JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Determination of the Alkylpyrazine Composition of Coffee Using Stable Isotope Dilution–Gas Chromatography–Mass Spectrometry (SIDA-GC-MS)

Stephanie Pickard, Irina Becker, Karl-Heinz Merz, and Elke Richling*

Food Chemistry and Toxicology, Department of Chemistry, University of Kaiserslautern, Erwin-Schroedinger-Strasse 52, 67663 Kaiserslautern, Germany

ABSTRACT: A stable isotope dilution analysis based on gas chromatography–mass spectrometry analysis (SIDA-GC-MS) was developed for the quantitative analysis of 12 alkylpyrazines found in commercially available coffee samples. These compounds contribute to coffee flavor. The accuracy of this method was tested by analyzing model mixtures of alkylpyrazines. Comparisons of alkylpyrazine-concentrations suggested that water as extraction solvent was superior to dichloromethane. The distribution patterns of alkylpyrazines in different roasted coffees were quite similar. The most abundant alkylpyrazine in each coffee sample was 2-methylpyrazine, followed by 2,6-dimethylpyrazine, 2,5-dimethylpyrazine, 2-ethylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, and 2,3,5-trimethylpyrazine, respectively. Among the alkylpyrazines tested, 2,3-dimethylpyrazine, 2-ethyl-3-methylpyrazine, 2-ethyl-3,6-dimethylpyrazine, and 2-ethyl-3,5-dimethylpyrazine revealed the lowest concentrations in roasted coffee. By the use of isotope dilution analysis, the total concentrations of alkylpyrazines in commercially available ground coffee ranged between 82.1 and 211.6 mg/kg, respectively. Decaffeinated coffee samples were found to contain lower amounts of alkylpyrazines than regular coffee samples by a factor of 0.3–0.7, which might be a result of the decaffeination procedure.

KEYWORDS: *coffee, alkylpyrazines, SIDA, GC-MS, decaffeinated coffee, extraction solvent*

■ INTRODUCTION

Coffee is one of the most popular beverages, and it is estimated that more than two billion cups are consumed worldwide every day.[1] The aroma of coffee is important for the quality and success of this popular drink and has been the subject of much research for more than two centuries. Most aroma compounds such as alkylpyrazines are formed during the roasting process as a result of the Maillard reaction between sugar and amino acids.[2] Alkylpyrazines can also arise from the pyrolysis of serine and threonine[3] and partially contribute to coffee flavor.[4] Among the steam-volatile compounds in coffee, alkylpyrazines are quantitatively the second largest fraction, accounting for 25–30%, furans being the largest.[5] In 2001 Sanz et al. identified 22 different pyrazines in coffee samples using static headspace–gas chromatography–mass spectrometry (SH-GC-MS).[6] The disubstituted derivates 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, and 2-ethyl-3-methylpyrazine showed in gas chromatography–olfactometry experiments a nutty odor and the trisubstituted derivates 2-ethyl-3,6-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2-ethyl-3,5-dimethylpyrazine a roasty or roasty/earthy odor.[7] The latter two have already been identified as potent odorants in coffee powder and brew.[4,8−11]

There are several publications describing the importance of Maillard reaction products as antioxidative compounds in coffee extracts,[12,13] but it is still not fully elucidated which group of Maillard reaction products exhibit antioxidative properties. Tetramethylpyrazine, also designated ligustrazine, is one of the most important active ingredients of the Chinese herb *Ligusticum wallichii*. In vitro experiments indicated that tetramethylpyrazine has antioxidative and anti-inflammatory properties, and in vivo experiments revealed its neuroprotective properties.[14−18] Therefore, structure-related alkylpyrazines from coffee may exhibit similar properties and contribute to the diverse physiological action of coffee. To date, there are only a few publications dealing with the quantitative composition of alkylpyrazines in coffee.[5,10,11,19−22] The quantitative analysis of these compounds in the complex coffee matrix is difficult to handle. These difficulties can be overcome by the use of stable isotopically labeled standards to perform isotope dilution analysis.[23] In this paper we describe the synthesis of deuterium-labeled compounds and the optimization of the extraction procedure used for the quantitative analysis of 12 methyl- and ethyl-substituted alkylpyrazines, **1**–**12**, in 10 different commercially available ground coffee samples using stable isotope dilution analysis (SIDA-GC-MS).

■ MATERIALS

Coffee. Ten commercially available vacuum-packed ground coffee samples were purchased from local grocery markets in Germany. Among them, seven were declared as "regular" coffees including five of the Arabica variety (coffees 2, 3, 5, 6, and 10) and two Arabica/Robusta blends (coffees 1 and 4); three were declared as decaffeinated coffees including two of the Arabica variety (coffees 7 and 9) and one Arabica/Robusta blend (coffee 8). All of them were designed to be normal filter coffee with similar degrees of roast (medium) and also medium-scale grinding.

Semolina. Commercially available wheat semolina was purchased from a local grocery store in Germany. Semolina was used as model

Received: March 21, 2013
Revised: June 6, 2013
Accepted: June 8, 2013
Published: June 8, 2013

ACS Publications © 2013 American Chemical Society

matrix because it contains fat, proteins, and carbohydrates similar to coffee but none of the investigated alkylpyrazines.

Chemicals. Compounds **1**−**6**, **9**, and **10** (all of ≥98% purity) were purchased from Sigma-Aldrich (Munich, Germany). 2-Ethyl-6-methylpyrazine (**7**) and 2-ethyl-5-methylpyrazine (**8**) were purchased as a 60:40 mixture (both of ≥98% purity) from Sigma-Aldrich. 2-Ethyl-3,6-dimethylpyrazine (**11**) and 2-ethyl-3,5-dimethylpyrazine (**12**) were purchased as a 50:50 mixture (both of ≥98% purity) from Sigma-Aldrich. Stable isotope labeled standard [$^2H_4$]-pyrazine (98% isotopic purity) was obtained from Sigma-Aldrich. 3-[$^2H_5$]Ethyl-2,5-dimethylpyrazine (1 mg/mL in diethyl ether, 99% isotopic purity) was purchased from AromaLAB (Freising, Germany). *n*-Alkane standard solutions of C_8−C_{20} (mixture 04070) was purchased from Sigma-Aldrich.

Bromo[$^2H_5$]ethane (99% isotopic purity), 2-chloropyrazine, 2,3-dichloropyrazine, magnesium turnings, [$^2H_3$]-methylmagnesium iodide (99% isotopic purity), and sodium hydroxide were purchased from Sigma-Aldrich. 2-Bromo-5-methylpyrazine was from RihaChem (Prague, Czech Republic), and 2-chloro-3-methylpyrazine, 2-chloro-6-methylpyrazine, and iron(III) acetylacetonate were obtained from Thermo Fisher Scientific (Bremen, Germany). 3-Chloro-2,5-dimethylpyrazine was from TCI (Eschborn, Germany). Silica gel 60 (particle size 40−63 μm) was from Macherey Nagel (Dueren, Germany) and hydrochloric acid from J. T. Baker (Griesheim, Germany). Sodium sulfate was from Th. Geyer (Renningen, Germany). All solvents (diethyl ether, pentane, dichloromethane) were from Thermo Fisher Scientific, Merck (Darmstadt, Germany), Sigma-Aldrich, J. T. Baker, and Riedel-de Haën (Seelze, Germany).

EXPERIMENTAL PROCEDURES

Synthesis. The deuterated pyrazines were prepared and purified according to the method of Fuerstner et al.[24] with modifications.

