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High throughput and quantitative measurement of microbial metabolome by gas chromatography/mass spectrometry using automated alkyl chloroformate derivatization

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Abstract

The ability to identify and quantify small molecule metabolites derived from gut microbial-mammalian co-metabolism is essential for the understanding of the distinct metabolic functions of the microbiome. To date, analytical protocols that quantitatively measure a complete panel of microbial metabolites in biological samples have not been established, but urgently needed by the microbiome research community. Here, we report an automated high-throughput quantitative method using a gas chromatography/time-of-flight mass spectrometry (GC/TOFMS) platform to simultaneously measure over one hundred microbial metabolites in human serum, urine, feces and *Escherichia coli* cell samples within 15 minutes per sample. A reference library was developed consisting of 145 methyl and ethyl chloroformate (MCF and ECF) derivatized compounds with their mass spectral and retention index information for metabolite identification. These compounds encompass different chemical classes including fatty acids, amino acids, carboxylic acids, hydroxylic acids and phenolic acids, as well as, benzoyl and phenyl derivatives, indoles, etc., that are involved in a number of important metabolic pathways. Within an optimized range of concentrations and sample volumes, most derivatives of both reference standards and endogenous metabolites in biological samples exhibited satisfactory linearity ($R^2 > 0.99$), good intra-batch reproducibility and acceptable stability within 6 days (RSD<20%). This method was further validated by examination of the analytical variability of 76 paired human serum, urine, and fecal samples as well as quality control samples. Our method involved using high-throughput sample preparation, measurement with automated derivatization and rapid GC/TOFMS analysis. Both techniques are well suited for microbiome metabolomics studies.

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Supporting Information **Available:** Further details are given in the supplemental Figure S1–5 and Table S1–5. This material is available free of charge via the Internet at http://pubs.acs.org.

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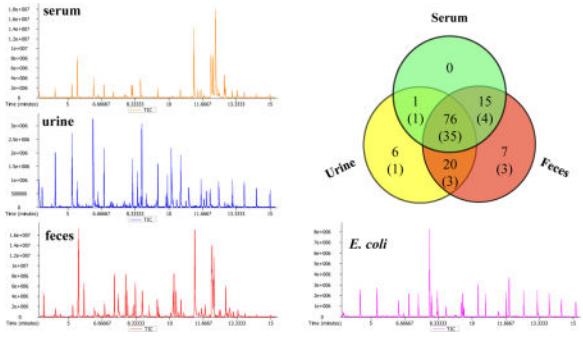

INTRODUCTION

Gut dysbiosis has been associated with various diseases, including obesity[1], diabetes[2], non-alcoholic fatty liver disease[3], inflammatory bowel diseases[4], and cancer[5]. A better understanding of the contribution that variations in gut microbiota metabolites make to host disease risk and health sustainability will assist in the development of new strategies for disease prevention and therapeutic intervention[6,7]. The host and symbiotic gut microbiota coproduce a large array of small molecule metabolites during the metabolism of food and xenobiotics, many of which play critical roles in shuttling information between host cells and the microbial symbionts[8]. Recent studies have indicated that the metabolic variations in host's body fluids and tissues were directly related to the activities of various microorganisms that coexist in the gut[9]. Any intercellular metabolic transformation (metabolic fingerprint) due to the differences in microbial communities could cause significant alterations of the extracellular metabolome in the host (metabolic footprints). While it is useful to understand changes in gut microbial phyla/species that affect host health, it is much more useful to characterize changes in microbial metabolites that can be analyzed in easily obtainable body fluids such as plasma or urine and correlate changes in microbial metabolites with a patient's condition. Such an approach adds functionality to the metagenomics analysis, thus linking meta-genotypes to their metabolic phenotypes of the host.

Important small molecule metabolites that regulate host-microbiota interactions include short-chain fatty acids[10], amino acids[11], phenolic, benzoyl and phenyl derivatives[12], indole derivatives[13], lipids[14], bile acids[15], choline[16], vitamin[17], polyamines[18], etc. Our previous study was a metabolic profile of the metabolic footprints of gut microbial-mammalian co-metabolism in rats exposed to antibiotic. A combined gas chromatography/ mass spectrometry (GC/MS) and liquid chromatography/ mass spectrometry (LC/MS) approach was used and the result was a panel containing 202 urinary and 223 fecal metabolites that were considered as potential readouts of the co-metabolism effect[12]. More recently, a strategy for the targeted metabolomics analysis of 11 gut microbiota-host co-metabolites in rat serum, urine and feces was developed and employed ultrahigh performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS)[19]. To our knowledge there has

been no publication that proposes a metabolomics method for the identification and quantification of a large set of microbial metabolites.

The simultaneous determination of numerous gut microbiota-host co-metabolites with as few platforms as possible in complex biological samples is challenging, due to the fact that they have diverse structures with varied chemical and physical properties. The derivatization technique employed in this GC/MS study was the alkyl chloroformate derivatization proposed by Husek[20], which allows simultaneous esterification of carboxylic group, amino group and hydroxyl group linked to benzene ring or joined to the side chain, creating alkyl esters or N(O)-alkoxycarbonyl ethers, respectively. In contrast to the popular derivatization approach of silylation, alkyl chloroformate derivatization has advantages in being faster (about 1 min), it involves milder reaction conditions (ie., aqueous medium and room temperature), has better reproducibility and greater stability[21]. The combination of these factors therefore makes the derivatization protocol achievable using an automated robotic workstation. Methods based on alkyl chloroformate derivatization for metabolomics application have been published by our lab[22–25] and others[26–29]. Most of them use methyl chloroformate (MCF)[26–28] or ethyl chloroformate (ECF)[22–24,29], but other chloroformate compounds have been used as well[25]. The performance for quantification of one or two chemical classes of compounds such as amino acids[26,28,29], non-amino organic acids[26,28,29], fatty acids[25], as well as phenolic acids[30] based on chloroformate derivatization has been reported previously in biological cells and fluids. However, no such method has been optimized for the simultaneous measurement of all the aforementioned compounds and the many more that exist related to gut microbiota.

In this work, we developed a practical and feasible method of targeted identification and quantification of as many metabolites as possible associated with gut microbiota-host co-metabolism. These results will enable us to acquire wider insights on the functioning of the symbiotic supraorganism system. To the best of our knowledge, the current study represents the first comprehensive alkyl chloroformate (methyl- and ethyl-) derivative library containing mass spectral/retention index (MS/RI) information for 145 structurally diverse compounds all of which were acquired using automated derivatization via a commercially available robotic workstation and GC/time-of-flight MS (GC/TOFMS) analysis. The sample preparation and GC separation parameters were optimized to produce a rapid, simple and sensitive method for simultaneous measurement of 92, 103, 118 and 52 compounds in human serum, urine, feces, and *Escherichia coli* (*E. coli*) cell, respectively within 15 minutes. This automated and high-throughput method, which has been validated using a large range of reference standards and biological samples, is well suited for future microbiome metabolomics research.

EXPERIMENTAL SECTION

Chemicals

The derivatization regents, MCF and ECF, as well as HPLC grade solvents including methanol, ethanol, chloroform and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, sodium bicarbonate and anhydrous sodium sulfate were of analytical grade and obtained from JT Baker Co. (Phillipsburg, NJ). All standard compounds

were commercially purchased from Sigma-Aldrich and Nu-Chek Prep (Elysian, MN, USA). Ultrapure water was prepared by the Milli-Q system (Millipore, Billerica, MA).

The stock solutions of all reference standards were prepared in HPLC grade methanol or ultrapure water with a concentration of either 5 mg mL^{-1} or 1 mg mL^{-1}. The mixed working standard solutions containing methanol-soluble or water-soluble standards were prepared by dilution with solvents of the same chemical class. 145 representative compounds from different chemical classes (amino acids, fatty acids, carboxylic acids, hydroxyl acids, phenolic acids, indoles, etc.) were used. Further serial dilutions of the working standard solutions were made to generate the calibration curves. A mixture of internal RI markers was prepared by combining equal volumes of 5 mg mL^{-1} chloroform stock solutions of thirteen normal alkanes with carbon chain lengths, C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, and C30.

Sample preparation and GC/TOFMS analysis

We selected de-identified human biological samples from our sample bank for the method development, evaluation, and validation. There were 76-paired human serum, urine and feces samples. The pooled quality control (QC) samples employed in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or collected from volunteers. All samples were stored at −80 °C until analysis.

