Development of an untargeted metabolomics analytical protocol for fecal samples by liquid chromatography–mass spectrometry

Tsai-Wei Ting [a], Chih-Ning Cheng [a], Chieh-Chang Chen [b,c], Ching-Hua Kuo [a,d,e,*]

[a] School of Pharmacy, College of Medicine, National Taiwan University, Taipei, Taiwan
[b] Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan
[c] Division of Gastroenterology and Hepatology, Department of Internal Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan
[d] The Metabolomics Core Laboratory, Centers of Genomic and Precision Medicine, National Taiwan University, Taiwan
[e] Department of Pharmacy, National Taiwan University Hospital, Taipei, Taiwan

Abstract

Gut microbiota has recently gained attention for its role in regulating multiple host pathways and contributing to disease developments. Fecal metabolomics using liquid chromatography–mass spectrometry (LC–MS) offers a promising approach to study gut microbial metabolites; however, it remains technically challenging due to the complex, heterogeneous nature of fecal samples and the lack of standardized protocols. This study aimed to establish a robust and reproducible untargeted fecal metabolomics workflow. We systematically evaluated sample preparation parameters—including sample amount, extraction solvent, numbers of extraction, and sample-to-solvent ratio—and assessed method reproducibility. Additionally, we compared three LC–MS data acquisition workflows using 10 samples from inflammatory bowel disease (IBD) patients and healthy controls (HC) to improve the identification of biologically relevant metabolites. In sample preparation, our results showed that 50 mg of lyophilized feces was sufficient to capture inter-individual metabolic variation. Additionally, methanol outperformed acetonitrile and showed comparable results to three binary solvent mixtures. A single extraction with methanol was sufficient, and a 1:20 (w/v) sample-to-solvent ratio maximized feature detection. Among the acquisition methods, data-dependent acquisition (DDA) with simultaneous MS1 and MS2 scans provided the highest metabolite coverage with acceptable annotation reliability. In summary, we recommend a single extraction of 50 mg lyophilized feces with 1 mL methanol and the use of DDA for sample acquisition to ensure comprehensive and reproducible untargeted analysis. This optimized protocol improves metabolite detection in human feces and offers a practical strategy to support future studies exploring gut microbial contributions to human health and disease.

Keywords: Analytic sample preparation methods, Feces, Inflammatory bowel diseases, Liquid chromatography-mass spectrometry, Metabolomics

1. Introduction

Microbes, composed of over 100 trillion microorganisms including bacteria, fungi, viruses, and archaea, reside in the human gastrointestinal tract [1]. Gut microbiota is associated with regulating multiple host pathways, including immune, signaling, and metabolic pathways [2]. Dysbiosis of the gut microbiota has been implicated in the development of chronic diseases, such as obesity, Parkinson's disease, and Alzheimer's disease [3,4]. While physically confined to the gastrointestinal lumen, gut microbes influence host physiology through bioactive metabolites that can circulate systemically [5]. Understanding these microbial metabolites provides valuable insights into

Received 12 August 2025; accepted 23 October 2025.
Available online 15 December 2025

* Corresponding author at: School of Pharmacy, College of Medicine, National Taiwan University, Rm. 418, 4F., No.33, Linsen S. Rd., Chongsheng Dist., Taipei City 100, Taiwan.
E-mail address: kuoch@ntu.edu.tw (C.-H. Kuo).

https://doi.org/10.38212/2224-6614.3571

microbiota function and host physiological modulation. For example, trimethylamine N-oxide (TMAO) and phenylacetylglutamine (PAGln) are gut microbial metabolites whose blood levels are associated with incident cardiovascular disease (CVD) risk [6].

Given their direct representation of gut microbial activity, fecal samples are considered optimal for gut metabolite analysis. This is due to several reasons: first, a strong association exists between the gut microbiome and the fecal metabolome, highlighting the extensive interplay between microbial communities and their metabolic products within the gut [7,8]. Second, the fecal metabolome exhibits the highest diversity and abundance of metabolic features compared to plasma and urine [9]. Furthermore, fecal sample collection is simple, non-invasive, and painless, making it suitable for large-scale studies. Despites its growing popularity, fecal metabolomics faces challenges due to the high complexity and heterogeneity of feces. Although numerous fecal-extraction protocols have been proposed [10], the diversity of sample preparation methods and the lack of heterogeneity evaluation make it difficult to determine a reliable protocol for fecal metabolomics.

To effectively analyze these complex fecal samples, liquid chromatography–mass spectrometry (LC–MS) is a powerful technique offering high sensitivity, selectivity, and robust metabolite identification. Both targeted and untargeted metabolomics analysis are employed to analyze gut metabolites. While targeted methods are designed to detect specific analytes, they may overlook unknown but critical compounds. Untargeted metabolomics analysis, conversely, provides a more comprehensive overview of the metabolome. However, processing and interpreting the vast data generated by untargeted analysis pose substantial challenges. Moreover, diverse approaches exist within untargeted metabolomics workflows, and different data processing methods can significantly impact results [11,12]. Hence, establishing a reliable and efficient workflow for untargeted analysis is critical.

To address these challenges, this study evaluated critical parameters in the fecal sample handling protocol for untargeted fecal metabolomics analysis. We evaluated the heterogeneity of fecal samples and optimized the sample preparation procedure to enhance metabolite coverage and method reproducibility. Finally, we applied the optimized protocol to investigate inflammatory bowel disease (IBD). Moreover, we compared different untargeted metabolomics data analysis workflows to enhance the coverage of metabolite identification. The proposed protocol is expected to provide more comprehensive and reliable results in untargeted metabolomic analysis of fecal samples.

2. Materials and methods

2.1. Chemicals and reagents

LC–MS grade acetonitrile (ACN) was purchased from J.T.Baker (Phillipsburg, NJ, USA). MS-grade water (H_2O) and methanol (MeOH) were obtained from Scharlau Chemie (Sentmenat, Barcelona, Spain). Formic acid was purchased from Honeywell (Charlotte, NC, USA).

2.2. Preparation of fecal samples

Each sample was manually homogenized, and approximately 5 g of the homogenized feces were collected for lyophilization. After lyophilization, the fecal powders underwent a second manual homogenization step. Each 50 mg aliquot of the powder was then transferred into a 2 mL centrifuge tube and stored at −80 °C.

