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GC–MS methods for metabolic profiling of microbial fermentation products of dietary polyphenols in human and *in vitro* intervention studies☆

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ABSTRACT

Flavonoids, a subclass of polyphenols, are major constituents of many plant-based foods and beverages, including tea, wine and chocolate. Epidemiological studies have shown that a flavonoid-rich diet is associated with reduced risk of cardiovascular diseases. The majority of the flavonoids survive intact until they reach the colon where they are then extensively metabolized into smaller fragments. Here, we describe the development of GC–MS-based methods for the profiling of phenolic microbial fermentation products in urine, plasma, and fecal water. Furthermore, the methods are applicable for profiling products obtained from *in vitro* batch culture fermentation models. The methods incorporate enzymatic deconjugation, liquid–liquid extraction, derivatization, and subsequent analysis by GC–MS. At the level of individual compounds, the methods gave recoveries better than 80% with inter-day precision being better than 20%, depending on the matrix. Limits of detection were below 0.1 μg/ml for most phenolic acids. The newly developed methods were successfully applied to samples from human and *in-vitro* intervention trials, studying the metabolic impact of flavonoid intake. In conclusion, the methods presented are robust and generally applicable to diverse biological fluids. Its profiling character is useful to investigate on a large scale the gut microbiome-mediated bioavailability of flavonoids.

1. Introduction

Flavonoids are compounds present at high concentrations in many food products, such as fruit and vegetables, tea, cocoa, and wine [1]. A diet rich in flavonoids is associated with beneficial health effects, e.g. reduced risk of cardiovascular disease. In particular consumption of tea, grape (juice), and red wine has been shown to improve markers of vascular function [2]. For improving our understanding of the fate of polyphenols and their final clinical effects, gaining insight into their metabolism is essential. As yet, polyphenol metabolism has remained almost completely unexplored. It is known that the bioavailability of intact flavonoids is low [3]. Absorption primarily occurs after the polyphenol compounds underwent extensive metabolism by the resident gut microbiota in the colon where they are degraded into smaller phenolic acids [1,4]. In order to obtain a holistic view of microbiome-mediated bioavailability of polyphenols, analytical methods are required that can assess polyphenol gut-fermentation products in a comprehensive manner. Potential methods include NMR spectroscopy [5] and techniques based on LC and GC. NMR spectroscopy is a global technique requiring minimal sample pretreatment, but suffers from low sensitivity. Most phenolic acids are present in human biofluids at micromolar to sub-micromolar levels (ng/ml to μg/ml concentrations), requiring sensitive analytical methods, such as LC with electrochemical detection, LC–MS or GC–MS [6–14]. LC-based methods have been proven to be robust methods for quantification of selected metabolites [15] and only recently with the development of high-resolution LC systems utilizing sub 2 μm particles and highly sensitive mass spectrometric detection, non-targeted analysis by LC–MS is becoming more widespread. GC–MS has long been preferred for profiling (unknown) metabolites due to its superior separation properties and high sensitivity. Additionally, mass spectral libraries are available, facilitating the identification of metabolites.

Although a number of papers were published that describe the (targeted) analysis of phenolic acids in selected matrices, there is a clear lack of analytical methods that approach polyphenol gut

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fermentation products in an unbiased way. For successful targeted and non-targeted analysis of phenolic acids by GC–MS, the sample preparation is essential. It generally includes a deconjugation step, extraction of metabolites from the matrix and derivatization. Extraction methods can primarily be divided into those that are based on solid-phase extraction (SPE) [7,8,10–13,15,16] and those that apply liquid–liquid extraction (LLE) [6,11,14,17–20]. An advantage of SPE over LLE is that it is suited for automation. Disadvantages include the rather time consuming nature of developing an SPE method and its selectivity. Selectivity may be an advantage when targeted analysis is the main objective, but it is a distinct disadvantage in the discovery of unknown metabolites. Further requirements for a profiling method are: good reproducibility of the method (<20% variation) and high sensitivity. Levels of phenolic acids in human bodyfluids are typically in the low micromolar range (0.1–20 μg/ml [10,14]), therefore, limits of detection should be better than 0.1 μg/ml.

Here we describe the development and application of methods for the profiling and discovery of phenolic acids in human urine, plasma, feces as well as in an *in vitro* colonic fermentation model. The methods described in the present contribution are based on liquid–liquid extraction followed by GC–MS. The various steps in the methods (deconjugation, acidification, derivatization) are optimized in order to obtain the best recoveries and to minimize experimental variation. The methods are applied to human intervention studies as well as to *in vitro* gut fermentation experiments.

