JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantitative Investigation of Trigonelline, Nicotinic Acid, and Nicotinamide in Foods, Urine, and Plasma by Means of LC-MS/MS and Stable Isotope Dilution Analysis

ROMAN LANG,[†] ERKAN FIRAT YAGAR,[‡] RUDOLF EGGERS,[‡] AND THOMAS HOFMANN[†,*]

Chair of Food Chemistry and Molecular Sensory Science, Technische Universität München, Lise-Meitner-Strasse 34, D-85354 Freising, Germany, and Chair of Thermal and Separation Processes, Technische Universität Hamburg Harburg, Eissendorfer Str. 38, D-21073 Hamburg, Germany

A straightforward stable isotope dilution analysis (SIDA) for the quantitative determination of trigonelline, nicotinic acid, and nicotinamide in foods such as coffee, as well as in biological samples by means of LC-MS/MS (MRM) has been developed. The coefficients of variation for their quantitative analysis in a coffee sample were 2.1% for trigonelline, 1.1% for nicotinic acid, and 3.1% for nicotinamide, and recovery experiments showed good results between 98.5 and 104.5%. Application of this SIDA for the quantification of trigonelline, nicotinic acid, and nicotinamide in coffee samples of different roasting degrees revealed a drastic degradation of trigonelline as well as the generation of nicotinic acid accounting for 4−6% of the initial trigonelline content, whereas nicotinamide remained rather constant at a low level. Besides the analysis of coffee samples, the feasibility of the developed SIDA was verified by analysis of other foods including breakfast cereals, rice, liver, and herring, as well as human urine and plasma samples.

KEYWORDS: Nicotinic acid; nicotinamide; trigonelline; methyl nicotinate; coffee; urine; plasma; stable isotope dilution analysis; LC-MS/MS

INTRODUCTION

Besides caffeine, the betaine *N*-methyl nicotinic acid, also known as trigonelline (**1**), (**Figure 1**), is commonly reported as the second most abundant alkaloid in raw coffee beans. Upon bean roasting, trigonelline was found to be partially decomposed to give volatile degradation products such as pyridines (*1*) as well as nonvolatile compounds such as *N*-methylpyridinium (NMP) ions which were recently discovered in roasted coffee (*2*−*4*) and found to exhibit phase I/II biotransformation enzyme modulating activity in vitro as well as in vivo (*4*). Whereas NMP is thermally generated from trigonelline upon decarboxylation, demethylation of trigonelline is known to yield nicotinic acid (**2**) which, together with nicotinamide (**3**), is well proven to exhibit vitamin B3 activity. Furthermore, it has been reported that thermal degradation of trigonelline induces the intermolecular $N \rightarrow C$ as well as $N \rightarrow O$ shift of the nitrogen-bound methyl group to yield *N*-methylated picolinium derivatives (*2*), methylated pyridines (*1*), as well as methyl nicotinate (**4**).

Nicotinamide, the functional component of the coenzymes nicotine adenine dinucleotide (NAD) and nicotine adenine dinucleotide phosphate (NADP), is known to play an essential role in biochemical electron transport and oxidation/reduction reactions, respectively. The Expert group on Vitamins and

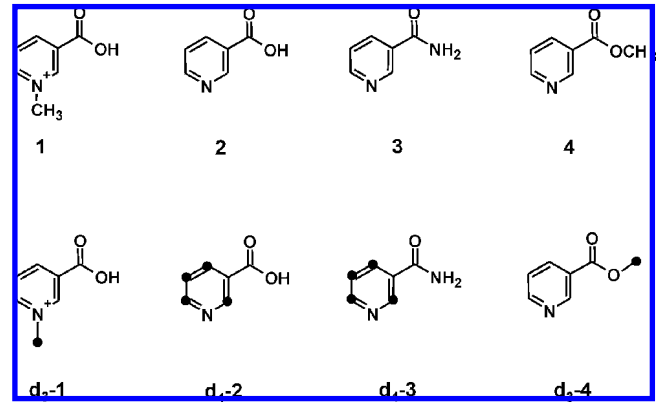

Figure 1. Chemical structures of trigonelline (**1**), nicotinic acid (**2**), nicotinamide (**3**), methyl nicotinate (**4**), and the internal standards d_3-trigonelline (**d_3-1**), d_4-nicotinic acid (**d_4-2**), d_4-nicotinamide (**d_4-3**), and d_3-methyl nicotinate (**d_3-4**), respectively. Deuterium-labeled carbon atoms are indicated by black dots.

* Corresponding author phone: +49-8161/71-2902; fax +49-8161/71-2949; e-mail: thomas.hofmann@wzw.tum.de.
[†] Technische Universität München,
[‡] Technische Universität Hamburg Harburg

10.1021/jf802838s CCC: $40.75
Published on Web 11/14/2008

Table 1. Monitored Mass Transitions, MS/MS Parameters, and Retention Times of Trigonelline (**1**), Nicotinic acid (**2**), Nicotinamide (**3**), Methyl Nicotinate (**4**), and the Corresponding Stable Isotope Labeled Internal Standards

compound	mass transition (*m/z*)	DP[a]	CEP[b]	CE[c]	CXP[d]	RT (min)[e]
trigonelline (**1**)	138→94[f] 138→92	46	10	8[g] 27[h]	4	1.69
d_3-trigonelline (**d_3-1**)	141→97[f] 141→95	46	10	8[g] 27[h]	3	1.67
nicotinic acid (**2**)	124→80[f] 124→78	41	11.5	29 31	4	2.05
d_4-nicotinic acid (**d_4-2**)	128→84[f] 128→81	36	10	29	4	2.01
nicotinamide (**3**)	123→80[f] 123→78	46	10	25 33	4	3.10
d_4-nicotinamide (**d_4-3**)	127→84[f] 127→83	36	4	27 29	4	2.96
methylnicotinate (**4**)	138→79[f] 138→78	46	7.5	37 31	4	8.23[l]
d_3-methylnicotinate (**d_3-4**)	141→79[f] 141→78	46	7.5	37 31	4	8.22[l]

[a] Declustering potential. [b] Cell entrance potential. [c] collision energy. [d] Cell exit potential. [e] Retention time. [f] Mass transition used for quantitation. [g] Collision energy used for the analysis of trigonelline in coffee, food, and urine. [h] Optimized collision energy used for plasma analysis. [l] Retention time taken from the extended HPLC-method.