2-[$^2H_3$]Methylpyrazine ([$^2H_3$]-2). 2-Chloropyrazine (1.60 g, 14.0 mmol) and iron(III) acetylacetonate (325 mg, 0.9 mmol) were dissolved in diethyl ether (80 mL). A solution of [$^2H_3$]-methylmagnesium iodide (1 M in Et_2O, 25 mL, 25 mmol) was added dropwise with stirring to the ice-cooled red solution, causing an immediate color change to dark brown. The mixture was stirred for 30 min at room temperature, diluted with Et_2O (20 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ∼25 mL), resulting in a yellow mixture. After extraction with diethyl ether (10 × 50 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 7 × 20 cm) using diethyl ether as eluent to achieve 0.72 g (52.9% yield) of the product as a yellow liquid: ^1H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, ^3J = 2.36 Hz, 2H), 8.38 (d, ^3J = 2.40 Hz, 1H); GC RI (DB-WAX) = 1246; MS (EI) *m*/*z* (relative intensity, %) 97 (100, $[M]^{•+}$), 70 (51), 53 (16), 45 (13), 43 (17), 42 (16), 41 (20), 40 (10), 28 (15), 26 (14).

2-Methyl-5-[$^2H_3$]methylpyrazine ([$^2H_3$]-3). 2-Bromo-5-methylpyrazine (1.99 g, 11.5 mmol) and iron(III) acetylacetonate (270 mg, 0.8 mmol) were dissolved in diethyl ether (70 mL). A solution of [$^2H_3$]-methylmagnesium iodide (1 M in Et_2O, 20 mL, 20 mmol) was added dropwise with stirring to the ice-cooled red solution, causing an immediate color change to brown. The mixture was stirred for 1.5 h at room temperature, diluted with Et_2O (20 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ∼20 mL), resulting in a yellow mixture. After extraction with diethyl ether (10 × 50 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel 7 × 18 cm) using a mixture of diethyl ether and pentane (1:1 v/v) as eluent to give 0.73 g (54.5% yield) of the product as a brown liquid: ^1H NMR (400 MHz, $CDCl_3$) δ 8.33 (s, 2H), 2.51 (s, 3H); GC RI (DB-WAX) = 1299; MS (EI) *m*/*z* (relative intensity, %) 111 (100, $[M]^{•+}$), 84 (14), 45 (52), 43 (56), 42 (16), 41 (20), 40 (16), 39 (27), 38 (10), 28 (26).

2-Methyl-6-[$^2H_3$]methylpyrazine ([$^2H_3$]-4). 2-Chloro-6-methylpyrazine (1.14 g, 8.8 mmol) and iron(III) acetylacetonate (220 mg, 0.6 mmol) were dissolved in diethyl ether (40 mL). A solution of [$^2H_3$]-methylmagnesium iodide (1 M in Et_2O, 14 mL, 14 mmol) was added dropwise with stirring to the ice-cooled red solution, causing an immediate color change to brown. The mixture was stirred for 1.5 h at room temperature, diluted with Et_2O (20 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ∼20 mL), resulting in a yellow solution. After extraction with diethyl ether (8 × 30 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5.5 × 17 cm) using diethyl ether as eluent to give 2.00 g (52.7% yield) of the product as an orange-brown liquid: ^1H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 2H), 2.53 (s, 3H); GC RI (DB-WAX) = 1304; MS (EI) *m*/*z* (relative intensity, %) 112 (8), 111 (100, $[M]^{•+}$), 45 (35), 43 (46), 42 (15), 41 (20), 40 (22), 39 (26), 38 (9), 28 (25).

2,3-Bis[[$^2H_3$]methyl]pyrazine ([$^2H_6$]-6). 2,3-Dichloropyrazine (0.86 g, 5.8 mmol) and iron(III) acetylacetonate (200 mg, 0.6 mmol) were dissolved in diethyl ether (40 mL). A solution of [$^2H_3$]-methylmagnesium iodide (1 M in Et_2O, 27 mL, 27 mmol) was added dropwise with stirring to the ice-cooled red solution, causing an immediate color change to brown. The mixture was stirred for 1.5 h at room temperature, diluted with Et_2O (20 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ∼20 mL), resulting in a yellow mixture. After extraction with diethyl ether (8 × 30 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5.5 × 17 cm) using diethyl ether as eluent to give 0.55 g (83.2% yield) of the product as an orange liquid: ^1H NMR (400 MHz, $CDCl_3$) δ 8.26 (s, 2H); GC RI (DB-WAX) = 1308; MS (EI) *m*/*z* (relative intensity, %) 114 (100, $[M]^{•+}$), 70 (99), 45 (25), 43 (22), 42 (24), 32 (16), 30 (15), 28 (52), 26 (12), 18 (13).

2,5-Dimethyl-3-[$^2H_3$]methylpyrazine ([$^2H_3$]-10). 3-Chloro-2,5-dimethylpyrazine (0.94 g, 6.6 mmol) and iron(III) acetylacetonate (164 mg, 0.5 mmol) were dissolved in diethyl ether (25 mL). A solution of [$^2H_3$]-methylmagnesium iodide (1 M in Et_2O, 15 mL, 15 mmol) was added dropwise with stirring to the ice-cooled red solution, causing an immediate color change to black. The mixture was stirred for 5 h at room temperature, diluted with Et_2O (20 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ∼20 mL), resulting in a yellow mixture. After extraction with diethyl ether (8 × 25 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5 × 30 cm) using a mixture of diethyl ether and pentane (1:1 v/v) as eluent to give 0.48 g (59.0% yield) of the product as an orange-brown liquid: ^1H NMR (400 MHz, $CDCl_3$) δ = 8.14 (s, 1H), 2.48 (s, 3H), 2.47 (s, 3H); GC RI (DB-WAX) = 1380; MS (EI) *m*/*z* (relative intensity, %) 125 (100, $[M]^{•+}$), 81 (20), 57 (11), 52 (9), 45 (40), 42 (79), 41 (9), 40 (21), 39 (32), 28 (19).

2-[$^2H_5$]Ethylpyrazine ([$^2H_5$]-5). To a stirred mixture of magnesium metal turnings (360 mg, 14.8 mmol) in diethyl ether (7 mL) were added a few drops of a solution containing bromo[$^2H_5$]-ethane (1.53 g, 13.4 mmol) in diethyl ether (5 mL) and a few crystals of iodine to initiate the reaction. Once the reaction began, the remaining solution of bromo[$^2H_5$]ethane in diethyl ether was added dropwise with cooling at a rate so as to maintain the exothermic reaction. The mixture was refluxed with stirring for 1 h and then cooled to room temperature. The resulting Grignard reagent was added dropwise to a stirred solution of 2-chloropyrazine (0.68 g, 5.9 mmol) and iron(III) acetylacetonate (180 mg, 0.5 mmol) in diethyl ether (30 mL) with cooling, causing an immediate color change to brown. The reaction mixture was stirred for 1 h at room temperature, diluted with Et_2O (15 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ∼20 mL), resulting in a yellow mixture. After extraction with diethyl ether (6 × 30 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5.5 × 30 cm) using a mixture of diethyl ether and pentane (1:1 v/v) as eluent to give 0.30 g (43.9% yield) of the product as an orange liquid: ^1H NMR (400 MHz, $CDCl_3$) δ 8.48 (m, 2H), 8.39 (d, ^3J = 2.32 Hz, 1H); GC RI (DB-WAX) = 1307; MS (EI) *m*/*z* (relative intensity, %) 113 (64, $[M]^{+•}$), 112 (14), 111 (100), 84 (12), 81 (16), 53 (13), 52 (15), 42 (12), 41 (15), 28 (34).