For sample preparation, 1000 μL of MeOH was added to 50 mg of lyophilized fecal powder, and the mixture was extracted using Geno/Grinder 2010 (OPS Diagnostics, Lebanon, NJ, USA) at 1000 rpm for 3 min, followed by 10 min of sonication. The extract was centrifuged at 18000 rcf for 10 min at 4 °C. A 750 μL aliquot of the supernatant was filtered through a 0.2 μm filter and stored at −80 °C until LC–MS analysis.

2.3. LC–MS analysis

Liquid chromatography was performed on an Agilent 1290 ultra-high-performance liquid chromatography (UHPLC) (Agilent Technologies, Santa Clara, CA, USA) with a Waters ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm). The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.3 mL/min. The gradient was as follows: 0–1.5 min, 2% B; 1.5–9 min, 2–50% B; 9–14 min, 50–95% B; 14–17 min, 95% B; and a 3 min re-equilibration to 2% B. The sample reservoir and the column oven was set to 4 °C and 45 °C, respectively. The injection volume was 2 μL.

Mass spectrometry was performed on an Agilent AdvanceBio 6545XT LC/QTOF MS. Data acquired in positive and negative electrospray ionization (ESI) modes with the following parameters: 250 °C gas temperature, 8 L/min gas flow, 35 psi nebulizer,

200 °C sheath gas temperature, 10 L/min sheath gas flow, 4000 V in the positive and negative mode for capillary voltage, and 120 fragmentation voltage. Mass range was m/z 50−1500 for both MS and MS/MS acquisition. For MS scan only, scan rate was 2 Hz. For data-dependent acquisition (DDA), MS and MS/MS scan rates were 3 Hz and 13 Hz, respectively, with up to 8 precursors per cycle. For iterative DDA mode, MS and MS/MS scan rates were 3 Hz and 6 Hz, respectively, with up to 3 precursors per cycle. Three sequential injections were conducted, and the iterative exclusion list was updated after each run. Reference masses (121.05087 and 922.00978 in positive mode, and 112.98559 and 1033.98811 in negative mode) were used for mass accuracy checking. Collision energy was ramped from 10 eV to 40 eV.

2.4. Optimization of fecal sample preparation

For sample heterogeneity evaluation, fecal samples from five volunteers were lyophilized, and six aliquots per sample (three of 50 mg and three of 100 mg) were prepared.

For extraction optimization, various parameters were examined: (1) five solvent systems (MeOH, ACN, MeOH/H_2O 1:1, ACN/H_2O 1:1, ACN/MeOH/H_2O 1:1:1), (2) single vs. double extraction, and (3) sample-to-solvent ratios ranging from 1:20 to 1:80 (w/v). Injection volumes were adjusted to normalize metabolite concentrations. The number of detected features and signal intensity distributions were compared across triplicate analyses for each condition.

For consistency assessment, fecal samples from three IBD patients were split into paired portions, extracted on different days with the same preparation protocol and subjected to LC−MS analysis on the same day.

2.5. Collection of fecal samples from patients with IBD

To assess the applicability of the protocol, a total of ten samples were collected, including five from healthy controls (HC) and five from IBD patients. This study was approved by the Research Ethics Committee of the National Taiwan University Hospital, and signed informed consent was obtained from all patients.

2.6. Data processing and statistical analysis

LC−MS data were acquired using Agilent ChemStation software (Santa Clara, CA, USA), converted to ABF format using AnalysisBaseFileConverter, and processed into MS-DIAL for feature detection and alignment. Peak detection parameters were defined as follows: an MS1 mass tolerance of 0.025 Da and a minimum peak height of 6500 amplitude. For peak alignment, the retention time tolerance was set to 0.2 min, and the MS1 tolerance was 0.015 Da. For peak identification, the MS-DIAL metabolomics MSP spectral kit, which includes ESI(±)-MS/MS spectra from authentic standards, MassBank, MassBankEU, Fiehn HILIC, MetaboBASE, and RIKEN PlaSMA authentic standards, was utilized in combination with in-house databases. Manual curation was additionally performed to ensure accuracy and reliability.

Bar chart and Principal Component Analysis (PCA) plots were conducted using Python version 3.11.7 (Python Software Foundation, Wilmington, Delaware, USA). Group comparisons of feature numbers and signal intensity used Student's t-tests or ANOVA, with Bonferroni correction. The significance level was set at p-value <0.05. Pearson correlation analysis was conducted using Excel 2016. For clinical applications, Mann−Whitney U test was performed for the univariate test with using SAS version 9.4 (SAS Institute, Inc., Cary, NC). Statistical significance was defined as $p < 0.015$.

3. Results

3.1. Evaluation of the heterogeneity of fecal samples

Fecal samples inherently exhibit heterogeneity. To assess and minimize this variability, we evaluated samples collected from three distinct sites (both ends and the middle of each specimen) of each specimen. We compared interindividual and intraindividual variation using both 50 mg and 100 mg sample weight. As shown in Fig. 1, both weights demonstrated clear separation and clustering of HC into five groups. It indicated that the intraindividual variance was notably smaller than the interindividual differences. Our findings confirmed that 50 mg is sufficient to differentiate fecal samples, and this quantity was selected as optimal.

3.2. Optimization of fecal sample preparation

To maximize metabolome coverage, we optimized several parameters in sample preparation, including types of extraction solvent, number of extractions, and various sample-to-solvent ratios.

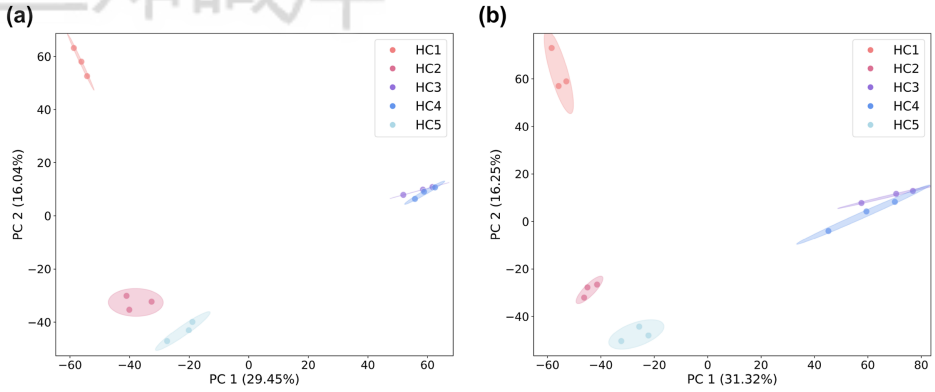

Fig. 1. PCA scores plot showing clustering of the same subjects using (a) 50 mg or (b) 100 mg fecal samples. Different colors represent fecal samples obtained from different subjects. Fecal samples were obtained from three different locations for each subject (n = 5). HC: healthy controls.

