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Determination of Microbial Phenolic Acids in Human Faeces by UPLC-ESI-TQ MS

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Supporting Information

ABSTRACT: The aim of the present work was to develop a reproducible, sensitive, and rapid UPLC-ESI-TQ MS analytical method for determination of microbial phenolic acids and other related compounds in faeces. A total of 47 phenolic compounds including hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxymandelic acids and simple phenols were considered. To prepare an optimum pool standard solution, analytes were classified in 5 different groups with different starting concentrations according to their MS response. The developed UPLC method allowed a high resolution of the pool standard solution within an 18 min injection run time. The LOD of phenolic compounds ranged from 0.001 to 0.107 μg/mL and LOQ from 0.003 to 0.233 μg/mL. The method precision met acceptance criteria (<15% RSD) for all analytes, and accuracy was >80%. The method was applied to faecal samples collected before and after the intake of a flavan-3-ol supplement by a healthy volunteer. Both external and internal calibration methods were considered for quantification purposes, using 4-hydroxybenzoic-2,3,4,5-d_4 acid as internal standard. For most analytes and samples, the level of microbial phenolic acids did not differ by using one or another calibration method. The results revealed an increase in protocatechuic, syringic, benzoic, *p*-coumaric, phenylpropionic, 3-hydroxyphenylacetic, and 3-hydroxyphenylpropionic acids, although differences due to the intake were only significant for the latter compound. In conclusion, the UPLC-DAD-ESI-TQ MS method developed is suitable for targeted analysis of microbial-derived phenolic metabolites in faecal samples from human intervention or in vitro fermentation studies, which requires high sensitivity and throughput.

KEYWORDS: UPLC-DAD-ESI-TQ MS, polyphenols, gut microbiota, microbial phenolic acids, metabolites, faeces

INTRODUCTION

In the last decades, polyphenols have attracted considerable interest because of their numerous health effects, including antioxidant, anticarcinogenic, cardioprotective, antimicrobial, and neuro-protective activities.[1] Despite their abundance in numerous plant foods, many polyphenols are not bioaccesible and have scarce absorption in the small intestine but reach the colon where they are catabolized by the colonic microbiota. It has been estimated that 90−95% of the dietary polyphenols are not absorbed in the small intestine and, therefore, accumulate in the colon.[2] Among these polyphenols are oligomeric and polymeric proanthocyanidins, polyphenols glycosylated with rhamnose, hydroxycinnamic acid esters, and lignans.[3,4] Polyphenols presenting gastrointestinal absorption and excreted in the bile could also reach the colon by enterohepatic recirculation and be subjected to further colonic catabolism. Therefore, the colon is an active site for polyphenol metabolism. Colonic catabolism involves the biotransformation to simple phenols, phenolic acids, aromatics, and lactones with different degrees of hydroxylation and side chain length.[3,4] Once originated by the microbiota, phenolic metabolites can be absorbed into the enterocytes via passive diffusion and reach the liver to be further metabolized by phase II enzymes, before entering the circulation or being eliminated in the urine. Nonabsorbed phenolic metabolites are eliminated through the faeces. Microbial phenolic metabolites excreted in urine represent the largest proportion of polyphenol intake.[5] The health-promoting activities of microbial metabolites as well as the microbiota−phenolic interactions and their implications in overall gut health are of current research interest.

Considering these facts, rapid, sensitive, and reliable analytical methods are needed for profiling microbe-derived phenolic metabolites in biological fluids in order to determine their contribution to the overall bioavailability of polyphenols and to allow identification of polyphenol-exposure biomarkers that could be further used to correlate dietary intake with particular health effects. Gas chromatography−mass spectrometry (GC-MS)[6−11] and liquid chromatography (LC) coupled to diode-array (DAD), electrochemical (ECD), and particularly tandem mass spectrometry (ESI-MS/MS)[12−18] are the most commonly used analytical methodologies reported for analysis of microbial phenolic metabolites in biological fluids, including plasma and urine samples collected after intake of polyphenol-rich sources and faecal samples from in vitro fermentation studies. GC methodologies provide higher resolution than LC methodologies but require a laborious sample preparation step which usually involves isolation of metabolites by liquid−liquid extraction (LLE) or other extraction procedures followed by further derivatization. In contrast, for LC analysis the sample preparation step is simpler since some samples (i.e., faecal water) may be diluted without a previous isolation step and provides a very sensitive method for

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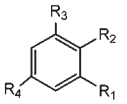
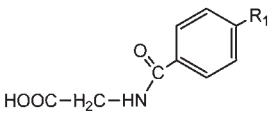
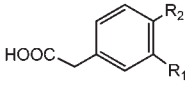


