Metabolite Analysis of Human Fecal Water by Gas Chromatography/Mass Spectrometry with Ethyl Chloroformate Derivatization

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Abstract:

Fecal water is a complex mixture of various metabolites with a wide range of physicochemical properties and boiling points. The analytical method developed here provides a qualitative and quantitative gas chromatography/mass spectrometry (GC/MS) analysis, with high sensitivity and efficiency, coupled with derivatization of ethyl chloroformate in aqueous medium. The water/ethanol/pyridine ratio was optimized to 12:6:1, and a two-step derivatization with an initial pH regulation of 0.1 M sodium bicarbonate was developed. The deionized water exhibited better extraction efficiency for fecal water compounds than did acidified and alkalized water. Furthermore, more amino acids were extracted from frozen fecal samples than from fresh samples based on multivariate statistical analysis and univariate statistical validation on GC/MS data. Method validation by 34 reference standards and fecal water samples showed a correlation coefficient higher than 0.99 for each of the standards, and the limit of detection (LOD) was from 10 to 500 pg on-column for most of the standards. The analytical equipment exhibited excellent repeatability, with the relative standard deviation (RSD) lower than 4% for standards and lower than 7% for fecal water. The derivatization method also demonstrated good repeatability, with the RSD lower than 6.4% for standards (except 3,4-dihydroxyphenylacetic acid) and lower than 10% for fecal water (except dicarboxylic acids). The qualitative means by searching the electron impact (EI) mass spectral database, chemical ionization (CI) mass spectra validation, and reference standards comparison totally identified and structurally confirmed 73 compounds, and the fecal water compounds of healthy humans were also quantified. This protocol shows a promising application in metabolome analysis based on human fecal water samples.

Article:

Humans are considered to be complex “superorganisms” because of the magnitude of symbiotic gut microflora [1]. The human colon constitutes an ecologically complicated microbiota community with more than 500 bacterial species and an aggregate biomass of 100 trillion [2]. Plant-derived foods contain a multiplicity of bioactive phytochemicals, such as dietary fibers and polyphenols, which pass indigestibly through the gut and are readily degraded into various

biologically active compounds by the resident gut microbiota in the colon [3-5]. These metabolites and their active derivatives, such as short-chain fatty acids, phenolic acids, and vitamins, modulate human nutrition and health by decreasing risk of developing gastrointestinal disorder [6], cancer [7], diabetes [8], and cardiovascular disease [9]. Recent research shows that human symbiotic gut microbiota modulate the variation of human metabolic phenotypes in fecal and urinary samples [10]. Thus, metabolite compositions and variations of feces not only reflect the status of the gut microbiome community but also bridge the relationships between symbiotic microbes and the host's (human) health. There is general consensus that the aqueous phase of human feces, fecal water, directly interacts with the colonic epithelium, and its composition and concentration appears to be an important factor for a healthy colon environment [11,12]. Today, fecal water has been a common medium in human nutrition and health studies.

Characterizing the metabolite profile of human fecal water requires a holistic metabolite analysis. Metabonomics is "the quantitative measurement of the multiparametric metabolic responses of a living system to pathophysiological stimuli or genetic modification," as described by Nicholson and coworkers [13]. Combining a robust instrumental analysis method with holistically extracted metabolite information and multivariate statistical analysis, such as principal component analysis (PCA)[1] and partial least squares discriminant analysis (PLS–DA) [14], metabonomics is an efficient means to elucidate significant differences and screen the potential "biomarkers" account for such variance within majorities of metabolites. However, a comprehensive metabolite analysis is difficult to achieve due to the wide range of structural diversity of compounds in human fecal water. Potential analytical methods include nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) techniques based on high-performance liquid chromatography (LC), capillary electrophoresis (CE), and gas chromatography (GC).

NMR is a global and nondestructive technique requiring minimal sample pretreatment, and the comprehensive metabolite analysis of fecal water based on NMR has been attempted [15-17]. GC/MS was shown to be a robust method for quantification of selected metabolites, with more satisfactory sensitivity and resolution than the conventional NMR approach and better reliability in structure identification of candidate biomarkers than LC/MS [18], and was applied in metabonomic research of urinary and serum samples and brain tissue extracts [19-22]. In addition, mass spectral databases, such as NIST05, Wiley, and "Agilent Fiehn GC/MS Metabolomics RTL Library" (commercially available), further facilitated the identification of metabolites. In spite of such advantages, the GC/MS method hitherto concerned the analysis of only a certain class of metabolites such as fatty acids or phenolic acids in fecal water [23,24]. To date, simultaneous analysis of multiple classes of human fecal compositions with varied physicochemical properties has not been reported.

For comprehensive metabolome analysis based on GC/MS, derivatization is an effective method of rendering highly polar materials sufficiently volatile and narrowing the boiling point window so that they can be eluted at reasonable temperatures without thermal decomposition or molecular rearrangement. Derivatization also improves compound ionization. The active functional groups, such as carboxylic, amide, amino, and hydroxyl groups, are either alkylated, acylated, or silylated [25,26]. The silylation derivatization procedure needs a nonaqueous environment for the reaction, whereas some nonsilylating derivatization techniques, such as

chloroformate derivatization, can be performed in the presence of water. Chloroformates have been proven to be strong and rapid derivatizing reagents, and in contrast to trimethylsilylation (TMS) derivatization, alkyl chloroformate derivatization reactions occurred directly in aqueous media without the requirement of heating, thereby simplifying the sample pretreatment and derivatization procedure and consequently improving the batch repeatability [27,28]. Recent work indicates that the revised ethyl chloroformate (ECF) derivatization procedure can be well applied in holistically analyzing the metabolites of urinary and serum samples [20,21]. In the current study, we attempted the GC/MS analysis method coupled with chloroformate derivatization to describe the metabolome of human fecal water. The derivatization parameters and the pH of extraction solvent were studied emphatically in terms of relative derivatization efficiency and multivariate statistical analysis, respectively. Such a method was validated by different categories of 34 standards as well as by fecal water samples. More than 70 metabolites were identified and structurally confirmed by comparing with standards, searching database libraries, and making structural identifications based on mass spectral data of electron impact (EI) and chemical ionization (CI).

MATERIALS AND METHODS

Chemicals and materials

ECF, pyridine, and sodium hydroxide (NaOH) from Fluka (Saint Quentin en Yvelines, France) and anhydrous ethanol and *n*-hexane from Riedel-de Haën (Seelze, Germany) were used for derivatization reagents. l-2-Chloro-phenylalanine (a Sigma product) was used as an internal standard (IS) for batch quality control. Sodium bicarbonate and sodium sulfate were analytical reagent grade. All standards (Table 1), which were commercially obtained from Sigma–Aldrich (St. Louis, MO, USA), were prepared in ultrapure water (Milli-Q system, Millipore, Billerica, MA, USA) or ethanol solution (Riedel-de Haën). Human fecal water samples were prepared as follows: the homogenized stool samples (5–8 g) were ultracentrifuged at 4 °C and 50,000 rpm (equivalent to 171,500*g* average on Ti rotor angular 70.1 Beckman) for 2 h, and 2 µl of NaN_3 (100 mg ml^{-1}) as an antimicrobial agent per gram of fecal water was added to the supernatants, which were stored at −80 °C as 1-ml fecal water aliquots prior to the derivatization.

Effects of extraction solvents on human feces metabolome

Three kinds of extraction solvents—pure water, formic acid (0.2 M), and NaOH aqueous solution (0.15 M)—were selected to evaluate extraction effects on metabolome of human feces. The homogenized cold feces (1 g) was mixed with 1 ml of extraction solvent (4 °C), vortexed for 30 s, ultrasonicated for 2 min (4 °C), and vortexed for 30 s prior to ultracentrifugation (171,500*g*) for 30 min at 4 °C. A 1-ml aliquot of supernatant was collected for a diluted fecal water sample. To eliminate the effects of pH differences between fecal water samples on derivatization efficiency, all diluted fecal water samples were further adjusted isometrically to the same pH value as that extracted by deionized water using NaOH or formic acid aqueous solution before derivatization.

Effects of frozen storage of feces on recovery of fecal water compounds

Fecal water was extracted from either fresh or frozen–thawed aliquots of the same six stool specimens (from six healthy individuals). The frozen aliquots had been stored at −80 °C for 3 months. Each fecal water sample was derivatized in triplicate, and its derivatives in *n*-hexane were analyzed by GC/MS.

Table 1: Effects of initial pH regulation on relative derivatization efficiencies of test compounds in contrast to control two-step derivatization.

