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Targeted analysis of microbial-generated phenolic acid metabolites derived from grape flavanols by gas chromatography-triple quadrupole mass spectrometry

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Abstract

Grape-derived products contain a wide array of bioactive phenolic compounds which are of significant interest to consumers and researchers for their multiple health benefits. The majority of bioavailable grape polyphenols, including the most abundant flavan-3-ols, *i.e.*(+)-catechin and (−)-epicatechin, undergo extensive microbial metabolism in the gut, forming metabolites that can be highly bioavailable and bioactive. To gain a better understanding in microbial metabolism of grape polyphenols and to identify bioactive metabolites, advanced analytical methods are needed to accurately quantitate microbial-derived metabolites, particularly at trace levels, in addition to their precursors. This work describes the development and validation of a high-throughput, sensitive and reproducible GC-QqQ/MS method operated under MRM mode that allowed the identification and quantification of 16 phenolic acid metabolites, along with (+)-catechin and (−)-epicatechin, in flavanol-enriched broth samples anaerobically fermented with human intestinal bacteria. Excellent sensitivity was achieved with low limits of detection and low limits of quantification in the range of 0.24–6.18 ng/mL and 0.480–12.37 ng/mL, respectively. With the exception of hippuric acid, recoveries of most analytes were greater than 85%. The percent accuracies for almost all analytes were within ±23% and precision results were all below 18%. Application of the developed method to *in vitro* samples fermented with different human gut microbiota revealed distinct variations in the extent of flavanol catabolism, as well as production of bioactive phenolic acid metabolites. These results support that intestinal microbiota have a significant impact on the production of flavanol

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Appendix A. Supplementary data

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metabolites. The successful application of the established method demonstrates its applicability and robustness for analysis of grape flavanols and their microbial metabolites in biological samples.

Keywords

GC-QqQ/MS; Grape polyphenol; Phenolic acid metabolite; Gut microbiota *in vitro* fermentation

1. Introduction

Accumulating evidence demonstrates that the polyphenols abundant in grape-derived products have great potential in preventing or attenuating a number of chronic diseases including neurodegenerative diseases, cardiovascular diseases, inflammatory diseases and cancers [1–4]. Due to their multiple health benefits, grape polyphenol-rich foods and nutraceuticals are increasingly popular worldwide [5] and as such, there is an imperative need in understanding the bioavailability and metabolism of grape polyphenols [6,7]. The primary bioactive constituents of grape-derived products include flavan-3-ols/proanthocyanidins, anthocyanins, flavonols, resveratrol and phenolic acids [1]. However, a factor limiting the use of flavanols, including the most abundant monomers, (+)-catechin (C) and (−)-epicatechin (EC), is the poor bioavailability. Flavanols in grape-derived products exist in monomeric, oligomeric and polymeric forms, with an estimated 40% of the monomers and oligomers absorbed in the upper intestine [9]. The substantial proportion of non-absorbed flavanols then go to the colon where they undergo extensive microbial catabolism before being absorbed as low-molecular-weight phenolic acids [8–10]. Mounting evidence demonstrates that microbial-generated phenolic acid metabolites are more bioavailable and exhibit diverse bioactivities, often to a greater extent than their precursors, considering their markedly higher abundances *in vivo* [9,10]. This adds complexity to the identification of the actual active agents from botanicals that exert specific health impacts. The variations in systemic absorption, metabolism, distribution and excretion of grape flavanols may also explain their varied bioefficacies in humans. One hypothesis is that the type and quantity of low-molecular-weight metabolites produced largely depend on gut microbiota, while the compositions of microbial community vary greatly among individuals [11]. This opens up a new avenue of research involving the identification of human-originated probiotic strains that are capable of producing wide varieties and high quantities of bioactive metabolites.

Analytical methods for the analysis of phenolic acid metabolites need to have high sensitivity and selectivity in view of the low concentrations of certain microbial-derived metabolites and the complex endogenous nature of biological matrices [12]. Polyphenol metabolites produced by microbial metabolism consist of mainly low molecular weight phenolic acids, generally comprised of hydroxybenzoic and hydroxycinnamic acids and their derivatives [6]. The most commonly used methods for the analysis of small molecules with moderate to high polarity include gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). Although LC-MS has been the most widely applied technique, problems with LC-based methods include difficulties in

chromatographic resolution of peaks and detection with MS due to in-source fragmentation, ion clustering and matrix effects [13,14]. With the advent of tandem mass spectrometry (MS/MS), sensitivity and selectivity of both LC and GC methods are greatly improved. Compared with LC-MS, advantages of GC-MS-based methods include better chromatographic separation with capillary columns, lower maintenance costs, fewer matrix effects, and reduced ionization suppression and adduct formation, leading to less instrumental variability [15–17]. In addition, interfacing with a triple quadrupole (QqQ) analyzer operated under multiple reaction monitoring (MRM) or selective reaction monitoring (SRM) mode provides additional analytical selectivity, sensitivity and accuracy for quantitative analysis of targeted compounds [18].

Although a number of LC-MS/MS methods have been reported [18–20], there are limited GC-MS/MS methods for the analysis of diet-derived microbial phenolic metabolites [12]. In the present work, we report a highly sensitive and reproducible GC-QqQ/MS method for the identification and quantification of 16 microbial-derived phenolic acid metabolites, along with two grape polyphenol precursors, C and EC. This method was then validated and successfully applied to an *in vitro* fermentation study.