Extraction of metabolites from human serum, urine, and feces—Serum and urine samples were thawed on ice and prepared using the following procedure. Each aliquot of 100 μL urine sample was transferred to an auto-sampler glass vial and lyophilized using a Labconco freeze-dryer (Kansas City, MO). Serum samples required protein precipitation before lypholization. Briefly, 100 μL of serum samples were extracted with 300 μL of cold methanol in an Eppendorf microcentrifuge tube, and placed in a −20 °C freezer for 30 min. The extracts were centrifuged at 16,000 rcf and 4 °C for 10 min and the supernatant was immediately transferred to an auto-sampler glass vial and lyophilized. For fecal samples, 10 mg of lyophilized feces was homogenized with 300 μL of NaOH (1M) solution and centrifuged at 16,000 rcf at 4 °C for 20 min. Each 200 μL of supernatant was transferred into an auto-sampler vial, and the residue was further exacted with 200 μL of cold methanol. After the second step of homogenization and centrifugation, 167 μL of supernatant was combined with the first supernatant in the sample vial. The solids from serum and urine samples after the lyophilization process and aqueous fecal extracts were sealed and stored at −80 °C for a subsequent automated derivatization assay.

Extraction of intracellular metabolites from E. coli—An *E. coli* BL 21 cell line was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture and quenching of the cells were carried out according to a previous report[31]. Briefly, cells were harvested in a 50 mL conical tube. After centrifugation at 200 *g* and 4 °C for 10 min (Allegra X15R, Beckman Coulter, Brea, CA), the culture media was carefully removed and the cells were washed twice with 50 mL freshly-prepared phosphate buffered saline (PBS). The cells were re-suspended with 1 mL of PBS and the number of cells was counted with a TC20 Automated Cell Counter (Bio-Rad Laboratories Inc., Hercules, CA). The average cell number ideal for

the quantitation of microbial metabolites was 1×10^7. The cell lysates were homogenized with 50 μL of Millipore ultrapure water and extracted with 200 μL of cold methanol. After centrifugation at 16,000 rcf and 4 °C for 10 min, the supernatant was carefully transferred to an autosampler vial, lyophilized and stored at −80 °C prior to use.

Automated chloroformate derivatization—The sample derivatization protocols with MCF and ECF were based on the method described by Villas-Boas *et al*[26] and our previously published procedures[22], with some minor modifications. For routine large-scale sample analysis, sample derivatization and all liquid handling were performed by a commercially available robotic workstation (GERSTEL MPS Autosampler). MCF and ECF derivatization procedures were processed under exactly the same parameters. The only difference was the use of methanol for MCF derivatization and ethanol for ECF derivatization respectively, in order to avoid the production of the mixture of methyl and ethyl chloroformate derivatives. Briefly, for serum and urine samples, the sealed glass vials containing solids after lyophilization were placed in a cooled tray at 4 °C for automated derivatization. The solids were first redissolved in 200 μL of sodium hydroxide solution (1M) and then mixed with 167 μL of methanol (or ethanol) and 34 μL of pyridine. 20 μL of MCF (or ECF) were added to the mixture and the samples were shaken vigorously for exactly 30 s. Another 20 μL of MCF (or ECF) were added again and samples shaken for another 30 s. Subsequently, 400 μL of chloroform/RIs mixture (385 μg mL^{-1} for each) (50:1 by vol.) were added and samples were shaken for 10 s followed by an addition of 400 μL of sodium bicarbonate solution (50 mM) and additional shaking for 10 s. Samples were then centrifuged at 2000 × g for 10 min at 4 °C in order to clearly visualize the double meniscus. The bottom chloroform phase was transferred to GC vials containing ~100 mg of anhydrous sodium sulfate.

Aqueous fecal extracts, after the above two-step extraction with sodium hydroxide solution followed by methanol (or ethanol) were then derivatized following the aforementioned procedure, omitting the initial addition of 200 μL of sodium hydroxide solution (1 M) and 167 μL of methanol or ethanol.

GC/TOFMS analysis—Samples were randomly analyzed by GC/TOFMS (Agilent 6890N gas chromatography coupled with a LECO Pegasus HT time-of-flight mass spectrometer) using our newly developed, optimized conditions. One μL of each derivatized sample was injected using a splitless injection technique into a DB-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; (5%-phenyl)-methylpolysiloxane bonded and cross-linked; Agilent J&W Scientific, Folsom, CA), with helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The solvent delay time was set to 2.5 min. The optimized temperature gradient was the following: 45 °C held for 1 min, then increased at a rate of 20 °C min^{-1} up to 260 °C and 40 °C min^{-1} to 320 °C, then held there for 2 min. The total time of analysis was 15.25 min. The temperature of the injection, transfer interface and ion source were set to 270, 270, and 220 °C, respectively. Electron impact ionization (70 eV) at examined m/z range of 38−650 was used. The acquisition rate was 20 spectra $second^{-1}$.

Data processing

Raw data from GC/TOFMS analysis were exported in NetCDF format to ChromaTOF software (v4.50, Leco Co., CA, USA) and subjected to the following preprocessing, baseline correction, smoothing, noise reduction, deconvolution, library searching, and area calculation. Individual compound identification was performed by comparing both MS similarity and Kovats RI distance with reference standards in the author-constructed alkyl chloroformate derivative library, utilizing a similarity score cutoff of more than 70%. Afterwards, data sets were exported to a CSV file where each datum was labeled with a sample name, compound name, Kovats RI, quantification mass, peak area and concentration. Multivariate analysis was performed using SIMCA 14 software (Umetrics AB, Umeå, Sweden).

RESULTS AND DISCUSSION

A MS/RI library of MCF and ECF derivatives

The application of mass spectra and retention index analysis has been proven to be an efficient technique for accurate compound identification in GC/MS-based metabolomics[32]. In this study, we compiled a MS/RI library consisting of MCF and ECF derivatives from 145 reference standards (Table 1). As shown in Figure 1A, these compounds span a large number of chemical classes, including fatty acids (29 %), amino acids and derivatives (26 %), carboxylic acids and derivatives (11 %), hydroxy acids and derivatives (6 %), phenols, phenylacetic acid, benzyl alcohols, benzoic acid and their derivatives (12 %), indoles (6 %), cinnamic acids, keto-acids, sugar acids and their derivatives (4 %), and other nitrogen-containing compounds generally found in human urine or feces (6 %). A detailed, tabulated analysis of the determined metabolites and their corresponding metabolic pathways are listed in Supporting Information Table S1. Our library enriched the number of MCF derivatives of amino acids and non-amino organic acids reported by Smart *et al*[27], especially previously unreported aromatic homocyclic or heterocyclic compounds. The inclusion of the classic Kovats RI parameter ensures that this newly compiled library is more reliable for unambiguous metabolite identification than previous libraries with only reference mass spectral or with both mass spectral and retention time. The classic Kovats RI parameter also makes possible a wider application by different laboratories in different GC separation conditions.

Reaction scheme was illustrated using a representative compound of tyrosine, which simultaneously contains amine (-NH_2), carboxyl (-COOH) and hydroxy (-OH) functional groups (Figure S1). Other compounds in Table 1, when treated with alkyl chloroformate, would react in the same way. The paired MCF/ECF derivatives for each compound have similar fragmentation patterns but slightly different RI, i.e., MCF derivatives with greater volatility had shorter chromatographic retention time than ECF derivatives. Figure S2 illustrated the identification process by interpretation of possible fragmentation mechanism and comparison of RIs of MCF/ECF derivatives for p-hydroxyphenylacetic acid, a microbial metabolite important for tyrosine metabolism. Different from the common methods that just rely on the similarity analysis of comparison with reference library, our library made it possible to mutually authenticate the fragmentation patterns and RIs between MCF/ECF

derivatives, which greatly increased the accuracy of compound identification in biological samples. Moreover, the accumulation of fragmentation mechanisms provides information that may be used to solve structure problems for unknown metabolites that have no available authentic standards but have similar chemical structures to known metabolites.

Microbial metabolites identified in human and microbial samples

Based on our library of MCF and ECF derivatives, a subset of 125 metabolites were identified in human samples, including 92 metabolites in serum, 103 in urine, and 118 in feces samples (Figure 1B). A total of 61% of the compounds were identified in all three body fluids, such as 3-(3-hydroxyphenyl)-3-hydroxypropanoic acid, 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, butyric acid, hippuric acid, phenylacetic acid, etc. A total of 29% were detected in two fluids (ie., 3-indolepropionic acid, 4-hydroxycinnamic acid, 4-hydroxyphenylpyruvic acid, putrescine, salicyluric acid, vanillic acid, etc.), and 10 % were unique for a specific fluid (ie., indole, cresol, pipecolic acid, ortho-hydroxyphenylacetic acid, N-methylnicotinamide, hydroxyphenyllactic acid, etc.).