2. Experimental

2.1. Materials

β-D-Glucuronidase containing ~10% sulfatase activity from *Helix pomatia* Type H-5 (G1512, 500 kU), phenolphthalein-β-D-glucuronide (≥98%), benzoic acid (BA, 99.5%), sodium azide (p.a.), phenylacetic acid (PAA, 99%), *m*-toluic acid (*m*TA, 99%), 3-phenylpropionic acid (3-PPA, 99%), mandelic acid (MA, 99%), 2-hydroxybenzoic acid (2-HBA, 99%), 3-hydroxybenzoic acid (3-HBA, ≥99%), 4-hydroxybenzoic acid (4-HBA, ≥99%), 2,3-dihydroxybenzoic acid (2,3-DHBA, 99%), 2,4-dihydroxybenzoic acid (2,4-DHBA, ≥98%), 2,6-dihydroxybenzoic acid (2,6-DHBA, 98%), 3,4-dihydroxybenzoic acid (3,4-DHBA, ≥97%), 3,5-dihydroxybenzoic acid (3,5-DHBA, ≥97%), 2,4,6-trihydroxybenzoic acid (2,4,6-THBA, ≥90%), 3-hydroxyphenylacetic acid (3-HPAA, ≥97%), *trans*-cinnamic acid (*t*CA, 99%), *trans*-2-hydroxycinnamic acid (2-HCA, ≥97%), *trans*-3-hydroxycinnamic acid (3-HCA, 99%), *trans*-4-hydroxycinnamic acid (4-HCA, ≥98%),, 4-methoxybenzoic acid (4-MBA, 99%), gallic acid (GA, 98%), 3-*O*-methylgallic acid (3-OMGA), 4-*O*-methylgallic acid (4-OMGA), vanillic acid (VA, ≥97%), homovanillic acid (HVA, ≥99%), syringic acid (SA, 98%), hydrocaffeic acid (HCA, ≥98%), ferulic acid (FA, 99%), caffeic acid (CA, 99%), phloroglucinol (PG, ≥99%), catechin (98%), 4-hydroxyphenylacetic acid (4-HPAA, 98%), 3,4-dimethoxyphenylacetic acid (3,4-DMPAA, 98%), 3-(4-hydroxyphenyl)-propionic acid (4-HPPA, 98%), *trans*-cinnamic acid-d_6 (≥98 at.% *D*), 3-phenylpropionic acid-d_9 (≥98 atom %D), 2-hydroxybenzoic acid-d_4 (≥98 at.% *D*), 4-hydroxybenzoic acid-d_6 (≥98 at.% *D*), 2,4,6-tris(trifluoromethyl)-1,3,5-triazine, and perfluorotributylamine were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Syringic acid-d_6 and ferulic acid-d_3 were synthesized in-house. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS) was purchased in 1-ml ampules from Pierce (Perbio Science Nederland B.V., Etten-Leur, The Netherlands, p.a.). Glacial acetic acid, methanol, ethyl acetate, dichloromethane, *n*-hexane, sodium acetate, sodium bicarbonate, sodium chloride, hydrochloric acid, and sodium hydroxide (50%) were from Merck (VWR International, Amsterdam, The Netherlands) and were all of analytical purity. Glycine was from Acros Organics (Fisher Emergo BV, Landsmeer, The Netherlands).

2.2. Sample collection and storage

Urine and plasma samples were collected in a dietary intervention trial run at the Unilever Vlaardingen Center for Nutritional Intervention Trials. In brief, 26 adult human volunteers (15 men and 11 women) participated in a double-blind, placebo-controlled randomized cross-over study consisting of a two-week run-in period followed by two four-week treatment periods. Treatments consisted of either cellulose capsules with a polyphenol-rich mix of red wine and red grape juice extracts (daily dose of 800 mg polyphenols) or placebo (empty) capsules. At the end of the treatment periods fasted blood samples were collected in heparinized tubes. 6 ml of blood was drawn in tubes containing lithium heparin as anticoagulant. Plasma samples were prepared by centrifugation at 1500 × *g* for 10 min at 4 °C and were immediately stored at −80 °C. 24-h urine samples were collected, weighed, and stored at −20 °C. Stool samples from a single, healthy volunteer were collected at day 0, 1 and 2 after the consumption of eight cups containing 200 ml of green tea (5-min extractions of 2 g of green tea in hot water). Samples were frozen directly after collection. The *in vitro* batch culture fermentations were basically performed as described by Tzounis et al. [21]. For the *in vitro* batch culture fermentation, fecal samples were obtained from one healthy human volunteer who had not been prescribed antibiotics for at least 6 months prior to the study and had no history of any gastrointestinal disease. Samples were collected and used immediately after collection. A 1:10 dilution in anaerobic phosphate buffered saline (PBS) (8 g NaCl, 0.2 g KCL, 1.44 g $Na_2HPO_4H_2O$, 0.24 g KH_2PO_4, pH 7.4) was prepared and the samples were homogenized in a stomacher for 2 min. For the anaerobic batch fermentation, 800 mg of Provinol wine extract (Seppic, France) was used.

2.3. Sample preparation

2.3.1. Urine

To 500 μl urine, 50 μl 1.5 M sodium acetate, and 30 μl of β-D-glucuronidase (1500 units in 150 mM sodium acetate) was added. The samples were incubated at 50 °C. Optimum incubation times were determined by incubating known concentrations of phenolphthalein-β-D-glucuronide for up to 4 h and measuring the absorption of liberated phenolphthalein as described previously [22]. A solution of phenolphthalein-β-D-glucuronide in buffer (150 mM sodium acetate, pH 4.8) was used as a control. After allowing to cool to room temperature (RT) samples were acidified prior to extraction. Different acidification procedures were tested. Either no acid, 40 μl of glacial acetic acid (final concentration 1.4 M), 20 μl of trifluoroacetic acid (final concentration 0.5 M) or 65 μl of 1 M hydrochloric acid was added (final concentration 0.1 M). The best procedure was selected by comparing recoveries of urine samples that were spiked with known amounts of phenolic acid standards. After the addition of internal standard (50 μl of 100 μg/ml *trans*-cinnamic acid-d_6 in 1:1 methanol/water (v/v)), the samples were placed at 4 °C for 10 min. 2 ml of ethyl acetate was added, the samples were vortexed for 30 s, and centrifuged at 3000 × *g* for 10 min. The supernatants were transferred and the extraction was repeated twice. Extracts were combined and dried under a stream of nitrogen at 40 °C. Samples were further dried by the subsequent addition and evaporation of 1 ml of dichloromethane. To the samples 100 μl of BSTFA/TMCS was added. The samples were incubated at RT, 50, 70, or 90 °C for 30 min or at 70 °C for 4 h. From the results, the

best derivatization condition was chosen. After cooling down to RT, 400 μl of hexane was added and the samples were vortex-mixed. 200 μl of the derivatized samples was transferred to GC vials and analyzed by GC–MS.

