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Microbial metabolism of catechin stereoisomers by human faecal microbiota: Comparison of targeted analysis and a non-targeted metabolomics method

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Abstract

Microbial metabolism of the stereoisomers (+)-catechin and (−)-epicatechin was compared by two analytical techniques, GC/MS for quantitative targeted analysis and GC×GC-TOF for global characterization of the metabolome, using human faecal microbiota as an inoculum of converting microbiota. The ring-fission site changed when the inocula originated from two different groups of donors, but dehydroxylation progressed similarly regardless of the inoculum. Whereas GC/MS proved to be an appropriate tool for the study of specific expected metabolites of catechin stereoisomers, GC×GC-TOF-based metabolomics analysis also revealed new metabolites not included in the targeted analyses. Quantitation and verification of identification can also be performed in a metabolomics platform, if authentic standards are available.

Keywords: Metabolomics; (+)-Catechin; (−)-Epicatechin; GC/MS; GC×GC-TOF; Colonic microbiota

1. Introduction

Dietary phenolic compounds, including catechins, are ubiquitous plant-derived secondary metabolites which are metabolised extensively during digestion. Monomeric catechins are abundant in tea as (+)-catechin and (−)-epicatechin (Fig. 1) and their galloylated derivatives and condensed catechins (proanthocyanins) are abundant in red wine, tea and cocoa and in various fruits and berries (Hollman & Arts, 2000; Santos-Buelga & Scalbert, 2000). Catechins undergo ring-fission in the colon by the microbiota and are known to be transformed to phenolic acids and lactone derivatives. Eleven (+)-catechin metabolites have hitherto been identified in human urine. The three major metabolites are 3-hydroxyphenylpropionic acid, δ-(3,4-dihydroxyphenyl)-γ-valerolactone and δ-(3-hydroxyphenyl)-γ-valerolactone. The metabolites are excreted in urine in both free and glucuronidated forms and to a lesser degree as ethereal sulphates (Das, 1971). (−)-Epigallocatechin gallate, the major green tea catechin, is converted to di- and trihydroxyphenyl derivatives of γ-valerolactone and excreted in human urine after consumption of green tea solids. Galloylation affects the hydroxylation pattern of the metabolites. The ring-fission metabolites account for 1.5–16% of the ingested catechins. Consumption of both black and green tea results in an increase in the excretion of hippuric acid, which is a glycinated benzoic acid, into human urine. The results suggest both ring-fission in the colon and β-oxidation of the resulting phenolic acids (Meng et al., 2002).

3-Hydroxyphenylpropionic acid and 3-hydroxyhippuric acid were also identified in rat urine and faeces after ingestion of (+)-catechin (Griffiths, 1964). Das and Sothy (1971) showed that 3- and 4-hydroxyphenylpropionic acids as well as δ-(3,4-dihydroxyphenyl)- and δ-(3-hydroxyphenyl)-γ-valerolactones originate from the action of intestinal microorganisms and that they undergo enterohepatic circulation, reflected by conjugation of the metabolites. Identification of the metabolites in the early studies was performed by paper chromatography and semi-quantitatively using spectrophotometry (Griffiths, 1964; Das & Griffiths, 1969; Das, 1971; Das & Sothy, 1971), whereas the later studies involved NMR techniques (Meselhy, Nakamura, & Hattori, 1997) and LC/MS (Meng et al., 2002; Mulder, Rietveld, & van Amelsvoort, 2005).

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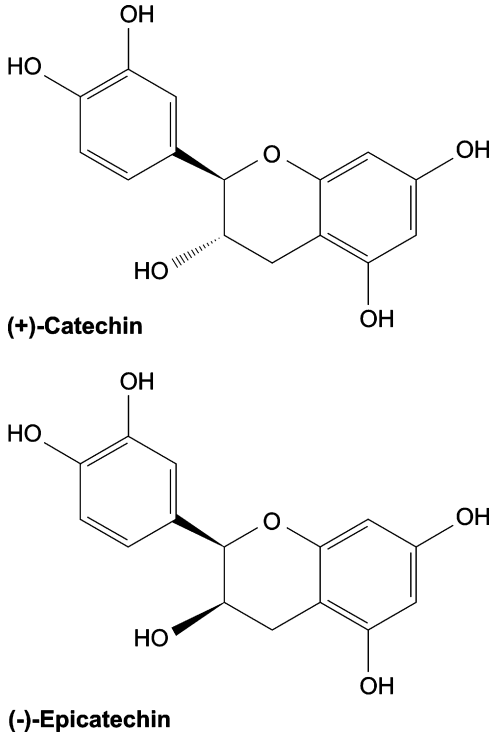

