Convenient Synthesis of Stable Deuterium-Labeled Alkylpyrazines for Use in Stable Isotope Dilution Assays

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ABSTRACT: Stable isotope dilution assays (SIDA) provide for accurate and precise quantitation of aroma components, such as alkylpyrazines, which are often present in low concentrations in complex food matrices. The unavailability of labeled standards is the main limitation to the widespread use of SIDA. This study describes the chlorination of several alkylpyrazines to form the corresponding chloroalkylpyrazine compounds, which are efficient starting materials for the synthesis of deuterium-labeled alkylpyrazines, namely $[^2H_3]$-2-methylpyrazine (**d-1**), $[^2H_5]$-2-ethylpyrazine (**d-2**), $[^2H_3]$-2,3(or 6)-dimethylpyrazine (**d-3$_A$, d-3$_B$**), $[^2H_3]$-2,$[^2H_3]$-6-dimethylpyrazine (**d-3$_C$**), $[^2H_5]$-2,$[^2H_5]$-6-diethylpyrazine (**d-4**), $[^2H_5]$-2-ethyl-3(or 6)-methylpyrazine (**d-5$_A$, d-5$_B$**), 2,$[^2H_3]$-3,5-trimethylpyrazine (**d-6**), $[^2H_5]$-2-ethyl-3,6-dimethylpyrazine (**d-7**), $[^2H_5]$-2-ethyl-3,5-dimethylpyrazine (**d-8**), and 2,3-diethyl-$[^2H_3]$-5-methylpyrazine (**d-9**), which were obtained in good yields (57–100%) and high purities (86–98%). These stable isotopes were used as internal standards in SIDA to accurately and precisely determine selected alkylpyrazines in commercial peanut butter, cocoa powder, and instant coffee. 2,3-Diethyl-5-methylpyrazine (**p-9**) and 2-ethyl-3,5-dimethylpyrazine (**p-8**), despite their low abundance, had the highest odor-active values among the 13 pyrazines quantified in all products due to their very low odor thresholds.

KEYWORDS: *stable isotope dilution assay, pyrazines, chloropyrazines, synthesis, flavor*

■ INTRODUCTION

Pyrazines impart pleasant roasted and nut-like aroma notes to foods and have been reported as predominant aroma components of cooked beef,[1] French fries,[2] coffee,[3] and roasted peanuts.[4] Pyrazines have been used as food flavoring agents since the early 18th century, for example, in the preparation of an artificial coffee oil.[5] Most naturally occurring pyrazines are generated during food preparation, so-called reaction flavors, and often are used as indicators for monitoring the degree of processing/roasting for cocoa beans,[6] coffee,[7] and peanut products.[8] Alkylpyrazine derivatives contain only hydrocarbon side groups. The annual use of naturally occurring alkylpyrazine derivatives in foods is estimated to be 300 tons and 860 kg for the artificial derivatives.[9] The odor thresholds for alkylpyrazines are in the range from 23 ppb for trimethylpyrazine (**p-6**) to 0.04 ppb for 2-ethyl-3,5-dimethylpyraizne (**p-8**).[10]

Some sensorially relevant pyrazine compounds, such as 2,3-diethyl-5-methylpyrazine (**p-9**) and **p-8**, are often naturally present in low concentrations in foods. In general, foods represent very complex matrices containing high levels of proteins, sugars/carbohydrates, and fats. This makes quantitation of minor constituents, such as aroma components, rather difficult. Therefore, great care and special efforts are usually needed to obtain reliable quantitative results. Stable isotope dilution assay (SIDA) is a state-of-the-art quantitative method enabling both high precision and accuracy and has been successfully applied for the detection and quantitation of food contaminants,[11] food flavors,[12] and pesticide residues.[13] The isotopically labeled internal standard, which is spiked into the sample matrices prior to sample preparation, has great similarity in both chemical and physical properties with its unlabeled counterpart, which represents the target compound. After equilibration, the ratio of natural substance (unlabeled target compound) and the labeled internal standard is maintained throughout the entire analysis. The abundance of the target analyte is determined by mass spectrometry in relation to the known amount of isotope initially added to the sample.

The unavailability of labeled alkylpyrazines is the main limitation to the widespread use of SIDA. Several attempts have been made to synthesize labeled alkylpyrazines. Schieberle and Grosch condensed 2,3-pentanedione and $[^2H_4]$-ethylenediamine to obtain labeled 2-ethyl-3-methylpyrazine ($[^2H_{1-2}]$-**p-5$_A$**) for use in SIDA in wheat and rye bread crusts.[14] Other deuterium-labeled alkylpyrazines, such as $[^2H_3]$-**p-9**, $[^2H_3]$-**p-8**, $[^2H_3]$-**p-1**, dimethylpyrazine isomers ($[^2H_3]$-**p-3$_{A,B,C}$**), and $[^2H_3]$-**p-6**, were prepared by nucleophilic addition via the organolithium reagent $[^2H_3]$-alkyl-lithium, with the corresponding alkylpyrazines. The target compounds were isolated in low yield via complicated chromatography procedures such as HPLC or preparative GC.[15,16] The main drawback of the above-mentioned synthesis is the generation of numerous side products, thus resulting in low overall yield of target compounds. The organolithium reagent is a highly reactive nucleophile, which preferentially reacts with electrophilic substrates. However, due to the fact that an alkylpyrazine is not an electrophilic substrate, this synthesis usually results in very low yields.

Recently, our laboratory demonstrated the advantage of using chloropyrazines as substrates for the synthesis of three

deuterated alkyl pyrazines.[17] The present work describes the synthesis of various types of chloroalkylpyrazines for use as starting materials for the preparation of 12 isotopically labeled alkylpyrazine compounds. The chlorine group on chloroalkylpyrazines, acting as an electrophile, is attacked selectively and efficiently by a deuterated alkyl magnesium halide (Grignard reagent). This procedure promotes efficient production of isotopically labeled alkylpyrazines.[18] The synthesized isotopes were used as internal standards in SIDA for the quantitation of selected alkylpyrazines in several types of foods. Two distinctly different extraction techniques for sample preparation were compared to access the precision and accuracy of SIDA.

■ MATERIALS AND METHODS

Peanut butter (Jif creamy peanut butter 18 oz; The J. M. Smucker Co., Orrville, OH, USA), instant coffee (Maxwell House instant coffee original; Kraft Foods Global Inc., Northfield, IL, USA), and cocoa powder (Ghirardelli premium hot cocoa; Ghirardeli Chocolate Co., San Leandro, CA, USA) were obtained from a local retailer (Champaign, IL, USA). Phosphoryl chloride, [1,3-bis-(diphenylphosphino propane] nickel(II) chloride, hydrogen peroxide (35 wt %), acetic acid, [^{2}H$_3$]-iodomethane (99.5+ atom % D), [^{2}H$_5$]-bromoethane (99 atom % D), vinylmagnesium bromide (1.0 M in tetrahydrofuran), iodine, magnesium, 2-chloro-3,6-dimethylpyrazine (c-4), pyrazine, pentane, hexane, chloroform, deuterated chloroform (99.8 atom % D), methylene chloride, and silica gel (grade 923, 100–200 mesh) were from Sigma-Aldrich (St. Louis, MO, USA). 2-Methylpyrazine (p-1), 2-ethylpyrazine (p-2), 2,3-dimethylpyraizne (p-3$_A$), 2,6-dimethylpyrazine (p-3$_B$), 2,5-dimethylpyrazine (p-3$_C$), 2,3-diethylpyrazine (p-4$_A$), 2-ethyl-3-methylpyrazine (p-5$_A$), 2-ethyl-5(or 6)-methylpyrazine (p-5$_C$, p-5$_B$), 2,3,5-trimethylpyrazine (p-6), 2-ethyl-3,5(or 6)-dimethylpyrazine (p-8, p-7), and 2,3-diethyl-5-methylpyrazine (p-9) used in quantitative analysis as authentic standards (purity > 98%), were purchased form Sigma-Aldrich. Diethyl ether (anhydrous) was from Fisher Scientific (Fair Lawn, NJ, USA). Chlorine (99.9%) was from Matheson (Basking Ridge, NJ, USA).