3.2.1. Selection of extraction solvent

To determine the optimal extraction solvent, we evaluated five solvent systems: two pure solvents, including MeOH and ACN, and three binary solvent mixtures of MeOH/H_2O (1:1), ACN/H_2O (1:1), and ACN/MeOH/H_2O (1:1:1). Fig. 2a shows that MeOH yielded a significantly higher number of feature compared to ACN, while all three binary solvent extraction were comparable to MeOH. Furthermore, the distribution of average signal intensities across all measured features did not differ significantly among the five extraction solvents (data not shown). Based on its superior performance and simplicity in sample preparation, MeOH was selected as the extraction solvent.

3.2.2. Evaluation of the number of extractions

We compared single versus double extractions to assess whether a single extraction was sufficient for metabolite recovery. As shown in Fig. 2b, single extraction yielded significantly more features compared to double extraction (mean 2358 features vs. 2246 features; $p < 0.001$). The small standard deviation in both groups may have contributed to the statistical significance. Additionally, the distribution of signal intensities did not differ significantly between the two methods (data not shown). Therefore, we chose single extraction for subsequent sample preparation.

3.2.3. Assessment of sample-to-solvent ratios

The sample-to-solvent ratio is crucial, as excessively high concentrations can lead to signal saturation and cause carry-over, while overly diluted samples may result in fewer detectable features. We evaluated sample-to-solvent ratio (w/v) of 1:20, 1:40, 1:60, and 1:80. As shown in Fig. 2c, the 1:20 (w/v) ratio significantly increased the number of features (passing Bonferroni-corrected $p < 0.001$). Higher dilution led to a clear declines. Ratios greater than 1:20 were not examined due to the formation of irremovable brownish precipitates. Additionally, the overall distribution of signal intensities did differ markedly across the sample-to-solvent ratio when considering all detected features. However, when restricting the comparison to the shared features detectable at the 1:80 ratio (right panel), the 1:20 ratio yields significantly higher intensities than the other ratios (Fig. 2d). Consequently, we selected the 1:20 (w/v) ratio for sample preparation.

3.3. Assessment of sample preparation consistency

After optimization of sample preparation, we aimed to ensure the consistency of the sample preparation process. Samples from three patients were extracted on different days and analyzed using Pearson correlation. Fig. 2e presents two different scenarios: samples from the same patient extracted on different days showed much higher correlation than samples from different patients. The regression lines further demonstrate this consistency. As summarized in Table 1, correlation coefficients for the same patient across different days were all above 0.99, whereas correlations between different patients were substantially lower. Collectively, these findings confirm the high consistency, robustness, and reproducibility of the sample preparation process across extraction days.

3.4. Optimization metabolite workflow for untargeted fecal metabolomics in IBD research

Different workflows can influence the metabolite identification in untargeted metabolomics. Fecal

