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Quantitation of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides in Coffee by Means of LC-MS/MS-SIDA and Assessment of Their Gastric Acid Secretion Potential Using the HGT-1 Cell Assay

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A straightforward stable isotope dilution analysis (SIDA) for the reliable quantitative determination of $^{\beta}N$-$C_{18:0}$- to $^{\beta}N$-$C_{24:0}$-alkanoyl-5-hydroxytryptamides (C5HTs) in coffee powder and beverages by means of LC-MS/MS was developed. The developed SIDA showing accuracy values of 92.6−107% and precision between 0.5 and 7% relative standard deviation for the individual derivatives allowed the sensitive and selective quantification of the target compounds in coffee beverages. Depending on the type of coffee, quantitation revealed C5HT levels between 65 and 144 μg/L in filtered coffee and up to 3500 μg/L in a French press beverage, thus indicating that about 0.3 or 7.2% of the C5HTs were extracted from the coffee powder into the beverage when using the cellulose filter method or the French press, respectively. To estimate the potential contribution of the C5HTs to the phenomenon of stomach irritation after ingestion of coffee brew, *in vitro* cell studies were performed with pure individual 5-hydroxytryptamides and a mixture of the predominating derivatives in ratios matching those found in coffee. All substances tested induced a decrease in the intracellular proton index (IPX) coined as an indicator of stomach acid secretion. While the biomimetic C5HT mixture was highest in its inducing effect, the individual stearic acid, oleic acid, and linoleic acid 5-hydroxytryptamide did not differ significantly from each other, but showed a less pronounced effect compared to arachinic acid 5-hydroxytryptamide. In conclusion, not the grade of saturation seems to determine the C5HT's mode of action in driving the stomach acid secretion, rather than the fatty acid chain length.

KEYWORDS: **$^{\beta}N$-Alkanoyl-5-hydroxytryptamide; coffee; stable isotope dilution analysis; intracellular proton index; HGT-1 cells; stomach irritation**

INTRODUCTION

Besides its pleasing overall aroma and its typical taste, a freshly prepared coffee brew is appreciated by the consumer for its stimulatory effect. However, the number of consumers experiencing undesirable physiological symptoms, for example, stomach irritation or gastroesophageal reflux after ingestion is increasing (*1*−*6*). As a consequence, these sensitive individuals have to avoid coffee consumption completely. Induced by this observation, developments were focused on making coffee more "stomach-friendly". Due to their less stomach irritating potential, the consumption of decaffeinated and/or steam-treated coffees is increasing and currently accounts for a total of about 18% of the roast coffee market. Although the nature of the coffee constituents inducing gastric acid secretion after coffee ingestion is not completely defined on a molecular level, compounds generated upon coffee bean roasting such as di- and trihydroxybenzenes are discussed to be one of the factors causing stomach discomfort after coffee consumption (*7*, *8*). The observation that beverages prepared from steam-treated or dewaxed coffee beans produce less stomach irritations put substances in the waxy layer of coffee beans into focus of research. A considerable amount of the coffee wax was found to consist of the $^{\beta}N$-alkanoyl-5-hydroxytryptamides $C_{18:0}$-5HT to $C_{24:0}$-5HT (**Figure 1**), the fatty acid amides of serotonin (*9*−*11*, *16*−*18*).

Since the content of $^{\beta}N$-alkanoyl-5-hydroxytryptamides is decreased by both the steam treatment used for preparation of "stomach-friendly" coffee and the solvent-assisted decaffeination process (*9*−*16*), these lipophilic amides were supposed to cause phenomena of dyspepsia and gastrooesophageal reflux. Feeding

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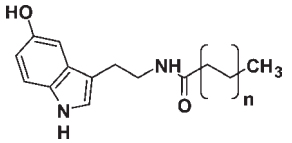

Figure 1. Chemical structure of $^{\beta}N$-alkanoyl-5-hydroxytryptamides: n = 16, $^{\beta}N$-stearoyl-5-hydroxytryptamide ($C_{18:0}$-5HT); n = 18, $^{\beta}N$-arachinoyl-5-hydroxytryptamide ($C_{20:0}$-5HT); n = 19, $^{\beta}N$-heneicosanoyl-5-hydroxytryptamide ($C_{21:0}$-5HT); n = 20, $^{\beta}N$-behenoyl-5-hydroxytryptamide ($C_{22:0}$-5HT); n = 21, $^{\beta}N$-tricosanoyl-5-hydroxytryptamide ($C_{23:0}$-5HT); and n = 22, $^{\beta}N$-lignoceroyl-5-hydroxytryptamide ($C_{24:0}$-5HT).

experiments on rats showed evidence that high doses of C5HTs do have a degenerative effect on the gastric mucosa, thus strengthening the suggested stomach irritating potential of these compounds (*19*). Since these C5HTs are suspected to account for stomach irritations experienced by sensitive subjects after coffee consumption and are reported to cause gastric lesions, ulcerogenic effects, and undesirable physiological effects on bile and liver (*14*, *15*, *18*), there is an increasing interest in the exact concentrations of these substances in coffee and coffee products.

Aimed at using the C5HTs as analytical indicators for the steam-treatment in manufacturing of mild and stomach-friendly coffee (*9*−*13*), analytical procedures have been developed for their quantification. However, laborious cleanup procedures are necessary to isolate and purify the C5HTs prior to analysis (*9*, *12*). For example, the C5HTs have been first extracted from coffee beans by means of solvent extraction, purified by column chromatography and thin layer chromatography, and finally analyzed by means of UV−vis spectrometry after derivatization with Gibb's reagent (*9*−*12*, *20*−*22*). Besides thin layer chromatography, normal phase HPLC-UV (*23*, *24*) as well as reversed phased HPLC, either coupled to a UV−vis detector (*18*, *25*, *26*) or a fluorescence detector (*17*, *27*, *28*), have been used to determine the C5HT content in methanol extracts of coffee beans. However, the observation that the C5HTs exhibit rather low water solubility induced severe doubts on whether significant amounts of these compounds are extracted from the coffee powder upon preparing the aqueous brew using standard household procedures (*12*, *16*). In contrast to the information available on raw and roasted ground coffee, there are mainly contradictory data on the levels of C5HTs in coffee brews prepared by the common methods. For example, some groups were not able to detect any trace amounts of C5HTs in filtered coffee beverages (*16*), whereas others reported amounts of about 1 mg/L in rather concentrated brews prepared without using any paper filter (*27*).

The objective of the present investigation was, therefore, to develop a sensitive HPLC-MS/MS method enabling the accurate and reliable quantitative analysis of $^{\beta}N$-alkanoyl-5-hydroxytryptamides in coffee beverages by means of a stable isotope dilution analysis, and to evaluate the activity of these compounds on gastric acid (GA) secretion by using an *in vitro* HGT-1 cell assay, which has recently been developed and successfully applied to assess the GA secretion induced by coffee beverages (*29*).

MATERIALS AND METHODS

Chemicals and Materials. The following compounds were obtained commercially: tris(triphenylphosphin)-rhodium(I)-chloride, nervonic acid (Fluka AG, Buchs, Germany); stearic acid, arachinic acid, behenic acid, lignocerinic acid, oleic acid, erucic acid, 11-*cis*-eicosenic acid, oleoyl chloride, linoloyl chloride (Sigma-Aldrich, Steinheim, Germany); serotonin hydrochloride (Alfa Aesar GmbH & Co. KG, Karlsruhe, Germany); triethylamin, *N*,*N*-dimethylformamide (DMF), dichloromethane, tetrahydrofuran (THF), methanol, pentane, petroleum ether (40−60 °C), diethylether, silica gel (70 − 230 mesh), formic acid, thionylchloride (Merck KGaA, Darmstadt, Germany); carboxy-SNARF-AM (Invitrogen, Karlsruhe, Germany); nigericin, Dulbecco's modified Eagle's medium, fetal calf serum, L-glutamine, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, accutase, trypan blue (PAA, Coelbe, Germany). Deuterium (99.999) was supplied by Air Liquide (Düsseldorf, Germany). Methanol was of HPLC grade, and water was Millipore grade. Deuterated solvents were supplied by Euriso-Top (Gif-sûr-Yvette, France). The human parietal carcinoma cell line HGT-1 was obtained from Dr. C. Laboisse (Laboratory of Pathological Anatomy, Nantes France). Coffee beans, Arabica Brazil and Robusta Vietnam, were provided by the German coffee industry. Aliquots of the Arabica Brazil coffee was steam-treated (2 bar) for 60 min at 110−120 °C, and an aliquot of the Robusta Vietnam was steam-treated (5.5 bar) for 60 min at 130−150 °C. Coffee powders had a mean particle size of 465 ± 35 μm.

Synthesis of d_2-Labeled Fatty Acids. Using the procedure reported earlier (*30*) with some modifications, the catalyst tris(triphenylphosphine)-rhodium(I)-chloride (0.2 mmol) in dichloromethane (10 mL) was injected through a septum into a three-necked, brown glass round-bottom flask equipped with a gas balloon containing deuterium gas. After stirring until the color of the solution turned from deep red to yellow, a solution of either oleic acid (2.0 mmol), 11-*cis*-eicosenic acid (2.0 mmol), erucic acid (2.0 mmol), or nervonic acid (0.27 mmol) in dry dichloromethane (10 mL) was added by injection through the septum. After 12 and 36 h of stirring, another aliquot of the catalyst (0.2 mmol, in dichloromethane) as well as another portion of deuterium were added. After a total reaction time of 48 h, silica gel (5 g) was added and the suspension was freed from solvent in a vacuum. The residue was placed onto the top of a water-cooled glass column (55 × 2 cm) filled with a slurry of silica gel in pentane. After flushing the column with pentane (200 mL), pentane/diethyl ether (90/10, v/v; 2 × 200 mL), and pentane/diethyl ether (80/20, v/v; 2 × 200 mL), the target compounds were eluted with a mixture (80/20/0.1, v/v/v; 5 × 200 mL) of pentane, diethyl ether, and formic acid. This fraction was freed from solvents in vacuum, and the residue was rechromatographed using the same conditions as given above to afford the d_2-labeled fatty acids as white needles in a purity of more than 99% (GC-FID). The incorporation of the deuterium atoms was confirmed by comparing the MS and ^{1}H NMR data with those obtained for the corresponding nonlabeled isotopologues.