2. Materials and methods

2.1. Standards and reagents

All phenolic standards (19 in total including an internal standard) used to develop and validate the method were ≥98% pure. Gallic acid (GA), caffeic acid (CA),*trans*-p-coumaric acid (p-CA), dihydrocoumaric acid (diHCA), 3-(3,4-dihydroxyphenyl) propionic acid (3, 4-diHPPA), 3, 4-dihydroxybenzoic acid (3, 4-diHBA), hip-puric acid (HA), homovanillic acid (HVA), 3-hydroxybenzoic acid (3-HBA), 4-hydroxybenzoic acid (4-HBA), 3-hydroxyphenylacetic acid (3-HPAA), 3-(3-hydroxyphenyl)propionic acid (3-HPPA), vanillic acid (VA),*trans-c*innamic acid-d7(internal standard, IS), catechin (C) hydrate and epicatechin (EC) were purchased from Sigma-Aldrich (St Louis, MO, USA). 3, 4-Dihydroxyphenylacetic acid (3, 4-diHPAA) and ferulic acid (FA) were purchased from ChromaDex Inc. (Irvine, CA), and 5-(4-hydroxyphenyl)valeric acid (4-HPVA) from Alfa Aeser (Lancashire, UK). Formic acid, methanol (MeOH), ethyl acetate, hexane and isopropanol were purchased from Fischer Scientific (Pittsburgh, PA, USA). The derivatizing cocktail Sylon™ HTP, hexamethyldisilazane, trimethylchlorosilane and pyridine (HMDS+ TMCS +Pyridine, 3:1:9), was purchased from Sigma-Aldrich (St Louis, MO).

2.2. Preparation of standards

The stock solutions of all 19 standards, including an IS, were prepared in 70% aqueous MeOH with 0.1% formic acid to make a solution of*ca*.500 μg/mL (the actual concentration of individual standards varied from 440 μg/mL to 739 μg/mL). For compound identification and determination of retention times, each phenolic standard stock solution (including the IS) was diluted with MeOH containing 0.1% formic acid to *ca*.1 μg/mL for GC-MS/MS analysis as described below using full scan mode. For preparing standard working solutions used for calibration and method validation, all 18 standards excluding the IS were mixed and diluted with MeOH containing 0.1% formic acid to establish 16 concentration levels ranging

from *ca*.0.25 ng/mL to 7000 ng/mL. The IS stock was diluted with 100% MeOH containing 0.1% formic acid to a final concentration of 2 μg/mL. Each of the calibration standard mixture (200 μL) was spiked with the 2 μg/mL IS solution (40 μL). The mixture was then transferred to a HPLC vial and dried under a gentle stream of N2. Once dried, ethyl acetate (200 μL) was added to the vial and re-dried under N_2stream to ensure complete removal of water. The derivatizing cocktail (200 μL) was then added to each vial and incubated at 70 °C for 4 h prior to analysis.

2.3. GC-QqQ/MS method development and parameter optimization

2.3.1. Instrumentation—Phenolic compounds were analyzed using a Shimadzu GCMS-TQ8040 gas chromatography triple quadrupole mass spectrometer interfaced with a Shimadzu AOC6000autosampler (Shimadzu Corporation, Kyoto, Japan). Data were collected and analyzed using the GCMS Solution Software (Version 4.30, Shimadzu). Chromatographic separations were performed using a Shimadzu RP-5SilMS capillary column (30.0 m × 0.25 μm × 0.25 mm) (Shimadzu, Kyoto, Japan). Helium was used as the carrier gas and argon as the collision-induced dissociation (CID) gas.

2.3.2. Optimization of GC-QqQ/MS parameters—To ensure optimal performance of the GC-MS system for targeted analytes, column oven temperature, temperature gradient, split ratio and MRM parameters were carefully optimized. Prior to optimization, all phenolic standards were prepared at a concentration of *ca*.1μg/mL and derivatized individually as aforementioned. Each derivatized standard was individually injected into the GC/MS and analyzed under full scan mode to confirm the analyte identity and retention time. A standard mixture containing all 19 compounds was then analyzed under full scan mode to assess peak resolution among analytes. The column oven temperature was optimized by first exploring a range of oven programs with different temperature gradients. The temperature gradient with a specific column temperature that collectively delivered the best peak resolution, peak shape, and the highest signal to noise ratio was then selected. The final program was: initial column oven temperature set at 80 °C, held for 1 min then raised to 220 °C at a rate of 10 °C/min and held for 3.5 min, then raised to 310 °C at a rate of 20 °C/min and held for 15 min for a total run time of 38 min. The injection temperature was set at 280 °C, the ion source temperature at 200 °C and the interface temperature at 280 °C. The detector voltage was set at 0.04 kV with a signal threshold at 1000. Helium was used as the carrier gas with a column flow rate at 0.95 mL/min and a purge flow rate at 1.0 mL/min.

To enhance analytical accuracy and sensitivity, a MRM method for the QqQ analyzer was created and optimized utilizing the Shimadzu Smart MRM program. Data collected from the analysis of individual compounds with aid of the software, optimal ion transitions were selected to include in the MRM method. Two of the most intense precursor-to-product ion transitions were registered for each analyte. For the two selected product ions of each analyte, the more intense one was used as the target ion for quantification and the second one as the reference ion for structural confirmation. Preference was given for higher molecular weight product ions since they can be more easily distinguished from other small fragment ions invariably generated from endogenous compounds in the matrix. Using the Smart MRM program, collision energy for each analyte was automatically tested at 15

different levels, escalating stepwise from 3 to 45 eV to select the optimal collision energy for each transition. Following this, a MRM method with seven different time frames was set up based on the optimal collision energies for each analyte and the retention time upon acquisition of the retention times and optimal parent-to-target/reference ion transitions.

Following MRM optimization, the effects of injection split ratios on detection sensitivity, peak resolution and sharpness were examined. The analysis was performed using the optimized MRM method with injection of 1.0 μL of a standard mixture (ca.1μg/mL) at various split ratios including splitless injection and split ratios of 2, 10 and 100. For analyzing standard mixtures and samples, an injection volume of 1.0 μL with a split ratio of 2 was employed.

2.4. Method validation

2.4.1. Linearity and sensitivity—To establish linearity and sensitivity, standards were analyzed at 16 different concentration levels ranging from *ca.*0.25–7000 ng/mL. The standard mixtures were all spiked with IS at a concentration of 400 ng/mL. The calibration set was then analyzed in duplicate with the optimized MRM method. Calibration curves were established by plotting the analyte-to-IS peak area ratios against the exact concentration of individual analytes in the 16-point calibration set, using linear regression and the origins were not forced through zero. Linearity was determined by evaluating the coefficient of determination (R^2) of each calibration curve. Analytical sensitivity for each analyte was determined by measuring the lower limit of detection (LLOD) and lower limit of quantification (LLOQ), *i.e.* the lowest concentration of an analyte that reaches a signal-to-noise ratio ≥3 and ≥10, respectively.

