAO, N^1-acetylpolyamine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase (SSAT). The biosynthetic pathway of N1N8 is not fully understood [69].

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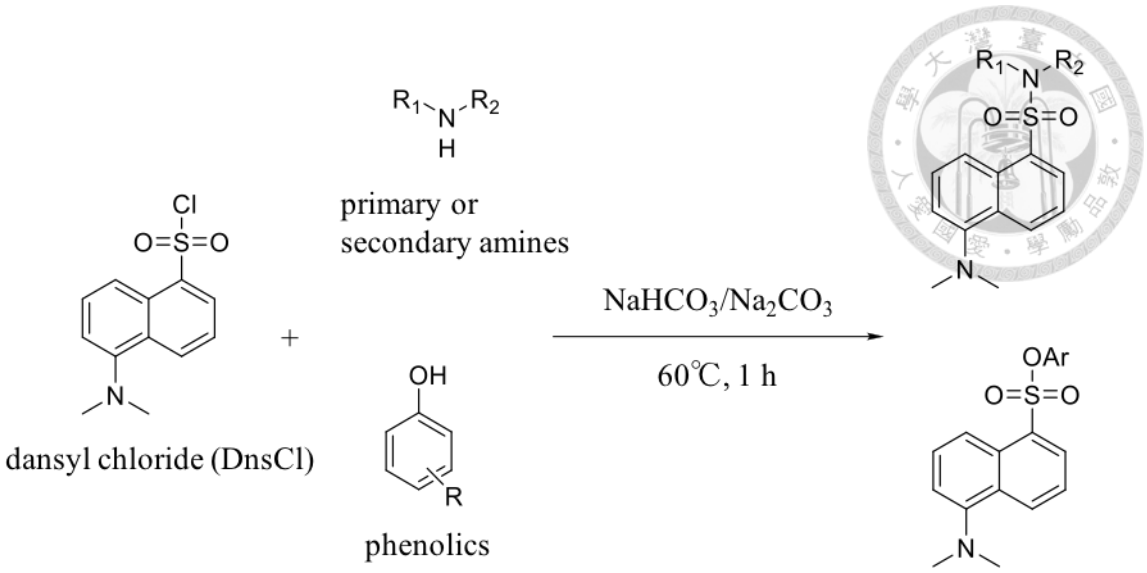

Figure 8. The reaction scheme of dansyl chloride with primary, secondary amines, and phenolic compounds.

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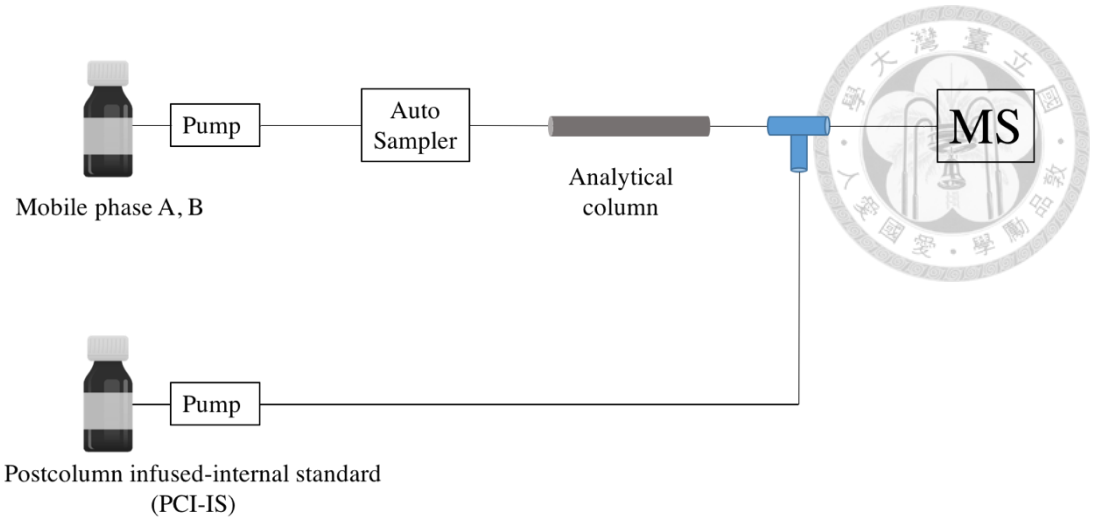

Figure 9. Scheme of the postcolumn infusion system.