Synthesis of 2-Chloropyrazine (c-1). Pyrazine-1N-oxide:[19] Pyrazine, acetic acid, and hydrogen peroxide in the mole ratio of 1:5:2 were placed into a reaction flask and heated at 75 °C for 7 h. The reaction mixture was then alkalized (pH ≥ 10, 20% NaOH) and extracted with methylene chloride. After removal of solvent, pyrazine-1N-oxide was obtained as a fine white powder: GC retention indices (RI), RTX-5, 1750; Innowax, 2124; MS electron impact (EI), m/z (%) 96 (100, M$^+$), 80 (67), 53 (50), 41 (33), 52 (32), 39 (31), 40 (27), 51 (21), 38 (13), 42 (6). UV absorption agreed with Klein et al.[19] 2-Chloropyrazine (c-1):[20] Pyrazine-1N-oxide was placed into a dry three-neck flask equipped with a magnet stir bar, and 2 mol ratio of phosphoryl chloride was added. The system was kept under nitrogen atmosphere to avoid moisture and maintained at 55 °C for 45 min. When the reaction was complete, the reaction mixture was poured onto crushed ice and alkalized (20% NaOH). The solution was then extracted with diethyl ether (3 × 20 mL). The combined organic solution was passed through a short pad of silica gel, and the solvent was dried over anhydrous sodium sulfate and removed through a Vigreux column (20 × 1 cm, 43 °C) to obtain c-1 as a clear colorless oil: GC RI, RTX-5 = 876, Innowax = 1432; MS(EI), m/z (%) 114 (100), 79 (81), 52 (46), 51 (34), 116 (33, M$^+$), 60 (30), 87 (14), 62 (12), 53 (11), 38 (9).

Synthesis of 2,6-Dichloropyrazine (c-2). Pyrazine-1N,4N-dioxide:[19] Pyrazine, acetic acid, and hydrogen peroxide in the mole ratio of 1:5:2 were heated at 95 °C overnight. The acetic acid was evaporated by repeated heating and by addition of water. The residue was washed with hot chloroform (20 mL, ∼60 °C) to remove pyrazine-1N-oxide and then filtered. The residue was washed with cold methanol (20 mL × 2) and then filtered. Pure pyrazine-1N,4N-dioxide was obtained as a fine white powder. 2,6-Dichloropyrazine (c-2):[20] To pyrazine-1N,4N-dioxide was added 3 mol ratio of phosphoryl chloride, and the mixture was heated at 70 °C for 1 h. Then, the reaction
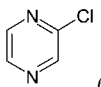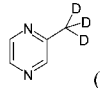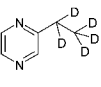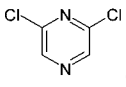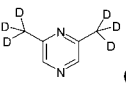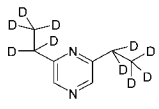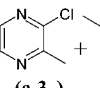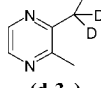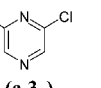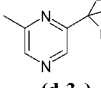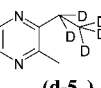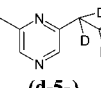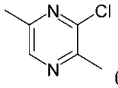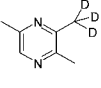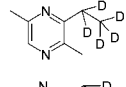
mixture was alkalized (20% NaOH) and extracted with diethyl ether. The organic solution was passed through a short pad of silica gel, and the solvent was dried and removed to obtain c-2: GC RI, RTX-5 = 1373, Innowax = 1540; MS(EI), m/z (%) 148 (100, M$^+$), 51 (83), 113 (82), 60 (76), 150 (63), 86 (56), 87 (34), 62 (30), 115 (24), 38 (20).

Synthesis of 2-Chloro-3(or 6)-methylpyrazine (c-3$_A$, c-3$_B$). The synthesis method was modified from that of Gainer et al.[21] Chlorine gas was bubbled at a rate of 2 bubbles/s into 10 mL of carbon tetrachloride in a test tube maintained in a −15 °C ice/salt bath for 60 min. P-1 (2 g) was then added. The suspension was allowed to warm to room temperature for another 2 h. The white precipitate was collected and washed with carbon tetrachloride. After removal of the solvent by heating at 50 °C, 2.1 g of crude product was obtained. The white powder was then alkalized (20% NaOH) and then extracted with diethyl ether (15 mL × 3). The solvent layers were collected, and the solvent was removed using a Vigreux column (43 °C). The residue was dissolved in a mixed solvent of pentane and diethyl ether (90:10, P/E, v/v) passed through 10 g of silica gel and eluted with same solvent (50 mL). Eluate was dried over anhydrous sodium sulfate, and solvent was evaporated. A mixture of c-3$_A$ (GC RI, RTX-5 = 967, Innowax = 1486; MS(EI), m/z (%) 93 (100), 128 (79, M$^+$), 42 (53), 66 (39), 39 (30), 130 (26), 52 (24), 38 (22), 37 (17), 40 (16)) and c-3$_B$ (GC RI, RTX-5 = 972, In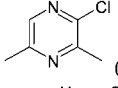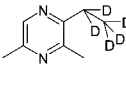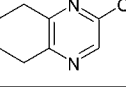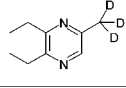nowax = 1493; MS(EI), m/z (%) 128 (100, M$^+$), 66 (59), 39 (46), 60 (39), 87 (38), 130 (33), 38 (25), 93 (25), 40 (24), 62 (16)) was obtained. Purity = 98.2% (82.5% for c-3$_A$ and 15.6% for c-3$_B$, by GC-FID).

Synthesis of 2-Chloro-3,5-dimethylpyrazine (c-5). 2,6-Dimethylpyrazine-4N-oxide:[19] A solution of p-3$_B$, acetic acid, and hydrogen peroxide at a mole ratio of 1:9:1.5 was heated at 60 °C in a round-bottom flask equipped with a magnet stir bar and condenser for 6 h. To minimize the formation of 2,6-dimethylpyrazine-1N,4N-dioxide, the temperature and heating time should not exceed 60 °C and 6 h, respectively. When finished, this solution was cooled and alkalinized (20% NaOH) in an ice bath. The solution was then extracted with chloroform (15 mL × 3). Solvent layers were pooled and dried over anhydrous sodium sulfate. White needle-like crystals formed after evaporation of the solvent under reduced pressure at 50 °C. The crystalline material was washed with boiling hexane and then filtered (30 mL × 2) to obtain 2,6-dimethylpyrazine-4N-oxide: purity = 93.6% measured by GC-FID; GC RI, RTX-5 = 1277, Innowax = 2188; MS(EI), m/z (%) 42 (100), 124 (88, M$^+$), 39 (68), 108 (66), 40 (50), 54 (35), 38 (26), 68 (18), 37 (13), 52 (13). 2-Chloro-3,5-dimethylpyrazine (c-5):[20] 2,6-Dimethylpyrazine-4N-oxide and phosphoryl chloride were place in a round-bottom flask at a mole ratio of 1:3 at 60 °C for 1 h. The reaction mixture was cooled and poured onto crushed ice, and the pH was adjusted (≥10, 20% NaOH). The dark solution was extracted with diethyl ether (20 mL × 3). Solvent layers were combined, dried, and evaporated. The residue was dissolved in mixed solvent (90:10, P/E, v/v) and then passed through a short pad of silica gel. The solvent was collected and evaporated, yielding c-5: purity = 99% by GC-FID; GC RI, RTX-5 = 1059, Innowax = 1556; MS(EI), m/z (%) 142 (100), 42 (90), 107 (79), 39 (72), 66 (72), 144 (32, M$^+$), 38 (28), 40 (21), 37 (16), 143 (10).