Fig. 1. Structures of catechin stereoisomers (+)-catechin and (−)-epicatechin.

The majority of the colonic microbial species are strict anaerobes which actively participate in the degradation and decomposition of the non-absorbable intake (Kleessen, Bezirtzoglou, & Mättö, 2000). The individual variation of microbiota in faeces is affected by age, diet, intestinal diseases and medication, particularly antibiotics (Rowland, Wiseman, Sanders, Adlercreutz, & Bowe, 1999; Kilkkinen et al., 2002). The differences in microbiota also lead to varying concentrations of microbial metabolites (Rowland et al., 1999; Cerda, Tomas-Barneran, & Espin, 2005). Involvement of the microbiota in the metabolism of catechins has been confirmed by *in vitro* incubations with human faecal or animal caecal suspensions (Scheline, 1970; Meselhy et al., 1997). Furthermore, there are differences in the degradation products of catechins between microbiota from humans and different animals (Das & Griffiths, 1969; Meselhy et al., 1997). Thus differences in metabolism occur due to the origin of the microbiota and possibly due to the structure of the precursor.

In this paper, an *in vitro* colon model was used to compare the microbial metabolism of two stereoisomers, (+)-catechin and (−)-epicatechin. The stereoisomer metabolites were analysed using targeted quantitative gas chromatography coupled to mass spectrometry (GC/MS) and a metabolomics strategy was applied utilizing the two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-TOF) technology. The advantages and limitations of these two different analytical approaches are discussed.

2. Results and discussion

Selected microbial metabolites were first analysed using GC/MS analysis. Transient formation of 3,4-dihydroxyphenylpropionic acid (3,4-diOHPPr) was observed for (+)-catechin, but was absent from samples containing (−)-epicatechin (Fig. 2a). The main microbial metabolites of both (+)-catechin and (−)-epicatechin were 3-hydroxyphenylpropionic acid (3-OH-PPr) and 3-phenylpropionic acid (3-PPr), showing differences in metabolite concentrations during the first 8 h of incubation (Fig. 2b and c). The differences in metabolite concentrations at the latest time point 24 h were most likely due to dying microbiota. 3,4-dihydroxyphenylacetic acid (3,4-diOHPAc) and 3-hydroxyphenylacetic acid (3-OHPAc) were not formed in the presence of either of the monomeric catechins (data not shown). Extent of fermentation for (+)-catechin and (−)-epicatechin were 91.4% and 69.8%, respectively.

The experiment was repeated using faeces from a new group of donors and analysis was performed using GC×GC-TOF coupled with bioinformatics tools. GC×GC-TOF has major advantages over GC/MS. The instrument is equipped with a non-polar and a polar column to produce a three-dimensional chromatogram. This means improved resolution and more accurate identifications (Welthagen et al., 2005). The two-column set-up also enhances sensitivity, which enables the use of scan measurement.

The alignment programme was able to separate 980 peaks, of which 161 were unknown peaks. Derivatives such as lipids and nitrogen heterocycles, expected to be formed from the faecal microbiota, were excluded. A total of 448 metabolites, of which 287 were identified, were selected for multivariate statistical analysis using partial least squares discriminant analysis (PLS/DA). PLS/DA is a pattern recognition technique that correlates variation in the data set with class membership. The projection model showed five latent variables (LV; $Q^2 = 63\%$) focusing on maximum separation (“discrimination”), which could be clearly distinguished based on substrate (i.e. (+)-catechin, (−)-epicatechin and faecal suspension “No addition”) and time points of 0–24 h. As shown in Fig. 3, the two first LV scores followed a time course within the same substrate group.