E. coli strains are commonly present in human gut microbiota and the species has been the most widely studied prokaryotic model organism in microbiological research[33]. In this study, *E. coli* was used as a model to validate the microbial metabolites that were identified from human biospecimens. A total of 52 metabolites were detected in *E. coli* cells cultured in *vitro*, and 47 of them (90%) were found to overlap with human samples (Figure 1B). The total ion current (TIC) chromatograms of representative human and microbial samples, standards mixture and internal RI markers are illustrated in Figure 2.

Optimization of sample preparation and GC separation

Given that the influences of solvent-to-catalyst ratio, reaction temperature, reaction time and pH on derivatization efficiencies have been thoroughly studied in many previous publications[22,26], the focus of this work was to develop a fast, sensitive and reliable approach for high-throughput and large-scale microbiome metabolomics research. In our pilot study, methyl- and ethyl-chloroformate both yielded satisfactory derivatization efficiency in standard mixtures and biological samples (Figure S3). Therefore, for this current protocol, we chose MCF derivatization and performed the following optimization experiments.

Determination of the appropriate sampling amount range—Appropriate sampling amount helps to avoid GC column overloading and mass detector oversaturation and therefore improves the accuracy of relative quantification protocols. We examined the linear correlation of mass intensities of a wide range of volume/weight ratios for urine and feces samples. Table S2 shows that the majority of metabolites exhibited a good correlation coefficient (greater than 0.9900) within an appropriate range of sample loading. In this work, the optimal column loading volume of urine and lyophilized weight of feces was 100 μL and 10 mg, respectively.

Influence of the lyophilization process on metabolite analysis in urine and cell samples—Dehydration of samples via lyophilization has only recently been introduced for

use in metabolomics studies[26,27]. Lyophilization is an easy and safe way to effectively concentrate samples making it a very useful tool for metabolite profiling. However, the influence of the lyophilization process on the physical integrity of metabolites isolated from biological fluids and cells has not been completely studied. In this work, we compared the number of identified metabolites and their peak abundance, using fresh and lyophilized urine and *E. coli* cell samples. We found that the lyophilization process produced stronger signal intensities for most of metabolites identified in urine and cell samples, and as a result, a greater number of metabolites were identified with lyophilization compared to the procedure without lyophilization (data not shown). A possible explanation for this is that lyophilization increased compound solubility in the medium of derivatization, and thus, reduced the loss of volatile compounds.

Combination of preprocessing and derivatization of fecal samples—Human fecal samples, especially the aqueous extract, have recently received attention due to increased interest in exploring the relationships between symbiotic gut microflora and human health. In many previous studies[34], before being subject to derivatization, homogenization in water without pH adjustment was commonly applied in the preparation of fecal water. The protocol for MCF derivatization employed in our experiments allowed us to develop a simplified procedure that combined two steps, the preprocessing and derivatization of fecal samples as one. This combination processing protocol makes it more amenable for large-scale sample analyses that are common in metabolomics studies. We also did a comparison experiment of the one-step extraction with sodium hydroxide solution and the two-step extraction using sodium hydroxide solution followed by methanol (based on optimized ratio for MCF derivatization[26]), with the aim of increasing the extraction efficiency. Results showed that, compared to the one-step extraction, the two-step method improved the relative extraction efficiency of some metabolites, especially the medium and long-chain fatty acids (Figure S4).

Optimization of GC separation parameters—Large-scale metabolomics studies often have problems with large analytical variations over a long time due to the large sample size, but a fast analysis method can help to reduce this effect. In order to achieve the separation of as many metabolites in as short a run time as possible, programmed temperature parameters in GC were optimized, as shown in Table S3. In condition 1 which has a single run time of 28.85 min and only one stage of temperature raise, we found that a majority of compounds in Table 1 mainly distributed before 20 min, and only a small number of metabolites appeared after 20 min. So we changed the temperature gradient program from one-stage to two-stage, and compared the separation efficiencies of three temperature gradient rates in the first stage (condition 2, 3 and 4). Results showed that when the temperature gradient was increased from 10 °C/min to 20 °C/min, more metabolites were detected in both pooled serum and urine samples, with higher peak height, smaller peak width at half height (PWH) and higher peak purity (PP). As a consequence, condition 4 was chosen as the optimal analysis condition and the analysis time of a single run was reduced to 15.25 min from 28.85 min. (Table S3).

There is increasing interest in using short-chain fatty acids (SCFA) as biomarkers to study the relationship between gut microbial activity and the host's health status, particular in the area of obesity and metabolic disorder[35]. Therefore, a reliable method for the accurate separation and measurement of SCFA has gained importance. In this study the separation and identification of 12 SCFAs was achieved with good separation in less than 3 minutes (Figure S5), a superior result compared to our previously reported method[25].

Method Validation

Linearity and quantification limits—The linearity of response was determined by linear regression modeling according to a series of standards at different concentrations in solvent (Table S4). The correlation coefficient (R^2) value was greater than 0.9900 for most of compounds investigated with the ability to detect a wide concentration range. To be noted, some compounds such as 3-hydroxybutyric acid, 3-indolepropionic acid, 4-hydroxyphenylpyruvic acid, 5-hydroxy-*L*-tryptophan, etc., could not be detected at lower concentrations due to the detection limit, and some compounds such as hydrocinnamic acid, *L*-phenylalanine, *L*-glutamic acid, *L*-cysteine, etc., had quadratic regression at higher concentrations. Thus, these compounds were not reported in the result. Additionally, the quantification limit of each compound was determined by analyzing the signal-to-noise ratio (S/N) provided by ChromaTOF software.

Reproducibility of results—The reproducibility of the automated derivatization technique and the GC/TOFMS analysis were investigated by using both the standard mixtures and biological samples. Six independently prepared standard mixtures and samples were analyzed by successive replicate measurements, respectively. As showed in Table S4, most of the test compounds and metabolites identified in human serum, urine and fecal samples exhibited acceptable reproducibility with relative standard deviations (RSDs) smaller than 15%, excepting some compounds whose concentrations were close to the quantification detection limit.

Stability—The stability of derivatized analytes under different storage conditions was evaluated using human serum, urine and fecal samples. Samples, after automated derivatization, were separated into four aliquots and stored under four different sets of conditions including room temperature, 4 °C, −20 °C, and −80 °C, each for 0, 1, 2, 3, 4 and 6 days. The analysis error due to drift of instrument detector responses over long time periods were corrected using internal RI standards. Results indicated that better stability could be achieved under lower temperature (data not shown). Nearly 80% of the derivatized metabolites showed acceptable stability with RSD% less than 20% within 6 days when stored at −80 °C, in all of three different biological sample types (Table S5).

Application

Finally, we applied our method to comprehensively analyze 76 paired human serum, urine and fecal samples and *E. coli* BL 21 cellular extracts as well. Each of three kinds of human samples was derivatized using automation and analyzed in 5 batches. During each batch, there was a quality control (QC) sample for every 17 study samples. We assessed the variability of the derivatization and instrument analysis across batches using QC samples,

which were either commercially obtained or self-prepared using pooled samples from volunteers. As shown in the PCA scores plots (Figure 3), the QC samples were clustered closely relative to the rest of serum, urine and fecal samples, indicating the good reproducibility of our method. Table 2 shows the quantification results of over one hundred compounds in human and *E. coli* cell samples. Only those metabolites that were identified in over 80% of the human samples were included and quantified. This big panel of human and gut microbiota co-metabolites, particularly those metabolites that were simultaneously identified in multiple matrices, are likely to be of great importance in exploring host-gut microbiota metabolic interactions.