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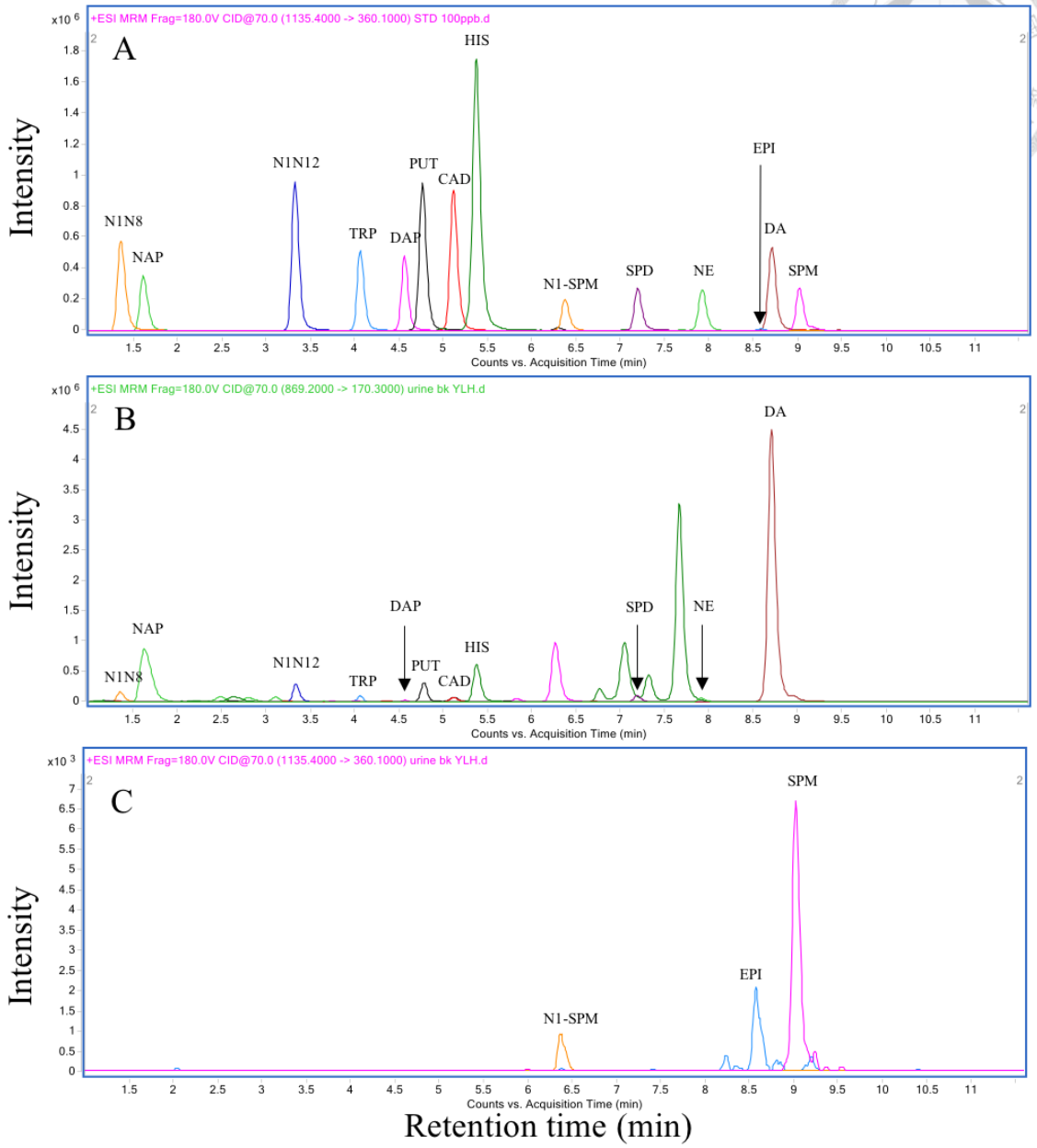

Figure 10. The MRM chromatograms of 14 dansylated amines obtained from standard mixture at 100 ng ml^{-1} (A) and urine sample from a healthy individual (B) (C). Due to large concentration differences between different biogenic amine, the chromatogram of urine sample was separated into figure B and C to express each signal more clearly.

