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Research Article

Absorption of *N*-phenylpropenoyl-L-amino acids in healthy humans by oral administration of cocoa (*Theobroma cacao*)

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Besides flavan-3-ols, a family of *N*-phenylpropenoyl-L-amino acids (NPAs) has been recently identified as polyphenol/amino acid conjugates in the seeds of *Theobroma cacao* as well as in a variety of herbal drugs. Stimulated by reports on their biological activity, the purpose of this study was to investigate if these amides are absorbed by healthy volunteers after administration of a cocoa drink. For the first time, 12 NPAs were quantified in human urine by means of a stable isotope dilution analysis with LC-MS/MS (MRM) detection. A maximum amount was found in the urine taken 2 h after the cocoa consumption. The highest absolute amount of NPAs excreted with the urine was found for *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5), but the highest recovery rate (57.3 and 22.8%), that means the percentage amount of ingested amides excreted with the urine, were determined for *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6) and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (13). In order to gain first insights into the NPA metabolism *in vivo*, urine samples were analyzed by LC-MS/MS before and after β-glucuronidase/sulfatase treatment. As independent of the enzyme treatment the same NPA amounts were found in urine, there is strong evidence that these amides are metabolized neither *via* their *O*-glucuronides nor their *O*-sulfates. In order to screen for caffeic acid *O*-glucuronides as potential NPA metabolites, urine samples were screened by means of LC-MS/MS for caffeic acid 3-*O*-β-D-glucuronide and 4-*O*-β-D-glucuronide. But not even trace amounts of one of these glucuronides were detectable, thus excluding them as major NPA metabolites and underlining the importance of future investigations on a potential *O*-methylation or reduction of the *N*-phenylpropenoyl moiety in NPAs.

Keywords: Cocoa / (*E*)-caffeoyl-3-*O*-β-D-glucuronide / (*E*)-caffeoyl-4-*O*-β-D-glucuronide / *N*-phenylpropenoyl-L-amino acids / Polyphenols

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1 Introduction

Epidemiological and medical anthropological investigations suggest that flavanol-rich foods exert cardiovascular health benefits. Recent dietary interventions in humans using polyphenol-containing foods have substantiated epidemiological data on an inverse relationship between flavanol intake and the risk of cardiovascular disease, indicating various potential flavanol-mediated bioactivities, including the improvement of vasodilation [1 – 3], blood pressure [4], insulin resistance, and glucose tolerance [5], the attenuation of platelet reactivity [6], as well as the improvement of immune responses and antioxidant defense systems [7, 8].

The seeds of *Theobroma cacao* are an important source of polyphenols comprising 12 – 18% of its total weight on dry basis. Among the cocoa polyphenols, epicatechin (249.4 mg/100 g), and procyanidins such as procyanidin B2 (121.4 mg/100 g), procyanidin C1 (138.6 mg/100 g), and procyanidin B5 (46.4 mg/100 g) were found as the quantitatively predominating flavan-3-ols in cocoa nibs used as the key ingredient for the manufacturing of chocolate and chocolate confectionary [9]. Besides these flavanoids, a family of *N*-phenylpropenoyl-L-amino acids 1 – 10 (Fig. 1) has been

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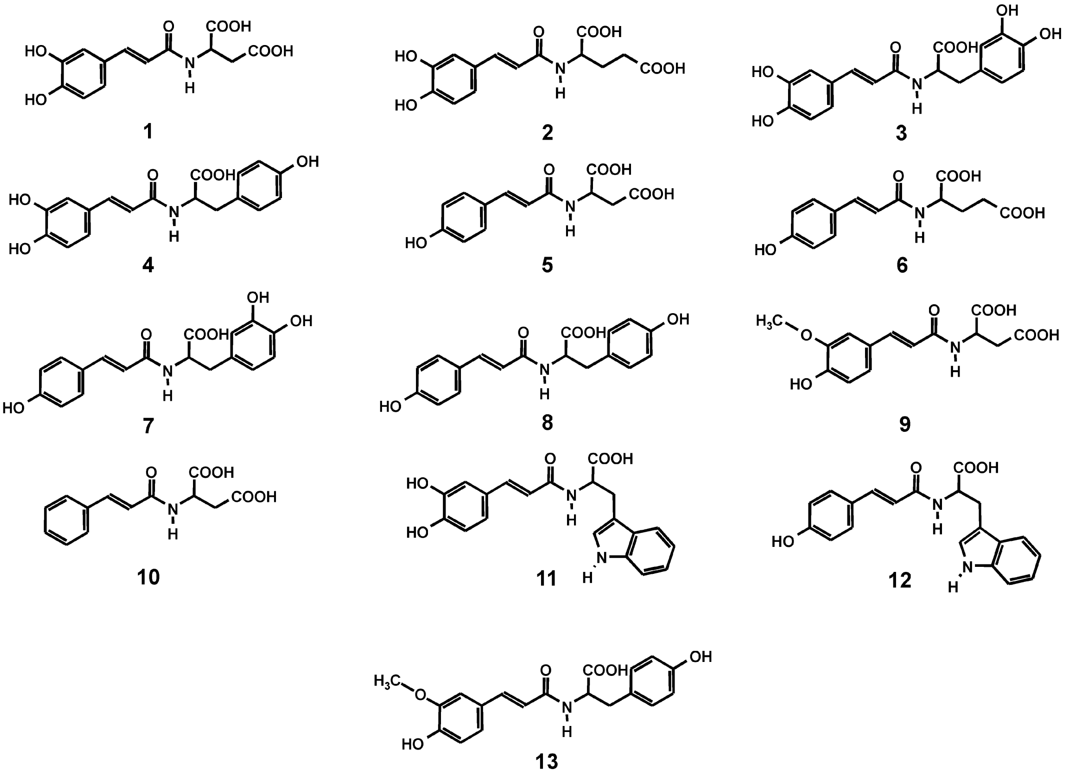
Abbreviations: HMBC, heteronuclear multiple bond correlation spectroscopy; **HMQC,** heteronuclear multiple-quantum correlation spectroscopy; **MRM,** multiple reaction monitoring; **NPAs,** *N*-phenylpropenoyl-L-amino acids; **SIDA,** stable isotope dilution assay recently identified as polyphenol/amino acid conjugates in cocoa [10, 11]. Among these compounds, *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (1, 47.2 mg/100 g) (–)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (3, 28.7 mg/100 g), and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5, 13.9 mg/100 g) were found as predomi-



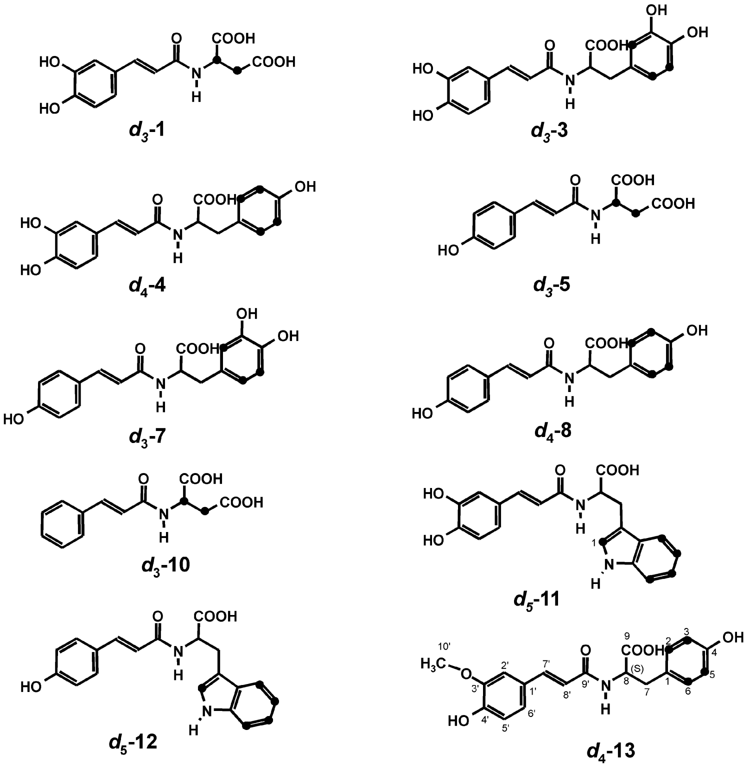


Figure 1. Chemical structures of *N*-phenylpropenoyl-L-amino acids 1–13 and their deuterium-labeled isotopologues d_3-1–d_4-13.

nating polyphenolic amides in cocoa nibs [9, 12]. Furthermore, amide 3 was identified in red clover (*Trifolium pratense*) [13–15], and together with (–)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8) was reported as an antioxidant in cocoa [16] and as a constituent of the bark of African blackwood (*Dalbergia melanoxylon*) [17]. In addition (–)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (4) has been previously identified in cocoa flowers [18] and together with the tryptophane derivatives 11 [19] and 12 [20], as well as the tyrosine conjugate 13 were detected in raw robusta coffee beans [21]. Very recently, a comprehensive LC-MS/MS screening enabled the identification of several amides in a variety of herbal drugs; for example, compounds 1, 5, 6, 9, and 10 were found in fruits of *Coriandrum sativum* and flowers of *Sambucus nigra* as well as *Lavandula* species, and compounds 5 and 10 in roots of *Angelica archangelica* and fruits of *Phytostigma venenosum* [22].