d_2-Stearic Acid. Yield: 1.48 mmol, 74%. MS(ESI)$^-$: m/z (%) 331.1 (100, [M + HCOO]$^-$), 285.1 (90, [M − H]$^-$). ^{1}H NMR ($CDCl_3$): δ/ppm 0.88 (t, 3H, J = 6.8 Hz, H−C(18)), 1.17−1.39 (m, 13 × 2H, H−C(4−17)), 1.64 (m, 2H, J = 7.2 Hz, H−C(3)), 2.37 (t, 2H, J = 7.2 Hz, H−C(2)).

d_2-Arachinic Acid. Yield: 1.89 mmol, 95%. MS(ESI)$^-$: m/z (%) 359.3 (100, [M + HCOO]$^-$). ^{1}H NMR ($CDCl_3$): δ/ppm 0.88 (t, 3H, J = 6.8 Hz, H−C(20)), 1.18−1.38 (m, 15 × 2H, H−C(4−19)), 1.63 (m, 2H, J = 7.2 Hz, H−C(3)), 2.35 (t, 2H, J = 7.2 Hz, H−C(2)).

d_2-Behenic Acid. Yield: 1.69 mmol, 85%. MS(ESI)$^-$: m/z (%) 387.5, (100, [M + HCOO]$^-$). ^{1}H NMR ($CDCl_3$): δ/ppm 0.88 (t, 3H, J = 6.8 Hz, H−C(22)), 1.18−1.38 (m, 17 × 2H, H−C(4−21)), 1.63 (m, 2H, J = 7.2 Hz, H−C(3)), 2.35 (t, 2H, J = 7.2 Hz, H−C(2)).

d_2-Lignoceric Acid. Yield: 0.24 mmol, 89%. MS(ESI)$^-$: m/z (%) 415.5, (100, [M + HCOO]$^-$). ^{1}H NMR ($CDCl_3$): δ/ppm 0.88 (t, 3H, J = 6.8 Hz, H−C(24)), 1.18−1.37 (m, 19 × 2H, H−C(4−23)), 1.63 (m, 2H, J = 7.2 Hz, H−C(3)), 2.35 (t, 2H, J = 7.2 Hz, H−C(2)).

Synthesis of d_2-Labeled $^{\beta}N$-Alkanoyl-5-hydroxytryptamides. A solution of the corresponding d_2-labeled fatty acid (0.5 mmol) in thionylchloride (3 mL) was refluxed for 1 h, the excess of thionylchloride was removed in vacuum, and the residue was taken up in dried dichloromethane (5 mL) and added dropwise to a solution of serotonin hydrochloride (0.75 mmol) and triethylamine (1.5 mmol) in dried DMF (5 mL). After stirring for 4 h at 45 °C, the solution was diluted with dichloromethane (250 mL) and extracted with aqueous hydrochloric acid (0.1 mol/L, 5 × 100 mL) to remove excess amounts of serotonin and triethylamine, followed by water (100 mL). After drying over Na_2SO_4, the organic solution was filtered, evaporated, and taken up in THF (1 mL). Addition of petroleum ether (20 mL) led to the precipitation of the target compounds which were isolated by centrifugation and recrystallized from methanol, yielding the $^{\beta}N$-d_2-alkanoyl-5-hydroxytryptamides in a purity of more than 99% (LC-MS, ^{1}H NMR).

$^{\beta}N$-d_2-Stearoyl-5-hydroxytryptamide (d_2-$C_{18:0}$-C5HT). Yield: 37.1%. MS(ESI)$^+$: m/z (%) 445.6 (100, [M + H]$^+$). HPLC-MS/MS (ESI)$^+$:

m/*z* (%) 160.1 (100), 177 (12). ^{1}H NMR (400 MHz, d_6-DMSO): δ/ppm 0.84 (t, 3H, J = 7.1 Hz, H−C(28)), 1.15−1.30 (m, 12 × 2H, 2 × 1H, H−C(14−18, 20−27), H−C(19, 20)), 1.47 (m, 2H, H−C(13)), 2.03 (t, 2H, J = 7.4 Hz, H−C(12)), 2.69 (t, 2H, J = 7.4 Hz, H−C(8)), 3.25 (q, 2H, J = 6.6 Hz, H−C(9)), 6.56 (dd, 1H, J = 8.56 Hz, J = 2.02 Hz, H−C(6)), 6.80 (d, 1H, J = 2.02 Hz, H−C(4)), 6.99 (d, 1H, J = 1.77 Hz, H−C(2)), 7.10 (d, 1H, J = 8.6 Hz, H−C(7)), 7.82 (t, 1H, J = 5.56 Hz, H−N(10)), 8.54 (s, 1H, HO-C(5)), 10.44 (d, 1H, J = 1.77 Hz, H−N(1)). ^{13}C NMR (100 MHz, d_6-DMSO): δ/ppm 14.08 (CH_3, C(28)), 25.35 (CH_2, C(8,13)), 22.17−34.47 (CDH, CH_2, C(14−27)), 35.53 (CH_2, C(12)), 39.75 (CH_2, C(9)), 102.66 (CH, C(4)), 111.80 (CH, C(6,7)), 112.66 (C, C(3)), 123.39 (CH, C(2)), 128.06 (C, C(3′)), 131.07 (C, C(7′)), 150.84 (C−OH, C(5)), 172.32 (C═O, C(11)).

$^{\beta}$N-d$_2$-Arachinoyl-5-hydroxytryptamide (*d$_2$-C$_{20:0}$-C5HT*). Yield: 57.8%. MS(ESI)$^+$: *m*/*z* (%) 473.6 (100, [M + H]$^+$). HPLC-MS/MS(ESI)$^+$: *m*/*z* (%) 160.1 (100), 177 (24). ^{1}H NMR (400 MHz, d_6-DMSO): δ/ppm 0.84 (t, 3H, J = 7.0 Hz, H−C(30), 1.13−1.29 (m, 14 × 2H, 2 × 1H, H−C(14−20, 23−29), H−C(21, 22)), 1.49 (m, 2H, H−C(13)), 2.03 (t, 2H, J = 7.5 Hz, H−C(12)), 2.68 (t, 2H, J = 7.0 Hz, H−C(8)), 3.25 (m, 2H, H−C(9)), 6.56 (dd, 1H, J = 2.2 Hz, J = 8.7 Hz, H−C(6)), 6.80 (d, 1H, J = 2.2 Hz, H−C(4)), 7.00 (d, 1H, J = 2.2 Hz, H−C(2)), 7.10 (d, 1H, J = 8.5 Hz, H−C(7)), 7.83 (t, 1H, J = 5.5 Hz, H−N(10)), 8.55 (s, 1H, HO-C(5)), 10.45 (d, 1H, J = 1.54 Hz, H−N(1)). ^{13}C NMR (100 MHz, d_6-DMSO): δ/ppm 14.20 (CH_3, C(30)), 25.52 (CH_2, C(8)), 25.85 (CH_2, C(13)), 21.96−34.89 (CDH, CH_2, C(14−29)), 35.87 (CH_2, C(12)), 39.43 (CH_2, C(9)), 102.81 (CH, C(4)), 111.54 (CH, C(6)), 111.86 (CH_2, C(7)), 112.66 (C, C(3)), 123.13 (CH, C(2)), 127.99 (C, C(3′)), 131.03 (C, C(7′)), 150.95 (C−OH, C(5)), 172.18 (C═O, C(11)).

$^{\beta}$N-d$_2$-Behenoyl-5-hydroxytryptamide (*d$_2$-C$_{22:0}$-C5HT*). Yield: 52.2%. MS(ESI)$^+$: *m*/*z* (%) 501.5 (100, [M + H]$^+$). HPLC-MS/MS(ESI)$^+$: *m*/*z* (%) 160.1 (100), 177 (34). ^{1}H NMR (400 MHz, d_6-DMSO): δ/ppm 0.84 (t, 3H, J = 6.98 Hz, H−C(32)), 1.11−1.33 (m, 16 × 2H, 2 × 1H, H−C(14−22, 25−31), H−C(23, 24)), 1.47 (m, 2H, H−C(13)), 2.03 (t, 2H, J = 7.43 Hz, H−C(12)), 2.68 (t, 2H, J = 7.43 Hz, H−C(8)), 3.25 (m, 2H, H−C(9)), 6.56 (dd, 1H, J = 8.67 Hz, J = 1.91 Hz, H−C(6)), 6.80 (d, 1H, J = 1.91 Hz, H−C(4)), 6.99 (d, 1H, J = 1.32 Hz, H−C(2)), 7.10 (d, 1H, J = 8.53 Hz, H−C(7)), 7.82 (t, 1H, J = 5.15 Hz, H−N(10)), 8.55 (s, 1H, HO-C(5)), 10.44 (d, 1H, J = 1.77 Hz, H−N(1)). ^{13}C NMR (100 MHz, d_6-DMSO): δ/ppm 13.62 (CH_3, C(32)), 24.94 (CH_2, C(13)), 25.26 (CH_2, C(8)), 21.38−32.63 (CDH, CH_2, C(14−31)), 35.29 (CH_2, C(12)), 39.17 (CH_2, C(9)), 101.89 (CH, C(4)), 110.95 (CH, C(6)), 111.28 (CH, C(7)), 112.66 (C, C(3)), 122.27 (CH, C(2)), 128.43 (C, C(3′)), 132.33 (C, C(7′)), 151.38 (C−OH, C(5)), 172.17 (C═O, C(11)).

