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Study on the Role of Precursors in Coffee Flavor Formation Using In-Bean Experiments

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The formation of several key odorants, such as 2-furfurylthiol (FFT), alkylpyrazines, and diketones, was studied upon coffee roasting. The approach involved the incorporation of potential precursors in green coffee beans by means of biomimetic in-bean and spiking experiments. Both labeled and unlabeled precursor molecules were used, and the target analytes in the roasted coffee samples were characterized in terms of their isotope labeling pattern and abundance. The biomimetic in-bean experiments ruled out the 2-furaldehyde route to FFT as suggested by model studies. Furthermore, no evidence was found for the incorporation of the arabinose C5 skeleton into FFT. Pathways proposed for the formation of alkylpyrazines and diketones were confirmed, and a new mechanism is suggested for the formation of 2-ethenyl-3-ethyl-5-methylpyrazine. The role of amino acids, for example, alanine, and free sugars was substantiated. The results underscore the potential of this methodology to provide better understanding of the formation pathways occurring in complex food systems, which may be different from those obtained in model experiments.

KEYWORDS: Coffee; flavor; precursor; Maillard reaction; mechanistic study

INTRODUCTION

The desirable aroma of coffee is foremost formed during the roasting step at high temperature beyond 200 °C from precursors that are present in the green beans. Sugars, proteins, free amino acids, chlorogenic acids, and trigonelline are the principal flavor precursor compounds in green coffee (reviews in refs *1* and *2*). Maillard-type reactions play a central role, among many other chemical transformations taking place during roasting of coffee, and therefore special emphasis has been devoted to studying the generation of Maillard-derived aroma compounds such as thiols, diketones, and pyrazines. Their formation has been extensively studied in model systems under dry heating conditions (*3*−*6*).

2-Furfurylthiol (FFT) is probably the most important sulfur-containing odorant in roasted coffee that has been suggested as one of the character impact compounds (*7*−*9*). Model systems reacting pentoses (e.g., ribose, xylose) or hexoses (e.g., glucose, rhamnose) with cysteine under roasting conditions usually result in high levels of FFT (*10*−*13*). As shown in arabinose/cysteine model experiments, FFT is formed via 3-deoxypentosone and 2-furaldehyde while maintaining the intact carbon chain (*6*). 2-Furaldehyde is a well-known dehydration product of pentoses formed in high yield at low pH (*14*), and hydrogen sulfide is released by the degradation of cysteine (*15*). Model reactions of arabinogalactane/cysteine models (*16*) and more refined experiments with polysaccharides isolated from green coffee and roasted in the presence of cysteine (*4*) suggested arabinogalactanes as key precursors of FFT.

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Alkylpyrazines such as 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine were reported to contribute to the earthy-roasty character of roasted coffee (*7*, *17*). Czerny et al. (*18*) identified unsaturated alkylpyrazines, such as 2-ethenyl-3,5-dimethylpyrazine, as further potent earthy-smelling compounds in roasted coffee. Amrani-Hemaimi et al. (*3*) showed in different model studies that C6 and C5 sugars (e.g., fructose, glucose, arabinose) are potential precursors for alkylpyrazines. α-Amino carbonyl fragments, which are formed through Strecker reaction of sugar degradation compounds (e.g., dicarbonyls) and α-amino acids, yield upon condensation dihydropyrazines. These are subsequently oxidized to the corresponding alkylpyrazines. In addition, alanine and glycine play a key role as their Strecker degradation products, for example, acetaldehyde and formaldehyde, are incorporated into the side chain of the alkylpyrazine molecule (*3*, *19*)).

According to Yaylayan et al. (*5*), α-diketones are formed from mono-, di-, and oligosaccharides by Maillard-type reactions in the presence of amino acids such as glycine and alanine. Besides the direct formation from the carbohydrate skeleton, the recombination of transient intermediates has been discussed (*20*).

Despite numerous studies performed in model systems (reviews in refs *1*, *21*, and *22*), flavor formation pathways in coffee are still not well understood, mainly because results of model systems can hardly be extrapolated to complex food products. The complexity of the green coffee composition as well as the chemical and physical transformations that the coffee beans undergo during roasting cannot be sufficiently well reproduced in model systems. For example, an extensive pressure increase takes place in the