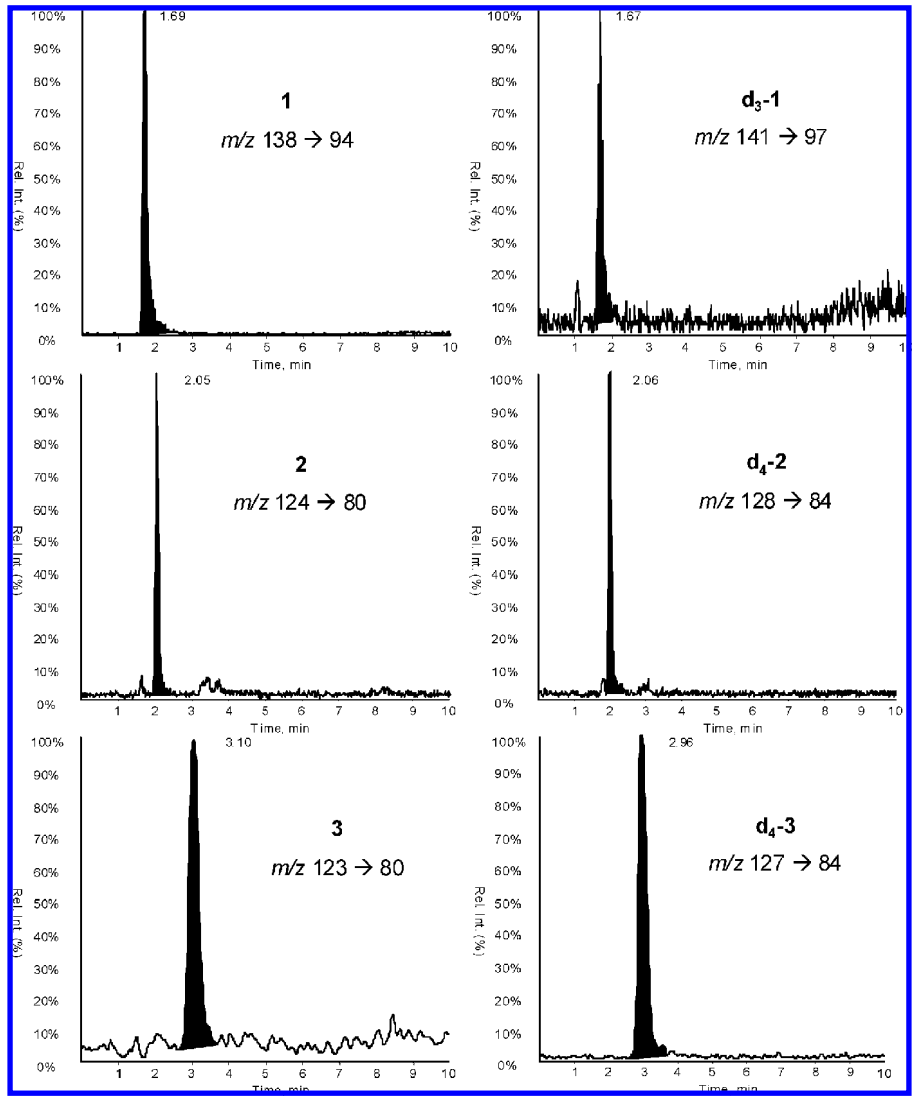

Figure 2. MS/MS chromatogram of trigonelline (**1**), nicotinic acid (**2**), nicotinamide (**3**) and the internal standards d_3-trigonelline (**d_3-1**), d_4-nicotinic acid (**d_4-2**), and d_4-nicotinamide (**d_4-3**) in HPLC-MS/MS analysis of a coffee extract after RP18-SPE cleanup.

Minerals recommend an intake of at least 17 mg/day of niacin as sum of nicotinamide and nicotinic acid for males and 13 mg/day for females to prevent hypovitaminoses (*5*). As nicotinic acid (**2**) is known to be liberated from the alkaloid trigonelline (**1**) upon roasting of coffee beans (*6*), the consumption of coffee beverages as part of our diet might contribute to the daily vitamin B3 uptake. To answer the question as to how much the content of nicotinic acid (**2**) and its amide (**3**) in roasted coffee is accounting for the daily dietary uptake of vitamin B3 active compounds, accurate quantitative data are needed.

Quantitative analysis of niacin in foods is typically performed by means of RP-HPLC with UV detection after extensive sample cleanup (*7*). Furthermore, ion chromatography was introduced to analyze nicotinic acid and nicotinamide in meat products (*8*). Another working group performed HPLC-MS for the simultaneous quantitation of niacin, niacinamide, and nicotinuric acid in human plasma (*9*) utilizing quinoline-3-carboxylic acid as internal standard. Recently, HPLC-MS/MS approaches were reported to quantify nicotinamide among other water soluble vitamins using external calibration techniques or deuterium

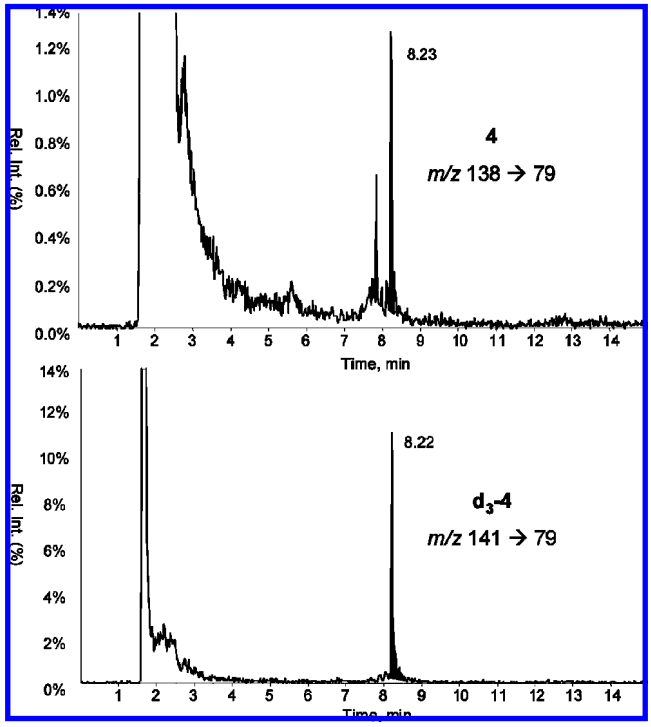

Figure 3. MS/MS chromatogram of methyl nicotinate (**4**) and d_3-methyl nicotinate (**d_3-4**) in HPLC-MS/MS analysis of directly injected coffee extract.

labeled nicotinamide as internal standard (*10, 11*). These methods were applied for the assessment of the vitamin content in multivitamin tablets, but not in foods or complex biological samples. Moreover, LC-MS with external calibration has been employed for quantitation of nicotinic acid beside trigonelline, sucrose and caffeine in coffee (*12*). However, in LC-MS analysis of challenging matrices, e.g., roast coffee or plasma samples, stable isotopically labeled internal standards are indispensable for the accurate quantitation, as they compensate the effects of coextracted compounds.

The purpose of the present investigation therefore was to develop a versatile and reliable stable isotope dilution assay (SIDA) for the quantitative determination of trigonelline, nicotinic acid, and its amide in complex samples such as roasted coffee. Thereafter, this method should be applied to quantitatively determine the influence of roasting time and temperature on the amounts of trigonelline, nicotinic acid, and nicotinamide in coffee. Finally, the compounds should be analyzed in some additional food samples as well as body fluids in order to confirm the feasibility of the SIDA developed.