$^{\beta}$N-d$_2$-Lignoceroyl-5-hydroxytryptamide (*d$_2$-C$_{24:0}$-C5HT*). Yield: 36.4%. MS(ESI)$^+$: *m*/*z* (%) 529.3 (100, [M + H]$^+$). HPLC-MS/MS(ESI)$^+$: *m*/*z* (%) 160.1 (100), 177 (34). ^{1}H NMR (400 MHz, d_6-DMSO/CDCl, 1/1): δ/ppm 0.84 (t, 3H, J = 6.98 Hz, H−C(34)), 1.11−1.33 (m, 18 × 2H, 2 × 1H, H−C(14−24, 27−33), H−C(25, 26)), 1.47 (m, 2H, H−C(13)), 2.03 (t, 2H, J = 7.43 Hz, H−C(12)), 2.68 (t, 2H, J = 7.43 Hz, H−C(8)), 3.25 (m, 2H, H−C(9)), 6.56 (dd, 1H, J = 8.67 Hz, J = 1.91 Hz, H−C(6)), 6.80 (d, 1H, J = 1.91 Hz, H−C(4)), 6.99 (d, 1H, J = 1.32 Hz, H−C(2)), 7.10 (d, 1H, J = 8.53 Hz, H−C(7)), 7.82 (t, 1H, J = 5.15 Hz, H−N(10)), 8.55 (s, 1H, HO−C(5)), 10.44 (d, 1H, J = 1.77 Hz, H−N(1)). ^{13}C NMR (100 MHz, d_6-DMSO+CDCl, 1 + 1): δ/ppm 13.62 (CH_3, C(34)), 24.94 (CH_2, C(13)), 25.22 (CH_2, C(8)), 21.18−32.42 (CDH, CH_2, C(14−33)), 35.29 (CH_2, C(12)), 39.17 (CH_2, C(9)), 101.89 (CH, C(4)), 110.92 (CH, C(6)), 111.28 (CH, C(7)), 112.66 (C, C(3)), 122.27 (CH, C(2)), 128.41 (C, C(3′)), 132.13 (C, C(7′)), 151.41 (C−OH, C(5)), 171.21(C═O, C(11)).

Synthesis of Unsaturated $^{\beta}$N-Alkanoyl-5-hydroxytryptamides. A solution of linoloyl chloride (1 mmol) or oleoyl chloride (1 mmol) in dry dichloromethane (10 mL) was added dropwise to a solution of serotonin hydrochloride (1.5 mmol) and triethylamine (2.5 mmol) in dry DMF (10 mL). After stirring for 4 h under an atmosphere of nitrogen at room temperature, the solution was diluted with diethyl ether (250 mL) and extracted with aqueous hydrochloric acid (0.1 mol/L; 5 × 100 mL) to remove excess amounts of serotonin and triethylamin, followed by water (100 mL). The organic layer was filtered, the solvent was removed in vacuum, and the residue was taken up in methanol (5 mL) and then separated by preparative HPLC on a 250 × 21.2 mm Microsorb-RP-18 column (5 μm, Varian, Darmstadt, Germany). Chromatography was performed isocratically with a mixture of methanol/water/formic acid (88/12/1, v/v/v) using a flow of 18 mL/min. The peaks of the target compounds were collected, freed from solvent on a rotary evaporator, and then applied onto an SPE-cartridge (RP-18, Varian, Darmstadt, Germany) preconditioned with methanol (10 mL), followed by water (10 mL). After drying the stationary phase by sucking a stream of nitrogen through the cartridge, the target compound was eluted with methanol (50 mL). The solvent was removed in a vacuum, followed by lyophilization (48 h, 0.77 mbar) to afford the target compounds in a purity of more than 95%.

$^{\beta}$N-Oleoyl-5-hydroxytryptamide (*C$_{18:1}$-C5HT*). Yield 44.2%. MS-(ESI)$^+$: *m*/*z* (%) 441.7 (100, [M + H]$^+$), 463.7 (73, [M + Na]$^+$). MS/MS(ESI$^+$): *m*/*z* (%) 160 (100), 177 (41). UV/vis (MeOH): λ_{max} = 224, 278, 302 nm. ^{1}H NMR (400 MHz, d_4-MeOD): δ/ppm 0.83 (t, 3H, J = 7.1 Hz, H−C(28)), 1.14−1.31 (m, 11 × 2H, H−C(14−17, 22−27)), 1.49 (m, 2H, J = 7.3 Hz, H−C(13)), 1.96 (m, 2 × 2H, J = 6.2 Hz, H−C(18, 21)), 2.08 (t, 2H, J = 7.6 Hz, H−C(12)), 2.79 (t, 2H, J = 7.3 Hz, H−C(8)), 3.38 (t, 2H, J = 7.3 Hz, H−C(9)), 5.27 (t, 2H, J = 4.5 Hz, H−C(19,20)), 6.59 (dd, 1H, J = 2.3 Hz, 8.6 Hz, H−C(6)), 6.87 (d, 1H, J = 2.3 Hz, H−C(4)), 6.92 (s, 1H, H−C(2)), 7.08 (d, 1H, J = 8.6 Hz, H−C(7)). ^{13}C NMR (100 MHz, d_4-MeOD): δ/ppm 14.76 (CH_3, C(28)), 24.03 (CH_2, C(27)), 26.66 (CH_2, C(13)), 27.36 (CH_2, C(8)), 28.44 (CH_2, C(18,21)), 30.52 (CH_2, C(25)), 30.58 (CH_2, C(24)), 30.64 (CH_2, C(17,22)), 30.74 (CH_2, C(23)), 30.91 (CH_2, C(16)), 31.14 (CH_2, C(14,15)), 33.35 (CH_2, C(26)), 37.52 (CH_2, C(12)), 41.54 (CH_2, C(9)), 103.83 (CH, C(4)), 112.66 (C, C(3)), 112.79 (CH, C(6)), 112.93 (CH, C(7)), 124.47 (CH, C(2)), 129.77 (C, C(3′)), 131.10 (CH, C(20)), 131.14 (CH, C(19)), 133.39 (C,C(7′)), 151.41 (C−OH, C(5)), 176.54 (C═O, C(11)).

$^{\beta}$N-Linoloyl-5-hydroxytryptamide (*C$_{18:2}$-C5HT*). Yield 41.3%. MS-(ESI)$^+$: *m*/*z* 439.7 (10, [M + H]$^+$), 461.7 (100, [M + Na]$^+$). MS/MS(ESI)$^+$: *m*/*z* (%) 160 (100), 177 (33). UV/vis (MeOH): λ_{max} = 224, 278, 302 nm. ^{1}H NMR (400 MHz, d_4-MeOD, COSY): δ/ppm 0.83 (t, 3H, J = 7.1 Hz, H−C(28)), 1.21−1.40 (m, 7 × 2H, H−C(14−17, 25−27)), 1.57 (m, 2H, J = 7.1 Hz, H−C(13)), 2.00 (m, ×2H, J = 6.9 Hz, H−C(18, 24)), 2.08 (t, 2H, J = 7.4 Hz, H−C(12)), 2.71 (t, 2H, J = 6.4 Hz, H−C(21)), 2.79 (t, 2H, J = 7.2 Hz, H−C(8)), 3.38 (t, 2H, J = 7.4 Hz, H−C(9)), 5.27 (m, 4 × 1H, H−C(19, 20, 22, 23)), 6.59 (dd, 1H, J = 2.3 Hz, 8.7 Hz, H−C(6)), 6.87 (d, 1H, J = 2.3 Hz, H−C(4)), 6.93 (s, 1H, H−C(2)), 7.08 (d, 1H, J = 8.7 Hz, H−C(7)). ^{13}C NMR (100 MHz, d_4-MeOD, DEPT, HMBC, HMQC): δ/ppm 14.76 (CH_3, C(28)), 23.91 (CH_2, C(27)), 26.65 (CH_2, C(13)), 26.84 (CH_2, C(21)), 27.36 (CH_2, C(8)), 28.46 (CH_2, C(18,24)), 30.53 (CH_2, C(14)), 30.56 (CH_2, C(15)), 30.63 (CH_2, C(16)), 30.76 (CH_2, C(17)), 31.04 (CH_2, C(25)), 32.95 (CH_2, C(26)), 37.52 (CH_2, C(12)), 41.53 (CH_2, C(9)), 103.83 (CH, C(4)), 112.66 (C, C(3)), 112.80 (CH, C(6)), 112.93 (CH, C(7)), 124.48 (CH, C(2)), 129.33 (CH, C(23)), 129.36 (CH, C(22)), 129.77 (C, C(3′)), 131.20 (CH, C(20)), 131.23 (CH, C(19)), 133.40 (C, C(7′), 151.41 (C−OH, C(5)), 176.54 (C═O, C(11)).

Preparation of Coffee Brew Samples. *Filter Coffee*. Roast and ground coffee (5.40 g) was placed in a folded cellulose filter and percolated with boiling tap water in portions of approximately 20 mL each. The filtrate was collected in a measured flask (100 mL) and cooled to room temperature in an ice-bath. An aliquot (1 mL) was taken for analysis.

French Press. Coffee powder (54.0 g) was suspended in boiling tap water (1.0 L) in a French press device (Bodum, Germany) and maintained for 5 min. Thereafter, the metallic sieve was passed through the suspension, the supernatant was decanted and cooled to room temperature in an ice-bath, and an aliquot (0.2 mL) was taken for analysis.

Machine-Made Espresso. Coffee powder (150 g) was infused with hot water (1.0 L) using an automated espresso machine (Saeco, Italy), the resulting brew was cooled to room temperature in an ice-bath, and an aliquot (0.2 mL) was taken for analysis.

Hand-Made Espresso. Coffee powder (15 g) was placed in the powder input of the aluminum can (Dürkop Espresso Kanne, Dürkop, Germany), and water (300 mL) was placed in the bottom part of the device and, after closing the system, heated on a hot plate. The resulting beverage was cooled to room temperature in an ice-bath, and an aliquot (0.2 mL) was taken for analysis.

Turkish Mokka. Coffee powder (15 g) was placed in a Cevze made of copper (Orient Life GbR, Möchengladbach, Germany), water (300 mL) was added, and the suspension was heated to the boiling point on a heating place. Thereafter, the device was taken from the hot plate, and the suspension was stirred for 2 min. The brew was heated again until boiling,

removed from the heat, and stirred. Finally, the brew was decanted, and an aliquot (0.2 mL) was taken for analysis.

Preparation of Standard Solutions. *Stock Solutions*. The deuterium labeled and unlabeled ^{ss}N-alkanoyl-5-hydoxytryptamides were individually dissolved in methanol/THF (95/5, v/v) at a concentration of 15 μg/mL.

Internal Standard Working Solution. Aliquots of the individual stock solutions were appropriately combined and diluted with methanol to afford a working solution containing the internal standards d_2-$C_{18:0}$-C5HT (15 ng/mL), d_2-$C_{20:0}$-C5HT (240 ng/mL), d_2-$C_{22:0}$-C5HT (500 ng/mL), and d_2-$C_{24:0}$-C5HT (100 ng/mL). This solution was kept in brown glass vials at −20 °C until use.