2-[$^2H_5$]Ethyl-6-methylpyrazine ([$^2H_5$]-7). To a stirred mixture consisting of magnesium metal turnings (428 mg, 17.6 mmol) in diethyl ether (10 mL) were added a few drops of a solution containing bromo[$^2H_5$]ethane (2.14 g, 18.8 mmol) in diethyl ether (7 mL) and a few crystals of iodine to initiate the reaction. Once the reaction began, the remaining solution of bromo[$^2H_5$]ethane in diethyl ether was added dropwise with cooling at a rate so as to maintain the exothermic reaction. The mixture was refluxed with stirring for 1 h and then cooled to room temperature. The resulting Grignard reagent was added dropwise to a stirred solution of 2-chloro-6-methylpyrazine (1.54 g, 11.9 mmol) and iron(III) acetylacetonate (213 mg, 0.6 mmol) in diethyl ether (40 mL) with cooling, causing an immediate color change to brown. The mixture was stirred for 1 h at room temperature, diluted with Et_2O (20 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ~20 mL), resulting in a yellow mixture. After extraction with diethyl ether (10 × 30 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5.5 × 18 cm) using a mixture of diethyl ether and pentane (1:3 up to 1:2 v/v) as eluent to give 0.67 g (43.8% yield) of the product as a yellow-orange liquid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (m, 2H), 2.53 (s, 3H); GC RI (DB-WAX) = 1358; MS (EI) m/z (relative intensity, %) 127 (62, $[M]^{\bullet+}$), 126 (12), 125 (100), 95 (17), 61 (12), 43 (7), 42 (9), 41 (12), 40 (11), 39 (19).

2-[$^2H_5$]Ethyl-5-methylpyrazine ([$^2H_5$]-8). To a stirred mixture consisting of magnesium metal turnings (1164 mg, 47.9 mmol) in diethyl ether (25 mL) were added a few drops of a solution containing bromo[$^2H_5$]ethane (5.00 g, 43.9 mmol) in diethyl ether (15 mL) and a few crystals of iodine to initiate the reaction. Once the reaction began, the remaining solution of bromo[$^2H_5$]ethane in diethyl ether was added dropwise with cooling at a rate so as to maintain the exothermic reaction. The mixture was refluxed with stirring for 30 min and then cooled to room temperature. The resulting Grignard reagent (25 mL) was added dropwise to a stirred solution of 2-bromo-5-methylpyrazine (1.00 g, 5.8 mmol) and iron(III) acetylacetonate (145 mg, 0.4 mmol) in diethyl ether (25 mL) with cooling, causing an immediate color change to brown. The mixture was stirred for 30 min at room temperature, diluted with Et_2O (20 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ~12 mL), resulting in a yellow mixture. After extraction with diethyl ether (8 × 15 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5.5 × 30 cm) using a mixture of diethyl ether and pentane (1:1 v/v) as eluent to give 0.16 g (22.2% yield) of the product as an orange-brown liquid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (m, 2H), 2.53 (s, 3H); GC RI (DB-WAX) = 1365; MS (EI) m/z (relative intensity, %) 127 (24, $[M]^{+\bullet}$), 126 (7), 125 (36), 61 (9), 41 (6), 40 (6), 39 (9), 32 (25), 28 (100), 18 (11).

2-[$^2H_5$]Ethyl-3-methylpyrazine ([$^2H_5$]-9). To a stirred mixture consisting of magnesium metal turnings (400 mg, 16.5 mmol) in diethyl ether (7 mL) were added a few drops of a solution containing bromo[$^2H_5$]ethane (1.78 g, 15.6 mmol) in diethyl ether (5 mL) and a few crystals of iodine to initiate the reaction. Once the reaction began, the remaining solution of bromo[$^2H_5$]ethane in diethyl ether was added dropwise with cooling at a rate so as to maintain the exothermic reaction. The mixture was refluxed with stirring for 30 min and then cooled to room temperature. The resulting Grignard reagent was added dropwise to a stirred solution of 2-chloro-3-methylpyrazine (1.15 g, 8.9 mmol) and iron(III) acetylacetonate (186 mg, 0.5 mmol) in diethyl ether (25 mL) with cooling, causing an immediate color change to brown. The mixture was stirred for 1.5 h at room temperature, diluted with Et_2O (50 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ~20 mL), resulting in a yellow mixture. After extraction with diethyl ether (10 × 30 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5 × 30 cm) using a mixture of diethyl ether and pentane (1:2 v/v) as eluent to give (0.25 g, 21.0% yield) of the product as a yellow liquid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (s, 1H), 8.28 (s, 1H), 2.57 (s, 3H); GC RI (DB-WAX) = 1376; MS (EI) m/z (relative intensity, %) 127 (100, $[M]^{\bullet+}$), 126 (52), 125 (66), 96 (9), 95 (9), 86 (10), 84 (15), 67 (21), 42 (14), 40 (9).

3-[$^2H_5$]Ethyl-2,5-dimethylpyrazine ([$^2H_5$]-11). To a stirred mixture consisting of magnesium metal turnings (1164 mg, 47.9 mmol) in diethyl ether (25 mL) were added a few drops of a solution containing bromo[$^2H_5$]ethane (5.00 g, 43.9 mmol) in diethyl ether (15 mL) and a few crystals of iodine to initiate the reaction. Once the reaction began, the remaining solution of bromo[$^2H_5$]ethane in diethyl ether was added dropwise with cooling at a rate so as to maintain the exothermic reaction. The mixture was refluxed with stirring for 30 min and then cooled to room temperature. The resulting Grignard reagent (15 mL) was added dropwise to a stirred solution of 3-chloro-2,5-dimethylpyrazine (1.18 g, 8.3 mmol) and iron(III) acetylacetonate (211 mg, 0.6 mmol) in diethyl ether (20 mL) with cooling, causing an immediate color change to black. The mixture was stirred for 1.5 h at room temperature, diluted with Et_2O (15 mL), and carefully quenched upon the addition of aqueous HCl (1 M, ~15 mL), resulting in a yellow mixture. After extraction with diethyl ether (10 × 15 mL), the organic layer was dried over sodium sulfate and concentrated. The crude product was purified by column chromatography (silica gel, 5.5 × 30 cm) using a mixture of diethyl ether and pentane (1:3 up to 1:1 v/v) as eluent to give 0.56 g (47.5% yield) of the product as a yellow liquid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H), 2.52 (s, 3H), 2.48 (s, 3H); GC RI (DB-WAX) = 1417; MS (EI) m/z (relative intensity, %) 141 (100, $[M]^{\bullet+}$), 140 (58), 139 (72), 110 (13), 109 (13), 61 (24), 42 (52), 40 (17), 39 (31), 28 (35).

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were recorded with a Bruker DPX 400 MHz spectrometer using $CDCl_3$ as solvent. NMR data were analyzed using the Bruker WIN-NMR program.

