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Quantitative Analysis of *N*-Phenylpropenoyl-L-amino Acids in Roasted Coffee and Cocoa Powder by Means of a Stable Isotope Dilution Assay

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Since recent reports on the role of *N*-phenylpropenoyl-L-amino acids as powerful antioxidants and key contributors to the astringent taste of cocoa nibs, there is an increasing interest in the concentrations of these phytochemicals in plant-derived foods. A versatile analytical method for the accurate quantitative analysis of *N*-phenylpropenoyl-L-amino acids in plant-derived foods by means of HPLC-MS/MS and synthetic stable isotope labeled *N*-phenylpropenoyl-L-amino acids as internal standards was developed. By means of the developed stable isotope dilution assay (SIDA), showing recovery rates of 95−102%, 14 *N*-phenylpropenoyl-L-amino acids were quantified for the first time in cocoa and coffee samples. On the basis of the results of LC-MS/MS experiments as well as cochromatography with the synthetic reference compounds *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophan, *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tryptophan, and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine, respectively, were detected for the first time in cocoa powder, and (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine, (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine, *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine, (+)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid, (+)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid, *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophan, *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tryptophan, and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tryptophan, respectively, were detected for the first time in coffee beverages.

KEYWORDS: ***N*-Phenylpropenoyl-L-amino acids; taste; cocoa; coffee; stable isotope dilution assay; SIDA; *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid**

INTRODUCTION

Recently, application of taste dilution analyses (*1*) revealed besides procyanidins the *N*-phenylpropenoyl amino acids **1**−**10** (**Figure 1**) as the key contributors to the typical astringent taste of cocoa beans as well as roasted cocoa nibs. Using the so-called half-tongue test, human recognition thresholds for the astringent and mouth-drying oral sensation were determined to be between 26 and 220 μmol/L (water) depending on the amino acid moiety (*2*, *3*).

Besides reports of its taste activity, (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**3**), named clovamide, was earlier identified in red clover (*Trifolium pratense*) (*4*−*6*), and together with (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (**8**), named deoxyclovamide, was earlier found as an antioxidant in cocoa (*7*) and as a constituent of the bark of African blackwood (*Dalbergia melanoxylon*) (*8*). Moreover, (+)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**5**) and (+)-*N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (**9**) have been earlier isolated from cell suspension cultures of *Arabidopsis thaliana* (*9*) and cell cultures of *Beta vulgaris* (*10*), respectively. In addition, (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (**4**) has been previously identified in cocoa flowers (*11*), and together with the tryptophan derivatives **11** (*13*), **12** (*14*), and **13** (*15*) as well as the tyrosine conjugate **14** was detected in raw Robusta coffee beans (*12*).

Although these *N*-phenylpropenoyl amino acids are biologically active as astringent chemosensates (*2*, *3*), phytoalexins (*16*), and plant antioxidants (*7*), suitable methodologies for the rapid and accurate quantification of these unstable, highly polar phytochemicals are still missing. As the use of stable isotopomers of analytes is known to enable the correction of compound discrimination during extraction, cleanup, chromatographic separation, and MS detection, the purpose of the present investigation was to develop a versatile and reliable stable isotope dilution assay (SIDA) for the quantitative determination of *N*-phenylpropenoyl amino acids in plant foods such as roasted cocoa and coffee.

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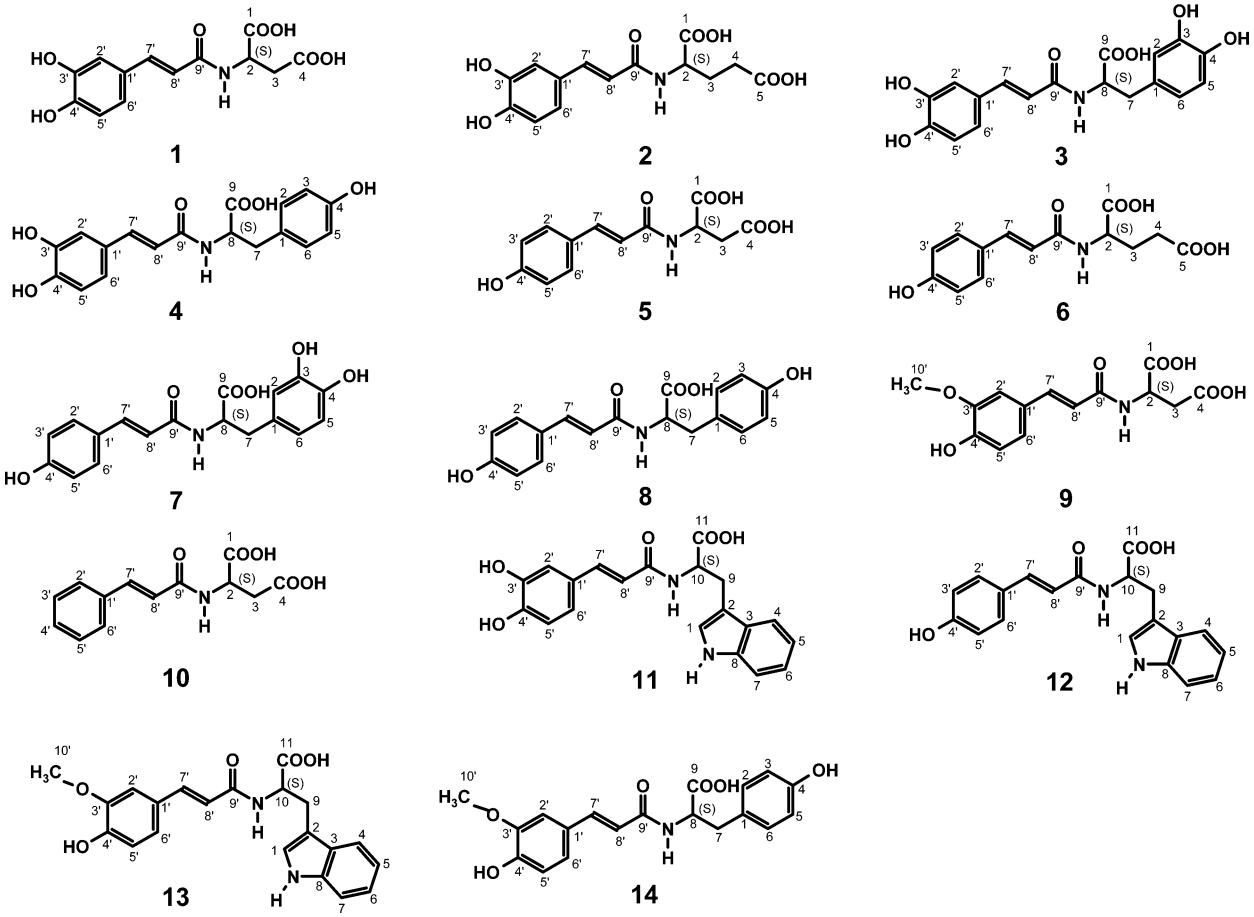

Figure 1. Chemical structures of *N*-phenylpropenoyl-L-amino acids **1**–**14**.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: Amberlyst 15 (H^+, 20−50 mesh) and (dimethylamino)pyridine (Fluka, Neu-Ulm, Germany); acetic anhydride, (*E*)-4-hydroxycinnamic acid, thionyl chloride, and (*E*)-4-hydroxy-3-methoxycinnamic acid (Sigma-Aldrich, Steinheim, Germany); (*E*)-3,4-dihydroxycinnamic acid, potassium carbonate, l-aspartic acid, L-dopa, L-glutamic acid, L-tyrosine, L-tryptophan, sodium sulfate, pyridine, and tetrahydrofuran (Merck, Darmstadt, Germany); [2,3,3-^{2}H]-L-aspartic acid, [2,5,6-^{2}H]-L-dopa, [2,3,5,6-^{2}H]-L-tyrosine, and [1,4,5,6,7-^{2}H]-L-tryptophan (Cambridge Isotope Laboratories, Andover, MA). Solvents were of HPLC grade (Merck). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). Roasted and nonalkalized cocoa powder and roasted coffee (Arabica Columbia) as well as decaffeinated coffee (70% Arabica, 30% Robusta) were obtained from the food industry. (+)-*N*-[3′,4′-Dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**) (**Figure 1**), (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**2**), (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**3**), (−)-*N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine (**4**, **Figure 1**), (+)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**5**), (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-glutamic acid (**6**), (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**7**), (−)-*N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (**8**), (+)-*N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-aspartic acid (**9**), and (+)-*N*-[(*E*)-cinnamoyl]-L-aspartic acid (**10**) were synthesized following the procedure reported recently (*3*).