2.4.2. Preparation and extraction of quality control samples—Blank nutrient broth samples (detailed composition provided in Supplementary Material) were previously stored at −80 °C and transferred to −20 °C 24 h prior to analysis. The broth was conditioned to room temperature before processing. All spiked and blank control samples were prepared in replicates of six. The blank broth (200 μL) and 2 μg/mL IS (40 μL) were mixed in an Eppendorf tube and spiked with a standard mixture (50 μL) (diluted from a stock solution in 100% MeOH with 0.1% formic acid) to a final concentration at *ca.*200 ng/mL as the low quality control (LQC) level, or *ca.*2000 ng/mL as the high quality control (HQC) level. Control samples were only mixed with IS. Six replicates of LQC, HQC and Control samples were prepared for analysis. All samples were dried under N_2 and then reconstituted with 500 μL deionized water. A reconstituted sample was transferred into an Eppendorf tube followed by addition of 4 M HC1 (100 μL). Ethyl acetate (500 μL) was added and the mixture was vigorously vortexed for1min followed by centrifugation for 5 min at 3000 × *g.*The upper organic layer was transferred into a HPLC vial. The extraction process was repeated two more times and the combined organic fractions were dried under a gentle stream of N_2. The derivatizing cocktail (200 μL) was then added to each vial and incubated for 4 h at 70 °C prior to analysis. Original blank broth without IS nor standards were similarly extracted and derivatized to reveal the endogenous phenolic composition.

2.4.3. Recovery—Recovery of each analyte was determined in samples spiked at both LQC (ca. 200 ng/mL) and HQC (ca. 2000 ng/mL) levels with an IS concentration at *ca.*400 ng/mL. Blank broth spiked with IS only was also analyzed in replicates of 6 to measure endogenous phenolic compounds in the broth. All samples were analyzed in duplicate. Recovery was calculated by evaluating the analyte-to-IS response ratio of the analyte in the spiked samples *versus* that in standards prepared in pure solvent at corresponding concentrations. The formula below was used to calculate recovery:

$$\text{Recovery}(\%) = [(\text{SR}-\text{BR})/\text{CR}] \times 100$$

where SR = IS ratio in spiked sample, BR=IS ratio in blank broth samples, and CR = IS ratio in the corresponding standard used for calibration.

2.4.4. Precision and accuracy—To measure accuracy and precision, broth samples were spiked with standard mixture and IS same as the recovery test and analyzed in replicates of six. Precision was determined as the percent relative standard deviation (%RSD) of the measured analytes in spiked samples. Accuracy was determined by comparing measured concentration in samples with the actual spiked concentrations. Since many phenolic acids were found to exist endogenously in blank broth samples, the formula was adjusted to account for this factor as below:

$$\text{Accuracy}(\%) = [(\text{S}-\text{M}-\text{MB})/\text{S}] \times 100$$

where S = spiked concentration, M = measured concentration, and MB = endogenous concentration in blank broth samples.

2.5. *In vitro* microbial fermentation of grape flavanols and method application

To assess the applicability and efficiency of this new method, sample extracts procured from an *in vitro* microbial fermentation study were analyzed using the proposed GC-QqQ/MS method. Three sets of representative *in vitro* samples were analyzed: control samples were blank broth supplemented with C and EC and incubated without bacteria; Donor 1 and Donor 2 samples were blank broth supplemented with C and EC and fermented with gut microbiota isolated from fecal excreta of Donor 1 and Donor 2, respectively. All phenolic extracts of *in vitro* samples were prepared in duplicate and injected twice into the GC-MS for analysis.

2.5.1. *In vitro* anaerobic fermentation—Nutrient broth samples enriched with C and EC (detailed composition included in Supplementary Material) were inoculated with gut microbiota from two healthy human donors and incubated at 37 °C for 24 h. The control samples without bacteria were similarly incubated. Fermented broth samples were recovered by centrifuging at 4000 × *g* for 5 min. The supernatants were then acidified with 2% formic acid to a final concentration of 0.2% and stored at −80 °C.

2.5.2. Extraction of phenolic compounds from *in vitro* samples—Bacterial broth samples were previously stored at −80 °C and were transferred to −20 °C 24 h prior to

analysis. Samples were thawed on ice and conditioned to room temperature before processing. Bacterial broth (500 μL) was mixed with 2μg/mL IS (40 μL), and then extracted and derivatized in the same manner as for quality control samples (Section 2.4.2). For each sample, 1.0 *μL* was injected into the GC-MS system with a split ratio of 2. Peak identity was confirmed by comparing the MS characteristics of both target and reference ions with those of authentic standards. Quantitation was achieved with calibration curves established using the analyte-to-IS peak area of target ions, and final concentrations were divided by 2.5 to compensate for the concentration change following derivatization.

3. Results and discussion

3.1. Development and optimization of GC-QqQ/MS method

To clearly identify individual analytes and to determine retention times, individual standards at a concentration of *ca.*1 μg/mL were first analyzed under full scan mode. The established method demonstrated satisfactory chromatographic separation of most analytes. For two compounds that overlapped in retention time (3, 4-diHPPA and *p-C*A), their identification was resolved by MS operated under MRM mode. With this approach, all analytes were specifically identified and then accurately quantified. The representative chromatograms of compounds with parent-to-target ion transitions are presented in Fig. 1.