Validation Standard Solution. Aliquots of the individual stock solutions of the d_2-labeled *N*-alkanoyl-5-hydroxytryptamides d_2-$C_{18:0}$-C5HT, d_2-$C_{20:0}$-C5HT, d_2-$C_{22:0}$-C5HT, and d_2-$C_{24:0}$-C5HT were combined and diluted with methanol to afford a validation standard solution containing each of the internal standards in a concentration of 125 ng/mL. This solution was kept in brown glass vials at −20 °C until use.

Spiking Solution. Aliquots of the individual stock solutions of unlabeled *N*-alkanoyl-5-hydroxytryptamides C_{18}-5HT, C_{20}-5HT, C_{22}-5HT, and C_{24}-5HT, were combined and diluted with methanol to give a spiking solution containing each of the analytes at a concentration of 3000 ng/mL.

Quantification of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides in Ground Coffee. Tetrahydrofuran (5 mL) was added to a sample of roast coffee powder (100 mg) placed in a centrifugation tube (10 mL, Schott, Mainz, Germany), and the mixture was heated for 10 min at 65 °C and centrifuged (3000 rpm, 5 min). The supernatant (100 μL) was diluted with methanol (900 μL), and an aliquot (100 μL) was spiked with the internal standard working solution (200 μL) and diluted with methanol to a final volume of 1 mL. After membrane filtration, an aliquot (5 μL) was injected into the HPLC-MS/MS system.

Quantitative Analysis of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides Coffee Brews. An aliquot of the freshly prepared coffee brew was mixed with the internal standard working solution and incubated at room temperature for equilibration (10 min). Then an aliquot of the solution (100 μL) was diluted with methanol (900 μL) and centrifuged (30 min, 12 000 rpm). The supernatant was injected into the HPLC-MS/MS (5 μL) system without further treatment.

Precision and Accuracy. Dewaxed roast and ground coffee (5.40 g) was placed in a folded cellulose filter and percolated with boiling tap water in portions of approximately 20 mL each. The filtrate was collected in a measured flask (100 mL) and cooled to room temperature in an ice-bath. The brew was doted with aliquots of the spiking solution to yield a coffee beverage fortified with $^{\beta}N$-stearoyl-5-hydroxytryptamide, $^{\beta}N$-arachinoyl-5-hydroxytryptamide, $^{\beta}N$-behenoyl-5-hydroxytryptamide, and $^{\beta}N$-lignoceroyl-5-hydroxytryptamide at a concentration of 1, 5, 10, 50, and 300 μg/L, respectively. Aliquots of the fortified coffee beverage (1 mL) were spiked with the validation standard solution (100−500 μL) and treated as detailed above.

High Performance Liquid Chromatography/Mass Spectrometry (HPLC-MS/MS). The Agilent 1200 series HPLC system consisted of a pump, a degasser, and an autosampler (Agilent, Waldbronn, Germany) and was connected to a 4000 Q triple quadrupole (Applied Biosystems/MDS Sciex, Darmstadt) with an electrospray ionization (ESI) device running in positive ionization mode (ion spray voltage 5500 V). Zero grade air served as nebulizer gas (50 psi) and as turbo gas (60 psi, 370 °C) for solvent drying. Nitrogen served as curtain (35 psi) and collision gas (8.5×10^{-7} psi). Detection was performed in multiple reaction monitoring (MRM) mode recording the mass transition from the positively charged pseudomolecular ion ($[M + H]^+$) to the fragments *m*/*z* 160.1 (quantifier) and 177.0 (qualifier) after collision-induced dissociation. The dwell time for each mass transition was 35 ms. The declustering potential (DP), the cell exit potential (CXP), and the collision energy (CE) were set as given in **Table 1**. The quadrupoles operated at unit mass resolution. For instrumentation control and data collection, Sciex Analyst software v1.4.2 was used. After sample injection (5 μL), chromatographic separation was carried out on a 50 × 2 mm i.d., 3 μm, Gemini C18 110A (Phenomenex, Aschaffenburg, Germany) with gradient elution at a flow rate of 300 μL/min. Eluent A was 1% formic acid in methanol, and eluent B was 1% formic acid in water. For chromatography, eluent A was held at 93% for 1 min, then increased linearly to 100% within 1 min, followed by isocratic elution for 4 min. Within 0.2 min, the starting conditions were re-established and held for equilibration of the column (4 min).

Table 1. Overview of Ionization and Fragmentation Parameters and Retention Times

	MW (Da)	mass transitions[a]	DP[b]	CE[c]	CXP[d]	RT[e]
		analytes				
$C_{18:0}$-5HT	442	*m*/*z* 443→160*; 443→177	81	35; 23	12; 4	1.55
$C_{20:0}$-5HT	470	*m*/*z* 471→160*; 471→177	106	37; 25	12; 4	2.22
$C_{21:0}$-5HT	484	*m*/*z* 485→160*; 485→177	96	33; 27	12; 4	3.10
$C_{22:0}$-5HT	498	*m*/*z* 499→160*; 499→177	116	43; 27	12; 4	3.33
$C_{23:0}$-5HT	512	*m*/*z* 513→160*; 513→177	96	33; 27	12; 4	4.72
$C_{24:0}$-5HT	526	*m*/*z* 527→160*; 527→177	111	43; 25	12; 4	5.08
		internal standards				
d_2-$C_{18:0}$-5HT	444	*m*/*z* 445→160*; 445→177	81	35; 23	12; 4	1.54
d_2-$C_{20:0}$-5HT	472	*m*/*z* 473→160*; 473→177	106	37; 25	12; 4	2.20
d_2-$C_{22:0}$-5HT	500	*m*/*z* 501→160*; 501→177	116	43; 27	12; 4	3.30
d_2-$C_{24:0}$-5HT	528	*m*/*z* 529→160*; 529→177	111	43; 25	12; 4	4.99

[a] Recorded mass transitions, quantifier is marked with an asterisk. [b] Declustering potential. [c] Collision energy. [d] Cell exit potential. [e] Retention time.

Calibration. Combinations of the deuterated standards and the analytes were prepared in seven mass ratios from 0.1 to 10, and analysis was performed in triplicates. The concentration of the internal standards was fixed at 50 ng/10 mL. Calibration curves were prepared by plotting peak area ratios of analyte to internal standard against concentration ratios of each analyte to the internal standard using quadratic regression. $^{\beta}N$-Heneicosanoyl-5-hydroxytryptamide ($C_{21:0}$-C5HT) and $^{\beta}N$-tricosanoyl-5-hydroxytryptamide ($C_{23:0}$-C5HT) were calibrated and quantified using d_2-$C_{18:0}$-C5HT as the internal standard with linear regression. The equations used are as follows: $y = -0.022x^2 + 1.316x + 0.049$ ($C_{18:0}$-C5HT/d_2-$C_{18:0}$-C5HT, $R^2 = 0.9996$), $y = -0.011x^2 + 0.708x + 0.018$ ($C_{20:0}$-C5HT/d_2-$C_{20:0}$-C5HT, $R^2 = 0.9995$), $y = 1.434x + 0.012$ ($C_{21:0}$-C5HT/d_2-$C_{18:0}$-C5HT, $R^2 = 0.9911$), $y = -0.032x^2 + 1.369x + 0.023$ ($C_{22:0}$-C5HT/d_2-$C_{22:0}$-C5HT, $R^2 = 0.9995$), $y = 1.482x - 0.032$ ($C_{23:0}$-C5HT/d_2-$C_{18:0}$-C5HT, $R^2 = 0.9962$), $y = -0.047x^2 + 1.531x + 0.099$ ($C_{24:0}$-C5HT/d_2-$C_{24:0}$-C5HT, $R^2 = 0.9994$).

High-Resolution Gas Chromatography. The system consisted of a HP6890 series gas chromatograph with flame ionization detector (Hewlett-Packard), an Agilent 7683 series autosampler (Agilent, Waldbronn, Germany) and a HP7683 injector. Separations were performed on a DB-FFAP column (30 m × 0.25 mm, 0.25 μm, J&W, Agilent, Waldbronn, Germany). The injector and detector temperature were set to 260 °C, the injector split was 1:1, and 1 μL was injected. Carrier gas was hydrogen initially set at 30 cm/s for 14 min followed by an increase of 0.4 mL/min to 120 cm/s held for 5 min. For chromatography, the temperature was set to 140 °C for 5 min, then increased to 230 °C with a rate of 10 °C/min, and then maintained for 5 min at 230 °C.

HGT-1 Cell Culture Assay. As reported recently (*29*), HGT-1 cells were cultured under standard tissue culture conditions at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium high in glucose (4%) and supplemented with 20% fetal calf serum, 2% L-glutamine, 2% penicillin streptomycin, and 2% 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) buffer. For the experiments, cells were plated into T/75 flasks and grown for 5 days. For the flow cytometry assay, the cells were washed once with phosphate buffered saline (0.9% w/v) and were harvested with a cell scraper after accutase treatment. The suspended cells were counted using a hemocytometer (Neubauer, VWR, Germany), and the viability was assessed by trypan blue staining and an automated cell counter (Schärfe System, Reutlingen, Germany). The cells were set to a concentration of 1×10^6 viable cells per milliliter of PBS. The gastric acid secretory acitvity of the cells was measured by the determination of the intracellular proton index (IPX) using the fluorescence dye carboxy-SNARF-AM as a probe for the measurement of the intracellular pH value (*29*). HGT-1 cells were loaded with the dye (3 μmol/L) for 30 min on ice. The intracellular pH was calculated referring to a calibration curve with 2 μmol/L nigericin treated HGT-1 cells in K^+ clamp buffer consisting of 20 mmol/L NaCl, 110 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L

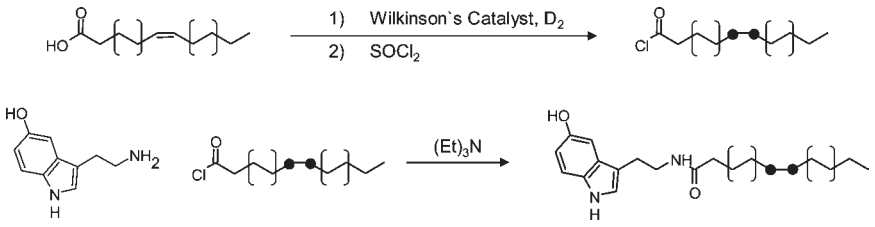

Figure 2. Reaction scheme for the synthesis of d_2-labeled $^{\beta}N$-alkanoyl-5-hydroxytryptamides.