MATERIALS AND METHODS

Chemicals. Nicotinic acid, nicotinic acid chloride hydrochloride, trigonelline hydrochloride, methyl nicotinate, nicotinamide, thionyl chloride, ammonia (2.0 mol/L in isopropanol), dichloroethane, ammonium acetate, d_4-nicotinic acid, d_3-methyl iodide, and d_4-methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, acetonitrile, ethanol, diethyl ether, sodium sulfate, formic acid, and sodium carbonate were from Merck (Darmstadt, Germany). Water was Millipore grade, methanol and acetonitrile were HPLC grade. Commercial food samples were purchased from a local retailer. Raw coffee beans (Arabica Brasil, Santos) used for preparation of the roasting series, and samples of raw and roasted ground coffee (Arabica Columbia) used for method development and quantitative analysis were provided by the German coffee industry. A standard coffee beverage was prepared using ground roast coffee (48 g) and water (900 mL) by means of a drip filter coffee machine (TCM, Germany). The plasma and urine samples were obtained from a healthy, voluntary coffee drinker.

Roasting of Coffee Beans. Aliquots (300 g) of coffee beans were roasted in a laboratory fluidized bed roaster (Novopak, Germany). In a first set of roasting experiments, the roast gas temperature was varied between 200 and 280 °C, and the roasting duration was kept constant for 240 s. In a second set of experiments, the beans were roasted for 60 s up to 600 s at a constant roast gas temperature of 260 °C. Air was used as a roast gas with a flow rate of 150 m^3/h. After roasting, the coffee beans were cooled to room temperature within 2 min by using air as the quench gas and were then stored in vacuum packages at 6 °C until use. Roasted coffee samples were weighed before and after roasting to determine the roast loss on a wet and dry basis, respectively. Prior to each roasting series as well as after each roasting experiment, the water content of the coffee beans was calculated from the weight difference of 10 g of the powdered coffee (five replicates) before and after drying in an oven for 16 h at 105 °C.

Synthesis of d_3-Trigonelline (d_3-1). Following a method reported in the literature (*13*), a mixture of d_3-methyl iodide (20 mmol) and nicotinic acid (5 mmol) in ethanol (25 mL) was refluxed for 4 h resulting in a clear yellow solution. The solution was cooled in an ice-bath, and the resulting suspension was filtered to obtain a yellowish crystalline solid. After recrystallization from ethanol, followed by a recrystallization from ethanol/water (95/5, v/v) and lyophilization, d_3-trigonelline hydroiodide (2.0 mmol, 40% in yield) was obtained as a yellowish powder with a high purity of more than 99%.

d_3-Trigonelline Hydroiodide, d_3-1 (Figure 1). MS(ESI^+): *m/z* (%) 141 (100, $[M]^+$); LC-MS/MS (ESI^+) (0.1% formic acid in water): *m/z* (%) 97 (88), 95 (74); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.23 [dd, 1H, $J = 8.04$ Hz, $J = 7.99$ Hz, H−C(5)], 8.90 [dd, 1H, $J = 8.08$ Hz, $J = 1.52$ Hz, H−C(4)], 9.17 [dd, 1H, $J = 6.06$ Hz, $J = 1.26$ Hz, H−C(6)], 9.45 [s, 1H, H−C(2)], 14.25 [bs, 1H, HOOC(7)]; ^{13}C NMR (100 MHz): δ (ppm) 47.47 [C(8)], 127.49 [C(6)], 130.19 [C(3)], 144.63 [C(5)], 146.57 [C(2)], 148.19 [C(4)], 162.70 [C(7)].

Synthesis of d_4-Nicotinamide (d_4-3). A mixture of d_4-nicotinic acid (1 mmol) and thionyl chloride (2 mL) was heated under reflux for 1 h. Thereafter, the excess of thionyl chloride was distilled off in a stream of nitrogen at 60 °C. The residue was suspended in a solution (5 mL) of ammonia in isopropanol (2.0 mol/L) and, again, refluxed for 1 h. Evaporation of the solvent in vacuum yielded an amorphous solid which was dissolved in water (5 mL) and, then, separated by preparative HPLC on a 250 × 21.5 mm i.d., 5 μm, Microsorb column (Varian, Darmstadt). Using a flow rate of 20 mL/min and monitoring the effluent at 254 nm, pure d_4-nicotinamide (0.72 mmol, 72% in yield) was obtained by isocratic chromatography with a mixture (99/1, v/v) of aqueous formic acid (0.1% in water) and methanol.

d_4-Nicotinamide d_4-3, Figure 1. MS(ESI^+): *m/z* (%) 127 (100, $[M+H]^+$); LC-MS/MS (ESI^+) (0.1% formic acid in water): *m/z* (%) 84 (25), 83 (20), 53 (5); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.62 [bs, N−H_a], 8.18 [bs, N−H_b]; ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 123.45 [C(5), $J_{C\text{-}D}\sim$ 29 Hz], 129.91 [C(3)], 135.22 [C(4), $J_{C\text{-}D}\sim$ 29 Hz], 148.78 [C(6), $J_{C\text{-}D}\sim$ 29 Hz], 151.92 [C(2), $J_{C\text{-}D}\sim$ 29 Hz], 166.87 [C(7)].

Synthesis of d_3-Methyl Nicotinate (d_3-4). d_4-Methanol (2 mL) was added to a suspension of nicotinic acid chloride hydrochloride (1 mmol) in dichloroethane (2 mL), and the mixture was refluxed for 2 h while stirring. After cooling, the solution was diluted with diethyl ether (100 mL), an aqueous solution of sodium carbonate (100 mL; 0.5 mol/L) was added, and after phase separation, the organic layer was washed with aqueous sodium carbonate (0.5 mol/L; 50 mL), followed by water (50 mL). The organic layer was freed from solvent in vacuum, the residue was taken up in water (10 mL), and then applied onto the top of a RP18 SPE-cartridge (1 g) (Varian, Darmstadt) conditioned with methanol (10 mL), followed by water (10 mL). After rinsing twice with water (10 mL each), the title compound d_3-methylnicotinate was eluted by flushing the cartridge twice with methanol (20 mL each). The methanolic effluent was freed from solvent in vacuum to yield the title compound (0.74 mmol; 74% in yield) as a crystalline powder in a purity of more than 98%.

Table 2. Results from Precision and Accuracy Experiments of the Developed Stable Isotope Dilution Analysis.

		compound no.		
		1	**2**	**3**
intraday precision (%)[a]		2.1%	1.1%	3.1%
interday precision (%)[b]		2.3%	1.5%	0.7%
accuracy (%)[c] addition level	+10%	104.5 (±2.06)	103.0 (±2.16)	104.3 (±2.91)
	+25%	102.2 (±2.58)	100.7 (±1.48)	100.6 (±3.65)
	+50%	104.3 (±1.81)	102.5 (±3.89)	98.5 (±2.64)
	+75%	103.5 (±1.45)	101.2 (±5.66)	99.4 (±2.85)
	+100%	103.8 (±4.91)	103.7 (±1.85)	101.0 (±3.24)

[a] Expressed as coefficient of variance ($n = 5$). [b] Expressed as deviation of mean values. [c] Agreement of measured and calculated concentration (±RSD, $n = 3$).