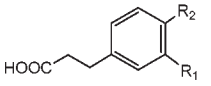
	Simple phenols	R_1	R_2	R_3	R_4
12	Catechol/Pyrocatechol	OH	OH	H	H
27	4-Methylcatechol	OH	OH	H	OCH_3
41	4-Ethylcatechol	OH	OH	H	OC_2H_5
2	Phloroglucinol	OH	H	OH	OH
5	Pyrogallol	OH	OH	OH	H



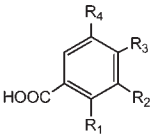
	Glycinated benzoic acids	R_1
11	4-Hydroxyhippuric acid	OH
21	Hippuric acid	H



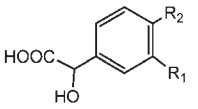
	Phenylacetic acid derivatives	R_1	R_2
13	3,4-Dihydroxyphenylacetic acid	OH	OH
24	3-Hydroxyphenylacetic acid	OH	H
18	4-Hydroxyphenylacetic acid	H	OH
26	4-Hydroxy-3-methoxyphenylacetic acid	OCH_3	OH
39	4-Methoxyphenylacetic acid	H	OCH_3
38	3,4-Dimethoxyphenylacetic acid	OCH_3	OCH_3
36	Phenylacetic acid	H	H



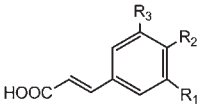
	Phenylpropionic acid derivatives	R_1	R_2
19	3-(3,4-Dihydroxyphenyl)-propionic acid	OH	OH
30	3-(3-Hydroxyphenyl)-propionic acid	OH	H
28	3-(4-Hydroxyphenyl)-propionic acid	H	OH
44	3-(3,4-Dimethoxyphenyl)-propionic acid	OCH_3	OCH_3
45	Phenylpropionic acid	H	H



	Benzoic acid derivatives	R_1	R_2	R_3	R_4
4	Gallic acid	H	OH	OH	OH
14	3-*O*-methylgallic acid	H	OCH_3	OH	OH
16	4-*O*-methylgallic acid	H	OH	OCH_3	OH
8	3,5-Dihydroxybenzoic acid	H	OH	H	OH
42	3,4,5-Trimethoxybenzoic acid	H	OCH_3	OCH_3	OCH_3
9	Protocatechuic acid	H	OH	OH	H
33	3,4-Dimethoxybenzoic acid	H	OCH_3	OCH_3	H
20	3-Hydroxybenzoic acid	H	OH	H	H
15	4-Hydroxybenzoic acid	H	H	OH	H
40	4-Methoxybenzoic acid	H	H	OCH_3	H
43	3-Methoxybenzoic acid	H	OCH_3	H	H
34	Benzoic acid	H	H	H	H
23	Vanillic acid	H	OCH_3	OH	H
25	Syringic acid	H	OCH_3	OH	OCH_3
37	Salicylic acid	OH	H	H	H



	Mandelic acid derivatives	R_1	R_2
1	3,4-Dihydroxymandelic acid	OH	OH
7	3-Hydroxymandelic acid	OH	H
10	3-Hydroxy-4-methoxymandelic acid	OH	OCH_3
3	4-Hydroxymandelic acid	H	OH
6	4-Hydroxy-3-methoxymandelic acid	OCH_3	OH
17	Mandelic acid	H	H



	Cinnamic acid derivatives	R_1	R_2	R_3
47	*t*-Cinnamic acid	H	H	H
32	*m*-Coumaric acid	OH	H	H
29	*p*-Coumaric acid	H	OH	H
22	Caffeic acid	OH	OH	H
31	Ferulic acid	OCH_3	OH	H
35	Isoferulic acid	OH	OCH_3	H
46	3,4,5-Trimethoxycinnamic acid	OCH_3	OCH_3	OCH_3

Figure 1. Chemical structures of the phenolic compounds studied. Compound numbers refer to Table 1.

quantification of selected metabolites when coupled to MS/MS. The efficiency of separation and the sensitivity of MS detection of LC-MS/MS methodologies can be largely improved by the use of ultraperformance liquid chromatography (UPLC), which operates with smaller particle size (<2 μm) sorbent materials and internal diameter columns (1−2.1 mm) than LC at very high pressures (up to 15 000 psi). This results in a marked decrease in separation time and, therefore, in a very high throughput which is required for analysis of complex samples. The UPLC technique coupled to MS detection is finding good application in the analysis of phenolic compounds in biological fluids and food matrixes.[19−21]

To our knowledge, the UPLC technique has not been used before for analysis of microbial phenolic acids derived from gut catabolism. The aim of the present work was to develop and validate a reproducible, sensitive, and rapid UPLC-ESI-MS/MS analytical method for simultaneous determination of 47 microbe-derived phenolic metabolites (Figure 1) in human faeces.