Gas Chromatography−Mass Spectrometry (GC-MS). GC-MS analysis was performed using an Agilent Technologies 6890 N Network GC System with split injection (220 °C; 1:5), directly coupled to an HP Agilent 5973 Network Mass Selective Detector device (Agilent Technologies Inc., Waldbronn, Germany). Gas chromatographic separation was performed on a 60 m DB-WAX column (0.25 mm i.d., df = 0.25 μm, J&W, Agilent, Waldbronn, Germany) using helium as carrier gas (flow = 1.7 mL/min). The initial oven temperature was 50 °C for 2 min, raised at a rate of 3 °C/min to 100 °C, then raised at a rate of 1 °C/min to 120 °C, and subsequently raised at 8 °C/min to 220 °C, followed by a 20 min hold. Linear retention indices (RI) of the compounds were calculated by using the retention times of n-alkanes as reference.[25] Mass spectra and selected ion monitoring were recorded in positive-ion mode at electron energy of 70 eV (EI, m/z 10−250 amu). Identification was performed by comparison of linear retention indices and mass spectrometric data for sample constituents with data of authentic reference compounds. Mass charge ratios (m/z) used for quantitation are listed in Table 2.

Calibration Curves. Calibration for each compound was performed as follows: Mixtures at seven analyte/internal standard mole ratios (concentration of the analyte = 1−250 μg/mL at 50 μg/mL of the associated internal standard) in dichloromethane were analyzed by GC-MS. The ratios of the peak areas of the ions selected for quantitation (Table 2) were plotted against the weight ratio of analyte/internal standard. The linearity of each calibration regression line was $R^2 > 0.99$. Limits of detection ranged from 0.06 to 0.20 μg/mL with a signal to-noise ratio of 3:1 and limits of quantitation (10:1) from 0.19 to 0.68 μg/mL.[26]

Standard Solution. The concentration of each internal standard in the standard solution was scaled according to the concentration of each analyte expected in coffee samples to achieve optimal accuracy and precision. Standard stock solutions of [$^2H_4$]-**1** (3 mg/mL), [$^2H_3$]-**2** (25 mg/mL), [$^2H_3$]-**3** (6 mg/mL), [$^2H_3$]-**4** (5 mg/mL), [$^2H_5$]-**5** (2 mg/mL), [$^2H_6$]-**6** (1 mg/mL), [$^2H_5$]-**7** (2 mg/mL), [$^2H_5$]-**8** (1 mg/mL), [$^2H_5$]-**9** (1 mg/mL),[$^2H_3$]-**10** (1 mg/mL), and [$^2H_5$]-**11**(1 mg/mL) were prepared in dichloromethane. Equal volumes of each standard stock solution and [$^2H_5$]-**12** (1 mg/mL in diethyl ether) were mixed to obtain the standard solution. The final concentrations of the standard solution were 250 μg/mL for [$^2H_4$]-**1**, 2083 μg/mL for [$^2H_3$]-**2**, 500 μg/mL for [$^2H_3$]-**3**, 417 μg/mL for [$^2H_3$]-**4**, 167 μg/mL

for [$^2H_5$]-**5**, 83 μg/mL for [$^2H_6$]-**6**, 167 μg/mL for [$^2H_5$]-**7**, and 83 μg/mL for [$^2H_5$]-**8**, [$^2H_5$]-**9**, [$^2H_3$]-**10**, [$^2H_5$]-**11**, and [$^2H_5$]-**12**.

Recovery and Precision. Standard stock solutions of **1** (1 mg/mL), **2** (30 mg/mL), **3** (10 mg/mL), **4** (10 mg/mL), **5** (1 mg/mL), **6** (1 mg/mL), **7** (**8**) (2 mg/mL, containing 60% of 7 and 40% of **8**), **9** (1 mg/mL), **10** (1 mg/mL), and **11** (**12**) (2 mg/mL, containing 50% of **11** and 50% of **12**) were prepared in dichloromethane. Equal volumes of each standard stock solution were mixed. The final concentrations of the analytes were 100 μg/mL for **1**, 3000 μg/mL for **2**, 1000 μg/mL for **3** and **4**, 100 μg/mL for **5** and **6**, 120 μg/mL for 7, 80 μg/mL for **8**, and 100 μg/mL for **9**−**12**. One hundred microliters of this mixture was added to semolina (15 g) and then analyzed according to procedure A or B. As summarized in Table 3, the recovery of each alkylpyrazine was determined by using the SIDA developed here.

Analysis and Quantitation of Alkylpyrazines in Ground Coffee Samples. *Procedure A.* Ground roasted coffee (15 g) was suspended in boiling water (400 mL) and stirred for 10 min while the flask was kept closed. After cooling to room temperature, the obtained slurry was spiked with the standard solution (150 μL), containing defined quantities of isotopically labeled internal standards of the analytes (Table 2), and was stirred for an additional 10 min. This mixture was filtered using a Büchner funnel, and the acidic filtrate was basified to pH 11−14 with aqueous sodium hydroxide solution (1 M) and extracted with dichloromethane (400 mL) using a liquid−liquid continuous extractor for 2 h. The organic layer was concentrated to a final volume of 3−5 mL by an IKA RV10 control rotary evaporator (VWR, Darmstadt, Germany) at 40 °C and 900 hPa and analyzed by GC-MS for alkylpyrazine quantitation.

Procedure B. Ground roasted coffee (15 g) was spiked with the standard solution (150 μL) containing the corresponding isotopically labeled standards of the analytes (Table 2) and extracted in a Soxhlet apparatus for 5 h with dichloromethane (250 mL). For quantitative GC-MS analysis of alkylpyrazines, the extract was evaporated to a final volume of 3−5 mL by an IKA RV10 control rotary evaporator (VWR) at 40 °C and 900 hPa.

Extraction Efficiency. To get information about the extraction efficiency of the native analytes bound in the matrix of ground coffee compared to their presolved corresponding isotopically labeled standards, procedure A was chosen because it provided higher alkylpyrazine concentrations in coffee samples and therefore seemed to be more complete than procedure B. Ground roasted coffee (15 g) was suspended in boiling water (400 mL) and stirred for 10 min while the flask was kept closed. After cooling to room temperature, the obtained slurry was spiked with the standard solution (150 μL) and stirred for an additional 10 min. The coffee powder was separated from the first aqueous extract using a Büchner funnel and then subjected in the same way to a second extraction procedure with fresh boiling water and without addition of standard. After the second mixture had been filtered analogously, both aqueous extracts were basified and extracted with dichloromethane as described under Procedure A. The organic layers were evaporated to dryness using an IKA RV10 control rotary evaporator (VWR) at 40 °C and 900 hPa. Both extracts were resuspended in dichloromethane (first extract in 2 mL, second in 1 mL) and used for GC-MS analysis. The percentage amount of each native alkylpyrazine and its associated internal standard in the second extract according to the first extract was calculated from their peak area ratio.

RESULTS AND DISCUSSION

Stable Isotope Dilution Assay. *Identification of Alkylpyrazines.* The main alkylpyrazines from an extract containing the volatiles of an Arabica coffee sample were identified as pyrazines **1**−**12** by comparison of GC retention times, mass spectra, and linear retention indices (Table 1) with those of authentic standards.

Synthesis. Although the syntheses of deuterium-labeled pyrazine standards have already been described,[7,27] we have chosen a different route of synthesis, which was already known for introducing alkyl groups to heteroaromatic compounds. The deuterated alkylpyrazines [$^2H_3$]-**2**, [$^2H_3$]-**3**, [$^2H_3$]-**4**, [$^2H_5$]-**5**, [$^2H_6$]-**6**, [$^2H_5$]-**7**, [$^2H_5$]-**8**, [$^2H_5$]-**9**, [$^2H_3$]-**10**, and [$^2H_5$]-**11** used as internal standards were prepared via iron-catalyzed alkyl-aryl cross-coupling reactions described by Fuerstner[24] according to the experimental procedures. After purification, the structures were corroborated by mass spectrometry and ^{1}H NMR.