Besides their astringent taste activity [9–11] as well as their antioxidant properties [16], some of these *N*-phenylpropenoyl-L-amino acids were found to be biologically active as phytoallexins [23], as inducers of mitochondrial activity and proliferation rate in human liver cell lines as well as human keratinocytes [22], and as potent inhibitor of the adhesion of *Helicobacter pylori* to human stomach tissue [22]. In consequence, there is a growing interest in the human bioavailability of these nitrogen-containing phytochemicals from foods. Although it is generally believed that the bioavailability of intact polyphenols including hydroxycinnamic acid esters is rather poor [24], there are no data available on the human bioavailability of *N*-phenylpropenoyl-L-amino acids consisting of a hydroxycinnamic acid linked to an amino acid *via* an amide bond.

The objective of the present study was, therefore, to administer a cocoa drink containing defined amounts of *N*-phenylpropenoyl-L-amino acids to healthy volunteers and to quantitatively follow the urinary excretion of these amides over time. As the use of stable isotopologues of analytes is known to enable the correction of compound discriminating during extraction, cleanup, chromatographic separation, as well as MS detection, the recently developed stable isotope dilution assay (SIDA) with LC-MS/MS multiple reaction monitoring (MRM) detection [12] was used as a versatile and reliable method to ensure the accurate quantification of these phytochemicals.

2 Materials and methods

2.1 Chemicals

The following chemicals and reagents were obtained commercially: caffeic acid, methyl-acetobromo-α-D-glucuronate, 1,2-dichloroethane, 2,6-di-*tert*-butyl-4-methylpyridine, silver trifluoromethane sulfonate, barium hydroxide (Sigma–Aldrich, Steinheim, Germany), methanol, sulfuric acid, diethyl ether, toluene, ethyl acetate, acetone, sodium chloride, silica gel (70–230 mesh), and formic acid (Merck, Darmstadt, Germany). [2,3,5,6-$^2H_4$]-L-tyrosine was purchased from Cambridge Isotope Laboratories (MA, USA). The enzyme solution containing β-glucuronidase (~100 unit/μL) and sulfatase (~7 unit/μL) from Helix pomatia Type HP-2 was purchased from Sigma–Aldrich. One unit of β-glucuronidase liberated 1.0 μg of phenolphthalein from phenolphthalein *O*-glucuronide *per* hour at pH 5.0 and 37°C, one unit of sulfatase hydrolyzed 1.0 μmol of *p*-nitrocatechol *O*-sulfate *per* hour at pH 5.0 and 37°C. Water for chromatographic separations was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany), and solvents used were of HPLC grade (Merck). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). Table water (Evian) from a local supplier was used for the preparation of the cocoa drink. Alkalized cocoa powder prepared from cocoa from Ghana (West Africa) was obtained from the food industry.

2.2 Preparation of the cocoa drink and analysis of *N*-phenylpropenoyl-L-amino acids

A mixture of alkalized cocoa powder (20.3 g) and sucrose (3.0 g) was suspended in table water (400 mL) at 50°C for 10 min whilst stirring. After cooling, this cocoa drink was used for the human intervention study as well as for chemical analysis.

For quantitative determination of *N*-phenylpropenoyl-L-amino acids by means of a recently developed stable isotope dilution analysis [12], an aliquot of the cocoa drink (105.0 g) was spiked with solutions (100 μL, each) of the labeled internal standards d_3-1, d_3-3, d_4-4, d_3-5, d_3-7, d_4-8, d_3-10, d_5-11, d_5-12, and d_4-13 in methanol (1.0 mg/mL) and the mixture was homogenized and equilibrated for 30 min whilst moving in a lab shaker. Thereafter, the cocoa drink was freeze-dried, the powder was extracted with *n*-pentane (5 × 30 mL) for 30 min, and the residual cocoa material was extracted five times with acetone/water (70:30 v/v; 30 mL each) for 45 min at room temperature whilst stirring. After centrifugation, the liquid layer was freed from acetone under reduced pressure at 30°C, and then freeze-dried to give the acetone/water extract. Aliquots (about 40 mg) of the acetone/water extract were taken up in methanol/water (1:1 v/v; 10 mL) and adjusted to pH 2.5 with formic acid. After membrane filtration, aliquots (5–10 μL) were analyzed by means of LC-MS/MS, which was equipped with a 2 × 150 mm, 5 μm, phenylhexyl column (Phenomenex, Germany) operated with a flow rate of 0.2 mL/min. Chromatography was performed starting with a mixture (20:80 v/v) of methanol and aqueous formic acid (0.1% in water; pH 2.5) for 5 min, then increasing the methanol content to 60% within 35 min, and finally, to 100% within 5 min.

The labeled compounds d_3-1, d_3-3, d_4-4, d_3-5, d_3-7, d_4-8, d_3-10, d_5-11, d_5-12, and d_4-13 were used as internal standards for the quantitative analysis of the corresponding analytes. In addition, compound 2 was quantified *via* d_3–1, and compounds 6 and 9 *via* d_3-5 as the internal standard. The amounts of the individual *N*-phenylpropenoyl amino acids were calculated using the response factors (given in brackets) for the individual compounds 1 (1.00), 2 (0.80), 3 (0.92), 4 (0.94), 5 (0.99), 6 (0.85), 7 (0.86), 8 (0.99), 9 (0.86), 10 (0.85), 11 (0.95), 12 (0.96), 13 (0.90) which had been determined by analysis of solutions containing defined amounts of the internal standards and the target compounds in five mass ratios from 0.2 to 5.0.

2.3 Experimental design of human study

Eight healthy volunteers aging between 24 and 30 years (four females and four males) participated in the intervention study. The protocol was fully explained to the volunteers who were nonsmokers and gave their written informed consent to participate the present study and had no history of cocoa noncompatibility. Exclusion criteria involved a vegetarian diet, weight-reducing dietary regimen, alcoholism, consumption of vitamin supplements or nutraceuticals, diabetes, hyperlipidemia, cardiovascular disease, history of gastrointestinal disease, or any other chronic disease. The volunteers followed a controlled diet in which chocolate, cocoa, coffee, as well as any coffee or cocoa containing food products were strictly forbidden at least for 48 h prior to the intervention.

In the morning, 1 h before the intervention, blank urine samples were collected in plastic bottles, and 30 min before the study, the volunteers consumed a glass of table water (200 mL) on an empty stomach. Then, a bolus of the cocoa drink, which was freshly prepared as described above, was administered to the eight volunteers. After 1, 2, 3, 4, 6, and 8 h, the volunteers collected urine samples which were immediately ice-cooled and, then, used for quantitative analysis. After the first urine collection (after 1 h), the volunteers consumed 300 mL of table water, and after the following urine collections the volunteers consumed 500 mL of table water within 5 min. After the first and the fourth urine collection, the volunteers had meal together containing rolls, butter, jam, salami, and bacon.

2.4 Analysis of *N*-phenylpropenoyl-L-amino acids in excreted urine

For the quantitative analysis of *N*-phenylpropenoyl-L-amino acids in urine, aliquots (5.0 mL) of the urine samples were spiked with solutions (100 µL each) of the labeled internal standards d_3-1, d_3-3, d_4-4, d_3-5, d_3-7, d_4-8, d_3-10, d_5-11, d_5-12, and d_4-13 dissolved in methanol (10 µg/mL) and equilibrated for 30 min at room temperature on a laboratory shaker. Aliquots of the samples (500 µL each) were ultracentrifuged using a Vivaspin 500 filter cartridge (Satorius Group, Hannover, Germany) at 12 000 rpm by means of a Napco 2019R centrifuge for 15 min at 4°C. An aliquot (20 µL) of the ultrafiltrate obtained was analyzed by means of LC-MS/MS using the conditions given above for analysis of the cocoa sample. In order to investigate the liberation of *N*-phenylpropenoyl-L-amino acids from corresponding *O*-glucuronides and/or *O*-sulfates, aliquots (4 mL) of the urine samples were adjusted to pH 5.0 with hydrochloric acid (1 mol/L) and, after addition of 10 µL of the β-glucuronidase/sulfatase solution, were incubated for 4 h at 37°C prior to ultracentrifugation and subsequent LC-MS/MS analysis.