MATERIALS AND METHODS

Standards. Phenolic acid standards used in the study are listed in Table 1. They were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany), or Extrasynthèse (Genay, France). The compound 4-hydroxybenzoic 2,3,5,6-d_4 acid, used as internal standard, was purchased from Sigma-Aldrich Chemical Co. LC-grade solvents were purchased from Lab-Science (Sowinskiego, Poland) or Scharlau (Barcelona, Spain).

Table 1. Validation Parameters of the UPLC-DAD-ESI-TQ MS Method

no.	compound	R_t (min)	tested concentration range (μg/mL)[a]	LOD (μg/mL)	LOQ (μg/mL)	linear concentration range (μg/mL)	r^2	accuracy (%)	precision (% RSD)[b]
1	3,4-dihydroxymandelic acid	0.57	A	0.007	0.020	0.05−12.5	0.996	108.00	13.35
2	phloroglucinol	0.77	C	0.013	0.026	0.02−5	0.990	116.67	5.71
3	4-hydroxymandelic acid	0.86	C	0.065	0.147	0.2−20	0.994	103.67	7.80
4	gallic acid	0.91	A	0.004	0.015	0.05−12.5	0.994	109.33	7.62
5	pyrogallol	1.04	C	0.015	0.030	0.1−5	0.984	122.00	0.01
6	4-hydroxy-3-methoxymandelic acid	1.22	B	0.006	0.017	0.05−25	0.996	107.33	4.30
7	3-hydroxymandelic acid	1.32	A	0.005	0.013	0.05−25	0.997	98.67	10.20
8	3,5-dihydroxybenzoic acid	1.57	C	0.002	0.005	0.01−5	0.997	110.00	4.55
9	protocatechuic acid	1.76	B	0.001	0.003	0.01−10	0.994	112.00	5.36
10	3-hydroxy-4-methoxymandelic acid	2.01	C	0.008	0.020	0.02−20	0.998	105.67	1.45
11	4-hydroxyhippuric acid	2.22	A	0.003	0.005	0.025−12.5	0.996	108.00	0.01
12	catechol/pyrocatechol	2.45	B	0.012	0.037	0.05−5	0.988	121.33	5.30
13	3,4-dihydroxyphenylacetic acid	2.79	A	0.004	0.009	0.0025−12.5	0.993	114.67	5.33
14	3-*O*-methylgallic acid	2.98	A	0.003	0.008	0.025−5	0.996	112.00	3.57
15	4-hydroxybenzoic acid	3.04	A	0.008	0.022	0.025−5	0.997	110.67	2.09
16	4-*O*-methylgallic acid	3.25	A	0.001	0.003	0.005−5	0.997	105.33	2.19
17	mandelic acid	3.4	C	0.005	0.012	0.02−20	0.996	111.00	3.93
18	4-hydroxyphenylacetic acid	4.03	D	0.008	0.020	0.04−10	0.993	111.83	4.90
19	3-(3,4-dihydroxyphenyl)-propionic acid	4.09	B	0.008	0.027	0.050−10	0.994	114.67	2.01
20	3-hydroxybenzoic acid	4.16	C	0.032	0.068	0.1−10	0.996	112.00	3.22
21	hippuric acid	4.26	C	0.014	0.039	0.1−5	0.995	111.67	2.25
22	caffeic acid	4.26	A	0.004	0.008	0.025−5	0.995	80.00	0.01
23	vanillic acid	4.34	A	0.008	0.020	0.025−2.5	0.995	113.33	2.04
24	3-hydroxyphenylacetic acid	4.56	C	0.009	0.025	0.1−20	0.993	115.67	2.50
25	syringic acid	5.03	A	0.007	0.017	0.025−1.25	0.990	108.00	3.70
26	4-hydroxy-3-methoxyphenylacetic acid	5.03	C	0.022	0.045	0.1−10	0.993	119.67	4.90
27	4-methylcatechol	5.27	C	0.016	0.044	0.1−10	0.985	126.00	2.10
28	3-(4-hydroxyphenyl)-propionic acid	5.45	E	0.058	0.133	0.5−25	0.987	117.60	3.12
29	*p*-coumaric acid	5.62	A	0.003	0.007	0.025−12.5	0.990	118.67	5.15
30	3-(3-hydroxyphenyl)-propionic acid	6.26	C	0.005	0.012	0.02−10	0.995	112.33	2.24
31	ferulic acid	6.72	A	0.006	0.019	0.025−5	0.993	117.33	7.10
32	*m*-coumaric acid	6.91	B	0.005	0.013	0.05−10	0.994	116.67	1.98
33	3,4-dimethoxybenzoic acid	7.18	C	0.024	0.080	0.1−10	0.985	123.67	5.26
34	benzoic acid	7.31	E	0.050	0.107	0.1−25	0.987	114.53	2.71
35	isoferulic acid	7.32	C	0.023	0.092	0.1−5	0.987	118.33	2.44
36	phenylacetic acid	7.50	D	0.032	0.102	0.2−10	0.988	116.17	2.93
37	salicylic acid	7.54	C	0.007	0.017	0.02−20	0.988	124.00	2.13
38	3,4-dimethoxyphenylacetic acid	7.55	A	0.005	0.011	0.025−1.25	0.988	117.33	3.94
39	4-methoxyphenylacetic acid	8.52	D	0.058	0.137	0.2−20	0.990	112.50	10.41
40	4-methoxybenzoic acid	8.56	C	0.037	0.117	0.2−10	0.978	123.33	12.28
41	4-ethylcatechol	8.77	D	0.005	0.013	0.04−20	0.988	119.67	3.88
42	3,4,5-trimethoxybenzoic acid	8.89	C	0.016	0.050	0.1−10	0.990	117.67	4.36
43	3-methoxybenzoic acid	9.03	D	0.009	0.023	0.04−20	0.987	122.00	2.96
44	3-(3,4-dimethoxyphenyl)-propionic acid	9.50	C	0.027	0.095	0.1−10	0.995	108.67	2.32
45	phenylpropionic acid	11.14	E	0.107	0.233	0.5−50	0.987	121.13	3.74
46	3,4,5-trimethoxycinnamic acid	12.26	C	0.010	0.028	0.1−20	0.991	121.00	2.48
47	*t*-cinnamic acid	12.24	C	0.012	0.030	0.1−10	0.994	112.00	4.64