Table 1. Linear Retention Indices for Alkylpyrazines on the Polar Column (DB-WAX) Calculated According to the Method of Van den Dool and Kratz[25]

analyte (A)	RI
pyrazine (**1**)	1194
2-methylpyrazine (**2**)	1249
2,5-dimethylpyrazine (**3**)	1301
2,6-dimethylpyrazine (**4**)	1307
2-ethylpyrazine (**5**)	1312
2,3-dimethylpyrazine (**6**)	1325
2-ethyl-6-methylpyrazine (**7**)	1363
2-ethyl-5-methylpyrazine (**8**)	1369
2-ethyl-3-methylpyrazine (**9**)	1381
2,3,5-trimethylpyrazine (**10**)	1382
2-ethyl-3,6-dimethylpyrazine (**11**)	1422
2-ethyl-3,5-dimethylpyrazine (**12**)	1438

Gas Chromatography−Mass Spectrometry. GC-MS analyses of alkylpyrazines were carried out according to the method described under Experimental Procedures. With the selection of the mass charge ratios shown in Table 2 we achieved the best results during method development. For each investigated alkylpyrazine there was a linear relationship between the concentrations of analyte and associated internal standard with coefficients of linear correlation R^2 > 0.99 of the calibration lines (regression lines not shown).

Table 2. Selected Ions (m/z Ratios) of the Analytes (A) and Their Corresponding Deuterium-Labeled Internal Standards (IS) Used for Quantitation of the 12 Alkylpyrazines

analyte (A)	selected ion (m/z)	internal standard (IS)	selected ion (m/z) of IS
pyrazine (**1**)	80	[$^2H_4$]-**1**	84
2-methylpyrazine (**2**)	94	[$^2H_3$]-**2**	97
2,5-dimethylpyrazine (**3**)	108	[$^2H_3$]-**3**	111
2,6-dimethylpyrazine (**4**)	108	[$^2H_3$]-**4**	111
2-ethylpyrazine (**5**)	107	[$^2H_5$]-**5**	114
2,3-dimethylpyrazine (**6**)	108	[$^2H_6$]-**6**	114
2-ethyl-6-methylpyrazine (**7**)	121	[$^2H_5$]-**7**	125
2-ethyl-5-methylpyrazine (**8**)	121	[$^2H_5$]-**8**	125
2-ethyl-3-methylpyrazine (**9**)	121	[$^2H_5$]-**9**	127
2,3,5-trimethylpyrazine (**10**)	122	[$^2H_3$]-**10**	125
2-ethyl-3,6-dimethylpyrazine (**11**)	135	[$^2H_5$]-**11**	139
2-ethyl-3,5-dimethylpyrazine (**12**)	135	[$^2H_5$]-**12**	139

Isotope dilution methods for quantitating substances in biological samples are complicated by isotopic overlap between the isotopic variants to be quantified. Often a correction for isotopic overlap of the peak areas used for quantitation is needed. Unfortunately, 2-ethyl-3-methylpyrazine and 2,3,5-trimethylpyrazine exhibited isotope overlaps at m/z 122 because of coelution and their identical molecular masses. The relative intensities of the ion fragments in the mass spectra

were 122 (77, $[M]^{\bullet+}$), 121 (100), 94 (16), 80 (23), 67 (25), 52 (15), 42 (17), 41 (15) 40 (15), and 39 (22) for 2-ethyl-3-methylpyrazine and 122 (92, $[M]^{\bullet+}$), 81 (20), 54 (12), 53 (11), 52 (10), 42 (100), 40 (16), 39 (33), 28 (33), and 27 (13) for 2,3,5-trimethylpyrazine. The content of 2-ethyl-3-methylpyrazine was calculated with the peak area at m/z 121 and that of 2,3,5-trimethylpyrazine with the peak area at m/z 122 (Table 2). To correct the peak area at m/z 122 of 2,3,5-trimethylpyrazine used for quantitation, the contribution of 2-ethyl-3-methylpyrazine at m/z 122 was calculated with the content of 2-ethyl-3-methylpyrazine an additional calibration curve for 2-ethyl-3-methylpyrazine at m/z 122 and then subtracted from the peak area of 2,3,5-trimethylpyrazine at m/z 122. In contrast, the peak area (m/z 121) used to quantitate 2-ethyl-3-methylpyrazine required no correction, because the mass spectrum of 2,3,5-trimethylpyrazine showed only a low intensity for the m/z 121 fragment.

Recovery and Precision. Model experiments have been carried out to test the accuracy of the method. A mixture consisting of the alkylpyrazines **1**−**12** spiked with isotopically labeled internal standards $[^2H_4]$-**1**−$[^2H_5]$-**12** was added to semolina and analyzed according to experimental procedures A and B. The recovery of each compound was determined by the stable isotope dilution assay. The amounts for the alkylpyrazines added and measured did not differ more than 27% (Table 3). Recoveries of analytes **1**−**12** ranged between 82 and 127% for procedure A and between 78 and 115% for procedure B. High deviations are observed for alkylpyrazines, where the selected mass charge ratios (m/z) used for quantitation showed only low intensity. For some alkylpyrazines, especially 2-ethyl-3-methylpyrazine and 2,3,5-trimethylpyrazine, this deviation is partially due to the interference of coeluting compounds, forming ions with identical masses used for quantitation. Additional variations may be caused by the volatility of alkylpyrazines when the samples are handled.

Table 3. Recovery of a Model Mixture of the Compounds after Isolation of Alkylpyrazines by Extraction Procedures A and B

compound	recovery[a] (%) procedure A	recovery[a] (%) procedure B
pyrazine (**1**)	81.6 ± 7.8	91.6 ± 2.3
2-methylpyrazine (**2**)	92.2 ± 7.1	100.2 ± 8.5
2,5-dimethylpyrazine (**3**)	111.6 ± 6.0	111.6 ± 6.4
2,6-dimethylpyrazine (**4**)	114.1 ± 3.6	115.4 ± 4.9
2-ethylpyrazine (**5**)	87.2 ± 7.2	78.1 ± 6.2
2,3-dimethylpyrazine (**6**)	84.8 ± 7.7	87.0 ± 0.9
2-ethyl-6-methylpyrazine (**7**)	107.8 ± 2.6	105.9 ± 4.5
2-ethyl-5-methylpyrazine (**8**)	116.2 ± 1.9	112.8 ± 5.2
2-ethyl-3-methylpyrazine (**9**)	127.1 ± 4.6	114.1 ± 2.1
2,3,5-trimethylpyrazine (**10**)	120.9 ± 5.2	106.2 ± 3.1
3-ethyl-2,5-dimethylpyrazine (**11**)	110.5 ± 0.9	102.7 ± 0.6
2-ethyl-3,5-dimethylpyrazine (**12**)	93.4 ± 22.5	102.4 ± 1.0

[a]Data are means of at least three assays.