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bean during roasting, caused by the high amounts of water vapor generated (*23*), the pH changes continuously, and additional reactions may take place in the gas phase or at the cell wall surface (*24*). The importance of the bean structure integrity has been shown by the fact that roasting of ground green coffee resulted in different analytical data as compared to whole green coffee (*24*). Hence, the so-called biomimetic in-bean experiments have been developed to study the importance of precursors for the formation of key aroma compounds during coffee roasting under realistic conditions. This approach is based on the use of green coffee beans as "minireactors" for model reactions, which allows a more realistic evaluation of potential precursors and provides a more precise insight into formation pathways (*25*, *26*). It consists of (i) extraction of the soluble part of the green coffee beans with hot water and (ii) drying of both the depleted beans and the obtained extract. Various subsequent studies can be envisaged with this "raw material". To test the overall concept of in-bean experiments, a complete compounded equivalent of the natural extract can be incorporated into the exhausted beans. This biomimetic recombinate can be selectively omitted or fortified in certain precursors or, for mechanistic studies, labeled precursors may be incorporated. In addition, spiking of untreated green coffee beans with precursors or precursor groups (sugars, amino acids, chlorogenic acids, etc.) represents another straightforward approach to study modulation of coffee flavor.

The aim of this study was to better understand the formation of several key odorants such as 2-furfurylthiol, alkylpyrazines, and diketones upon coffee roasting by employing the biomimetic in-bean experiment approach. Labeled precursors were used to substantiate formation pathways and evaluate the relevance of data obtained in model studies.

MATERIALS AND METHODS

Raw Material. Green Arabica coffee beans (*Coffea arabica*, Colombia, class 1.1, wet processed) were used.

Chemicals. The following chemicals were commercially available: caffeine (99.5%), copper(II) sulfate (99%), dichloromethane (99.8%), D-(−)-arabinose (99%), D-(+)-galactose (99%), D-(+)-glucose (99.5%), D-(+)-mannose (99%), D-(−)-quinic acid (98%), ethanol (99.5%), iron-(III) chloride (97%), L-alanine (99.5%), L-arginine (99.5%), L-asparagine (98.5%), L-aspartic acid (99.5%), L-cysteine (99.5%), L-glutamic acid (99.5%), L-glutamine (99.9%), L-glycine (98.5%), L-histidine (99.5%), L-isoleucine (99.5%), L-(+)-lactic acid (99%), L-leucine (99.5%), L-lysine (98%), L-methionine (99.5%), L-phenylalanine (98%), L-proline (99.5%), L-(+)-rhamnose (99%), L-serine (98.5%), L-threonine (98.5%), L-tyrosine (99%), L-valine (99.5%), malic acid (99.5%), manganese(II) chloride (98%), potassium acetate (99%), potassium hydroxide (85%), trigonelline hydrochloride (98%), zinc sulfate heptahydrate (99%) (Sigma-Aldrich, Buchs, Switzerland); chlorogenic acid (98%, APIN Chemicals, Oxon, U.K.); D-(+)-sucrose (99.5%), potassium citrate (99%, Riedel de Haen, Buchs, Switzerland); D-[U-$^{13}C_5$]-arabinose (98%), [U-$^{13}C_6$fructose]-sucrose (98%) (Omicrometer Biochemicals, Inc., South Bend, IN); [$^2H_4$]-furfural (98%), and L-[3-^{13}C]-alanine (99%) (Cambridge Isotope Laboratories Schweiz, Innerberg, Switzerland).

Reference Compounds. The following reference compounds were used in this study: 2-furaldehyde (**1**), 2-furfurylthiol (FFT, **2**), methanethiol (**3**), dimethyl sulfide (**4**), 2,3-butanedione (**6**), 2,3-pentanedione (**7**), 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (**8**), 2-ethyl-3,6-dimethylpyrazine (**9**), 2-ethyl-3,5-dimethylpyrazine (**10**), 2,3-diethyl-5-methylpyrazine (**11**), 3-isobutyl-2-methoxypyrazine (**12**), 2-acetylpyrazine (**15**) (Sigma-Aldrich, Buchs, Switzerland); 3-mercapto-3-methylbutyl formate (**5**) (Oxford Chemicals, Hartlepool, U.K.); and 2-ethenyl-3-ethyl-5-methylpyrazine (**14**) (Toronto Research Chemicals, North York, Canada).

Internal Standards. The following isotopically labeled standards were used for quantification: [$^{13}C_4$]-2,3-butanedione, [$^{13}C_2$]-2,3-pentanedione, [$^2H_2$]-3-mercapto-3-methylbutyl formate, [$^2H_5$]-2-ethyl-3,5-dimethylpyrazine, [$^{13}C_2$]-4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (aromaLAB, Munich, Germany); [$^2H_6$]-dimethyl sulfide, [$^2H_4$]-2-furaldehyde, [$^2H_3$]-2,3-diethyl-5-methylpyrazine (CDN Dr. Ehrenstorfer GmbH, Augsburg, Germany); [$^2H_2$]-2-furfurylthiol (Toronto Research Chemicals); [$^2H_3$]-methanethiol (Sigma-Aldrich, Buchs, Switzerland); and [$^2H_3$]-3-isobutyl-2-methoxypyrazine (WITEGA Laboratorien, Berlin, Germany).