[a] A, B, C, D, and E refer to groups in Table S2, Supporting Information. [b] $n = 3$ for the % RSD.

Preparation of Standard Solutions. For the 47 analytes, individual stock solutions (250 μg/mL in acetonitrile/water (1:4, v/v)) were first prepared and diluted to obtain 50 and 5 μg/mL solutions in order to optimize the MS/MS parameters (Table S1, Supporting Information) and to initially assess the MS response of each compound under optimized conditions. According to their response, analytes were classified in five different groups (A, B, C, D, and E, from highest to lowest response) for preparing a stock standard pool solution by weighting individual compounds to achieve the following starting concentrations: 25, 50, 100, 200, and 500 μg/mL, respectively (Table S2,

Supporting Information). Dilutions were made from the standard pool to prepare an injection range comprising 11 different calibration levels (from 1:10 to 1:10 000). Compounds included in each group are presented in Table 1.

Collection of Human Faeces and Preparation of Faecal Waters. For application of the method, faecal samples from a healthy volunteer who followed normal dietary habits were collected at the different days before ($n = 3$) and after ($n = 3$) daily intake (2 capsules, 700 mg each; minimum content of catechins/dose 140 mg) of a commercial flavan-3-ol supplement composed of grape seed and green tea catechins (Inneox Capillary Mass, Nestlè-L′Oreal. Madrid). Informed consent was obtained from this volunteer. Samples were stored at −80 °C until analysis. For preparation of faecal waters, samples were thawed at room temperature and weighted (0.5 g) in 50 mL sterile conical tubes. Five milliliters (5 mL) of sterile saline solution (NaCl, 0.9%; Fresenius Kabi, Spain) was added, vortexed, and stored in the fridge for hydration. Samples were then centrifuged (10 min, 1000 rpm, 4 °C) and the supernatant (faecal water) collected in 2 mL aliquots. For UPLC analysis, faecal water samples were diluted 1:2 with acetonitrile/water (2:4, v/v) and filtered (0.22 μm). Samples (190 μL) were spiked (10 μL) with a stock IS solution (50 μg/mL in acetonitrile/water (1:4, v/v)) to achieve a final IS concentration of 2.5 μg/mL.

UPLC-DAD-ESI-TQ MS. An UPLC system coupled to a Acquity PDA eλ photodiode array detector (DAD) and a Acquity TQD tandem quadrupole mass spectrometer equipped with Z-spray electrospray interfece (UPLC-DAD-ESI-TQ MS) (Waters, Milford, MA) was used. Separation was performed on a 2.1 × 100 mm id., 1.7 μm, BEH C18 column (Waters, Milford, MA) at 40 °C. A gradient composed of solvents A (water:acetic acid, 98:2 v/v) and B (acetonitrile:acetic acid, 98:2 v/v) was applied at a flow rate of 0.5 mL/min as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B; 18 min, 0.1% B. The DAD was operated in the 250−420 nm wavelength range at a 20 points/s rate and 1.2 nm resolution. The ESI parameters were as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative mode. The MS/MS parameters (cone voltage and collision energy) of each analyte were initially optimized by direct infusion experiments using 10 μg/mL solutions at a flow rate of 5 μL/min. The most sensitive transition (precursor and product ions) was selected for quantification purposes using the multiple reaction monitoring (MRM) mode. Data acquisition and processing was carried out by the MassLynx 4.1 software.