To establish the MRM method, target and reference ion transitions were selected based on the MS response of product ions, with preference taken for higher molecular weight ions with the strongest response to avoid matrix interference. Initially, the first MRM method was established with different parent to target and reference ion transitions, excluding the more prevalent *m/z* values that were observed for multiple compounds,*i.e.*73.1, 73.2, 177.0 and 149.0. However, this approach rendered reduced sensitivity, so a second MRM method was set up including these values. The comparative analysis of standard mixture using two methods revealed comparable selectivity, while the second method was more sensitive and given the study objectives was then adopted. Optimal collision energies were chosen based on those giving the greatest intensity for each transition. The use of MRM mode with multiple time frames ensures higher sensitivity and selectivity, as only the selected parent-to-target/reference ion transitions with retention times falling in the same defined time frame are scanned and allows longer dwell time for each transition. Retention times, analyte-specific parent-to-product ion transitions, dwell times and optimal collision energy for each transition are presented in Table 1. The MRM chromatograms of all 19 compounds at their selected ion transitions, obtained from a combined standard mixture are presented in Fig. 1. All peaks were well resolved, including 3,4-diHPPA and *p-C*A, due to the exclusive advantages of the MRM mode with defined time frames (Fig. 1). In addition, the amount of sample actually injected into the column, determined by both the inlet and outlet split ratios, can also markedly affect peak resolution and sharpness. By monitoring elution of standards with injection set to splitless, or a split ratio of2,10 or 100, we found that splitless injections led to inter-injection carryover as the transfer of the analyte vapor (diluted in carrier gas) from the inlet is much slower compared with that in split injection, while split ratios greater than 2 reduced sensitivity and trace constituents may thus be overlooked. Taking into

account both peak shape and detection sensitivity, injection of 1 μL sample with a split ratio of 2 was determined to be optimal.

3.2. Method validation

3.2.1. Linearity and sensitivity—Dynamic linear ranges, LLODs and LLOQs were determined by analysis of the 16-point calibration set and results are presented in Table 2. All linear regression coefficient were >0.996, indicating excellent linearity. LLODs were in the range of 0.24–6.18 ng/mL and LLOQs were 0.48–12.37 ng/mL. These values demonstrate excellent sensitivity of the proposed method, especially when compared to other GC-MS methods for analyzing phenolic acid metabolites derived from dietary polyphenols [21,22]. For example, Grün et al. analyzed microbial-generated phenolic acid metabolites present in biofluids from human subjects treated with a grape polyphenol-rich extract and reported much higher LLODs, ranging from 50 to 50,000 ng/mL [21]. This study used GC-TOF/MS without targeted analysis in MRM mode, which could explain the much lower sensitivity. In a recent investigation closer to the present work, a GC-QqQ/MS method was established for the analysis of phenolic acid metabolites in plasma samples from gerbils administered with a berry (*Barneris microphylla*) extract [12]. Comparable LLOQs were reported, ranging from 0.5 to 16.9 ng/mL when a liquid-liquid extraction method was applied. Same as in our study, an injection volume of 1 $\mu L$was used, but this study used splitless injection. We found that although splitless injection lead to improved sensitivity for a few analytes, it caused significant inter-sample carryover, which compromised accuracy in identification and quantitation of more compounds, and therefore splitless injection was not adopted. Even with a split ratio of 2, we achieved even lower LLOQs with complete separation of all target peaks.

As highlighted in this study, the use of tandem mass spectroscopy offers many advantages such as enhanced selectivity and sensitivity, for the analysis of phenolic acid metabolites which are generally low-molecular-weight and polar. High selectivity is necessary for the analysis of low quantities of phenolic acid metabolites in complex biological matrices such as plasma, urine and digesta of gut microbiota to avoid high background noise and to reduce interferences from matrix compounds that can generate same product ions as our target analytes. While methods without the use of GC-MS in MRM mode may be adequate for the analysis of changes in phenolic acid metabolites at higher concentrations, such methods are less likely to detect subtle variations in metabolite profiles across different biological samples.

3.2.2. Recovery—Percent recoveries were determined by comparing MS response ratio (peak area) of the analyte measured in spiked samples to that in standards prepared in pure solvent. For analytes existing endogenously in the broth matrix, response for components in blank broth was subtracted from that in spiked broth samples to reveal the net change in response following bacterial fermentation. Recoveries were all above 85% at HQC level (ca. 2000 ng/mL), except for C and EC, which were around 73% and HA which was 58%. At LQC level (ca. 200 ng/mL), recoveries for 3-HBA, 4-HBA, C and EC were lower than other target compounds but were all above 71% (Table 2). During our first assay, much lower recoveries of C and EC were obtained (<10%), which could be attributed to insufficient

extraction from the broth into the organic solvent. During the first extraction process, we did not dry the spiked sample completely before reconstituting in deionized water. Since broth samples were spiked with standards prepared in MeOH, the methanol content introduced to the reconstituted sample mixture could have affected the extraction efficiency. Very likely, the MeOH constituent in the sample mix-tures increased the affinity of the more hydrophilic compounds, such as C and EC, to the aqueous layer and reduced their affinity for the organic layer, *i.e.* ethyl acetate. For this reason, we re-prepared all spiked and blank broth samples. Spiked broth samples were first dried under $N_2$and then reconstituted in deionized water. Samples were reconstituted with 500 μL of water to closely resemble the preparation of *in vitro* samples, where 500 μL of broth sample was used (section 2.5.2). By using the modified extraction protocol, we obtained much higher recoveries, especially for C, EC and HA. Furthermore, a lack of salt in the aqueous layer may also reduce extraction efficacy. Viñas et al. observed that increasing salt concentrations in aqueous layer can increase the affinity of polyphenols including C and EC for organic solvent during liquid-phase extraction [23]. Future investigations into the effects of ionic strength on the extraction efficiency of polyphenols and phenolic acids during liquid-liquid extraction are warranted.

Regarding the derivatization reagent, trimethylsilyl (TMS) derived reagents including trimethylsilyl chloride (TMCS) have been successfully applied to the analysis of polyphenol-derived metabolites in plasma and other biological matrices [12,21,24]. In our study, HMDS-TMCS-pyridine (3:1:9, v /v /v) was used due to its feasibility to silyate weakly acidic polar compounds, whereas no validation study has been conducted to evaluate the efficacy of the derivatization. This is also worthy of further examination to exclude the adverse influence on recovery due to lack of efficiency or repeatability of the derivatization procedure. While the extra step of derivatization required to make phenolic compounds volatile is a drawback of GC analysis, this step can also greatly improve detection specificity, sensitivity and analyte stability [25]. In contrast to native phenolic compounds, their trimethylsilyl derivatives are more thermostable and photostable [26]. As part of an on-going project involving further investigation into microbial metabolism of grape polyphenols, we also analyzed the same phenolic acid metabolites in their native form using a LC-MS methodology. Marked degradation of phenolic acids and precursor flavanols stored at 4°C occurred on the third day in an inter-day precision study using LC-MS, while the derivatized phenolic compounds analyzed directly from the anhydrous derivatizing agent for GC-MS analysis were all well retained when stored at room temperature. (D. Zhao and E. Carry, unpublished data). This is a major advantage for analyzing a large amount of such samples as degradation can occur during a lengthy analytical sequence. Furthermore, the stability of derivatized phenolic compounds can be improved through use of different protecting groups, such as*tert*-butyldimethylsilyl (TBDMS). TBDMS derivatization enhances the hydrolytic stability as compared to TMS derivatization [26] and has been successfully applied for GC analysis of phenolic compounds [27].