Table 3. Trigonelline (**1**), Nicotinic acid (**2**), Nicotinamide (**3**), and Methyl Nicotinate (**4**) in Coffee Samples[a]

	concentration (μmol/g) of compound no.			
coffee samples	**1**	**2**	**3**	4
roasted coffee (Arabica Columbia)[b]	41.82 (±2.1)	0.617 (±1.1)	0.029 (±3.1)	0.002 (±8.4)
raw coffee (Arabica Columbia)	55.67	0.057	0.006	na
commercial blend A	34.42	0.373	0.017	na
commercial blend B	57.01	0.941	0.019	na

[a] Data are means of duplicates with RSD < 5%. [b] Concentration based on fresh weight (±RSD, $n = 5$).

d_3-Methyl nicotinate, d_3-4, (Figure 1). MS(ESI^+): *m/z* (%) 141 (100, $[M+H]^+$); LC-MS/MS (ESI^+) (0.1% formic acid in water): *m/z* (%) 78 (45), 79 (40), 51 (25); ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.59 [dd, 1H, $J = 8.20$ Hz $J = 8.20$ Hz, H−C(5)], 8.30 [dd, 1H, $J = 8.0$ Hz, 2.0 Hz, H−C(4)], 8.84 [dd, 1H, $J = 4.7$ Hz, 1.5 Hz, H−C(6)], 9.10 [d, 1H, $J = 1.8$ Hz, H−C(2)]; ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 51.8 [C(8), $J_{C\text{-}D} = \sim 29$ Hz], 123.9 [C(5)], 125.5 [C(3)], 136.8 [C(4)], 149.9 [C(2)], 153.6 [C(6)], 165.2 [C(7)].

High Performance Liquid Chromatography/Mass spectrometry (HPLC-MS/MS). *Mass Spectrometry.* Analytes and internal standards were tuned using the automatic tuning tool provided by the Analyst 1.4.1 software (Sciex). Solutions of the individual compounds prepared in methanol/water (1/1, v/v) were infused using a syringe pump at a flow rate of 10 μL/min. For each compound two mass transitions were recorded. The ion source parameters were optimized for nicotinamide using flow injection analysis.

The Agilent 1200 Series HPLC-system (Agilent, Waldbronn, Germany) consisting of a pump, a degasser, and an autosampler was connected to a 3200 API triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt) equipped with an electrospray ionization (ESI) source running in positive ionization mode. The ion spray voltage was 5500 V. Nebulizer gas and curtain gas were nitrogen and set at 65 and 25 psi, respectively. Heater gas was set at 60 psi, the temperature was 450 °C. Detection was performed in multiple reaction monitoring (MRM) mode, recording the transition from the positively charged parent ion $[M+H]^+$ to the fragment ions after collision-induced dissociation. The declustering potential (DP), the cell entrance potential (CEP), the cell exit potential (CXP), and the collision energy (CE) were set as detailed in **Table 1**. The dwell time for each mass transition was 35 ms. The quadrupoles operated at unit mass resolution.

Chromatography. After sample injection (5 μL), chromatographic separation was carried out on a 150 × 2.5 mm i.d., 4 μm, Synergi Fusion RP80 Polar column (Phenomenex, Aschaffenburg, Germany) with gradient elution at a flow rate of 350 μL/min. Eluent A was 0.1% formic acid in methanol containing 5 mmol/L ammonium acetate, and eluent B was 0.1% formic acid in water containing 5 mmol/L ammonium acetate. For samples (coffee samples, rice, herring, liver), which were prefractionated by means of a RP18 SPE cartridge, eluent B was held at 100% for 1 min, then eluent A was increased linearly to 8% within 2.5 min, then to 60% within additional 1 min, followed by isocratic elution for 2 min.

Coffee extracts directly injected into the LC-MS/MS system for analysis of methyl nicotinate were chromatographed as follows: eluent B was held at 100% for 1 min, then eluent A was increased linearly to 8% within 2.5 min, then to 100% within additional 1 min, followed by isocratic elution for 4.5 min.

Standard Solutions. Standard solutions (1 mmol/L) of d_4-nicotinic acid (**d_4-2**), d_4-nicotinamide (**d_4-3**), and d_3-methylnicotinate (**d_3-4**) were prepared in acetonitrile/water (50/50, v/v), d_3-trigonelline (**d_3-1**) was dissolved in Millipore water and diluted to a final concentration of 5 or 1 mmol/L for analysis of coffee and plasma samples, respectively.

Calibration. The deuterated standards and the analytes were mixed in seven molar ratios from 0.1 to 10 keeping constant concentrations of the calibration standards **d_3-1** (100 nmol/mL), **d_4-2** (4 nmol/mL), **d_4-3** (2 nmol/mL), and **d_3-4** (0.4 nmol/mL). After LC-MS/MS analysis, 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 linear regression. The equations obtained were $y = 1.617x + 0.111$ (**1/d_3-1**, $R^2 = 0.999$), $y = 0.745x + 0.072$ (**2/d_4-2**, $R^2 = 1.000$), $y = 1.199x + 0.072$ (**3/d_4-3**, $R^2 = 0.999$), and $y = 0.991x + 0.052$ (**4/d_3-4**, $R^2 = 0.993$), respectively.

Quantitative Analysis of Compounds 1−4 in Food and Human Samples. *Analysis of* **1-3** *in Coffee Samples.* The powdered coffee sample (0.5−1 g) was placed in a centrifugation tube (18 mL) (Schott AG, Mainz, Germany), spiked with the internal standards **d_3-1** (5 μmol), **d_4-2** (200 nmol), and **d_4-3** (100 nmol) and, after adding water (9 mL) and hydrochloric acid (100 μL; 1 mol/L), the centrifugation tube was tightly closed and incubated for 10 min at 60 °C with vigorous stirring. Thereafter, the suspension was pressed through a membrane filter (0.45 μm) to give a clear filtrate. An aliquot (1 mL) of the filtrate was applied onto the top of a RP18 SPE-cartridge (1 g, 6 mL) (Varian, Darmstadt) conditioned with methanol and water (5 mL each). The eluate obtained by rinsing the cartridge twice with aqueous formic acid (15% in water, 3 mL each) was diluted with water to a final volume of 10 mL, and an aliquot (5 μL) was analyzed by means of LC-MS/MS.

Analysis of ***4*** *in Coffee Samples.* For analysis of methyl nicotinate (4), coffee powder (0.5−1 g) was spiked with d_3-4 (10 nmol), stirred with aqueous hydrochloric acid (10 mmol/L, 10 mL) for 20 min. After membrane-filtration (0.45 μm), an aliquot (5 μL) was injected into the LC-MS/MS-system.