Compound	Formic acid (mol L^{-1})		Sodium bicarbonate (mol L^{-1})	
	0.01	**0.1**	**0.1**	**1.0**
Monocarboxylic acids				
2-Methylbutyric acid	−2.89	−21.53	4.19	−21.90
Hexanoic acid	0.08	−4.80	2.33	4.66
Nonanoic acid	−2.40	3.07	6.10	3.70
Myristic acid	0.49	0.10	9.77	15.51
Oleic acid	−0.94	−3.98	4.03	8.32
Stearic acid	−2.87	−10.69	8.56	20.18
Dicarboxylic acids				
Succinic acid	−12.91	−14.82	−1.64	−58.54
Methylsuccinic acid	−14.96	−24.89	−5.02	−53.27
Glutaric acid	−9.03	−14.81	−3.71	−41.86
Malic acid	−9.43	−42.07	12.42	−55.14
Suberic acid	0.77	7.03	8.58	0.15
Sebecic acid	2.97	1.56	3.91	5.75
Phenolics				
Benzoic acid	−9.52	−27.65	−5.45	−61.32
Phenylacetic acid	−0.79	3.87	0.17	−5.38
3-Phenylpropionic acid	0.04	3.29	3.63	4.26
4-Hydroxybenzoic acid	−3.68	−23.02	−1.37	−64.18
4-Hydroxyphenylacetic acid	−0.90	−1.76	6.11	−12.83
3,4-Dihydroxyphenylacetic acid	1.47	7.44	2.25	−36.54
Amino acids				
Alanine	−12.51	−68.98	4.51	−64.04
Valine	−9.63	−63.77	1.04	−58.86
Leucine	−12.72	−66.17	5.04	−38.72
Proline	−2.99	−39.35	1.05	−62.57
Phenylalanine	−12.73	−59.26	8.08	−49.92
Tyrosine	−10.31	−66.19	10.62	−69.23

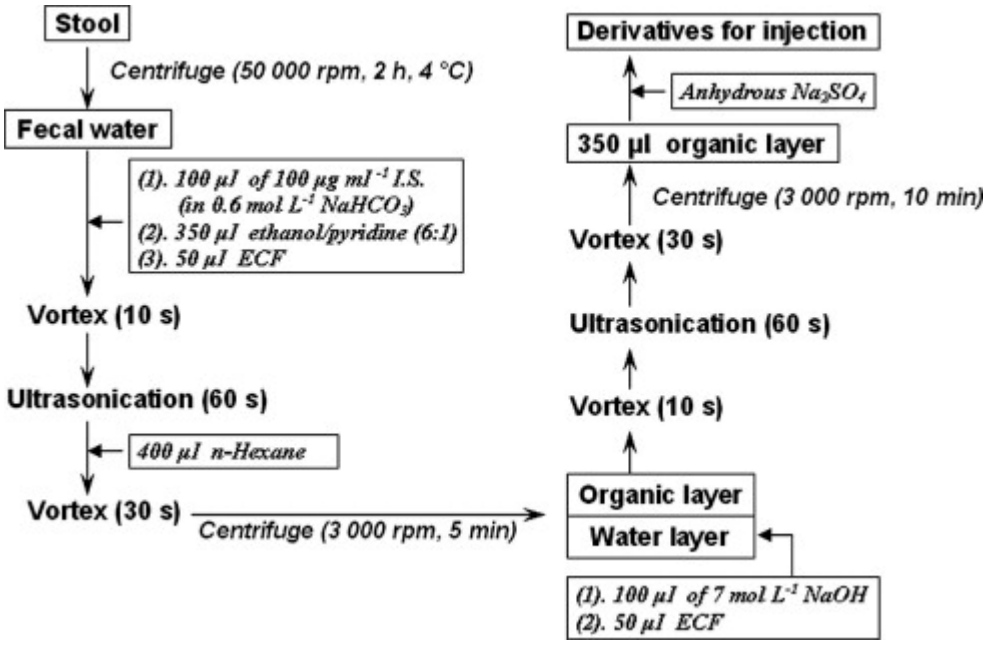
Note. The increases (%) of relative derivatization efficiency compared with control two-step derivatization were calculated by the following formula: 100∗ (peak area of two-step derivatization with pH regulation − peak area of control two-step derivatization)/peak area of control two-step derivatization.

Sample derivatization

In the first step of derivatization reaction, 100 µl of l-2-chloro-phenylalanine (0.1 mg ml^{-1} in 0.6 M $NaHCO_3$), 350 µl of ethanol/pyridine (6:1), and 50 µl of ECF were added to each 500-µl aliquot of diluted fecal water sample or the mixture of the 34 test standards in a Corning disposable glass centrifuge tube with a screw cap. The reaction mixture was then vortexed for 10 s and ultrasonicated for 60 s to accelerate the reaction at room temperature. The derivatization products were vortexed with 400 µl of *n*-hexane for 30 s and centrifuged for 5 min at 3000 rpm. Then 100 µl of NaOH (7 M) was added to the aqueous layer to adjust the pH to 9 to 10, followed by an additional 50 µl of ECF to trigger the second step of the derivatization procedure. The derivatization mixture was vortexed for 10 s, ultrasonicated for 60 s, and again vortexed for 30 s prior to centrifugation at 3000 rpm for 10 min.

To compare the relative derivatization efficiency caused by varying derivatization parameters, 100 µl of 200 µg ml^{-1} ethyl 3-(methylthio)propionate (EMP, in *n*-hexane) was added into the derivatization medium before the last vortex. A total of 350 µl of derivatives (organic layer) was transferred to a small crimp top glass vial with anhydrous granular sodium sulfate to remove traces of water. The anhydrous derivatives in *n*-hexane were subjected to GC/MS assay. The procedure for preparing ECF derivatives of fecal water is illustrated in Fig. 1.

Figure 1: Procedure for preparing ECF derivatives of fecal water.



GC/MS analysis conditions

Each 1-µl aliquot of the derivatives was injected in a splitless mode into an Agilent 7890A GC system coupled to an Agilent 5975C inert XL EI/CI mass spectrometric detector (MSD) system (Agilent Technologies, Santa Clara, CA, USA). An HP-5MS capillary column coated with 5% phenyl/95% methylpolysiloxane (30 m × 250 µm i.d., 0.25 µm film thickness, Agilent J & W Scientific, Folsom, CA, USA) was used to separate the derivatives. The initial oven temperature was held at 40 °C for 3 min; ramped to 60 °C at a rate of 10 °C min^{-1}, to 140 °C at a rate of 8 °C min^{-1}, to 240 °C at a rate of 5 °C min^{-1}, and to 280 °C at a rate of 20 °C min^{-1}; and finally held at 280 °C for 3 min. Helium was used as a carrier gas at a constant flow rate of 1 ml min^{-1} through the column. The initial inlet gas pressure was 7.069 psi. The temperatures of the EI ion source and injector were 200 and 280 °C, respectively. The electron energy was 70 eV, and mass data were collected in a full-scan mode (*m*/*z* 30–550). The solvent delay was set at 5 min. Agilent "retention time locking" (RTL) was applied to control the accuracy of retention time (RT), where phenylalanine was selected as the calibrated compound. For CI mode, the same capillary column and GC parameters were set. Pure methane was used as reagent gas.

Method validation

A total of 34 standard mixture and fecal water samples were used to validate the developed method. Each stock solution of test standard was carefully prepared in the deionized water (2 mg ml^{-1}) or pure ethanol (2 mg ml^{-1}) and stored at −20 °C. The spiked standard solution was obtained accordingly by adding 500 µl of each aliquot of stock solution to water or ethanol, respectively, to obtain 50 µg ml^{-1} of the spiked standard solution. The spiked standard solution was diluted in water or ethanol, and then two types of spiked standard solutions with the same concentration were combined into different concentrations of spiked standard solution in 50%

ethanol for the determination of linear range, regression coefficient (R^2), limit of detection (LOD), and repeatability.

Extraction and pretreatment of GC/MS raw data

Raw GC/MS data were converted into CDF format (NetCDF) files by Agilent GC/MS 5975 Data Analysis software and subsequently processed by the XCMS toolbox (version 1.14.0, http://masspec.scripps.edu/xcms/xcms.php) using XCMS's default settings with the following changes: xcmsSet (fwhm = 8, snthresh = 6, max = 200), retcor (method = "linear," family = "gaussian," plottype = "mdevden"), bandwidth (bw) of 8 for first grouping and 4 for second grouping [29,30]. For multivariate statistical analysis, the XCMS output was further processed using Microsoft Excel (Microsoft, Redmond, WA, USA). The data of IS and impurity peaks from column bleeds and vessels were excluded, and only ion features between RTs 320 and 2400 s were normalized within the sample and arranged on a three-dimensional matrix consisting of arbitrary peak index (RT–*m*/*z* pair), sample names (observations), and peak area (variables). For comparing relative derivatization efficiency, the base peak areas of standards or test compounds in fecal samples were selected and calibrated by EMP.