CONCLUSION

In this work, we developed an automated high-throughput sample derivatization and analysis method for the simultaneous identification of 92, 103, 118 and 52 microbial metabolites in human serum, urine, feces and *E. coli* cell samples, respectively, in a single run analysis of ~15 minutes. A combined MS/RI library of MCF and ECF derivatives from 145 structurally diverse compounds was constructed to aid in metabolite identification. The identified metabolites participate in multiple metabolic pathways related to host-gut microbiota co-metabolism. Our proposed method exhibited good linearity, reproducibility and stability. This method has potential as a powerful tool for quantitative microbiome metabolomics studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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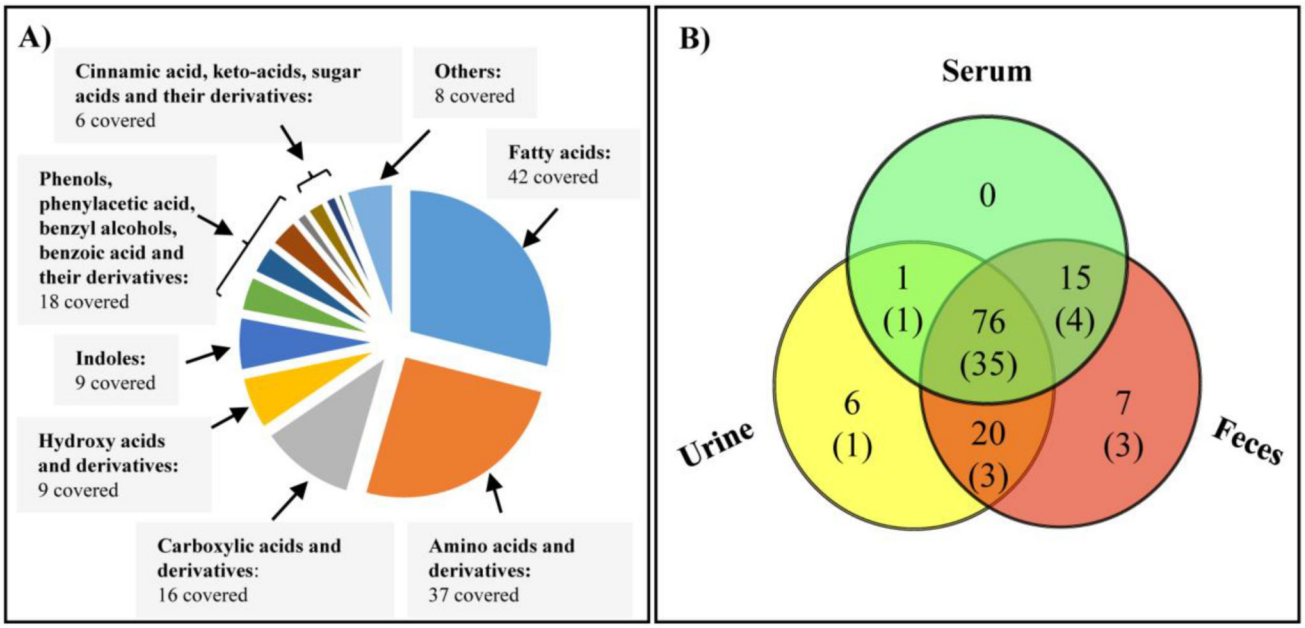

Figure 1.

(A) Pie chart showing chemical classification of the covering 145 compounds in author-constructed MCF & ECF derivatives library. (B) Venn diagram of a subset of 125 metabolites identified in human samples, including 92 in serum, 103 in urine, and 118 in feces. A total of 61% of the compounds were identified in all three body fluids, 29% were detected in two fluids, and 10 % were unique for a specific fluid. Among them, a total of 47 metabolites were also identified in *E. coli* cell, as the numbers in parenthesis shown.

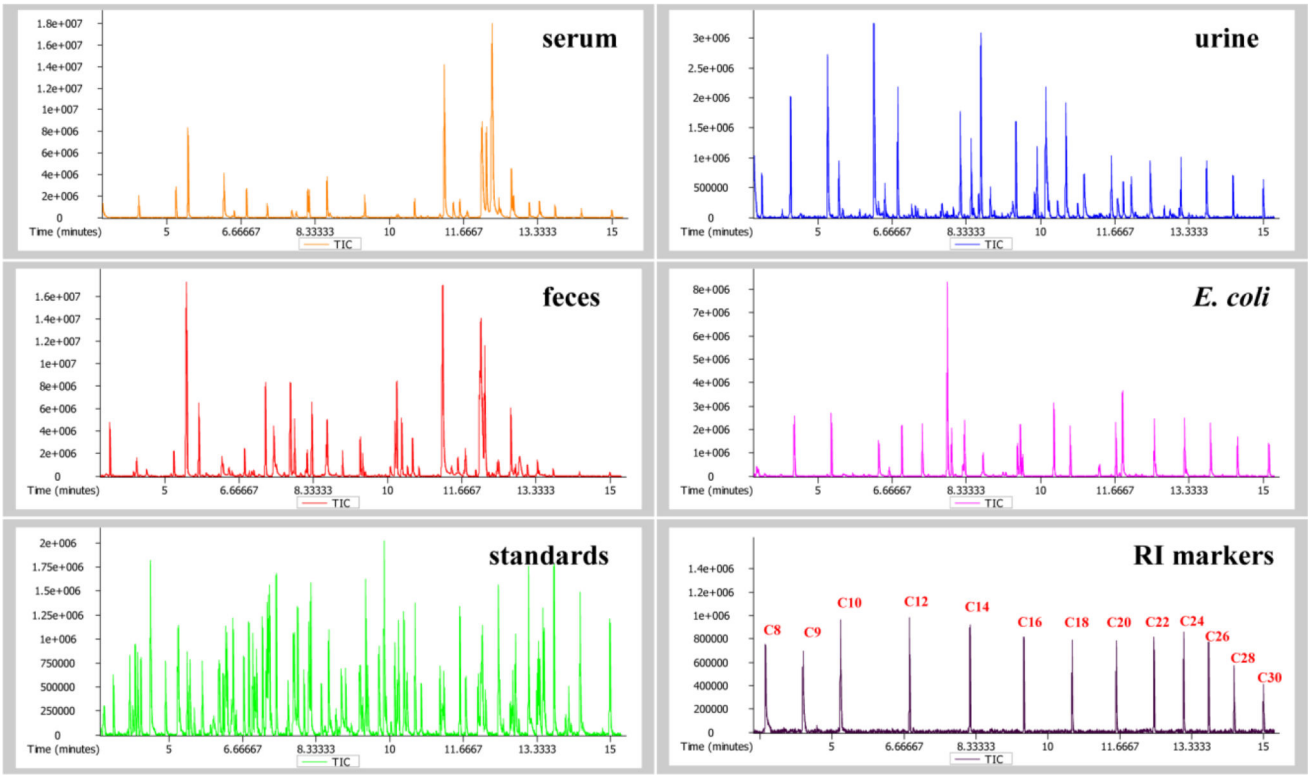

Figure 2.

GC/TOFMS total ion current (TIC) chromatograms of MCF derivatives in human serum, urine and feces samples, a sample of intracellular metabolites extracted from *E. coli* cell, a mixture of reference standards, and a mixture of 13 alkanes (C8-C30) which act as internal RI markers for the conversion of retention times to classic Kovats RI.

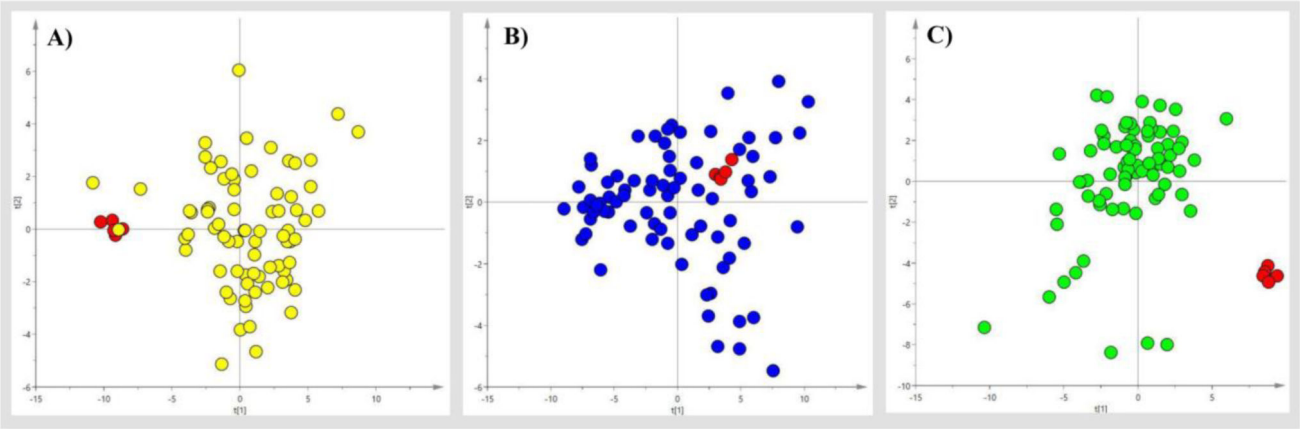

Figure 3.