RESULTS AND DISCUSSION

Optimization of MS/MS Parameters. A total of 47 standards of phenolic acids (hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxymandelic acids and simple phenols) was used for method development. The MS/MS parameters (parent and daughter ions, cone voltage, and collision energy) optimized for each compound by performing direct infusion experiments are listed in Table S1 (Supporting Information). Negative-ion ESI showed better sensitivity than positive-ion ESI for all compounds. Cone voltage was optimized by acquiring the full-scan spectra and verifying the abundance of the $[M - H]^-$ ions. The MS/MS fragmentation behavior of each compound was then studied by recording the product or daughter ion spectra at different collision energies. The cone voltage ranged from 20 (3,4-dihidroxyphenylacetic acid) to 44 V (catechol/pyrocatechol) and the collision energy from 6 (4-hydroxyphenylacetic acid) to 30 V (3,4-dimethoxybenzoic acid).

Development of the Mass Spectrometry Method Acquisition. The selected UPLC conditions allowed separation of the 47 different analytes contained in the standard pool with high resolution within an 18 min injection run time. Due to the large number of analytes, a retention time-dependent MRM method was developed grouping the 47 transitions in 17 different MRM functions (Figure S1, Supporting Information). The number of compounds included in a particular MRM function varied from 1 to 9 compounds according to the dynamism of run time range. A dwell time of 7 ms was used for each compound, which allowed collection of an average of at least 20 points (as a mean) for each peak in the MRM chromatogram. To provide better visualization of the complete chromatogram, the MRM transitions have been separated in 4 different overlapped chromatograms (Figure 2). As can be observed, a high peak resolution was obtained for a wide range of analytes under the chromatographic conditions employed; however, MS detection was useful for correct identification and quantification of some other analytes presenting peak overlapping, particularly for analytes with very different responses.

Method Validation. The method validation was carried out in terms of limits of detection (LOD) and quantification (LOQ), calibration range and linearity, and precision and accuracy, according to official methods.[22]

Limits of Detection and Quantification. The instrumental limits of detection (LOD) and quantification (LOQ) following the criterion of signal-to-noise ratio ≥ 3 ($S/N \geq 3$) and $S/N \geq 8$, respectively, were determined by injection of the pool standard dilutions. LOD varied considerably (ca. 100 times) among the different analytes ranging from 0.001 (protocatechuic and 4-*O*-methylgallic acid) to 0.107 μg/mL (phenylpropionic acid) (Table 1), but it was <0.015 μg/mL for most analytes and therefore lower than the range found in most biological samples (>0.1 μg/mL).[10,18] Similarly, LOQ varied from 0.003 (protocatechuic acid) to 0.233 μg/mL (phenylpropionic acid). These limits seem to be in agreement with those obtained for phenolic acids by other LC-MS/MS and GC-MS/MS methods[10,18] and lower than other previous LC-MS/MS methods.[13] Some methoxylated (i.e., compounds **26, 27, 33, 35, 39, 40, 42, 44**), monohydroxylated, in particular C-4 monohydroxylated (i.e., compounds **3, 20, 28**), and nonhydroxylated (i.e., compounds **34, 36, 45**) compounds were among the ones presenting the highest LOD. Urpi-Sarda et al.[18] also found that compounds presenting these structural features, particularly 4-hydroxy-methoxy phenylacetic, 3-(4-hydroxyphenyl)-propionic, and phenylacetic acids, had the highest LOD. This could be associated with their low ionization efficiency under ESI in negative-ion mode.[13]

Linearity. Calibration curves were prepared by the method of external standard using 5 different starting concentrations (A−E) at 11 different calibration levels each. Weighted ($1/x^2$) least-squares regression analysis was applied to obtain the equation regression lines and correlation coefficients (r^2) (Table 1). As expected from the differences observed in MS responses and LODs, the linear concentration range was found to largely vary among the different analytes and inclusive among the ones included in the same calibration range (A−E).