In our study, we obtained averaged recoveries of 98.9% and 104.7% for high and low QC levels, respectively, and all recoveries were above 71% with the only exception being HA in HQC samples. Overall, the recoveries obtained in our study were satisfactory compared to other related GC-MS studies. Previous GC-MS studies related to the analysis of phenolic acids reported averaged recoveries in the range of 70–80%, while for some compounds

recoveries were as low as 20% [12,21,24]. The varied recovery values for different phenolic acids determined in our study and previous work demonstrate the challenge in simultaneous extraction and profiling of multiple phenolic analytes in biosamples.

3.2.3. Precision and accuracy—Precision and accuracy were determined by analyzing six replicates of spiked samples at both high and low concentrations. Concentration of endogenous compounds in blank broth matrix was subtracted from the measured concentration of corresponding analyte in spiked samples. Precision for analyzing each compound was evaluated by the %RSD of the measured concentrations of six replicates of QC samples. As shown in Table 2, all %RSDs were below 19%, with most being less than 10%, indicating adequate reproducibility. Percent accuracy was determined by the ratio of the measured concentration to the actual spiked concentration. For almost all phenolic compounds, percent accuracies were <23%, except for FA, C, EC and HA at HQC level, and only EC at LQC level (Table 2). While percent accuracies are adequate for in *vitro* analysis, possible explanations for the observed variation includes incomplete extraction from the broth sample, inefficient derivatization (especially for C and EC who have multiple hydroxyl groups, making sialylation less feasible) and the presence of considerable amount of endogenous phenolic acids. A total of nine compounds, including, 3-HBA, 4-HBA, diHCA, VA, 3, 4-diHBA, HA, p-CA, GA and FA were determined to exist endogenously at moderate concentrations. The average %RSD of these compounds in the blank broth samples was 8.4%. While this is more than acceptable, slight variations in these concentrations could have a big impact on the concentration determined in the spiked samples. Except for C and EC, other analytes with lower accuracies were among those compounds present endogenously in broth samples.

3.3. Method application to *in vitro* fermentation studies involving human gut microbiota

The developed and validated GC-QqQ/MS method was applied to identify and quantify 16 phenolic acids metabolites and their polyphenol precursors in bacterial fermented flavanol-enriched broth samples. The 16 phenolic acids targeted for analysis were chosen since they have been widely reported as the major microbial polyphenol catabolites and are purported with diverse bioactivities [8,20,28,29]. These 16 targeted phenolic acids have been shown to be generated from microbial metabolism of the major grape flavanols, C and EC. C and EC can be found in a variety of dietary sources, particularly in grapes, apples, tea, wines and cocoa [8]. Since the majority of C and EC are not absorbed in the small intestine, the selected phenolic acids are expected to be generated by microbiota in the colon following ingestion of flavanol-rich dietaries.

This is the first study employing GC coupled with QqQ/MS to investigate microbial-derived phenolic metabolites in *in vitro* fermentation studies involving human gut microbiota. In this study, C/EC-enriched nutrient broth was incubated with gut microbiota from two healthy donors for 24 h to reveal microbial activity towards grape flavanols. Clear indication of concentration variations of C, EC and their phenolic metabolites following fermentation (Table 3) verifies that the proposed method is efficient and robust. In general, all phenolic acid metabolites were detected at ng/mL level, with the lowest concentration observed for 3-HBA at 4.66 ng/mL in the control sample (Table 3). Inspection of the changes in precursor

polyphenol concentrations reveals that gut microbiota originating from different individuals greatly influenced the extent of flavanol metabolism. Fermentation with Donor 1 microbiota resulted in degradation of 63% of C and 80% of EC, while with Donor 2 microbiota, over 99% of both C and EC were metabolized. This suggests that the bacterial culture from Donor 1 was less efficient at metabolizing C/EC than Donor 2. Regarding the catabolites, fermentation with bacterial culture from both donors led to significant increases in 3-HBA, 4-HBA, 4-HPVA and 3, 4-diHPPA levels (Fig. 2). Microbiota from Donor 1 were more active than Donor 2 in generating 3-HBA, 3, 4-diHPPA and 4-HPVA while decreasing HA content. In contrast, increases in 3,4-diHBA and HA were only observed in samples fermented with Donor 2 microbiota. Moreover, differences in the production of these metabolites are of particular interest due to multifarious bioactivities of selected phenolic acids. For instance, 3-HBA, 3, 4-diHBA and 4-HBA have been shown to have significant analgesic and anti-inflammatory activity [28,30], while studies have demonstrated 3,4-diHPPA to have anti-thrombolytic activity [8]. 3-HBA has been shown to accumulate in the brain and interferes with β-amyloid oligomerization which partly underlies the pathology of Alzheimer's disease [29]. In this regard, our findings further support the well-accepted viewpoint that inter-individual differences in gut microbiota account for the varied bioefficacy of orally ingested polyphenols in the body [31]. As such, application of advanced GC-MS/MS methods for screening human bacterial culture with remarkable potentials to produce high levels and types of bioactive phenolic acid metabolites may extend a new dimension in maximizing the health benefits of grape-derived polyphenols. It is also important to note that phenolic acids are converted to phase II conjugates following enteric absorption although they are not expected to be produced by gut microorganisms in our *in vitro* fermentation model. In fact, GC-MS has been employed to quantitate phase II phenolic acid metabolites in urine, feces and/or plasma from human subjects and rats administered to red wine or red grape juice extracts [32,33]. However, *in vivo* samples in related studies were all analyzed by GC-MS following enzymatic digestion to deconjugate phase II metabolites. No GC-MS method is available for analyzing conjugated phenolic metabolites, possibly due to the difficulty in converting them into volatile compounds by derivatization. Thus, this may represent a limitation of GC-based methodology for direct analysis of conjugated phenolic acid metabolites. In addition, the advent of hydrophilic interaction liquid chromatography (HILIC), allowing better separation of polar compounds compared with conventional reversed-phase LC and GC methods [12], presents another promising option for analyzing microbial-generated polyphenol metabolites. However, as demonstrated in our study, the reproducibility, sensitivity and minimal injection volume requirement of GC-based methods warrant further investigation into their use for high-throughput analysis of phenolic acid metabolites.