Furthermore 61 metabolites were selected for closer investigation. Data from the selected compounds were drawn as a profile on a time course and compared with the faecal suspension without added catechin isomers. The time course of 3,4-dihydroxyphenylvaleric acid formation (Fig. 4a) and that of a corresponding dehydroxylation product, 3-hydroxyphenylvaleric acid (Fig. 4b), were profiled. Notably, only (−)-epicatechin was a precursor of 3-hydroxyphenylvaleric acid. In a previous study Scheline (1970) was able to detect hydroxyphenylvaleric acid as a fermentation product of (+)-catechin by rabbit intestinal microbiota *in vitro*, but its formation by human microbiota has not been shown before. In the presented study hydroxyphenylpropionic acids formed using the inoculum A were not detected using the inoculum B, indicating inoculum-dependent change in the ring-fission site.

In another previous study by Das and Griffiths (1969) ^{14}C-labelled (+)-catechin showed that labelled phenyl-γ-valerolactones and carbon dioxide were derived from the A-ring and phenolic acids from the B-ring. Administration of [^{14}C]δ-(3-hydroxyphenyl)-γ-valerolactone gave rise to a labelled

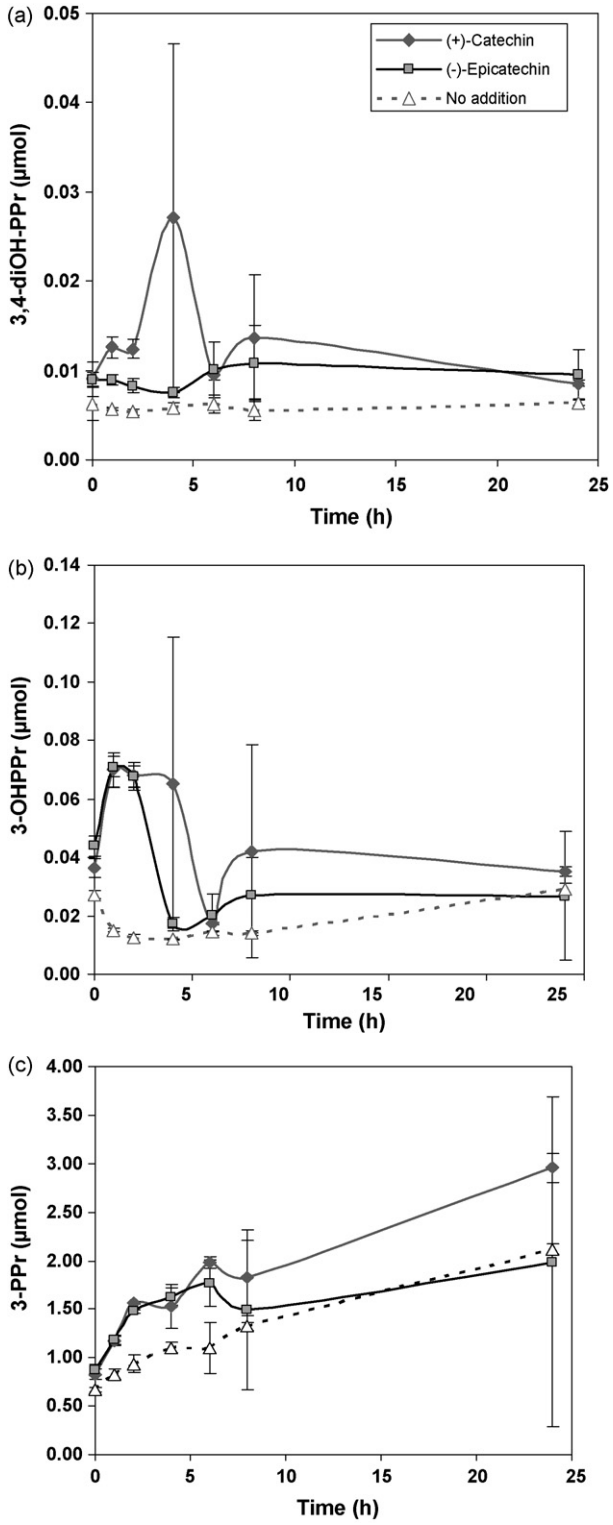