Synthesis of 2,3-Diethyl-5-chloropyraizne (c-6). A solution of p-4$_A$ (2 g, 15 mmol), hydrogen peroxide (30 mmol), and acetic acid (0.15 mol) was heated at 65–75 °C for 10.5 h. Then solution was cooled and alkalinized (20% NaOH). After extraction with chloroform, solvent was dried and evaporated under reduced pressure. Crude 2,3-diethylpyrazine-1N-oxide (1.8 g) was obtained, yield 80%: GC RI, RTX-5 = 1748, Innowax = 2152; MS(EI), m/z (%) 135 (100), 119 (39), 152 (34, M$^+$), 39 (26), 120 (24), 52 (20), 41 (17), 53 (17), 107 (16), 79 (15). C-6 was synthesized by adding crude 2,3-diethylpyrazine-1N-oxide (1.8g, 11 mmol) into phosphoryl chloride (88 mmol) in a round-bottom flask at 65 °C for 40 min. The mixture was cooled and poured onto crushed ice. The solution was alkalinized and then extracted with diethyl ether (15 mL × 3). Solvent layers were collected, and the solvent was evaporated. The residue was separated via silica gel chromatography (10 g, column: 15 cm length × 2.1 cm i.d.) conditioned with mixed solvent (90:10, P/E, v/v). After washing with the same solvent, C-6 was obtained in the first fraction (20 mL):

Table 1. Synthesis, Yields, Purities, and Reaction Times of Alkylpyrazine Derivatives

Grignard reagent from	Substrate (D = ^{2}H)	product	Approximate[a] reaction yield	Final[b] yield	Purity %	Reaction[d] time
(c-1)	CD$_3$	(d-1)	~84%	14%	86.2	1 d
	C$_2$D$_5$	(d-2)	~57%	12%	96.3	6 h
(c-2)	CD$_3$	(d-3$_C$)	~75%	42%	91.4	3 d
	C$_2$D$_5$	(d-4)	~100%	35%	93.4	3 h
(c-3$_A$) + (c-3$_B$)	CD$_3$	(d-3$_A$) (d-3$_B$) 80.6% + 16.5%	~92%	22%	97.1[c]	3 d
	C$_2$D$_5$	(d-5$_A$) (d-5$_B$) 88.7% + 9.5%	~100%	55%	98.2[c]	3 h
(c-4)	CD$_3$	(d-6)	~75%	25%	96.6	3 d
	C$_2$D$_5$	(d-7)	~100%	82%	98.0	3 h
(c-5)	C$_2$D$_5$	(d-8)	~100%	23%	91.6	3 h
(c-6)	CD$_3$	(d-9)	~88%	40%	91.2	29 h

[a]Approximate reaction yield is based on the area ratio of [product(s)/(starting material(s) + product(s))] × 100% on GC-MS. [b]Final yield: the mole yield after purification. [c]Purity is the amount of total isomers. [d]Reaction times are not optimized.

GC RI, RTX-5 = 1223, Innowax = 1666; MS(EI), *m/z* (%) 170 (100), 155 (93), 80 (71), 169 (66), 39 (34), 172 (32, M$^+$), 171 (31), 157 (27), 60 (26), 51 (25).

General Procedure for the Synthesis of Alkylpyrazines. Grignard reagents: An etheric solution of deuterium-labeled (or unlabeled) iodomethane (or bromoethane) was added by a syringe (dropwise) into a flask containing magnesium and a small amount of iodine in diethyl ether. The mixture was kept in an atmosphere of nitrogen for 2 h with good stirring at room temperature and then cooled to 0–5 °C. Alkylpyrazines: Freshly prepared Grignard reagent (120% mol) was placed under nitrogen, and [1,3-bis-(diphenylphosphino)propane] nickel(II) chloride[18] as catalyst was added (1% mol). The chloroalkylpyrazine in diethyl ether was then added dropwise by a syringe at 0 °C during a period of 5 min. The reaction mixture was allowed to warm to room temperature for time periods as described in Table 1. Then, water was added to quench the reaction. The solution was extracted with diethyl ether (3 × 15 mL). The combined solvent layers were washed with brine and then dried over anhydrous sodium sulfate and concentrated to 1 mL using a Vigreux column (43 °C). The target compound was purified by silica gel chromatography. Silica gel (10 g) was prepared in a column (15 cm length × 2.1 cm i.d.) and conditioned with a mixed solvent (90:10, P/E, v/v). Target compound was collected by elution with a mixed

solvent (90:10–70:30, P/E, v/v) and then was concentrated to 20 mL. After vacuum distillation/transfer (HVT), the distillate was dried over anhydrous sodium sulfate and solvent was removed by means of a Vigreux column (43 °C) and nitrogen purge. An oil-like liquid with an appearance from colorless to yellow was obtained.

[^{2}H$_3$]-Methylpyrazine (d-1): GC RI, SAC-5 = 817, Stabilwax = 1252; MS(EI), *m/z* (%) 97 (100, M$^+$), 70 (60), 41 (29), 43 (27), 42 (25), 53 (21), 45 (19), 40 (15), 52 (13), 39 (12); ^{1}H NMR δ 8.47 (m, *J* = 1.7 Hz, 2H, 2-CH), 8.39 (d, *J* = 2.4 Hz, 1H, 1-CH); isotope purity = 99.7%.

[^{2}H$_5$]-Ethylpyrazine (d-2): GC RI, SAC-5 = 906, Stabilwax = 1317; MS(EI), *m/z* (%) 111 (100), 113 (69, M$^+$), 81 (16), 112 (11), 41 (10), 61 (9), 53 (9), 52 (9), 84 (7), 42 (7); ^{1}H NMR δ 8.49 (m, *J* = 1.7 Hz, 2H, 2-CH), 8.41 (d, *j* = 2.5 Hz, 1H, 1-CH); isotope purity = 99.5%.

[^{2}H$_3$]-2,[^{2}H$_3$]-6-Dimethylpyrazine (d-3$_C$): GC RI, RTX-5 = 876; Innowax = 1333; MS(EI), *m/z* (%) 46 (100), 114 (90, M$^+$), 41 (57), 43 (56), 42 (37), 40 (9), 39 (16), 38 (11), 70 (9), 44 (8); ^{1}H NMR δ 8.36 (s, 2H, 2-CH); isotope purity = 99.2%.

[^{2}H$_5$]-2,[^{2}H$_5$]-6-Diethylpyrazine (d-4): GC RI, SAC-5 = 1067, Stabilwax = 1411; MS(EI), *m/z* (%) 144 (100), 146 (56, M$^+$), 114 (18), 145 (13), 41 (12), 57 (8), 62 (7), 42 (7), 147 (5), 113 (4); ^{1}H NMR δ 8.34 (s, 2H, 2-CH); isotope purity = 99.2%.

[²H₃]-2,3-Dimethylpyrazine (d-3ₐ): GC RI, SAC-5 = 911, Stabilwax = 1331; MS(EI), *m/z* (%) 111 (100, M⁺), 67 (40), 70 (37), 42 (15), 40 (14), 112 (12), 43 (11), 41 (10), 45 (10), 52 (5); ¹H NMR δ 8.29 (s, 2H, 2-CH), 2.55 (s, 3H, 1-CH₃); isotope purity = 98.9%.

[²H₃]-2,6-Dimethylpyrazine (d-3ᵦ): GC RI, SAC-5 = 906, Stabilwax = 1313; MS(EI), *m/z* (%) 111 (100, M⁺), 43 (46), 45 (33), 40 (20), 39 (16), 41 (13), 42 (10), 112 (7), 38 (5), 84 (4); ¹H NMR δ 8.27 (s, 2H, 2-CH), 2.53 (s, 3H, 1-CH₃); isotope purity = 99.5%.

[²H₅]-2-Ethyl-3-methylpyrazine (d-5ₐ): GC RI, SAC-5 = 990, Innowax = 1386; MS(EI), *m/z* (%) 127 (100, M⁺), 125 (69), 126 (55), 67 (24), 42 (19), 84 (16), 40 (12), 41 (11), 86 (11), 95 (11); ¹H NMR δ 8.34 (d, *J* = 2.5 Hz, 1H, 1-CH), 8.29 (d, *J* = 2.7 Hz, 1H, 1-CH), 2.58 (s, 3H, 1-CH₃); isotope purity = 97.0%.