Syntheses of *N*-Phenylpropenoyl-L-Amino Acids. Closely following the synthetic route reported recently for the synthesis of *N*-phenylpropenoyl-L-amino acids (*3*), *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophan (**11**), *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tryptophan (**12**), *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tryptophan (**13**), and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (**14**) were prepared starting from (*E*)-caffeic acid, (*E*)-*p*-coumaric acid, or (*E*)-ferulic acid and L-tryptophan or L-tyrosine, respectively. The target compounds were then applied onto the top of a water-cooled glass column (40 × 140 mm) filled with a slurry of LiChroprep 25−40 μm RP-18 material (Merck) in aqueous formic acid (0.1% in water; pH 2.5). Chromatography was performed using aqueous formic acid (0.1% in water; pH 2.5) as the effluent, followed by aqueous formic acid (0.1% in water; pH 2.5) containing increasing amounts of methanol. The target compounds were detected by monitoring the effluent at 300 nm, and the fractions containing these compounds were confirmed by RP-HPLC-DAD and RP-HPLC-MS/MS. After the individual fractions had been freeze-dried three times, the corresponding *N*-phenylpropenoyl-L-amino acids were obtained as white, amorphous powders in high purities of >99%.

*N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-L-tryptophan, **11**:* 0.29 mmol; 29% yield; UV_{max} = 221, 290, 323 nm; LC-MS (ESI^+), *m/z* 367 (100%, $[M + 1]^+$), 733 (15%, $[2M + 1]^+$), 389 (10%, $[M + Na]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 3.07 [dd, 1H, J = 8.8, 14.4 Hz, H−C(9a)], 3.23 [dd, 1H, J = 5.0, 14.4 Hz, H−C(9b)], 4.62 [m, 1H, J = 5.0, 8.8 Hz, H−C(10)], 6.44 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.75 [d, 1H, J = 8.0 Hz, H−C(5′)], 6.83 [dd, 1H, J = 2.0, 8.0 Hz, H−C(6′)], 6.94 [d, 1H, J = 1.6 Hz, H−C(2′)], 6.97 [m, 1H, J = 0.8 Hz, J = 8.0 Hz, H−C(5)], 7.07 [m, 1H, J = 0.8 Hz, J = 8.0 Hz, H−C(6)], 7.16 [d, 1H, J = 2.4 Hz, H−C(1)], 7.22 [d, 1H, J = 15.6 Hz, H−C(7′)], 7.34 [d, 1H, J = 8.0 Hz, H−C(7)], 7.56 [d, 1H, J = 8.0 Hz, H−C(4)], 8.26 [d, 1H, J = 7.6 Hz, H−N], 9.13 [s, 1OH, HO−C(3′)], 9.37 [s, 1OH, HO−C(4′)], 10.84 [d, 1H, H−N(indole)], 12.66 [s, 1OH, HOO−C(11)]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 27.8 [C-9], 53.5 [C-10], 110.4 [C-2], 111.8 [C-7], 114.3 [C-2′], 116.1 [C-5′], 118.5 [C-4], 118.6 [C-8′], 118.9 [C-5], 121.0 [C-6′], 121.3 [C-6], 124.0 [C-1], 126.8 [C-1′], 127.7 [C-3], 136.4 [C-8], 140.0 [C-7′], 146.0 [C-3′], 147.8 [C-4′], 165.8 [C-9′], 174.2 [C-11].

*N-[4′-Hydroxy-(E)-cinnamoyl]-L-tryptophan, **12**:* 0.41 mmol; 41% yield; UV_{max} = 219, 292, 307 nm; LC-MS (ESI^+), *m/z* 351 (100%, $[M + 1]^+$), 701 (24%, $[2M + 1]^+$), 373 (22%, $[M + Na]^+$), 723 (17%, $[2M + Na]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 3.05 [dd, 1H, J = 9.0, 14.8 Hz, H−C(9a)], 3.09 [dd, 1H, J = 5.0, 14.8 Hz, H−C(9b)],

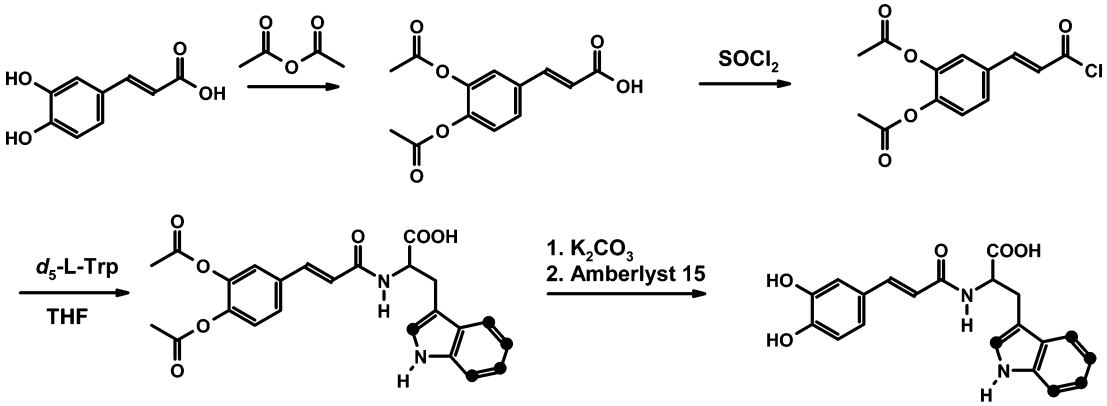

Figure 2. Synthetic sequence used for the preparation of *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan (**d_5-11**).

4.62 [m, 1H, J = 5.0, 9.0 Hz, H−C(10)], 6.52 [d, 1H, J = 16.0 Hz, H−C(8′)], 6.79 [d, 2H, J = 8.4 Hz, H−C(3′, 5′)], 6.99 [m, 1H, J = 1.2, 8.0 Hz, H−C(5)], 7.05 [m, 1H, J = 1.2, 8.0 Hz, H−C(6)], 7.16 [d, 1H, J = 2.0 Hz, H−C(1)], 7.30 [d, 1H, J = 16.0 Hz, H−C(7′)], 7.32 [d, 1H, J = 8.4 Hz, H−C(7)], 7.38 [d, 2H, J = 8.4 Hz, H−C(2′, 6′)], 7.56 [d, 1H, J = 8.4 Hz, H−C(4)], 8.23 [d, 1H, J = 7.6 Hz, H−N], 9.84 [s, 1OH, HO−C(4′)], 10.84 [d, 1H, J = 1.6 Hz, H−N(indole)], 12.65 [s, 1OH, HOO−C(11)]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 27.6 [C-9], 53.5 [C-10], 110.3 [C-2], 111.7 [C-7], 116.0 [C-3′, 5′], 118.1 [C-4], 118.2 [C-8′], 118.3 [C-5], 121.4 [C-6], 124.0 [C-1], 126.3 [C-1′], 127.6 [C-3], 129.8 [C-2′, 6′], 136.5 [C-8], 139.1 [C-7′], 159.2 [C-4′], 165.9 [C-9′], 174.0 [C-11].