$MgSO_4$, 18 mmol/L D-glucose, and 20 mmol/L HEPES that was set to different pH calibration points (6.2−8.2) by titration with NaOH. Then the intracellular proton index (IPX) was calculated by $\log_2$ transformation of the intracellular proton concentration ratio between treated cells and control cells. Higher IPX values indicate a higher concentration of protons remaining in the cell and imply a lower proton secretory activity. For the experiments, cells were treated either with histamine as physiological stimulant of stomach acid secretion or with different $^{\beta}N$-alkanoyl-5-hydroxytryptamides in their "natural" coffee concentrations for 10 min prior to the load with the fluorescent probe. Results were generated in three independent experiments and are displayed as mean ± standard deviation. Student's *t* test was applied to test whether treated cells showed different effects from each other or from untreated control cells. A *p*-value of at least < 0.05 was considered as significantly different.

Nuclear Magnetic Resonance Spectrometry (NMR). NMR experiments were performed on a Bruker AMX-400 instrument (Bruker, Rheinstetten, Germany). Chemical shifts are related to the proton signals of the solvent used.

RESULTS AND DISCUSSION

To accurately determine the amounts of $^{\beta}N$-alkanoyl-5-hydroxytryptamides in coffee powders and beverages, a stable isotope dilution assay (SIDA) with LC-MS/MS detection was developed. To achieve this, first, corresponding labeled internal standards needed to be synthesized.

Synthesis of Deuterated $^{\beta}N$-Alkanoyl-5-hydroxytryptamides. For the synthesis of deuterated $^{\beta}N$-alkanoyl-5-hydroxytryptamides (**Figure 2**), in a first step, the double bonds of the monounsaturated fatty acids oleic acid, 11-*cis*-eicosenic acid, erucic acid, and nervonic acid were deuterated by using the Wilkinson's catalyst tris(triphenylphosphin)-rhodium(I)- chloride (*30*) in an atmosphere of deuterium gas to give pure d_2-stearic acid, d_2-arachinic acid, d_2-behenic acid, and d_2-lignoceric acid after purification by means of column chromatography on silica gel. The d_2-labeled acids were converted into the corresponding acid chlorides by treatment with thionyl chloride and then coupled to the primary amino function of serotonin as recently shown for the nonlabeled analogues (*28*, *31*). The deuterated target compounds were precipitated from a solution in THF by addition of petroleum ether and were obtained in overall yields between 36.4 and 57.8%.

Development of a SIDA and Quantitation of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides in Coffee. In order to analyze the target compounds with high selectivity by using the multiple reaction monitoring (MRM) mode, methanolic solutions of the analytes and internal standards were individually infused into the MS/MS system using a syringe pump, and the mass range from 100 to 600 amu in full scan mode was monitored. Analysis of the fragmentation behavior of the C5HT confirmed previous findings (*16*, *28*) that the fragments obtained by collision induced dissociation of the pseudomolecular ions $[M + H]^+$ were independent of the fatty acid moiety. The serotonin moiety (*m*/*z* 177) as well as the fragment ion *m*/*z* 160 formed by additional elimination of ammonia were found as the most intense daughter ions (**Figure 3**). The intensities of the detected pseudomolecular ions $[M + H]^+$ and the respective fragments were optimized by software-assisted ramping of the ion path and collision cell settings. Optimal results were obtained with declustering potential between 81 and 116, collision energy between 23 and 27 for qualifier $[M + H]^+ \rightarrow m/z$ 177 and between 33 and 37 for quantifier $[M + H]^+ \rightarrow m/z$ 160, and cell exit potential at 4 and 12, respectively, resulting in maximized product ion intensities (**Table 1**). The mass transition from the mother ions to *m*/*z* 160 was chosen for quantitation purposes, as it proved to be the most selective one for the analysis of roast coffee samples (**Figure 5**).

To convert the measured ion intensities into the mass ratios of labeled and nonlabeled $^{\beta}N$-alkanoyl-5-hydroxytryptamides, mixtures of the analytes varying in their concentration and internal standards ranging from ratios of 0.1 to 10 were analyzed and the area ratios were plotted against the concentration ratios. Since the internal standards featured only two deuterium labels resulting in a mass shift of 2 Da, spectral overlap originating from the second isotope peak of the analytes was observed to a small extent of roughly 2.5% for each of the internal standards. As found in the fragmentation experiments, the daughter ion of both C5HT and d_2-C5HT was *m*/*z* 160 (**Figure 3**). Therefore, the spectral overlap resulted in the observation that the analyte simulated the internal standard due to the natural ^{13}C abundance. With increasing concentration of the nonlabeled analytes, the ratio between the area under the curve measured for the analytes and the corresponding deuterated internal standards decreased, because the ^{13}C isotope peak of the nondeuterated analyte added to the area of the deuterated internal standard. As a result, the calibration function showed successive leveling, a decrease of slope. However, the dependency followed a quadratic function, which allowed proper compensation of the spectral overlap. As an example, the calibration function obtained for $C_{20:0}$-C5HT/d_2-$C_{20:0}$-C5HT is shown in **Figure 4**. The individual equations obtained for the C5HT featured $R^2 > 0.999$.

For the quantitative analysis of the $^{\beta}N$-alkanoyl-5-hydroxytryptamides by means of a SIDA, coffee beverage was spiked with defined amounts of d_2-$C_{18:0}$-C5HT, d_2-$C_{20:0}$-C5HT, d_2-$C_{22:0}$-C5HT, and d_2-$C_{24:0}$-C5HT as the internal standards. The amounts of the labeled internal standard added were chosen to meet a concentration ratio of analyte versus internal standard in the sample which was covered by the respective calibration function. After equilibration, the mixture was diluted with methanol to precipitate polysaccharides and some melanoidins, and the solution was then centrifuged. Finally, mass chromatography was performed by analytical RP-HPLC-MS/MS(ESI) running in the multiple reaction monitoring (MRM) mode using the mass transitions detailed in **Table 1**. $^{\beta}N$-Heneicosanoyl-5-hydroxytryptamide ($C_{21:0}$-C5HT) and $^{\beta}N$-tricosanoyl-5-hydroxytryptamide ($C_{23:0}$-C5HT) were calibrated and quantified using d_2-stearoyl-5-hydroxytryptamide as the internal standard, because the concentrations of these minor components were in the concentration range of $C_{18:0}$-5HT. A representative analytical run including all mass transitions of a filter coffee brew sample spiked with the internal standards is given in **Figure 5**.

Performance of the SIDA Method. In order to check the performance of the developed stable isotope dilution analysis,

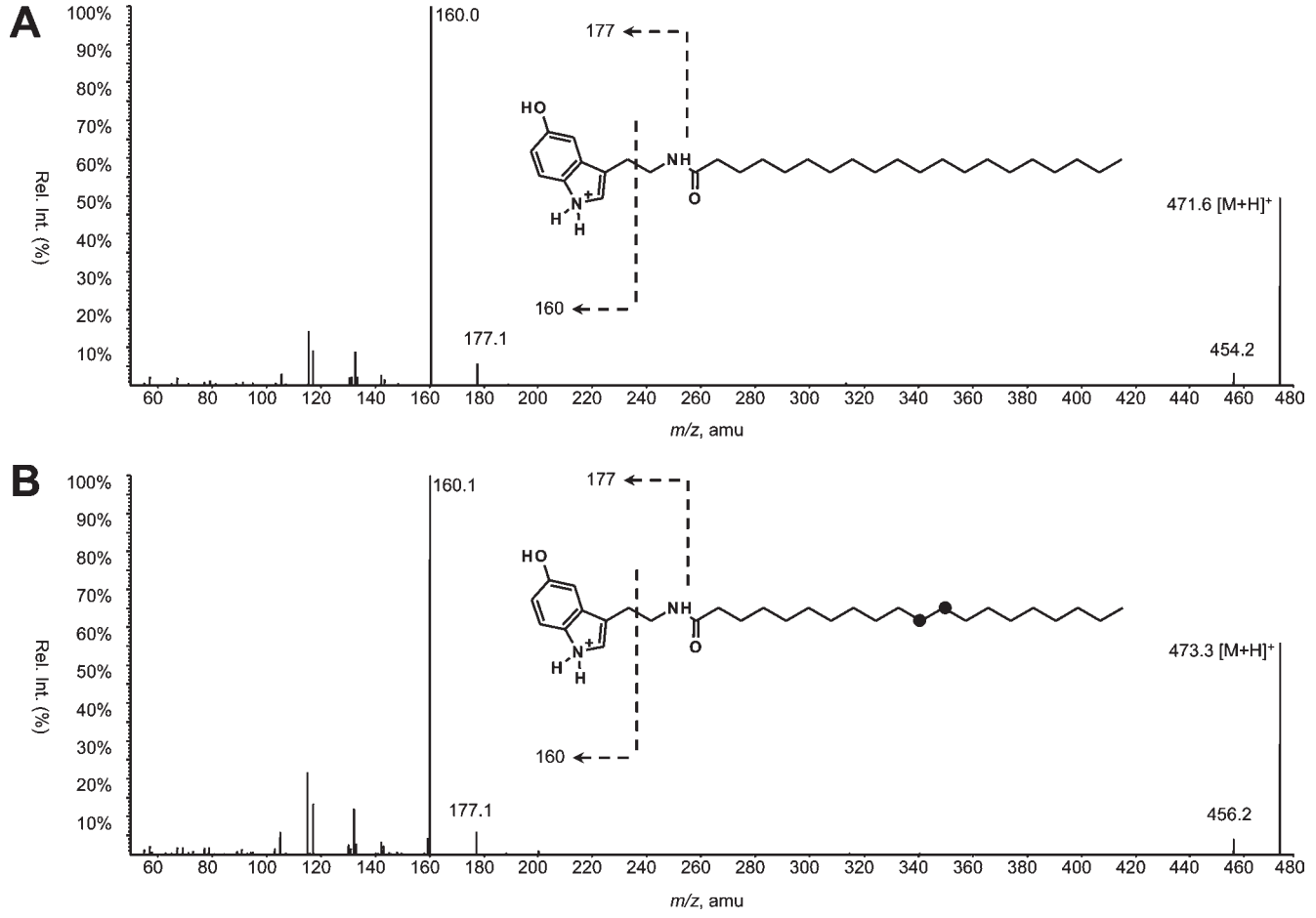

Figure 3. Product ion spectra (MS/MS) of (**A**) $^{\beta}N$-arachinoyl-5-hydroxytryptamide (*m*/*z* 471.6) and (**B**) d_2-$^{\beta}N$-arachinoyl-5-hydroxytryptamide (*m*/*z* 473.3) in positive electrospray ionization (ESI^+).