Analysis of ***1−3*** *in Other Food Samples.* Samples of liver, herring, cereals, and rice were homogenized in a blender, aliquots (0.5−1 g) were spiked with the internal standards **d_3-1** (5 μmol), **d_4-2** (200 nmol), and **d_4-3** (100 nmol), water (9 mL) and hydrochloric acid (1 mol/L, 100 μL) were added, and, after thorough mixing, the samples were incubated for 10 min at 60 °C. After a RP18-SPE cartridge cleanup as detailed above, an aliquot (5 μL) was analyzed by means of LC-MS/MS.

Analysis of ***1−3*** *in Plasma and Urine.* A human urine sample (1 mL) was spiked with the internal standards **d_3-1** (5 μmol), **d_4-2** (200 nmol), and **d_4-3** (100 nmol), diluted with water in a ratio of 1:100, and an aliquot (5 μL) was injected into the LC-MS/MS system. A human plasma sample (100 μL) was spiked with the internal standards **d_3-1**

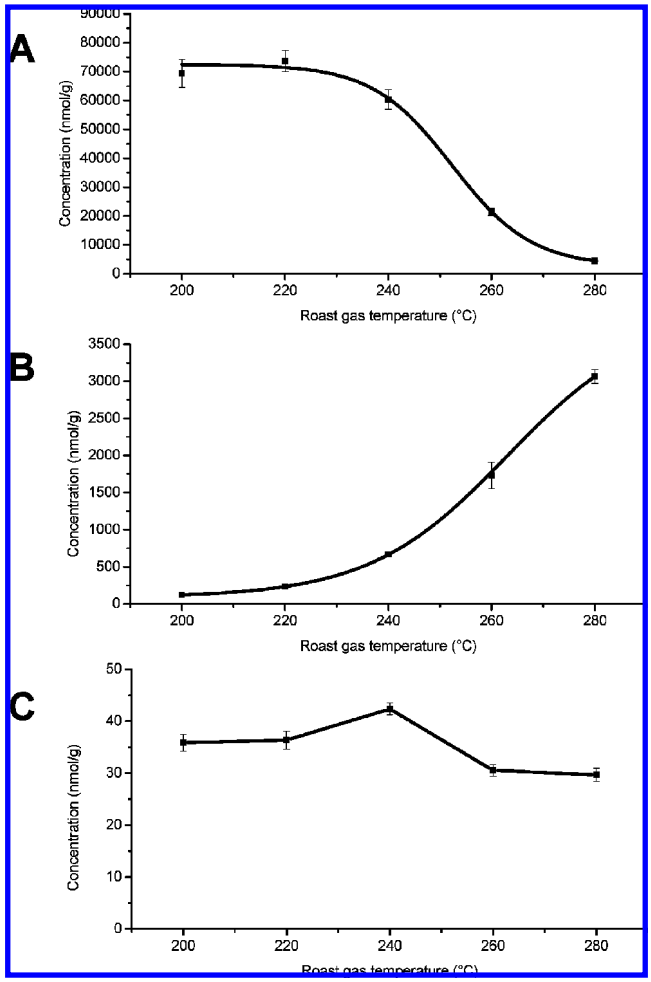

Figure 4. Influence of the roast gas temperature on the concentrations of (**A**) trigonelline, (**B**) nicotinic acid, and (**C**) nicotinamide in coffee (Arabica Brazil Santos) roasted for 240 s; the roast loss of the beans obtained by roasting at 200 (6.999/1.893), 220 (8.586/1.770), 240 (12.230/4.347), 260 (15.864/9.407) and 280 °C (23.717/16.990) are given in brackets (% based on wet/dry weight). Concentrations are related to dry weight (±RSD; $n = 3$).

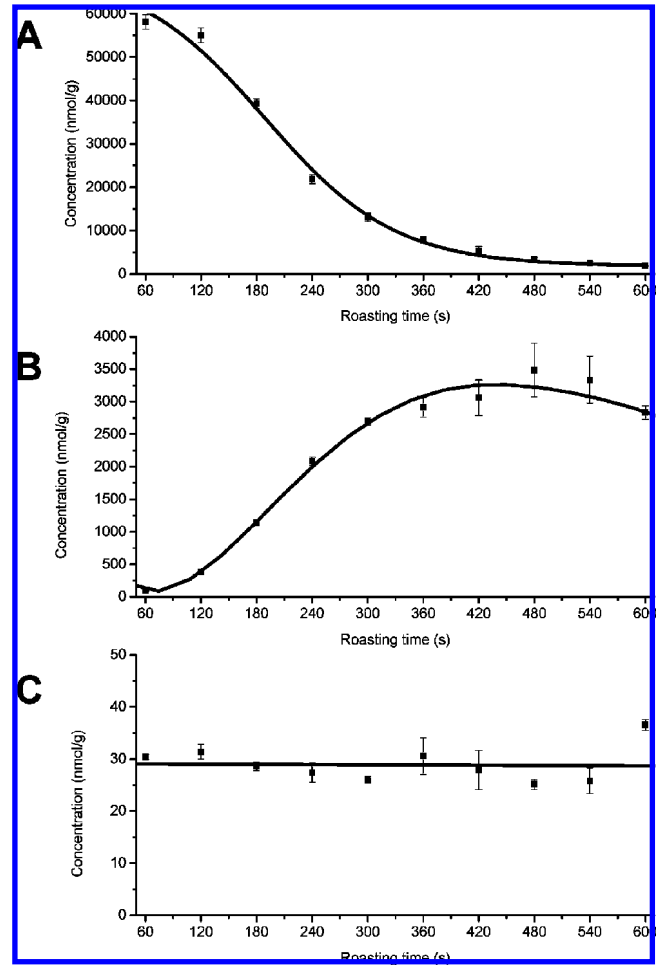

Figure 5. Influence of the roasting time on the concentrations of (**A**) trigonelline, (**B**) nicotinic acid, and (**C**) nicotinamide in coffee (Arabica Brazil Santos) roasted at 260 °C; the roast loss of the beans obtained by roasting at 260 °C for 60 (4.840/1.591), 120 (10.604/4.348), 180 (13.426/6.453), 240 (16.135/9.442), 300 (18.295/11.850), 360 (20.002/13.353), 420 (19.654/12.960), 480 (22.575/15.925), 540 (23.648/16.857), and 600 s (22.963/16.437) are given in brackets (% based on wet/dry weight). Concentrations are related to dry weight (±RSD; $n = 3$).

(20 nmol), **d_4-2** (20 nmol), and **d_4-3** (2 nmol) and stirred at room temperature for 10 min. Proteinaceous material was precipitated by the addition of methanol (400 μL) and, after membrane filtration, the solution was evaporated in vacuum. The residue was taken up in water (200 μL), and aliquots (5−10 μL) were analyzed by means of LC-MS/MS. For these analyses, the collision energy of trigonelline and d_3-trigonelline was set to its optimum as given in **Table 1**.