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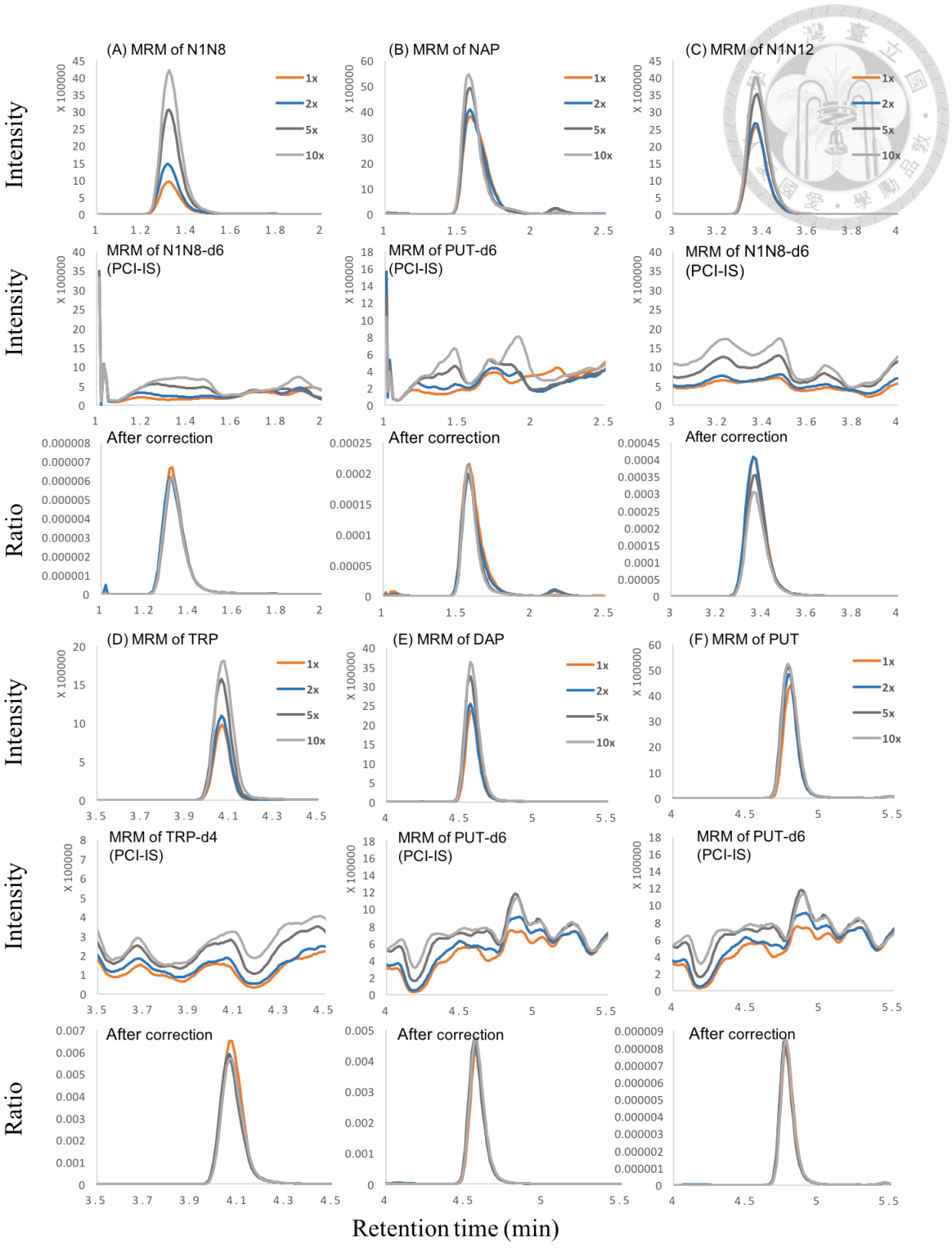

Figure 11. The MRM chromatograms of (A) N1N8, (B) NAP, (C) N1N12, (D) TRP, (E) DAP, (F) PUT after corrected by the corresponding dansylated PCI-IS in the 1, 2, 5, 10-dilution of urine sample post-spiked with high concentration amine standards. The ratio indicated the peak intensity of target amine divided by the peak intensity of the PCI-IS.

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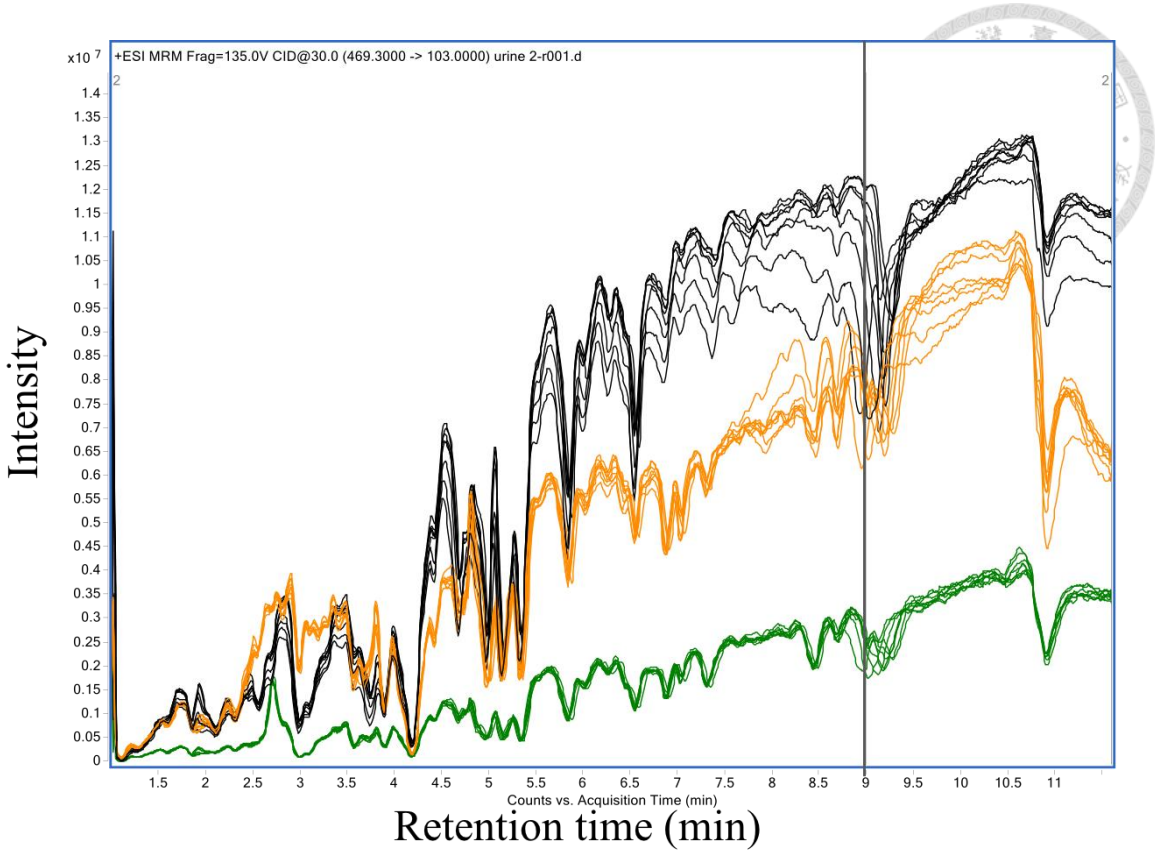