[²H₅]-2-Ethyl-6-methylpyrazine (d-5ᵦ): GC RI, SAC-5 = 990, Innowax = 1365; MS(EI), *m/z* (%) 125 (100), 127 (64, M⁺), 95 (17), 39 (14), 126 (12), 61 (11), 41 (10), 40 (9), 42 (8), 43 (7); ¹H NMR δ 8.34 (d, *J* = 2.5 Hz, 1H, 1-CH), 8.29 (d, *J* = 2.7 Hz, 1H, 1-CH), 2.55 (s, 3H, 1-CH₃); isotope purity = 97.0%. (A mixture of d-5ₐ (88%) and d-5ᵦ (10%) isomers was obtained. The ¹H NMR signal of d-5ᵦ may be covered due to its low abundance relative to d-5ₐ.)

2,[²H₃]-3,5-Trimethylpyrazine (d-6): GC RI, SAC-5 = 995, Stabilwax = 1387; MS(EI), *m/z* (%) 125 (100, M⁺), 42 (94), 45 (42), 39 (31), 40 (22), 81 (21), 57 (11), 38 (10), 126 (9), 43 (9); ¹H NMR δ 8.16 (s, 1H, 1-CH), 2.50 (s, 3H, 1-CH₃), 2.48 (s, 3H, 1-CH₃); isotope purity = 97.4%.

[²H₅]-2-Ethyl-3,6-dimethylpyrazine (d-7): GC RI, SAC-5 = 1072, Stabilwax = 1427; MS(EI), *m/z* (%) 141 (100, M⁺), 139 (76), 140 (57), 42 (41), 61 (19), 39 (19), 110 (13), 109 (13), 40 (11), 108 (10); ¹H NMR δ 8.18 (s, 1H, 1-CH), 2.56 (s, 3H, 1-CH₃), 2.52 (s, 3H, 1-CH₃); isotope purity = 98.3%.

[²H₅]-2-Ethyl-3,5-dimethylpyrazine (d-8): GC RI, SAC-5 = 1077, Stabilwax = 1442; MS(EI), *m/z* (%) 141 (100, M⁺), 139 (71), 140 (56), 61 (37), 42 (26), 39 (19), 110 (11), 40 (11), 109 (10), 142 (9); ¹H NMR δ 8.20 (s, 1H, 1-CH), 2.54 (s, 3H, 1-CH₃), 2.49 (s, 3H, 1-CH₃); isotope purity = 99.2%.

2,3-Diethyl-5-[²H₃]-methylpyrazine (d-9): GC RI, SAC-5 = 1150, Stabilwax = 1476; MS(EI), *m/z* (%) 153 (100, M⁺), 138 (74), 152 (62), 57 (31), 124 (25), 41 (17), 154 (10), 39 (8), 43 (7), 139 (7); ¹H NMR δ 8.20 (s, 1H, 1-CH), 2.82 (dq, *J* = 1, 7.5 Hz, 4H, 2-CH₂), 1.28 (dt, *J* = 1, 7.5 Hz, 6H, 2-CH₃); isotope purity = 99.5%.

2,6-Diethylpyrazine (p-4ᵦ): synthesized from ethylmagnesium bromide and c-2; purity (94.8%) was checked by GC-FID; GC RI, SAC-5 = 1072, Stabilwax = 1420; MS(EI), *m/z* (%) 135 (100), 136 (51, M⁺), 39 (13), 108 (11), 53 (10), 56 (7), 107 (6), 54 (5), 120 (5), 52 (5); ¹H NMR δ 8.32 (s, 2H, 2-CH), 2.86 (q, *J* = 7.6 Hz, 4H, 2-CH₂), 1.34 (t, *J* = 8.0 Hz, 6H, 2-CH₃).

2-Vinyl-3,6-dimethylpyrazine (p-10): synthesized from c-4 (0.5 g, 3.5 mmol), triethylamine (0.5g, 5 mmol), and vinylmagnesium bromide (5 mmol); a large amount of triethylamine was necessary to prevent pyrazine dimer (or polymer) formation;²² purity = 94.4% (GC-FID); GC RI, SAC-5 = 1169, Stabilwax = 1525; MS(EI), *m/z* (%) 133 (100), 134 (65, M⁺), 42 (45), 39 (27), 54 (20), 40 (12), 66 (8), 65 (7), 135 (5), 52 (5); ¹H NMR δ 8.21 (s, 1H, 1-CH), 6.96 (q, *J*av = 9 Hz, 1H, 1-CH), 6.42 (cis) (dd, *J* = 17, 1 Hz, 2H, 1-CH₂), 5.58 (trans) (dd, *J* = 11, 1 Hz, 2H, 1-CH₂), 2.57(s, 3H, 1-CH₃), 2.52 (s, 3H, 1-CH₃).

2-Ethyl-3,6-dimethylpyrazine (p-7): synthesized from c-4 and ethylmagnesium bromide (120% mol) (commercially available ethyl-dimethylpyrazine is a mixture of p-7 and p-8); purity = 99.4% (GC-FID); GC RI, RTX-5 = 1083, Innowax = 1469; MS(EI), *m/z* (%) 135 (100), 136 (71, M⁺), 42 (58), 39 (44), 56 (23), 108 (18), 40 (18), 41 (17), 53 (11); ¹H NMR δ 8.17 (s, 1H, 1-CH), 2.82 (q, *J* = 7.6 Hz, 2H, 1-CH₂), 2.54 (s, 3H, 1-CH₃), 2.51 (s, 3H, 1-CH₃), 1.28 (t, *J* = 7.6 Hz, 3H, 1-CH₃).

Compound Identification. Deuterium-labeled compounds were identified by comparing their mass spectra with unlabeled authentic standard compounds. For example, Figures 1 and 2 show a similar pattern for the EI mass spectra of the two isotopologues. Note that molecular weight is shifted by several units due to the higher mass of deuterium. ¹H NMR was also applied for further confirmation. The
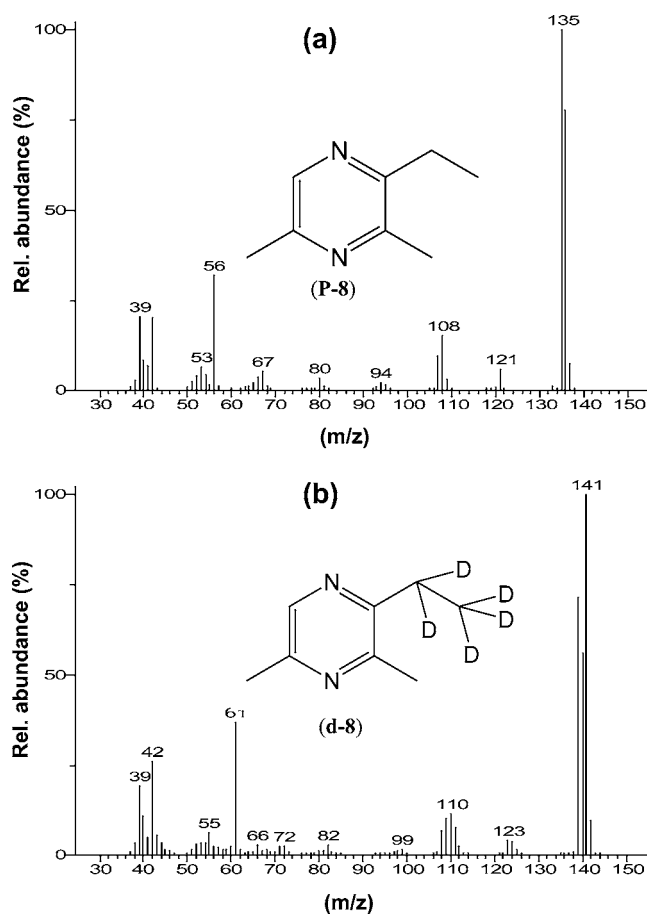

Figure 1. MS(EI) of unlabeled (a) and of ²H₅-labeled 2-ethyl-3,5-dimethylpyraizne (b).

missing signals from deuterated side chains on labeled pyrazine compounds indicate the positions of deuterium compared to the authentic unlabeled standard compounds (as shown in Figure 3).

Compound Purities. The purities of the deuterated compounds were determined by gas chromatography coupled with a flame ionization detector (GC-FID) using a 5890 series II GC (Agilent Technologies Inc., Palo Alto, CA, USA). Compounds were injected directly into the GC inlet (250 °C, hot split, 50:1). Purities were obtained by calculating target peak area divided by total area. Separations were performed using an Innowax column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies Inc.). The oven was programmed from 40 to 225 °C at the rate of 10 °C/min and holding at the end for 10 min. Isotope purities for deuterated compounds were based on the integrated area of selected side chain groups as follows: isotope purity (%) = [(¹H signal for authentic standard − ¹H residue for the deuterated compound)/(¹H signal for authentic standard)] × 100%.