Precision and Accuracy Experiments. Five aliquots of the same sample of roast coffee powder (Arabica Columbia) were pretreated and analyzed for trigonelline (**1**), nicotinic acid (**2**), and nicotinamide (**3**) as detailed above. This experiment was repeated on the following day to investigate interday precision. For determination of accuracy, aliquots of coffee powder (10 g) were spiked with aqueous solutions containing defined amounts of **1**−**3** and, after homogenization, the samples were lyophilized for 48 h and aliquots (500 mg) of the spiked coffee samples were analyzed in triplicate using the protocol detailed above.

Nuclear Magnetic Resonance Spectrometry (NMR). ^{1}H- and ^{13}C-experiments were performed on a Bruker AMX-400 instrument (Bruker, Rheinstetten, Germany). Chemical shifts are related to d_5-DMSO (quintet at 2.49 ppm relative to tetramethylsilane).

RESULTS AND DISCUSSION

To accurately determine the amounts of trigonelline (**1**), nicotinic acid (**2**), nicotinamide (**3**), and methyl nicotinate (**4**) in foods and biological samples, a stable isotope dilution assay (SIDA) with LC-MS/MS detection should be developed. Since only nicotinic acid was commercially available as a deuterated isotopologue, first, corresponding isotopologues of **1**, **3**, and **4** needed to be synthesized.

Table 4. Trigonelline (1), Nicotinic acid (2), Nicotinamide (3) in Selected Food Samples, Human Urine, and Plasma

	concentration of compound (no).[a]		
samples	1	2	3
breakfast cereals	0.180	0.028	1.323
parboiled rice	0.010	0.046	
pickled herring		0.023	0.158
beef liver		0.047	1.426
human plasma	0.001 (±2.3)[b,c]		0.001 (±7.8)[b]
human urine	0.530 (±4.9)[b]	0.203 (±5.3)[b]	0.004 (±7.7)[b]

[a] Concentration given in μmol/g, data are means of duplicates (RSD <5%) unless otherwise indicated. [b] Concentration in μmol/mL (±RSD, $n = 3$). [c] Collision energy of trigonelline mass transitions were set to the optimum for enhanced sensitivity.

Synthesis of Deuterated Internal Standards. For the preparation of deuterated trigonelline, nicotinic acid (**2**) was *N*-methylated by heating in the presence of excess amounts of d_3-methyl iodide. After several recrystallization steps, the d_3-

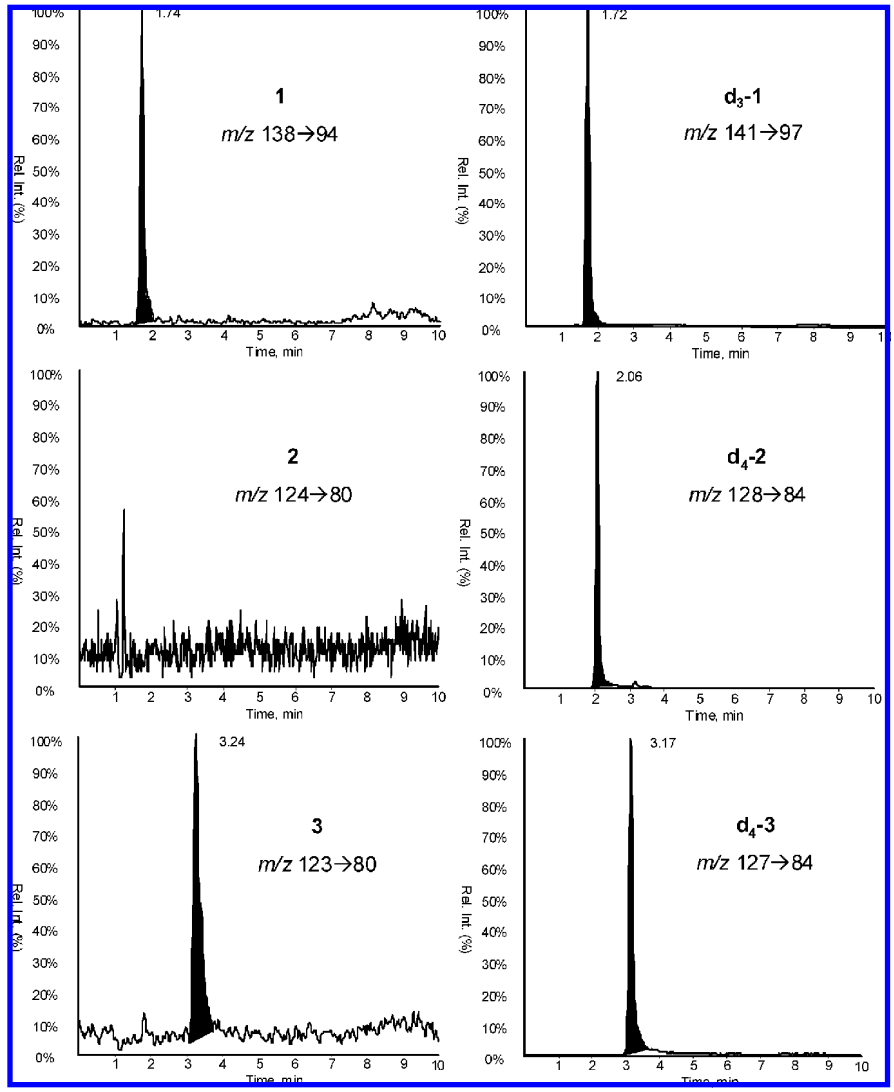

Figure 6. MS/MS chromatograms of trigonelline (**1**), nicotinic acid (**2**), nicotinamide (**3**) and the internal standards d_3-trigonelline (**d_3-1**), d_4-nicotinic acid (**d_4-2**), and d_4-nicotinamide (**d_4-3**) in human plasma.

trigonelline hydroiodide (**d_3-1**) was obtained in a yield of 40% with a purity of more than 99.9% as checked by HPLC-MS/MS. After activation of d_4-nicotinic acid with thionyl chloride, reaction with ammonia in isopropanol enabled the synthesis of d_4-nicotinamide (**d_4-3**) which was purified by means of preparative RP-HPLC to afford the target compound in a yield of 72.4%. In addition, d_3-methyl nicotinate (**d_3-4**) was prepared by methanolysis of nicotinic acid chloride hydrochloride with d_3-MeOD, followed by a RP18 SPE cartridge cleanup.

To check the stability of the labeled compounds under aqueous acidic conditions, the deuterated compounds were individually incubated in aqueous hydrochloric acid (10 mmol/L) and incubated for 10 min at 60 °C, and, after lyophilization, were then analyzed by means of ^{1}H NMR spectroscopy (data not shown). As no significant C-D/C−H exchange could be observed under these conditions, the suitability of the labeled compounds as internal standards for the stable isotope dilution assay (SIDA) could be unequivocally confirmed.