Figure 12. The overlaid MRM chromatograms of dansylated N1N8-d6 (469.3 > 103.0) (*top*), PUT-d6 (563.3 > 170.1) (*middle*), and TRP-d4 (398.2 > 148.1) (*bottom*) as PCI-IS in the repeatedly analyzed of the same urine sample during an 8-hour period. The last target compound eluted at RT = 9 min, which was represented by the straight line.

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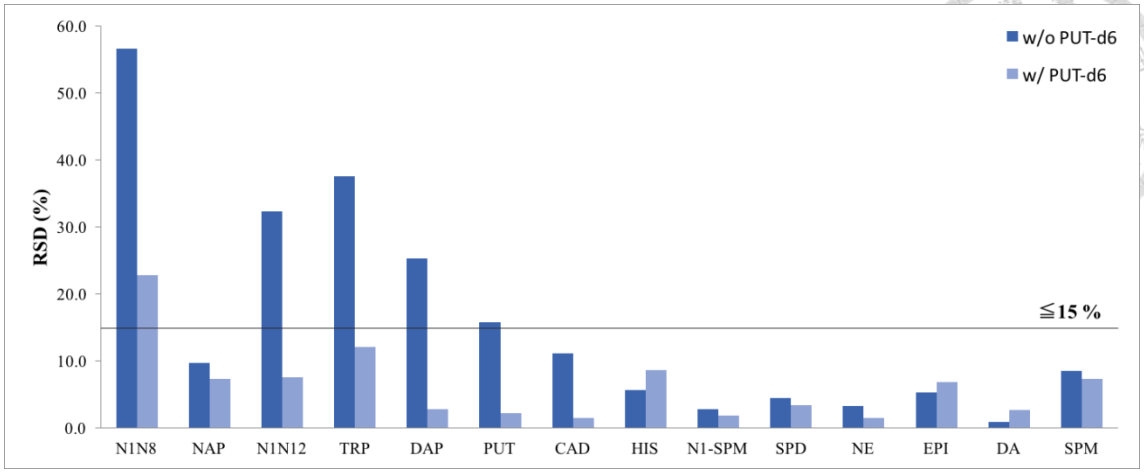

Figure 13. The average relative standard deviations (RSD %) for responses of target amines in four concentration of urine samples (n = 3) with and without correction by the dansylated PUT-d6. The four dilutions of urine samples were spiked with same concentration of amine standard mixture.