Hot Water Extraction of Green Coffee Beans. Green coffee beans were extracted with hot water as reported in the literature (*25*) using some modifications. Seventy kilograms of green coffee beans was extracted consequently four times with 280 L of demineralized water at 95 °C for a total of 4 h to obtain the water-soluble substances. The extracts were combined and concentrated by a climbing film evaporator (LCI, Charlotte, NC) to a total solid (TS) content of approximately 27%. In a second step, both the exhausted beans and the aqueous natural green bean extract were freeze-dried in an Atlas Cabinet freeze-dryer (NIRO, Søborg, Denmark) and stored at −40 °C until use.

Reincorporation of the Whole Natural Green Coffee Extract. On the basis of the yield of the hot water extraction, a defined amount (33.95 g) of the dried green coffee extract was dissolved in 65 g of demineralized water at 80 °C. The dark brown extract was added to 125 g of the freeze-dried, exhausted beans (WEB) in a 1 L flask. The mixture was slowly stirred for 4 h at 70 °C and subsequently overnight at room temperature (14 h) on a Rotavapor (Buechi, Flawil, Switzerland).

Incorporation of Biomimetic Recombinate. For the preparation of the biomimetic recombinate the single components (based on analytical results of the water-soluble green coffee composition, see **Table 1**) were dissolved in demineralized water at 80 °C. Fifty grams of water was used for 125 g of exhausted beans (WEB) to guarantee a complete incorporation of the model solution into the coffee beans. The pH value of the compounded water-soluble fraction was adjusted to 5.5 (corresponding to the pH of the natural extract) with a 16.5% w/w solution of KOH, and water-exhausted green coffee beans were soaked with the biomimetic recombinate at 50 °C for at least 5 h. During soaking, the beans were gently stirred using a Rotavapor.

Omission Experiments. The biomimetic recombinate was incorporated into the WEB coffee as described above with the exception of certain precursor groups. In two separate experiments, the compounded extracts were omitted either in all free sugars or in all free amino acids.

Spiking of Precursor Compounds. For the flavor modulation experiments, untreated green beans were fortified with different sugars such as glucose, arabinose, and rhamnose (0.025 mol; approximately 4 g of each sugar) or sucrose (4 g; about 34% of the natural amount in coffee). To reveal the role of free amino acids, L-cysteine (0.45 g/150 g of beans) and L-alanine (0.48 g/150 g of beans; 70% of total natural content), respectively, were also incorporated in the reference green beans. Each of the precursors was dissolved in 60 g of demineralized water at room temperature, and green coffee beans (150 g) were soaked with the prepared solution for 2 h at 50 °C and for 2 h at room temperature.

Mechanistic Studies. For the mechanistic studies, D-[U-$^{13}C_5$]-arabinose (0.9 g, 16% of total natural content) or [U-$^{13}C_6$-fructose]-sucrose (2 g; 17% of total natural content) was added to the recombined model extract, which was omitted in all sugars, and incorporated into the exhausted green beans (WEB). In addition, untreated green coffee beans were spiked with L-[3-^{13}C]-alanine (0.48 g/150 g of beans, 70% of total natural content) and [$^2H_4$]-2-furaldehyde (30 mg/150 g of beans).

Drying of Reconstituted Green Coffee Beans. The wet coffee beans were frozen to −80 °C and then freeze-dried at −53 °C. The drying time depended on the efficiency of the reincorporation and had to be identified in each trial by determination of the moisture content. The first moisture content was measured after 24 h, and freezing was then extended as a function of the obtained result to reach a moisture content of 10 ± 0.5 g/ 100 g wb (on weight basis).

Laboratory Roasting Trials. A standard procedure using the same roasting conditions (without considering the final color) was defined for roasting of the coffee samples. Consequently, the same thermal energy was provided to all coffee beans, which allowed an appropriate comparison of all samples. After freeze-drying, the green beans were roasted under same conditions on a "Signum" rotating fluidized bed roaster (Neuhaus-Neotec, Germany) for 380 s at 236 °C. The color of the roasted coffee samples was measured with a Colortest II instrument (Neuhaus-Neotec). The measured color test number (CTN) gives information about the roasting degree of