2.5 Syntheses

2.5.1 *N*-phenylpropenoyl-L-amino acids

The *N*-phenylpropenoyl-L-amino acids 1–13 as well as the isotopologues d_3-1, d_3-3, d_4-4, d_3-5, d_3-7, d_4-8, d_3-10, d_5-11, and d_5-12 (Fig. 1) were synthesized as reported earlier in the literature [11, 12]. Closely following the same procedure [12], *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-[2,3,5,6-^{2}H]-L-tyrosine (d_4-13) was synthesized in a yield of 40% starting from (*E*)-ferulic acid and [2,3,5,6-^{2}H]-L-tyrosine.

2.5.2 Caffeic acid methylester (14)

Concentrated sulfuric acid (1 mL) was added to a solution of caffeic acid (20 mmol) in methanol (100 mL) and the mixture was maintained under nitrogen at room temperature whilst stirring. After 24 h, diethyl ether (100 mL) and water (500 mL) were added, the organic layer was separated, and the aqueous phase was extracted with diethyl ether (5 × 100 mL). The combined organic layers were extracted with water (2 × 200 mL), the organic phase was freed from solvent in vacuum, and the residue was taken up in methanol (10 mL). Upon addition of water (50 mL), caffeic acid methylester (14, yield 91%) was obtained as a white precipitate, which was isolated by filtration, washed with water (5 × 50 mL), and freeze-dried.

2.5.3 Caffeic acid *O*-β-D-glucuronide (15) and 4-*O*-β-D-glucuronide (16)

Following a modification of the Koenigs-Knorr procedure developed for glucoside synthesis [25], solid caffeic acid methylester (14) (1 mmol), and methyl-acetobromo-α-D-glucuronate (2 mmol) were added in one portion to a suspension of powdered molecular sieve (5 g, 4 Å) and silver trifluoromethane sulfonate (2 mmol) in anhydrous 1,2-dichloroethane (30 mL) cooled to −20°C with stirring in the dark. After stirring the mixture at −20°C for 30 min, a solution of 2,6-di-*tert*-butyl-4-methylpyridine (2 mmol) in 1,2-dichloroethane (5 mL) was added, the cooling bath was removed, and the mixture was stirred for additional 14 h at r.t. Thereafter, the suspension was filtered, the residue washed with 1,2-dichloroethane (2 × 20 mL), silica gel (1 g) was added to the organic solution, and the solvent was carefully evaporated in vacuum. The resulting powder was placed onto the top of a glass column (50×3 cm^2) filled with a slurry of silica gel (5% water) in toluene. Chromatography was performed using toluene, followed by toluene/ethyl acetate mixtures with stepwise increasing content of ethyl acetate. The fractions eluting with toluene/ethylacetate (70:30 and 60:40 v/v) were combined, freed from the solvent in vacuum, and taken up in acetone (50 mL). After ice-cooling to 0°C, an aqueous Ba(OH)$_2$ solution (0.12 mol/L, 250 mL) was added and the mixture was shaken for 10 min. After maintaing the mixture at 0°C for 1 h, another aliquot (150 mL) of an aqueous Ba(OH)$_2$ solution (0.12 mol/L) was added and the mixture was maintained for 12 h in the dark under an atmosphere of argon at 0°C. Then, the solution was acidified with aqueous sulfuric acid (1 mM), precipitated BaSO$_4$ was removed by filtration, the filtrate was concentrated to about 10 mL in vacuum, and aliquots (1 mL) were separated by means of HPLC on RP18 (Varian, Microsorb 100-5, 250×21.2 mm^2, 5 μm) using acidified water (0.1% formic acid) as eluent A and methanol (0.1% formic acid) as eluent at a flow rate of 20 mL/min. After isocratic elution with 95% eluent A for 5 min, the content of eluent B was increased to 10% within 5 min, then to 20% within 10 min, to 25% within 13 min, and finally, to 100% within 2 min, followed by isocratic elution for 5 min. Monitoring the effluent at 324 nm, the fractions containing caffeic acid 3-*O*-β-D-glucuronid (15, yield: ~10%) and 4-*O*-β-D-glucuronid (16, yield, ~6%) were collected individually, freed from solvent in vacuum, and freeze-dried for 48 h.

2.6 Spectroscopic data

2.6.1 *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-[2,3,5,6-^{2}H]-L-tyrosine (d_4-13)

UV/Vis (MeOH/water, 1:1 v/v; pH 2.5) λ_{max} = 221, 297, 322 nm; LC/MS (ESI$^+$): *m/z* 362 (100%, [M + 1]$^+$), 384 (55%, [M + 23]$^+$), 177 (30%, [M – 184]$^+$); MS/MS (ESI$^+$): 177 (100%, [M–184]$^+$), 89 (76%, [M–272]$^+$), 145 (70%, [M–216]$^+$), 117 (51%, [M–244]$^+$), 362 (1%, [M + 1]$^+$); exact mass: *m/z* 384.1352$^+$ (Calcd. for C$_{19}$H$_{15}$D$_4$NO$_6$Na$^+$ 384.1356$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY; the assignment of the NMR-signals refers to the numbering of the chemical structure as displayed in Fig. 1): δ = 2.80 [dd, 1H, *J* = 9.2, 14.0 Hz, H–C(7a)], 2.98 [dd, 1H, *J* = 4.6, 14.0 Hz, H–C(7b)], 3.81 [s, 3H, H–C(10′)], 4.46 [ddd, 1H, *J* = 4.6, 8.8, 14.0 Hz, H–C(8)], 6.54 [d, 1H, *J* = 15.6 Hz, H–C(8′)], 6.79 [d, 1H, *J* = 8.0 Hz, H–C(5′)], 6.98 [dd, 1H, *J* = 1.6, 8.0 Hz, H–C(6′)], 7.12 [d, 1H, *J* = 1.6 Hz, H–C(2′)], 7.28 [d, 1H, *J* = 15.6 Hz, H–C(7′)], 8.15 [d, 1H, *J* = 8.0 Hz, H–N], 9.21 [brs, 1OH, HO–C(4)], 9.46 [brs, 1OH, HO–C(4′)], 12.72 [brs, 1OH, HOO–C(9)].

2.6.2 Caffeic acid methylester (14)

UV-Vis (MeOH, 0.1% formic acid): λ_{max} = 294, 329 nm; LC/MS (ESI$^-$): *m/z* 193 (100%, [M–H]$^-$); ^{1}H-NMR (400 MHz, DMSO-d_6, COSY; the assignment of the NMR-signals refers to the numbering of the chemical structure given in Fig. 2): δ = 3.67 [s, 3 × H, H–C(10)], 6.26 [d, 1 × H, *J* = 15.66 Hz, H–C(8)], 6.75 [d, 1 × H, *J* = 8.34 Hz, H–C(5)], 6.99 [dd, 1 × H, *J* = 8.08 Hz, *J* = 2.02 Hz, H–C(6)], 7.04 [d, 1 × H, *J* = 2.02, H–C(2)], 7.47 [d, 1 × H, *J* = 15.66 Hz, H–C(7)], 9.13 [brs, 1 × OH, HO–C(3)], 9.58 [brs, 1 × OH, HO–C(4)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HMBC, HMQC): δ = 51.3 [CH$_3$, C(10)], 113.3 [CH, C(8)], 114.7 [CH, C(2)], 115.3 [CH, C(5)], 121.4 [CH, C(6)], 125.4 [C, C(1)], 144.9 [CH, C(7)], 145.4 [HO–C, C(3)], 148.4 [HO–C, C(4)], 167.0 [COO, C(9)].