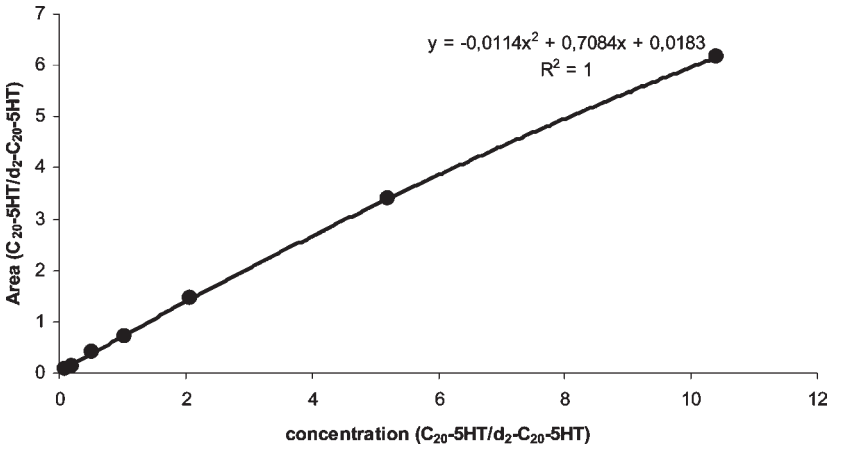

Figure 4. Function (quadratic regression) obtained by plotting the area ratios versus the concentration ratios of $C_{20:0}$-5HT (analyte) and d_2-$C_{20:0}$-5HT (internal standard) for calibration and compensation of spectral overlap.

several validation criteria were evaluated for the $^{\beta}N$-alkanoyl-5-hydroxytryptamides $C_{18:0}$-C5HT, $C_{20:0}$-C5HT, $C_{22:0}$-C5HT, and $C_{24:0}$-C5HT (**Table 2**). Percolation of dewaxed ground roast coffee powder and filtration by a cellulose filter yielded a brew which was void of $^{\beta}N$-alkanoyl-5-hydroxytryptamides. To this matrix, the derivatives $C_{18:0}$-C5HT, $C_{20:0}$-C5HT, $C_{22:0}$-C5HT, and $C_{24:0}$-C5HT were added in defined concentrations from 1 to 300 μg/L, delivering spiked coffee samples. The precision of the SIDA, expressed by the relative standard deviation (RSD, %), obtained by triplicate analysis of each spiking level ranged between 0.3 and 7.0%. The accuracy of the analytical method, defined by agreement (%) of calculated concentration in the spiked coffee brew samples and the measured concentration, was between 92.6 and 107.4%. These data clearly demonstrate the developed SIDA as a reliable tool enabling a rapid and accurate quantitative determination of $^{\beta}N$-alkanoyl-5-hydroxytryptamides in coffee.

Quantitative analysis of the coffee beverage revealed signal-to-noise ratios (S/N) of >15 for the minor components $C_{18:0}$-5HT, $C_{21:0}$-5HT, and $C_{23:0}$-5HT. That means that the volume of

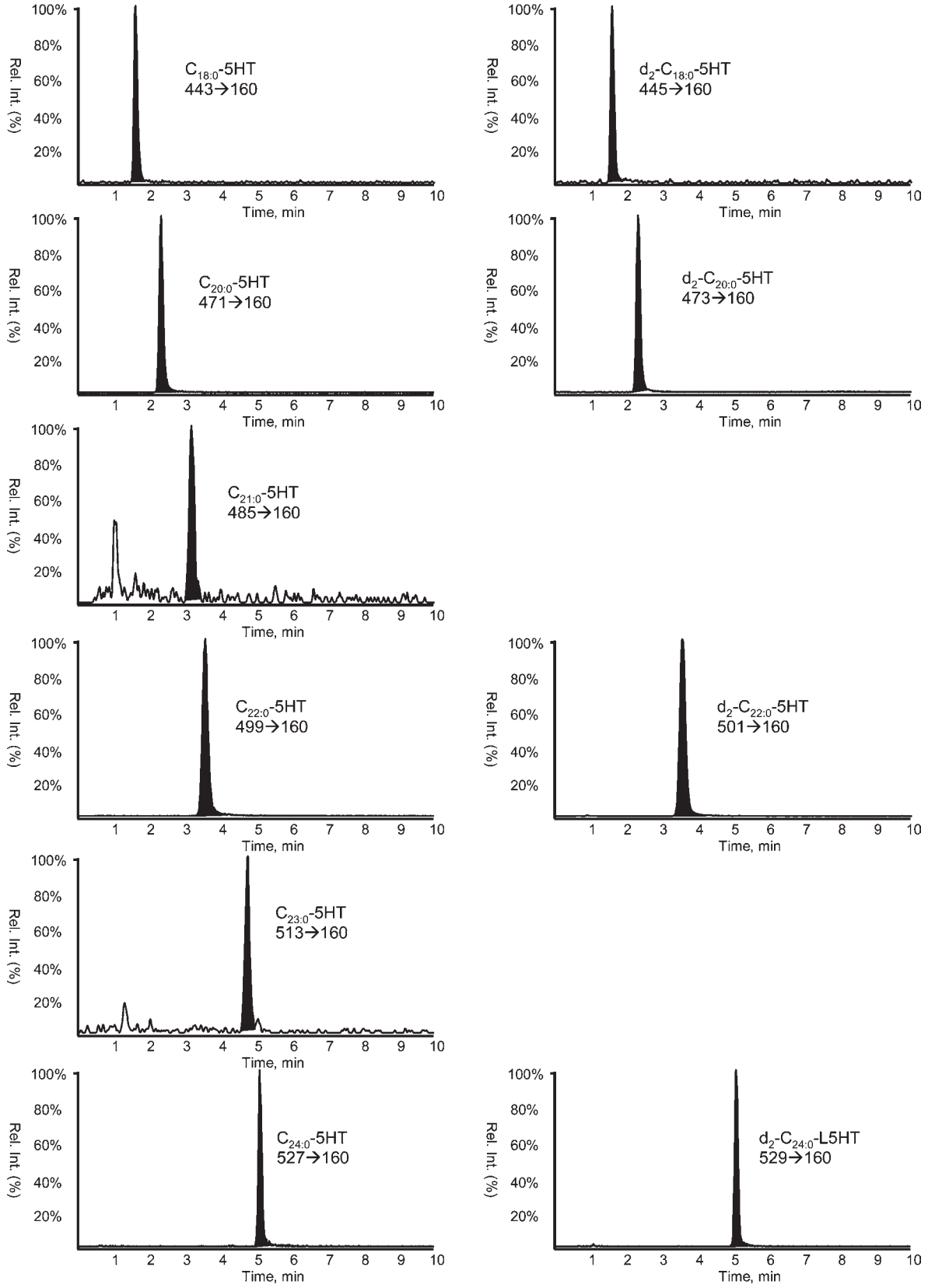

Figure 5. Mass transitions for the individual C5HTs and the respective d_2-labeled internal standards recorded by HPLC-MS/MS analysis of a filter coffee beverage.

5 μL of the coffee extract containing these compounds in absolute amounts of 0.5−1.0 pg and injected into the LC-MS system resulted in an S/N ratio of >15. Samples with signals below an S/N ratio of 15 were reinjected with higher injection volumes.

Influence of Steam Treatment on the Amounts of $^{\beta}$*N*-Alkanoyl-5-hydroxytryptamides in Coffee Powder and Filter Coffee Beverage. In order to quantitatively determine the influence of the Lendrich steam treatment (*8*, *32*) on the concentrations of $^{\beta}$*N*-alkanoyl-5-hydroxytryptamides in coffee powder, the compounds $C_{18:0}$-C5HT,

Table 2. Precision and Accuracy for the Individual $^{\beta}N$-Alkanoyl-5-hydroxytryptamides (C5HTs) Quantified in C5HT-Spiked Filter Coffee Brew Prepared from Dewaxed Roast Coffee[a]

	precision (%)				accuracy (%)			
addition level (μg/L)	$C_{18:0}$-5HT	$C_{20:0}$-5HT	$C_{22:0}$-5HT	$C_{24:0}$-5HT	$C_{18:0}$-5HT	$C_{20:0}$-5HT	$C_{22:0}$-5HT	$C_{24:0}$-5HT
1	0.3	7.0	0.5	1.9	97.5	104.1	99.4	97.4
5	1.9	1.9	3.7	0.9	92.6	94.3	98.8	103.6
10	4.2	4.9	1.3	3.6	98.7	95.2	96.0	99.6
50	2.4	3.0	1.3	1.4	97.5	107.4	106.1	104.5
300	5.9	4.0	6.9	3.9	99.4	100.3	103.5	106.2

[a] Data are means of triplicates. Precision is expressed as relative standard deviation. Accuracy is expressed as agreement of nominal and measured concentration based on the concentration in the zero coffee sample.