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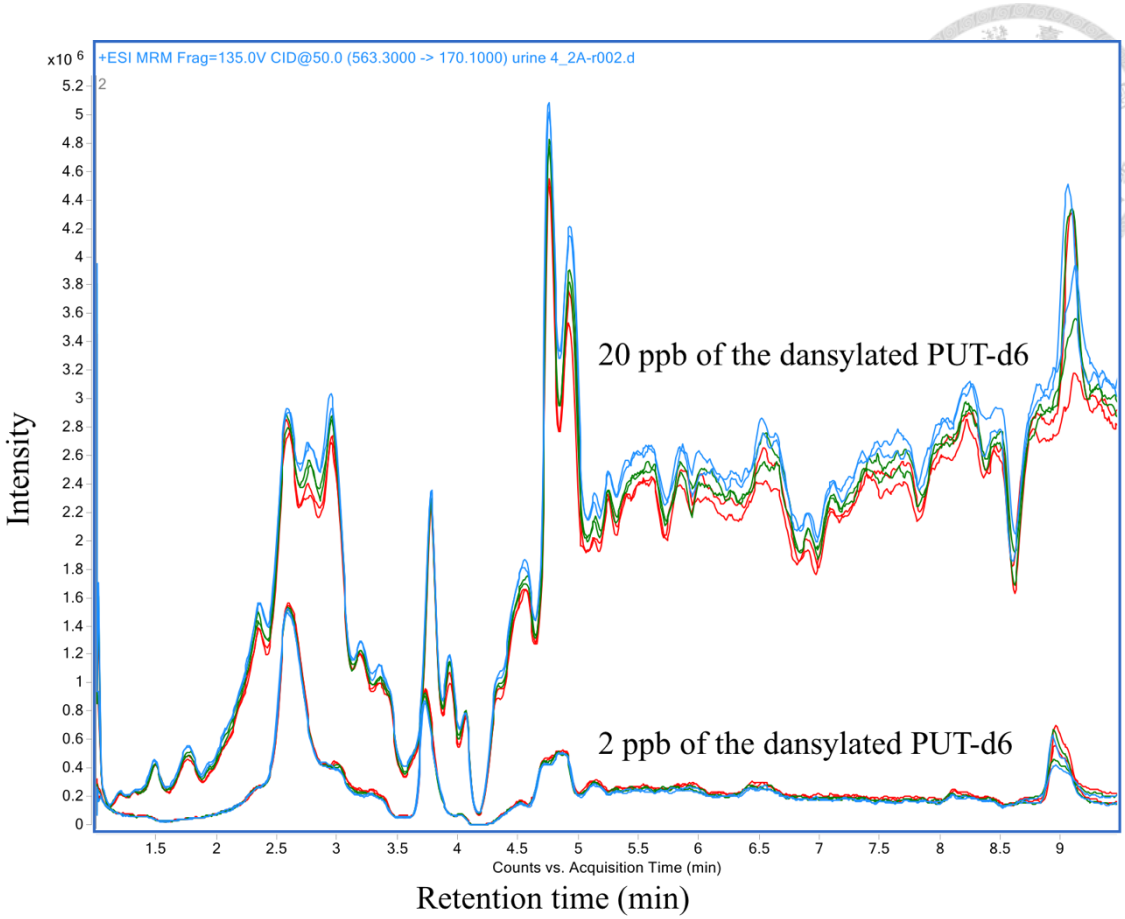

Figure 14. The overlaid MRM chromatograms of the PCI-IS obtained by six times measurement of a urine sample during a 3-hour period.

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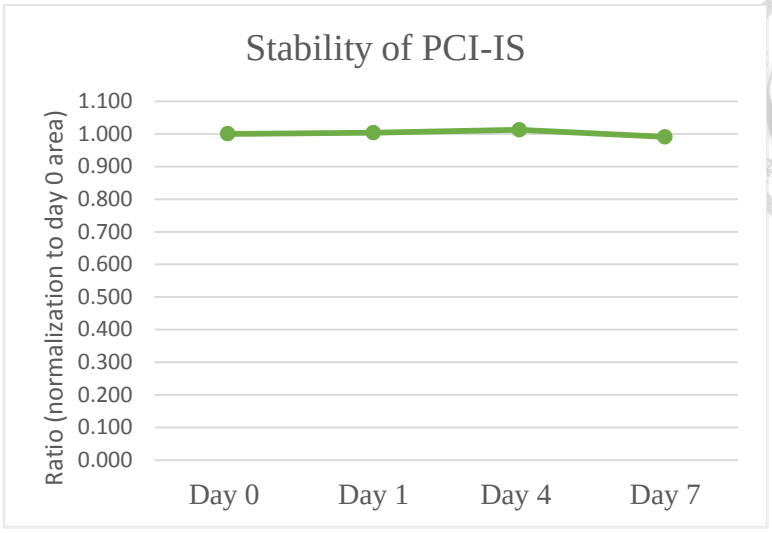

Figure 15. The stability of dansylated PUT-d6 after storage at room temperature for one week.