¹H Nuclear Magnetic Resonance (NMR). Compounds were prepared in deuterated chloroform (C²HCl₃) in Wilmad 528-PP 5 mm NMR tubes. NMR spectra were acquired on a Varian Unity 500 spectrometer (499.693 MHz), equipped with a 5 mm Nalorac QUAD probe (¹H,¹⁹F, ¹³C, ³¹P). The probe temperature was 20.4 °C for the proton spectra. Chemical shift δ values are cited in parts per million (ppm) in reference to CHCl₃ as 7.26 ppm. The multiplicity abbreviations used to describe NMR signals are s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, dt = double triplet, dq = double quartet, *J* = *J* coupling, and *J*av = average of *J* coupling.

Quantitation of Selected Pyrazines. Compound identification in the quantitative study was based on matching retention indices on both polar (Stabilwax or Innowax) and nonpolar (RTX-5 or SAC-5) columns and on comparison of mass spectra to authentic standards.
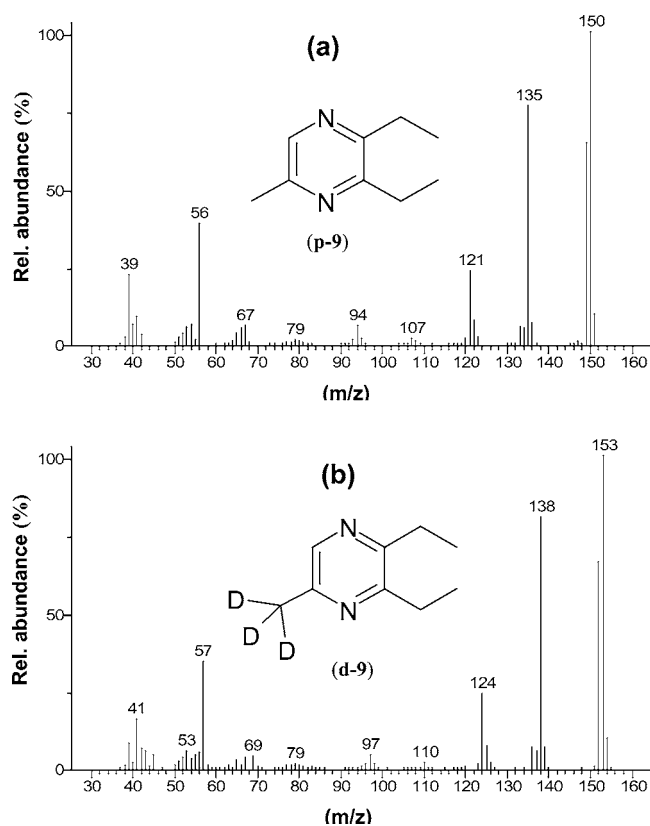
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Figure 2. MS(EI) of unlabeled (a) and of $^2H_3$-labeled 2,3-diethyl-5-methylpyraizne (b).

The concentrations of selected pyrazines in food samples were based on their area response ratio corrected by response factors (Table 2), as follows: mass of analyte = [(extracted ion chromatogram area of analyte)/(area of labeled internal standard) × mass of internal standard × response factor]. Response factors were determined by

analyzing mixtures of known amounts of the unlabeled and labeled compounds at five different mass ratios (1:10, 1:5, 1:1, 5:1, and 10:1) by GC-MS.

Direct Solvent Extraction–Solvent-Assisted Flavor Evaporation (DSE-SAFE). A sample (100 g) of peanut butter, cocoa powder, or instant coffee was stirred in 100 mL of saturated aqueous sodium chloride solution, and then 150 mL of diethyl ether was added for the purpose of extraction. Isotopically labeled internal standards in diethyl ether were added (based on preliminary experiments). This mixture was stirred for 3 h to achieve equilibrium between the internal standards and analytes. After centrifuging, the solvent layer was collected and the residue was extracted two more times with 100 mL of diethyl ether (1 h of solvent contact time for each extraction). The combined solvent extracts were concentrated to 150 mL using a Vigreux column (43 °C) prior to SAFE. Volatile compounds were collected after distillation at $(5-9) \times 10^{-5}$ Torr for 3 h as previously described.[23] The SAFE apparatus was kept at 45 °C with a circulating water bath. The SAFE distillate was concentrated to 30 mL and then fractionated to neutral/basic (NB) fraction and acidic (AC) fractions.[24] Due to the fact that pyrazines are weakly basic compounds, only the NB fraction was analyzed by GC-MS. Sample extraction/analysis was performed in duplicate.

Solid Phase Microextraction (SPME). A sample, consisting of 0.3–10 g of peanut butter, cocoa powder, or instant coffee, was placed in a 22 mL vial containing 0.6 g of NaCl and 3 mL of water. The vial was spiked with a 20 µL mixture of internal standards and sealed with an aluminum crimp cap and Teflon-lined silicon septum. SPME was performed using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supleco, Bellefonte, PA, USA). The sample vial was incubated at 60 °C for 20 min for equilibration, and then the SPME fiber was exposed to the headspace of the sample for 30 min. Volatiles were transferred immediately via the injection port (hot splitless; 260 °C; 4 min valve delay) of the gas chromatograph and desorbed for 10 min for subsequent analysis by GC-MS.

Gas Chromatography–Mass Spectrometry (GC-MS). GC-MS was performed using a 6890 GC-5973N mass selective detector (Agilent Technologies Inc.). Separations were performed on a Stabilwax column (30 m × 0.25 mm × 0.25 µm film thickness, Restek, Bellefonte, PA, USA). Helium was the carrier at a constant flow of 1 mL/min. To minimize loss of any labile constituents, DSE-
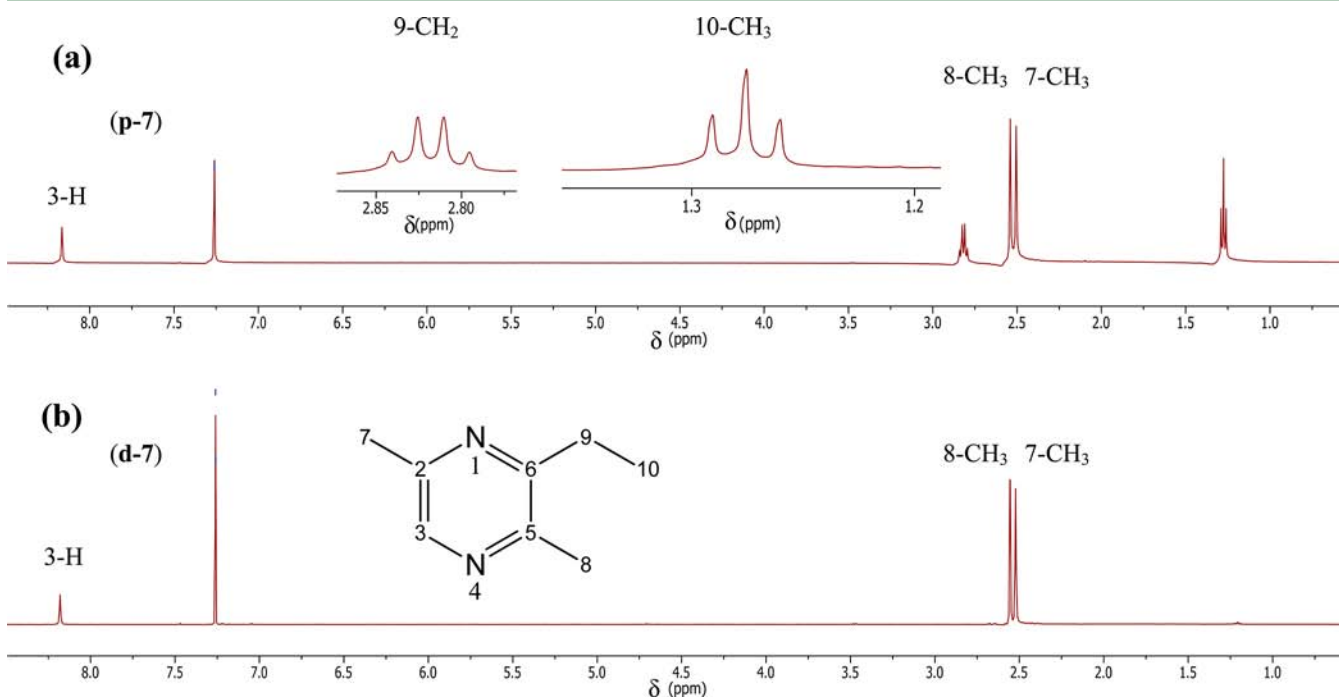

Figure 3. ^1H NMR spectra of (a) and of $^2H_5$-labeled 2-ethyl-3,6-dimethylpyrazine (b).