N-[4′-Hydroxy-3′-methoxy-(E)-cinnamoyl]-L-tryptophan, ***13****:* 0.26 mmol; 26% yield; UV_{max} = 229, 291, 322 nm; LC-MS (ESI$^+$), *m/z* 381 (100%, $[M + 1]^+$), 761 (39%, $[2M + 1]^+$), 403 (35%, $[M + Na]^+$), 783 (18%, $[2M + Na]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 3.07 [dd, 1H, J = 8.8, 14.8 Hz, H−C(9a)], 3.23 [dd, 1H, J = 4.8, 14.8 Hz, H−C(9b)], 3.80 [s, 3H, H−C(10′)], 4.61 [m, 1H, J = 4.8, 8.8 Hz, H−C(10)], 6.56 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.78 [d, 1H, J = 8.0 Hz, H−C(5′)], 6.97 [dd, 1H, J = 2.0, 8.0 Hz, H−C(6′)], 6.99 [m, 1H, H−C(5)], 7.06 [m, 1H, H−C(6)], 7.12 [d, 1H, J = 2.0 Hz, H−C(2′)], 7.15 [d, 1H, J = 2.4 Hz, H−C(1)], 7.30 [d, 1H, J = 15.6 Hz, H−C(7′)], 7.33 [d, 1H, J = 8.0 Hz, H−C(7)], 7.55 [d, 1H, J = 7.6 Hz, H−C(4)], 8.16 [d, 1H, J = 8.4 Hz, H−N], 9.42 [s, 1OH, HO−C(4′)], 10.83 [s, 1H, H−N(indole)], 12.69 [s, 1OH, HOO−C(11)]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 27.7 [C-9], 53.5 [C-10], 56.0 [C-10′], 110.3 [C-2], 111.2 [C-2′], 111.7 [C-7], 116.1 [C-5′], 118.6 [C-4], 118.7 [C-5], 119.2 [C-8′], 121.2 [C-6], 122.2 [C-6′], 124.0 [C-1], 126.6 [C-1′], 127.5 [C-3], 136.5 [C-8], 140.0 [C-7′], 147.8 [C-3′], 148.3 [C-4′], 165.7 [C-9′], 174.0 [C-11].

N-[4′-Hydroxy-3′-methoxy-(E)-cinnamoyl]-L-tyrosine, ***14****:* 0.32 mmol; 32% yield; UV_{max} = 221, 297, 322 nm; LC-MS (ESI$^+$), *m/z* 358 (100%, $[M + 1]^+$), 715 (22%, $[2M + 1]^+$), 380 (18%, $[M + Na]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.81 [dd, 1H, J = 9.2, 14.0 Hz, H−C(7a)], 2.99 [dd, 1H, J = 4.8, 14.0 Hz, H−C(7b)], 3.80 [s, 3H, H−C(10′)], 4.48 [m, 1H, J = 4.8, 14.0 Hz, H−C(8)], 6.54 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.66 [d, 2H, J = 8.4 Hz, H−C(3, 5)], 6.79 [d, 1H, J = 8.0 Hz, H−C(5′)], 6.99 [dd, 1H, J = 1.6, 8.0 Hz, H−C(6′)], 7.04 [d, 2H, J = 8.4 Hz, H−C(2, 6)], 7.13 [d, 1H, J = 1.6 Hz, H−C(2′)], 7.29 [d, 1H, J = 15.6 Hz, H−C(7′)], 8.13 [d, 1H, J = 8.0 Hz, H−N], 9.20 [s, 1OH, HO−C(4)], 9.44 [s, 1OH, HO−C(4′)], 12.70 [s, 1OH, HOO−C(9)]; ^{13}C NMR (100 MHz, DMSO-d_6, HMQC, HMBC) δ 36.5 [C-7], 54.4 [C-8], 56.0 [C-10′], 111.2 [C-2′], 115.4 [C-3, 5], 116.0 [C-5′], 119.0 [C-8′′], 122.1 [C-6′], 126.6 [C-1′], 128.2 [C-1], 130.4 [C-2, 6], 140.0 [C-7′], 148.0 [C-3′], 148.8 [C-4′], 156.3 [C-4], 165.7 [C-9′], 173.7 [C-9].

Syntheses of ^{2}H-Labeled *N*-Phenylpropenoyl-L-amino Acids. As exemplified for the preparation of *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan in **Figure 2**, acetic anhydride (25 mmol) was added dropwise to a solution of (*E*)-caffeic acid, (*E*)-*p*-coumaric acid, or (*E*)-ferulic acid (10 mmol each), respectively, in pyridine (5 mL) and (dimethylamino)pyridine (0.25 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and then poured onto crushed ice, and the aqueous phase was acidified to pH 2.0 with aqueous hydrochloric acid (2 mol/L). After extraction with a mixture of ethyl acetate and tetrahydrofuran (3:1, v/v; 3 × 50 mL), the combined organic extracts were dried over Na_2SO_4, filtered, and freed from solvent in a vacuum affording the acetylated phenylpropenoic acid derivative. An aliquot (1.0 mmol) of this product was mixed with thionyl chloride (1.0 mmol) and was then heated under reflux until no further formation of hydrogen chloride was observable. After cooling to room temperature, the reaction mixture was dried under a stream of nitrogen, and either [2,3,3-^{2}H]-L-aspartic acid (for **d_3-1** and **d_3-5**), [2,5,6-^{2}H]-L-dopa (for **d_3-3** and **d_3-7**), [2,3,5,6-^{2}H]-L-tyrosine (for **d_4-4** and **d_4-8**), or [1,4,5,6,7-^{2}H]-L-tryptophan (for **d_5-11**−**d_5-13**) (each 1.0 mmol) dissolved in dry tetrahydrofuran (30 mL) was added. After this solution had been stirred for up to 72 h at room temperature, the solvent was evaporated in a vacuum, and the residue was dissolved in a solution of K_2CO_3 (0.25 mol/L; 20 mL) in water/methanol (1:1; v/v) and stirred at room temperature. After 20 min, Amberlyst 15 ion-exchange resin was added with stirring until a pH value of 3.5 was reached. The resin was then filtered off and washed with water (20 mL), and the filtrate was freed from methanol in a vacuum and, finally, freeze-dried. The residue was taken up in water/methanol (1:1, v/v; 3 mL) and applied onto the top of a water-cooled glass column (40 × 140 mm) filled with a slurry of LiChroprep 25−40 μm RP-18 material (Merck) in aqueous formic acid (0.1% in water; pH 2.5). Chromatography was performed using aqueous formic acid (0.1% in water; pH 2.5) as the effluent, followed by aqueous formic acid (0.1% in water; pH 2.5) containing increasing amounts of methanol. The target compounds were detected by monitoring the effluent at 300 nm, and the fractions containing these compounds were confirmed by RP-HPLC-DAD. After the individual fractions had been freeze-dried three times, the corresponding deuterium-labeled *N*-phenylpropenoyl-L-amino acids were obtained as white, amorphous powders in high purities of >99%.

(+)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-[2,3,3-^{2}H]-L-aspartic acid, ***d_3-1****:* 0.28 mmol; 28% yield (**Figure 3**); UV_{max} = 207, 285, 309 nm; LC-MS (ESI$^+$), *m/z* 619 (100%, $[2M + Na]^+$), 321 (55%, $[M + Na]^+$), 163 (52%, $[M - 136]^+$), 933 (32%, $[3M + K]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 6.44 [d, 1H, J = 15.7, H−C(8′)], 6.74 [d, 1H, J = 8.2 Hz, H−C(5′)], 6.85 [dd, 1H, J = 1.9, 8.2 Hz, H−C(6′)], 6.95 [d, 1H, J = 1.9 Hz, H−C(2′)], 7.24 [d, 1H, J = 15.7 Hz, H−C(7′)], 8.19 [s, 1H, H−N], 9.10 [s, 1OH, HO−C(3′)], 9.32 [s, 1OH, HO−C(4′)].