Accuracy and Precision. The accuracy and precision were tested using the 1:100 dilution for each calibration level (0.25 μg/mL for group A; 0.5 μg/mL for group B; 1.0 μg/mL for group C; 2.0 for group D; and 5.0 for group E) (Table S2, Supporting Information), performing 3 different injections/day on 3 different days. The accuracy of the method, expressed as the

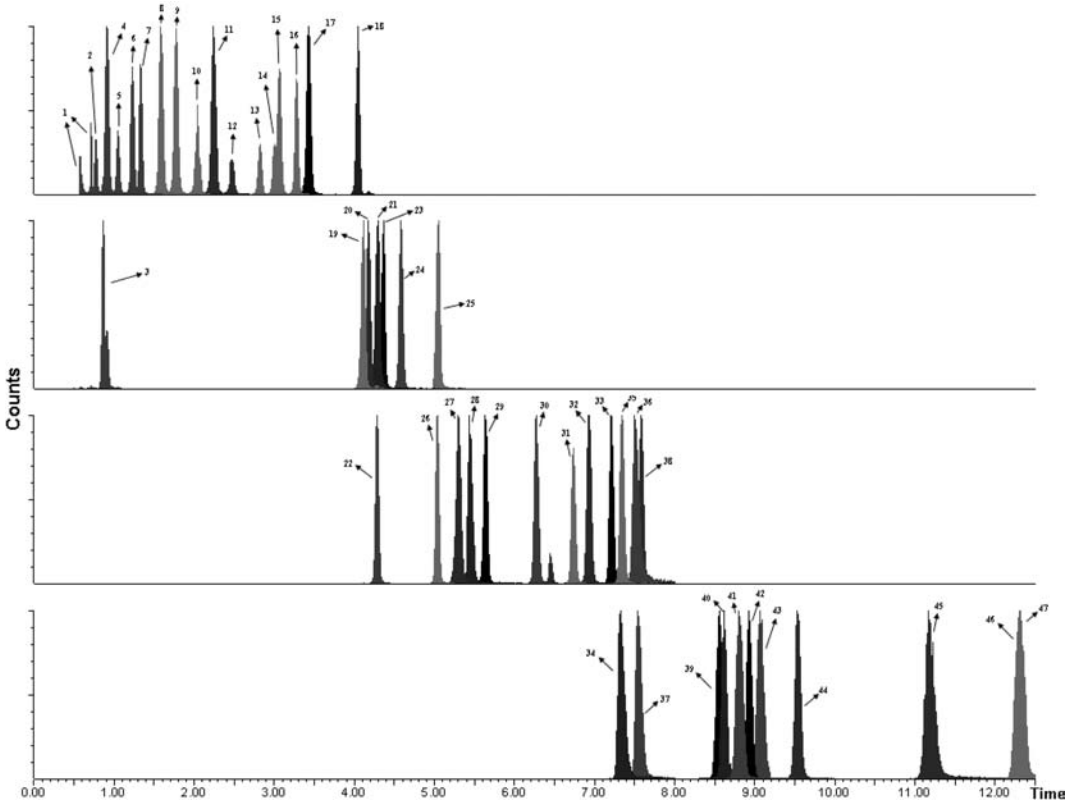


Figure 2. Overlapped MRM chromatograms (n = 47) of the pool standard solution.

nondeviation from added concentrations (mean observed concentration/added concentration ×100), ranged between 80% (caffeic acid) and 126% (4-methylcatechol) and met acceptance criteria (<20%)[22] for most of the phenolic compounds. The precision (interday precision or reproducibility), expressed as the relative standard deviation (% RSD), was lower than 15% ranging from 0.01% (4-hydroxyhippuric acid, pyrogallol and caffeic acid) to 13.4% (3,4-dihydroxymandelic acid) and therefore met acceptance criteria.[22]

Use of Internal Standard vs External Standard Calibration for Determination of the Content of Microbial Phenolic Acid in Faecal Samples. The use of an internal standard (IS) calibration method was also considered. After optimization of the MS/MS parameters (Table S1, Supporting Information), the MS response of the IS (4-hydroxybenzoic 2,3,5,6-d_4 acid) (R_t= 3.05 min) was first determined using 5 and 50 μg/mL solutions, as for the rest of the analytes. Considering the MS response, a calibration curve was prepared using dilutions in the range of 0.0025−25 μg/mL in acetronitrile/water (1:4, v/v) (results not shown). The LOD and LOQ were 0.010 and 0.030 μg/mL, respectively, which was is in the range of the analytes having the best MS response (Table 1). From these limits, a final concentration of IS of 2.5 μg/mL was considered to provide an optimum IS response for quantification purposes.

Standard curves were prepared by both external and internal standard calibration methods, and the faecal samples were processed by each method on 3 different days (Table S3, Supporting Information). Main metabolites detected in faecal water include: phenylpropionic acids (3-hydroxyphenylpropionic and phenylpropionic acids), phenylacetic acids (4- and 3-hydroxyphenylacetic, and phenylacetic acids), benzoic acids (protocatechuic, 3,5-dihydroxybenzoic, syringic and benzoic acids), and hydroxycinnamic acids (p-coumaric acid) (Figure 3). For each sample (1−6), one-way ANOVA was used to determine differences in metabolite levels as quantified by the external or internal standard calibration methods and F test to compare the variances of data obtained by the two calibration methods (Table S3, Supporting Information). No significant differences were detected in the level of microbial phenolic metabolites between the external or the internal standard calibration methods (both curves prepared in acetronitrile/water, 1:4, v/v), with the exception of 4 particular cases (protocatechuic acid in samples 3 and 5, p-coumaric acid in sample 5, and benzoic acid in sample 3) (Table S3, Supporting Information).