4. Conclusions

This study presents a highly sensitive and reproducible GC-QqQ/MS method for the analysis of 16 phenolic acid metabolites derived from two major grape flavanols, C and EC. This is the first study that developed and validated a GC-QqQ/MS method operated under MRM mode with multiple time frames for targeted analysis of phenolic acid metabolites derived from grape flavanols with implications on metabolic activity from human gut microbiota.

This method proved to be highly sensitive, accurate and efficient for the analysis of phenolic compounds, and was successfully applied to a study of *in vitro* fermented bacterial broth supplemented with two major grape flavanols. Analysis of these samples revealed intensive metabolism of precursor polyphenols by human intestinal bacteria, resulting in depleted polyphenol precursors and elevated phenolic acid concentrations. Marked differences in the production of diverse phenolic acid metabolites were observed between flavanol-enriched broths fermented with microbiota from different human donors. Although not fully comprehensive, accurate and valuable information in relation to the complex interactions between gut microbiota and grape flavanols can be obtained by quantifying the phenolic acid metabolites using the proposed method. Given the increased interest in linking possible health benefits to the consumption of polyphenol-rich foods and critical roles of gut microbiota in modulating the bioefficacy of dietary polyphenols, the method described in this work may be widely applied to investigate microbial biotransformation of other dietary polyphenols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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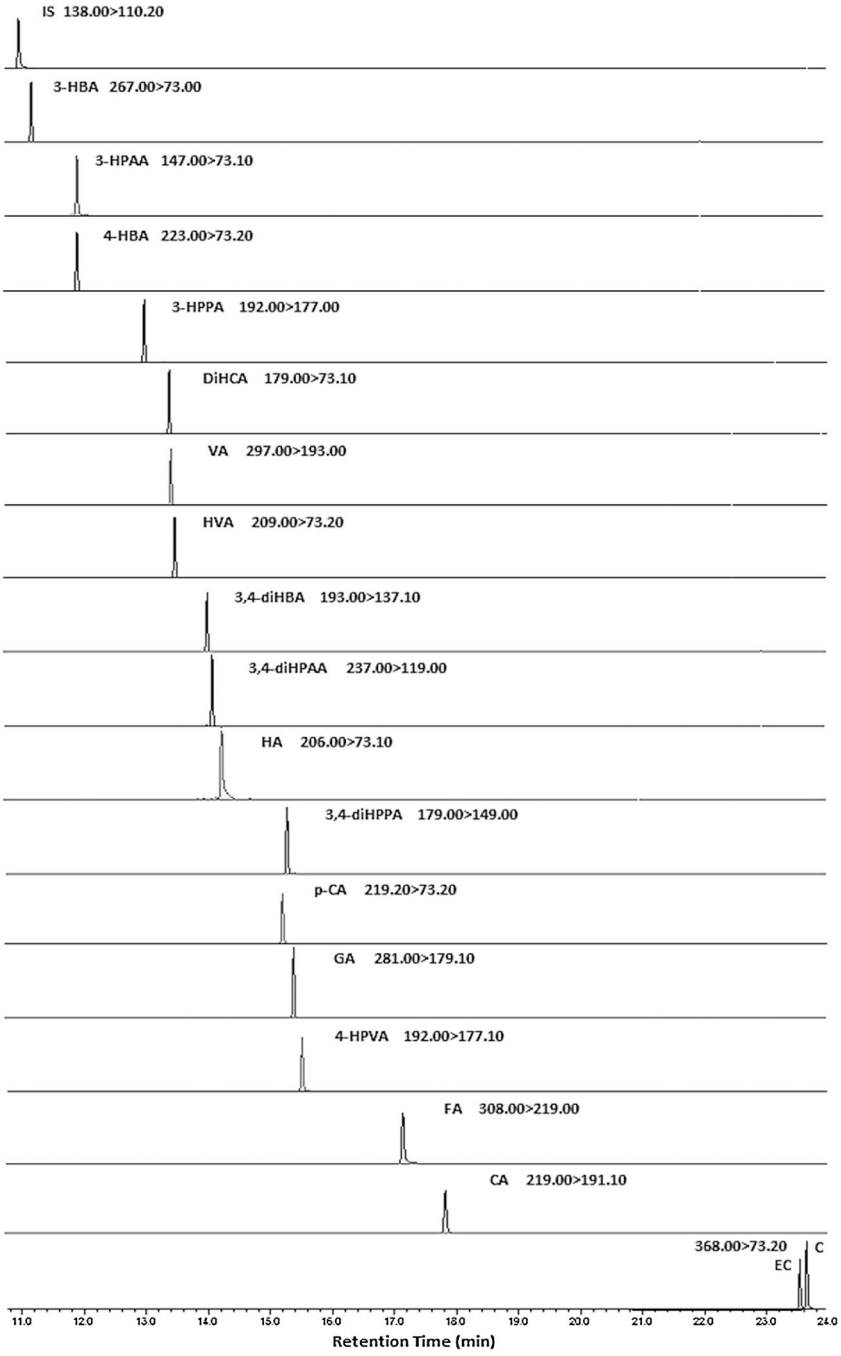

Fig.1.

Representative mass chromatograms displaying parent-to-target ion transitions for select phenolic acids, catechin, epicatechin and an internal standard. The standard mixture injected was at a concentration of *ca.*4000 ng/mL. Abbreviations are the same as in Table 1.