Table 1. Amounts of Components Used for the Preparation of Biomimetic Recombinate[a]

component	amount (g)	component	amount (g)
phenols[b]		amino acids (continued)[b]	
chlorogenic acid	8.849	L-proline	0.105
trigonelline[b]	1.584	L-tyrosine	0.049
caffeine[b]	1.031	L-valine	0.042
organic acids[b]		L-isoleucine	0.063
D-(−)-quinic acid	0.865	L-leucine	0.052
L-(+)-lactic acid	0.0034	L-phenylalanine	0.076
potassium acetate	0.081	L-lysine	0.031
malic acid	0.398	sugars[b]	
potassium citrate	2.620	D-(−)-arabinose	0.011
amino acids[b]		L-(+)-rhamnose	0.0036
L-alanine	0.028	D-(+)-galactose	0.198
L-threonine	0.109	D-(+)-sucrose	12.48
L-serine	0.070	D-(+)-mannose	0.029
L-aspartic acid	0.286	metals[c]	
L-glutamic acid	0.205	iron(III) chloride	0.0084
L-glycine	0.044	manganese(II) chloride	0.0048
L-histidine	0.015	copper(II) sulfate	0.0053
L-arginine	0.075	zinc(II) sulfate, heptahydrate	0.0008

[a] For 125 g of exhausted beans. [b] Data from Dr. Karin Kraehenbuehl (Nestle Research Center, Lausanne) using standard analytical methods (personal communication). [c] Initial weight of metals was adopted from Mueller and Hofmann (*36*).

Table 2. Roasting Degree of Model Systems

	model system[a]											
	1	2	3	4	5	6	7	8	9	10	11	12
roasting degree (CTN)[b]	88	119	83	81	77	144	66	62	60	67	79	77
humidity (%)	6.1	5.0	6.8	5.8	5.0	5.0	7.8	6.7	6.8	7.3	6.2	6.2

[a] Referring to **Table 4**. [b] CTN, color test number (Neuhaus).

the coffee sample; that is, dark samples are indicated by low CTN, whereas lighter samples show higher CTN values. The obtained CTN values varied within 3 units among replicates (**Table 2**). After roasting, all swollen green beans returned to a smaller size, which was close to the roasted coffee beans from untreated green coffee.

Quantification of Coffee Aroma Compounds. The following odorants were analyzed: 2-furaldehyde (**1**), 2-furfurylthiol (FFT, **2**), methanethiol (**3**), dimethyl sulfide (**4**), 3-mercapto-3-methylbutyl formate (**5**), 2,3-butanedione (**6**), 2,3-pentanedione (**7**), 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (HDMF, **8**), 2-ethyl-3,6-dimethylpyrazine (**9**), 2-ethyl-3,5-dimethylpyrazine (**10**), 2,3-diethyl-5-methylpyrazine (**11**), and 3-isobutyl-2-methoxypyrazine (**12**).

Roast and ground (R&G) coffee (5 g for compounds **2**−**5** and **8**; 1 g for compounds **1**, **6**, **7**, and **9**−**12**) was suspended in 100 mL of boiling water and stirred for 10 min. After cooling, the obtained slurry was spiked with defined quantities of labeled isotopes of the analytes. Cysteine (25 mg/g of R&G coffee) was added to the suspension for the quantification of 2-furfurylthiol (to suppress reactions of FFT with coffee matrix; see also ref *25*), and the mixture was stirred for a further 30 min. The coffee solution was subsequently stirred during 10 min, and 7 mL was transferred to a 20 mL headspace vial.

Sample preparation was carried out in duplicates. Single preparation was performed for compounds **5**, **8**, and **10** (**Table 5**). Relative standard deviation (RSD) of duplicates was < 20%, except for compound **6** with an RSD of 24% in sample 12 and compound **11** with and RSD of 30% in samples 8 and 9 (**Table 6**).

SPME-GC-MS Analysis. Coffee aroma compounds were sampled by solid phase microextraction (SPME) at 40 °C during 10 min (compounds **1**−**7** and **9**−**12**) and at 60 °C during 30 min (compound **8**) using a Supelco 50/30 μm StableFlex DVB/CAR/PDMS fiber (Supelco, Buchs, Switzerland). Separation was carried out on a 60 m × 0.25 mm × 0.25 μm polar ZB-Wax column (Phenomenex, Brechbühler, Switzerland) using a Trace gas chromatograph (Thermo, Brechbühler, Switzerland). Detection of aroma compounds was carried out on a quadrupole mass spectrometer DSQ (Thermo). Quantitative analyses were performed in the selective ion monitoring (SIM) mode as well as in full scan mode (m/z 30−200).

The experiments with the labeled precursors were analyzed in only the full scan mode. All results were corrected for the ^{13}C content of the natural isotope and, if relevant, for the loss of one hydrogen atom (compounds **1**, **9**−**11**, **13**, **14**). An obtained percentage after correction of < 0.5% was set to 0% by definition.