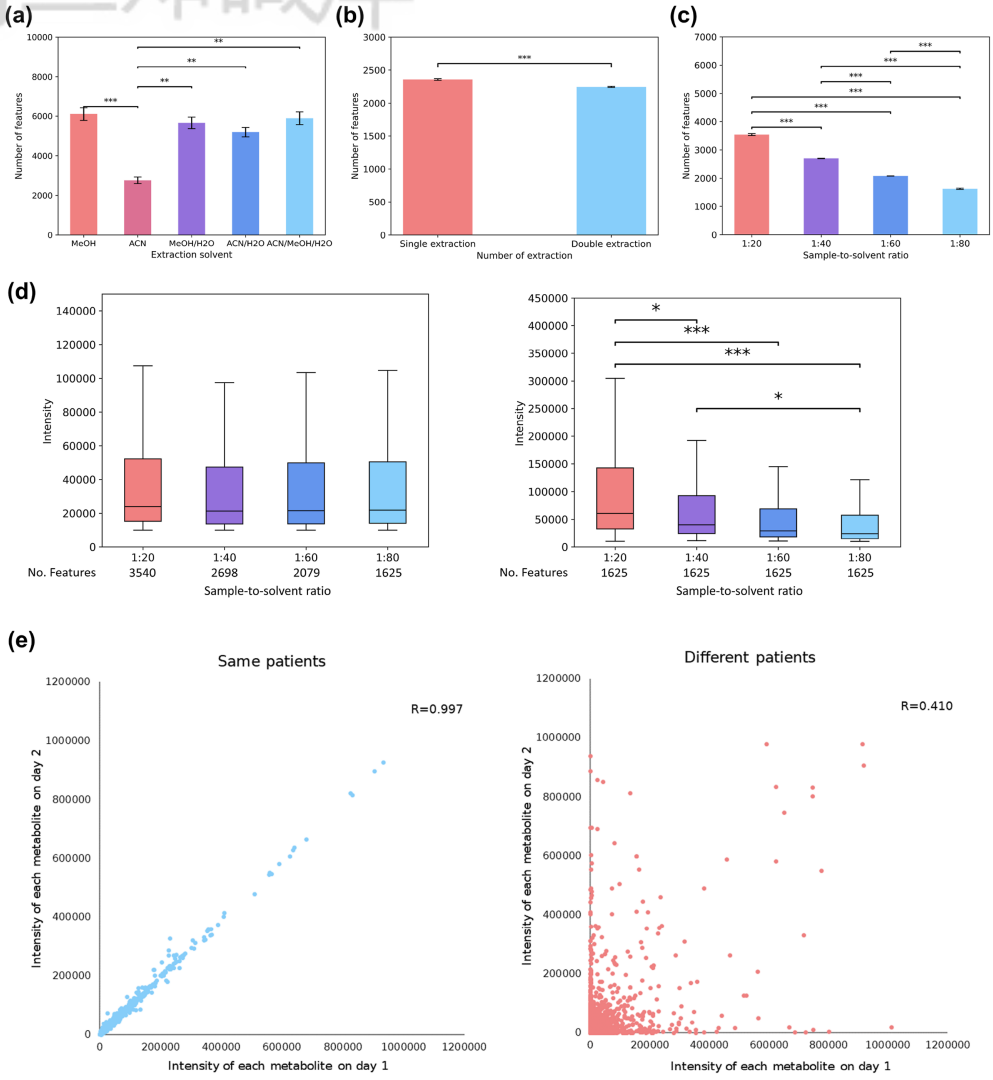

Fig. 2. The effect of (a) extraction solvent (b) the number of extractions (c) sample-to-solvent ratio on features of metabolomic analysis. Different colors represent fecal samples obtained from different subjects. Fecal samples were obtained from three different locations for each subject (n = 5). (d) Signal intensity comparisons of different sample-to-solvent ratio (left panel: all features; right panel: shared features detected at 1:80) (e) Regression line of analytical results from different days of the same patients and different patients.

samples from five IBD patients and five HC were prepared using the optimized protocol. We compared three distinct data-acquisition and processing workflows. To streamline the comparison, only metabolites with $p < 0.015$ were retained. Finally, we compared the number of significantly altered metabolites identified by each workflow.

Table 1. The Pearson correlation analysis for analytical results from different days or sample origins.

		Day 1		
		Patient 1	Patient 2	Patient 3
Day 2	Patient 1	0.997	0.414	0.359
	Patient 2	0.410	0.995	0.400
	Patient 3	0.366	0.417	0.995

3.4.1. Optimization of MS parameters and the number of iterative DDA injections

Before comparing workflows, we optimized MS parameters, specifically collision energy and the number of iterative DDA injections. For collision energy, a linear ramp from 10 eV to 40 eV identified 94 metabolites, significantly more than the 67 detected with a fixed 20 eV collision energy. Consequently, we decided to set a linear collision energy ramp ranging from 10 eV to 40 eV for untargeted metabolomics analysis.

To determine the optimal number of iterative DDA injections needed to capture at least 90% of metabolite identifications, we performed 10 injections. A total of 408 total spectra were obtained, with the first four yielding 353, 28, 16, and 5 spectra

respectively. We observed a diminishing return with each additional injection. Notably, three injections covered 95% of all identified metabolites, indicating that further injections were unnecessary.

3.4.2. Comparison of workflows for optimal metabolite identification

We compared three workflows that differed in their MS/MS acquisition and reporting strategies (Fig. 3). In workflow (a), all samples were analyzed in MS1 full-scan mode to acquire molecular feature, which were then subjected to quality check and statistical analysis. Significant features were subsequently subjected to metabolite identification using iterative DDA on group-specific pooled QC samples. In contrast, workflow (b) employed a single-run DDA for all samples, with direct online database matching and metabolite identification performed prior to statistical analysis. Workflow (c) followed a similar strategy to (b) but was further supplemented with iterative DDA on group-specific pooled QC samples to enhance metabolite coverage. Table 2 summarized the number of molecular features, significant features or metabolites, and identified metabolites across the three workflows. Finally, a total of 51 statistically significant metabolites were identified, with 23, 45, and 42 significant metabolites identified in workflows (a), (b), and (c), respectively. Specifically, the number of Level 1 identifications was comparable, with five metabolites commonly detected across all workflows (Fig. 4a). However, workflows (b) and (c) demonstrated a clear advantage in the number of Level 2 identifications, with at least ten additional significant metabolites compared to workflow (a) (Fig. 4b). Interestingly, while workflow (c) incorporated QC iterative DDA to maximize metabolite identification, the incremental gain in unique metabolite coverage over workflow (b) was limited. Considering both metabolite coverage and acquisition efficiency, workflow (b) was selected for subsequent data acquisition and processing. Fig. 5

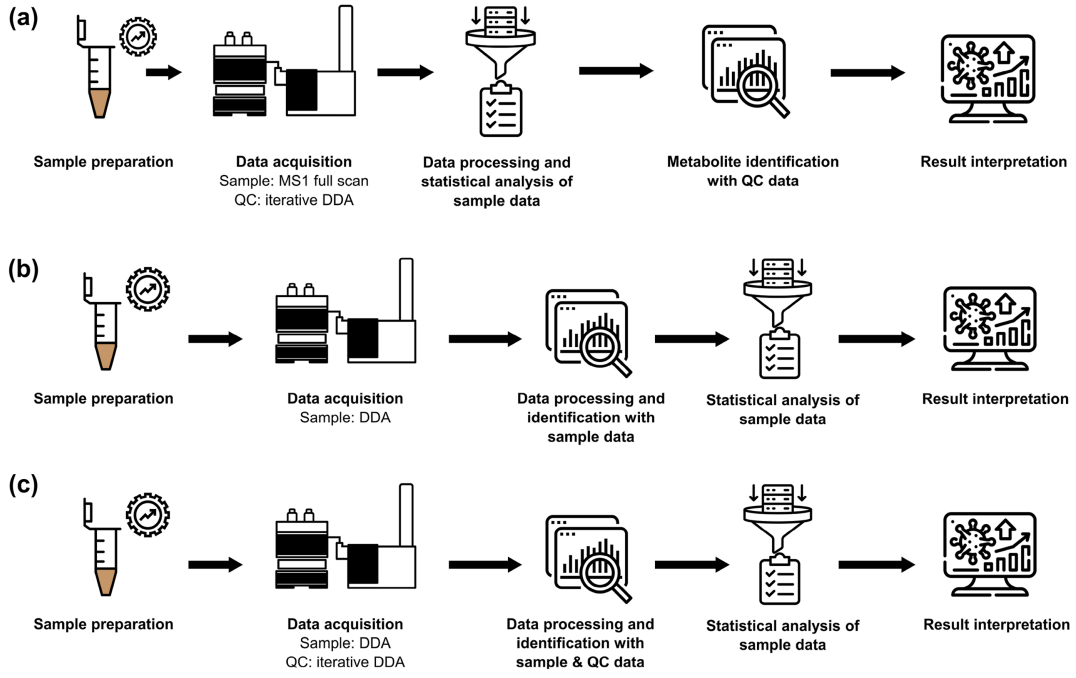

Fig. 3. Three workflows for untargeted metabolomics analysis.