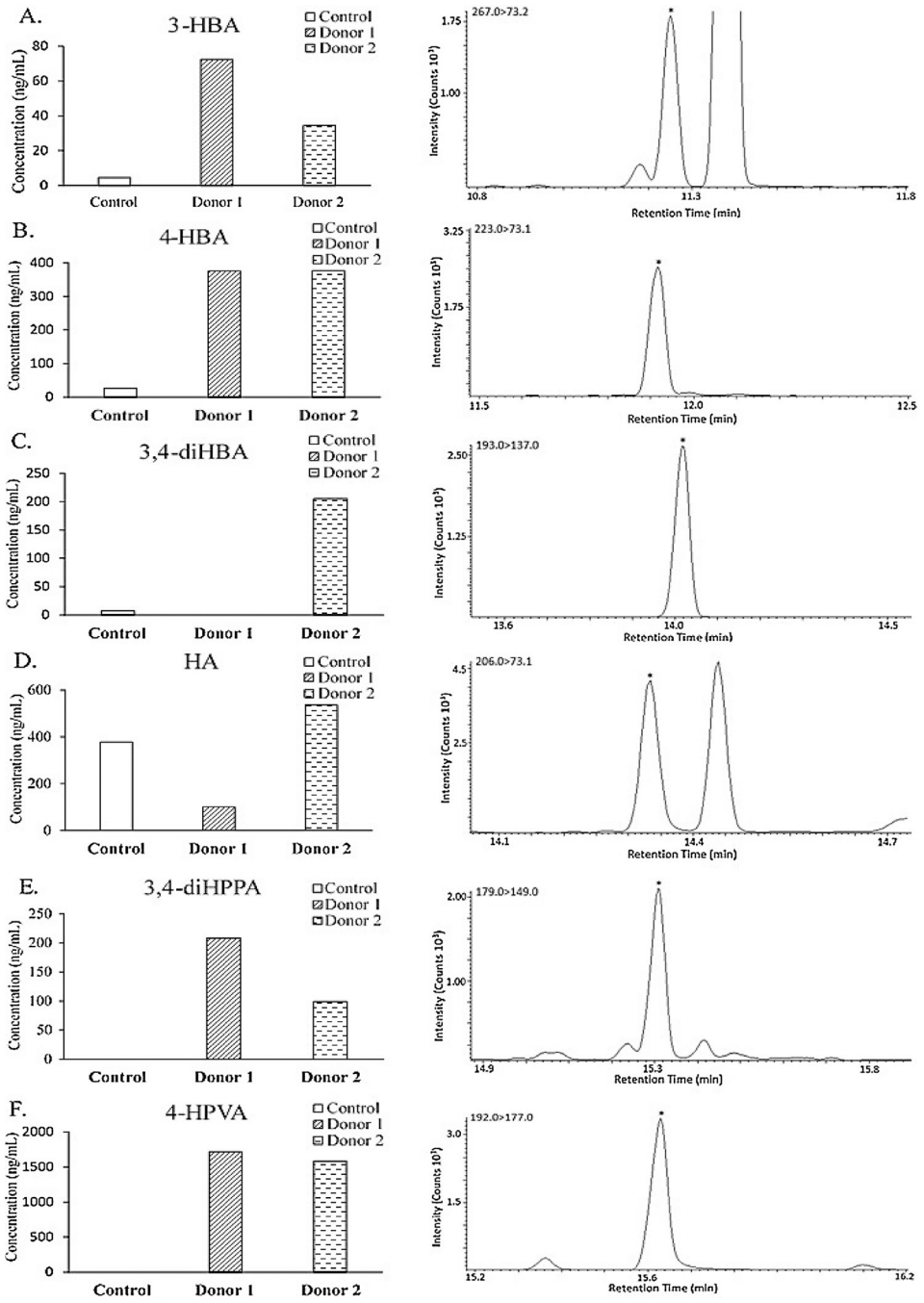

Fig. 2.

Concentration changes and MS identification of select phenolic acid metabolites derived from catechin/epicatechin in broth samples fermented with human gut microbiota ***in vitro.***Left column (charts A-F): average concentrations (ng/mL) of phenolic acid metabolites in control and samples fermented with microbiota from Donor 1 and 2. Right column: mass chromatograms showing the corresponding peaks of parent-to-target ion transition for identifying the target analyte, with chromatograms A, B, D, E and F selected from a

representative Donor 1 sample and chromatogram C from a representative Donor 2 sample. Abbreviations are the same as in Table 1.

Table 1

GC-QqQ/MS parameters of the optimized method and retention times of targeted phenolic compounds.

Compound[a]	Abbreviation	Retention Time (min)	Dwell Time (ms)	Target Ion Transition	Collision Energy (eV)	Reference Ion Transition	Collision Energ (eV)
Trans-cinnamic acid-d_7	IS	11.00	25.7	212.0 > 110.2	27.0	110.0 > 82.1	15.0
3-Hydroxybenzoic acid	3-HBA	11.21	26.0	267.0 > 73.2	30.0	267.0 > 193.0	18.0
3-Hydroxyphenylacetic acid	3-HPAA	11.75	26.0	147.0 > 73.1	15.0	296.0 > 73.1	21.0
4-Hydroxybenzoic acid	4-HBA	11.95	25.7	223.0 > 73.1	27.0	193.0 > 73.0	21.0
3-(3-Hydroxyphenyl)propionic acid	3-HPPA	13.08	20.3	192.0 > 177.0	9.0	192.0 > 73.1	21.0
Dihydrocoumaric acid	diHCA	13.44	20.3	179.0 > 73.1	21.0	192.0 > 177.0	12.0
Vanillic acid	VA	13.47	20.0	297.0 > 193.0	30.0	297.0 > 267.0	15.0
Homovanillic acid	HVA	13.54	19.5	209.0 > 73.2	21.0	179.0 > 149.0	27.0
3,4-Dihydroxybenzoic acid	3,4-diHBA	14.05	20.0	193.0 > 137.0	21.0	193.0 > 165.0	15.0
3, 4-Dihydroxyphenylacetic acid	3,4-diHPAA	14.13	31.5	237.0 > 119.0	12.0	237.0 > 209.0	9.0
Hippuric acid	HA	14.33	31.5	206.0 > 73.1	21.0	206.0 > 73.1	21.0
Trans-p-coumaric acid	p-CA	15.34	32.0	219.0 > 73.1	27.0	249.0 > 73.1	30.0
3-(3,4-Dihydroxyphenyl)propionic acid	3,4-diHPPA	15.33	32.0	179.0 > 149.0	30.0	219.2 > 73.2	15.0
Gallic acid	GA	15.45	32.0	281.0 > 179.0	21.0	458.0 > 179.0	42.0
5-(4-Hydroxyphenyl)valeric acid	4-HPVA	15.60	31.5	192.0 > 177.0	15.0	192.0 > 73.2	30.0
Ferulic acid	FA	17.22	98.7	308.0 > 219.0	21.0	338.0 > 249.0	21.0
Caffeic acid	CA	17.90	98.7	219.0 > 191.0	15.0	396.0 > 73.0	27.0
Epicatechin	EC	23.62	73.5	368.0 > 73.2	27.0	179.0 > 149.0	21.0
Catechin	C	23.73	73.5	368.0 > 73.2	30.0	179.0 > 149.0	21.0