(−)-N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-[2,5,6-^{2}H]-3-hydroxy-L-tyrosine, ***d_3-3****:* 0.25 mmol; 25% yield (**Figure 3**); UV_{max} = 225, 255, 297 nm; LC-MS (ESI$^+$), *m/z* 725 (100%, $[2M + 1]^+$), 363 (45%, $[M + 1]^+$) 163 (25%, $[M - 200]^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.73 [dd, 1H, J = 9.1, 13.9 Hz, H−C(7a)], 2.91 [dd, 1H, J = 5.0, 13.9 Hz, H−C(7b)], 4.44 [m, 1H, H−C(8)], 6.41 [d, 1H, J = 15.7 Hz, H−C(8′)], 6.74 [d, 1H, J = 8.1 Hz, H−C(5′)], 6.82 [dd, 1H, J = 2.0, 8.1 Hz, H−C(6′)], 6.94 [d, 1H, J = 2.0 Hz, H−C(2′)], 7.19 [d, 1H, J = 15.7 Hz, H−C(7′)], 8.15 [d, 1H, J = 8.0, H−N], 8.63, 8.68, 9.08, 9.32 [4s, 4OH, HO−C], 12.56 [s, 1OH, HOOC(9)].

N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-[2,3,5,6-^{2}H]-L-tyrosine, ***d_4-4****:* 0.18 mmol; 18% yield (**Figure 3**); UV_{max} = 216, 297, 323 nm; LC-MS

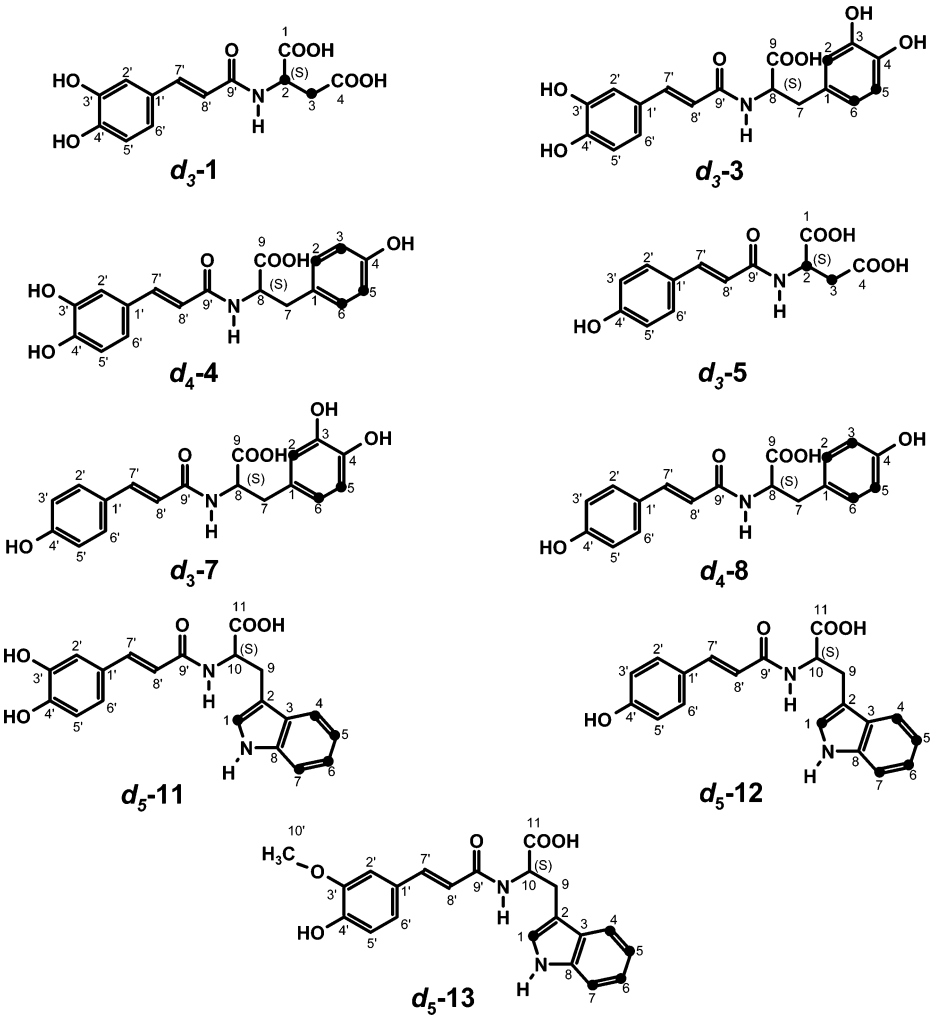

Figure 3. Structures of deuterated *N*-phenylpropenoyl-L-amino acids **d_3-1**, **d_3-3**, **d_4-4**, **d_3-5**, **d_3-7**, **d_4-8**, and **d_5-11**–**d_5-13**.

(ESI$^+$), *m/z* 348 (100%, [M + 1]$^+$), 370 (33%, [M + Na]$^+$), 695 (12%, [2M + 1]$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.83 [dd, 1H, J = 9.6, 14.0 Hz, H−C(7a)], 3.00 [dd, 1H, J = 4.8, 14.0 Hz, H−C(7b)], 4.49 [m, 1H, J = 4.8, 9.6, 14.0 Hz, H−C(8)], 6.52 [d, 1H, J = 15.8 Hz, H−C(8′)], 6.78 [d, 1H, J = 8.4 Hz, H−C(5′)], 6.97 [d, 1H, J = 2.0 Hz, H−C(2′)], 7.23 [d, 1H, J = 15.8 Hz, H−C(7′)], 7.86 [dd, 1H, J = 2.0, 8.0 Hz, H−C(6′)], 8.24 [d, 1H, J = 8.0 Hz, H−N], 9.17 [s, 1OH, HO−C(3′)], 9.23 [s, 1OH, HO−C(4)], 9.41 [s, 1OH, HO−C(4′)], 12.68 [s, 1OH, HOOC(9)].

(+)-N-[4′-Hydroxy-(E)-cinnamoyl]-[2,3,3-^{2}H]-L-aspartic acid, ***d_3-5****:* 0.30 mmol; 30% yield (**Figure 3**); UV$_{max}$ = 207, 285, 309 nm; LC-MS (ESI$^+$), *m/z* 305 (100%, [M + Na]$^+$), 587 (93%, [2M + Na]$^+$), 603 (60%, [2M + K]$^+$) 885 (40%, [3M + K]$^+$), 147 (20%, [M − 136]$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 6.52 [d, 1H, J = 15.7 Hz, H−C(8′)], 6.79 [d, 2H, J = 8.6 Hz, H−C(3′, 5′)], 7.33 [d, 1H, J = 15.7 Hz, H−C(7′)], 7.40 [d, 2H, J = 8.6 Hz, H−C(2′, 6′)], 8.18 [s, 1H, H−N], 9.81 [s, 1OH, HO−C(4′)], 12.82 [s, 2OH, HOOC-(1, 4)].

(−)-N-[4′-Hydroxy-(E)-cinnamoyl]-[2,5,6-^{2}H]-3-hydroxy-L-tyrosine, ***d_3-7****:* 0.25 mmol; 25% yield (**Figure 3**); UV$_{max}$ = 225, 255, 297 nm; LC-MS (ESI$^+$), *m/z* 1407 (100%, [4M + Na]$^+$), 1077 (85%, [3M + K]$^+$), 731 (46%, [2M + K]$^+$), 1423 (44%, [4M + K]$^+$), 147 (23%, [M − 200]$^+$), 347 (15%, [M + 1]$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.73 [dd, 1H, J = 8.7, 13.8 Hz, H−C(7a)], 2.91 [dd, 1H, J = 5.1, 13.8 Hz, H−C(7b)], 4.41 [m, 1H, H−C(8)], 6.49 [d, 1H, J = 15.7 Hz, H−C(8′)], 6.78 [d, 2H, J = 8.6 Hz, H−C(3′, 5′)], 7.27 [d, 1H, J = 15.7 Hz, H−C(7′)], 7.38 [d, 2H, J = 8.6 Hz, H−C(2′, 6′)], 8.06 [d, 1H, J = 8.0 Hz, H−N], 8.62, 8.67, 9.82 [3s, 3OH, HO−C].