Table 2. Summary of molecular features, significant features, and identified metabolites across the three untargeted metabolomics workflows.

Number	Workflow (a)	Workflow (b)	Workflow (c)
Molecular features	42,243	33,124	35,493
Molecular features after data pre-processing (background subtraction and quality check)	4943	Not applicable	Not applicable
Identified metabolites	Not applicable	818	840
Significant features[a]/metabolites[b]	319[a]	45[b]	42[b]
Identified metabolites	23	Not applicable	Not applicable

The letters a and b refer to features and metabolites, respectively

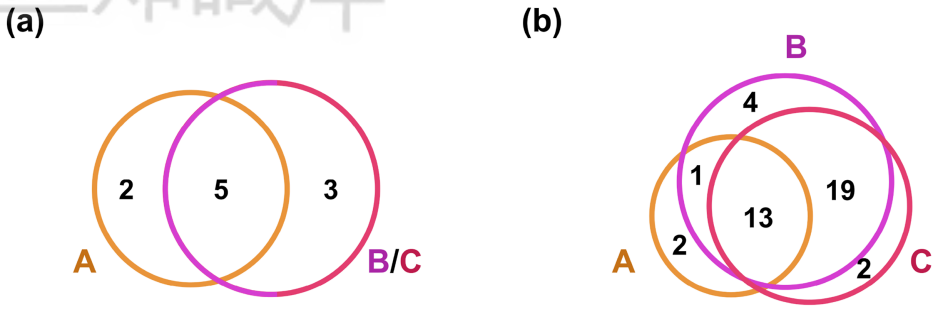

Fig. 4. Venn diagram of significantly identified metabolites ($p < 0.015$) between HC and IBD patients across three workflows. (a) Level 1 identification and (b) level 2 identification. HC: healthy controls; IBD: Inflammatory Bowel Disease.

presents the 45 significant metabolites identified by workflow (b), of which 38 were elevated in IBD patients and 7 were increased in healthy volunteers. Among these, eight metabolites were confidently identified as Level 1 using our in-house standards: adenine (fold change [FC]: 253.3), p-courmaric acid (FC: 7.0), desaminotyrosine (FC: 5.5), mandelic acid (FC: 4.4), itaconic acid (FC: 3.7), 2-hydroxy-2-methylbutyric acid (FC: 3.5), N-Acetyl-L-methionine (FC: 2.7), and 5-methoxytryptophan (FC: 2.1). While these Level 1 metabolites represent confidently identified metabolic changes, the most prominent fold changes among IBD patients were observed for the therapeutic agent mesalazine (FC: 1825.1) and its major metabolite, N-acetyl mesalazine (FC: 1336.1), highlighting the ability of the workflow to capture disease- and treatment-associated metabolic signatures.

4. Discussion

In recent years, various methods for fecal sample preparation have emerged, yet a standardized workflow has not been established [10]. In our study, we aimed to establish an effective and reliable untargeted metabolomics protocol, covering sample input quantity, preparation methods, verification of sample preparation consistency, and data acquisition and processing workflows.

Fecal samples serve as the ideal substrate for gut metabolome analysis. However, fecal samples are inherently heterogeneous. Gratton et al. showed significant differences between the entire fecal sample homogenate and the specific topographical positions [13]. Trost et al. found that spot sampling can introduce substantial metabolic variability [14]. Another study indicated that each fecal sample as large as 1 g was necessary to analyze SCFAs and distinguish between different sampling sites from the same individual [15]. These studies highlight the need to homogenize and evaluate how sample weight affects measurement variability, particularly considering that interindividual differences were substantially greater than intraindividual variation. By assessing clustering patterns in the PCA score plot, we confirmed that 50 mg is an adequate and reliable sample weight for untargeted metabolomics. These results reflected that even 50 mg of feces contains sufficient metabolite diversity for PCA to cluster an individual's samples tightly to address the intrinsic local heterogeneity of fecal specimens. The finding is consistent with the recommendations proposed by Kelly et al. [16].

We also evaluated critical parameters for fecal sample extraction. First, we considered the extraction solvent. Previous studies have commonly used water, ACN, MeOH, or their mixtures [16–19]. However, a systematic comparison of these solvents and their combinations has not been thoroughly investigated. Therefore, we first compared five extraction solvents and found that using only ACN significantly decreased the number of detected features. We attribute this to our targets being small, polar metabolites rather than nonpolar compounds. As a result, more nonpolar metabolites, which lack hydrogen-bonding capacity, may exhibit lower extraction efficiency. A study assessing extraction solvents for panda feces exhibited a similar trend [20]. Second, we evaluated the number of extractions and found that a single extraction is sufficient to extract most metabolites compared to a double extraction. Third, we assessed the sample-to-solvent ratios (w/v) from 1:20 (w/v) to 1:80 (w/v), and determined 1:20 (w/v) as the optimized ratio. This study did not test ratios lower than 1:20 (w/v) due to the formation of brownish precipitates. Similarly, the comparison results reported by Kelly et al. showed that a 1:20 (w/v) ratio yielded a significantly higher mean number of m/z features and putatively identified metabolites compared to 1:5 and 1:10 ratios [16]. Finally, to verify the robustness of the optimized sample preparation protocol, we evaluated its consistency across different extraction days. High intra-patient