Another question that arises about MS quantification of phenolic acids in faeces concerns the possible effects of faecal matrix. In relation to this, we carried out some experiments to compare the MS response of the internal standard (4-hydroxybenzoic 2,3,5,6-d_4 acid, a compound that is, for sure, absent from faeces) dissolved in acetonitrile/water (1:4, v/v) and in faecal solutions at concentrations ranging from 0.025 to 25 μg/mL (results not shown). A general decrease in the MS response of the IS dissolved in faecal solution was observed in comparison to that in acetonitrile/water solution, being of 16% decrease at the standard concentration of 2.5 μg/mL. Therefore, faecal matrix could also underestimate the concentration of phenolic acids in faecal samples that were not subjected to any purification step. This is a matter that deserves further investigation.

Influence of the Intake of the Flavan-3-ol Supplement on the Content of Faecal Microbial Phenolic Acids. Considering that both internal and external standard methods gave similar quantitative results (Table S3, Supporting Information), the influence of the intake of the flavan-3-ol supplement on the content of faecal microbial acids was determined using the external

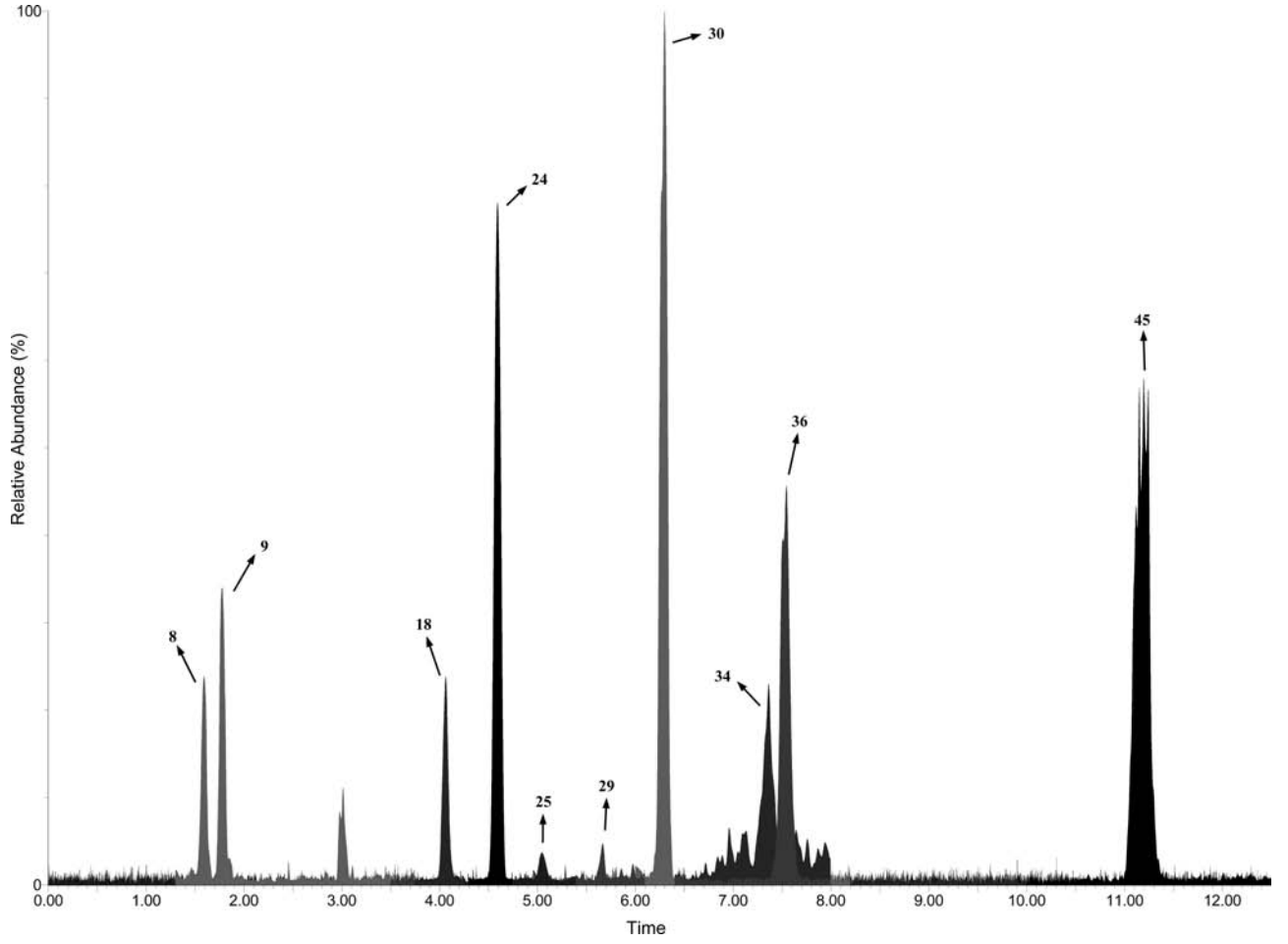


Figure 3. Overlapped MRM chromatograms (n = 47) of faecal sample 6.

calibration procedure. Among microbial metabolites, phenylacetic acid was the most abundant compound in faecal waters followed by phenylpropionic acid and by 4-hydroxyphenylacetic acid (Table 2), which is in line with the baseline profile from volunteers following normal dietary habits.[7] In a study carried out with five volunteers, these authors found a concentration range of 16−149 mg/L of phenylacetic acid in faecal water (this is, the upper water layer after homogenization of faeces in a stomacher), which is equivalent to a content of 10.4−96.9 μg/g of fresh faeces considering an average faecal water content of 65%. These data are in line with our findings since the content of phenylacetic acid varied from 58.3 to 211.4 μg/g (Table S3, Supporting Information).

Two sample t test was used to compare means of the content of phenolic acids in faeces before and after intake of the flavan-3-ol supplement (Table 2). With the exception of 3,5-dihydroxybenzoic, 4-hydroxyphenylacetic, and phenylacetic acids, consumption of the flavan-3-ol supplement increased the level of microbial acids, although differences were only significant (p < 0.05) for 3-(3-hydroxyphenyl)-propionic acid. The profile of phenolic acids found in faecal samples seems to be