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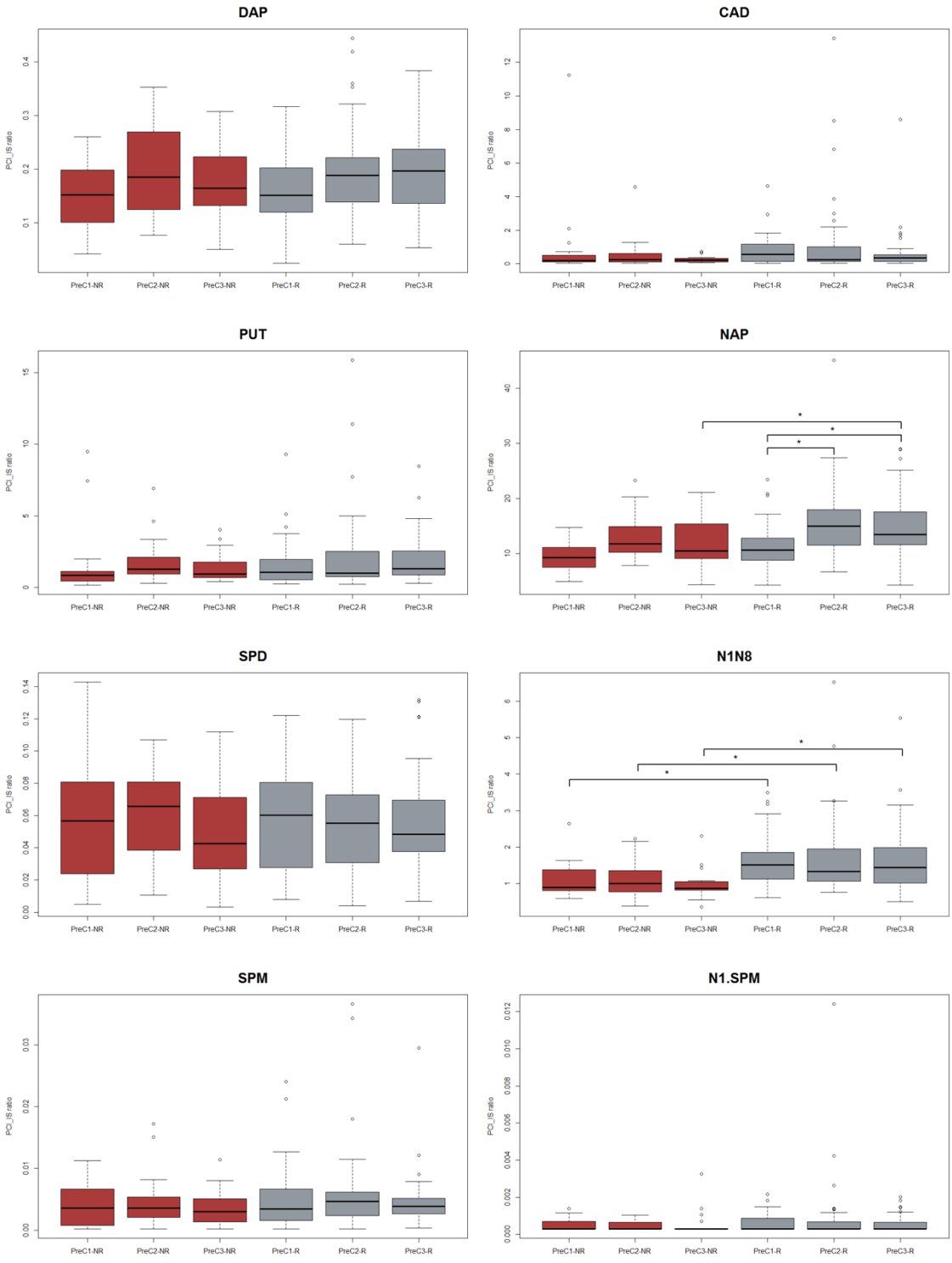

Figure 16. Boxplot of the levels of 14 urinary biogenic amines measured at three time points (pre-C1, pre-C2, and pre-C3) in nonresponders (NR) and responder (R) groups. *p-value < 0.05 by Fisher's LSD test