N-[4′-Hydroxy-(E)-cinnamoyl]-[2,3,5,6-^{2}H]-L-tyrosine, ***d_4-8****:* 0.33 mmol; 33% yield (**Figure 3**); UV$_{max}$ = 224, 299, 309 nm; LC-MS (ESI$^+$), *m/z* 332 (100%, [M + 1]$^+$), 354 (38%, [M + Na]$^+$), 663 (25%, [2M + 1]$^+$), 685 (18%, [2M + Na]$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 2.83 [dd, 1H, J = 9.2, 14.0 Hz, H−C(7a)], 3.01 [dd, 1H, J = 4.8, 14.0 Hz, H−C(7b)], 4.51 [m, 1H, J = 4.8, 8.4, 8.8, 9.6, 13.2 Hz, H−C(8)], 6.52 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.82 [d, 1H, J = 8.8 Hz, H−C(3′, 5′)], 7.33 [d, 1H, J = 15.6 Hz, H−C(7′)], 7.42 [d, 1H, J = 8.4 Hz, H−C(2′, 6′)], 8.13 [d, 1H, J = 8.4 Hz, H−N], 9.23 [s, 1OH, HO−C(4)], 9.89 [s, 1OH, HO−C(4′)], 12.72 [s, 1OH, HOOC-(9)].

N-[3′,4′-Dihydroxy-(E)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan, ***d_5-11****:* 0.27 mmol; 27% yield (**Figure 3**); UV$_{max}$ = 221, 290, 323 nm; LC-MS (ESI$^+$), *m/z* 372 (100%, [M + 1]$^+$), 394 (49%, [M + Na]$^+$), 765 (33%, [2M + Na]$^+$), 743 (28%, [2M + 1]$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 3.10 [dd, 1H, J = 8.8, 14.4 Hz, H−C(9a)], 3.25 [dd, 1H, J = 4.8, 14.4 Hz, H−C(9b)], 4.64 [m, 1H, J = 4.8, 8.0, 13.2 Hz, H−C(10)], 6.47 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.78 [d, 1H, J = 8.0 Hz, H−C(5′)], 6.86 [dd, 1H, J = 1.6, 8.0 Hz, H−C(6′)], 6.97 [d, 1H, J = 1.6 Hz, H−C(2′)], 7.25 [d, 1H, J = 15.6 Hz, H−C(7′)], 8.29 [d, 1H, J = 8.0 Hz, H−N], 9.18 [s, 1OH, HO−C(3′)], 9.42 [s, 1OH, HO−C(4′)], 10.80 [s, 1H, H−N(indole)], 12.70 [s, 1OH, HOOC(11)].

N-[4′-Hydroxy-(E)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan, ***d_5-12****:* 0.43 mmol; 43% yield (**Figure 3**); UV$_{max}$ = 219, 292, 307 nm; LC-MS (ESI$^+$), *m/z* 356 (100%, [M + 1]$^+$), 378 (32%, [M + Na]$^+$), 711 (18%, [2M + 1]$^+$); ^{1}H NMR (400 MHz, DMSO-d_6, COSY) δ 3.09 [dd, 1H, J = 8.6, 14.6 Hz, H−C(9a)], 3.26 [dd, 1H, J = 5.0, 14.6 Hz, H−C(9b)], 4.64 [m, 1H, J = 5.0, 8.6, 13.2 Hz, H−C(10)], 6.55 [d, 1H, J = 16.0 Hz, H−C(8′)], 6.82 [d, 2H, J = 8.4 Hz, H−C(3′, 5′)], 7.33 [d, 1H, J = 16.0 Hz, H−C(7′)], 7.41 [d, 2H, J = 8.4 Hz, H−C(2′, 6′)], 8.26 [d, 1H, J = 7.6 Hz, H−N], 9.89 [s, 1OH, HO−C(4′)], 10.87 [s, 1H, H−N(indole)], 12.72 [s, 1OH, HOOC(11)].

N-[4′-Hydroxy-3′-methoxy-(E)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryp-

*tophan, **d$_5$-13**:* 0.26 mmol; 26% yield (**Figure 3**); UV_{max} = 229, 291, 322 nm; LC-MS (ESI^+), *m/z* 386 (100%, $[M + 1]^+$), 408 (38%, $[M + Na]^+$), 771 (23%, $[2M + 1]^+$), 793 (12%, $[2M + Na]^+$); ^1H NMR (400 MHz, DMSO-d_6, COSY) δ 3.11 [dd, 1H, J = 8.6, 14.6 Hz, H−C(9a)], 3.27 [dd, 1H, J = 5.0, 14.6 Hz, H−C(9b)], 3.84 [s, 3H, H−C(10′)], 4.65 [m, 1H, J = 5.0, 8.0, 8.6, 13.2 Hz, H−C(10)], 6.59 [d, 1H, J = 16.0 Hz, H−C(8′)], 6.82 [d, 1H, J = 8.0 Hz, H−C(5′)], 7.03 [dd, 1H, J = 1.6, 8.0 Hz, H−C(6′)], 7.15 [d, 1H, J = 1.6 Hz, H−C(2′)], 7.32 [d, 1H, J = 16.0 Hz, H−C(7′)], 8.20 [d, 1H, J = 8.0 Hz, H−N], 9.49 [s, 1OH, HO−C(4′)], 10.87 [s, 1H, H−N(indole)], 12.77 [s, 1OH, HOOC(11)].

Quantitative Analysis of *N*-Phenylpropenoyl Amino Acids in Roasted Cocoa and Coffee. *Roasted Cocoa.* Cocoa powder (5.0 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$_5$-11**, and **d$_5$-12** in methanol (1.0 mg/mL), and the mixture was homogenized in a laboratory shaker for 30 min. After equilibration, the cocoa powder was extracted with *n*-pentane (5 × 30 mL) at room temperature for 30 min. The residual cocoa material was then extracted five times with acetone/water (70:30, v/v; 30 mL each) for 45 min at room temperature with 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 (~40 mg) of the acetone/water extract were taken up in a methanol/water mixture (1:1, v/v; 10 mL), which was acidified 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 150 × 2 mm i.d., 4 μm, RP-18 Synergi Fusion 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) for 5 min; the methanol content was increased 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**, and **d$_5$-11**−**d$_5$-13** were used as the internal standards for the quantitative analysis of the corresponding analytes. In addition, compound **2** was quantified via **d$_3$-1**, compounds **6**, **9**, and **10** were quantified via **d$_3$-5**, and compound **14** was quantified via **d$_3$-8** as the internal standard. The amounts of the individual *N*-phenylpropenoyl amino acids were calculated using the response factors (given in parentheses) 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** (0.95), **11** (0.95), **12** (0.96), **13** (0.91), and **14** (1.00), 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.

Roasted Coffee. Roast coffee beans were frozen in liquid nitrogen, ground in a mill, and passed through a sieve (pore size = 0.5 mm). Coffee powder (5.4 g) was spiked with the labeled internal standards **d$_3$-1**, **d$_4$-4**, **d$_3$-5**, **d$_4$-8**, and **d$_5$-11**−**d$_5$-13** in methanol (1.0 mg/mL), and the mixture was homogenized by means of a laboratory shaker for 30 min. After equilibration, the coffee powder was percolated with hot water (110 mL) using a no. 4 cellulose filter (Melitta), and the brew obtained (100 mL) was analyzed by means of LC-MS/MS as described above.