Multivariate statistical analysis and univariate statistical validation

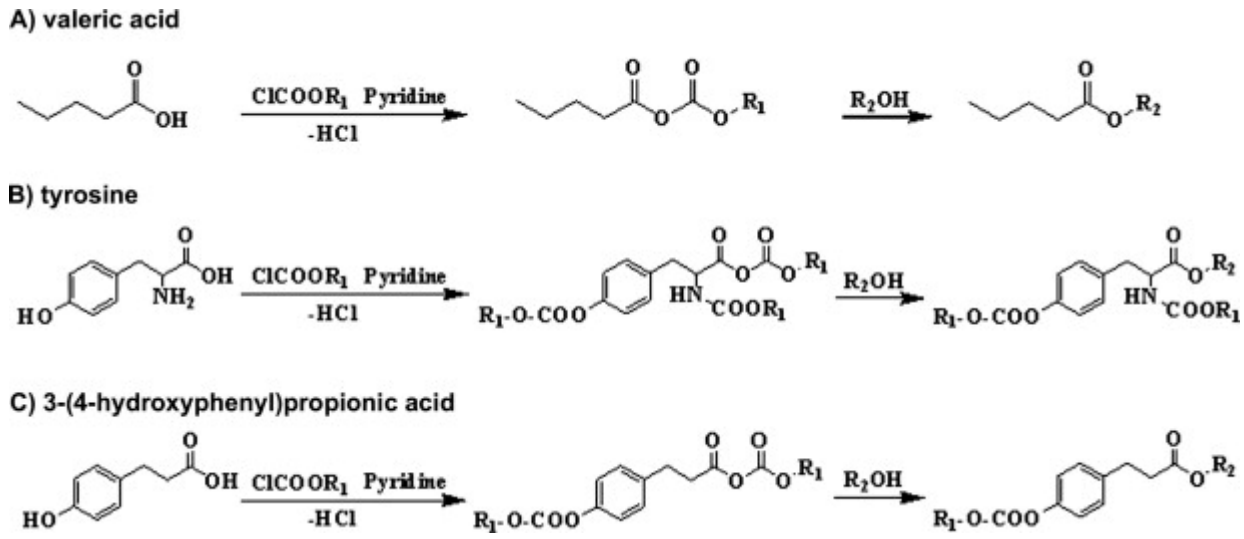
The resulting three-dimensional data table was entered into the SIMCA-P 11.0 software package (Umetrics, Umeå, Sweden), where multivariate statistical analyses, including PCA and PLS–DA, were performed. All data was mean-centered and unit variance (UV)-scaled before PCA and PLS–DA. PCA was used to observe general clustering and trends among all samples extracted from feces by acidified, alkalized, and deionized water, respectively, or between fresh and frozen feces samples. Meanwhile, the PLS–DA model, as a method derived from PLS analysis where the Y matrix was set as a dummy descriptor by Simca-P, was used to maximize metabolite variations and identify significantly altered metabolites responsible for such variations. R^2X represents the cumulative modeled variation in X, R^2Y is the cumulative modeled variation in Y, and Q^2Y is the cumulative predicted variation in Y. The values of these parameters approaching 1.0 indicated a stable model with a predictive reliability. These discriminating metabolites were obtained by using a statistically significant threshold of variable influence on projection (VIP > 1.0) values obtained from the PLS–DA model and were further validated by Student's *t* test. The metabolites with VIP values greater than 1.0 and *P* values less than 0.05 (threshold) were selected as discriminating metabolites between two classes of samples. Fold was calculated as the logarithm of the average mass response (area) ratio between two arbitrary classes, where the positive value means that the average mass response of class 1 is larger than that of class 2.

Identification and quantification of human fecal water metabolites

Compound identification was performed by comparing the RTs and mass spectra of each peak with those of reference standards available in our lab. The remaining compounds were identified by search against commercially available libraries such as NIST05, NBS, and Wiley databases. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS) before comparing with the databases. Then the mass spectra of individual components were transferred to the NIST Mass Spectral Search Program MS Search 2.0, where they were matched against reference compounds of the NIST Mass Spectral Library 2005. GC/MS data from unidentified compounds were further processed by TurboMass 5.3.0 for comparison of their mass spectra with NBS and Wiley databases. Further validation of

these candidate compounds by library research was performed by checking CI mass spectra, and this helped to obtain the molecular weight of the derivative, the reaction laws (Fig. 2), and the EI mass spectra. Calibration curves of 34 reference standards were used to quantify the compounds of fecal water.

Figure 2: Reaction scheme of three representative compounds treated with ECF in current aqueous reaction system: (A) valeric acid; (B) tyrosine; (C) 3-(4-hydroxyphenyl)propionic acid. R_1 or R_2 represents alkyl group such as $–CH_3$, $–C_2H_5$, $–C_3H_7$, or $–C_4H_9$.



RESULTS AND DISCUSSION

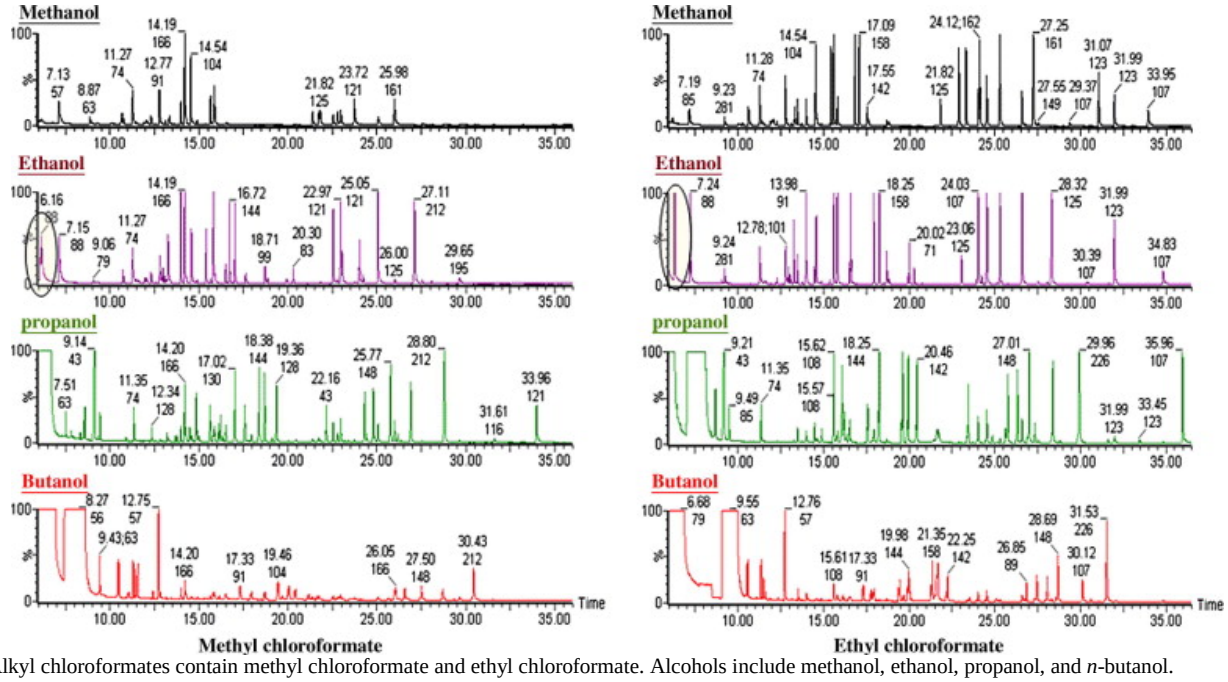
Chromatographic separation

Fecal water is a complex mixture of various metabolites, including fatty acids, amino acids, amines, and phenolic compounds, with a wide range of boiling points. The application of ECF derivatization in fecal water is beneficial not only to decrease the boiling points of metabolites but also to narrow the boiling point window of the derivatives. Consequently, capillary gas chromatography separation can cover as many compounds as possible. When the initial oven temperature was decreased from 80 to 40 °C, a large number of short-chain fatty acids, such as 2-methylbutyric, isovaleric, and valeric acids, were identified as important because many short-chain fatty acids exist abundantly in fecal water [9,31]. The Agilent RTL technique is a powerful means for reproducing compound RTs of gas chromatograms, and this is important for accurately assigning peaks in processing data. It was found that RTL efficiently controlled the RT accuracy within batch and between different batches. But we found that the increased variation of RTs occurred in the low oven temperature region, and this usually happened in the initial two runs. The optimized oven temperature program and RTL technique allowed good chromatographic separation and repeatability of RTs.

Derivatization reagents

The reaction system was generally composed of alkyl chloroformate, water, alcohol, and pyridine. Here pyridine triggers derivatization reaction, whereas both the length of the alkyl group and the kinds of alcohol affect the structures of derivatives. Therefore, two kinds of alkyl chloroformates (methyl- and ethyl-) and four kinds of alcohols (methanol, ethanol, propanol, and *n*-butanol) were evaluated for the derivatization of test compounds aiming to obtain better separation and sensitivity (Fig. 3). If methanol was selected, the short-chain fatty acids (2-methylbutyric acid and isovaleric acid) were overlapped by solvent peaks even if the initial column temperature was decreased to 40 °C. The application of ethanol increased the RT and peak intensity. Propanol and *n*-butanol further enhanced the RT, but the intensity and purity of derivatives were seriously affected. Alkyl chloroformate principally affected the peak intensity; ECF showed better peak intensity of derivatives, especially amino acids, than did methyl chloroformate.

Figure 3: Effects of alkyl chloroformate and alcohol on derivatization of water-soluble standards.



Alkyl chloroformates contain methyl chloroformate and ethyl chloroformate. Alcohols include methanol, ethanol, propanol, and *n*-butanol.