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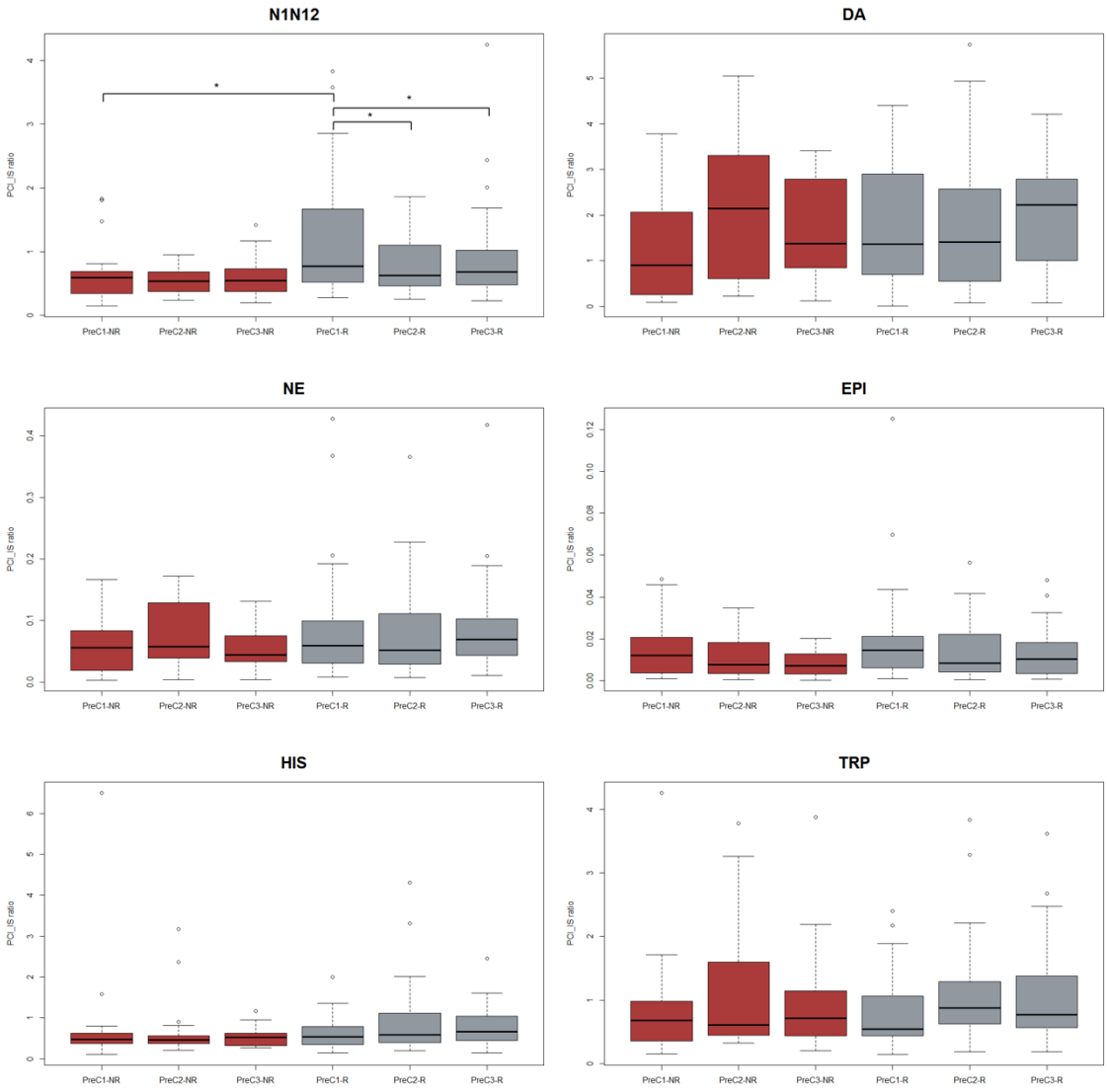

Figure 16. Boxplot of the levels of 14 urinary biogenic amines measured at three time points (pre-C1, pre-C2, and pre-C3) in nonresponders (NR) and responder (R) groups.
* p-value < 0.05 by Fisher's LSD test

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6. Tables

Table 6. The mass parameters and retention time (RT) of the 14 targeted amines.

Compound Name	Number of dansyl tags	Quantifier (m/z)	Collision Energy (V)	Qualifier (m/z)	Collision Energy (V)	Dwell (s)	Fragmentor (V)	RT (min)
N^1,N^8-diacetylspermidine	1	463.2 > 100.0	30	463.2 > 71.9	30	15	135	1.36
N-acetylputrescine	1	364.2 > 170.1	50	364.2 > 72	50	15	135	1.62
N^1,N^{12}-diacetylspermine	2	753.3 > 100.1	30	753.3 > 502.2	30	15	180	3.32
tryptamine	1	394.2 > 144.0	30	394.2 > 170	30	15	135	4.07
1,3-diaminopropane	2	541.2 > 170.3	50	541.2 > 307	30	15	135	4.56
putrescine	2	555.2 > 170.1	50	555.2 > 70.1	50	15	135	4.77
cadaverine	2	569.2 > 169.9	50	569.2 > 186.2	50	15	135	5.11
histamine	2	578.2 > 170.1	30	578.2 > 155	70	15	135	5.37
N^1-acetylspermine	3	944.4 > 100.1	50	944.4 > 170.1	70	15	180	6.37
spermidine	3	845.3 > 360.3	50	845.3 > 170.3	70	15	180	7.19
norepinephrine	3	869.2 > 170.3	70	869.2 > 383	50	15	180	7.92
epinephrine	3	883.3 > 289.9	70	883.3 > 170	70	50	180	8.58
dopamine	3	853.2 > 619.2	30	853.2 > 170.1	50	15	180	8.71
spermine	4	1135.4 > 360.1	70	1135.4 > 84.3	80	15	180	9.02

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Table 7. The matrix effects (ME) of the 14 targeted amines in a mixed urine sample.

Compound Name	RT (min)	ME (%)
1-Dns N^1,N^8-diacetylspermidine	1.36	18.3
1-Dns *N*-acetylputrescine	1.62	26.6
2-Dns N^1,N^{12}-diacetylspermine	3.32	37.6
1-Dns tryptamine	4.07	20.1
2-Dns 1,3-diaminopropane	4.56	23.0
2-Dns putrescine	4.77	36.4
2-Dns cadaverine	5.11	53.7
2-Dns histamine	5.37	87.2
3-Dns N^1-acetylspermine	6.37	74.1
3-Dns spermidine	7.19	47.6
3-Dns norepinephrine	7.92	42.6
3-Dns epinephrine	8.58	73.9
3-Dns dopamine	8.71	32.1
4-Dns spermine	9.02	60.3

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Table 8. The correction performance of the same concentration of amine standard mixture in four dilutions of urine samples (n = 3) using the combination of dansylated PUT-d6, TRP-d4, and N1N8-d6 as PCI-IS. Data were expressed as relative standard deviations (RSD %). The grey highlight indicated RSD values >15% before correction. Considering the chain length and the structure similarity of polyamines, N1N8, N1N12, N1-SPM, SPD, and SPM were corrected by N1N8-d6; NAP, DAP, PUT, CAD were corrected by PUT-d6; TRP, HIS, NE, EPI, DA were corrected by TRP-d4.