2.6.3 Caffeic acid 3-*O*-β-D-glucuronide (15)

UV/Vis (water/MeOH, 28:72 v/v; 0.1% formic acid): λ_{max} = 238, 292, 317 nm; LC/MS (ESI)$^-$: *m/z* (%) 355 (100, [M–H]$^-$); MS/MS (ESI$^-$): *m/z* 178.9 (100%), 135 (47%), 175.0 (23%), 113 (23%); exact mass: *m/z* 379.0637$^+$ (calcd. for C$_{15}$H$_{16}$O$_{10}$Na$^+$: 379.0636); ^{1}H-NMR (400 MHz, DMSO-d_6, COSY; the assignment of the NMR-signals refers to the numbering of the chemical structure given in Fig. 2): δ = 3.33 [m, 2 × H, H–C(2′), H–C(3′)], 3.40 [pt, 1H, *J* = 9.2 Hz, H–C(4′)], 3.98 (d, 1H, *J* = 9.6 Hz, H–C(5′)), 4.49 [brs, 1OH, HO–C(4′)], 5.07 [d, 1H, *J* = 7.2 Hz, H–C(1′)], 5.22 [brs, 1OH, HO–C(2′) or HO–C(3′)], 5.47 [brs, 1OH, HO–C(2′), or HO–C(3′)], 6.31 [d, 1H, *J* = 15.9 Hz, H–C(8)], 6.84 [d, 1H, *J* = 8.4 Hz, H–C(5)], 7.19 [dd, 1H, *J* = 8.4 Hz, *J* = 1.8 Hz, H–C(6)], 7.36 [d, 1H, *J* = 1.8 Hz, H–C(2)], 7.42 [d, 1H, *J* = 15.9 Hz, H–C(7)], 9.30 [brs, 1OH, HO–C(4)], 12.25 [brs, 2OH, HOOC(9), HOOC(6′)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HMBC, HMQC, DEPT-135): δ = 71.3 [CH, C(4′)], 72.9 [CH, C(2′)], 75.2 [CH, C(3′)], 75.3 [CH, C(5′)], 100.5 [CH, C(1′)], 115.0 [CH, C(2)], 115.9 [CH, C(8)], 116.2 [CH, C(5)], 124.1 [CH, C(6)], 125.7 [C, C(1)], 144.1 [CH, C(7)], 145.0 [C, C(3)], 149.0 [C, C(4)], 167.8 [COO, C(9)], 170.1 [COO, C(6′)].

2.6.4 Caffeic acid 4-*O*-β-D-glucuronide (16)

UV-Vis (water/MeOH, 37:63 v/v; 0.1% formic acid): λ_{max} = 240, 289, 321 nm; LC/MS (ESI)$^-$: *m/z* 355 (100%, [M–H]$^-$); MS/MS (ESI$^-$): *m/z* 179.0 (100%), 135 (47%), 175.0 (24%) 113 (23%); exact mass: *m/z* 379.0637$^+$ (Calcd. for C$_{15}$H$_{16}$O$_{10}$Na$^+$: 379.0636); ^{1}H-NMR (400 MHz, DMSO-d_6, COSY; the assignment of the NMR-signals refers to the numbering of the chemical structure given in Fig. 2): δ = 3.33 [m, 2 × H, H–C(2′), H–C(3′)], 3.38 [m, 1 H, H–C(4′)], 3.82 [d, 1H, *J* = 9.2 Hz, H–C(5′)], 4.93 [d, 1H, *J* = 7.2 Hz, H–C(1′)], 5.19 [brs, 1OH, HO–C(2′) or HO–C(3′)], 5.46 [brs, 1OH, HO–C(2′) or HO–C(3′)], 5.78 [brs, 1OH, HO–C(4′)], 6.31 [d, 1H, *J* = 16.0 Hz, H–C(8)], 7.05 [d, 1H, *J* = 8.4 Hz, H–C(5)], 7.09 [dd, 1H, *J* = 8.4 Hz, *J* = 1.6 Hz, H–C(6)], 7.13 [d, 1H, *J* = 1.6 Hz, H–C(2)], 7.45 [d, 1H, *J* = 1.6 Hz, H–C(7)], 8.90 [brs, 1H, HO–C(3)], 12.25 [brs, 2OH, HOOC(9), HOOC(6′)]; ^{13}C-NMR (100 MHz, DMSO-d_6, HMBC, HMQC, DEPT-135):

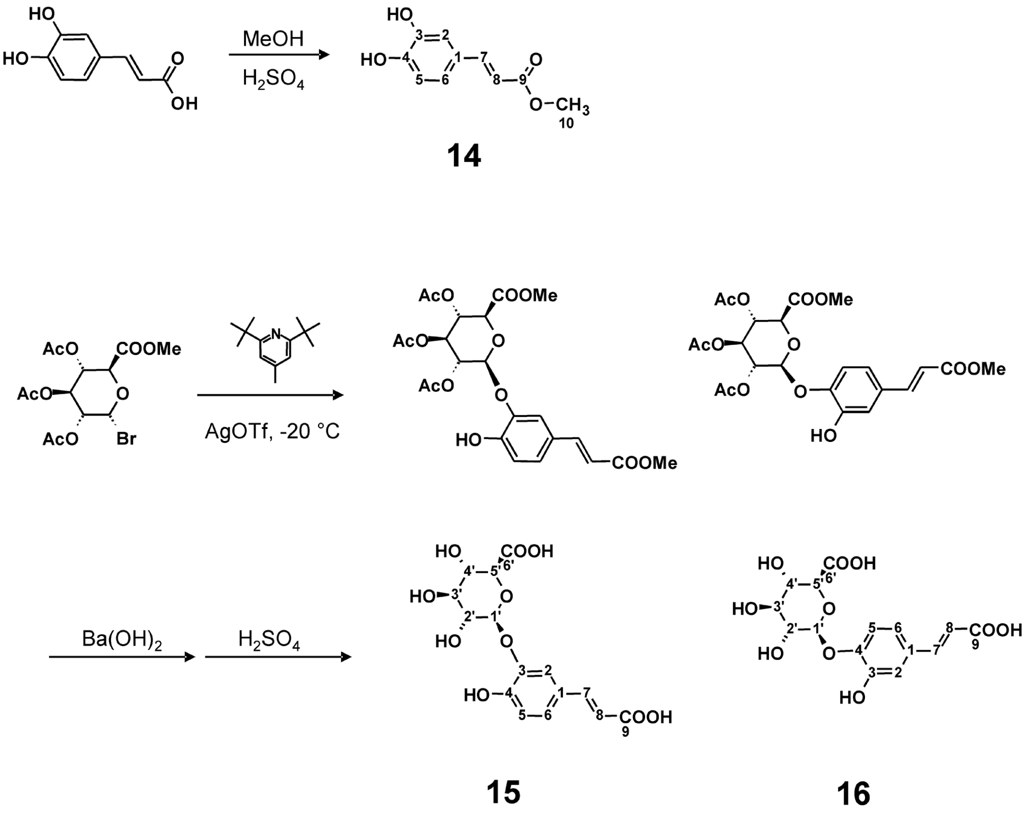


Figure 2. Synthetic sequence used for the preparation of caffeoyl-3-*O*-β-D-glucuronide (15) and caffeoyl-4-*O*-β-D-glucuronide (16).

δ = 71.5 [CH, C(4′)], 72.9 [CH, C(2′)], 75.1 [CH, C(3′)], 75.2 [CH, C(5′)], 101.0 [CH, C(1′)], 115.0 [CH, C(2)], 116.0 [CH, C(5)], 117.2 [CH, C(8)], 120.4 [CH, C(6)], 128.9 [C, C(1)], 143.8 [CH, C(7)], 146.8 [C, C(3)], 146.9 [C, C(4)], 167.6 [COO, C(9)], 170.5 [COO, C(6′)].

2.7 Enzymatic hydrolysis of caffeic acid *O*-β-D-glucuronides

A solution of the caffeic acid *O*-β-D-glucuronides 15 or 16 (1 mg, each) in water (10 mL) was adjusted to pH 5.0 with aqueous hydrochloric acid (1 mol/L), spiked with 10 μL of the β-glucuronidase/sulfatase solution, and then, incubated for 6 h at 37°C. After 0.5, 1, 2, 3, 4, and 6 h, respectively the mixtures were ice-cooled and aliquots (5 μL) were immediately analyzed by means of HPLC-MS/MS equipped with a 150 × 2 mm id, 5 μm, RP-phenylhexyl column (Phenomenex) operated with a flow rate of 0.2 mL/min. Chromatography was performed starting with a mixture (20:80 v/v) of methanol and aqueous formic acid (0.1% in water, pH 2.5), then the methanol content was increased to 100% within 25 min, and, finally, held at 100% for 5 min. Using the MRM with ESI$^-$ ionization, the individual caffeic acid *O*-β-D-glucuronides 15 and 16 were analyzed using the mass transitions *m/z* 355.2 → 179.0, *m/z* 355.2 → 135.0, and *m/z* 355.2 → 113.0 monitored for a duration of 250 ms. The HPLC-MS/MS parameters used were the same as applied for the analysis of caffeic acid *O*-β-D-glucuronides in urine samples.