RESULTS AND DISCUSSION

Evaluation of the Biomimetic In-Bean Approach. The yield of soluble solids was determined as 19.4% by hot water extraction at 95 °C. On a dry matter basis, the yields amounted to 21.7% for the water extract and 78.3% for the depleted coffee beans. The water content was found to be 5.0% for the dried, water-exhausted beans (WEB) and 3.1% for the freeze-dried water extract. On the basis of the results of the chemical composition, a mass balance was calculated for all groups of components in the reference green coffee beans (RGB), the WEB, and the freeze-dried water extract (**Table 3**). Free amino acids (97%; corresponds to 3% of total amino acids) and trigonelline (96%) were almost quantitatively removed from RGB. High extraction yields were also obtained for caffeine (91%), sucrose (83%; corresponds to 93% of extractable sugars), and organic acids (80%). About 95% of the total amino acids, 82% of the total sugars, and 83% of the analyzed metals remained in the water-insoluble fraction of the green beans. The incorporation efficiency of the soaking of hydrophilic components in the depleted coffee beans was only evaluated visually. A successful incorporation was supposed to be achieved when beans presented no residual components on the surface. **Table 4** provides an overview of the performed biomimetic in-bean as well as spiking experiments.

Table 5 summarizes the results of key aroma compounds quantitated in roasted samples of reference green beans (RGB, sample 1), water-exhausted beans (WEB, sample 2), exhausted beans fortified with the natural extract (NREB, sample 3), exhausted beans reconstituted with the biomimetic recombinate (BREB, sample 4), and the latter in the absence of free amino acids or water-extractable sugars. In general, most of the analyzed aroma compounds decreased considerably in the exhausted beans as a result of the water extraction of soluble precursors, which is well in line with previously reported data (*25*). In particular, key compounds such as dimethyl sulfide (**4**, −77%), 3-mercapto-3-methylbutyl formate (**5**, −98%), diketones (−40% for 2,3-butanedione **6** and −83% for 2,3-pentanedione **7**), 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (**8**, −35%), and the pyrazines (**9**−**12**, up to −89%) were affected. Noticeable increase was found for only FFT (**2**, +61%) and methanethiol (**3**, +86%).

To prove the efficiency of the in-bean incorporation, the extracted beans were soaked with the natural extract and the beans were roasted at the same temperature and for the same period of time as the roasted green beans. Quantitative analysis of this sample resulted in similar levels of assessed odorants as compared to the roasted RGB. Thus, it can be assumed that hot water extraction and subsequent re-incorporation of the whole extract into the green coffee bean did not provoke major changes, either in the freeze-dried water extract or in the bean structure. Exceptions were dimethyl sulfide (**4**) and 3-isobutyl-2-methoxypyrazine (**12**), the levels of which declined to about half of that of the roasted green beans. The latter component is already present in the aromatic fraction of green coffee and is partly removed by the hot water extraction. Unlike the alkylpyrazines, no subsequent generation of odorant **12** occurs during roasting (*27*).

Different results were obtained when reconstituting WEB with a biomimetic recombinate (BREB) (see **Table 1** for the

Table 3. Percentage Composition of the Green Coffee Extract[a]

component[b]	standard green beans	water extract[c]	exhausted beans[d]	extracted[e]	recovery[f]
lipids	13.3	0	13.1	0	98.1
ashes	3.63	2.68	0.96	73.8	100.3
total phenols	7.15	5.85	0.76	81.8	92.4
caffeine	1.28	1.16	0.04	90.6	93.8
trigonelline	0.71	0.68	0.02	95.8	98.6
total organic acids	2.25	1.81	0.35	80.4	96.0
total amino acids	10.65	0.50	10.32	4.7	101.6
free amino acids	0.31	0.30	0.01	96.8	100.0
total sugars	44.50	7.87	32.61	17.7	91.0
sucrose	8.81	7.30	0.76	82.9	91.5
metals	0.0081	0.0014	0.0069	17.3	102.5

[a] Values are expressed on percent dry matter basis. [b] Main ingredients in green coffee. [c] From 100 g of green beans; TS at 21.7%. [d] From 100 g of green beans (on dry matter basis). [e] Extraction yield (%) from green beans. [f] Recovery (%) = (exhausted + water extracted)/standard green beans × 100.