Recovery. Aliquots (5.0 g) of cocoa powder were spiked with known amounts of a mixture of selected *N*-phenylpropenoyl amino acids (**1**−**9**, **11**, **12**, **14**) dissolved in methanol. After stirring and equilibration for 30 min, the quantitative analysis of the *N*-phenylpropenoyl amino acids was done as detailed above. As the basis for the calculation of the recovery rate, the content of the *N*-phenylpropenoyl amino acids in cocoa powder, which was not spiked with additional *N*-phenylpropenoyl amino acids (control), was determined as the mean of triplicates.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a PU 1580 HPLC pump system with a DG-1580-53 in-line degasser, an LG-1580-02 low-pressure gradient unit, and an MD 1515 diode array detector (DAD). Chromatographic separations were performed on stainless steel columns packed with ODS-Hypersil, 5 μm (ThermoHypersil, Kleinostheim, Germany) in either an analytical (250 × 4.6 mm i.d., flow rate = 0.8 mL/min) or a semipreparative scale (250 × 10 mm i.d., flow rate = 3.0 mL/min).

Liquid Chromatography−Mass Spectrometry (LC-MS/MS). LC-MS/MS analysis was performed using an Agilent 1100 HPLC system connected to the API 3200 LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the positive electrospray ionization mode. By means of the selected reaction monitoring (SRM) mode, the individual *N*-phenylpropenoyl amino acids were analyzed using the following mass transitions given in parentheses: **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), **11** (*m/z* 367.3→163.0), **d$_5$-11** (*m/z* 372.3→163.0), **12** (*m/z* 351.3→147.0), **d$_5$-12** (*m/z* 356.3→147.0), **13** (*m/z* 381.3→177.0), **d$_5$-13** (*m/z* 386.3→177.0), **14** (*m/z* 358.3→177.0).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^1H, ^{13}C, DEPT-135, COSY, HMQC, and HMBC measurements were performed on an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). Evaluation of the experiments was carried out using 1D- and 2D-WIN-NMR (version 6.1) as well as XWin-NMR software (version 3.5; Bruker). Tetramethylsilane was used as the internal standard.

RESULTS AND DISCUSSION

Recent investigations revealed the *N*-phenylpropenoyl-L-amino acids **1**−**10** (**Figure 1**) as the key compounds contributing to the astringent taste of roasted cocoa nibs (*2*, *3*). In addition, the structurally related *N*-phenylpropenoyl-L-tryptophans **11**−**13**, as well as *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (**14**), have been detected in coffee beans (*12*−*15*). To investigate the natural occurrence of all these compounds in roasted cocoa and coffee, reference compounds needed to be synthesized for compounds **11**−**14** (**Figure 1**). Closely following the procedure reported recently for the synthesis of the *N*-phenylpropenoyl-L-amino acids **1**−**10** (*3*), compounds **11**−**14** were prepared starting from (*E*)-caffeic acid, (*E*)-*p*-coumaric acid, or (*E*)-ferulic acid and L-tryptophan or L-tyrosine, respectively.

Identification of *N*-Phenylpropenoyl-L-Amino Acids in Cocoa and Coffee. To identify the *N*-phenylpropenoyl-L-amino acids **1**−**14** in cocoa and coffee, an aqueous acetone extract isolated from cocoa powder as well as an aqueous brew prepared from roasted ground coffee, respectively, was analyzed by RP-HPLC coupled to an LC-MS/MS instrument running in the positive electrospray ionization mode. By means of the SRM mode, the mass transitions *m/z* 296.3→163.0 (**1**), *m/z* 310.3→163.0 (**2**), *m/z* 360.3→163.0 (**3**), *m/z* 344.3→163.0 (**4**), *m/z* 280.3→147.0 (**5**), *m/z* 294.3→147.0 (**6**), *m/z* 344.3→147.0 (**7**), *m/z* 328.3→147.0 (**8**), *m/z* 310.3→177.0 (**9**), *m/z* 264.3→131.0 (**10**), *m/z* 367.3→163.0 (**11**), *m/z* 351.3→147.0 (**12**), *m/z* 381.3→177.0 (**13**), and *m/z* 358.3→177.0 (**14**) were recorded for the individual *N*-phenylpropenoyl-L-amino acids.

Analysis of the cocoa sample demonstrated for the first time that, besides the previously reported compounds **1**−**10**, also *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophan (**11**), *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tryptophan (**12**), and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tyrosine (**14**) are naturally occurring in cocoa. These data were further confirmed by comparing the mass spectra and retention times (RP-HPLC) with those obtained for the synthetic reference compounds as well as by cochromatography. In contrast, *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tryptophan (**13**) was not detectable in significant amounts.

LC-MS/MS analysis of the coffee brew revealed besides the previously reported compounds **4**, **8**, and **11**−**13**, the L-aspartic acid derivatives **1** and **5** as phytochemicals in coffee. These data were further confirmed by comparing the mass spectra and retention times (RP-HPLC) with those obtained for the synthetic reference compounds as well as by cochromatography.

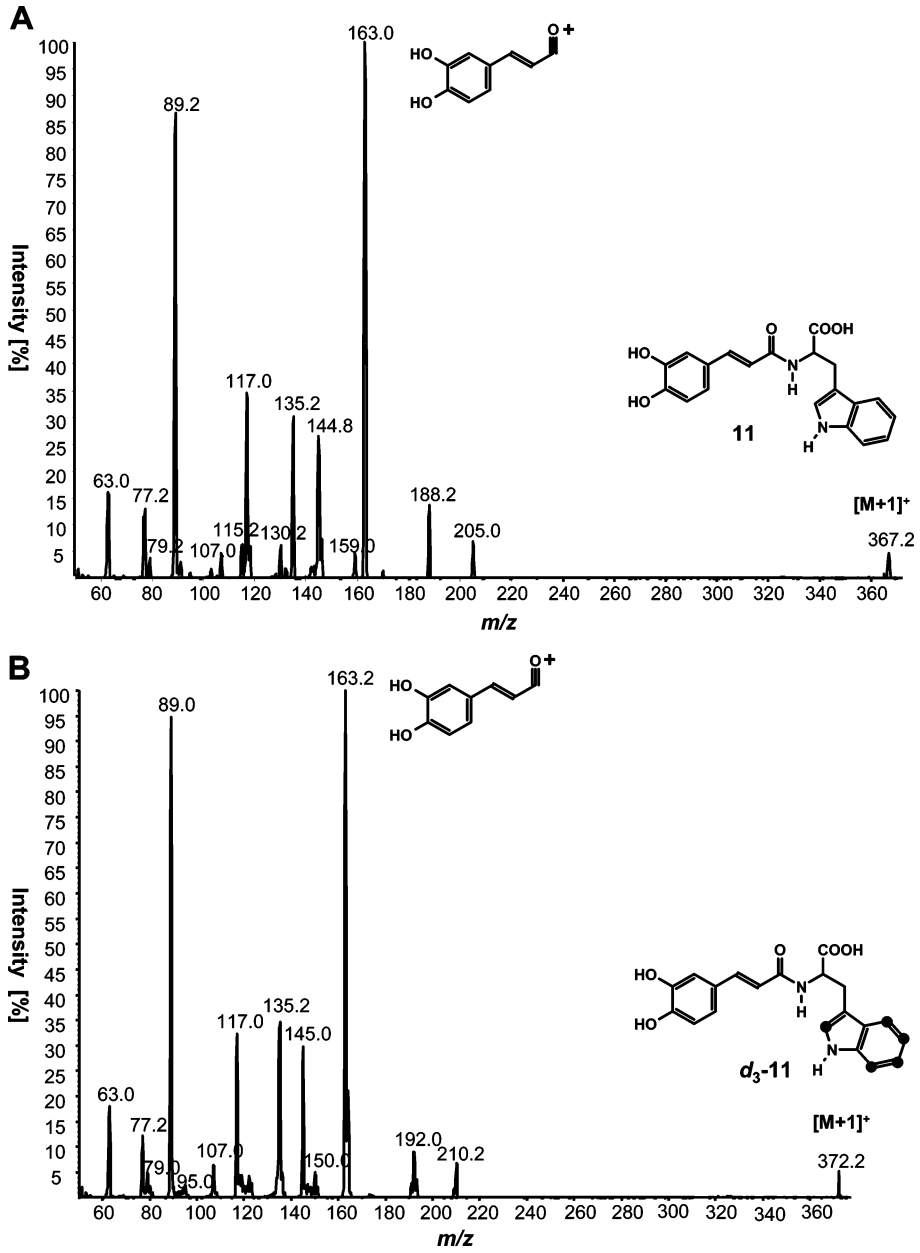

Figure 4. MS/MS (ESI+) spectrum of (**A**) *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-[1,4,5,6,7-H]-L-tryptophan (**11**) and (**B**) *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan (**d_5-11**).