Table 2. Pyrazines, Respective Isotopologues, Selected Ions, and MS(EI) Response Factors Used in Stable Isotope Dilution Assays

| | | | | mass trace (m/z) | | |
				analyte	internal standard	response factor
2-methyl-	(**p-1**)	[$^2H_3$]-methyl-	(**d-1**)	94	97	1.013
2-ethyl-	(**p-2**)	[$^2H_5$]-ethyl-	(**d-2**)	108	113	1.514
2,3-dimethyl-	(**p-3$_A$**)	[$^2H_3$]-2,3-dimethyl-	(**d-3$_A$**)	108	111	1.097
2,6-dimethyl-	(**p-3$_B$**)	[$^2H_3$]-2,6-dimethyl-	(**d-3$_B$**)	108	111	1.267
2,5-dimethyl-	(**p-3$_C$**)	[$^2H_3$]-2,6-dimethyl-	(**d-3$_B$**)	108	111	0.719
2,3-diethyl-	(**p-4$_A$**)	[$^2H_{10}$]-2,6-diethyl-	(**d-4**)	136	146	0.936
2,6-diethyl-	(**p-4$_B$**)	[$^2H_{10}$]-2,6-diethyl-	(**d-4**)	136	146	0.694
2-ethyl-3-methyl-	(**p-5$_A$**)	[$^2H_5$]-2-ethyl-3-methyl-	(**d-5$_A$**)	122	127	1.149
2-ethyl-6-methyl-	(**p-5$_B$**)	[$^2H_5$]-2-ethyl-6-methyl-	(**d-5$_B$**)	122	127	0.631
2-ethyl-5-methyl-	(**p-5$_C$**)	[$^2H_5$]-2-ethyl-6-methyl-	(**d-5$_B$**)	122	127	1.149
2,3,5-trimethyl-	(**p-6**)	2,[$^2H_3$]-3,5-trimethyl-	(**d-6**)	122	125	1.069
2-ethyl-3,6-dimethyl-	(**p-7**)	[$^2H_5$]-2-ethyl-3,6-dimethyl-	(**d-7**)	136	141	1.080
2-ethyl-3,5-dimethyl-	(**p-8**)	[$^2H_5$]-2-ethyl-3,5-dimethyl-	(**d-8**)	136	141	0.913
2,3-diethyl-5-methyl-	(**p-9**)	[$^2H_3$]-2,3-diethyl-5-methyl-	(**d-9**)	150	153	1.088
2-vinyl-3,6-dimethyl-	(**p-10**)	[$^2H_5$]-2-ethyl-3,6-dimethyl-	(**d-7**)	134	141	1.544

Scheme 1. Synthesis of [$^2H_5$]-2-Ethyl-3,5-dimethylpyrazine
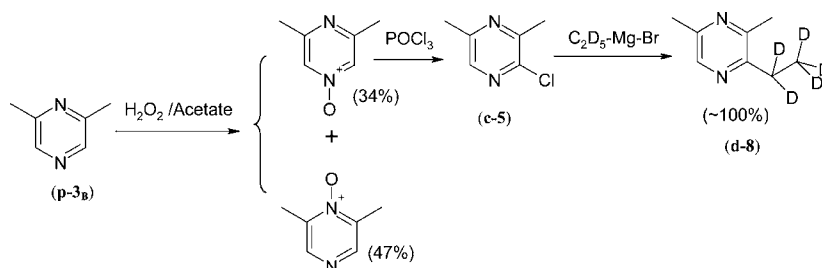
SAFE extracts were injected using a CIS4 (Gerstel GmbH & Co. KG, Germany) programmable temperature vaporization (PTV) inlet in the cold splitless mode (−50 °C for 0.1 min, then ramped at 12 °C/s, and held at 260 °C). GC oven temperature was programmed from 35 to 225 °C at 6 °C/min with initial and final hold times of 5 and 20 min, respectively. Other conditions were as follows: MSD interface temperature, 260 °C; ionization energy, 70 eV; mass range, 35−350 amu; EM voltage, Autotune +165 V; scan rate, 4.45 scans/s.

■ RESULTS AND DISCUSSION

Reaction of chloroalkylpyrazines with deuterium-labeled alkyl Grignard reagents provides an improved method for the preparation of isotopically labeled alkylpyrazines. The chloroalkylpyrazine compounds used as starting materials were synthesized either by direct chlorination or by chlorination of alkylpyrazine-N-oxide compounds.

Direct Chlorination. Passing chlorine gas through a carbon tetrachloride (CCl$_4$) solution of **p-1** resulted in a dense white precipitate being formed. The precipitate was the hydrochloride salt of **c-3$_A$** and **c-3$_B$**. This chlorination was not a free radical process, but an electrophilic substitution.[25] During chlorination, the inductive effect of the positively charged nitrogen atom in the pyrazine−perchloride complex caused the nearby carbons on **p-1** to be relatively electronegative; thus, the chlorination occurred on the 3-carbon or 6-carbon. The same approach could be applied in case of the chlorination of the 2,5-disubstituted pyrazine compounds.[25] However, treating the 2,6-disubstituted pyrazines or the 2,3-disubstituted pyrazines directly with chlorine in CCl$_4$ solution appeared to be a free radical process and, thus, the chlorination occurred on the alkyl side chain.[25] Because alkylpyrazines containing side-chain

halogens cannot be used as starting materials for the purpose of preparation of labeled pyrazines, another approach was developed for the chlorination of the 2,6-disubstituted pyrazines.

Chlorination of 2,6-Dimethylpyrazine (p-3$_B$). As mentioned above, the direct chlorination of **p-3$_B$** formed a side-chain halogenated product. To obtain a ring chlorinated product, **p-3$_B$** was treated with hydrogen peroxide in glacial acetic acid. During the oxidation process, a mixture of **p-3$_B$**, 2,6-dimethylpyrazine-1N-oxide, and 2,6-dimethylpyrazine-4N-oxide was formed at the ratio of 19:47:34 (by GC-FID). This mixture was purified by washing with boiling hexane. Because 2,6-dimethylpyrazine-4N-oxide has a high melting point (mp ∼ 110 °C)[19] and low solubility in hexane, it can be collected after filtering. On the other hand, **p-3$_B$** and 2,6-dimethylpyrazine-1N-oxide (mp ∼ 55 °C)[19] passed through the filter. The reaction of 2,6-dimethylpyrazine-4N-oxide with phosphoryl chloride first resulted in the formation of an N,O-dichlorophosphite. Then, the electron-deficient carbon (adjacent to the nitrogen) was attacked by chloride, followed by oxygen removal[20] to form **c-5** (Scheme 1).