Development of a HPLC-MS/MS SIDA Method. For tuning the LC-MS system, the internal standards **d_3-1, d_4-2, d_4-3**, and **d_3-4** and the analytes **1**−**4** were individually infused into the mass spectrometer using a syringe pump. Optimum intensities of pseudomolecular ions and respective fragments (**Table 1**) were obtained by software-assisted ramping of the declustering potential, the cell entrance potential, the collision energy, as well as the cell exit potential. The ion source parameters including the flow and temperature of the curtain gas, heater gas, and nebulizer gas were automatically optimized for nicotinamide using flow injection analysis. Because of the expected high abundance of trigonelline relative to nicotinic acid and nicotinamide in roasted coffee, the collision energy for the mass transitions of trigonelline was manually decreased to reduce the detected frequency of daughter ions as well as the signal intensity for trigonelline in the mass chromatogram. By doing this, it was possible to quantitatively determine all the analytes within a single run without exceeding the range of linearity of the MS detector for trigonelline.

To convert the measured ion intensities into the mass ratios of labeled and nonlabeled analytes, a graph was calculated from calibration mixtures of known mass ratios and the corresponding peak area ratios in HPLC-MS/MS. To achieve this, fixed amounts of the labeled compounds were mixed with the analytes in ratios of 0.1−10 on a molar basis and the quotients of area internal standard/area analyte obtained by HPLC-MS/MS were plotted against the quotient concentration analyte/concentration internal standard.

Analysis of 1−4 in Coffee Powder. For the quantitative analysis of nicotinic acid, trigonelline, nicotinamide, and methyl nicotinate, ground roasted coffee was spiked with defined amounts of the internal standards **d_3-1**, **d_4-2**, **d_4-3**, and **d_3-4**,

and, after the addition of aqueous hydrochloric acid, was stirred for 10 min at 60 °C. After filtration, lipophilic components as well as brown colored melanoidins were removed from the liquid extract by means of RP18 SPE cleanup. The colorless eluate obtained was then directly analyzed by means of HPLC-MS/MS. RP-HPLC-MS/MS analysis showed that even without any further sample cleanup, the mass chromatograms obtained for the individual analytes **1**−**3** and the corresponding internal standards looked rather clean as no major peaks appeared within the respective traces of analytes and internal standards (**Figure 2**). However, the concentration of methyl nicotinate in the coffee sample was found to be below the detection limit (data not shown).

In order to gain a first estimation of the amounts of methyl nicotinate in roasted coffee, the solution obtained by extracting coffee with aqueous hydrochloric acid was directly injected into the HPLC-MS/MS system after membrane filtration. As illustrated in **Figure 3**, methyl nicotinate (**4**) was detectable at a retention time of 8.2 min. The comparison of the small peak of the methyl nicotinate with the huge signal detected for trigonelline between 1.5 and 3.0 min, however, showed first evidence that this methyl ester is present in coffee only in trace amounts. From the area of the peak of the analyte **4** and the peak area of **d_3-4** spiked to the coffee in defined amounts, an average concentration of 0.002 μmol/g (±8.4%, $n = 5$) was found for methyl nicotinate in roasted coffee. As the analysis of other coffee samples revealed this methyl ester in similar low concentrations (data not shown), the studies on the performance assessment of the method as well as further quantitative analyses were focused on the analysis of trigonelline, nicotinic acid, and nicotinamide, respectively.

Method Performance. In order to check the performance of the analytical method, intra-assay precision of the SIDA was determined by analysis of trigonelline, nicotinic acid, and nicotinamide in five aliquots of the same sample of coffee powder. The coefficient of variation was 2.1% for trigonelline, 1.1% for nicotinic acid, and 3.1% for nicotinamide (**Table 2**). For assessment of the interday precision, the same coffee sample was analyzed on the following day. The results deviated by 2.3% (trigonelline), 1.5% (nicotinic acid), and 0.7% (nicotinamide).

In addition, accuracy experiments were conducted by spiking 10, 25, 50, 75, or 100% of the initial content of the analytes to roast coffee powder samples and, after adding water, the samples were homogenized and lyophilized. The unspiked as well as the spiked samples were then analyzed in triplicate, and the measured concentrations were compared with the calculated concentration. Accuracy, calculated as the agreement (%) of measured and calculated concentration was 102.2 −104.5% for trigonelline, 100.7−103.7% for nicotinic acid, and 98.5−104.3% for nicotinamide (**Table 2**). These data clearly demonstrate the developed SIDA as a reliable tool enabling a rapid and accurate quantitative determination of trigonelline, nicotinic acid, and nicotinamide in foods.

Using the developed SIDA, compounds **1**−**3** were quantitatively determined in the raw coffee beans as well as in two commercial coffee blends. In all the coffee samples, trigonelline was found in high concentrations ranging from 34.42 to 57.01 μmol/g (**Table 3**). In comparison, about 100-fold lower amounts were found for nicotinic acid (0.057−0.941 μmol/g), and nicotinamide was present just in trace amounts. Interestingly, comparison of the raw and roasted coffee sample indicated that nicotinic acid and, to a minor extent, also nicotinamide seem to be generated upon roasting.

Influence of Roasting on the Concentrations of 1−3 in Coffee. In order to gain a more detailed insight into the influence of the roast gas temperature on the amounts of trigonelline, nicotinic acid, and nicotinamide, respectively, in a first set of experiments, five equal coffee samples were roasted for 240 s at 200, 220, 240, 260, and 280 °C, respectively, and compounds **1**−**3** were quantitatively determined by means of the developed SIDA (**Figure 4**). The results showed that roasting for 240 s at 200 and 220 °C, respectively, did not result in a significant difference of the trigonelline content in the coffee sample (**Figure 4A**). However, increasing the roast gas temperature to 240 °C and above induced a substantial decomposition of **1** (**Figure 4A**) running in parallel with a nearly exponential increase of the concentration of nicotinic acid (**Figure 4B**), e.g., raising the roast gas temperature from 200 to 280 °C increased the concentration of nicotinic acid (**2**) from 0.120 (±5.9%) to 3.070 (±3%) μmol per g coffee. In contrast, the concentration of nicotinamide was found to be rather stable and seem not to be strongly influenced by the roasting temperature, e.g., only a slight decrease from 0.035 (±4.5%) to 0.029 (±11%) μmol/g was observed when the roasting temperature was increased from 200 to 280 °C (**Figure 4C**).

In a second set of experiments, ten coffee samples were roasted at a constant roast gas temperature of 260 °C for 60−600 s and the target compounds **1**−**3** were quantitatively determined by means of the developed SIDA (**Figure 5**). Trigonelline was found to be rather unstable when the roasting time is increased, e.g., the concentration of **1** was reduced by about 38% after 240 s and approximated a minimum concentration of 2 μmol/g above roasting times of 8 min (**Figure 5A**). In parallel to the trigonelline degradation, a strong increase in the amount of nicotinic acid (**2**) was observed when the roasting time was increased from 120 to 360 s, e.g., the concentration of **2** increased by a factor of about 8 from 0.380 (±0.5%) to 2.910 (±5.7%) μmol/g (**Figure 5B**). In contrast, the amount of nicotinamide in the coffee samples centered around 0.030 μmol/g and was not strongly influenced by the roasting time (**Figure 5C**); for example, the minimum amount found was 0.025 μmol/g (±13%) after 480 s and the maximum level was 0.036 μmol/g (±2.9%) measured after roasting for 600 s.