[a]Listed in order of elution. IS, internal standard.

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Table 2

Calibration parameters, sensitivity, recovery, accuracy and precision of the optimized GC-QqQ/MS method.

Compound	LOQ (ng/mL)	LOQ (ng/mL)	Dynamic Linear Range	Correlation Coefficient	%Accuracy 2000ng/mL	%Recovery 2000ng/ml	%Accuracy 200ng/mL	%Recovery 200ng/ml	% RSD 2000ng/mL	%RSD 200ng/mL
3-HBA	0.312	0.624	0.312–10222.5	0.9986	−5.97	87.35	−13.35	73.62	8.50	3.36
3-HPAA	3.77	7.54	1.600–1638.9	0.9974	5.58	91.99	−12.86	100.93	12.87	16.82
4-HBA	0.240	0.480	0.970–7944.5	0.9982	−7.69	94.49	−18.38	71.75	5.61	4.83
3-HPPA	3.28	6.56	0.448–7333.3	0.9967	−0.88	101.13	−13.88	96.65	6.65	4.49
diHCA	1.57	3.15	0.393–6444.5	0.9995	−2.43	101.11	−10.97	99.11	7.23	4.96
VA	1.00	3.99	0.498–8166.8	0.9988	0.11	102.04	−6.47	93.83	9.89	8.19
HVA	1.17	2.33	0.583–9555.5	0.9999	2.35	97.83	−10.62	119.16	7.70	5.12
3,4-diHBA	1.17	2.35	0.587–9611.1	0.9981	9.07	97.58	−2.79	89.04	14.27	8.79
3,4-diHPAA	3.50	7.00	0.875–7166.8	1.0000	16.7	108.18	−5.27	134.82	18.80	8.61
HA	6.18	12.37	1.546–6333.25	0.9984	−41.86	57.9	−15.53	93.72	18.72	15.70
p-CA	1.87	3.74	0.468–7666.8	0.9994	18.08	105.55	−4.79	145.26	18.01	9.82
3,4-diHPPA	0.94	3.74	0.468–7666.8	0.9995	9.19	113.37	−22.42	132.14	18.89	6.58
GA	0.51	1.02	0.507–8333.25	0.9999	1.36	91.08	−5.30	121.62	14.29	11.07
4-HPVA	0.96	1.93	0.963–7889.0	0.9993	22.85	115.79	5.71	114.14	12.65	6.54
FA	3.28	6.56	1.641–1680.6	0.9989	50.5	137.31	0.68	93.04	11.22	11.31
CA	1.49	5.97	1.491–1527.8	0.9969	15.40	125.38	−7.09	153.58	4.35	11.31
EC	0.98	3.91	0.488–8000.0	0.9976	−42.39	78.17	−29.94	72.16	6.39	6.82
C	1.13	2.25	0.563–9222.3	0.9997	−45.11	73.53	−18.99	79.24	12.84	7.63

Method was validated for *in vitro* analysis of phenolic acids in bacterial broth samples. Blank broth samples were spiked with concentrations of *ca.*200 ng/mL and*ca.*2000 ng/mL, with accuracy (%), recovery (%) and RSD (%) determined at both concentrations. Abbreviations are the same as in Table 1.

Table 3

Concentrations of phenolic acid metabolites and precursor polyphenols (cate-chin/epicatechin) in *in vitro* bacterial broth samples.

Compound	Blank	Control	Donor 1	Donor 2
3-HBA	4.91	4.66	72.4	34.5
3-HPAA	N.D.	N.D.	N.D.	N.D.
4-HBA	35.5	25.9	375	377
3-HPPA	0.0	N.D.	N.D.	N.D.
diHCA	23.4	22.5	41.3	97.2
VA	67.6	61.8	15.7	15.1
HVA	N.D.	N.D.	N.D.	N.D.
3,4-diHBA	17.7	7.40	N.D.	205
3,4-diHPAA	N.D.	N.D.	N.D.	N.D.
HA	731	379	101	536
p-CA	89.2	62.7	146	69.4
3,4-diHPPA	N.D.	N.D.	208	99.0
GA	72.1	60.0	89.8	107
4-HPVA	N.D.	N.D.	1717	1586
FA	69.0	62.3	196	58.0
CA	N.D.	N.D.	92.3	N.D.
EC	N.D.	4145	814	235
C	N.D.	3968	1478	177

Data is expressed in ng/mL. Abbreviations are the same as in Table 1. N.D. = not detected.

The **Blank** sample refers to non-incubated nutrient broth, used to determine endogenous levels of phenolic acids. The **Control** sample represents broth sample enriched with EC and C only.**Donor 1** and **Donor 2** represent EC and C enriched broth samples inoculated with intestinal microbiota from human donors 1 and 2, respectively. Control and donor samples were incubated for 24 h at 37 °C prior to processing. Abbreviations are the same as in Table 1.