2.3.2. Plasma

To 200 μl plasma, 40 μl 0.1% Na_2EDTA, 20 μl 1.5 M sodium acetate, and 500 U glucuronidase (10 μl) were added. The samples were incubated at 37 °C. Similar to the urine samples, optimum incubation times were determined by measuring the hydrolysis of phenolphthalein-β-D-glucuronide. After cooling to RT, 300 μl of water, 10 μl 1 N HCl and 40 μl of internal standard mixture (5 μg/ml of *trans*-cinnamic acid-d_6, 3-phenylpropionic acid-d_9, 2-hydroxybenzoic acid-d_4, and 4-hydroxybenzoic acid-d_6, syringic acid-d_6 and ferulic acid-d_3 in 1:1 methanol/water (v/v)) was added. Samples were placed at 4 °C for 10 min. Phenolic acids were extracted three times by vortexing for 30 s with 1 ml of ethyl acetate. The extracts were further processed as described above. After derivatization, the samples were diluted with 100 μl of hexane.

2.3.3. Fecal extracts

Fecal extracts were prepared by adding two volumes (v/w) of water to a weighed sample of thawed stool material. The mixture was homogenized by vortex mixing for 60 s. The fecal slurry was centrifuged at 2200 × *g* and 8 °C for 35 min. The supernatant was filtered through a Millipore Millex-FH sterile 0.45 μm syringe filter. The filtered fecal water was stored at −20 °C prior to analysis. Phenolic acids were extracted and derivatized as described for urine samples except for the enzymatic hydrolysis, which was omitted.

2.3.4. In vitro batch culture fermentation

The extraction and analysis of phenolic acids was as described for urine samples except for the enzymatic hydrolysis, which was omitted.

2.4. GC–MS analysis

Derivatized samples were analyzed on a Waters GCT mass spectrometer (Waters, Etten-Leur, The Netherlands) equipped with an Agilent 6890 gas chromatograph and an Agilent 7683 autosampler (Agilent, Amstelveen, The Netherlands). Three fused-silica columns were evaluated for separating the phenolic acids: a VF-5 ms (30 m × 0.25 mm, d.f. = 0.25 μm), a VF-17 ht column (30 m × 0.25 mm, d.f. = 0.10 μm) (both from Varian, Middelburg, The Netherlands), and a Phenomenex ZB-5 ms (30 m × 0.25 mm; d.f. = 0.25 μm) (Bester, Amstelveen, The Netherlands). Helium was the carrier gas and was used at a flow rate of 1 ml/min. The sample (1 μl) was injected into a liquid-nitrogen-cooled CIS-4 injector (Gerstel, Mülheim an der Ruhr, Germany) operated in split mode at a split ratio of 20:1 (urine, fecal water and batch culture fermentations) or 5:1 (plasma). The injector used a temperature gradient from 55 °C (held for 0.05 min) to 300 °C (held for 5 min) at 8 °C/s. The temperature program of the column oven was as follows: 0–1 min isothermal at 45 °C, 1–6.5 min at 10 °C/min to 100 °C, 6.5–26.5 min at 7.5 °C/min to 250 °C, and 26.5–29 min at 10 °C/min to 300 °C, where it was held for 6 min. For plasma extracts, the final temperature was increased to 350 °C and was held at that temperature for 10 min before returning to the initial conditions. The interface and source temperature of the mass spectrometer were 250 °C and 180 °C, respectively. Mass spectra were recorded in EI mode from 6 to 30 min at a scan time of 0.3 s, an interscan time of 0.1 s, and a scan range of *m*/*z* 50–600. The mass spectrometer was tuned with 2,4,6-tris(trifluoromethyl)-1,3,5-triazine and calibrated with perfluorotributylamine. Samples were analyzed batchwise. All samples of an individual were grouped. A batch typically consisted of ~20 samples and included two reference and two quality control (QC) samples. The reference samples consisted of a set of reference phenolic acid standards that were derivatized and analyzed. The QC samples consisted of the appropriate bodyfluid that was spiked with phenolic acid standards. The QC samples were processed and analyzed as described. The recoveries and repeatability of the extraction procedure were determined on a Hewlett Packard 5973 MSD mass spectrometer equipped with an Agilent 6890 gas chromatograph and an Agilent 7683 autosampler. The analysis conditions were similar to those described for the GC–TOF–MS analyses.

2.5. Data analysis

MS-based metabolomics typically requires initial alignment of the data to correct for peak shifts in subsequent profiles when they are collected over prolonged periods of time. Visual inspection of the total ion chromatograms (TICs) showed very good alignment for the majority of the signals, owing to the relatively short period of the measurement schemes (2–12 weeks). Therefore alignment was not deemed necessary and simple binning of the TICs prior to multivariate data analysis was preferred over more sophisticated alignment algorithms that attempt to align molecular fragments using both retention time and *m*/*z* dimensions. GC–MS raw data were baseline corrected and converted to NetCDF format using MetAlign software (http://www.metalign.nl) and then imported into AMIX software (Bruker, Karlsruhe, Germany), which was used to segment the TICs in equally sized bins of 0.01 min. Individual binned TICs were then normalized to the peak of the internal standard *trans*-cinnamic acid-d_6 at 12.96 min and corrected for the total volume of the 24-h urine sample. Multivariate data analysis based on principal component analysis (PCA) and orthogonal projection to latent structures (OPLS) [23] was performed using Simca-P software (version 11.0, Umetrics, Umeå, Sweden) to differentiate the phenolic profiles of treated and placebo samples. Since in human nutrition studies the inter-individual variation in metabolic profile is typically much greater than the intra-individual variation (i.e. the treatment effect) and to exploit the paired data structure, the data were mean centered per individual and then Pareto-scaled. OPLS discriminant analysis (OPLS-DA [24,25]) was applied to specifically focus on the polyphenol treatment effect. The quality of the models was assessed using the parameter R^2, which reflects the ability to fit the data, and Q^2, which was determined by seven rounds of internal cross-validation and reflects the model's ability to predict the data (with $Q^2 > 0$ indicating a significant model).