Table 4. Coffee Bean Experiments To Study the Formation of Key Aroma Compounds in Coffee

sample[a]	sample[b]
coffee samples	*mechanistic studies*
(1) reference green beans (RGB)	(13) BREB including D-[U-$^{13}C_5$]-arabinose
(2) water-extracted, exhausted beans (WEB)	(14) BREB including [U-$^{13}C_6$-fructose]-sucrose
biomimetic in-bean experiments	(15) RGB + [$^2H_4$]-2-furaldehyde
(3) WEB + whole natural extract (NREB)	(16) RGB + L-[3-^{13}C]-alanine
(4) WEB + biomimetic recombinate (BREB)	
(5) BREB omitted in amino acids	
(6) BREB omitted in sugars	
spiking with hydrophilic precursors	
(7) reference green beans (RGB) + sucrose	
(8) RGB + glucose	
(9) RGB + arabinose	
(10) RGB + rhamnose	
(11) RGB + L-alanine	
(12) RGB + L-cysteine	

[a] Experiments 3−6 were carried out with water-extracted (exhausted) coffee beans (WEB). Experiments 7−12 were carried out with reference green coffee beans (RGB). [b] Samples 13 and 14 were obtained by adding the biomimetic recombinate omitted in residual sugars to the water-extracted beans; samples 15 and 16 were obtained by spiking reference green coffee beans with labeled compounds.

composition of the biomimetic recombinate). As shown in **Table 5**, much higher concentrations of the alkylpyrazines **9**−**11** were found in BREB as compared to the roasted RGB. In contrast, dimethyl sulfide (**4**), 3-mercapto-3-methylbutyl formate (**5**), 2-furaldehyde (**1**), and methanethiol (**3**) were present in considerably lower amounts in roasted BREB. A third group of odorants comprising 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (**8**), FFT (**2**), and the diketones **6** and **7** was determined at similar concentrations. Incorporation of aqueous solutions of several flavor precursors (e.g., sucrose) into green coffee has been studied by fluorescence microscopy (*28*). The authors proved the entire penetration of sucrose into the green coffee bean within 5−6 h. Hence, it can be assumed that most of the hydrophilic precursors used in this study were well distributed in RGB. However, our results suppose that the BREB composition does not correspond exactly to that of the natural extract, which is very difficult to achieve. Therefore, all results of the biomimetic in-bean experiments (samples 5 and 6) were compared with those of sample 4 (BREB). On the other hand, sample 1 (RGB) was taken as reference for the spiking experiments.

Formation of 2-Furaldehyde and 2-Furfurylthiol. The role of 2-furaldehyde (**1**) as potential intermediate in the formation of FFT (**2**) was evaluated by quantitative analysis of both compounds in roasted coffee samples. As shown in **Table 5** (sample 2), roasted WEB contained significantly higher amounts of FFT (+61%) than reference sample 1, which is well in line with the findings of Milo et al. (*25*). The authors explained this phenomenon by the absence of competing reactions and concluded that FFT is mainly formed from water-nonsoluble precursors. In contrast to FFT, 2-furaldehyde was by 19% lower in roasted exhausted beans (sample 2) as compared to the reference (sample 1). The omission of all water-soluble sugars in the biomimetic recombined green coffee (sample 6) resulted again in significantly higher amounts of FFT (+62%), whereas the 2-furaldehyde content was highly suppressed to $<40\%$ as compared to sample 4. In the absence of all free amino acids (sample 5) 2-furaldehyde increased by 29%, whereas the level of FFT remained constant. Furthermore, spiking experiments did not show a link between the formation of FFT and 2-furaldehyde, as fortification of green beans with sucrose (34% of natural content) increased 2-furaldehyde amounts up to 161%, whereas concentrations of FFT considerably decreased by 32% (**Table 6**). On the other hand, the addition of labeled [^{2}H]$_4$-2-furaldehyde (30 mg/150 g) to green beans did not result in any [^{2}H]$_4$-FFT (0% m/z 118) as shown in **Table 7**.

Neither fully labeled 2-furaldehyde nor fully labeled FFT was generated by the addition of D-[U-$^{13}C_5$]-arabinose to the biomimetic recombinate that was omitted in all free sugars, but partially labeled FFT with molecular masses of 115 (3%), 116 (4%) and 117 (4%; **Table 7**) was . Consistently, spiking of green coffee with arabinose did not result in increased amounts of either FFT or 2-furaldehyde, thus corroborating that FFT is mainly formed from the non-water-soluble fraction. In contrast, spiking experiment with L-cysteine resulted in enhanced FFT amounts (+49%; **Table 6**), showing that cysteine is one important S source in the formation of FFT. As amounts of free cysteine are insignificant in green coffee (*29*, *30*) and FFT quantities were found to be similar in roasted WEB spiked with the biomimetic recombinate (sample 4, no cysteine added) as compared to roasted RGB (1.07 and 1.15 mg/kg, respectively), bound cysteine seems to be the key precursor. However, it cannot be excluded that additional S sources exist, which is also related to the fact that the yield of FFT in roasted coffee (as per mol % total cysteine) is relatively high as compared to cysteine-based Maillard model systems (data not shown). Overall, it seems that the formation of FFT during coffee roasting via 3-deoxypentosone and 2-furaldehyde as intermediate compounds represents only a minor pathway. FFT is mainly generated from the water-nonsoluble part of precursors, which is valid for both the ring skeleton of the molecule and the sulfur source. Furthermore, it can be assumed either that FFT formation takes place in a region of the coffee beans that cannot be simulated by in-bean experiments (i.e., by simple incorporation of arabinose and/or cysteine) or that other types of precursors and/or pathways have to be considered. Such precursors could be matrix-bound *S*-glycosides composed of a sugar and cysteine or a cysteine derivate. In terms of sugar-derived key intermediates for FFT, the importance of furfuryl alcohol (FF−OH) should also be evaluated. Although it is known that the formation yields of FFT from FF−OH are by a factor of >10 lower relative to furfural (*37*), FF−OH contents in roasted coffee are by a factor of >10 higher as compared to furfural (e.g., own data, not published). In