Fig. 2. Microbial metabolite profiles of (+)catechin and (−)-epicatechin monomers using human faecal inoculum (A) and analysed with GC/MS. (a) 3,4-dihydroxyphenylpropionic acid (3,4-diOHPPr); (b) 3-hydroxyphenylpropionic acid (3-OHPPr); (c) 3-phenylpropionic acid (3-PPr). The connecting lines are drawn as guides. $n = 3$. Faecal suspension without addition of catechins was used as a control.

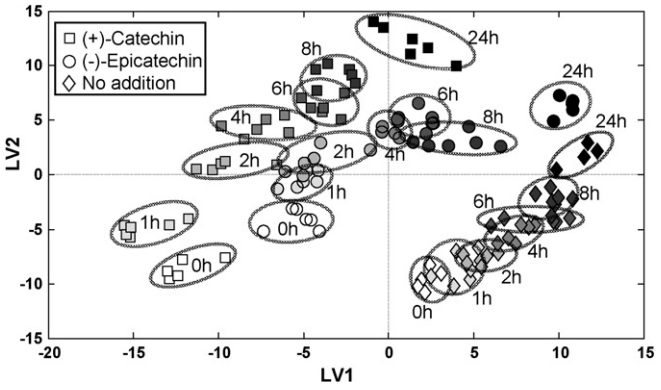

Fig. 3. Scores plot for the first two latent variables (LV1 and LV2) of 448 metabolites across all time points and substrate groups ((+)-catechin, (−)-epicatechin and faecal suspension alone as a control).

3-hydroxyphenylpropionic acid and administration of [^{14}C]3-hydroxyphenylpropionic acid to labelled 3-hydroxybenzoic acid. 3-hydroxybenzoic acid was present only in guinea pig urine and 3-hydroxyphenylpropionic acid only in rat urine. However, both animals showed urinary excretion of hydroxylated and methylated valerolactone derivatives and

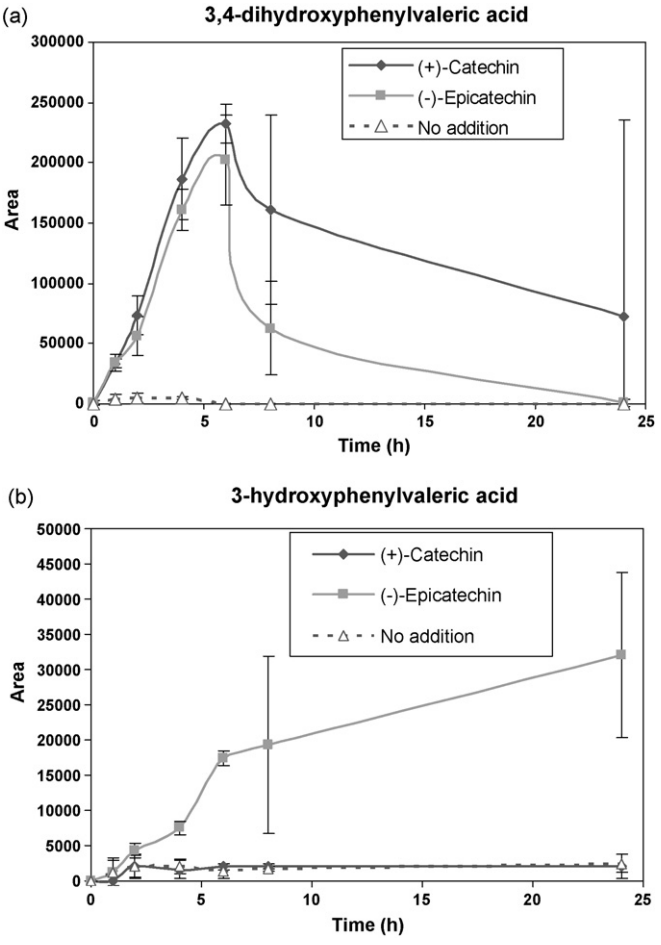

Fig. 4. Microbial metabolites of (+)catechin and (−)-epicatechin monomers using human faecal inoculum (B) and analysed with GC×GC-TOF: (a) 3,4-dihydroxyphenylvaleric acid; (b) 3-hydroxyphenylvaleric acid. The connecting lines are drawn as guides. $n = 6$. Faecal suspension without addition of catechins was used as a control.