accordance with other studies carried out with other flavan-3-ol rich sources or extracts. According to Grun et al.,[10] 3- and 4-hydroxybenzoic, 4-hydroxyphenylacetic, and 4-hydroxyphenylpropionic acids were among the microbial acids detected in faeces after consumption of green tea. Faecal fermentations of cider and apple proanthocyanidins also resulted in production of 3,4-dihydroxyphenylpropionic, 3-hydroxyphenylpropionic, phenylpropionic, 3,4-dihydroxyphenylacetic, 3-hydroxyphenylacetic, and benzoic acids.[8] In another study, 3- and 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, and 3,4-dihydroxyphenylacetic acid were detected after in vitro fermentation of a dimeric procyanidin fraction from grape seeds by human microbiota.[12]

Table 2. Level of Microbial Phenolic Acids (μg/g) in Human Faeces before and after Consumption of the Flavan-3-ol Supplement[a]

no.	compound	before (n = 3)	after (n = 3)
8	3,5-dihydroxybenzoic acid	0.233 ± 0.034	0.217 ± 0.189
9	protocatechuic acid	0.433 ± 0.289	0.825 ± 0.266
18	4-hydroxyphenylacetic acid	4.850 ± 1.314	2.451 ± 1.488
24	3-hydroxyphenylacetic acid	2.882 ± 0.476	4.278 ± 7.165
25	syringic acid	0.337 ± 0.228	0.660 ± 0.172
29	p-coumaric acid	0.208 ± 0.046	0.447 ± 0.222
30	3-(3-hydroxyphenyl)-propionic acid	1.152[b] ± 0.763	7.259 ± 2.900
34	benzoic acid	0.936 ± 0.748	2.861 ± 2.511
36	phenylacetic acid	160.115 ± 25.442	144.417 ± 74.349
45	phenylpropionic acid	54.833 ± 5.149	55.629 ± 29.228

[a] Mean (n = 3) ± standard deviation (SD). [b] Mean values between before and after consumption significantly differed at p < 0.05.

In conclusion, a reproducible, sensitive, and rapid UPLC-ESI-TQ MS analytical method was developed allowing determination of 47 microbial-derived phenolic acids in faeces with high resolution within an 18 min injection run time. The LOD (0.001−0.107 μg/mL) of the different analytes is in the range found in biological samples, and therefore, it is useful for application in the targeted analysis of microbial phenolic acids in faeces from human intervention and in vitro fermentation studies.

■ ASSOCIATED CONTENT

Supporting Information. Optimized MS/MS conditions for detection of microbial phenolic acids; concentration range (μg/mL) of each calibration level of the pool standard solution; application of external and internal calibration methods for quantification of microbial phenolic acids in human faeces; diagram of the time-dependent MRM mode acquisition method developed. This material is available free of charge via the Internet at http://pubs.acs.org.

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