PCA scores plots of 76 human serum (yellow circle), urine (blue circle) and feces samples (green circle) and their 5 QC samples that were either purchased commercially or collected from volunteers (red circle). (A) R^2X=0.504, two principal components; (B) R^2X=0.479, two principal components; and (C) R^2X=0.267, two principal components.

Table 1

Main fragments and Kovats RIs of 145 compounds in our library that were produced using MCF and ECF derivatization for GC/TOF-MS analysis.

No.	Compounds	MCF Derivatives		ECF Derivatives		Notes	No.	Compounds	MCF Derivatives		ECF Derivatives		Notes
		main fragments (m/z)#	RI	main fragments (m/z)#	RI				main fragments (m/z)#	RI	main fragments (m/z)#	RI	
1	(±)-2-Methylpentanoic acid	88, 43, 57, 71, 101	885	74, 43, 102, 55, 87	947	c	75_1	L-2-Hydroxyglutaric acid (m)	85, 47, 144	1241	85, 131,159, 57, 203	1684	bcd
2_1	2-Hydroxybutyric acid (m)	59, 45, 73, 117, 100	1108	59, 131, 87, 159, 176	1250	abc	75_2	L-2-Hydroxyglutaric acid (s)	71, 59, 99, 131, 175	1502	85, 57, 159	1314	
2_2	2-Hydroxybutyric acid (s)	59, 41, 89, 69	851	59, 41, 75, 89, 103	919		76	L-Alanine	70, 102, 129, 59, 42	1132	116, 44, 70, 88, 144	1279	abcd
3	2-Methylhexanoic acid	88, 43, 57, 69, 101	966	74, 43, 102, 56, 85	1029		77	L-Alpha-aminobutyric acid	84, 56, 116, 72, 103	1217	130, 58, 102, 86, 74	1362	abcd
4_1	2-Phenylglycine (m)	77, 132, 51, 164, 104	1657	132, 178, 77, 105, 205	1785		78	L-Asparagine	127, 59, 83, 95, 146	1407	141, 69, 95, 56, 113	1537	abcd
4_2	2-Phenylglycine (s)	77, 132, 51, 105, 191	1439	132, 77, 91, 105, 177	2029		79	L-Aspartic acid	86, 59, 128, 160, 96	1481	188, 70, 142, 100, 88	1661	abcd
5	2-Phenylpropionate	105, 77, 164, 51, 63	1219	77, 105, 178, 63, 91	1295		80	L-Cysteine	160, 59, 116, 132, 146	1707	220, 74,102, 132, 174	1885	abcd
6_1	3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid (m)	91, 178, 59,134, 238	1751	120, 91, 77, 149, 194	1886	abc	81_1	L-Cystine (m)	160, 59, 100, 132, 192	2382	74, 188, 90, 174, 220	2793	
6_2	3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid (s)	120, 77, 91, 107, 180	1595	91, 119, 120, 65, 50	1082		81_2	L-Cystine (s)	160, 59, 192, 100, 76	2601	74, 146, 174, 188, 102	2545	
7	3-Aminoisobutanoic acid	88, 56, 96, 115, 144	1262	102, 56, 74, 112, 129	1402	bc	82	L-Glutamic acid	114, 142, 174, 59, 82	1599	128, 84, 156, 56, 202	1772	abcd
8_1	3-Hydroxybutyric acid (m)	43, 74, 59, 87, 103	874	60, 43, 71, 87, 117	944	abc	83_1	L-Glutamine (m)	141, 109, 59, 68, 82	1546	155, 83, 56, 111, 43	1667	
8_2	3-Hydroxybutyric acid (s)	59, 69, 100, 85, 75	1124	69, 114, 131, 159, 99	1274		83_2	L-Glutamine (s)	128, 84, 56, 143, 70	1152	84, 56, 128, 173, 156	1934	
9	3-Hydroxyhippuric acid	179, 92, 135, 107, 208	2174	121, 149, 92, 193, 223	2365		84	L-Histidine	81, 59, 139, 194, 210	2084	81, 136, 154, 238, 254	2272	abcd
10_1	3-Hydroxyisovaleric acid (m)	43, 59, 85, 117, 74	891	43, 59, 85, 131, 103	959	abc	85	L-Homoserine	56, 100, 115, 83, 70	1378	100, 56, 70, 129, 115	1473	abcd
10_2	3-Hydroxyisovaleric acid (s)	73, 44, 56, 117, 90	1149	43, 59, 83, 128, 173	1301		86	Linoleic acid	55, 67, 81, 95, 294	2096	67, 55, 81, 95, 109	2158	ac
11	3-Hydroxyphenylacetic acid	121, 59, 91, 78, 180	1657	107, 77, 180, 135, 90	1784	abc	87	L-Isoleucine	115, 144, 88, 70, 59	1370	101, 129, 158, 70, 112	1499	abc
12	3-Indoleacetonitrile	121, 59, 78, 224, 165	1682	107, 77, 135, 180, 252	1808	d	88_1	L-Kynurenine (m)	146, 92, 119, 248, 205	2355	146, 92, 119, 262, 205	2477	bd
13	3-Indolepropionic acid	130, 203, 77, 115, 143	1934	130, 217, 143, 115, 77	2031	ac	88_2	L-Kynurenine (s)	117, 90, 63, 145, 173	1847	146, 120, 92, 65, 175	2459	
14	3-Methyl-2-oxovaleric acid	57, 41, 85, 69, 144	972	57, 41, 85, 102, 158	1047	abc	89	L-Lactic acid	59, 103, 43, 130, 87	1030	45, 73, 117, 145, 56	1167	
15	3-Methylindole *	130, 131, 77, 51, 103	1416	130, 131, 77, 51, 103	1430	c	90	L-Leucine	88, 115, 144, 128, 69	1357	158, 102, 43, 112, 69	1483	abcd
16	3-Methylpentanoic acid	74, 43, 59, 101, 55	899	88, 60, 43, 70, 55	964	bc	91	L-Lysine	142, 212, 244, 59, 88	2021	156, 56, 84, 102, 128	2207	abcd
17_1	4-Hydroxybenzoic acid (m)	135, 59, 77, 92, 107	1581	121, 138, 65, 93, 166	1731	abc	92	L-Methionine	115, 61, 147, 128, 162	1621	129, 61, 101, 175, 114	1737	abcd
17_2	4-Hydroxybenzoic acid (s)	121, 65, 93, 152, 74	1472	121, 152, 65, 93, 193	1658		93_1	L-Norleucine (m)	88, 69, 144, 59, 112	1406	69, 56, 112, 158, 86	1538	cd
18_1	4-Hydroxycinnamic acid (m)	161, 59, 89, 133, 236	1883	147, 120, 91, 192, 164	2041	bc	93_2	L-Norleucine (s)	69, 112, 59, 83, 128	1171	158, 230, 74, 86, 114	1746	
18_2	4-Hydroxycinnamic acid (s)	147, 91, 119, 178, 65	1760	147, 120, 91, 192, 164	1926		94_1	L-Phenylalanine (m)	91, 162, 65, 128, 146	1730	91, 176, 65, 128, 77	1850	abcd
19	4-Hydroxyphenylpyruvic acid	135, 77, 92, 107, 180	1449	121, 65, 93, 77, 51	1531	bc	94_2	L-Phenylalanine (s)	91, 162, 65, 128, 77	1528	91, 128, 176, 65, 148	1586	