Table 3. Concentrations of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides in Coffee Powders and Filter Coffee Beverages

	concentration[a]						
sample	$C_{18:0}$-5HT	$C_{20:0}$-5HT	$C_{21:0}$-5HT	$C_{22:0}$-5HT	$C_{23:0}$-5HT	$C_{24:0}$-5HT	Σ
Arabica Brazil (untreated)							
coffee powder (μg/g)	11.2 (±4)	221.2 (±2)	3.88 (±13)	589.3 (±1)	11.2 (±4)	61.8 (±1)	898.1 (±1)
coffee beverage (μg/L)	0.8 (±10)	34.7 (±9)	0.50 (±14)	91.3 (±17)	1.22 (±13)	12.2 (±24)	143.8 (±13)
extraction rate (%)	0.14	0.29	0.24	0.29	0.21	0.37	0.29
Arabica Brazil (steam-treated)							
coffee powder (μg/g)	7.97 (±9)	165.3 (±1)	2.71 (±26)	352.2 (±1)	10.5 (±3)	38.1 (±5)	577.0 (±2)
coffee beverage (μg/L)	0.65 (±21)	23.5 (±11)	0.24 (±33)	44.1 (±12)	0.72 (±17)	5.69 (±13)	74.2 (±12)
extraction rate (%)	0.15	0.26	0.16	0.23	0.13	0.28	0.24
Robusta Vietnam (untreated)							
coffee powder (μg/g)	5.56 (±6)	153.9 (±2)	1.02 (±32)	231.0 (±2)	9.66 (±9)	27.2 (±18)	428.3 (±3)
coffee beverage (μg/L)	0.46 (±24)	24.3 (±11)	0.13 (±48)	36.7 (±10)	0.33 (±20)	6.28 (±17)	68.2 (±10)
extraction rate (%)	0.15	0.29	0.24	0.29	0.06	0.43	0.29
Robusta Vietnam (steam-treated)							
coffee powder (μg/g)	5.25 (±9)	120.7 (±8)	1.05 (±8)	173.1 (±8)	7.40 (±11)	23.5 (±3)	330.9 (±7)
coffee beverage (μg/L)	0.49 (±19)	23.6 (±6)	0.08 (±37)	35.2 (±8)	0.39 (±29)	5.54 (±13)	65.4 (±5)
extraction rate (%)	0.17	0.36	0.14	0.38	0.09	0.44	0.37

[a] Concentrations are given as the mean of five independent brew preparations and sample workups ($n = 5$) ± relative standard deviation (%); data on coffee powder are based on dry matter. For each brew, 5.40 g of coffee powder/100 mL of coffee brew were used. The basis for calculation of the extraction rate is the total amount of C-5HT in the powder versus the total amount in the beverage.

$C_{20:0}$-C5HT, $C_{21:0}$-C5HT, $C_{22:0}$-C5HT, $C_{23:0}$-C5HT, and $C_{24:0}$-C5HT were quantitatively determined in two roasted coffee samples, namely, an Arabica Brazil Santos and a Robusta Vietnam coffee, which were either roasted or steam-treated prior to roasting (**Table 3**). As preliminary studies demonstrated an almost quantitative extraction of the C5HTs from coffee powder by tetrahydrofuran (*33*), the roast and ground coffee extracted with tetrahydrofuran, the extract was freed from solvent in vacuum, taken up in methanol, and then spiked with the internal standard working solution. The solution obtained was injected into the LC-MS/MS instrument. Quantitative analyses revealed $C_{22:0}$-C5HT and $C_{20:0}$-C5HT as the major $^{\beta}N$-alkanoyl-5-hydroxytryptamides in both nontreated coffee samples, although the Arabica coffee contained significantly higher amounts of the C5HTs when compared to the Robusta sample. For example, 589.3 and 221.2 μg/g of $C_{22:0}$-C5HT and $C_{20:0}$-C5HT, respectively, were determined in the Arabica coffee, whereas 231.0 and 153.9 μg/g of these C5HTs were found in the Robusta. Furthermore, a significant decrease in the concentration of $^{\beta}N$-alkanoyl-5-hydroxytryptamides was observed when the coffee beans were steam-treated prior to roasting; for example, with a total amount of 577.0 μg/g, the steam-treated Arabica coffee contained about 36% less C5HTs than the corresponding nontreated coffee sample (898.1 μg/g).

In order to gain first insights into the extraction yields of the $^{\beta}N$-alkanoyl-5-hydroxytryptamides upon preparation of a cellulose filtered coffee beverage, coffee brews were prepared from the four coffee powders and the C5HTs were quantified by means of the stable isotope dilution analysis. As given in **Table 3**, all the $^{\beta}N$-alkanoyl-5-hydroxytryptamides were detectable in the filter coffee beverages and could be quantitatively determined to be present in rather low concentrations ranging from 0.08 to 91.3 μg/L depending on the structure of the C5HT as well as the coffee sample used for preparing the beverage. The total amount of the six C5HTs in the filter coffee brews was found to be between 65 and 144 μg/L, equaling an extraction rate between 0.24 and 0.37% of the total concentration present in the corresponding coffee powders. As already found for the coffee powder, the highest concentration of 91.3 or 36.7 μg/L and 34.7 or 24.3 μg/L were found for $C_{22:0}$-C5HT and $C_{20:0}$-C5HT, respectively, in the nontreated beverage of the Arabica or the Robusta coffee. In comparison, the beverages prepared from the steam-treated coffees contained somewhat lower amounts of C5HTs; for example, 44.1 μg/L of $C_{22:0}$-C5HT and 23.5 μg/L of $C_{20:0}$-C5HT were found in the beverage made from steam-treated Arabica coffee (**Table 3**).

Influence of the Preparation Method on the Concentrations of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides in Coffee Beverages. To answer the question as to how the amounts of $^{\beta}N$-alkanoyl-5-hydroxytryptamides in a coffee beverage are influenced by the procedure of coffee making, coffee brews were prepared from

Table 4. Influence of the Type of Beverage Preparation on the Concentrations of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides in the Beverage and the Extraction Rate from Coffee Powder[a]

		concentration in beverage (μg/L)[c]								
preparation method	C/W ratio (g/L)[b]	$C_{18:0}$-5HT	$C_{20:0}$-5HT	$C_{21:0}$-5HT	$C_{22:0}$-5HT	$C_{23:0}$-5HT	$C_{24:0}$-5HT	total	total amount (μg)[d]	extraction rate (%)[e]
filter coffee	54	0.86	34.7	0.50	91.3	1.22	12.2	143.8	48 495	0.3
Turkish mokka (Cevze)	50	50.2	237.8	2.22	522.2	5.09	176.5	993.9	44 903	2.2
espresso (tin can)	50	52.4	239.5	3.45	707.2	10.2	197.8	1210.6	44 903	2.7
espresso (coffee automate)	150	42.4	2080.0	47.1	5436.8	105.3	700.6	8412.2	134 710	6.2
French press	54	35.6	886.1	21.2	2207.0	50.6	301.1	3501.6	48 495	7.2

[a] Arabica Brazil Santos (not steam-treated) was used for the study. [b] Coffee/water ratio used for beverage preparation. [c] Data are given as the mean of triplicates. [d] Absolute amount of C5HTs in the coffee powder used for preparing the beverage. [e] Extraction rate was determined from the total amount of C5HTs found in the coffee powder and the corresponding beverage.

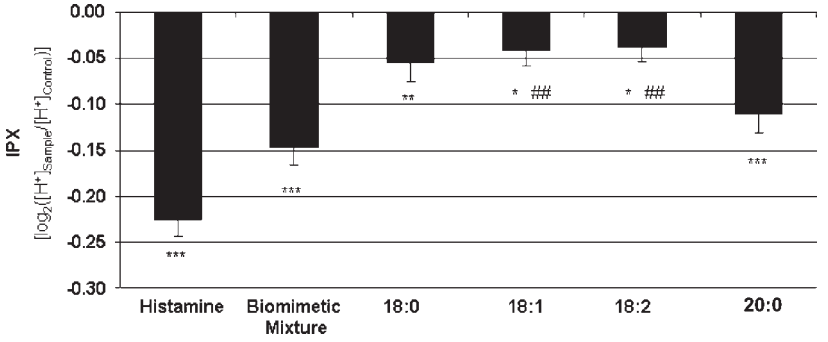

Figure 6. Intracellular proton index (IPX) calculated by $\log_2$ transformation of the intracellular proton concentration ratio between cells treated with either histamine (1.0 mmol/L), a mixture containing $C_{18:0}$-5HT (0.86 μg/L), $C_{20:0}$-5HT (34.71 μg/L), $C_{22:0}$-5HT (91.28 μg/L), and $C_{24:0}$-5HT (12.21 μg/L) in the concentrations found in the Arabica filter coffee sample (**Table 3**), or the single $^{\beta}N$-alkanoyl-5-hydroxytryptamides $C_{18:0}$-5HT, $C_{18:1}$-5HT, $C_{18:2}$-5HT, or $C_{20:0}$-5HT in concentrations of 140 μg/L each. Data are given as mean ± standard deviation from three independent experiments; ∗, $p < 0.05$ versus untreated control cells; ∗∗, $p < 0.01$ versus untreated control cells; ∗∗∗, $p < 0.001$ versus untreated control cells; ##, $p < 0.01$ versus cells treated with arachinoyl-5-hydroxytryptamide.

the non-steam-treated Arabica Brazil sample by using the cellulose filter paper method, the French press method, the Turkish mokka procedure using a copper device, named Cevze, and an espresso made by using either a tin cup or a fully automatic espresso machine (**Table 4**). HPLC-MS/MS-SIDA analysis of the C5HTs in the coffee beverages revealed the lowest total amount of 143.8 μg/L and the lowest extraction rate of 0.3% for the hydroxytryptamides in the filter coffee, followed by the Turkish mokka and the handmade espresso with about 10 times higher extraction rates of 2.2 and 2.7%, respectively. By far, the highest extraction rates of 7.2 and 6.2% were found for the beverages made by means of a French press and an espresso machine, respectively (**Table 4**). These data clearly indicate that the $^{\beta}N$-alkanoyl-5-hydroxytryptamides are most strongly retained by filtration using a cellulose filter, whereas espresso-type as well as French press beverages were found to contain more than 20 times higher amounts of C5HTs. This is well in line with the amphiphilic character of the C5HTs which are expected to solubilize at the water/oil surface of the tiny coffee oil droplets and seem to be removed by cellulose filtration.

In conclusion, the data show that the method of preparation has a far more important influence on the concentration of these putative stomach irritant serotonin derivatives in the brew than the steam treatment.