2.8 HPLC

The HPLC apparatus (Kontron, Eching, Germany) consisted of low pressure gradient system 525 HPLC pump, an M800 gradient mixer, a type 560 autosampler, and a DAD type 540+ diode array detector. Chromatography was performed on 250 × 4.6 mm stainless-steel columns packed with RP-phenylhexyl material, 5 μm (Phenomenex) operated with a flow rate of 0.8 mL/min.

2.9 LC-MS/MS

LC-MS/MS analysis was performed using an Agilent 1100 HPLC-system connected to an API3200-type LC-MS/MS system (Applied Biosystems, Darmstadt, Germany) running in the ESI mode. For ESI$^+$, the ion spray voltage was set at +5500 V in the positive mode and for ESI$^-$ at –4500 V in the negative mode. Zero grade air served as nebulizer gas (35 psi), and as turbo gas (300°C) for solvent drying (45 psi). Nitrogen served as curtain (20 psi) and collision gas (4.5×10^{-5} Torr). Both quadrupols were set at unit resolution. By means of the MRM mode, the individual

N-phenylpropenoyl amino acids were analyzed using the following mass transitions monitored for a duration of 75 ms as given in brackets: 1 (*m*/*z* 296.3 → 163.0), d_3-1 (*m*/*z* 299.3 → 163.0), 2 (*m*/*z* 310.3 → 163.0), 3 (*m*/*z* 360.3 → 163.0), d_3-3 (*m*/*z* 363.3 → 163.0), 4 (*m*/*z* 344.3 → 163.0), d_4-4 (*m*/*z* 348.3 → 163.0), 5 (*m*/*z* 280.3 → 147.0), d_3-5 (*m*/*z* 283.3 → 147.0), 6 (*m*/*z* 294.3 → 147.0), 7 (*m*/*z* 344.3 → 147.0), d_3-7 (*m*/*z* 347.3 → 147.0), 8 (*m*/*z* 328.3 → 147.0), d_4-8 (*m*/*z* 332.3 → 147.0), 9 (*m*/*z* 310.3 → 177.0), 10 (*m*/*z* 264.3 → 131.0), d_3-10 (*m*/*z* 267.3 → 131.0), 11 (*m*/*z* 367.3 → 163.0), d_5-11 (*m*/*z* 72.3 → 163.0), 12 (*m*/*z* 351.3 → 147.0), d_5-12 (*m*/*z* 356.3 → 147.0) 13 (*m*/*z* 358.3 → 177.0), and d_4-13 (*m*/*z* 362.3 → 177.0).

2.10 LC/TOF-MS

Mass spectra of the target compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer with flow injection referenced on sodium formiate and PEG 600, respectively. The compounds were dissolved in 1 mL of MeOH and 10 µL of a saturated solution of $NaBF_4$ in MeOH was added to measure the exact mass of the sodium adducts.

2.11 NMR

^{1}H, COSY, heteronuclear multiple-quantum correlation spectroscopy (HMQC), heteronuclear multiple bond correlation spectroscopy (HMBC), ^{13}C, and DEPT-135 NMR measurements were performed on a DMX 400 spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts were referenced to the solvent signal. Data processing was performed by using XWin-NMR software (version 3.5; Bruker) as well as Mestre-C software (Mestrelab Research, Santiago de Compostela, Spain).

3 Results

3.1 Quantification of *N*-phenylpropenoyl-L-amino acids in administered cocoa drink and urine samples

For the accurate quantitative analysis of the *N*-phenylpropenoyl-L-amino acids in the cocoa drink to be used for the intervention study, a freshly prepared cocoa drink was spiked with defined amounts of d_3-1, d_3-3, d_4-4, d_3-5, d_3-7, d_4-8, d_3-10, d_5-11, d_5-12, and d_4-13 as the internal standards, followed by homogenization and equilibration at room temperature. After de-fatting and acetone/water extraction of the *N*-phenylpropenoyl-L-amino acids, mass chromatography was performed by means of analytical RP-HPLC-MS/MS. By far the highest amounts of 1196.2 and 473.3 µg *per* 100 g of cocoa drink were found for *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (1) and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5), followed by *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8) and *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (3) with somewhat lower amounts of 195.7 and 106.2 µg *per* 100 g of cocoa drink, respectively (Table 1). All the other *N*-phenylpropenoyl-L-amino acids were present in amounts below 61.4 µg *per* 100 g of cocoa drink.

Table 1. Concentrations of *N*-phenylpropenoyl-L-amino acids in the cocoa drink[a)] used for the intervention study

Compound (no.)	Conc. (µg/100g)[b)]
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (1)	1196.2
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5)	473.3
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8)	195.7
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-dopa (3)	106.2
N-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (9)	61.4
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (4)	39.8
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-dopa (7)	26.1
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6)	18.4
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (2)	16.0
N-[cinnamoyl]-L-aspartic acid (10)	10.6
N-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (13)	3.7
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophane (11)	1.9
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-tryptophane (12)	1.7

a) A mixture of alkalized cocoa powder (20.3 g) and sucrose (3.0 g) was suspended in table water (400 mL) at 50°C for 10 min. After cooling, this drink was used for quantitative analysis of amides.
b) Concentrations are given as the mean of three independent clean-ups and measurements of the sample.

In order to gain first insights into the absorption of these polyphenolic amides upon cocoa consumption, a bolus of the freshly prepared cocoa drink was administered to eight healthy volunteers who followed a controlled diet lacking any chocolate, cocoa, coffee, as well as any coffee or cocoa containing food products for at least 48 h prior to the intervention. Urine samples were collected in plastic bottles 1 h before starting the study, and 1, 2, 3, 4, 6, and 8 h after ingestion of the cocoa drink. After cooling, the urine samples were spiked with defined amounts of d_3-1, d_3-3, d_4-4, d_3-5, d_3-7, d_4-8, d_3-10, d_5-11, d_5-12, and d_4-13 as the internal standards, followed by equilibration, and clean-up by ultracentrifugation. Analysis of the *N*-phenylpropenoyl-L-amino acids was performed by means of analytical RP-HPLC-MS/MS (MRM) as exemplified for compounds 1, 5, and 8 in Fig. 3. As given in Table 2, the blank urine taken 1 h prior to the intervention did not contain any detectable amounts of *N*-phenylpropenoyl-L-amino acids. Independent from the chemical structure of the *N*-phenylpropenoyl-L-amino acid analyzed, a maximum amount was found in the urine samples taken 2 h after the cocoa consumption. Thereafter, the amount of *N*-phenylpropenoyl-L-amino acid was found to decrease again with increasing time, *e.g.*, after 8 h the target amides were not detectable or present just in trace amounts, respectively. Among all urine samples analyzed, by far the highest amount of 64.37 ± 20.22 µg was measured for

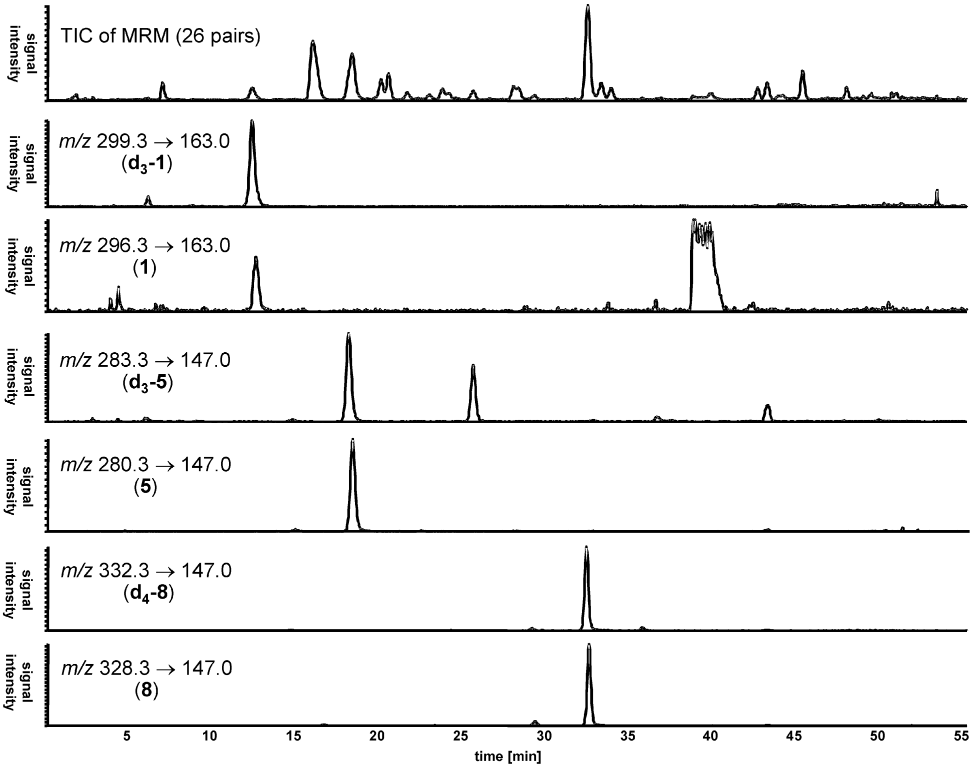


Figure 3. RP-HPLC-MS/MS(ESI$^+$)-MRM analysis for the quantitative determination of the *N*-phenylpropenoyl-L-amino acids 1, 5, and 8 in urine excreted after 1 h using the internal standards *d*$_3$-1, *d*$_3$-5, and *d*$_4$-8.