Analysis of Ground Coffee Samples. Pyrazines are quantitatively the second most important volatile compounds in steam of roasted coffee.[5] The amounts of 12 different methyl- and ethyl-substituted alkylpyrazines found in medium-scale ground coffee, as usually applied for filter coffee, were determined by stable isotope dilution analysis, whereby pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-6-pyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-3-methylpyrazine, and 2-ethyl-3,6-dimethylpyrazine were quantitated with this method for the first time. Obviously, the concentrations of the alkylpyrazines depend on the extraction solvent employed. Extraction with water (procedure A) provided alkylpyrazine concentrations that were approximately twice as high as those obtained by extraction with dichloromethane (procedure B). In the literature there was no evidence to suggest that under these conditions large amounts of pyrazines are formed.[28−30] The use of dichloromethane probably results in an incomplete extraction of naturally occurring alkylpyrazines. When the isotope dilution technique was applied, isotopically labeled standards dissolved in dichloromethane were added prior the extraction of the samples, in contrast, the native alkylpyrazines being tightly bound to or even enclosed by the matrix. With dichloromethane possibly only alkylpyrazines at the coffee particle surface could have been extracted from ground coffee under the applied conditions.[31] In contrast, water would induce swelling of the coffee particles, resulting in an alteration of the matrix and thus enabling extraction of now accessible alkylpyrazines.[32] Therefore, water was probably used in many studies to extract coffee aroma compounds.[4,10,11] The findings indicate that the practice of using only dichloromethane as extraction solvent for the extraction of pyrazines from ground coffee in the literature apparently underestimated the amounts of alkylpyrazines in the past.[33,34]

Table 4. Percentage of Amounts of Alkylpyrazine 2−4 and Their Internal Standards in the Second Extract According to the Amounts in the First Extract, Calculated from Their Peak Area Ratio

compound[b]	amounts in the second extract[a] (% amounts in the first extract) analyte	amounts in the second extract[a] (% amounts in the first extract) internal standard
2-methylpyrazine (**2**)	2.7 ± 2.0	2.5 ± 1.9
2,5-dimethylpyrazine (**3**)	3.1 ± 2.2	3.0 ± 2.2
2,6-dimethylpyrazine (**4**)	3.0 ± 2.1	3.1 ± 2.2

[a]Data are means of at least three assays. [b]The amounts of alkylpyrazines **1** and **5**−**12** in the second extract were below under the limit of quantitation.

Extraction Efficiency. Application of procedure A to roasted ground coffee, using water as extraction solvent, delivered alkylpyrazine concentrations that were approximately twice as high as those obtained by procedure B using dichloromethane as extraction solvent. This raises the question of whether procedure A led to a complete extraction of the alkylpyrazines **1**−**12**. To answer this question, a second extraction of the same ground coffee sample was performed. At first, the sample was spiked with isotopically labeled internal standards and extracted according procedure A. Then the extraction was repeated without addition of standard and with fresh solvent (water). Both extracts were purified and analyzed. According to the literature the extraction procedure is correct if the second extract does not contain >10% of native and labeled compounds; otherwise, a second extraction would be necessary. Furthermore, the extraction rate of standard and analyte should be in the same range. If this is not the case, the extraction has to be optimized.[35] Table 4 shows the percentage of amounts of alkylpyrazine **2**−**4** and their internal standards in the second extract according to the amounts in the first extract, calculated from their peak area ratio. Not all alkylpyrazines could be

Table 5. Individual and Total Concentrations of Compounds 1−12 in Roasted Coffee Samples after Isolation by Extraction Procedure A

	concentrations[a] (mg/kg) in coffee									
compound	1	2	3	4	5	6	7[b]	8[b]	9[b]	10
pyrazine (**1**)	19.5	11.9	12.6	12.9[c]	11.1	12.5	7.7	8.7	6.7	5.6
2-methylpyrazine (**2**)	91.6	68.2	70.6	68.1	66.2	64.0	46.8	40.8	38.9	34.8
2,5-dimethylpyrazine (**3**)	28.1	22.3	22.5	20.0	21.7	22.1	15.2	9.3	11.1	10.3
2,6-dimethylpyrazine (**4**)	30.0	24.6	24.4	23.7	24.0	24.4	19.8	15.0	15.2	14.3
2-ethylpyrazine (**5**)	9.8	8.2[c]	8.6	9.1	9.0	7.1	5.8[c]	5.1	4.3	4.1
2,3-dimethylpyrazine (**6**)	5.2	4.7	4.1	4.4	4.0	4.3	2.6	2.1	2.0	1.9
2-ethyl-6-methylpyrazine (**7**)	7.4	7.7	6.5	7.5	7.3	7.4	5.1	3.9	4.2	4.0
2-ethyl-5-methylpyrazine (**8**)	5.6	5.9	4.9	4.6	5.5	5.6	3.5	2.2	2.5	2.4
2-ethyl-3-methylpyrazine (**9**)	3.4	3.8	2.9	3.2	3.5	3.5	2.0	1.4	1.6	1.6
2,3,5-trimethylpyrazine (**10**)	6.7	6.7	5.5	5.8	5.9	6.1	3.0	1.7	2.0	1.8
2-ethyl-3,6-dimethylpyrazine (**11**)	2.9	4.1	2.5	3.2	3.4	3.6	1.3	0.7	1.1	1.0
2-ethyl-3,5-dimethylpyrazine (**12**)	1.4	1.9	1.1	1.6	1.6	1.7	0.7	0.4	0.5	0.4
total	211.6	170.1	166.2	164.2	163.1	162.2	113.4	91.5	90.1	82.1

[a]Data are means of at least three assays: maximum SD ± 15%. [b]Decaffeinated coffee. [c]Data are means of at least three assays: maximum SD ± 30%.

Table 6. Individual and Total Concentrations of Compounds 1−12 in Roasted Coffee Samples after Isolation by Extraction Procedure B

	concentrations[a] (mg/kg) in coffee									
compound	1	2	3	4	5	6	7[b]	8[b]	9[b]	10
pyrazine (**1**)	6.0[c]	4.0	5.3	3.8	3.7	5.1[c]	2.2[c]	2.8	1.6	1.8
2-methylpyrazine (**2**)	39.5	27.5	35.2	25.4	27.6	32.3[c]	17.3[c]	17.0	13.4	14.6
2,5-dimethylpyrazine (**3**)	15.8	12.2	13.2	9.3	11.6	11.1[c]	7.5[c]	4.6	5.5	4.9
2,6-dimethylpyrazine (**4**)	16.5	13.7	14.1	10.9	12.8	12.1[c]	9.7[c]	7.6	7.7	7.4
2-ethylpyrazine (**5**)	5.3	4.8	5.1	4.2	5.0	4.3[c]	3.2[c]	2.9	2.9	2.8
2,3-dimethylpyrazine (**6**)	2.4[c]	2.3	2.2	1.8	2.0	1.9[c]	1.2[c]	0.9	0.8	0.7
2-ethyl-6-methylpyrazine (**7**)	5.5	5.2	4.6	4.4	5.2	4.4	3.7	2.5	3.2	3.0
2-ethyl-5-methylpyrazine (**8**)	4.3	4.0	3.6	3.0	4.1	3.2	2.7	1.5	2.2	2.0
2-ethyl-3-methylpyrazine (**9**)	2.1	2.2	1.8	1.8	2.0	1.9	1.1	0.9	1.0	0.8
2,3,5-trimethylpyrazine (**10**)	5.0	4.6	3.7	3.2	4.3	3.6	2.2	1.1	1.6	1.3
2-ethyl-3,6-dimethylpyrazine (**11**)	1.9	2.6	1.9	1.9	2.5	2.1	0.9	0.5	0.8	0.6
2-ethyl-3,5-dimethylpyrazine (**12**)	0.9	1.2	0.8	1.0	1.0	0.9	0.5	0.3	0.3	0.3
total	105.2	84.3	91.5	70.7	81.8	82.9	52.2	42.5	41.0	40.3