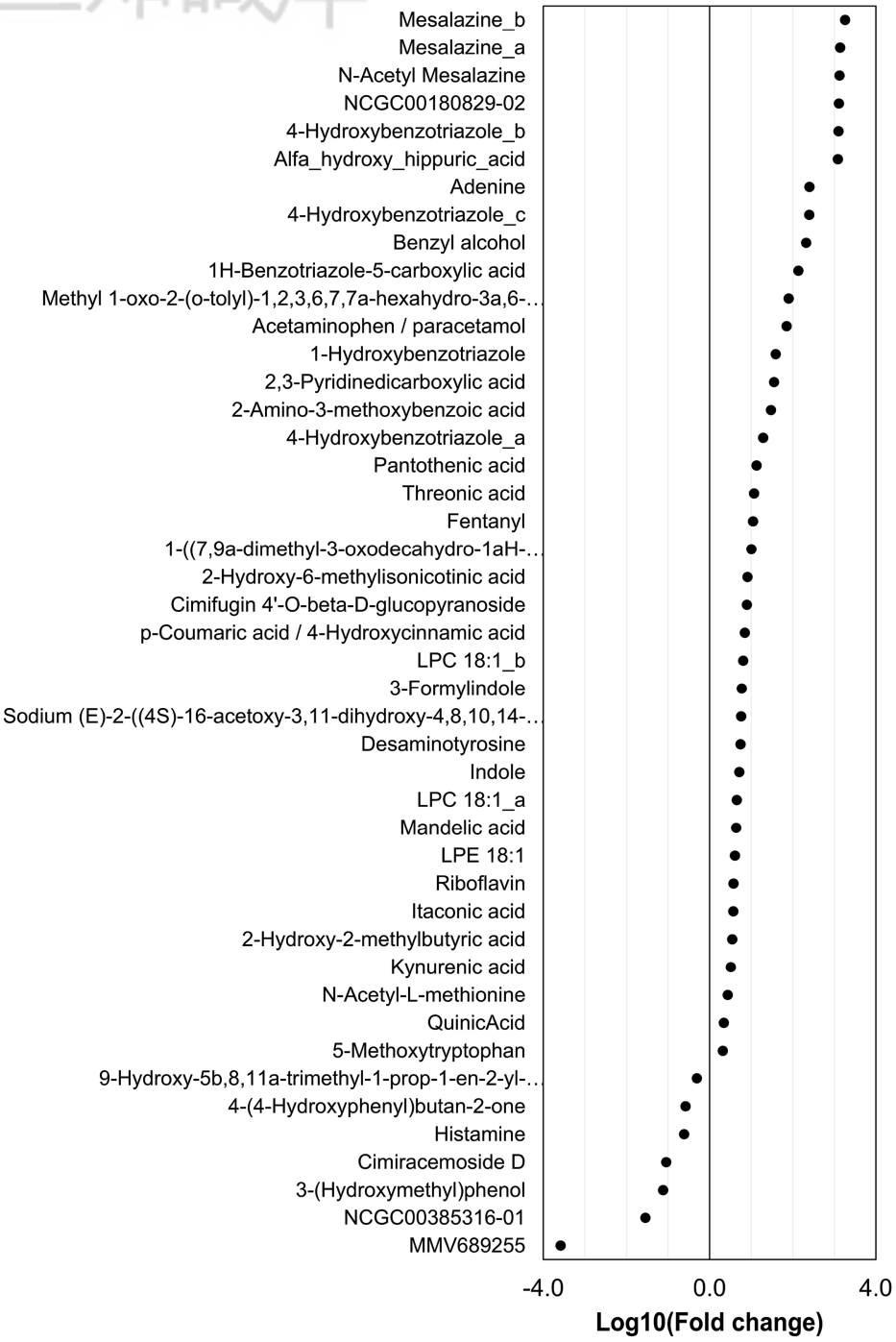

Fig. 5. Significant metabolites (n = 45) identified in workflow B.

correlation coefficients (r > 0.99) confirmed its stability and reproducibility, whereas low inter-patient correlations reflected biological variability.

Previous studies have demonstrated various approaches to data processing for untargeted metabolomics workflows, including using different acquisition modes such as full scan, targeted DDA, and DDA [12,21]. Nonetheless, no consensus exists on the optimal workflow for metabolite identification. Therefore, we proposed three distinct workflows to figure out the most effective and reliable one. Before comparing them rigorously, we optimized our mass spectrometry parameters to ensure fair and meaningful evaluation. Initially, to ensure that the collision energy is appropriate for maximizing the number of identified metabolites, we

selected two settings commonly referenced in the literature: a fixed collision energy and a linear collision energy ramp within a widely used range [22–24]. A ramp from 10 eV to 40 eV and a fixed collision energy of 20 eV were employed to balance fragmentation coverage and spectral quality, with the former resulting in the identification of more metabolites. Likewise, we evaluated the number of iterative DDA injections required to capture most metabolites and observed a progressive decline in spectra with each injection, with spectral number becoming negligible after the third injection. Taken together, the 10–40 eV ramp and three injections constituted an effective balance between coverage and efficiency.

To further evaluate different data acquisitions and processing strategies, we used an IBD study to compare three workflows. To reduce the complexity of data processing, we only focused on metabolites that showed significant differences ($p < 0.015$). Workflow (a), which performs statistical analysis prior to metabolite identification, effectively reduces the need for extensive manual inspection. Notably, this approach uniquely identified two Level 1 metabolites, while three metabolites exclusively found in other workflows were also detected in workflow (a) but excluded by the significance threshold. These observations suggest that full MS1 scans provide high-quality data for untargeted metabolomics acquisition. However, this workflow has several limitations. First, the large number of detected features increases the burden of multiple testing correction, which can obscure true positives and highlights the need for a better feature-reduction strategy before statistical analysis. Second, a huge difference was observed in Level 2 identification between workflows, with 16 metabolites identified in workflow (a) versus 37 in workflow (b). The higher number in workflow (b) may be attributed to the use of sample DDA acquisition. Third, the overall identification rate was also low—only 5.0% more identifications—reflecting a common challenge in untargeted metabolomics where annotation rates remain low [25,26]. It remains unclear whether the missing identifications were due to statistically insignificant features or simply to a failure in annotation. Additionally, a post-statistical targeted MS/MS acquisition workflow was evaluated but raised concerns about false identifications (data not shown). Between MS and MS/MS runs, metabolite degradation and potential retention time shifts reduced confidence in matching MS/MS spectra to the initially MS1 features. Taken together, both the limited gain from QC-based iterative DDA and the reliability issues with post-statistical targeted MS/MS led us to exclude these strategies from our final workflow selection.