	N1N8	NAP	N1N12	TRP	DAP	PUT	CAD	HIS	N1-SPM	SPD	NE	EPI	DA	SPM
U1_before correction	**59.5**	5.6	**24.0**	**34.0**	**21.5**	12.2	6.8	4.5	2.6	2.1	3.7	2.9	0.7	**16.6**
U1_with PUT-d6	17.8	**12.2**	6.5	11.6	**3.7**	**0.9**	**1.8**	7.8	1.7	3.7	1.3	4.6	2.0	17.5
U1_with TRP-d4	25.4	10.6	4.4	**6.2**	0.9	13.7	9.0	**17.0**	6.7	7.6	**2.2**	**5.1**	**2.8**	18.8
U1_with N1N8-d6	**4.1**	9.6	**13.5**	19.8	10.3	18.3	14.5	20.8	**8.0**	**7.0**	5.1	6.9	4.6	**19.6**
U2_before correction	**56.7**	11.1	**40.8**	**40.9**	**28.3**	**19.9**	**18.0**	9.5	0.5	6.9	2.2	7.4	1.2	5.4
U2_with PUT-d6	27.1	**4.5**	7.2	9.4	**2.4**	**3.7**	**1.1**	9.7	1.3	3.1	1.6	12.3	3.5	1.9
U2_with TRP-d4	30.0	6.3	1.6	**3.1**	2.3	16.2	6.1	**20.2**	8.1	7.9	**6.6**	**12.9**	**4.5**	4.0
U2_with N1N8-d6	**2.4**	1.6	**14.1**	18.2	10.7	19.8	12.4	29.2	**6.5**	**5.0**	4.3	13.6	3.0	**3.7**
U3_before correction	**53.6**	12.4	**32.3**	**37.8**	**26.1**	**15.4**	8.7	3.0	5.3	4.3	4.1	5.5	0.9	3.7
U3_with PUT-d6	23.5	**5.3**	9.1	15.5	**2.3**	**1.9**	**1.6**	8.3	2.7	3.6	1.6	3.8	2.5	2.6
U3_with TRP-d4	24.8	8.0	2.5	**5.5**	2.9	12.2	9.9	**19.0**	8.9	10.9	**5.6**	**4.6**	**3.5**	3.1
U3_with N1N8-d6	**2.4**	7.6	**11.0**	18.3	5.9	18.4	15.6	22.5	**8.3**	**8.4**	4.6	4.3	2.4	**3.3**

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Table 9. The repeatability of a urine sample analyzed within an 8-hour period before and after correction by the PCI-IS method. Data were expressed as relative standard deviations (% RSD).

	N1N8	NAP	N1N12	TRP	DAP	PUT	CAD	HIS	N1-SPM	SPD	NE	EPI	DA	SPM
Before correction	2.6	3.2	2.4	3.2	7.1	4.7	4.6	9.1	5.9	13.7	16.0	15.5	14.3	20.4
With PUT-d6	4.8	4.2	6.2	7.4	7.9	6.8	7.9	5.1	8.1	13.5	15.6	11.6	16.3	23.2
With TRP-d4	3.6	5.5	5.9	4.6	9.0	6.9	8.7	5.4	7.4	13.4	15.3	14.2	15.1	29.7
With N1N8-d6	13.0	8.7	12.0	14.4	19.2	11.1	18.4	13.6	10.7	15.0	18.2	23.6	18.2	36.4

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Table 10. The intra-day precision of the 14 target amines. Each urine sample (n = 3) was prepared in triplicate. Data were corrected by PCI-IS method and expressed as their relative standard deviations (% RSD).

	N1N8	NAP	N1N12	TRP	DAP	PUT	CAD	HIS	N1-SPM	SPD	NE	EPI	DA	SPM
Urine 1	5.9	9.6	**18.1**	**20.2**	14.0	7.6	10.6	**17.1**	11.1	5.6	7.4	13.0	2.7	4.4
Urine 2	4.5	7.5	5.4	**15.8**	13.0	**16.2**	14.8	**15.5**	14.4	**15.8**	14.4	13.1	7.4	7.1
Urine 3	5.5	4.3	5.9	7.7	7.3	**18.0**	9.1	6.4	6.5	10.9	9.6	12.3	2.9	**16.2**

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