[a]Data are means of at least three assays: maximum SD ± 15%. [b]Decaffeinated coffee. [c]Data are means of at least three assays: maximum SD ± 30%.

considered, because their concentrations in the second extract were below the limit of quantitation. The second extract contained <10% of the analytes and their internal standards in comparison to the first extract. Therefore, the first extraction seems to be complete and no second extraction procedure was necessary. In addition, the extraction rate of each analyte and its standard was in the same range, which means that the percentages of amounts of alkylpyrazine **2−4** and its internal standards in the second extract according to the amounts in the first extract were quite similar. The applied extraction method did not show a discrimination between alkylpyrazines and isotopically marked reference substances. As shown in Tables 5 and 6 the distribution patterns of alkylpyrazines in roasted coffee were quite similar for all coffee samples under study and, except for pyrazine and 2-ethyl-3-methylpyrazine, in agreement with the literature.[5] 2-Methylpyrazine was determined to be the major compound (33−45%) in all samples investigated, followed by 2,6-dimethylpyrazine (14−19%), 2,5-dimethylpyrazine (10−15%), and pyrazine (4−10%). Concentrations of 2-ethylpyrazine (4−7%), 2-ethyl-6-methylpyrazine (3−8%), 2-ethyl-5-methylpyrazine (2−5%), and 2,3,5-trimethylpyrazine (2−5%) were in the same concentration range. Among the alkylpyrazines analyzed, 2,3-dimethylpyrazine (2−3%), 2-ethyl-3-methylpyrazine (2−3%), 2-ethyl-3,6-dimethylpyrazine (1−3%) and 2-ethyl-3,5-dimethylpyrazine (0−1%) showed the lowest concentrations in roasted coffee. Total contents of alkylpyrazines in coffee determined by stable isotope dilution analysis−gas chromatography−mass spectrometry (SIDA-GC-MS) varied from 82.1 to 211.6 mg/kg after extraction using procedure A and from 40.3 to 105.2 mg/kg after extraction using procedure B (Tables 5 and 6). In our study, the three investigated decaffeinated coffee samples contain significantly lower amounts of alkylpyrazines than the regular coffee samples by factors of 0.3−0.7, which might be a result of the decaffeination procedure ($p < 0.05$, Student's t test). Fujioka et al.[36] found concentrations of pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethylpyrazine 2,3-dimethylpyrazine, and 2-ethyl-5-methylpyrazine in extracts of brewed coffee to be higher for decaffeinated coffee using a dichloromethane extraction procedure. However, Fujioka's results cannot be compared with our results directly, because the method used by this group did not compensate losses

during extraction. Moreover, only one regular and one decaffeinated coffee sample were compared in this study.

In the commercially available ground coffee samples under study, pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-3-methylpyrazine, and 2-ethyl-3,6-dimethylpyrazine were quantified with stable isotope dilution assay for the first time. However, compounds **1**−**12** were previously quantified by Silwar et al. in roasted coffee using a conventional internal standard method.[5] The quantitative data for the most abundant pyrazines reported in this publication, namely, 2-methylpyrazine (60.00−80.00 mg/kg), 2,5-dimethylpyrazine (25.00−35.00 mg/kg), and 2,6-dimethylpyrazine (30.00−35.00 mg/kg), were in agreement with those determined here by stable isotope dilution assay. With the exceptions of 2-ethyl-3-methylpyrazine (0.15−0.20 mg/kg) and 2-ethyl-3,6-dimethylpyrazine (0.70−0.85 mg/kg), the contents of the remaining minor present pyrazines reported in Silwar's publication, that is, pyrazine (3.50−6.00 mg/kg), 2,3-dimethylpyrazine (4.50−5.30 mg/kg), 2-ethylpyrazine (13.50−16.50 mg/kg), 2-ethyl-6-methylpyrazine (8.50−10.50 mg/kg), 2-ethyl-5-methylpyrazine (7.50−8.60 mg/kg), 2,3,5-trimethylpyrazine (8.00−10.00 mg/kg), and 2-ethyl-3,5-dimethylpyrazine (2.00−2.20 mg/kg), were found to be slightly higher when compared to the present study. There are varied reasons for these small discrepancies. One may be that the conventional internal standard method is not as sensitive as the stable isotope dilution assay, because the use of structurally different internal standards could alter the ratio between standard and analyte during sample preparation, resulting in systematic errors.

In previous studies, 2,3,5-trimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine, the latter being a key compound of coffee flavor,[8] were quantified by stable isotope dilution assay.[10,11,19−21] Semmelroch et al. and Mayer et al. determined lower concentrations of 2-ethyl-3,5-dimethylpyrazine (0.249−0.543 mg/kg in Arabica coffees, 0.94 mg/kg in Robusta coffee).[10,11,21] In contrast, recently Baggenstoss et al. have reported contents of 1.0−1.22 mg/kg for 2-ethyl-3,5-dimethylpyrazine and 3.5−4.9 mg/kg for 2,3,5-trimethylpyrazine in Arabica coffees, matching the range achieved in our study.[19,20] In our study we have investigated commercially available ground coffee samples to be used in traditional filter coffee machines. We did not investigate the relationship between degree of roasting of the samples and the pyrazine amounts. In the literature some groups have systematically addressed this question.[10,20]

In summary, a gas chromatography−mass spectrometry-based stable isotope dilution assay for the simultaneous determination of 12 alkylpyrazines in ground coffee samples was developed. The method consists of a simple sample extraction step followed by the principle of stable isotope dilution analysis. Water was considered to be the preferred solvent for the extraction of alkylpyrazines from roasted coffee. The method is simple and suitable for the routine analysis of alkylpyrazines.

AUTHOR INFORMATION

Corresponding Author

*(E.R.) E-mail: richling@chemie.uni-kl.de. Phone: +49-(0)631 205 4061. Fax: +49-(0)631 205 3085.

Funding

This work was supported by a grant of the German Federal Ministry of Education and Research (BMBF), Grant 0315692. We thank Tchibo GmbH for sponsoring part of this research.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank C. Mueller, Faculty of Chemistry (University of Kaiserslautern, Germany), for the NMR measurements. We give many thanks to Prof. Dr. Gerhard Eisenbrand for useful feedback and advice. We also thank G. Bytof, I. Lantz, and H. Stiebitz for their support.

REFERENCES

(1) International Coffee Organization. http://www.ico.org/about_coffee.asp?section=About_Coffee (accessed March 16, 2012).

(2) Shibamoto, T.; Bernhard, R. A. Investigation of pyrazine formation pathways in sugar-ammonia model systems. *J. Agric. Food. Chem.* **1977**, *25*, 609−614.

(3) Baltes, W.; Bochmann, G. Model reactions on roast aroma formation. 4. Mass-spectrometric identification of pyrazines from the reaction of serine an threonine with sucrose under the conditions of coffee roasting. *Z. Lebensm. Unters. Forsch.* **1987**, *184*, 485−493.

(4) Czerny, M.; Mayer, F.; Grosch, W. Sensory study on the character impact odorants of roasted Arabica coffee. *J. Agric. Food Chem.* **1999**, *47*, 695−699.

(5) Silwar, R., K. H.; Tressl, R. Gaschromatographisch-massenspektrometrische Untersuchungen des Röstkaffeearomas − Quantitative Bestimmung wasserdampfflüchtiger Aromastoffe. *Chem. Mikrobiol. Technol. Lebensm.* **1987**, *10*, 176−187.