In contrast, DDA is commonly employed in untargeted metabolomics due to its ability to simultaneously acquire both MS and MS/MS spectra within a single run. However, one inherent limitation of conventional DDA is that ions with low MS abundance may not be selected for fragmentation, resulting in a lack of corresponding MS/MS spectra [11]. To address this issue, we incorporated an iterative DDA strategy in workflow (c) to improve coverage of low-abundance features and enhance overall metabolite identification. However, the results demonstrated that the iterative DDA strategy in workflow (c) did not yield additional benefits. A careful evaluation revealed that the issue was due to sample pooling, which diluted the signals of high-intensity metabolites—especially those found in only a few samples. Even with iterative DDA acquisition, this dilution caused the signal intensity to be insufficient for MS/MS acquisition. Conversely, a single-sample DDA was successful because the metabolite's high-intensity signal was not diluted, remaining strong enough to trigger MS/MS acquisition for library matching. Notably, we applied conventional iterative DDA approach without further optimizing the settings across injections, such as adjusting acquisition thresholds based on prior runs. Future studies that dynamically optimize parameters for each injection may enhance the detection of low-abundance metabolites and improve MS2 coverage.

This study used fecal samples collected from 5 IBD patients and 5 HC to select an optimal untargeted metabolomics workflow. The results suggested using workflow (b), simultaneous acquisition of MS1 and MS2 data in a single DDA run. We observed that the concentrations of 45 metabolites differed significantly between the IBD and HC groups. Among these, mesalazine and its metabolite, N-acetyl mesalazine, were found at higher concentrations in IBD patients. This finding is consistent with its therapeutic use in IBD treatment, as many patients were prescribed medications containing this compound [27]. Furthermore, itaconic acid, with a Level 1 identification, was found at elevated levels in the IBD samples. Itaconic acid is an immunometabolite derived from activated macrophages, produced through the decarboxylation of the TCA cycle intermediate cis-aconitic acid by the enzyme aconitate decarboxylase 1 (ACOD1) [28,29]. Previous studies have shown that ACOD1 is significantly upregulated in inflamed intestinal tissues of IBD patients [30]. Consistently, our workflow detected elevated levels

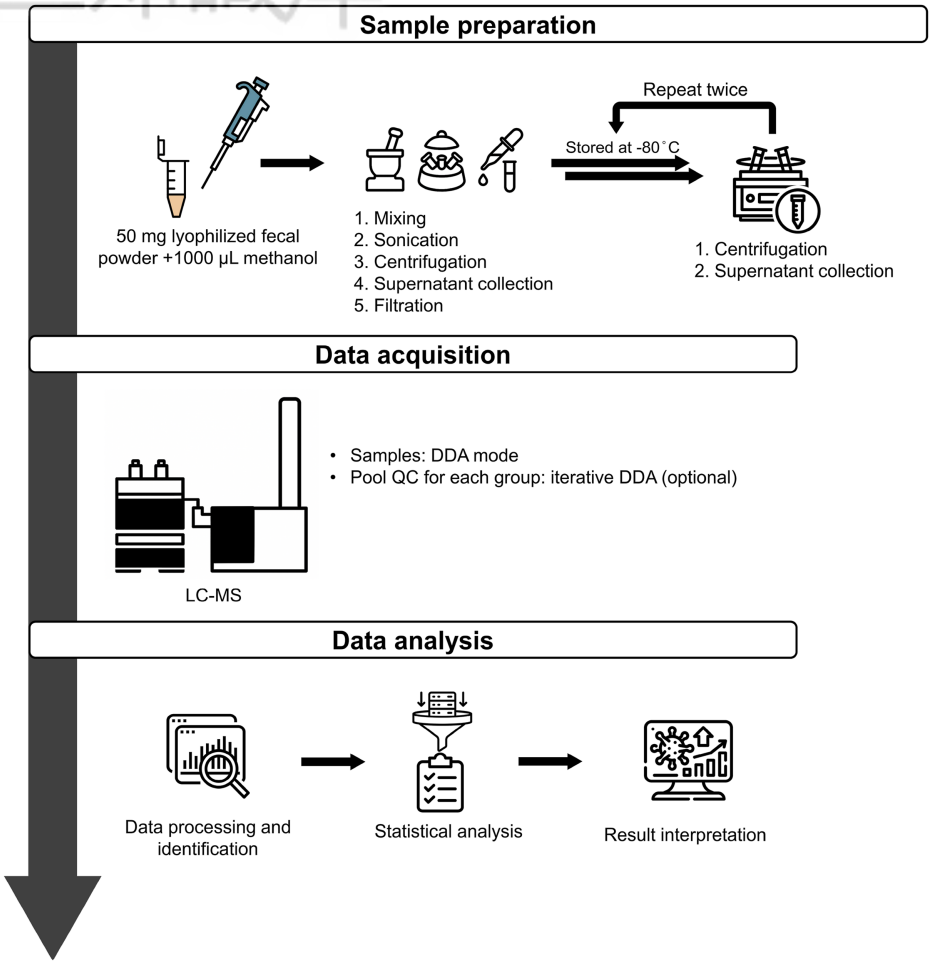

Fig. 6. The proposed analytical protocol for untargeted metabolomics.

of itaconic acid in IBD samples, demonstrating its capability to capture biologically relevant and disease-associated metabolic alterations. Ultimately, these results confirm that a single-run DDA workflow is an effective method for identifying biologically and therapeutically relevant metabolic changes associated with IBD.

The proposed analytical protocol for untargeted metabolomics is shown in Fig. 6. Our results suggest using 50 mg lyophilized fecal sample extract with 1000 µL MeOH in a single extraction to enhance metabolite coverage. We also recommend the simultaneous acquisition of MS1 and MS2 data in a single DDA run to obtain more reliable results in untargeted metabolomics.

In conclusion, we evaluated fecal sample heterogeneity, optimized preparation procedures, and demonstrated high reproducibility, thereby establishing a reliable and robust fecal sample preparation and LC–MS workflow for untargeted metabolomics. In the future, we anticipate that this protocol will assist in identifying critical metabolites associated with human diseases, thereby increasing our understanding of disease mechanisms and the role of gut microbial metabolites in diseases.

Funding statement

This work was supported by grants from National Taiwan University (NTU-110-A-CC-5400-64841) and Pharmacy Endowment Fund in support of the Platform for Clinical Mass Spectrometry and NMR Structure Elucidation.

Acknowledgements

We thank the staff of the Eighth Core Lab, Department of Medical Research, National Taiwan University Hospital for technical support during the study.

References

[1] Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124:837–48.