hippuric acid (Das & Griffiths, 1969). Das and Sothy (1971) showed that 3- and 4-hydroxyphenylpropionic acids and δ-(3,4-dihydroxyphenyl)- and δ-(3-hydroxyphenyl)-γ-valerolactones originated from the action of intestinal microorganisms. Meselhy et al. (1997) obtained several other microbial metabolites from (−)-epicatechin, for example 1,2,3-trihydroxybenzene (pyrogallol), 3,4,5-trihydroxybenzoic acid (gallic acid), 5-(3′,4′-dihydroxyphenyl)-valerolactone and 5-(3′,4′-dihydroxyphenyl)-valeric acid. These metabolites were not included in our GC/MS analysis, because some of the compounds are not commercially available and selected ion monitoring requires authentic standards.

Benzoic acid formation was not detected by GC×GC-TOF. 3,4,5-Trihydroxybenzoic acid (gallic acid) was identified and included in the metabolome, but the profiles did not indicate its formation from the catechin stereoisomers. Furthermore, γ-valerolactone structures were not indicated by the bioinformatics tools, because they were not among the compounds in the library. However, it is possible that, if they were formed, they were among the 161 unknown peaks. Previous chromatographic work indicating γ-valerolactones was performed using LC methods (Meng et al., 2002; Mulder et al., 2005). γ-Valerolactones should be tested by GC×GC-TOF using authentic standards in order to determine whether the compounds can be analysed using the technique and furthermore to recognize them from the unidentified peaks in the metabolome of catechin stereoisomers. However, γ-valerolactones are not commercially available and time consuming, expensive synthesis would be needed.

It is notable that when using the GC×GC-TOF analysis, the power of the method is in observing in an unbiased manner all the detectable peaks formed in the course of time. Identification is dependent on the extent of the library. Differences between substrates and faecal background can be observed semi-quantitatively. The responses of different compounds, the relative proportions or significances cannot be compared quantitatively, because the responses are compound-specific and not only proportional to the concentration. Thus authentic standards are needed for quantitation in both analytical approaches.

In conclusion, the two analytical methods with authentic standards provide a powerful tool for identification and quantitation of microbial metabolites, GC/MS as a quick method for known metabolites and GC×GC-TOF for observation of the whole metabolome. The experiments presented here were able to show that there are slight differences between the dehydroxylation of stereoisomers, but that the site of ring-fission is more dependent on the microbiota (inoculum) than the stereoisomer.

3. Experimental

(+)-Catechin and (−) epicatechin (Fig. 1) were purchased from Carl Roth GmbH+ Co (Karlsruhe, Germany). Reagents for analysis of the microbial metabolites of catechins were as follows:

Heptadecanoic acid and succinic-d_4 acid used as the internal standards, were purchased from Sigma–Aldrich Inc. (St. Louis, USA) and the following compounds were used as standards: benzoic acid, 3-hydroxybenzoic acid, 3-(2-hydroxyphenyl)-propionic acid, 3-(4-hydroxyphenyl)propionic acid and 3,4-dihydroxyphenylpropionic acid (**3,4-diOHPPr**) were from Aldrich (Steinheim, Germany). 4-hydroxybenzoic acid, 3-hydroxyphenylacetic acid (**3-OHPAc**) and 3,4-dihydroxyphenylacetic (**3,4-diOHPAc**) were purchased from Sigma (St. Louis, USA). 3-Phenylpropionic acid (**3-PPr**) and 3,4-dihydroxybenzoic acid were from Fluka (Buchs, Switzerland) and 3-(3-hydroxyphenyl)propionic acid (**3-OHPPr**) was purchased from Alfa Aesar (Karlsruhe, Germany). The abbreviated reagents in bold were the standards of the selected microbial metabolites according to previous experiments by Deprez et al. (2000) and by the authors. MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoracetamide) from Pierce (Rockford, USA) was used as the silylation reagent.