Table 2. Amount of *N*-phenylpropenoyl-L-amino acids excreted with urine over a time period of 8 h after intervention

Compound no.	Amount[a] (µg) excreted after						
	0 h	1 h	2 h	3 h	4 h	6 h	8 h
1	n.d.	3.59 ± 3.55	4.76 ± 4.01	3.64 ± 2.99	1.74 ± 1.80	0.83 ± 1.17	0.48 ± 1.05
2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	n.d.	0.52 ± 1.24	1.25 ± 1.19	<0.01	<0.01	n.d.	n.d.
4	n.d.	0.04 ± 0.12	0.30 ± 0.39	<0.01	<0.01	n.d.	n.d.
5	n.d.	22.92 ± 8.81	64.37 ± 20.22	31.96 ± 7.04	16.54 ± 5.69	11.10 ± 2.44	3.01 ± 1.93
6	n.d.	8.98 ± 4.60	22.17 ± 12.57	7.21 ± 2.79	3.22 ± 1.55	2.06 ± 1.54	0.92 ± 1.18
7	n.d.	0.26 ± 0.51	0.85 ± 0.96	<0.01	<0.01	n.d.	n.d.
8	n.d.	10.86 ± 5.48	18.91 ± 5.53	7.44 ± 1.44	3.56 ± 1.30	2.53 ± 0.66	0.73 ± 0.48
9	n.d.	4.67 ± 1.66	9.70 ± 2.93	4.11 ± 0.95	2.07 ± 0.57	1.02 ± 0.92	0.08 ± 0.21
10	n.d.	0.06 ± 0.18	0.43 ± 0.33	0.14 ± 0.20	0.07 ± 0.14	<0.01	n.d.
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	n.d.	<0.01	<0.01	<0.01	<0.01	n.d.	n.d.
13	n.d.	0.81 ± 0.39	1.91 ± 0.59	0.69 ± 0.44	0.23 ± 0.33	<0.01	n.d.

n.d.: not detectable.

a) The given amounts are calculated as the mean of the individual urinary excretion determined for each of the eight volunteers by three independent clean-up and measurements.

N-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5), followed by *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6) and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8) for which amounts of 22.17 ± 12.57 and 18.91 ± 5.52 µg were found (Table 2). *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (3), *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (4), *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-dopa (7), *N*-cinnamoyl-L-aspartic acid (10), and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (13) were present just in rather low amounts ranging between 0.3 and 1.9 µg (Table 2). In comparison, *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tryptophane (12) was detectable only in trace amounts below the LOQ, and *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (2) and *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophane (11) were not detectable at all.

Moreover, it is interesting to notice that five out of eight volunteers showed a significantly higher amount of amides in the excreted urine (Fig. 4A), whereas the other three vol-

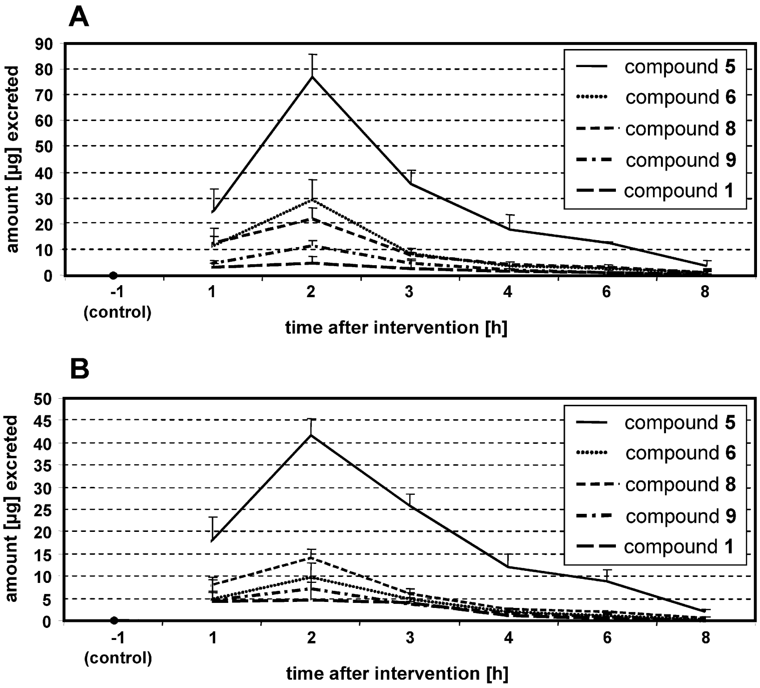


Figure 4. Influence of individuals on the absolute amounts (µg/urine sample) of *N*-phenylpropenoyl-L-amino acids 1, 5, 6, 8, and 9 in urine excreted over a time period of 8 h. Amounts and SD are given as average values obtained from (A) five individuals excreting higher amounts of amides and (B) three individuals excreting lower amounts of amides.

Table 3. Total amount of *N*-phenylpropenoyl-L-amino acids determined in the administered cocoa drink, the total amount of urine collected over 8 h, and the recovery rate

Compound (no.)	Amount (µg) ingested[a)]	Amount (µg) excreted[b)]	Recovery (%)[c)]
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6)	77.7	44.6	57.3
N-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (13)	15.8	3.6	22.8
N-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (9)	259.8	21.6	8.3
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5)	2003.4	149.9	7.5
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8)	828.2	44.0	5.3
N-[cinnamoyl]-L-aspartic acid (10)	44.7	0.7	1.6
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-dopa (7)	110.7	1.1	1.0
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-dopa (3)	449.5	1.8	0.4
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (1)	5063.6	15.0	0.3
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (4)	168.5	0.3	0.2
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (2)	67.6	n.d.	n.c.
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophane (11)	8.1	n.d.	n.c.
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-tryptophane (12)	7.1	<0.1	n.c.

n.d.: not detectable. n.c.: not calculated.

a) Amount of amides in the administered cocoa drink is given as the mean of three independent clean-ups and measurements of the sample.

b) Average amount of amide in total urine collected over 8 h for eight volunteers.

c) The recovery rate was calculated in percent from the ingested amount and the excreted amount of amides.

unteers excreted comparatively lower amounts (Fig. 4B), *e.g.*, the group A excreted 76.83 µg of 5 in the total urine collected after 2 h, whereas only 41.69 µg of that compound was detectable in the urine sample taken form group B.