No.	Compounds	MCF Derivatives		ECF Derivatives		Notes	No.	Compounds	MCF Derivatives		ECF Derivatives		Notes
		main fragments (m/z)#	RI	main fragments (m/z)#	RI				main fragments (m/z)#	RI	main fragments (m/z)#	RI	
20	4-Methylhexanoic acid	74, 43, 55, 87, 115	996	41, 61, 74, 101, 129	1064	bc	95	L-Proline	128, 59, 82, 187, 68	1408	142, 70, 98, 114, 215	1526	abcd
21	5-Dodecenoic acid	74, 55, 67, 96, 138	1520	88, 55, 96, 138, 180	1592	abcd	96_1	L-Serine (m)	86, 42, 58, 145	1434	114, 60, 74, 102, 204	1727	
22	5-Hydroxy-L-tryptophan	204, 117, 145, 350, 90	2894	146, 218, 117,174, 346	2824	b	96_2	L-Serine (s)	56, 144, 86, 70, 103	1542	86, 60, 132, 74, 102	1495	
23	Adipic acid	59, 55, 114, 101, 74	1248	111, 55, 73, 83, 157	1398	abcd	97_1	L-Tryptophan (m)	130, 77, 103, 276, 185	2410	130, 77, 103, 304, 258	2534	abcd
24	Alpha-Hydroxyisobutyric acid	73, 43, 59, 117, 101	1040	59, 43, 87, 131, 159	1169	bc	97_2	L-Tryptophan (s)	130, 77, 103, 244, 185	2163	130, 77, 103, 258, 185	2218	
25	Alpha-Linolenic acid	55, 67, 79, 93, 107	2100	79, 67, 55, 93, 108	2186	ac	98_1	L-Tyrosine (m)	121, 236, 59, 165, 77	2201	107, 192, 264, 74, 91	2408	abcd
26	Aminoadipic acid	114, 59, 156, 188, 124	1698	170, 98, 55, 128, 216	1870	abc	98_2	L-Tyrosine (s)	121, 165, 59, 77, 91	1985	107, 135, 192, 264, 77	2118	
27	Arachidic acid	74, 87, 43, 55, 283	2337	88, 43, 101, 55, 73	2404	ac	99	L-Valine	115, 98, 130, 55, 87	1270	144, 55, 101, 72, 129	1409	abcd
28	Arachidonic acid	79, 55, 67, 91, 203	2276	79, 55, 67, 91, 105	2335	abc	100	Malic acid	59, 75, 85,113, 101	1393	71, 43, 89, 117, 127	1587	abc
29	Behenic acid	74, 87, 43, 55, 311	2552	88, 43, 101, 55, 69	2614	acd	101	Malonic acid	59, 101, 74, 42, 69	939	115, 43, 88, 60, 133	1066	bc
30	Beta-Alanine	101, 56, 70, 74, 88	1222	115, 70, 98, 56, 88	1375	bc	102	m-Cresol	77, 91, 166, 107, 122	1256	180, 108, 77, 91, 136	1357	cd
31	Butyric acid	74, 43, 71, 59, 87	719	71, 43, 88, 60, 101	837	abc	103	Melatonin*	160, 173, 117, 145, 232	2477	173, 160, 145, 117, 232	2510	
32	Capric acid	143, 55, 87, 101, 129	1332	88, 101,73, 55, 157	1396	abcd	104	Methylsuccinic acid	59, 129, 101, 41, 69	1062	115, 43, 73, 87, 143	1204	bcd
33	Caproic acid	74, 43, 59, 55, 87	934	60, 43, 88, 73, 101	998	abc	105	Myristic acid	74, 87, 43, 55, 101	1728	88, 41, 55, 73, 101	1789	ac
34	Caprylic acid	74, 87, 43, 55, 101	1118	88, 41, 55, 70, 101	1192	abc	106	Myristoleic acid	55, 74, 87, 110, 137	1712	55, 69, 88, 101, 124	1777	abcd
35	Cinnamic acid	131, 103, 77, 51, 162	1409	103, 131, 77, 176, 147	1502	abc	107	N-acetyltryptophan	130, 77, 103, 201, 260	2412	130, 215, 77, 103, 143	2463	abc
36	cis-Aconitic acid	59, 153, 184, 125, 98	1453	112, 84, 139, 167, 213	1640	abc	108	Nervonic acid	55, 69, 83, 97, 111	2710	55, 69, 83, 97, 111	2778	acd
37_1	Citraconic acid (m)	59, 126, 68, 98, 53	1108	112, 84, 141, 68, 96	1267	bc	109	Nicotinic acid	78, 106, 51, 137	1137	78, 51, 106, 123, 151	1218	bc
37_2	Citraconic acid (s)	127, 59, 99, 69, 53	1089	113, 85, 141, 157, 171	1237		110	N-Methylnicotinamide*	78, 51, 106, 135, 136	1458	78, 51, 106, 135, 136	1478	b
38	Citramalic acid	43, 85, 117, 59, 75	1111	131, 43, 85, 103, 58	1252	abc	111	Nonadecanoic acid	74, 87, 55, 143, 312	2224	88, 101, 157, 115, 326	2333	acd
39_1	Citric acid (m)	143, 101, 59, 43, 175	1485	112, 84, 139, 167, 212	1639	abcd	112	Norvaline	88, 130, 55, 98, 115	1310	144, 55, 72, 98, 129	1450	cd
39_2	Citric acid (s)	59, 101, 143, 69, 126	1384	57, 71, 115, 157, 85	1511		113	Oleic acid	55, 74, 83, 97, 296	2106	55, 69, 88, 96, 111	2163	abcd
40_1	D-2-Hydroxyglutaric acid (m)	85, 57, 69, 144	1243	85, 57, 159, 101	1311	bc	114_1	Ornithine (m)	128, 59, 88, 115, 198	1913	142, 70, 56, 96, 212	2093	abc
40_2	D-2-Hydroxyglutaric acid (s)	71, 59, 99, 131, 175	1504	85, 131, 159, 57, 203	1683		114_2	Ornithine (s)	128, 59, 139, 70, 96	1651	142, 70, 56, 113, 129	1761	
41	Docosahexaenoic acid	79, 91, 67, 55, 105	2488	79, 91, 41, 67, 55	2544		115	Ortho-Hydroxyphenylacetic acid	91, 121, 78, 133, 148	1587	106, 134, 78, 180, 208	1706	b
42	Docosapentaenoic acid	55, 67, 79, 91, 105	2468	79, 91, 67, 55, 105	2529	ac	116_1	Oxoglutaric acid (m)	115, 55, 59, 87, 130	1272	101, 129, 55, 73, 158	1390	abc
43	Docosatrienoic acid	55, 67, 79, 95, 108	2523	79, 67, 55, 95, 108	2585	ac	116_2	Oxoglutaric acid (s)	115, 55, 59, 87, 143	1229	101, 129, 55, 73, 157	1379	
44	Dodecanoic acid	74, 87, 43, 55, 101	1538	88, 41, 55, 73, 101	1603	abcd	117	Palmitic acid	74, 87, 43, 55, 101	1919	88, 43, 101, 55, 73	1982	abcd
45	Dopamine	117, 201, 164, 166, 94	1074	117, 94, 201, 166, 129	1093	abcd	118	Palmitoleic acid	55, 41, 69, 74, 87	1905	55, 41, 69, 88, 236	1963	abcd