Synthesis of Labeled *N*-Phenylpropenoyl-L-Amino Acids. To accurately determine the amounts of *N*-phenylpropenoyl-L-amino acids in roasted coffee beverage and cocoa nibs, a SIDA should be developed in the following. To achieve this, first, corresponding labeled internal standards needed to be synthesized.

Exemplified for *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan (**d_5-11**), the synthetic sequence used for the preparation of deuterium-labeled *N*-phenylpropenoyl-L-amino acids is outlined in **Figure 2**. (*E*)-Caffeic acid was acetylated with acetic anhydride in pyridine and (dimethylamino)pyridine, converted into the corresponding chloride using thionyl chloride, and, finally, reacted with [1,4,5,6,7-^{2}H]-L-tryptophan in dry tetrahydrofuran. After K_2CO_3-catalyzed cleavage of the protecting groups and Amberlite treatment, the title compound was isolated by column chromatography on RP-18 material, followed by a final HPLC purification yielding *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan (**d_5-11**) as a white, amorphous powder in high purities of >99%. Following the same procedure, the labeled *N*-phenylpropenoyl-L-amino acids **d_3-1**, **d_3-3**, **d_4-4**, **d_3-5**, **d_3-7**, **d_4-8**, **d_5-12**, and **d_5-13** were synthesized (**Figure 3**). When compared to *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tryptophan (**11**), the MS/MS spectrum of *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan (**d_5-11**) measured in the ESI$^+$ mode revealed an increase of the pseudomolecular ion by 5 units, thus reflecting the incorporation of the five deuterium atoms in **d_5-11** (**Figure 4**). In addition, the daugther ion *m*/*z* 163 corresponding to caffeic acid was observed as the base peak. Similarily, the 4′-hydroxy-(*E*)-cinnamoyl and 4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl amino acids showed the fragments *m*/*z* 147 and 177 for *p*-coumaric acid and ferulic acid, respectively, as the corresponding base ions.

In addition, the incorporation of the deuterium atoms into the target molecules was confirmed by means of ^{1}H NMR spectroscopy. For example, *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tryptophan (**13**) showed 10 olefinic resonance signals each integrating for one proton (**Figure 5A**). In

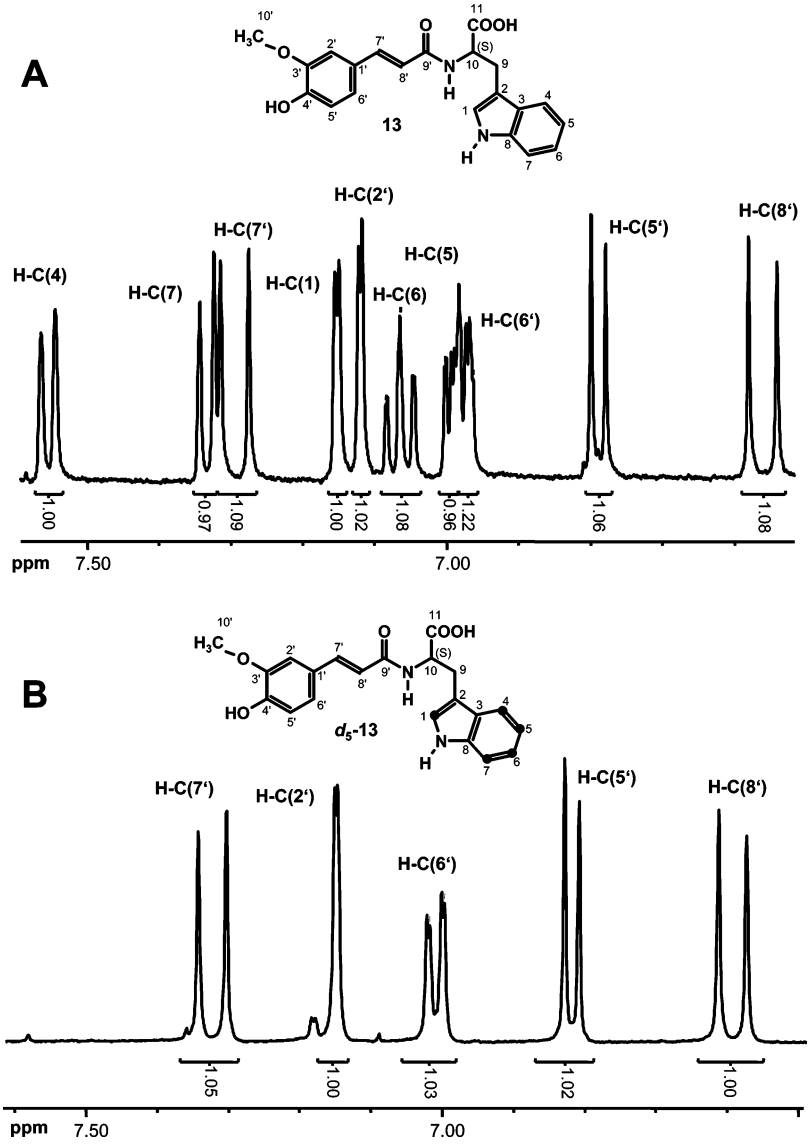

Figure 5. Excerpt of the ^{1}H NMR spectra (400 MHz; DMSO-d_6) of (**A**) *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-L-tryptophan (**13**) and (**B**) *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan (**d_5-13**).

comparison, the same segment of the ^{1}H NMR spectrum of *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-[1,4,5,6,7-^{2}H]-L-tryptophan (**d_5-13**) showed only five signals, each integrating for one proton as part of the ferulic acid moiety (**Figure 5B**). Taking all of the NMR and MS data into account, it was proven that no significant deuterium−protium exchange occurred during their chemical synthesis.

To check the stability of the labeled compounds under aqueous acidic conditions, the deuterated compounds were individually incubated in H_2O acidified with HCl to pH 4.0 for 4 h at room temperature and, after lyophylization, were then analyzed by means of ^{1}H NMR spectroscopy. However, the aromatic protons H−C(1) and H−C(4)−H−C(7) were still not observable for the labeled compound (data not shown), thus demonstrating the lack of any significant D/H exchange under workup conditions and confirming the suitability of the labeled compounds as internal standards for the SIDA.

Development of a SIDA and Quantitation of *N*-Phenylpropenoyl-L-Amino Acids. To convert the measured ion intensities into the mass ratios of labeled and nonlabeled *N*-phenylpropenyl-L-amino acids, a graph was calculated from calibration mixtures of known mass ratios and the corresponding peak area ratios in LC-MS/MS. Good linearity was found for mass ratios ranging from 0.2 to 5.0.

For the quantitative analysis of the *N*-phenylpropenoyl-L-amino acids by means of a SIDA, cocoa powder or ground roasted coffee was dosed with defined amounts of **d_3-1**, **d_3-3**, **d_4-4**, **d_3-5**, **d_3-7**, **d_4-8**, **d_5-11**, **d_5-12**, and **d_5-13** as the internal standards, followed by homogenization and equilibration at room temperature. After isolation of the *N*-phenylpropenoyl-L-amino acids from cocoa or coffee by acetone/water extraction or hot water percolation, respectively, mass chromatography was performed by analytical RP-HPLC-MS/MS as shown in **Figure 6**.