3. Results and discussion

3.1. Deconjugation

In both urine and plasma, phenolic acids are commonly conjugated to sulfate and glucuronic acid [4]. To reduce the total number of possible metabolites and to facilitate the analysis by GC–MS, an enzymatic deconjugation step using a mixture of β-glucuronidase and sulfatase was included in the sample preparation. First the optimum incubation time was determined by monitoring the hydrolysis of phenolphthalein-β-D-glucuronide. In urine, hydrolysis reached equilibrium after 2 h at 50 °C (data not shown). In plasma, hydrolysis was complete after 30 min, whereas in blank matrix (150 mM NaOAc, pH 4.8) complete hydrolysis was obtained within 10 min. As glucuronic acid or sulfate conjugates have not been reported in fecal extracts [7], the enzymatic hydrolysis step was omitted for fecal extracts and gut model fermentation samples.

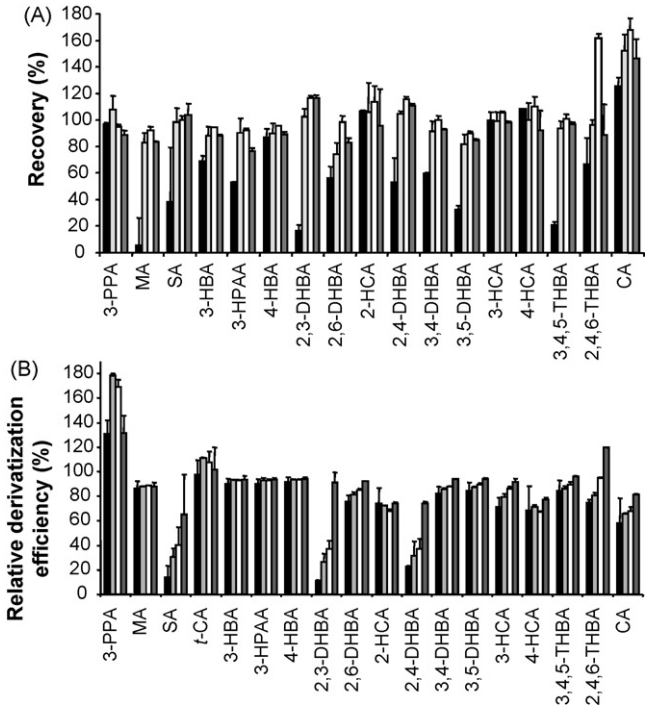

Fig. 1. Optimization of acidification and derivatization conditions. (A) Effect of acidification on recoveries of phenolic acids. Black: no acid; light grey: acetic acid; white: hydrochloric acid; dark grey: trifluoroacetic acid, (B) effect of temperature on derivatization efficiency. Black: 30 min at RT; light grey: 30 min at 50 °C; white: 30 min at 70 °C; dark grey: 30 min at 90 °C. Derivatization efficiency is displayed relative to that obtained for derivatizing a standard mixture at 70 °C for 4 h (100%). For abbreviations, refer to the Section 2.

3.2. Effect of acid during extraction

Prior to ethyl acetate extraction, samples were acidified to pH 2–3. Acidification of the samples was performed for three reasons. First, phenolic acids are less prone to degradation at a low pH. Second, acidification decreases interactions between the phenolics and protein, resulting in better overall extraction recoveries. Finally, lowering the pH to under the pK_a of the phenolic acids (which is between 4 and 5 [26]) will render them more hydrophobic, which is favorable for extraction with ethyl acetate. Several conditions and acids were evaluated to find the optimal extraction procedure. To determine the recoveries of phenolic acids from urine, urine was spiked with a mixture of phenolic acids and extracted with ethyl acetate using four different conditions: without acidification, or acidified with acetic acid (AcOH), hydrochloric acid (HCl), or trifluoroacetic acid (TFA). The results show that relative recoveries were 5–125% (average 64%) when omitting acidification (Fig. 1A). After acidification with AcOH, HCl, or TFA, recoveries of respectively 74–152% (average 97%), 89–176% (average 108%), and 76–146% (average 96%) were obtained. Recoveries exceeding 100% were due to the presence of endogenous phenolic acids. Although addition of any acid tested strongly increased the recoveries of phenolic acids, HCl was preferred over the others because of the high overall recoveries and the low experimental variations. Therefore, all other experiments were performed using HCl as acidifier.

3.3. Derivatization conditions

Phenolic acids should be derivatized prior to GC–MS analysis. Commonly, trimethylsilylation using BSTFA is used [26]. Due to its low reactivity, vicinal hydroxyls and secondary amines are best derivatized when a small percentage (1–10%) of TMCS is present in the BSTFA reagent. We used BSTFA containing 10% TMCS and optimized the incubation conditions. After thoroughly drying the ethyl acetate extracts, the derivatization reagent was added and the samples were incubated at RT, 50, 70, or 90 °C for 30 min or at 70 °C for 4 h. The results are graphically represented in Fig. 1B and show that in general a higher incubation temperature increased derivatization efficiency. Although an incubation at 70 °C for 4 h overall gives best results, we found that a 30-min incubation at 90 °C is a good compromise between derivatization completeness and time efficiency.

3.4. Recoveries

Recoveries from urine, plasma, and fecal water extracts were determined by spiking each of the biofluids with phenolic acid standards. Samples were extracted and analyzed by GC–MS. For urine spiked with phenolic acid standards at 4 or 10 μg/ml, the recoveries were better than 83% or 90%, respectively, with intra-experimental errors of 0.9–14.5%. Recoveries from plasma were better than 73% for most phenolic acids with intra-experimental errors of 2.1–11.6% when spiked with known standards at a concentration of 1 μg/ml. Some phenolic acids, particularly some dihydroxybenzoic acids and caffeic acid, gave poor recoveries (<20%). Recoveries of phenolic acids from fecal extracts were better than 75%, 86% or 85% when spiked at concentrations of 0.1, 1 or 10 μg/ml, respectively.