Fermentation experiments were performed under strictly anaerobic conditions according to Aura et al. (2002). Faecal suspension was prepared using a 0.11 M – carbonate – 0.02 M phosphate buffer (pH 5.5) by pooling the faeces of at least four healthy donors, suspending them to the buffer in a Warring–Blender and filtering through a 1 mm sieve. The suspension was then diluted to 5% (w/v) concentration and applied to the samples in strictly anaerobic conditions.

Samples were incubated in a water bath at 37 °C for 0, 1, 2, 4, 6, 8 and 24 h and stirred magnetically (250 rpm). Four 2 ml aliquots were drawn from the bottles and microbial metabolites were analysed from each aliquot according to a modified procedure from Deprez et al. (2000). Two milliliters of faecal suspension was acidified with 50 μl 6 M HCl in order to keep the analytes in de-ionized form. Internal standard (heptadecanoic acid for GC/MS and succinic-d_4 acid for GC×GC-TOF runs) was added and the samples were extracted twice with 3 ml ethyl acetate. The extracts were evaporated to dryness under nitrogen, dissolved in 100 μl dichloromethane and silylated with 30 μl MSTFA (5 min, 50 °C). In the first experiment (inoculum A; $n = 3$), the microbial metabolites of (+)-catechin and (−)-epicatechin were analysed by GC/MS using selected ion monitoring (SIM). For the second experiment (inoculum B; $n = 6$), metabolites were analysed using GC×GC-TOF.

The instrument used for GC/MS runs was an Agilent GC/MS (6890A GC and 5973N MS) from Agilent Technologies, USA. The capillary column used was a Nordion NB-54 (15.0 m × 0.2 mm × 0.25 μm) coated with poly(5% diphenyl/95% dimethylsiloxane) stationary phase (HNU-Nordion Ltd. Oy, Finland). Helium with a flow rate of 1 ml/min was used as carrier gas. Split injection mode with a 50:1 ratio was selected. Injection port temperature was 250 °C and the injection volume was 1.0 μl. The GC temperature program was as follows: initial temperature 50 °C rate10 °C/min → 100 °C, rate 15 °C/min → 235 °C, final time 16 min. The data was collected in SIM mode with every analyte having at least one characteristic ion measured.

The GC×GC-TOF instrument used was a Leco Pegasus 4D, equipped with an Agilent GC 6890N from Agilent

Technologies, USA and a Combi PAL autosampler from CTC Analytics AG, Switzerland. The modulator, secondary oven and time-of-flight mass spectrometer were from Leco Inc., USA. The GC was operated in split mode with a 1:20 ratio. Helium with a constant pressure of 39.6 psig was used as carrier gas. The first dimension GC column was a relatively non-polar RTX-5 column, 10 m × 0.18 mm × 0.20 μm (Restek Corp., USA), coupled to a polar BPX-50 column, 1.10 m × 0.10 mm × 0.10 μm (SGE, Australia). The temperature program was as follows: initial temperature 50 °C, 1 min → 280 °C, 7 °C/min, 5 min. The secondary oven was set to +10 °C above the oven temperature. Inlet and transfer line temperatures were set to 250 °C. The second dimension separation time was set to 4 s. The mass range used was 40–700 amu and the data collection speed was 100 spectra/second. Measurement data was first processed by ChromaTOF software, which identifies compounds by matching deconvoluted spectra against the Palisade Complete 600 K mass spectral library. The results were exported to text files containing the names of the compounds, retention times in both dimensions and the spectrum for each compound. In-house developed software written in Java programming language (http://java.sun.com) was used for aligning compounds in different files by using retention time and spectrum information. Multivariate statistical analysis using partial least squares discriminant analysis (PLS/DA) (Barker & Rayens, 2003) was used as a recognition technique that correlated variation in the dataset with class membership (catechin stereoisomers and faecal suspension alone, and time points 0–24 h).

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