Activity of $^{\beta}N$-Alkanoyl-5-hydroxytryptamides to Induce Acid Secretion in HGT-1 Cells. In order to investigate as to whether the low concentrations of C5HTs in the filter coffee are able to induce gastric acid secretion, a solution containing the four predominant $^{\beta}N$-alkanoyl-5-hydroxytryptamides $C_{18:0}$-5HT, $C_{20:0}$-5HT, $C_{22:0}$5HT, and $C_{24:0}$-5HT in their "natural" concentrations found in the Arabica filter coffee was tested for its effect on the intracellular proton concentration as a biomarker for stomach acid secretion by means of a recently developed *in vitro* assay using the human gastric cell line HGT-1 (*29*). In addition, histamine was tested as a physiological stimulant. The results, given in **Figure 6**, are expressed as intracellular proton index (IPX) which was defined to describe the intracellular proton concentration ratio between treated cells and nontreated cells (blank control). Treatment of the HGT-1 cells with histamine and the mixture of $^{\beta}N$-alkanoyl-5-hydroxytryptamides, respectively, resulted in a remarkable IPX decrease, indicating a decrease in intracellular proton concentrations caused by proton secretion. These data give a first evidence that, although their concentrations are rather low in the filter coffee beverage, the $^{\beta}N$-alkanoyl-5-hydroxytryptamides are able to induce some gastric acid secretion.

In order to gain first insight into the influence of saturation as well as the chain length of the alkanoyl moiety in the C5HTs, HGT-1 cells were treated with pure $^{\beta}N$-stearoyl-5-hydroxytryptamide ($C_{18:0}$-5HT), $^{\beta}N$-oleoyl-5-hydroxytryptamide ($C_{18:1}$-5HT), $^{\beta}N$-linoloyl-5-hydroxytryptamide ($C_{18:2}$-5HT), and $^{\beta}N$-arachinoyl-5-hydroxytryptamide ($C_{20:0}$-5HT). The data obtained give rise to the assumption that $^{\beta}N$-alkanoyl-5-hydroxytryptamides with a saturated chain length of 20 carbons induce a more pronounced effect on proton secretion than the corresponding C_{18} analogue. In contrast, the degree of unsaturation of the amide carbon chain does not seem to play an important role, since no significant difference was observed among cells treated with $C_{18:0}$-5HT, $C_{18:1}$-5HT, and $C_{18:2}$-5HT. In conclusion, the data obtained show, for the first time, a structure dependent decrease in intracellular proton concentration as an indicator of stomach acid secretion in human stomach cells treated with $^{\beta}N$-alkanoyl-5-hydroxytryptamides.

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LITERATURE CITED

(1) Acquaviva, F.; DeFrancesco, A.; Andriulli, A.; Piantino, P.; Arrigoni, A.; Massarenti, P.; Balzola, F. Effect of regular and decaffeinated coffee on serum gastrin levels. *J. Clin. Gastroenterol.* **1986**, *8*, 150–153.

(2) Boekma, P. J.; Samsom, M.; van Berge Henegouwen, G. P.; Smout, A. J. Coffee and gastrointestinal function: facts and fiction. A review. *Scand. J. Gastroenterol., Suppl.* **1999**, *230*, 35–39.

(3) Cohen, S. Pathogenesis of coffee-induced gastrointestinal symptoms. *N. Engl. J. Med.* **1980**, *303*, 122–124.

(4) McArthur, K. E.; Feldman, M. Gastric acid secretion, gastrin release, and gastric emptying in humans as affected by liquid temperature. *Am. J. Clin. Nutr.* **1989**, *49*, 51–55.

(5) Nilsson, M.; Johnson, R.; Ye, W.; Hveem, K.; Lagergren, J. Lifestyle related risk factors in the aetiology of gastro-oesophageal reflux. *Gut* **2004**, *53*, 1730–1735.

(6) Ehrlich, A.; Basse, H.; Henkel-Ernst, J.; Hey, B.; Menthe, J.; Lücker, P. W. Effect of differently processed coffee on the gastric activity difference and intragastric pH in healthy volunteers. *Methods Find. Exp. Clin. Pharmacol.* **1998**, *20*, 155–161.

(7) König, W. A.; Sturm, A. Gas chromatography and mass spectrometry as helpful tools to study high-boiling coffee components (in German). *ASIC, 10e Colloque*; Salvador, **1982**.

(8) Darboven A. Method fort he quality improvement of raw coffee by treatment with steam and water (in German). Europäisches Patentblatt, 1997/05/95109295.6, 1997.

(9) Wurziger, J. Carboxylic acid hydroxytryptamides for the evaluation of fresh and processed coffee (in German). *ASIC, 5e Colloque*; Lissabon, **1971**.

(10) Wurziger, J. Carboxylic acid 5-hydroxytryptamides or ether soluble extractables for the identification and evaluation of processed coffee (in German). *Kaffee- und Tee-Markt* **1972**, *22*, 3–11.

(11) Harms, U.; Wurziger, J. Contribution to the taste and stomach friendliness of roasted coffee. II. phenolic compounds in the wax of raw coffee of different species and origin (in german). *Kaffee- und Tee-Markt* **1969**, *19*, 6–9.

(12) Hubert, P.; Kwasny, H.; Werkhoff, P.; Turner, U. Analysis of hydroxytryptamides in coffee (in German). *Z. Anal. Chem.* **1977**, *285*, 242–250.

(13) Speer, K.; Kölling-Speer, I. The lipid fraction of the coffee bean. *Braz. J. Plant Physiol.* **2006**, *18* (1), 201–216.

(14) Buergin, E. Lowering the carboxylic acid-5-hydroxytryptamid content in unroasted coffee. German Office Patent DE-74−2429233 19740618, 1975.

(15) Pehl, C.; Pfeiffer, A.; Wendl, B; Kaess, H. The effect of decaffeination of coffee on gastro-oesophageal reflux in patients with reflux disease. *Aliment. Pharmacol. Ther.* **1997**, *11*, 483–486.

(16) Van der Steegen, G. H. D.; Noomen, P. J. Mass-Balance of carboxy-5-hydroxytryptamides (C5HT) in regular and treated coffee. *Lebensm. Wiss. Technol.* **1977**, *10*, 321–323.

(17) Hinkel, C.; Speer, K. New carboxylic acid-5-hydroxytryptamides in Coffee Wax. In *Proceedings of Euro Food Chemistry XIII*, 21−23 September, Hamburg, 2005; Eklund, T., Schwarz, M., Steinhart, H., Thier, H.-P., Winterhalter, P., Eds.; Vol 2, pp 487−490, ISBN: 3-936028-32-X.

(18) Folstar, P.; Schols, H. A.; van der Plas, H. C.; Pilnik, W.; Landheer, C. A.; van Veldhuizen, A. New Tryptamine Derivatives Isolated from Wax of Green Coffee Beans. *J. Agric. Food Chem.* **1980**, *28*, 872–874.

(19) Fehlau, R.; Netter, K. J. On the influence of untreated and treated coffee and carboxylic acid hydroxytryptamides on the stomach epithelium of the rat (in German). *Z. Gastroenterol.* **1990**, *28*, 234–238.

(20) Culmsee, O. A method for the quantitative analysis of carboxylic acid 5-hydroxytryptamides in coffee (in German). *Dtsch. Lebensm.-Rundsch.* **1975**, *71*, 425–427.

(21) Nebesny, E.; Budryn, G. Effect of the roasting method on the content of 5-hydroxytryptamides of carboxylic acids in roasted coffee beans. *Nahrung* **2002**, *46*, 279–282.

(22) Kummer, P.; Bürgin, E. Neue Erkenntnisse zur quantitativen Bestimmung der Carbonsäure-5-hydroxytryptamide in Kaffee. *Mitt. Geb. Lebensmittelunters. Hyg.* **1976**, *67*, 212–225.

(23) Hunziker, H. R.; Miserez, A. Analysis of 5-hydroxytryptamides in coffee by means of high-pressure liquid chromatography (in German). *Mitt. Geb. Lebensmittelunters. Hyg.* **1977**, *68*, 267–274.

(24) Hunziker, H. R.; Miserez, A. Determination of 5-hydroxytryptamides in coffee by means of high-pressure liquid chromatography (in German). *Mitt. Geb. Lebensmittelunters. Hyg.* **1979**, *70*, 142–152.

(25) Battini, M. L.; Careri, M.; Casoli, A.; Mangia, A.; Lugari, M. T. Determination of N-alkanoyl-5-hydroxytryptamides (C5HT) in coffee beans by means of HPLC and TLC. *Ann. Chim.* **1989**, *79*, 369–377.

(26) Chiacchierini, E.; Ruggeri, P. Dewaxed coffee: Determination of carboxylic acid 5-hydroxytryptamides (C5HT). *Riv. Soc. Ital. Sci. Aliment.* **1985**, *14*, 197–200.

(27) Lagana, A.; Curini, L.; De Angelis Curtis, S.; Marino, A. Rapid liquid chromatographic analysis of carboxylic acid 5-hydroxytryptamides in coffee. *Chromatographia* **1989**, *28*, 593–596.

(28) Lang, R.; Hofmann, T. A versatile method for the quantitative determination of $^{\beta}$*N*-Alkanoyl-5-hydroxytryptamides in roasted coffee. *Eur. Food Res. Technol.* **2005**, *220*, 638–643.

(29) Weiss, C.; Rubach, M.; Lang, R.; Seebach, E.; Blumberg, S.; Frank, O.; Hofmann, T.; Somoza, V. Measurement of the intracellular pH in human stomach cells: a novel approach to evaluate the gastric acid secretory potential of coffee beverages. *J. Agric. Food Chem.*, submitted.

(30) Osborn, J. A.; Jardine, F. H.; Young, J. F.; Wilkinson, G. The preparation and properties of tris-(triphenylphosphine)halogenorhodium(I) and some reactions thereof including catalytic homogeneous hydrogenation of olefins and acetylenes and their derivatives. *J. Chem. Soc. A* **1966**, 1711–1732.

(31) Shevchenko, V. P.; Nagaev, I. Y.; Myasoedov, N. F.; Yudushkin, I. A.; Gretskaya, N. M.; Bobrov, M. Y.; Bezuglov, V. V. Synthesis of tritium labelled arachidonic acid amide and ester derivatives with dopamine, serotonin, vannilylamine, and ethyleneglycol moieties. *J. Labelled Compd. Radiopharm.* **2002**, *46*, 187–193.

(32) Lendrich, P.; Wemmering, E.; Lendrich, E. German Office Patent, DE1927L070519D 19271222, 1933.

(33) Lang, R. Analytical Investigations on coffee derived modulators of gastric acid secretion (in German). Ph.D. Thesis, Westfälische Wilhelm-Universität, Münster, Germany, **2007**.

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