(6) Sanz, C.; Maeztu, L.; Zapelena, M. J.; Bello, J.; Cid, C. Profiles of volatile compounds and sensory analysis of three blends of coffee: influence of different proportions of Arabica and Robusta and influence of roasting coffee with sugar. *J. Sci. Food Agric.* **2002**, *82*, 840−847.

(7) Wagner, R.; Czerny, M.; Bielohradsky, J.; Grosch, W. Structure odour-activity relationships of alkylpyrazines. *Z. Lebensm. Unters. Forsch.* **1999**, *208*, 308−316.

(8) Blank, I.; Sen, A.; Grosch, W. Potent odorants of the roasted powder and brew of Arabica coffee. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 239−245.

(9) Czerny, M.; Wagner, R.; Grosch, W. Detection of odor-active ethenylalkylpyrazines in roasted coffee. *J. Agric. Food Chem.* **1996**, *44*, 3268−3272.

(10) Mayer, F.; Czerny, M.; Grosch, W. Influence of provenance and roast degree on the composition of potent odorants in Arabica coffees. *Eur. Food Res. Technol.* **1999**, *209*, 242−250.

(11) Semmelroch, P.; Laskawy, G.; Blank, I.; Grosch, W. Determination of potent odourants in roasted coffee by stable isotope dilution assays. *Flavour Fragrance J.* **1995**, *10*, 1−7.

(12) Gomez-Ruiz, J. A.; Ames, J. M.; Leake, D. S. Antioxidant activity and protective effects of green and dark coffee components against human low density lipoprotein oxidation. *Eur. Food Res. Technol.* **2008**, *227*, 1017−1024.

(13) Yen, W. J.; Wang, B. S.; Chang, L. W.; Duh, P. D. Antioxidant properties of roasted coffee residues. *J. Agric. Food Chem.* **2005**, *53*, 2658−2663.

(14) Fan, L. H.; Wang, K. Z.; Cheng, B.; Wang, C. S.; Dang, X. Q. Anti-apoptotic and neuroprotective effects of tetramethylpyrazine following spinal cord ischemia in rabbits. *BMC Neurosci.* **2006**, *7*, 48.

(15) Juan, S. H.; Chen, C. H.; Hsu, Y. H.; Hou, C. C.; Chen, T. H.; Lin, H.; Chu, Y. L.; Sue, Y. M. Tetramethylpyrazine protects rat renal tubular cell apoptosis induced by gentamicin. *Nephrol. Dial. Transpl.* **2007**, *22*, 732−739.

(16) Liao, S. L.; Kao, T. K.; Chen, W. Y.; Lin, Y. S.; Chen, S. Y.; Raung, S. L.; Wu, C. W.; Lu, H. C.; Chen, C. J. Tetramethylpyrazine reduces ischemic brain injury in rats. *Neurosci. Lett.* **2004**, *372*, 40−45.
(17) Sue, Y. M.; Cheng, C. F.; Chang, C. C.; Chou, Y.; Chen, C. H.; Juan, S. H. Antioxidation and anti-inflammation by haem oxygenase-1 contribute to protection by tetramethylpyrazine against gentamicin-induced apoptosis in murine renal tubular cells. *Nephrol. Dial. Transpl.* **2009**, *24*, 769−777.
(18) Zhang, Z. H.; Wei, T. T.; Hou, J. W.; Li, G. S.; Yu, S. Z.; Xin, W. J. Tetramethylpyrazine scavenges superoxide anion and decreases nitric oxide production in human polymorphonuclear leukocytes. *Life Sci.* **2003**, *72*, 2465−2472.
(19) Baggenstoss, J.; Poisson, L.; Kaegi, R.; Perren, R.; Escher, F. Roasting and aroma formation: effect of initial moisture content and steam treatment. *J. Agric. Food Chem.* **2008**, *56*, 5847−5851.
(20) Baggenstoss, J.; Poisson, L.; Kaegi, R.; Perren, R.; Escher, F. Coffee roasting and aroma formation: application of different time-temperature conditions. *J. Agric. Food Chem.* **2008**, *56*, 5836−5846.
(21) Mayer, F.; Czerny, M.; Grosch, W. Sensory study of the character impact aroma compounds of a coffee beverage. *Eur. Food Res. Technol.* **2000**, *211*, 272−276.
(22) Semmelroch, P.; Grosch, W. Studies on character impact odorants of coffee brews. *J. Agric. Food Chem.* **1996**, *44*, 537−543.
(23) Grosch, W. Analyse von Aromastoffen. *Chem. Unserer Zeit* **1990**, *24*, 82−89.
(24) Furstner, A.; Leitner, A.; Mendez, M.; Krause, H. Iron-catalyzed cross-coupling reactions. *J. Am. Chem. Soc.* **2002**, *124*, 13856−13863.
(25) van den Dool, H.; Kratz, P. D. A generalization of retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.* **1963**, *11*, 463−471.
(26) MacDougall, D.; Crummett, W. Guidelines for data acquisition and data quality evaluation in environmental chemistry. *Anal. Chem.* **1980**, *52*, 2242−2249.
(27) Czerny, M. *Untersuchungen ueber den Beitrag von Carbonylverbindungen, Pyrazinen und Furanonen zum Aroma von Arabica-Kaffee*. Ph.D. thesis, University of Munich (TU), Munich, Germany, 1999.
(28) Koehler, P. E.; Odell, G. V. Factors affecting formation of pyrazine compounds in sugar-amine reactions. *J. Agric. Food Chem.* **1970**, *18*, 895−898.
(29) Low, M. Y.; Parker, J. K.; Mottram, D. S. Mechanisms of alkylpyrazine formation in a potato model system containing added glycine. *J. Agric. Food Chem.* **2007**, *55*, 4087−4094.
(30) Lu, C. Y.; Hao, Z. G.; Payne, R.; Ho, C. T. Effects of water content on volatile generation and peptide degradation in the Maillard reaction of glycine, diglycine, and triglycine. *J. Agric. Food Chem.* **2005**, *53*, 6443−6447.
(31) Zanoni, B.; Pagliarini, E.; Peri, C. Modeling the aqueous extraction of soluble substances from ground roasted coffee. *J. Sci. Food Agric.* **1992**, *58*, 275−279.
(32) Wang, N.; Fu, Y.; Lim, L. T. Feasibility study on chemometric discrimination of roasted Arabica coffees by solvent extraction and Fourier transform infrared spectroscopy. *J. Agric. Food Chem.* **2011**, *59*, 3220−3226.
(33) Schieberle, P.; Grosch, W. Evaluation of the flavor of wheat and rye bread crusts by aroma extract dilution analysis. *Z. Lebensm. Unters. Forsch.* **1987**, *185*, 111−113.
(34) Czerny, M.; Grosch, W. Potent odorants of raw Arabica coffee. Their changes during roasting. *J. Agric. Food Chem.* **2000**, *48*, 868−72.
(35) Henkelmann, B.; Schramm, K. W.; Klimm, C.; Kettrup, A. Quality criteria for the isotope dilution method with HRGC/MS. *Fresenius' J. Anal. Chem.* **1996**, *354*, 818−822.
(36) Fujioka, K.; Shibamoto, T. Quantitation of volatiles and nonvolatile acids in an extract from coffee beverages: correlation with antioxidant activity. *J. Agric. Food Chem.* **2006**, *54*, 6054−6058.