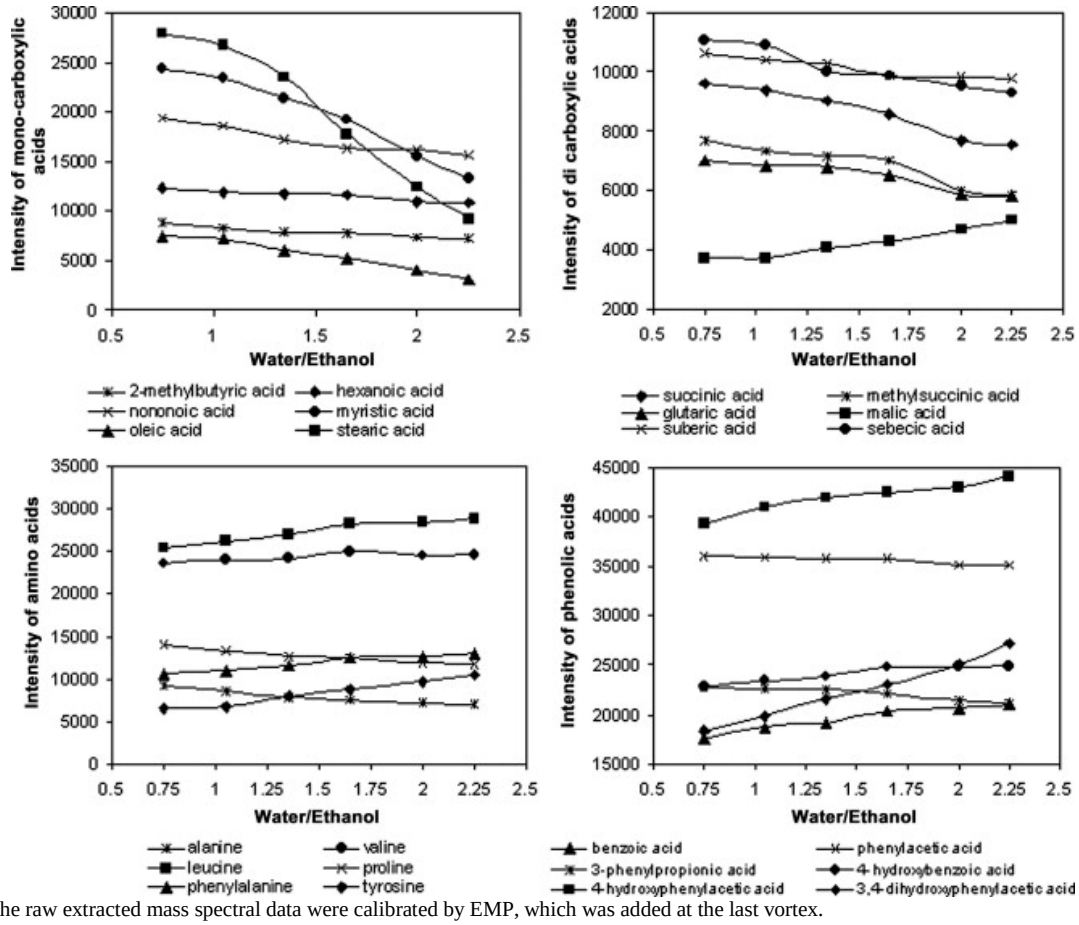
During the derivatization reaction, the amino and hydroxyl groups are converted into the structurally stable alkoxycarbonyl esters. But the carboxylic groups are transformed to the structurally unstable alkoxycarbonyl esters or stable alkyl esters by an exchange reaction with alcohol in the reaction medium. Thus, the final derivatives of compounds containing carboxylic groups were determined by alcohol types and reaction conditions such as the water/alcohol ratio. In this experiment, the solvent pair ECF and ethanol showed the better peak intensity, separation, and derivatization purity and was selected as the optimal reaction reagents.

Factors affecting the efficiency of derivatization

The water/ethanol/pyridine ratio. During the derivatization process, the content of ethanol or water affects the solubility of compounds in the reaction medium and the consequent reaction recovery. Fig. 4 indicates that water improved the derivatization efficiencies of most test amino acids except the hydrophobic amino acids (e.g., alanine, proline) and of all test compounds containing the active hydroxyl groups. In contrast, water inhibited the derivatization of carboxylic acids, especially long-chain fatty acids. Moreover, the decrease in reaction yield is proportional to the increase in carbon chain length of acids. Ethanol enhanced the derivatization efficiencies of all carboxylic acids without active hydroxylic groups, especially long chains of fatty acids (Fig. 4). It was known that ethanol promoted not only the dissolution of hydrophobic compounds in reaction mixture but also the esterification of the carboxylic group by exchanging the unstable alkoxycarbonyl esters with ethanol to the stable alkyl esters instantly. Therefore, high ethanol content was beneficial for the derivatization of carboxylic acids and hydrophobic compounds. Previously published data demonstrated that the excessive alcohol corresponding to the reagent alkyl (e.g., ethanol) promoted the esterification of di- or tricarboxylic acids favorably [32]. More water will decrease reaction yield [33] and [34], and then the amount of water should be as low as possible. Therefore, the water/ethanol ratio affected not only the solubility of compounds but also their esterification and purification of derivative products; thus, the precise water/ethanol ratio should be controlled to minimize the formation of side products. Pyridine is necessary for triggering catalyst reaction in the aqueous medium. It was found that 50 μl of pyridine received better effects for nearly all test compounds (data not shown). For the category analysis of metabolites in fecal water, we compromised by using a water/ethanol/pyridine ratio of 12:6:1 (total volume 950 μl).

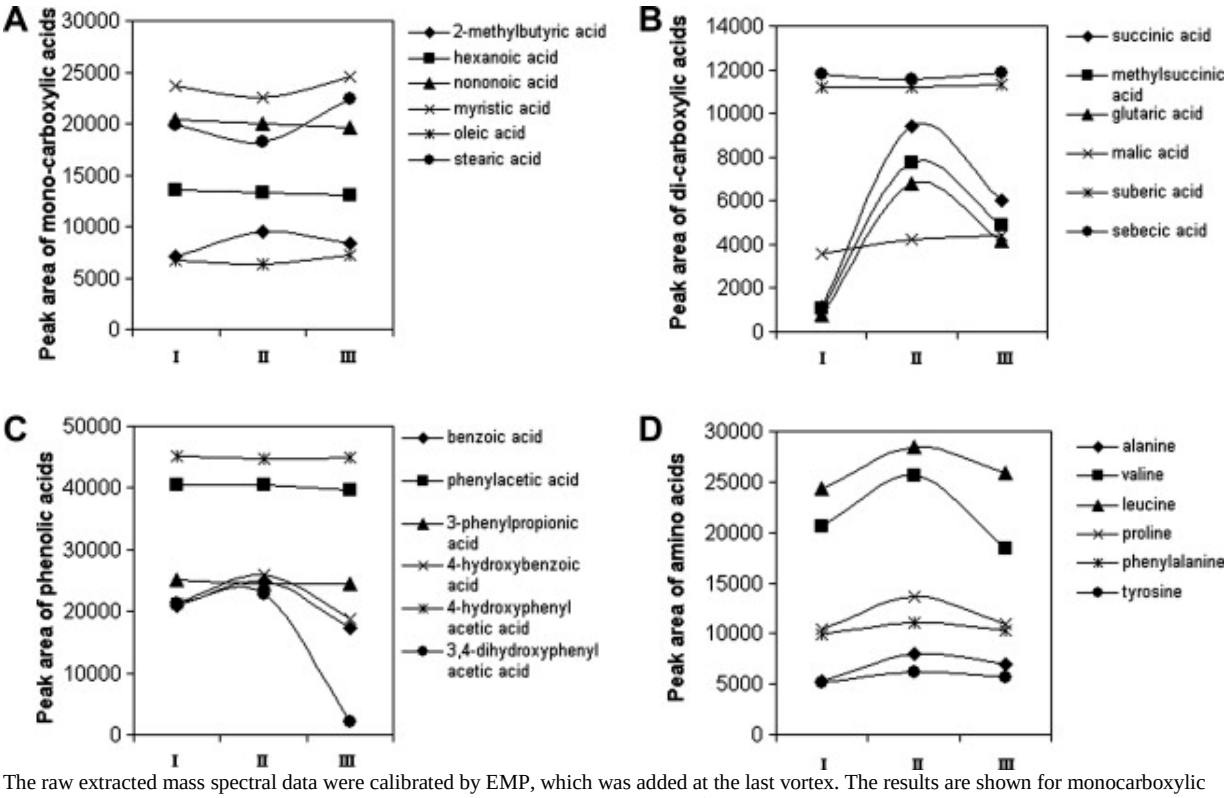
One-step versus two-step derivatization. In many publications, the classical one-step derivatization without pH adjustment is normally applied to obtain derivatives rapidly with regard to one given class of compounds. In our work, we found that dicarboxylic acids and glutamic acid (amino derivative of glutaric acid) produced very weak signals in one-step derivatization. Two-step derivatization [20] significantly improved the relative derivatization efficiency of amino acids, short-chain fatty acids and dicarboxylic acids, and phenolic compounds containing hydroxyl groups (phenolic acids), whereas only the long-chain fatty acids (mono- and dicarboxylic acids) were decreased slightly (Fig. 5). If the same content of NaOH and ECF that are added during the second step of the two-step method were added into the reaction medium for the one-step method, only long-chain fatty acids would obtain higher derivatization efficiencies than those of the one-step method without pH adjustment and the two-step derivatization. In addition, it was surprising to observe that the derivatization of phenolic acids, short-chain dicarboxylic acids, and amino acids was depressed in contrast to the two-step derivatization, where 3,4-dihydroxyphenylacetic acid was significantly inhibited or could not be detected, suggesting that these phenolic compounds were possibly transformed to quinone compounds by NaOH before starting derivatization. Consequently, the two-step derivatization with pH regulation by NaOH at the second step was superior to the one-step derivatization and the one-step derivatization with initial pH regulation.