Chlorination of 2,3-Diethylpyrazine (p-4$_A$). Ohta et al.[26] reported the N-oxidation and chlorination of some 2,3-disubstituted pyrazines such as dimethylpyrazine. Two relevant products, 2,3-dimethyl-5-chloropyrazine and 2-α-chloromethyl-3-methylpyrazine, were identified in that study. In the present work we started from **p-4$_A$**. The oxidation process gave a single product: 2,3-diethylpyrazine-1N-oxide. After chlorinating, two main products with m/z 144 were found, which were separated and purified into two fractions assumed to be **c-6** and 2-α-
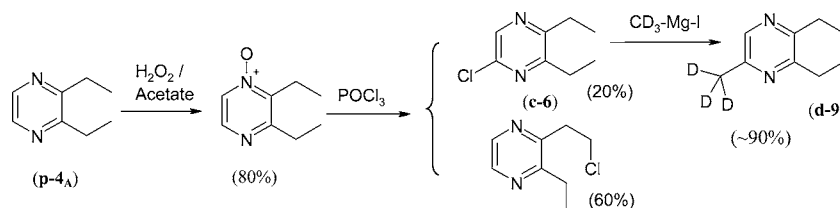
Scheme 2. Synthesis of 2,3-Diethyl-[^{2}H$_3$]-5-methylpyrazine

Table 3. Concentrations and OAVs for Selected Alkylpyrazines in Food Products by Two Extraction Techniques: Solvent-Assisted Flavor Evaporation (SAFE) and Solid-Phase Microextraction (SPME)

	odor threshold (μg/kg)		peanut butter			instant coffee			cocoa powder		
alkylpyrazine	water	oil	SAFE	SPME	OAV[a] (oil)	SAFE	SPME	OAV (water)	SAFE	SPME	OAV (water)
p-8	0.04[b]	2.2[c]	80 ± 3.3[d]	66 ± 2.6	36	402 ± 20	360 ± 1.7	10061	60 ± 1.0	55 ± 5.5	1500
p-9	0.5[c]	0.5[c]	12 ± 0.[c]	11 ± 1.0	24	166 ± 1.0	164 ± 3.2	333	57 ± 0	51 ± 4.4	113
p-7	8.6[b]	57[c]	328 ± 2.0	348 ± 30	5.7	988 ± 7.7	972 ± 32	115	159 ± 0.5	149 ± 13	18
p-6	23[b]	297[e]	475 ± 3.6	442 ± 0.5	1.6	1775 ± 64	1817 ± 118	77	310 ± 2.9	319 ± 31	13
p-3$_C$	1700[b]	2600[e]	1426 ± 45	1524 ± 72	0.55	2802 ± 12	3002 ± 15	1.65	360 ± 2.5	367 ± 25	0.21
p-5$_C$	100[b]	900[f]	336 ± 7.0	329 ± 5.0	0.37	1417 ± 45	1454 ± 55	14.2	130 ± 0.4	135 ± 12	1.30
p-5$_B$	NR[g]	900[f]	83 ± 3.4	81 ± 0.0	0.08	2468 ± 6.7	2465 ± 81		103 ± 0.6	103 ± 7.7	
p-3$_B$	1500[b]	8000[f]	272 ± 26	258 ± 18	0.03	1263 ± 45	1262 ± 7.1	0.84	167 ± 0.9	165 ± 13	0.11
p-2	4000[b]	17000[h]	142 ± 7.7	140 ± 11	0.01	2266 ± 18	2385 ± 81	0.57	160 ± 1.0	165 ± 5.4	0.04
p-3$_A$	2500[b]	NR	88 ± 2.5	87 ± 0.4		4097 ± 179	4548 ± 69	1.64	225 ± 1.2	238 ± 17	0.09
p-1	27000[f]	27000[h]	462 ± 2.6	459 ± 21	0.02	8477 ± 261	8756 ± 18	0.31	396 ± 11	419 ± 16	0.01
p-10	NR	NR	103 ± 6.3	116 ± 9.1		ND[i]	ND		ND	ND	
p-4$_B$	6[h]	NR	8 ± 0.3	7 ± 0.4		182 ± 2.6	187 ± 5.0	30.3	13 ± 0.1	12 ± 1.1	2.2

[a]OAV, odor activity values calculated on the basis of SAFE extraction. [b]Buttery et al.[10] [c]Wagnar et al.[2] [d]Mean ± SD, n = 2 (μg/kg). [e]Chetschik et al.[16] [f]Koehler et al.[34] [g]NR, not reported. [h]Fors.[35] [i]ND, not detected.

chloroethyl-3-ethylpyrazine (Scheme 2). To confirm this, the first fraction was treated with methylmagnesium iodide. A single product of 2,3-diethyl-5-methylpyrazine (p-9) was found for which the RI and mass spectrum matched the authentic standard compound. This confirmed our earlier assumptions. Treating c-6 with [^{2}H$_3$]-methylmagnesium iodide in diethyl ether for 29 h afforded deuterium-labeled d-9 (yield = 88%). The compound p-9 is an important flavor contributor in many foods due to its very low odor threshold (OT) of 0.5 ppb.[2]

Deuterated Alkylpyrazines. Twelve deuterated alkylpyrazines were synthesized using this convenient synthesis approach. The reactions are shown in Table 1. The average reaction yield was approximately 87%. The high yield was due to efficient and selective reaction of the Grignard reagent with the chloroalkylpyrazines. The unreacted starting material (chloroalkylpyrazine) was easy to separate from the desired labeled product (deuterated alkylpyrazine) because the chlorine group made these two compounds very different with respect to their elution through silica gel chromatography column. Chloropyrazine was not held by silica gel in a mixed solvent (90:10, P/E, v/v). Meanwhile, the labeled alkylpyrazines required a more polar mobile phase (80:20–70:30, P/E, v/v) to be eluted from the column. Therefore, the labeled alkylpyrazines could be obtained in high purities.

Quantitative Analysis. Peanut butter, instant coffee, and cocoa powder were chosen for quantitative studies on the basis of their complex matrices and because pyrazines are known to be important aroma components of these products.[27−29] Samples were prepared by SPME and SAFE to evaluate the breadth and utility of the SIDA technique.

Concentrations and odor-activity values (OAVs) for selected alkylpyrazines in commercial peanut butter, instant coffee, and cocoa powder are given in Table 3. The quantitative data in the literature concerning alkylpyrazines in peanut products were limited, and most of these reports focused only on the highly abundant alkylpyrazines, such as p-3$_C$ and p-3$_B$.[30,31] However, these highly abundant alkylpyrazines are not considered to be major contributors to roasted peanut flavor because of their relatively low OTs.[32] On the other hand, certain pyrazines with low OTs, such as p-7, p-8, and p-9, are reported to be important odorants despite their relatively low abundance in peanut products.[32] Previously, p-7, p-8, and p-9 were determined to be 352, 5534, and 2.2 ng/g,[31] respectively, in blanched oven-roasted peanuts (by use of an unlabeled internal standard) and 196, 23, and 13 ng/g, respectively, in pan-roasted peanuts (by SIDA).[16] The above concentrations are consistent with those determined in the present study for the commercial peanut butter, although some differences due to peanut variety, processing, and other factors are to be expected. In addition, low standard deviations (<10%) were observed in the SIDA of pyrazines in pan-roasted peanuts[16] and in the present study. Compounds p-8 and p-9 were reported to have the highest flavor dilution (FD) factors in coffee,[28] which agrees with the results of this study in which p-8 and p-9 had the highest OAVs among all pyrazines measured in instant coffee. Pyrazines comprised >40% of the cocoa powder essence. Among them, dimethylpyrazine isomers (DMPs; p-3$_A$, p-3$_B$, p-3$_C$) and trimethylpyrazine (TrMP; p-6) were present in highest abundance. The DMPs/TrMP index is often used to assess the degree of cocoa roasting.[33] P-3$_A$, p-3$_B$, p-3$_C$, and p-6 were found to be the most abundant alkylpyrazines in cocoa powder

in the present study. On the other hand, **p-8**, **p-9**, **p-7**, and **p-6** were reported to have high FD factors of 2048, 256, 256, and 128, respectively, over other compounds by molecular sensory analysis of cocoa powder.[29] This agrees with the results of the present study, in which the OAVs of these compounds were determined to be 1500, 113, 18, and 13, respectively. Therefore, SIDA appears to be a suitable means to accurately quantify alkylpyrazines in food products.