The data obtained demonstrate that the nicotinic acid content of coffee is strongly dependent on the time and temperature of coffee roasting and is accompanied by the degradation of its precursor trigonelline. As the levels of nicotinic acid are up to hundred times above the nicotinamide concentrations, depending on the roasting degree, it might be concluded that it is mainly the nicotinic acid which contributes to the niacin uptake upon coffee consumption.

Quantitative Analysis of 1−3 in Selected Foods. We next used the developed SIDA for the quantification of compounds **1**−**3** in some other foods (**Table 4**). As examples, the starch containing foods breakfast cereals and rice, and protein rich samples like beef liver and pickled herring were used. No interference of matrix components could be observed in the MRM-traces of any of the samples analyzed (data not shown). Application of the SIDA method revealed a content of 0.028 μmol/g nicotinic acid and 1.323 μmol/g nicotinamide in a sample of breakfast cereals representing a total of 16.5 mg/100 g niacin in the sample and matching well the amount declared by the manufacturer in the ingredients list. In addition, trigonelline could be found in breakfast cereals in amounts of 0.18 μmol/g. Analysis of a sample of parboiled rice revealed nicotinic acid and trigonelline in a concentration of 0.046 and 0.01 μmol/g, respectively, whereas nicotinamide was not detectable (**Table**

4). In contrast, the sample of beef liver was found to contain nicotinamide in a high concentration of 1.426 μmol/g, whereas pickled herring contained comparatively low amounts of nicotinamide (0.158 μmol/g) and nicotinic acid (0.023 μmol/g), respectively.

Quantitative Analysis of 1−3 in Plasma and Urine Samples. The following experiments were done to demonstrate that the SIDA method developed is also suitable for the quantitative analysis of **1**−**3** in biological samples. After tuning the collision energy of the spectrometer to its optimum (**Table 1**), a plasma sample obtained from a voluntary healthy coffee drinker was spiked with defined amounts of the internal standards **d_3-1**, **d_4-2**, and **d_4-3**, and after equilibration, proteinaceous material was precipitated by the addition of methanol. The clear filtrate was diluted with water, and then injected into the LC-MS/MS system. As shown in the LC-MS chromatograms in **Figure 6**, trigonelline (**1**) as well as nicotinamide (**3**) were detectable in the plasma sample, but not even trace amounts of nicotinic acid were found. Quantitative analysis revealed a concentration of 0.001 μmol/mL for trigonelline as well as nicotinamide (**Table 4**).

In addition, a human urine obtained from a voluntary healthy coffee drinker was spiked with defined amounts of the internal standards **d_3-1**, **d_4-2**, and **d_4-3**, diluted with water and was then injected into the LC-MS/MS system. The quantitative data obtained revealed trigonelline (**1**) and nicotinic acid (**2**) in rather high concentrations of 0.53 and 0.203 μmol/mL, whereas nicotinamide (**3**) was present in comparatively low levels of 0.004 μmol/mL.

Whereas previously reported LC/MS-SIDA methods were only applied to multivitamin tablets and were not validated for more complex food and biological samples (*11*), the stable isotope dilution assay developed in the present paper allows the quantitative determination of trigonelline (**1**), nicotinic acid (**2**), and nicotinamide (**3**) within 10 min in rather challenging samples such as, roasted coffee and human plasma, respectively.

ACKNOWLEDGMENT

We are grateful to the German federal ministry BMBF (project no. 0313843) for funding this research in part, and thank Ms. Christine Kotyczka, Deutsche Forschungsanstalt für Lebensmittelchemie (DFA), for the donation of plasma samples.

LITERATURE CITED

(1) Viani, R.; Horman, I. Thermal behaviour of trigonelline. *J. Food Sci.* **1974**, *39*, 1216–1217.
(2) Stadler, R. H.; Varga, N.; Hau, J.; Vera, F. A.; Welti, D. Alkylpyridiniums. 1. Formation in model systems via thermal degradation of trigonelline. *J. Agric. Food Chem.* **2002**, *50*, 1192–1199.
(3) Stadler, R. H.; Varga, N.; Milo, C.; Schilter, B.; Vera, F. A.; Welti, D. Alkylpyridiniums. 2. Isolation and quantification in roasted and ground coffees. *J. Agric. Food Chem.* **2002**, *50*, 1200–1206.
(4) Somoza, V.; Lindenmeier, M.; Wenzel, E.; Frank, O.; Erbersdobler, H. F.; Hofmann, T. Activity-guided identification of a chemopreventive compound in coffee using in vitro and in vivo techniques. *J. Agric. Food Chem.* **2003**, *51*, 6861–6869.
(5) *Experts Group on Vitamins and Minerals, Review of Niacin*; London Food Standard Agency: London, 2002; http://www.food.gov.uk/multimedia/pdfs/evm-01-11r.pdf.
(6) Smith, R. F. Niacin content in coffee. *Nature* **1963**, *197*, 1321.
(7) Juraja, S. M.; Trenerry, V. C.; Millar, R. G.; Scheelings, P.; Buick, D. R. Asia Pacific food analysis network (APFAN) training exercise: the detemination of niacin in cereals by alkaline extraction and high performance liquid chromatography. *J. Food Comp. Anal.* **2003**, *16*, 93–106.
(8) Saccani, G.; Tanzi, E.; Mallozzi, S.; Cavalli, S. Determination of niacin in fresh and dry cured pork products by ion chromatography: experimental design approach for the optimisation of nicotinic acid separation. *Food Chem.* **2005**, *92*, 373–379.
(9) Pfuhl, P.; Kärcher, U.; Häring, N.; Baumeister, A.; Tawab, M. A.; Schubert-Zsilavecz, M. Simultaneous determination of niacin, niacinamide and nicotinuric acid in human plasma. *J. Pharm. Biomed. Anal* **2005**, *36*, 1045–1052.
(10) Chen, P.; Wolf, W. R. LC/UV/MS-MRM for the simultaneous determination of water-soluble vitamins in multi-vitamin dietary supplements. *Anal. Bioanal. Chem.* **2007**, *387*, 2441–2448.
(11) Chen, P.; Ozcan, M.; Wolf, W. R. Contents of selected B vitamins in NIST SRM 3280 multivitamin/multielement tablets by liquid chromatography isotope dilution mass spectrometry. *Anal. Bioanal. Chem.* **2007**, *389*, 343–347.
(12) Perrone, D.; Donangelo, C. M.; Farah, A. Fast simultaneous analysis of caffeine, trigonelline, nicotinic acid and sucrose in coffee by liquid chromatography-mass spectrometry. *Food Chem.* **2008**, *110*, 1030–1035.
(13) Ciusa, W.; Nebbia, G. The preparation of salts of *N*-methylnicotinic acid. *Gaz. Chim. Ital.* **1950**, *80*, 98–99.

Received for review September 12, 2008. Revised manuscript received October 27, 2008. Accepted October 27, 2008.

JF802838S