Figure 4: Effects of water/ethanol ratio on derivatization of monocarboxylic and dicarboxylic acids, phenolic compounds, and amino acids.



The raw extracted mass spectral data were calibrated by EMP, which was added at the last vortex.

Figure 5: Comparison of derivatization efficiencies between one-step and two-step derivatizations.



The raw extracted mass spectral data were calibrated by EMP, which was added at the last vortex. The results are shown for monocarboxylic acids (A), dicarboxylic acids (B), phenolic compounds (C), and amino acids (D), for which traditional one-step derivatization (I), two-step derivatization (II), and one-step derivatization with NaOH regulation (III) were compared.

Initial pH. Based on the two-step method, the effects of initial pH of reaction medium on derivatization efficiency were evaluated. The effects of sodium bicarbonate (0.1–1.0 mol L^{-1}, final concentration) and formic acid (0.01–0.1 mol L^{-1}, final concentration) aqueous solution in initial reaction medium with control derivatization (deionized water) were compared (Table 1). Formic acid significantly inhibited the derivatization of most test compounds, especially amino acids, possibly due to the weak ionization of amino acids in acidic water. The low content of sodium bicarbonate (0.1 mol L^{-1}, final concentration) improved the reaction yields of most test compounds except benzoic acid and its analogs and short-chain dicarboxylic acids, whereas the high content of sodium bicarbonate (e.g., 1 mol L^{-1}, final concentration) seriously suppressed the reaction yields, especially for amino acids.

It is known that carboxylic groups are transformed either to unstable alkoxycarbonyl esters, which are decomposed slowly over time, or to the stable alkyl esters [35]. The low content of sodium bicarbonate not only promoted the ionization of the carboxylic groups in aqueous medium but also improved decarboxylation of alkoxycarbonyl esters to the desired alkyl esters. Meanwhile, the alkalized environment provided by sodium bicarbonate neutralized the free acids released during the decarboxylation reaction. The high content of sodium bicarbonate produced the excess free acids, especially short-chain dicarboxylic acids, during its competitive decarboxylation reaction with ethanol, and this probably caused feedback repression. Therefore,

the combination of adding sodium bicarbonate at the first step (0.1 mol L^{-1}, final concentration) and NaOH at the second step (1 mol L^{-1}, final concentration) was the optimum means of initial pH adjustment.

Effects of solvents on extraction yields of fecal water compounds

For metabonomics research, an appropriate extraction method not only covers more compounds but also obtains higher extraction yields of all compounds. Many factors affect the extraction yields of fecal water and even the final results. This work compared the extraction efficiencies of formic acid (0.2 mol L^{-1}), NaOH aqueous solution (0.15 mol L^{-1}), and deionized water on feces. Visual inspection of GC/MS TIC chromatograms revealed some differences in peak intensities. The raw data were calibrated by IS (l-2-chloro-phenylalanine) prior to PCA and PLS–DA analysis using Simca-P 11.0 software. A clear separation in scores plot of PCA occurred among the three groups of samples, where the cumulative R^2X of two principal components, 0.733, indicated the significantly different metabolite profiles caused by the pH of the extraction solvent. The separate PLS–DA models were conducted to reveal the variables that had the greatest contribution to the discrimination among the three pH conditions. Combining the results of VIP values (>1) of the PLS–DA model and the Student's *t* test ($P < 0.05$), it was found that both deionized water and NaOH solution showed better extraction efficiency on nearly all kinds of compounds than did formic acid aqueous solution (Table 2). The acidification probably inhibited the solubility of fecal compounds by affecting their ionization status in an aqueous medium. Water significantly improved the extraction yields of fatty acids and phenolic compounds compared with NaOH, whereas NaOH merely promoted the extraction of amino acids compared with deionized water. Such results indicate that the basic pH facilitates the solubility of the metabolites containing amino- or amide-functional groups, whereas fatty acids and phenolic compounds are preferably resolved in water. Considering that suitable extraction solvent must aim at recovering all classes of compounds**,** selecting water rather than acidified or alkalized water as the extraction solvent helped to obtain more information on fecal water metabolites.

Storage conditions of feces samples

For metabonomics research, the instant preparation of a large number of fecal water samples has been a hard task and a great challenge, and it may cause metabolite information distortion due to the metabolism of massive gut microorganisms and the alteration of their living environment in postcollection feces. Freezing the feces immediately after collection is an alternative method that can further benefit multicentric studies where the best standardization can be met if all samples are prepared in the same laboratory. It was found that the signals of amino acids were obviously stronger in fecal water prepared from frozen–thawed aliquots than from fresh aliquots of the same stool specimens. The scores plots of PCA ($R^2X = 0.944$) and PLS–DA ($R^2X = 0.521$, $R^2Y = 0.982$, $Q^2Y = 0.949$) models from UV-scaled data confirmed a clear separation between both preparations. The compounds with VIP values of the PLS–DA model greater than 1 were selected as the discriminating metabolites and further quantified. Table 3 indicates that all amino acids and two dicarboxylic acids (succinic acid and malic acid) were significantly increased in frozen fecal samples, and this was also supported by Saric and coworkers' work [36]. It is known that basic pH improves the extraction of amino acids, but this work indicates that no difference of pH occurred between fresh and frozen fecal water. It is presumed that these discriminating compounds were probably released from matrix during the freezing and thawing of fecal

Table 2: Differential compounds among samples extracted by deionized water, formic acid, and NaOH aqueous solution from the same human feces.

Compound	NaOH vs. formic acid			Water vs. formic acid			Water vs. NaOH		
	VIP value	*P* value (*t* test)	Fold	VIP value	*P* value (*t* test)	Fold	VIP value	*P* value (*t* test)	Fold
Fatty acids									
2-Methylbutyric acid	—	—	—	3.63	0.023	0.16 ↑	2.71	7.85×10^{-3}	0.15 ↑
Isovaleric acid	—	—	—	3.72	0.040	0.14 ↑	2.92	6.96×10^{-3}	0.16 ↑
Valeric acid	—	—	—	—	—	—	3.07	0.011	0.15 ↑
Hexanoic acid	—	—	—	5.53	0.016	0.19 ↑	3.22	0.024	0.13 ↑
Heptanoic acid	3.34	8.28×10^{-6}	0.28 ↑	3.97	1.34×10^{-3}	0.36 ↑	—	—	—
Succinic acid	—	—	—	6.61	2.34×10^{-5}	0.38 ↑	6.11	7.55×10^{-7}	0.40 ↑
Fumaric acid	—	—	—	1.556	1.37×10^{-3}	0.62 ↑	1.65	1.20×10^{-4}	0.91 ↑
Caprylic acid	2.40	3.54×10^{-9}	0.57 ↑	2.48	2.24×10^{-4}	0.57 ↑	—	—	—
Malic acid	—	—	—	2.12	5.70×10^{-4}	0.74 ↑	2.12	1.41×10^{-5}	1.04 ↑
Myristic acid	—	—	—	1.45	5.03×10^{-6}	0.53 ↑	—	—	—
Pentadecanoic acid	1.77	1.59×10^{-4}	0.29 ↑	3.29	3.67×10^{-7}	0.61 ↑	2.45	8.88×10^{-7}	0.32 ↑
Stearic acid	—	—	—	5.10	7.01×10^{-7}	0.40 ↑	5.59	1.90×10^{-7}	0.37 ↑
Heptadecanoic acid	—	—	—	1.60	1.32×10^{-6}	0.49 ↑	1.37	1.84×10^{-7}	0.39 ↑
Palmitic acid	1.83	0.010	−0.16 ↓	5.51	7.40×10^{-5}	0.29 ↑	4.79	5.92×10^{-8}	0.45 ↑
Phenolics									
Phenylacetic acid	4.52	0.021	0.04 ↑	6.18	0.0153	0.16 ↑	3.17	0.014	0.12 ↑
p-Cresol	—	—	—	—	—	—	1.00	0.034	0.09 ↑
3-Phenylpropionic acid	3.44	1.95×10^{-4}	0.15 ↑	4.21	3.8×10^{-3}	0.25 ↑	1.30	0.036	0.10 ↑
Amino acids									
Alanine	7.48	2.05×10^{-7}	0.55 ↑	6.169	9.63×10^{-6}	0.39 ↑	5.41	2.88×10^{-4}	−0.16 ↓
Valine	10.60	5.41×10^{-7}	0.70 ↑	8.08	2.50×10^{-5}	0.44 ↑	8.34	7.85×10^{-5}	−0.26 ↓
Leucine	11.47	1.08×10^{-9}	0.72 ↑	7.75	3.29×10^{-5}	0.37 ↑	9.86	1.05×10^{-7}	−0.35 ↓
Isoleucine	10.41	5.94×10^{-7}	0.71 ↑	7.96	2.20×10^{-5}	0.46 ↑	8.14	8.46×10^{-5}	−0.26 ↓
Proline	4.91	7.50×10^{-5}	0.55 ↑	4.42	1.92×10^{-5}	0.46 ↑	—	—	—
Aspartic acid	5.53	7.67×10^{-5}	0.49 ↑	—	—	—	5.94	7.48×10^{-5}	−0.50 ↓
Methionine	3.13	4.52×10^{-8}	0.71 ↑	1.76	1.58×10^{-5}	0.35 ↑	2.91	8.41×10^{-7}	−0.37 ↓
Glutamic acid	2.84	9.62×10^{-5}	0.29 ↑	2.51	5.89×10^{-6}	0.24 ↑	—	—	—
Phenylalanine	6.59	4.64×10^{-9}	0.73 ↑	3.84	5.85×10^{-4}	0.33 ↑	6.05	7.66×10^{-8}	−0.40 ↓
Lysine	3.52	5.99×10^{-8}	0.68 ↑	1.79	6.07×10^{-4}	0.35 ↑	3.27	5.36×10^{-6}	−0.33 ↓
Tyrosine	6.31	1.88×10^{-9}	0.84 ↑	2.84	5.74×10^{-6}	0.33 ↑	6.16	1.15×10^{-8}	−0.52 ↓
Unknown compounds									
Unknown-1	1.86	7.69×10^{-5}	0.74 ↑	1.50	1.02×10^{-3}	0.57 ↑	1.28	0.035	−0.17 ↓
Unknown-2	2.55	7.15×10^{-5}	0.59 ↑	1.82	1.61×10^{-6}	0.40 ↑	2.08	8.09×10^{-3}	−0.19 ↓
Unknown-3	—	—	—	1.34	1.31×10^{-7}	1.20 ↑	1.21	4.51×10^{-8}	0.61 ↑
Unknown-4	4.00	8.34×10^{-5}	1.22 ↑	2.59	1.48×10^{-7}	0.85 ↑	3.46	2.08×10^{-3}	−0.37 ↓
Unknown-5	1.13	7.11×10^{-6}	0.77 ↑	1.92	1.7×10^{-3}	0.46 ↑	1.01	5.54×10^{-4}	−0.31 ↓
Unknown-6	1.61	1.53×10^{-5}	0.31 ↑	2.37	1.13×10^{-6}	0.50 ↑	1.38	1.05×10^{-5}	0.19 ↑
Unknown-7	3.31	6.19×10^{-6}	0.50 ↑	—	—	—	3.70	1.16×10^{-5}	−0.65 ↓