3.5. Repeatability

The inter-day method repeatability was determined by analyzing urine or plasma samples spiked with 20 phenolic acid standards plus deuterated internal standard at a concentration of 10 or 1 μg/ml, respectively. Duplicate samples were prepared and analyzed on four different days. Relative standard deviations (RSD) in urine ranged from 1.5 to 21.4%, except for 2,4,6-trihydroxybenzoic acid, which had an RSD of 45.3%. In plasma, relative standard deviations ranged from 1.9 to 19.5%, except for some dihydroxybenzoic acids (not determined) and caffeic acid (39%), which gave very poor recoveries. Due to the limited availability of fecal extracts, inter-day repeatability was not determined for this matrix.

3.6. Limits of detection and linearity

Linearity and limits of detection (LODs) were determined for 20 phenolic acid standards in a blank matrix at concentrations ranging from 0.05 to 50 μg/ml. The LOD was defined as the lowest detectable chromatographic peak with a signal-to-noise ratio of 3:1 or higher. The LOD was better than 0.05 μg/ml for all standards except for 2,3-DHBA and GA, which had LODs of 0.5 μg/ml. The linearity of the GC–MS was determined for each of the 20 standards. For most compounds the mass spectrometer showed a reasonable linearity ($R^2 > 0.990$) only up to concentrations of 10 or 20 μg/ml. For 3-HPAA and 2,4,6-THBA the mass spectrometer was linear up to a concentration of 50 μg/ml.

From these results, we conclude that the sensitivity of the method is sufficient for the profiling of microbial fermentation products of dietary polyphenols in various bodyfluids. We further conclude that the limited dynamic range and linearity of the TOF mass analyzer make this type of analyzer not particularly suited for quantitative measurements. The latest generation of TOF analyzers is strongly improved and has an enhanced dynamic range. Still, when highest sensitivity or accurate mass recording are not required, a quadrupole mass analyzer may be preferred due to its high dynamic range and excellent linearity, and, not in the least, its ease in handling. For profiling, however, the advantages of the TOF

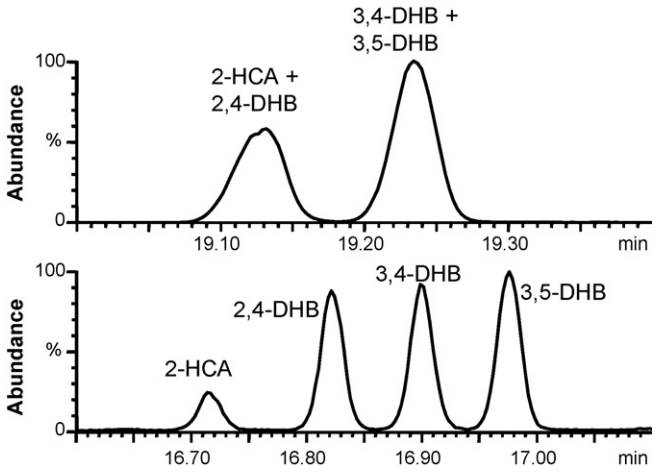

Fig. 2. Effect of stationary phase thickness on separation. Top chromatogram: 0.25 μm film thickness; bottom chromatogram: 0.10 μm film thickness.

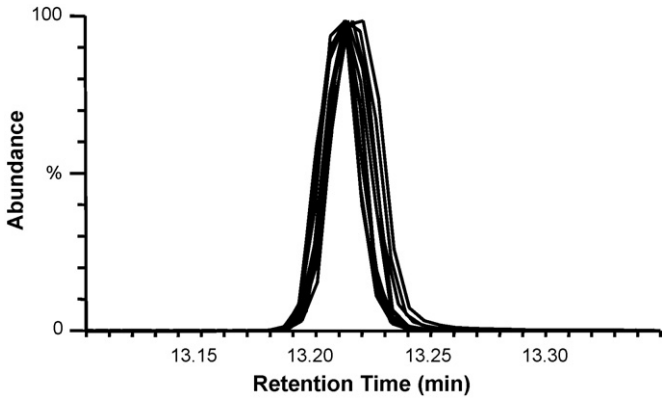

Fig. 3. Overlay of 12 extracted ion chromatograms randomly selected from the injection of over 200 urine samples. These data show that retention time drifts were hardly apparent.

mass analyzer as used here are its high sensitivity in combination with its ability to record accurate molecular masses.

3.7. Choice of column

For profiling phenolic acids, 25 to 30 m fused silica capillary columns with 5% phenyl methyl dimethylpolysiloxane are the most commonly employed [26]. The thickness of the stationary phase is typically 0.25 μm. To explore whether the film thickness influences the separation of phenolic acids, we compared columns that differed in film thickness: a VF-5 ms 30 m × 0.25 mm with 0.25 μm film thickness and a VF-5 ht 30 m × 0.25 mm with a 0.1 μm film (both from Varian). Fig. 2 represents a detailed section of the separation of 2-HCA, 2,4-DHB, 3,4-DHB, and 3,5-DHB on both columns. Using the same temperature program, on the VF-5 ht column the four phenolic acids are completely separated, whereas on the VF-5 ms column the compounds partly overlap. Also on a column from a different manufacturer (Phenomenex ZB-5 ms 30 m × 0.25 mm with 0.25 μm film thickness) the four compounds could not be well separated. From this, we conclude that a DB 5-type of fused silica capillary column with a film thickness of 0.1 μm is best suited for separating phenolic acids.

3.8. Retention time drifts

The statistical analysis of large data sets is primarily based on the retention time of a certain compound. Obviously, good alignment of the GC data is crucial. A number of alignment software packages exists, both non-commercial (e.g. MetAlign (http://www.metalign.nl), MzMine [27,28], xcms [29] and commercial software (e.g. MarkerLynx (Waters), GeneSpring (Agilent), and Expressionist (Genedata)). Internal standards may assist correct alignment, and for this, deuterium-labeled standards may be used. To determine the stability of the system described in the present paper, drifts in retention times were determined by monitoring the internal standard (*trans*-cinnamic acid-d_6) throughout the analyses. In Fig. 3, an overlay of 12 chromatograms is displayed, showing the chromatographic peak of the internal standard selected from over 200 injections of urine extracts. The drift in retention time between the first (injection # 3) and the last (injection # 212) of the monitored injections was only 0.01 min, demonstrating the excellent stability of the GC–MS system. Also the peak widths (average 0.023 ± 0.004 min), though varying slightly between the different experiments, did not increase with the number of injections. Therefore, in this study data alignment was omitted and a simpler and more rapid means of data pre-processing, i.e. binning of the total ion current chromatograms was chosen.