A crucial step in the determination of compounds that are responsible for the flavor of a food product is to select a suitable extraction method that allows for the extraction of all compounds and does not to alter the flavor profile of characteristic volatiles. To test the breadth of SIDA, two different extraction/isolation methods, solvent-assisted flavor evaporation and solid-phase microextraction, were tested in this study. The average difference between SAFE and SPME was <10% (4.9%). Only three single pyrazines (**p-3$_C$** in instant coffee and **p-6** and **p-8** in peanut butter) differed significantly ($p < 0.05$, t test). The precision of SIDA was also acceptable, with an average relative standard deviation (%RSD) of 3.6%, which ranged from 0.0 to 12.6%. The higher RSD for **p-9** was probably due to its low concentration (~10 μg/kg). Some other errors could have been caused by the difficulty in precisely spiking trace amounts of the labeled internal standards or by inconsistent integration of very small peaks during data analysis.

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Mussinan, C. J.; Wilson, R. A.; Katz, I. Isolation and identification of pyrazines present in pressure-cooked beef. *J. Agric. Food Chem.* **1973**, *21*, 871−872.

(2) Wagner, R. K.; Grosch, W. Key odorants of French fries. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1385−1392.

(3) Czerny, M.; Grosch, W. Potent odorants of raw *Arabica* coffee. Their changes during roasting. *J. Agric. Food Chem.* **2000**, *48*, 868−872.

(4) Burroni, L. V.; Grosso, N. R.; Guzman, C. A. Principal volatile compounds of raw, roasted, and fried Argentinean peanut flavors. *J. Agric. Food Chem.* **1997**, *45*, 3190−3192.

(5) Reichstein, T.; Staudinger, H. A new or improved method of producing artificial coffee oil. Br. Patent GB260960, 1928.

(6) Reineccius, G. A.; Keeney, P. G.; Weissberger, W. Factors affecting the concentration of pyrazines in cocoa beans. *J. Agric. Food Chem.* **1972**, *20*, 202−206.

(7) Hashim, L.; Chaveron, H. Use of methylpyrazine ratios to monitor the coffee roasting. *Food Res. Int.* **1996**, *28*, 619−623.

(8) Buckholz, L. L., Jr.; Daun, H.; Stier, E.; Trout, R. Influence of roasting time on sensory attributes of fresh roasted peanuts. *J. Food Sci.* **1980**, *45*, 547−554.

(9) Adams, T. B.; Doull, J.; Feron, V. J.; Goodman, J. I.; Marnett, L. J.; Munro, I. C.; Newberne, P. M.; Portoghese, P. S.; Smith, R. L.; Waddell, W. J.; Wagner, B. M. The FEMA GRAS assessment of pyrazine derivatives used as flavor ingredients. *Food Chem. Toxicol.* **2002**, *40*, 429−451.

(10) Buttery, R. G.; Ling, L. C.; Stern, D. J. Studies on popcorn aroma and flavor volatiles. *J. Agric. Food Chem.* **1997**, *45*, 837−843.

(11) USFDA. Detection and quantitation of acrylamide in foods, 2002; http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Acrylamide/default.htm (accessed Jan 9, 2013).

(12) Munch, P.; Schieberle, P. Quantitative studies on the formation of key odorants in thermally treated yeast extracts using stable isotope dilution assays. *J. Agric. Food Chem.* **1998**, *46*, 4695−4701.

(13) Cai, Z.; Wang, D.; Ma, W. T. Gas chromatography/ion trap mass spectrometry applied for the analysis of triazine herbicides in environmental water by an isotope dilution technique. *Anal. Chim. Acta* **2004**, *503*, 263−270.

(14) Schieberle, P.; Grosch, W. Quantitative analysis of aroma compounds in wheat and rye bread crusts using a stable isotope dilution assay. *J. Agric. Food Chem.* **1987**, *35*, 252−257.

(15) Cerny, C.; Grosch, W. Quantification of character-impact odour compounds of roasted beef. *Z. Lebensm. Unters. Forsch.* **1993**, *196*, 417−422.

(16) Chetschik, I.; Granvogl, M.; Schieberle, P. Quantitation of key peanut aroma compounds in raw peanuts and pan-roasted peanut meal. Aroma reconstitution and comparison with commercial peanut products. *J. Agric. Food Chem.* **2010**, *58*, 11018−11026.

(17) Fang, M.; Cadwallader, K. R. Improved synthesis of deuterium-labeled alkyl pyrazines for use in stable isotope dilution analysis. In *Recent Advances in the Analysis of Foods and Flavor*; Toth, S., Mussinan, C., Eds.; ACS Symposium Series 1098; American Chemical Society: Washington, DC, 2012; p 57.

(18) Sato, N.; Matsuura, T. Studies on pyrazines. Part 32. Synthesis of trisubstituted and tetrasubstituted pyrazines as ant pheromones. *J. Chem. Soc., Perkin Trans. 1* **1996**, 2345−2350.

(19) Klein, B.; Berkoeitz, J. Pyrazines. I. Pyrazine-*N*-oxide. Preparation and spectral characteristics. *J. Am. Chem. Soc.* **1959**, *81*, 5160−5166.

(20) Klein, B.; Hetman, N. E.; O'Donnel, M. E. Pyrazines III. The action of phosphoroyl chloride on pyrazine *N*-oxides. *J. Org. Chem.* **1963**, *28*, 1682−1686.

(21) Gainer, H.; Kokorudz, M.; Langdon, K. W. Chlorination of alkylpyrazines. *J. Org. Chem.* **1961**, *26*, 2360−2363.

(22) Gupta, P.; Naidu, S. V.; Kumar, P. A practice enantioselective synthesis of massoialactone via hydrolytic kinetic resolution. *Tetrahedron Lett.* **2004**, *45*, 849−851.

(23) Engel, W.; Bahr, W.; Schieberle, P. Solvent-assisted flavor evaporation-a new versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.* **1999**, *209*, 237−241.

(24) Potsatchakul, P.; Chaiseri, S.; Cadwallader, K. R. Identification of characteristic aroma components of Thai fried chili paste. *J. Agric. Food Chem.* **2008**, *56*, 528−536.

(25) Hirschberg, A.; Spoerri, P. E. Chlorination of some alkylpyrazines. *J. Org. Chem.* **1961**, *26*, 2356−2360.

(26) Ohta, A.; Masano, S.; Iwakura, S.; Tamura, A. Synthesis and reactions of some 2,3-disubstituted pyrazine monoxides. *J. Heterocycl. Chem.* **1982**, *19*, 465−472.

(27) Matsui, T.; Guth, H.; Grosch, W. A comparative study of potent odorants in peanut, hazelnut, and pumpkin seed oils on the basis of aroma extract dilution analysis (AEDA) and gas chromatography-olfactometry of headspace samples (GCOH). *Eur. J. Lipid Sci. Tech.* **1998**, *100*, 51−56.

(28) 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.

(29) Frauendorfer, F.; Schieberle, P. Identification of the key aroma compounds in cocoa powder based on molecular sensory correlations. *J. Agric. Food Chem.* **2006**, *54*, 5521−5529.

(30) Leunissen, M.; Davidson, V. J.; Kakuda, Y. Analysis of volatile flavor components in roasted peanuts using supercritical fluid extraction and gas chromatography-mass spectrometry. *J. Agric. Food Chem.* **1996**, *44*, 2694−2699.

(31) Baker, G. L.; Cornell, J. A.; Gorbet, D. W.; O'Keef, S. F.; Sims, C. A.; Talcott, S. T. Determination of pyrazine and flavor variations in peanut genotypes during roasting. *J. Food Sci.* **2003**, *68*, 394−400.

(32) Schirack, A. V.; Drake, M. A.; Sanders, T. H.; Sandeep, K. P. Characterization of aroma-active compounds in microwave blanched peanuts. *Food Chem. Toxicol.* **2006**, *71*, c513–c520.

(33) Bonvehi, J. S. Investigation of aromatic compounds in roasted cocoa powder. *Eur. Food Res. Technol.* **2005**, *221*, 19–29.

(34) Koehler, P. E.; Manson, M. E.; Odell, G. V. Odor threshold levels of pyrazine compounds and assessment of their role in the flavor of roasted foods. *J. Food Sci.* **1971**, *36*, 816–818.

(35) Fors, S. Sensory properties of volatile Maillard reaction products and related compounds. In *The Maillard Reaction in Foods and Nutrition*; Waller, G. R., Feather, M. S., Eds.; ACS Symposium Series 215; American Chemical Society: Washington, DC, 1983; p 185.