samples. Considering the variations introduced by freezing–thawing, we recommend preparing fecal water from freshly collected fecal samples. However, it would be prudent to use frozen feces when a large batch of samples must be analyzed in an acceptable time frame.

Table 3: Quantitative analysis of discriminating metabolites obtained by PLS–DA model (VIP > 1) between fresh and frozen fecal samples.

Compound	Content (μg ml^{-1})[a]		VIP value	Student's *t* test (*P* value)
	Fresh feces	Frozen feces		
Leucine[b]	1.48 ± 0.84	29.37 ± 19.04	3.94	3.958×10^{-6}
Valeric acid	151.43 ± 31.13	123.69 ± 45.34	3.85	0.182
Valine[b]	1.72 ± 0.57	22.34 ± 11.97	3.43	2.533×10^{-7}
Succinic acid[b]	10.74 ± 6.12	62.67 ± 40.54	3.23	2.994×10^{-5}
Isoleucine[b]	0.76 ± 0.43	16.07 ± 9.23	3.13	5.242×10^{-7}
Alanine[b]	7.88 ± 2.64	62.70 ± 33.82	2.92	7.141×10^{-7}
3-Phenylpropionic acid	40.58 ± 24.64	29.15 ± 21.52	2.86	0.322
Phenylacetic acid	46.03 ± 28.61	48.52 ± 47.14	2.79	0.594
Hexanoic acid	42.21 ± 70.95	33.81 ± 42.39	2.67	0.467
2-Methylbutyric acid	99.41 ± 53.31	93.74 ± 71.65	2.66	0.900
Glutamic acid[b]	59.98 ± 37.91	522.66 ± 154.09	2.43	4.214×10^{-12}
Proline[b]	1.98 ± 1.09	16.41 ± 7.23	2.21	1.932×10^{-8}
Isovaleric acid	72.91 ± 38.00	69.47 ± 54.40	2.16	0.851
Tyrosine[b]	8.38 ± 3.39	109.72 ± 58.91	2.11	2.696×10^{-7}
Malic acid[b]	1.476 ± 1.069	30.09 ± 11.97	2.1	4.655×10^{-10}
Phenylalanine[b]	1.75 ± 0.87	22.92 ± 13.88	1.91	1.956×10^{-6}
3-Hydroxyphenylacetic acid	13.20 ± 5.91	9.34 ± 6.41	1.24	0.349
Glutaric acid	31.25 ± 12.59	20.13 ± 13.94	1.21	0.350

[a]Values are means ± standard deviations.
[b]The discriminating compounds with VIP values of the PLS–DA model greater than 1 were further significantly validated by quantitative analysis with Student's *t* test ($P < 0.05$).

Method validation

The quantitative performance of the extraction and derivatization methods coupled with GC/MS analysis was investigated in terms of linearity and sensitivity. Using the optimized derivatization and GC/MS methods, a regression coefficient higher than 0.99 for each calibration curve from the spiked standards was obtained, indicating good linearity. Exceptions were three fatty acids (palmitic, stearic, and oleic acids) and two phenolic acids (4-hydroxybenzoic and 3-(4-hydroxyphenyl)propionic acids) that showed weak linearity at high concentrations, presumably due to the low solubility in an aqueous reaction system; thus, the data of high concentration were excluded in calculating R^2 for these substances. The LODs of most of the test standards were 10 to 500 pg on-column, indicating that this developed method is appropriate to analyze fecal water.

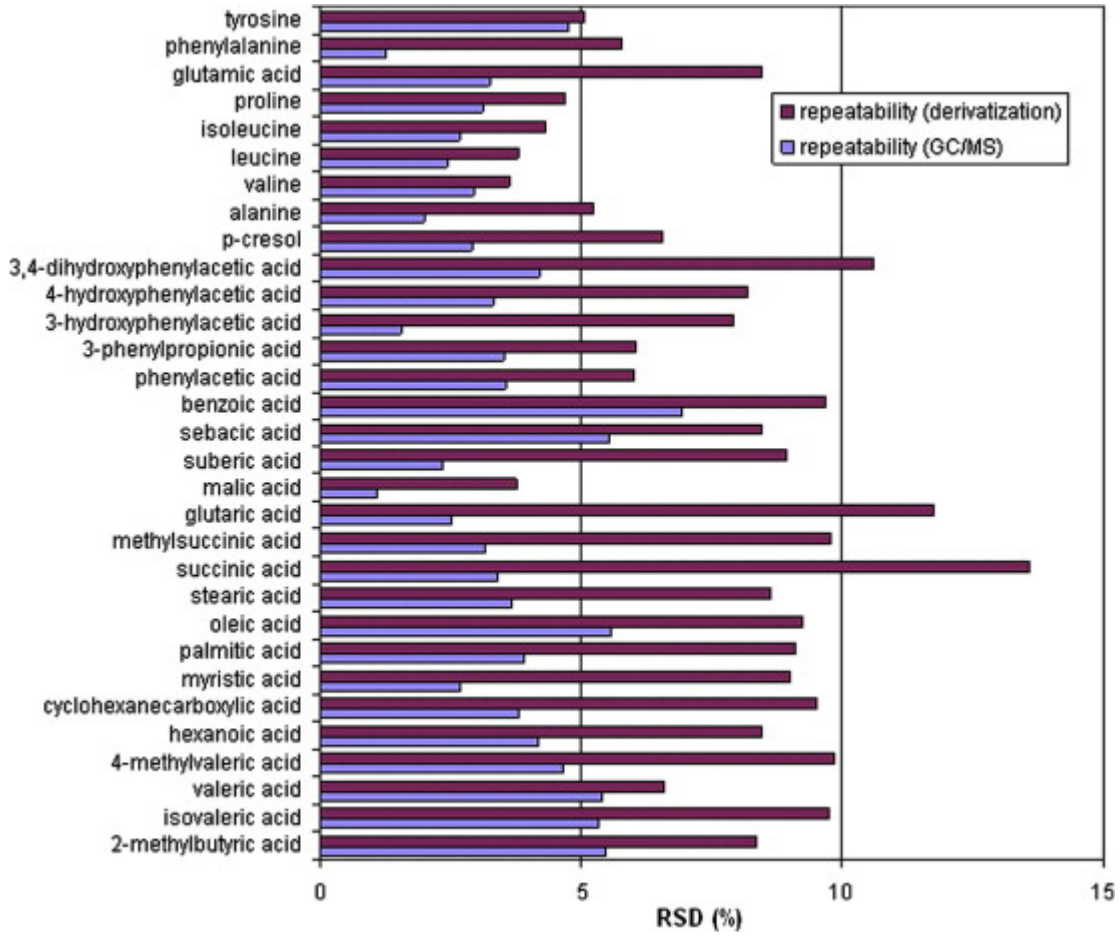
The analytical equipment exhibited excellent repeatability, with the relative standard deviations (RSDs) lower than 4% for all test standards (Table 4) and lower than 7% for 31 test compounds identified in fecal water samples (Fig. 6). The derivatization method also showed good repeatability, with RSDs lower than 6.4% for test standards (with the exception of 3,4-dihydroxyphenylacetic acid) (Table 4) and lower than 10% for 28 test compounds (except for two dicarboxylic acids and one phenolic compound) in fecal water from healthy volunteers (Fig. 6). The derivatives showed good stability for 48 h, and no obvious degradation occurred if stored at −80 °C for 2 months.