3.9. Profiling of phenolic metabolites in human urine

The profiling method was applied to urines of 26 volunteers that participated in a placebo-controlled cross-over study involving the consumption of a polyphenol-rich mixture of wine and grape juice extracts. In Fig. 4A, two representative total ion chromatograms (TIC) of a single volunteer are displayed showing urine profiles after placebo (top panel) and after intervention (lower panel). The most obvious differences between the chromatograms of placebo and treatment urines are indicated by arrow heads. Multivariate data analysis using OPLS-DA was performed on the complete set of urine GC–MS data to determine differences between control and intervention groups. Fig. 4B shows the OPLS-DA score plot of intervention vs. placebo, which had a predictive component t1[P] with Q^2 of 0.47. The complementary coefficient plot is shown in Fig. 4C and shows the relative contributions of each retention time variable to the model. The mass traces of the most relevant peaks were used for metabolite identification using the NIST02 library for EI-MS data. From the 67 peaks with the highest variable influence on the projection (VIP), i.e. strongest influence on the model, 11 peaks were identified as phenolic acids (Table 1). Other compounds include short-chain fatty acids and hitherto unidentified metabolites. Among the identified phenolic acids are 3-HPAA, 4-HPAA, vanillic acid, homovanillic acid, and several hydroxylated forms of hippuric acid that have been described in literature as metabo-

Table 1
Important phenolic acids that were increased in urine after grape juice/winepolyphenol-rich extract intake

RT (min)	Metabolite	VIP
14.14	3-Hydroxyphenylacetic acid	7.98
14.58	4-Hydroxyphenylacetic acid	2.51
15.86	3-(3-Hydroxyphenyl)propionic acid	1.94
16.38	Vanillic acid	1.31
16.48	Homovanillic acid	3.02
17.49	Hippuric acid	5.32
17.59	3-(3-Hydroxyphenyl)-3-hydroxypropionic acid	7.67
18.21	Syringic acid	1.63
21.31	3-Hydroxyhippuric acid	8.63
21.55	4-Hydroxymandelic acid	4.61
22.21	4-Hydroxyhippuric acid	8.29

VIP represents the variable influence on the projection, i.e. its contribution to the model.

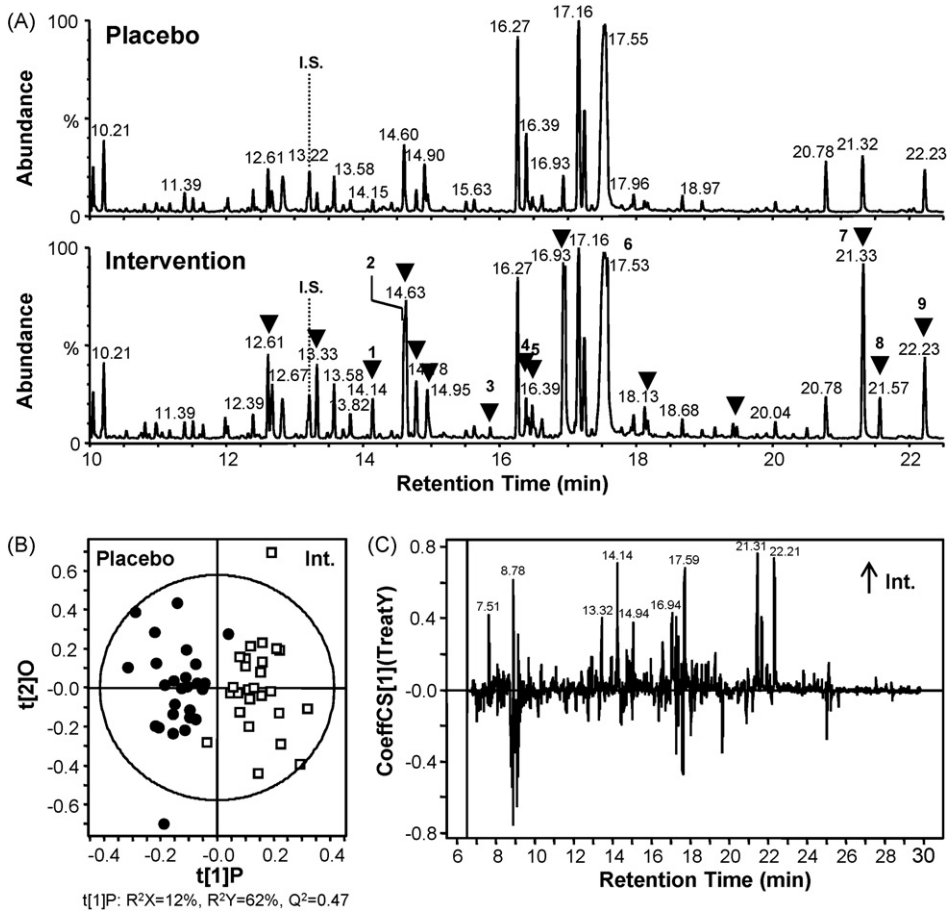

Fig. 4. Profiling of phenolic acids in urine after intake of grape juice/wine extract. (A) GC–MS profiles of ethyl acetate extracts of human urine of placebo (top chromatogram) and intervention (bottom chromatogram). (B) OPLS analysis of the GC–MS profiles of urine showing the different metabolic impact of intervention vs. placebo intake. (C) OPLS coefficients plots indicating the metabolites that increased after intervention. Identified phenolic acids are numbered: 1: 3-HPAA, 2: 4-HPAA, 3: 3-HPPA, 4: VA, 5: HVA, 6: hippuric acid (mono-TMS), 7: 3-hydroxyhippuric acid, 8: 4-hydroxymandelic acid, and 9: 4-hydroxyhippuric acid. For abbreviations, refer to the Section 2.

lites formed after ingestion of red wine or black tea polyphenols [10,30].