Table 5. Concentrations of Aroma Compounds in Roasted Reference Green Beans (RGB) Compared to Roasted Water-Exhausted Beans (WEB) and Roasted Water-Exhausted Beans Reconstituted with either the Natural Extract (NREB) or the Biomimetic Recombinate (BREB)

		concentrations (mg/kg of dm)					
no.	compound	(1) RGB[a]	(2) WEB[b]	(3) NREB[c]	(4) BREB[d]	(5) BREB (no amino acids)[e]	(6) BREB (no free sugars)[f]
1	2-furaldehyde	165.8	133.8	146.8	112.7	145.9	38.3
2	2-furfurylthiol (FFT)	1.15	1.85	1.05	1.07	1.12	1.73
3	methanethiol	1.90	3.54	2.50	1.23	1.77	3.41
4	dimethyl sulfide	1.56	0.36	0.67	0.12	0.24	0.16
5	3-mercapto-3-methylbutyl formate	0.33	0.005	0.36	0.013	0.019	0.021
6	2,3-butanedione	79.0	47.2	82.9	69.5	65.6	30.6
7	2,3-pentanedione	46.2	7.88	48.0	35.0	36.1	3.26
8	4-hydroxy-2,5-dimethyl-3[2*H*]-furanone	230.9	149.3	237.0	232.3	375.6	105.0
9	2-ethyl-3,6-dimethylpyrazine	2.32	1.18	2.20	5.47	1.10	7.84
10	2-ethyl-3,5-dimethylpyrazine	0.95	0.70	0.90	1.81	0.91	3.16
11	2,3-diethyl-5-methylpyrazine	0.35	0.13	0.34	0.88	0.18	1.25
12	3-isobutyl-2-methoxypyrazine	0.14	0.016	0.074	0.10	0.12	0.029

[a] Roasted reference green beans (RGB). [b] Roasted water-exhausted beans (WEB). [c] Roasted water-exhausted beans reconstituted with the natural extract. [d] Roasted water-exhausted beans with biomimetic recombinate incorporated. [e] Roasted exhausted beans with biomimetic recombinate, but all amino acids omitted. [f] Roasted exhausted beans with biomimetic recombinate, but all free sugars omitted.

Table 6. Concentrations of Aroma Compounds Obtained by Roasting of Reference Green Coffee Beans Spiked with Precursors (Sugars, Amino Acids)

	concentrations (mg/kg of dry matter) for model systems (green coffee beans spiked with precursors)[a]					
compound	(7) RGB + SUC	(8) RGB + GLU	(9) RGB + ARA	(10) RGB + RHA	(11) RGB + ALA	(12) RGB + CYS
2-furaldehyde (**1**)	266.9	196.4	156.8	173.6	152.2	163.1
2-furfurylthiol (**2**)	0.78	1.08	1.18	1.12	1.14	1.71
methanethiol (**3**)	1.84	2.54	2.57	2.66	2.25	2.89
dimethyl sulfide (**4**)	1.31	1.97	1.75	1.41	1.33	2.02
3-mercapto-3-methylbutyl formate (**5**)	0.52	0.52	0.34	0.32	0.31	0.30
2,3-butanedione (**6**)	88.3	85.5	98.8	89.0	84.3	81.4
2,3-pentanedione (**7**)	74.7	56.5	44.7	54.0	54.2	86.7
4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (**8**)	266.2	237.5	233.9	530.3	228.3	250.1
2-ethyl-3,6-dimethylpyrazine (**9**)	1.45	0.62	0.52	1.26	16.8	3.50
2-ethyl-3,5-dimethylpyrazine (**10**)	0.56	0.33	0.32	1.04	2.90	1.23
2,3-diethyl-5-methylpyrazine (**11**)	0.23	0.089	0.084	0.42	3.26	0.55

[a] Reference green beans (RGB), sucrose (SUC), glucose (GLU), arabinose (ARA), rhamnose (RHA), alanine (ALA), and cysteine (CYS).