To calculate the recovery of the individual *N*-phenylpropenoyl-L-amino acids 1 – 13 in urine, that means the percentage amount of ingested amides excreted with the urine, the sum amount of each amide was calculated for the total volume of urine collected over a time period of 8 h after intervention. As given in Table 3, the highest amount of 149.9 µg in the total urine volume was found for *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5), followed by 44.6 and 44.0 mg found for *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6) and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8), respectively. By far the highest recovery rates of 57.3 and 22.8% were found for *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6) and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (13), respectively. In comparison, only 8.3, 7.5, or 5.3% of the amount of *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (9), *N*-[4′-

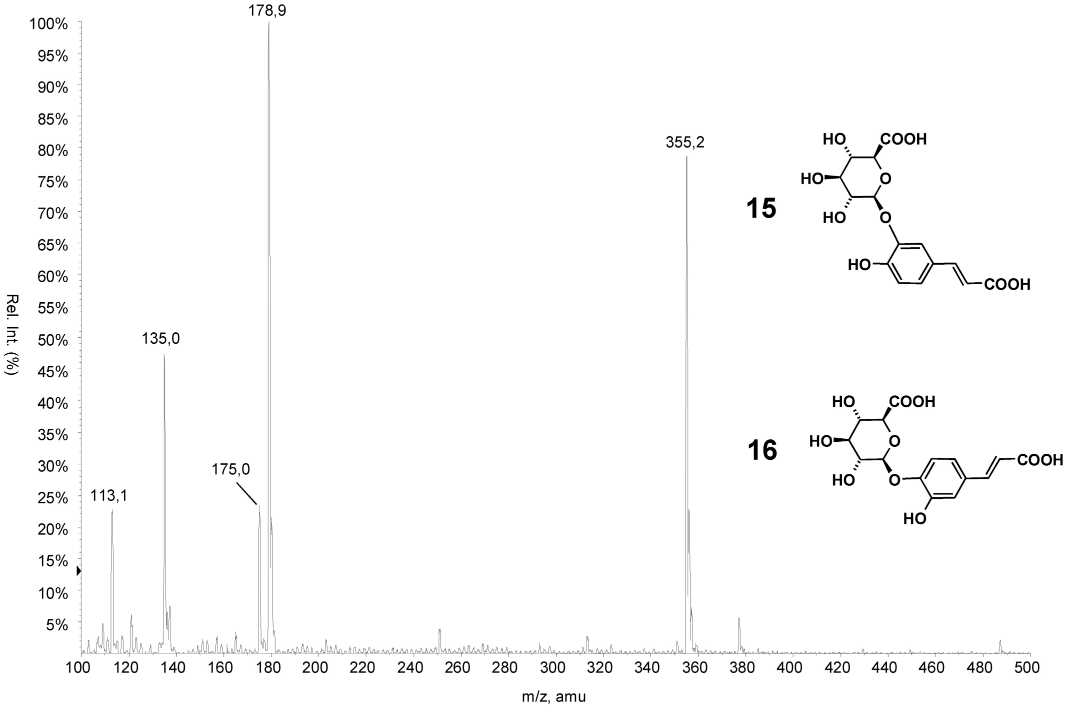


Figure 5. MS/MS (ESI⁻) spectrum obtained for synthetic caffeoyl-3-*O*-β-D-glucuronide (15) and caffeoyl-4-*O*-β-D-glucuronide (16), respectively.

hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5), and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8) present in the ingested cocoa drink were excreted with the urine (Table 3). In comparison, compounds 10 and 7 showed only a low recovery rate of 1.6 and 1.0%, respectively whereas the recovery rates of the remaining amides were found to be below 0.4%.

In order to gain first insights into the metabolism of *N*-phenylpropenoyl-L-amino acids *in vivo*, the urine samples taken from two volunteers, one of the group A and the other from group B (Fig. 4A and 4B), were treated with β-glucuronidase/sulfatase at pH 5.0 for 4 h at 37°C. Comparing the quantitative data obtained before and after enzyme treatment revealed exactly the same amounts of the *N*-phenylpropenoyl-L-amino acids in the corresponding urine samples, thus indicating that neither *O*-glucuronides nor *O*-sulfates of the polyphenolic amides were formed upon metabolic conjugation. In order to confirm the functionality of the β-glucuronidase/sulfatase enzyme solution used and to screen for caffeic acid *O*-glucuronides as potential metabolites of *N*-phenylpropenoyl-L-amino acids, the two potential candidates caffeic acid 3-*O*-β-D-glucuronide and caffeic acid 4-*O*-β-D-glucuronide needed to be synthesized as reference compounds.

3.2 Synthesis of caffeic acid *O*-β-D-glucuronides

In order to synthesize both caffeic acid *O*-β-D-glucuronides, caffeic acid methylester (14), prepared in good yield by acidic esterification of caffeic acid with methanol, was reacted with excess amounts of methyl acetobromo-α-D-glucuronate in presence of silver trifluoromethane sulfonate at −20°C (Fig. 2). Column chromatography on silica gel, followed by de-protection using aqueous barium hydroxide, and purification by means of preparative RP-HPLC yielded the target compounds caffeic acid 3-*O*-β-D-glucuronide (15) and caffeic acid 4-*O*-β-D-glucuronide (16) in a ratio of ∼2:1.

Measurements of the exact masses of conjugates 15 and 16 by means of high-resolution MS revealed the ion *m*/*z* 379.0637$^+$ fitting well with the calculated mass of 379.0636 and confirming the sodium adduct ($C_{15}H_{16}O_{10}Na^+$) of the target compounds. LC-MS analysis of 15 and 16 in the ESI$^-$ mode showed identical spectra exhibiting *m*/*z* 355 $[M - H]^-$ as the pseudomolecular ion. Collision-induced fragmentation produced *m*/*z* 179 $[M-\text{glucuronic acid}-H]^-$ as the most frequent daughter ion and *m*/*z* 135 as the second most frequent daughter ion (Fig. 5), thus indicating the release of a glucuronic acid moiety from the molecule as well as the decarboxylation of the caffeic acid moiety.

The ^{1}H NMR spectrum of compound 15 showed the five protons expected for the caffeic acid moiety. The doublets observed for H−C(7) and H−C(8) resonated at 7.42 and 6.84 ppm, respectively and exhibited a coupling constant of 16 Hz, thus indicating a *trans*-configured double bond in the molecule. The protons H−C(2), H−C(5), and H−C(6)

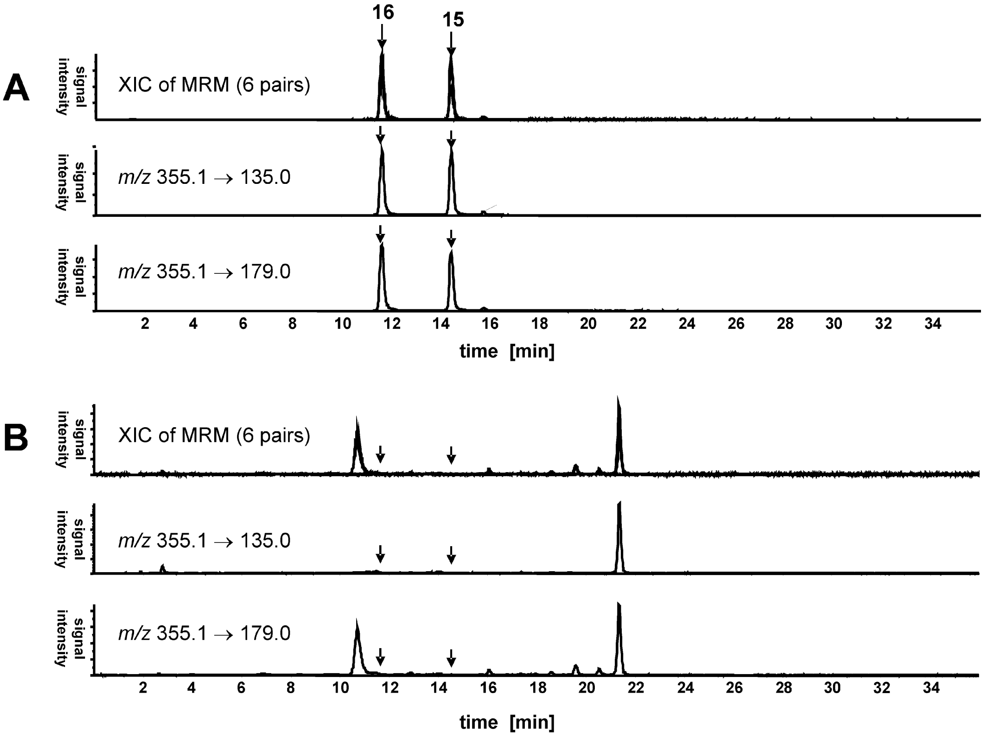


Figure 6. RP-HPLC-MS/MS (ESI$^-$)-MRM analysis of (A) a reference solution of caffeoyl-*O*-β-D-glucuronides 15 and 16, and (B) a pooled urine sample collected 2 h after the intervention.