When analyzing large numbers of samples over a prolonged period of time as in a metabolomics approach, experimental variation may become critical and potentially obscure treatment effects. In the present study, all samples of a single volunteer were processed in the same batch to reduce experimental variation. It is believed that within-batch variation is less strong than between-batch variation. Also the use of appropriate internal standards may reduce variations. We chose the deuterium-labeled *trans*-cinnamic acid-d_6 as an internal standard. This phenolic acid is commercially available, showed to be very stable, and since it is deuterated, it does not occur in nature.

3.10. Profiling of phenolic metabolites in human plasma

The method for extraction of phenolic acids from urine was slightly modified for human plasma samples. The major difference to urine is the high protein and lipid concentrations in plasma. To prevent interactions between proteins and phenolic metabolites, EDTA was added. In addition, we observed that extraction of the phenolics was facilitated when the plasma samples were diluted prior to extraction. Even then, plasma showed to be a rather difficult matrix. Some phenolics, especially some dihydroxybenzoic acids were extracted with very low recoveries. In Fig. 5, total ion chromatograms are displayed of trimethylsilyl derivatives of a mixture of 29 reference phenolic acid standards (Fig. 5A), an extract of plasma (Fig. 5B) and of an extract of plasma that was spiked with the phenolic acid standards (Fig. 5C). In addition to phenolic acids, lipids were identified which occasionally caused ion suppression of the lower-abundant phenolic acids. Overall, the method proved

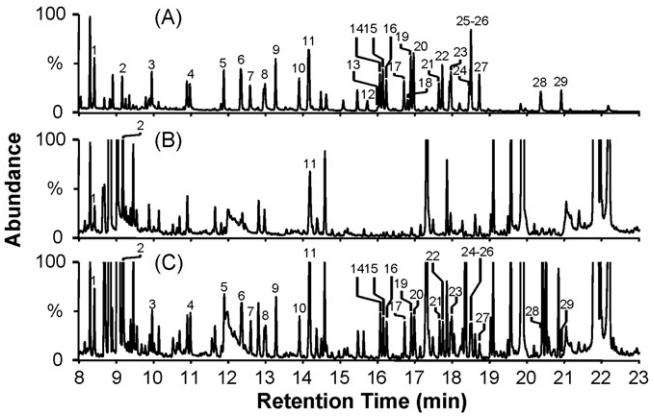

Fig. 5. Profiling of phenolic acids in plasma. GC–MS profiles of (A) 29 reference phenolic acid standards, (B) ethyl acetate extract of plasma, and (C) extract of plasma spiked with the phenolic acid standards at a concentration of 1 μg/ml. Peak annotation: 1: BA; 2: PAA; 3: *m*TA; 4: 3-PPA; 5: MA; 6: 2-HBA; 7: 4-MBA; 8: *t*CA; 9: 3-HBA; 10: 3-HPAA; 11: 4-HBA; 12: 2,3-DHBA; 13: 2,6-DHBA; 14: 4-HPPA; 15: VA; 16: HVA; 17: 2-HCA; 18: 2,4-DHBA; 19: 3,4-DHBA; 20: 3,5-DHBA; 21: 3-HCA; 22: 4-OMGA; 23: SA; 24: 4-HCA; 25: HCA; 26: 3-OMGA; 27: 3,4,5-THBA; 28: FA; 29: CA. For abbreviations, refer to the Section 2.

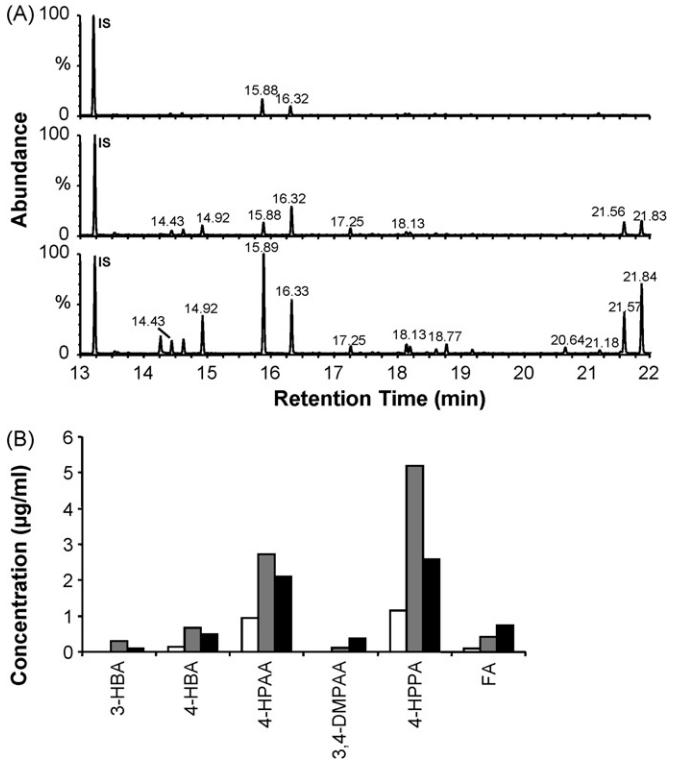

Fig. 6. Profiling of phenolic acids in fecal extracts after consumption of tea. (A) Extracted ion chromatograms at day 0 (top chromatogram), day 1 (middle chromatogram), and day 2 (bottom chromatogram). Note the increase of certain phenolic acids: 14.43 min, 4-HBA; 14.61 min, 4-HPAA; 15.89 min, 3,4-DMPAA; 16.32 min, 4-HPPA; 20.64 min, ferulic acid. (B) Quantification of metabolites in feces collected on day 0 (white), day 1 (grey), day 2 (black). For abbreviations, refer to the Section 2.