To check the accuracy of the analytical method, recovery experiments were performed (**Table 1**). To achieve this, synthetic reference material of the *N*-phenylpropenoyl-L-amino acids **1**−**9**, **11**, **12**, and **14** was added to cocoa powder in three different concentrations prior to quantitative analysis, and the amounts determined after cleanup were compared with those found in the blank cocoa sample (control). The recovery rates, calculated on the basis of the content of each *N*-phenylpropenoyl-L-amino acid added to the cocoa powder prior to cleanup, were found to range between 95 and 102% (**Table 1**). These

Table 1. Determination of the Recovery Rates for the Quantitative Analysis of *N*-Phenylpropenoyl-L-amino Acids in Cocoa Powder

compd no.	amount added (μg/g)	concn calcd (μg/g)	concn determined (μg/g)	recovery (%)
1			428.43	
	47.0	475.43	463.83	97.6
	94.0	522.43	526.21	100.7
	188.0	616.43	588.59	95.5
2			5.55	
	42.0	47.55	47.88	100.7
	84.0	89.55	88.71	99.1
	168.0	173.55	166.39	95.9
3			58.58	
	49.0	107.58	108.70	101.0
	98.0	156.58	159.08	101.6
	196.0	254.58	250.80	98.5
4			19.07	
	47.0	66.07	65.09	98.5
	94.0	113.07	111.17	98.3
	188.0	207.07	207.93	100.4
5			120.42	
	44.0	164.42	158.31	96.3
	88.0	208.42	208.88	100.2
	176.0	296.42	286.22	96.6
6			3.28	
	50.0	53.28	53.85	101.1
	100.0	103.28	103.63	100.3
	200.0	203.28	206.13	101.4
7			9.04	
	50.0	59.04	58.43	99.0
	100.0	109.04	106.96	98.1
	200.0	209.04	213.96	102.4
8			55.37	
	56.0	111.37	108.04	97.0
	112.0	167.37	163.44	97.7
	224.0	279.37	271.31	97.1
9			18.8	
	46.0	64.80	62.24	96.1
	92.0	110.8	111.64	100.8
	184.0	202.80	201.25	99.2
11			0.45	
	46.0	46.45	45.98	99.0
	92.0	92.45	90.94	98.4
	184.0	184.45	183.94	99.7
12			0.39	
	45.0	45.39	46.18	101.7
	90.0	90.39	88.55	98.0
	180.0	180.39	176.20	97.7
14			1.11	
	59.0	60.11	61.74	102.7
	118.0	119.11	116.86	98.1
	236.0	237.11	230.63	97.3

data clearly demonstrate the developed SIDA as a reliable tool enabling a rapid and accurate quantitative determination of *N*-phenylpropenoyl-L-amino acid concentration in foods.

Next, the concentrations of *N*-phenylpropenoyl-L-amino acids in roasted cocoa powder and a roasted decaffeinated coffee as well as a roasted regular coffee were quantitatively determined. As given in **Table 2**, independent of the coffee sample analyzed, *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl)-L-tryptophan (**11**) was found as the quantitatively predominating *N*-phenylpropenoyl-L-amino acid, accounting for up to 84% of the total amino acid amides in both of the coffee samples. In contrast, all of the other *N*-phenylpropenoyl-L-amino acids were present in significantly smaller amounts ranging between 0.01 and 0.55 mg/kg (**Table 2**). It is interesting to note that the Arabica Columbia showed higher concentrations for *N*-phenylpropenoyl-L-tryptophan and

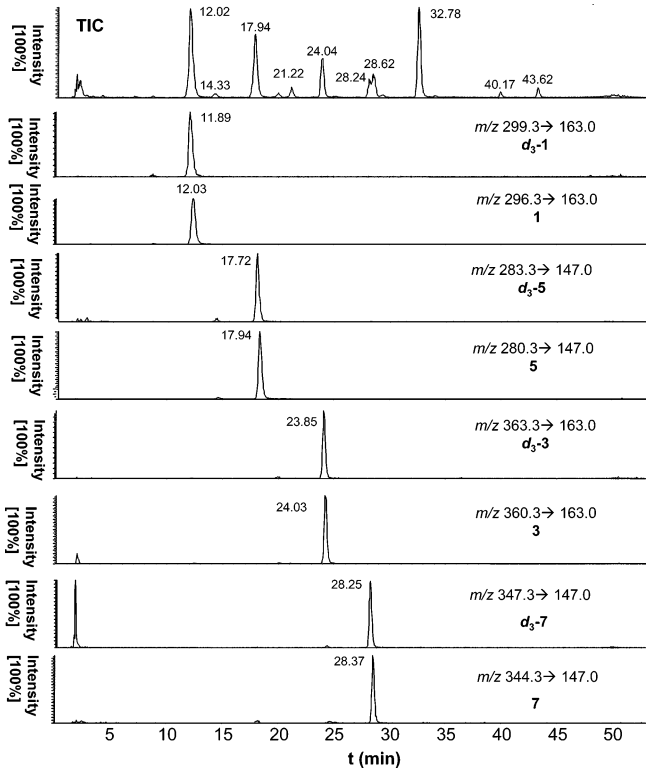

Figure 6. MS/MS chromatograms for the quantitative analysis of the *N*-phenylpropenoyl amino acids **1**, **3**, **5**, and **7** in cocoa powder via the internal standards **d_3-1**, **d_3-3**, **d_3-5**, and **d_3-7** by using the SRM mode.

Table 2. Concentrations of *N*-Phenylpropenoyl-L-amino Acids in Cocoa Powder and Coffee

	concn[a] (mg/kg) in		
compd no.	cocoa nibs	coffee[b]	decaffeinated coffee[c]
1	428.43 ± 10.11[d]	0.04	0.03
2	5.55 ± 0.32[d]	nd[e]	nd
3	58.58 ± 1.12[d]	nd	nd
4	19.07 ± 0.54[d]	0.10	0.13
5	120.42 ± 1.18[d]	0.01	0.01
6	3.28 ± 0.91[d]	nd	nd
7	9.04 ± 0.23[d]	nd	nd
8	55.37 ± 0.62[d]	0.01	0.05
9	18.80 ± 0.91[d]	nd	nd
10	2.26 ± 0.51[d]	nd	nd
11	0.45 ± 0.06[d]	3.32	3.11
12	0.39 ± 0.1[d]	0.55	0.32
13	nd	0.04	0.03
14	1.11 ± 0.04[d]	nd	nd

[a] Concentrations are given as the mean of triplicates. [b] Arabica Columbia. [c] Decaffeinated coffee consisted of 70% Arabica and 30% Robusta. [d] Standard deviation. [e] Not detectable.

aspartic acid, respectively, whereas in the decaffeinated coffee comparatively higher amounts of *N*-phenylpropenoyl-tyrosines were detectable (**Table 2**).

In comparison to the coffee samples, by far the highest concentrations of 428.43 and 120.42 mg/kg in cocoa were found for *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**1**) and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid (**5**). With somewhat lower amounts of 55.37 and 58.58 mg/kg *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine (**3**) and *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine (**8**), both of which have been reported earlier as cocoa antioxidants (*7*), were determined in cocoa, whereas all of the other *N*-phenylpropenoyl-L-amino acids

were present in amounts below 19.07 mg/kg. In particular, the tryptophan derivates were found as trace constituents in cocoa ranging from 0.39 to 0.45 mg/kg (**Table 2**).

In summary, a versatile analytical method enabling an accurate quantitative analysis of biologically active *N*-phenylpropenoyl-L-amino acids in foods such as cocoa and coffee by means of SIDA using RP-HPLC-MS/MS was developed. This sophisticated and reliable quantification method is the scientific basis to evaluate the contribution of these phytochemicals to the astringent taste of foods and natural plant materials such as coffee and cocoa and offers the possibility to bridge the gap between the antioxidant activity of cocoa and coffee products and their amounts of individual *N*-phenylpropenoyl-L-amino acids.

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