[2] Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. Science 2012;336:1262−7.
[3] Wilkins LJ, Monga M, Miller AW. Defining dysbiosis for a cluster of chronic diseases. Sci Rep 2019;9:12918.
[4] Benakis C, Martin-Gallausiaux C, Trezzi JP, Melton P, Liesz A, Wilmes P. The microbiome-gut-brain axis in acute and chronic brain diseases. Curr Opin Neurobiol 2020;61: 1−9.
[5] Liu J, Tan Y, Cheng H, Zhang D, Feng W, Peng C. Functions of gut microbiota metabolites, current status and future perspectives. Aging Dis 2022;13:1106−26.
[6] Witkowski M, Weeks TL, Hazen SL. Gut microbiota and cardiovascular disease. Circ Res 2020;127:553−70.
[7] Zierer J, Jackson MA, Kastenmüller G, Mangino M, Long T, Telenti A, et al. The fecal metabolome as a functional readout of the gut microbiome. Nat Genet 2018;50:790−5.
[8] Visconti A, Le Roy CI, Rosa F, Rossi N, Martin TC, Mohney RP, et al. Interplay between the human gut microbiome and host metabolism. Nat Commun 2019;10: 4505.
[9] Zhao L, Wang C, Peng S, Zhu X, Zhang Z, Zhao Y, et al. Pivotal interplays between fecal metabolome and gut microbiome reveal functional signatures in cerebral ischemic stroke. J Transl Med 2022;20:459.
[10] Karu N, Deng L, Slae M, Guo AC, Sajed T, Huynh H, et al. A review on human fecal metabolomics: methods, applications and the human fecal metabolome database. Anal Chim Acta 2018;1030:1−24.
[11] Guo J, Huan T. Comparison of full-scan, data-dependent, and data-independent acquisition modes in liquid chromatography-mass spectrometry based untargeted metabolomics. Anal Chem 2020;92:8072−80.
[12] Zhang Y, Liao J, Le W, Wu G, Zhang W. Improving the data quality of untargeted metabolomics through a targeted data-dependent acquisition based on an inclusion list of differential and preidentified ions. Anal Chem 2023;95:12964−73.
[13] Gratton J, Phetcharaburanin J, Mullish BH, Williams HR, Thursz M, Nicholson JK, et al. Optimized sample handling strategy for metabolic profiling of human feces. Anal Chem 2016;88:4661−8.
[14] Trošt K, Ahonen L, Suvitaival T, Christiansen N, Nielsen T, Thiele M, et al. Describing the fecal metabolome in cryogenically collected samples from healthy participants. Sci Rep 2020;10:885.
[15] Hsu YL, Chen CC, Lin YT, Wu WK, Chang LC, Lai CH, et al. Evaluation and optimization of sample handling methods for quantification of short-chain fatty acids in human fecal samples by GC-MS. J Proteome Res 2019;18:1948−57.
[16] Kelly PE, Ng HJ, Farrell G, McKirdy S, Russell RK, Hansen R, et al. An optimised monophasic faecal extraction method for LC-MS analysis and its application in gastrointestinal disease. Metabolites 2022;12.
[17] López-Bascón MA, Calderón-Santiago M, Argüello H, Morera L, Garrido JJ, Priego-Capote F. Comprehensive analysis of pig feces metabolome by chromatographic techniques coupled to mass spectrometry in high resolution mode: influence of sample preparation on the identification coverage. Talanta 2019;199:303−9.
[18] Moosmang S, Pitscheider M, Sturm S, Seger C, Tilg H, Halabalaki M, et al. Metabolomic analysis-Addressing NMR and LC-MS related problems in human feces sample preparation. Clin Chim Acta 2019;489:169−76.
[19] Cheng K, Brunius C, Fristedt R, Landberg R. An LC-QToF MS based method for untargeted metabolomics of human fecal samples. Metabolomics 2020;16:46.
[20] Yang Y, Yin Y, Chen X, Chen C, Xia Y, Qi H, et al. Evaluating different extraction solvents for GC-MS based metabolomic analysis of the fecal metabolome of adult and baby giant pandas. Sci Rep 2019;9:12017.
[21] Wang Y, Feng R, Wang R, Yang F, Li P, Wan JB. Enhanced MS/MS coverage for metabolite identification in LC-MS-based untargeted metabolomics by target-directed data dependent acquisition with time-staggered precursor ion list. Anal Chim Acta 2017;992:67−75.
[22] Yu M, Dolios G, Petrick L. Reproducible untargeted metabolomics workflow for exhaustive MS2 data acquisition of MS1 features. J Cheminf 2022;14:6.
[23] Nikolskiy I, Mahieu NG, Chen YJ, Tautenhahn R, Patti GJ. An untargeted metabolomic workflow to improve structural characterization of metabolites. Anal Chem 2013;85:7713−9.
[24] Gray N, Adesina-Georgiadis K, Chekmeneva E, Plumb RS, Wilson ID, Nicholson JK. Development of a rapid microbore metabolic profiling ultraperformance liquid chromatography-mass spectrometry approach for high-throughput phenotyping studies. Anal Chem 2016;88:5742−51.
[25] da Silva RR, Dorrestein PC, Quinn RA. Illuminating the dark matter in metabolomics. Proc Natl Acad Sci U S A 2015; 112:12549−50.
[26] Cajka T, Hricko J, Rakusanova S, Brejchova K, Novakova M, Rudl Kulhava L, et al. Hydrophilic interaction liquid chromatography-hydrogen/deuterium exchange-mass spectrometry (HILIC-HDX-MS) for untargeted metabolomics. Int J Mol Sci 2024;25.
[27] Herfarth H, Vavricka SR. 5-aminosalicylic acid chemoprevention in inflammatory bowel diseases: is it necessary in the age of biologics and small molecules? Inflamm Intest Dis 2022;7:28−35.
[28] Strelko CL, Lu W, Dufort FJ, Seyfried TN, Chiles TC, Rabinowitz JD, et al. Itaconic acid is a mammalian metabolite induced during macrophage activation. J Am Chem Soc 2011;133:16386−9.
[29] Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, et al. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. Proc Natl Acad Sci U S A 2013;110:7820−5.
[30] Zhang MN, Xie R, Wang HG, Wen X, Wang JY, He L, et al. Cepharanthine alleviates DSS-induced ulcerative colitis via regulating aconitate decarboxylase 1 expression and macrophage infiltration. Molecules 2023;28.