No.	Compounds	MCF Derivatives		ECF Derivatives		Notes	No.	Compounds	MCF Derivatives		ECF Derivatives		Notes
		main fragments (m/z)#	RI	main fragments (m/z)#	RI				main fragments (m/z)#	RI	main fragments (m/z)#	RI	
46	Eicosapentaenoic acid	55, 67, 79, 91, 105	2273	79, 67, 91, 55, 105	2355	abc	119	p-Cresol	77, 107, 121, 166, 91	1263	108, 77, 91, 180, 135	1366	c
47	Eicosatrienoic acid	55, 67, 79, 93, 107	2302	67, 79, 55, 93, 107	2367	abc	120	Pelargonic acid	74, 87, 43, 55, 101	1224	88, 41, 55, 73, 101	1299	abcd
48	Eicosenoic acid	55, 69, 79, 97, 111	2323	55, 69, 83, 97, 111	2386	abc	121	Pentadecanoic acid	74, 87, 43, 55, 213	1824	88, 41, 55, 73, 101	1884	ac
49	Epinephrine	117, 201, 166, 164, 94	1074	117, 94, 201, 166, 129	1093		122	Phenol	65, 78, 152, 108, 93	1146	94, 66, 77, 166, 121	1245	abcd
50	Erucic acid	55, 41, 69, 74, 83	2534	97, 55, 69, 83, 320	2605	abc	123	Phenylacetic acid	91, 65, 150, 51, 119	1181	91, 65, 164, 51, 119	1251	abc
51	Ethylmethylacetic acid	88, 57, 41, 101, 69	800	57, 74, 85, 102, 115	876	abcd	124	Phenylethylamine	91, 65, 147, 104, 179	1521	91, 102, 65, 147, 193	1588	d
52	Fumaric acid	59, 85, 54, 114, 144	1020	99, 127, 55, 71, 82	1181	abc	125_1	Phenyllactic acid (m)	91, 131, 162, 59, 103	1636	131, 91, 103, 148, 176	1768	abc
53	Gamma-Aminobutyric acid	102, 59, 88, 112, 143	1362	116, 56, 84, 69, 130	1504	abcd	125_2	Phenyllactic acid (s)	91, 65, 162, 103, 77	1393	131, 91, 162, 103, 121	1709	
54	Glutaric acid	59, 100, 129, 42, 55	1132	87, 42, 115, 143, 55	1284	bc	126	Phenylpyruvic acid	59, 90, 121, 75, 105	1735	118, 90, 192, 63, 147	1878	
55	Glutathione	142, 98, 70, 82, 59	1576	84, 128, 56, 156, 202	1770	abc	127_1	p-Hydroxyphenylacetic acid (m)	121, 59, 78, 91, 224	1673	107, 77, 135, 180, 252	1809	abcd
56_1	Glyceric acid (m)	43, 59, 87, 69, 102	1238	61, 91, 133, 105, 116	1653	abc	127_2	p-Hydroxyphenylacetic acid (s)	121, 149, 65, 138, 93	1488	107, 77, 135, 166, 238	1747	
56_2	Glyceric acid (s)	59, 91, 75, 103, 133	1463	61, 91, 133, 161, 205	1343		128	Pimelic acid	55, 74, 115, 43, 69	1357	101, 55, 69, 129, 171	1500	bc
57	Glycine	88, 115, 147, 44, 59	1128	102, 56, 74, 130, 175	1287	abc	129	Pipecolic acid	91, 174, 218, 65, 142	2099	91, 174, 218, 65, 156	2203	b
58	Glycolic acid	45, 59, 74, 117, 89	1002	103, 45, 59, 76, 131	1147	abc	130	Propionic acid	57, 88, 42	649	57, 74, 45, 102, 84	696	abc
59	Heptadecanoic acid	74, 87, 43, 55, 241	2021	88, 41, 101, 55, 73	2083	acd	131_1	Purine (m)	120, 133, 178, 80, 93	1548	120, 93, 148, 192, 66	1613	d
60	Heptanoic acid	74, 43, 55, 87, 101	1020	88, 43, 60, 73, 101	1091	abc	131_2	Purine (s)	178, 59, 65, 80, 107	1720	120, 192, 93, 66, 133	1810	
61_1	Hippuric acid (m)	105, 77, 51, 134, 161	1713	105, 77, 51, 134, 161	1781	abcd	132	Putrescine	88, 56, 44, 69, 128	1442	142, 102, 56, 70, 186	1856	ac
61_2	Hippuric acid (s)	105, 77, 51, 136, 92	1092	105, 77, 51, 122, 150	1171		133	Pyroglutamic acid	84, 41, 56, 143	1393	84, 41, 56, 157	1466	bc
62_1	Homocysteine (m)	59, 82, 115, 174, 142	1612	128, 56, 175, 234, 102	2032		134	Pyruvic acid	43, 89, 117, 57, 75	954	84, 56, 128, 173, 156	1963	
62_2	Homocysteine (s)	59, 114, 82, 174, 147	1824	133, 56, 161, 88, 115	1599		135_1	Salicyluric acid (m)	120, 92, 176, 235, 204	1898	120, 92, 176, 249, 204	1958	abd
63	Homogentisic acid	117, 94, 166, 201, 82	2010	117, 94, 201, 82, 166	1093	abc	135_2	Salicyluric acid (s)	44, 120, 56, 92, 77	2092	120, 92, 149, 193, 295	2272	
64	Hydrocinnamic acid	91, 104, 164, 51, 77	1288	91, 104, 77, 51, 178	1365	abc	136	Serotonin	204, 117, 145, 260, 90	2438	146, 159, 218, 174, 231	2909	
65	Hydroxyphenyllactic acid	59, 121, 161, 77, 236	2090	192, 107, 120, 147, 264	2327	b	137	Stearic acid	74, 87, 43, 55, 101	2123	88, 41, 101, 55, 73	2188	abc
66	Hydroxypropionic acid	45, 58, 71, 88, 103	1314	45, 87, 117, 102, 71	935	d	138	Suberic acid	55, 74, 97, 69, 138	1464	55, 69, 83, 139, 185	1598	bcd
67	Indole *	90, 117, 63, 50, 74	1311	117, 90, 63, 50, 74	1328	c	139	Succinic acid	55, 59, 87, 45, 116	1029	101, 55, 73, 129, 45	1175	abc
68	Indoleacetic acid	130, 189, 77, 103, 51	1839	130, 77, 103, 203, 51	1898	abc	140_1	Tartaric acid (m)	59, 85, 44, 115, 159	1689	115, 88, 71, 63, 131	1909	abc
69	Indoleacrylic acid	170, 143, 115, 215, 63	2213	170, 143, 115, 215, 89	2343	d	140_2	Tartaric acid (s)	59, 101, 145, 69, 85	1454	115, 133, 88, 105, 160	1061	
70	Isobutyric acid	43, 59, 71, 87, 102	680	71, 43, 88, 116, 101	809	abc	141	Tetracosanoic acid	74, 87, 43, 55, 339	2751	88, 43, 101, 55, 73	2804	ac
71	Isocaproic acid	74, 43, 55, 88, 101	905	88, 43, 101, 55, 73	969	bc	142	trans-Cinnamic acid	131, 103, 162, 77, 51	1409	131, 103, 77, 176, 147	1503	abcd

No.	Compounds	MCF Derivatives		ECF Derivatives		Notes	No.	Compounds	MCF Derivatives		ECF Derivatives		Notes
		main fragments (m/z)#	RI	main fragments (m/z)#	RI				main fragments (m/z)#	RI	main fragments (m/z)#	RI	
72_1	Isocitric acid (m)	115, 55, 143, 83, 99	1515	101, 129, 55, 157, 85	1636	abcd	143	Tryptamine	130, 143, 218, 103, 77	2185	130, 143, 232, 103, 77	2293	abcd
72_2	Isocitric acid (s)	59, 129, 75, 101, 157	1725	129, 157, 101, 55, 185	1941		144	Valeric acid	74, 43, 57, 87, 101	842	73, 41, 57, 88, 60	914	ac
73	Isovaleric acid	74, 43, 59, 101, 85	797	88, 60, 70, 41, 115	878	abc	145	Vanillic acid	165, 59, 79, 121, 196	1759	151, 168, 123, 196, 268	1898	bc
74_1	Itaconic acid (m)	59, 69, 99, 127, 113	1092	113, 86, 141, 68, 157	1230	bc							
74_2	Itaconic acid (s)	157, 59, 125, 98, 113	1387	90, 117, 189, 63, 133	1661								

Note: a–c, compounds identified in human serum (a), urine (b), feces (c) samples by two independent parameters of MS and Kovats-RI; d, compounds identified in intracellular extract of *E. coli*. m: main peak; s: secondary peak.

*These compounds cannot derivatize with MCF/ECF and elute as prototype.

#The top 5 ions for each compound were ordered by the decreasing intensity.

Table 2

Quantification results in human serum, urine, feces and *E. coli* cell samples (Median ± SE).