Table 4: Linearity, limit of detection (LOD), and repeatability for GC/MS analysis of 34 standards.

Compound	R^{2a}	Linear range (μg ml^{-1})	*n*	LOD[b]		Repeatability (RSD)[c]	
				Picograms on-column	Signal/noise	GC/MS	Derivatization
Monocarboxylic acids							
2-Methylbutyric acid	0.9983	0.1–25	9	100	26	3.29	2.93
Isovaleric acid	0.9978	0.1–25	9	100	31	2.89	5.54
Valeric acid	0.9976	0.25–25	8	250	37	2.57	3.39
4-Methylvaleric acid	0.9978	0.25–25	8	100	21	1.75	3.34
Hexanoic acid	0.9982	0.05–25	10	50	10	2.12	3.99
Cyclohexane carboxylic acid	0.9978	0.1–25	9	50	22	3.26	2.70
Nonanoic acid	0.9972	0.05–25	10	50	11	2.46	3.48
Myristic acid	0.9929	0.05–25	10	10	18	2.18	4.55
Palmitic acid	0.9871	0.05–12.5	9	10	8	2.10	4.95
Oleic acid	0.9951	0.5–12.5	6	250	3.6	3.87	5.92
Stearic acid	0.9829	0.05–12.5	9	10	19	2.80	5.75
Dicarboxylic acids							
Succinic acid	0.9942	0.5–25	7	500	23	1.68	4.97
Methylsuccinic acid	0.9946	0.25–25	8	250	26	1.16	6.40
Glutaric acid	0.9960	0.5–25	7	500	17	2.04	2.80
Malic acid	0.9943	0.5–25	7	250	3.5	2.75	3.26
Suberic acid	0.9997	0.5–25	7	250	7	1.61	2.31
Sebacic acid	0.9984	0.5–25	7	250	9	1.82	2.94
Phenolics							
p-Cresol	0.9985	0.25–25	8	100	8	1.95	2.60
Benzoic acid	0.9974	0.05–25	10	10	12	3.46	4.78
Phenylacetic acid	0.9990	0.25–25	8	250	25	1.96	2.90
3-Phenylpropionic acid	0.9990	0.25–25	8	100	5	1.39	2.67
4-Hydroxybenzoic acid	0.9982	0.5–12.5	6	250	8	1.46	3.62
3-Hydroxyphenylacetic acid	0.9995	0.5–25	7	250	4	1.27	2.78
4-Hydroxyphenylacetic acid	0.9937	0.5–25	7	250	8.6	1.10	2.79
3-(4-Hydroxyphenyl)propionic acid	0.9994	0.5–12.5	6	500	10	0.95	2.94
3,4-Dihydroxyphenylacetic acid	0.9908	2.5–25	5	2500	11	3.47	13.84
Amino acids							
Alanine	0.9977	1–25	6	1000	18	1.90	2.37
Valine	0.9993	0.25–25	8	100	7	1.53	2.04
Leucine	0.9996	0.25–25	8	100	10	1.32	1.45
Isoleucine	0.9993	0.1–25	9	100	13	1.80	2.54
Proline	0.9953	0.25–25	8	100	4	1.80	2.50
Glutamic acid	0.9936	2.5–25	5	2500	12	3.92	8.01
Phenylalanine	0.9998	0.5–25	7	250	5.6	1.35	0.69
Tyrosine	0.9987	2.5–25	5	1000	8.7	3.52	3.39

[a]Regression coefficients were calculated for linearity ranging at the concentration listed here.
[b]LOD (pg on-column) is the lowest calibration standard injected with a signal/noise ratio ⩾3, where the signal/noise ratio calculation was carried out to display the peak-to-peak values by Agilent ChemStation software.
[c]Repeatability of the test compounds from the spiked standard solution (5 μg ml^{-1}) with the same derivatization sample (GC/MS) and different derivatization samples (derivatization) with the same spiked standard solution (n = 7).

Figure 6: Repeatability of 31 test compounds from fecal water samples, including GC/MS instrument (n = 6) and derivatization method (n = 5).



Qualitative and quantitative analysis on human fecal water

Using the validated method combining sample derivatization of ECF and GC/MS analysis, fecal water samples from healthy human subjects were analyzed. Approximately 180 metabolites were identified from human fecal water samples; of these, 57 compounds were structurally confirmed by comparing their RTs and EI mass spectra with reference standards available in our lab (Table 5). The unknown peaks were further investigated by search against mass spectral databases such as NIST05, NBS, and Wiley, where candidate compounds were listed according to structural possibility. The EI ion source provided weak information about molecular ion. To further validate the results provided by library research, GC/MS in CI mode using methane reagent gas was conducted to obtain more information about molecular weights. At first, the reference standards were injected. The ions $[M+1]^+$, $[M+29]^+$, and $[M+41]^+$, which represent methane adducts [37], were clearly present in all CI spectra of test standards. Such characteristics positively confirmed the correct assignment of the $[M+1]^+$ ion in CI spectra. From the molecular weights (MWs) obtained from CI spectra and the reaction principle of ECF derivatization (Fig. 2), we preliminarily validated 16 candidate compounds provided by library research (summarized in Table 5).

Table 5: Compounds identified and confirmed in human fecal water.