were unequivocally assigned by means of homonuclear δ,δ-correlation (g-COSY), and HMQC optimized for $^1J_{C,H}$ coupling constants. The broad singlet detected at 9.30 ppm disappeared upon D_2O addition and, therefore, was identified as the residual aromatic hydroxyl proton. Carbon atoms C(3) and C(4) could be clearly distinguished by means of HMBC optimized for $^2J_{C,H}$ and $^3J_{C,H}$ (HMBC). Carbon atom C(3) showed an intense heteronuclear correlation to the H–C(5) in the *meta*-position and a weak $^2J_{C,H}$-coupling with H–C(2), whereas carbon C(4) coupled with the *meta*-protons H–C(2) and H–C(6), respectively. As carbon C(3) showed also coupling with the anomeric proton H–C(1′), compound 15 was identified as caffeoyl-3-*O*-β-D-glucuronide. The protons H–C(2′) and H–C(3′) of the glucuronic acid moiety resonated at 3.30 ppm and showed strong δ,δ-couplings to H–C(4′), which was slightly downfield shifted to 3.40 ppm and occurred as a pseudo triplet with a coupling constant of 9.2 Hz. Proton H–C(5′) resonated at 3.98 ppm as a doublet and coupled with H–C(4′) as confirmed by a COSY experiment. Furthermore, the coupling constant of 7.2 Hz observed for the anomeric proton H–C(1′) indicated the β-configuration of the glucuronide. The hydroxyl groups HO–C(2′) and HO–C(3′) resonated as broad singlets at 5.22 and 5.47 ppm but could not be further differentiated, whereas proton HO–C(4′) resonated as a small, broadened signal at 4.49 ppm. The acidic protons HOOC–(6′) and HOOC–(9) were detectable as a very broad coalescent signal resonating at around 12.3 ppm.

The isomer 16 showed similar NMR data as found for compound 15, however, the hydroxyl group HO–C(4′) was significantly downfield-shifted to 5.78 ppm and the residual phenolic proton HO–C(3) was visible at 8.90 ppm as a broad singlet. Also the pattern of the aromatic protons was slightly different. Proton H–C(6) resonating as double doublet at 7.09 ppm showed strong δ,δ-coupling to the neighboring proton H–C(5) and long-range coupling to H–C(2) in the COSY experiment. Taking all spectroscopic data into consideration, the structure of this compound was confirmed as caffeic acid 4-*O*-β-D-glucuronide (16, Fig. 5).

3.3 Enzymatic hydrolysis of caffeic acid *O*-β-D-glucuronides

In order to confirm the functionality of the β-glucuronidase/sulfatase enzyme solution, aqueous solutions of caffeic acid *O*-β-D-glucuronides 15 or 16, respectively were treated individually with β-glucuronidase/sulfatase at pH 5.0 for 6 h at 37°C and, after 0.5, 1, 2, 3, 4, and 6 h, aliquots were analyzed by means of HPLC-MS/MS. Already after 30 min, both caffeic acid *O*-β-D-glucuronides were nearly completely hydrolyzed and after 1 h not even trace amounts of the conjugates were detectable any more, thus confirming the functionality of the enzymes used (data not shown).

In order to screen for caffeic acid *O*-β-D-glucuronides as potential metabolites formed from *N*-phenylpropenoyl-L-amino acids, urine samples collected were screened for 15 and 16 by means of LC-MS/MS (Fig. 6). Independent of the urine sample collected, not even trace amounts of caffeoyl-*O*-β-D-glucuronides were detectable. These data clearly demonstrate that caffeic acid glucuronides are not the predominant metabolites of *N*-phenylpropenoyl-L-amino acids in humans.

4 Discussion

To date, there are no reports available on the absorption and bioavailability of *N*-phenylpropenoyl-L-amino acids, a recently identified family of major polyphenols in *T. cacao* [9–12]. First, the amounts of the polyphenolic amides 1–13 (Fig. 1) were quantitatively determined in a sweetened cocoa drink by means of a recently developed SIDA [12]. The data, given in Table 1, revealed *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (1) and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5), followed by *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8) and *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (3) as the quantitatively predominating polyphenolic amides in the cocoa drink, thus being well in line with earlier data on these compounds in roasted cocoa nibs [9, 12].

After administration of a bolus amount of the cocoa drink to eight healthy volunteers, the time-dependent urinary excretion of *N*-phenylpropenoyl-L-amino acid was quantitatively determined by means of LC-MS/MS-SIDA. The data obtained clearly demonstrated that independent from the structure of the *N*-phenylpropenoyl-L-amino acid, the highest concentrations of these phytochemicals were excreted with the urine already 2 h after consumption of the cocoa drink (Table 2). Most surprisingly, the predominant amide detected in urine was not the *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (1), which was found as the major *N*-phenylpropenoyl-L-amino acid in the cocoa drink used in the intervention study (Table 2). In contrast, *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5), *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6), and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8) were found as the quantitatively predominating amides in urine. Interestingly, five out of eight volunteers showed a significantly higher amount of amides in the excreted urine (Fig. 4A), whereas the other three volunteers excreted comparatively lower amounts (Fig. 4B). These findings give first evidence for major differences in the absorption of *N*-phenylpropenoyl-L-amino acids and/or the metabolic activity of individuals. However, further studies with a higher sample number are needed to justify the formation of dichotomic groups.

Calculating the percentage amount of ingested *N*-phenylpropenoyl-L-amino acids excreted with the urine, by far the highest recovery rates of 57.3, 22.8, and 8.3% were found for *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (6), *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (13), and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (9), followed by *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (5) and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (8), all of which are bearing either a *p*-coumaroyl or a feruloyl moiety in the molecule. It is interesting to notice, that all the *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-amino acids were found in much lower amounts in urine when compared to the amides bearing *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]- and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-moiety, respectively; for example, the recovery rates found for the caffeoyl derivatives 1–4, and 11 were found to be below 0.4% (Table 3).

Based on this finding, it might be concluded that after ingestion of *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-amino acids, these *N*-caffeoyl-amides are metabolically conjugated to give the corresponding *O*-glucuronides, *O*-sulfates, and/or *O*-methyl ethers as reported in the literature for polyphenols including caffeic acid and caffeic acid esters such as chlorogenic acid [24, 26–29]. Alternatively, it might be argued that the *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-amino acids are either not efficiently absorbed, or are enzymatically hydrolyzed to release caffeic acid which, upon further conjugation, might be conjugated to give its corresponding *O*-glucuronide [27]. In order to check, if *N*-phenylpropenoyl-L-amino acids are metabolized *in vivo* to give their corresponding *O*-glucuronide and/or *O*-sulfate conjugates, urine samples were comparatively analyzed by means of LC-MS/MS-SIDA before and after treatment with β-glucuronidase/sulfatase. Surprisingly, the concentrations of the *N*-phenylpropenoyl-L-amino acids in the excreted urine was not influenced by the enzymatic digestion, thus demonstrating that these polyphenolic amides are neither metabolized *via* their *O*-glucuronides nor their *O*-sulfates.

To confirm the enzymatic functionality of the enzymes used for the digestion experiment, caffeic acid 3-*O*-β-D-glucuronide (15) and caffeic acid 4-*O*-β-D-glucuronide (16) were synthesized for the first time, and then treated with β-glucuronidase/sulfatase. LC-MS/MS analysis of aqueous solutions of caffeic acid *O*-β-D-glucuronides before and after enzymatic digestion clearly demonstrated a rapid cleavage of the *O*-glucuronides to release caffeic acid and, in consequence, give strong evidence that *N*-phenylpropenoyl-L-amino acids are not enzymatically hydrolyzed and metabolically conjugated as *O*-glucuronides or *O*-sulfates.

In conclusion, this is the first human study demonstrating that the *N*-phenylpropenoyl-L-amino acids 1, 3–10, 12, and 13 are absorbed and excreted *via* the urine. LC-MS/MS experiments before and after β-glucuronidase/sulfatase treatment did not show any evidence that the *N*-phenylpropenoyl-L-amino acids are enzymatically hydrolyzed and then conjugated with glucuronic acid as reported for chlorogenic acids [27]. Bearing in mind that the *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]- and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-amino acids were found in urine with rather high recovery rates, it might be possible that these feruoyl- or *p*-coumaroyl-L-amino acids are formed by *O*-methylation or by reduction of the corresponding caffeoyl-L-amino acids. As chocolate intake was recently shown to increase the urinary excretion of *m*-hydroxyphenylpropionic acid, ferulic acid, 3,4-dihydroxyphenylacetic acid, *m*-hydroxyphenylacetic acid, vanillic acid, and *m*-hydroxybenzoic acid generated upon microbial metabolism of polyphenols [30], the intestinal microbial degradation of the *N*-phenylpropenoyl-L-amino acids has to be taken into account for future studies. Quantitative studies on the levels of *N*-phenylpropenoyl-L-amino acids as well as their putative metabolites in human plasma and urine are currently under investigation and will be published separately.

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