Compounds	Concentrations				Compounds	Concentrations			
	human serum (μg/mL)	human urine (μg/mL)	human feces (μg/10 mg)	*E. Coli* cell (μg/1×10^7 cells)		human serum (μg/mL)	human urine (μg/mL)	human feces (μg/10 mg)	*E. Coli* cell (μg/1×10^7 cells)
2-Hydroxybutyric acid	3.71 ± 0.22	0.19 ± 0.11	0.11 ± 0.01		*L*-2-Hydroxyglutaric acid		7.32 ± 0.43	0.83 ± 0.08	
3-(3-Hydroxyphenyl)-3-hydroxypropanoic acid			0.32 ± 0.28		*L*-Alanine	23.15 ± 1.00	13.90 ± 1.41	1.59 ± 0.20	1.51 ± 0.27
3-Aminoisobutanoic acid		3.91 ± 2.20			*L*-Alpha-aminobutyric acid	1.25 ± 0.05	0.70 ± 0.04	0.10 ± 0.05	
3-Hydroxyisovaleric acid		5.07 ± 0.53	0.20 ± 0.01		*L*-Asparagine	10.15 ± 0.36	13.11 ± 0.57		1.02 ± 0.1
3-Hydroxyphenylacetic acid		1.22 ± 0.35	0.26 ± 0.16		*L*-Aspartic acid		2.28 ± 0.03	3.13 ± 0.18	0.78 ± 0.11
3-Indolepropionic acid			3.17 ± 0.19		*L*-Cysteine	1.35 ± 0.05	4.55 ± 0.32	0.07 ± 0.20	0.04 ± 0.004
3-Methyl-2-oxovaleric acid	23.78 ± 0.93	10.90 ± 0.16	1.12 ± 0.02	1.07 ± 0.01	*L*-Glutamic acid	10.6 ± 0.76	6.85 ± 0.51	67.69 ± 4.38	6.21 ± 0.94
3-Methylpentanoic acid			0.04 ± 0.02		*L*-Histidine		63.24 ± 7.45	0.69 ± 0.14	0.78 ± 0.05
4-Hydroxybenzoic acid		0.60 ± 0.05	0.17 ± 0.03		Linoleic acid	810.87 ± 35.01		27.15 ± 4.35	0.11 ± 0.003
4-Hydroxycinnamic acid		3.40 ± 0.45	3.09 ± 0.76		*L*-Isoleucine	5.47 ± 0.28	0.90 ± 0.05	0.52 ± 0.11	5.98 ± 1.06
Adipic acid		4.99 ± 0.68	0.40 ± 0.16		*L*-Leucine	11.64 ± 0.64	1.65 ± 0.12	0.82 ± 0.21	4.91 ± 0.87
Alpha-Hydroxyisobutyric acid		3.84 ± 0.24			*L*-Lysine	17.79 ± 0.75	13.48 ± 1.89	7.56 ± 0.64	5.59 ± 1.08
Alpha-Linolenic acid	133.05 ± 5.73		5.27 ± 1.31		*L*-Methionine	2.03 ± 0.08	0.68 ± 0.02	0.37 ± 0.05	0.53 ± 0.08
Aminoadipic acid		9.83 ± 1.12	0.57 ± 0.13		*L*-Norleucine				3.46 ± 0.57
Arachidic acid	0.21 ± 0.02		1.56 ± 0.56		*L*-Phenylalanine	7.32 ± 0.26	5.21 ± 0.50	0.68 ± 0.22	2.86 ± 0.61
Arachidonic acid	78.26 ± 3.79		5.60 ± 2.45		*L*-Proline	16.6 ± 0.75	0.62 ± 0.03	0.63 ± 0.05	0.40 ± 0.06
Behenic acid			0.66 ± 0.17		*L*-Tryptophan	17.01 ± 0.37	15.33 ± 0.39	1.42 ± 0.05	1.56 ± 0.08
Beta-Alanine		5.32 ± 0.32	0.85 ± 0.05		*L*-Tyrosine	20.57 ± 0.94	12.39 ± 1.76	2.17 ± 0.35	1.09 ± 0.16
Butyric acid		0.35 ± 0.03	122.52 ± 18.54	0.03 ± 0.003	*L*-Valine	17.89 ± 0.71	2.16 ± 0.14	0.62 ± 0.16	1.55 ± 0.26
Capric acid	0.25 ± 0.02		0.07 ± 0.15		Malic acid	0.18 ± 0.05		1.36 ± 0.09	0.05 ± 0.01
Caproic acid			0.34 ± 0.31		Malonic acid		0.87 ± 0.04	0.24 ± 0.03	
Caprylic acid	0.28 ± 0.02		0.07 ± 0.02		m-Cresol			2.10 ± 0.18	
cis-Aconitic acid	1.98 ± 0.08	65.85 ± 5.63	0.17 ± 0.01		Methylsuccinic acid		0.95 ± 0.05	0.32 ± 0.06	
Citraconic acid		1.66 ± 0.02	0.17 ± 0.004		Myristic acid			1.04 ± 0.51	
Citramalic acid		13.62 ± 0.76	0.72 ± 0.02		Nervonic acid			0.35 ± 0.04	
Citric acid	39.14 ± 2.19	915.84 ± 76.1	2.41 ± 0.16	0.45 ± 0.01	Nicotinic acid		2.61 ± 0.01	0.67 ± 0.16	

Compounds	Concentrations				Compounds	Concentrations			
	human serum (μg/mL)	human urine (μg/mL)	human feces (μg/10 mg)	*E. Coli* cell (μg/1×10^7 cells)		human serum (μg/mL)	human urine (μg/mL)	human feces (μg/10 mg)	*E. Coli* cell (μg/1×10^7 cells)
Docosapentaenoic acid	3.22 ± 2.44		0.68 ± 0.33		Nonadecanoic acid			0.10 ± 0.01	
Docosatrienoic acid	14.32 ± 0.68		0.27 ± 0.07		Norvaline			0.33 ± 0.04	4.02 ± 0.65
Dodecanoic acid	0.28 ± 0.02	0.25 ± 0.02	0.22 ± 0.13	0.02 ± 0	Oleic acid	400.44 ± 13.75		38.04 ± 4.54	0.23 ± 0.01
Dopamine	13.17 ± 0.33	15.81 ± 1.66	6.27 ± 0.16	0.97 ± 0.04	Ornithine		5.72 ± 0.16		1.28 ± 0.17
Eicosapentaenoic acid	393.26 ± 17.83		6.81 ± 2.29		Ortho-Hydroxyphenylacetic acid		0.89 ± 0.04		
Eicosatrienoic acid	40.9 ± 3.25		3.08 ± 1.32		Oxoglutaric acid	2.76 ± 3.17	6.46 ± 0.50	0.19 ± 0.06	1.43 ± 0.74
Eicosenoic acid	5.35 ± 2.00		1.06 ± 0.30		Palmitic acid	167.74 ± 4.88		19.91 ± 1.53	0.24 ± 0.02
Erucic acid			0.57 ± 0.29		Palmitoleic acid	12.23 ± 1.04		2.05 ± 0.15	
Ethylmethylacetic acid		0.26 ± 0.01	1.75 ± 0.10	0.02 ± 0	p-Cresol			1.32 ± 0.11	
Fumaric acid			1.57 ± 0.03		Pelargonic acid		0.39 ± 0.01	0.044 ± 0.002	
Gamma-Aminobutyric acid		6.01 ± 0.05	1.22 ± 0.08	3.88 ± 0.48	Pentadecanoic acid	0.76 ± 0.02		1.12 ± 0.14	0.03 ± 0
Glutaric acid		2.51 ± 0.20	1.70 ± 0.24		Phenol		0.28 ± 0.001	0.07 ± 0.03	
Glyceric acid		37.68 ± 14.81	4.32 ± 0.34		Phenylacetic acid		1.58 ± 0.16	2.06 ± 0.16	
Glycine	11.54 ± 0.93	51.66 ± 3.94	1.16 ± 0.18		p-Hydroxyphenylacetic acid or 3-Indoleacetonitrile		4.33 ± 0.72	0.10 ± 0.06	
Glycolic acid		21.27 ± 2.64	2.03 ± 0.81		Pimelic acid		2.88 ± 0.12	0.31 ± 0.01	
Heptadecanoic acid	1.72 ± 0.06		0.62 ± 0.07		Propionic acid			10.98 ± 1.08	0.002 ± 0.001
Heptanoic acid			0.05 ± 0.08		Putrescine			0.74 ± 0.27	
Hippuric acid		218.77 ± 35.78			Pyroglutamic acid		33.68 ± 0.65		
Hydrocinnamic acid			0.71 ± 0.19		Salicyluric acid		2.67 ± 0.35		
Indole			0.67 ± 0.03		Stearic acid	125.55 ± 4.12		24.24 ± 1.87	
Indoleacetic acid		25.72 ± 1.65	1.56 ± 0.08		Suberic acid		12.39 ± 0.33	1.25 ± 0.08	
Isobutyric acid	0.002 ± 0.001	0.20 ± 0.03	1.87 ± 0.13	0.02 ± 0.002	Succinic acid		12.42 ± 1.83	3.80 ± 1.86	0.14 ± 0.07
Isocaproic acid			0.08 ± 0.04		Tartaric acid		10.71 ± 17.06		
Isocitric acid		160.52 ± 15.64	0.64 ± 0.04		Tetracosanoic acid			0.37 ± 0.04	
Isovaleric acid		0.21 ± 0.01	2.26 ± 0.14	0.02 ± 0.001	Valeric acid			3.90 ± 0.30	
Itaconic acid		6.11 ± 0.27	0.31 ± 0.01		Vanillic acid		1.73 ± 0.26	0.18 ± 0.07	

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