No.	Fecal compounds		ECF derivatives of fecal compounds					
	Name	MW	RT	MW	EI mass spectrum[a](*m/z*)	CI (*m/z*)		
						$[M+H]^+$	$[M+C_2H_5]^+$	$[M+C_3H_5]^+$
1	Butanoic acid	88	5.52	116	*71*, 88, 43, 60, 101	117	145	157
2	2-Methylbutyric acid	102	6.23	130	*57*, 102, 41, 74, 85	131	159	171
3	Isoveleric acid	102	6.29	130	*88*, 85, 57, 41, 60	131	159	171
4	Valeric acid	102	7.27	130	*88*, 85, 57, 41, 101	131	159	171
5	*N*-Methylalanine[b]	103	8.37	131	*116*, 44, 86, 72, 58	132	160	172
6	4-Methylvaleric acid	116	8.55	144	*88*, 101, 43, 81, 55	145	173	185
7	Hexanoic acid	116	9.25	144	*88*, 99, 43, 101, 60	145	173	185
8	*n*-Propylamine[b]	59	9.75	131	*102*, 43, 131, 58, 86	132	160	172
9	*N,N*-Dimethyl-l-alanine[b]	117	10.24	145	*116*, 44, 58, 72, 100	146	174	186
10	Propanedioic acid	104	10.70	160	*115*, 133, 43, 88, 60	161	189	201
11	Heptanoic acid[b]	130	11.22	158	*88*, 113, 43, 101, 60	159	187	199
12	3-(Methylthio)propionic acid	120	11.28	148	*74*, 148, 75, 61, 103	149	177	189
13	Butylamine[b]	73	11.67	145	*102*, 145, 115, 133	146	174	186
14	Cyclohexane carboxylic acid	128	11.91	156	*83*, 55, 101, 156, 111	157	185	197
15	Benzoic acid	122	12.64	150	*105*, 77, 122, 87, 150	151	179	191
16	Succinic acid	118	12.78	174	*101*, 129, 174	175	203	215
17	Fumaric acid	116	12.88	172	*127*, 99, 71, 54	173	201	213
18	Caprylic acid	144	13.09	172	*88*, 101, 127, 57, 73	173	201	213
19	Methylsuccinic acid	132	13.27	188	*143*, 115, 142, 73, 87	189	217	229
20	1-Piperidine carboxylic acid[b]	129	13.48	157	*128*, 84, 157, 56, 42	158	186	198
21	Phenylacetic acid	136	14.00	164	*91*, 164, 65	165	193	205
22	Alanine	89	14.46	189	*116*, 44, 72	190	218	230
23	Glutaric acid	132	14.59	188	*143*, 114, 87, 142, 188	189	217	229
24	Nonanoic acid	158	14.84	186	*88*, 101, 141	187	215	227
25	Indole[b]	117	14.90	—	*117*, 90, 89, 63, 57	118	146	158
26	Glycine	75	15.22	175	*102*, 90, 144, 73, 41	176	204	216
27	*p*-Cresol	108	15.62	180	*108*, 107, 77, 180	181	209	221
28	Cyclohexylamine[b]	99	15.76	171	*128*, 142, 171, 56	172	200	212
29	3-Phenylpropionic acid	150	15.87	178	*104*, 91, 178, 105	179	207	219
30	Hexanedioic acid[b]	146	16.42	202	*157*, 111, 128, 115, 55	203	231	243
31	Valine	117	16.62	217	*144*, 72, 116	218	246	258
32	Leucine	131	17.97	231	*158*, 102, 44, 58	232	260	272
33	Pimelic acid	160	18.25	216	*125*, 129, 171, 101, 69	217	245	257
34	Isoleucine	131	18.26	231	*158*, 102, 129, 69, 74	232	260	272
35	9-Oxo-nonanoic acid[b]	172	18.64	200	*88*, 157, 55, 101, 83	201	229	241
36	Proline	115	18.70	215	*142*, 70, 98, 41, 215	216	244	256
37	Malic acid	134	20.01	262	*71*, 117, 89, 43, 55	263	291	303
38	Suberic acid	174	20.22	230	*185*, 143, 138, 88, 115	231	259	271
39	Dodecanic acid	200	20.35	228	*88*, 101, 183, 73, 157	229	257	269
40	Aspartic acid	133	21.57	261	*188*, 116, 70, 142	262	290	302
41	Nonanedioic acid[b]	188	22.21	244	*199*, 152, 157, 55, 83	245	273	285
42	3- or 4-Hydroxybenzoic acid	138	22.86	238	*121*, 91, 138, 120, 149	239	267	279
43	Methionine	149	22.99	249	*61*, 175, 129, 176, 116	250	278	290
44	Glutamic acid	147	23.96	275	*202*, 128, 84, 156, 56	276	304	316
45	3-Hydroxyphenylacetic acid	152	24.07	252	*107*, 180, 108, 179	253	281	293
46	Sebacic acid	202	24.17	258	*213*, 171, 84, 240, 125	259	287	299
47	Myristic acid	228	24.28	256	*88*, 101, 43, 55, 157	257	285	297
48	4-Hydroxyphenylacetic acid	152	24.55	252	*107*, 135, 180	253	281	293
49	Phenylalanine	165	25.33	265	*176*, 192, 91, 102, 120	266	294	306
50	3-(2-Hydroxyphenyl)propionic acid	166	26.08	266	*120*, 149, 194, 91, 107	267	295	307
51	Pentadecanoic acid[b]	242	26.21	270	*88*, 168, 101, 43, 55	271	299	311
52	3-(3- or 4-Hydroxyphenyl)propionic acid	166	26.63	266	*120*, 107, 123, 135, 194	267	295	307
53	3-(3- or 4-Hydroxyphenyl)-2-propenoic acid[b]	164	27.02	264	*176*, 222, 107, 120, 148	265	293	305
54	Homovanillic acid	182	27.74	282	*137*, 210, 282, 165, 122	283	311	323
55	Palmitic acid	256	28.10	284	*88*, 101, 43, 284, 55	285	313	325
56	*p*-Coumaric acid	164	28.86	264	*147*, 192, 120, 164, 264	265	293	305
57	Hydroferulic acid	196	29.67	296	*137*, 150, 224, 296, 91	297	325	337
58	Heptadecanoic acid[b]	270	29.91	298	*88*, 101, 43, 55, 120	299	327	339
59	Pyrogallol	126	30.00	342	*126*, 97, 154, 225, 108	343	371	383
60	Tyramine	137	30.71	281	*120*, 107, 102, 192,	282	310	322
61	Linoleic acid	280	31.12	308	*67*, 81, 95, 55, 109, 308	309	337	349
62	Oleic acid	282	31.21	310	*55*, 69, 41, 88, 83	311	339	351
63	*cis*-11- or 13-Octadecenoic acid[b]	282	31.40	310	*55*, 69, 41, 83, 97	311	339	351

64	Stearic acid	284	31.67	312	*88*, 101, 312, 43, 55	313	341	353
65	Lysine	146	31.81	318	*156*, 84, 56, 102, 128	319	347	359
66	3,4-Dihydroxyphenylacetic acid	168	31.99	340	*123*, 196, 223, 135, 151	341	369	381
67	Ferulic acid	194	32.11	294	*222*, 150, 177, 145, 294	295	323	335
68	Phloroglucinol	126	32.82	342	*126*, 154, 98, 69, 110	343	371	383
69	2,3- or 2, 5- or 3, 5-Dihydroxyphenylacetic acid[b]	168	33.03	340	*123*, 196, 124, 267, 151	341	369	381
70	3,4-Dihydroxyphenylpropionic acid	182	33.78	354	*136*, 123, 210, 164, 237	355	383	395
71	Tyrosine	181	34.83	353	*107*, 192, 264, 135, 102	354	382	394
72	Sinapinic acid	224	35.15	324	*252*, 180, 207, 324, 280	325	353	365
73	Tryptophan	204	36.15	304	*130*, 55, 304	305	333	345

[a]Ions in italics were base peaks.
[b]Results were obtained by researching the EI mass spectral database and were further validated by CI mass spectra.

Table 5 indicates that the predominant compounds were carboxylic acids, especially fatty acids, amine, amino acids, and phenolic compounds. The current method could not derivatize the inactive compounds such as carbohydrates, alcohol, and trimethylamine. Formic acid, acetic acid, and propionic acid could not be detected because of the low boiling point of their derivatives. In contrast to the NMR method, the current method identified and qualified more compounds, especially metabolites present in low amounts in fecal water. Bile acids in fecal water are a class of important compounds affecting intestinal health. The current method can derivatize only carboxylic groups of bile acid rather than hydroxylic groups; as a result, their derivatives were eluted at the region of extremely high column temperature, which is out of the scope of this method. In addition, the mass intensity is significantly lower than that of the other kinds of compounds in this research.

The compounds present in healthy human fecal water were analyzed quantitatively using the calibration curves of reference standards. Table 6 showed that the predominant components were valeric, isovaleric, 2-methylbutyric, hexanoic, phenylacetic, 3-phenylpropionic, and glutamic acids in healthy human fecal water. A high standard deviation is a typical characteristic of human fecal specimens, and this may be a difficulty when searching for differences in fecal water metabolome of human subjects having pathologies.

Table 6: Quantitation of test standards in healthy human fecal water samples (n = 8, μg ml^{-1}).

Compound	Content[a]	Compound	Content[a]
Valeric acid	337.74 ± 128.32	Phenylalanine	6.90 ± 6.96
Isovaleric acid	165.74 ± 68.68	Leucine	6.66 ± 8.05
Phenylacetic acid	119.54 ± 57.96	Valine	5.81 ± 6.45
3-Phenylpropionic acid	77.30 ± 32.98	Suberic acid	4.06 ± 3.31
Hexanoic acid	77.00 ± 89.84	Cyclohexane carboxylic acid	3.73 ± 7.36
2-Methylbutyric acid	75.16 ± 23.11	3,4-Dihydroxyphenylacetic acid	3.72 ± 0.87
Glutamic acid	71.30 ± 46.31	4-Hydroxyphenylacetic acid	3.32 ± 1.36
Palmitic acid	31.34 ± 51.35	Isoleucine	3.26 ± 3.56
Glutaric acid	24.99 ± 16.57	Malic acid	3.01 ± 2.68
Tyrosine	20.70 ± 17.09	3-(4-Hydroxyphenyl)propionic acid	2.68 ± 2.52
Stearic acid	20.32 ± 36.73	4-Methylvaleric acid	2.38 ± 1.34
Succinic acid	15.65 ± 17.93	Sebacic acid	2.24 ± 2.07
Oleic acid	15.55 ± 22.61	Proline	2.20 ± 1.16
p-Cresol	14.77 ± 7.49	Benzoic acid	0.80 ± 0.59
3-Hydroxyphenylacetic acid	13.09 ± 8.54	4-Hydroxybenzoic acid	0.75 ± 0.61
Alanine	12.23 ± 8.29	Myristic acid	0.65 ± 1.62
Methylsuccinic acid	10.77 ± 6.19	Nonanoic acid	—

[a]Values are means and standard deviations.

Both dietary intake and physiological status affect the pH value and chemical composition of human fecal water. Such factors may cause interindividual variability [15]. Although this method has been validated in a wide range of concentrations, the data reported in the current work might

not reflect an average profile of fecal water of healthy subjects; therefore, great care should be taken when studying clinical samples.

CONCLUSIONS

Fecal water is a complex mixture of varieties of metabolites with a wide range of physicochemical properties and boiling points. The analytical method developed here provides a qualitative and quantitative GC/MS, with high sensitivity and efficiency, coupled with derivatization of ECF in aqueous medium. The parameters affecting derivatization efficiency and GC chromatographic separation were discussed and optimized. The pH of extraction solvent and storage conditions of fecal samples were investigated based on multivariate statistical analyses (PCA and PLS–DA) and univariate statistical validation (Student's *t* test), where deionized water exhibited better extraction properties. The proposed protocol was validated extensively on the basis of linearity, sensitivity, and repeatability of analytical equipment and the derivatization method. The qualitative means combining EI mass spectral database searching, CI mass spectra validation, and reference substance comparison identified and structurally confirmed 73 compounds in human fecal water, suggesting a promising approach of human fecal water metabolome characterization.

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