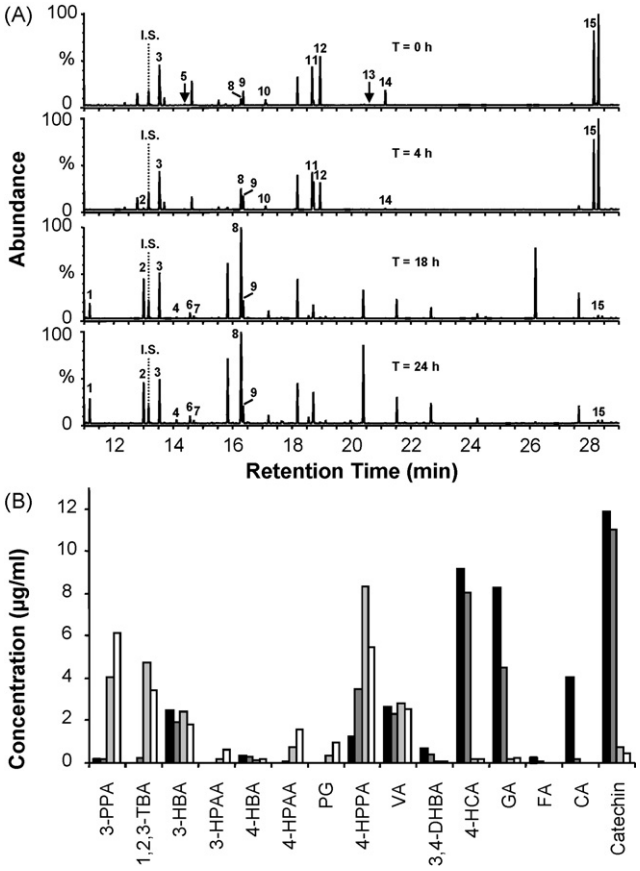

Fig. 7. Profiling of phenolic acids in a colonic gut model. (A) GC–MS chromatograms of red wine extracts fermented in a colonic gut model taken at time points 0, 4, 18, and 24 h. Peak annotation: 1: 3-PPA; 2: 1,2,3-TBA; 3: 3-HBA; 4: 3-HPAA; 5: 4-HBA; 6: 4-HPAA; 7: PG; 8: 4-HPPA; 9: VA; 10: 3,4-DHBA; 11: 4-HCA; 12: GA; 13: FA; 14: CA; 15: Catechin. (B) Quantification of identified metabolites from the *in vitro* fermentation of red wine extract. Black: $t = 0$; dark grey: $t = 4$; light grey: $t = 18$; white: $t = 24$. For abbreviations, refer to the Section 2.

useful for profiling phenolic acids in plasma, especially due to the good intra- and inter-day repeatabilities (refer to Section 3.5).

3.11. Profiling of phenolic metabolites in human feces

Feces of an individual that consumed eight cups of tea were collected at day 0, 1, and 2. Fecal extracts were prepared and phenolic acids were extracted and profiled by GC–MS. Fig. 6A shows three GC–MS profiles of fecal extracts at day 0 (top panel), day 1 (middle panel), and day 2 (bottom panel) after tea consumption. We particularly monitored the treatment-related change of phenolic acids and found six phenolic acids to increase after tea consumption. We quantified these six compounds by constructing calibration curves for each individual metabolite. In Fig. 6B the results are displayed, showing that 1 day after the consumption of tea, 3-HBA and 4-HBA, 4-HPAA acid and 4-HPPA reached its maximum concentration and decreased again on day 2. Two other compounds, 3,4-DMPAA and ferulic acid, were found to peak at day 2 after tea intake.

3.12. Profiling of phenolic metabolites in a colonic fermentation model

Red wine extract was incubated with fecal slurry and degradation products were collected at 0, 4, 18, and, 24 h, extracted and profiled by GC–MS. Fig. 7A displays four GC–MS chromatograms of extracts taken at the different time points. From the profiles we identified 15 phenolic acids (indicated by numbers). As shown in Fig. 7B, some compounds are formed during the fermentation process (e.g. 4-HPPA, 1,2,3-THB, and phloroglucinol), whereas others are initially present in the polyphenol-enriched starting material, but are gradually metabolized, such as catechin, 3,4-DHBA, 4-HCA, gallic acid, ferulic acid, and caffeic acid. A third group of compounds, containing compounds such as e.g. 3-HBA and vanillic acid, was not fermented and remained at a constant level. These findings are consistent with those described in literature for colonic fermentation of polyphenols [7]. Together, these results show that metabolic profiling of phenolics will help to understand the role of gut bacteria in metabolism of polyphenols.

4. Conclusion

In the present contribution we describe methods for the extraction, profiling by GC–TOF–MS, and discovery of gut fermentation products of ingested polyphenols in several biofluids as well as in an *in vitro* colonic fermentation model. Sample preparation methods are optimized for each of the matrices, i.e. urine, plasma or fecal water extracts using a large selection of phenolic acid standards. Best results are obtained after acidification with hydrochloric acid prior to extraction with ethyl acetate. Recoveries are generally better than 75% and intra- and inter-day relative standard deviations are generally below 25%, although matrix effects are observed. The linearity of the GC–TOF–MS system is acceptable for the profiling of metabolites, although it is only of limited use for quantification. Over 200 samples could be analyzed before the GC–MS system needed maintenance. Also retention time drifts were not observed,

making the methods suitable for application in (human) intervention trials.

The methods were applied to human and *in vitro* intervention studies. In an intervention study in which urine was collected and profiled, we identified 11 phenolic acids being significantly increased. The profiling of fecal extracts after consumption of tea revealed six phenolic acids, whereas 16 phenolic acids were identified in an *in vitro* colonic fermentation of red wine extract. The methods described here can be applied to studying the gut microbial impact on polyphenols via profiling of diverse biological fluids.

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