Table 7. Percentage Labeling Distribution of 2-Furaldehyde and 2-Furfurylthiol Generated from Isotope-Labeled Precursors upon Coffee Roasting

	2-furaldehyde (**1**)[a]				2-furfurylthiol (**2**)[a]			
ion	*m*/*z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [$^{2}H_4$]-2-FA	*m*/*z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [$^{2}H_4$]-2-FA
M	96	95	98	97	114	95	89	98
M + 1	97	1	1	3	115	0	3	0
M + 2	98	0	1	0	116	5	4	2
M + 3	99	0	0	0	117	0	4	0
M + 4	100	0	0	0	118	0	0	0
M + 5	101	4	0	0	119	0	0	0

[a] Sucrose (SUC), arabinose (ARA), 2-furaldehyde (2-FA). [b] Biomimetic recombinate omitted in free sugars.

addition, the formation kinetics of FF−OH are similar to those of FFT (*38*).

Formation of Alkylpyrazines. Quantification of pyrazines **9**−**11** in roasted coffee (obtained from green coffee that was either spiked with or devoid of precursors) aimed at evaluating the role of free amino acids in their formation. The absence of all free amino acids noticeably decreased the amounts of analyzed alkylpyrazines (**Table 5**, sample 5). As an example, the odor-relevant 2-ethyl-3,5-dimethylpyrazine (**10**) and 2,3-diethyl-5-methylpyrazine (**11**) were determined at levels of 50 and 21%, respectively, compared to the roasted exhausted beans spiked with the biomimetic recombinate (BREB, sample 4). The importance of the amino acids in pyrazine formation was further substantiated by spiking of green coffee with ^{13}C-labeled alanine, which was efficiently incorporated into the 2-ethyl-3,6-dimethylpyrazine molecule (**9**) (**Table 8**) with a 93% $[M + 1]^+$ (*m*/*z* 137) yield and of 2-ethyl-3,5-dimethylpyrazine (**10**), yet to a considerably lower extent with 33% of $[M + 1]^+$ ions. This difference in incorporation yields is in alignment with the 7.2-fold increase of pyrazine **9** in the spiking experiment with unlabeled alanine, whereas the amount of pyrazine **10** was just tripled (**Table 6**). Similar results were shown for 2,3-diethyl-5-methylpyrazine (**11**), which increased by a factor of 9.4 by spiking the green coffee with alanine, and where the addition of labeled alanine led to high amounts of single-labeled (44%, *m*/*z* 151) and double-labeled (34%, *m*/*z* 152) isotopomers of pyrazine **11** (**Tables 6** and **8**).

To study the impact of free sugars on pyrazine formation, recombined green coffee was devoid of all water-soluble sugars (mainly sucrose). This led to a considerable increase in alkylpyrazines (**Table 5**); especially the amounts of pyrazine **10** were enhanced by >70%. In addition, spiking of green beans with sucrose, glucose, and arabinose had a highly suppressing effect on

Table 8. Percentage Labeling Distribution of Pyrazines Generated from Isotope-Labeled Precursors[a] upon Coffee Roasting

	2-ethyl-3,6-dimethylpyrazine (**9**)				2-ethyl-3,5-dimethylpyrazine (**10**)			
ion	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [^{13}C]-ALA	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [^{13}C]-ALA
M	136	56	58	5	136	66	55	67
M + 1	137	0	0	93	137	5	9	33
M + 2	138	9	7	2	138	3	0	0
M + 3	139	28	18	0	139	13	16	0
M + 4	140	0	0	0	140	10	6	0
M + 5	141	3	7	0	141	2	8	0
M + 6	142	4	9	0	142	0	0	0
M + 7	143	0	0	0	143	1	5	0
M + 8	144	0	1	0	144	0	1	0
	2-ethenyl-3,5-dimethylpyrazine (**13**)				2-ethenyl-3-ethyl-5-methylpyrazine (**14**)			
ion	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [^{13}C]-ALA	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [^{13}C]-ALA
M	134	39	43	71	148	51	67	51
M + 1	135	17	16	28	149	6	0	49
M + 2	136	0	0	1	150	0	7	0
M + 3	137	37	16	0	151	22	12	0
M + 4	138	0	0	0	152	14	5	0
M + 5	139	7	11	0	153	0	0	0
M + 6	140	0	0	0	154	5	9	0
M + 7	141	0	8	0	155	2	0	0
M + 8	142	0	6	0	156	0	0	0
M + 9					157	0	0	0
	2,3-diethyl-5-methylpyrazine (**11**)				2-acetylpyrazine (**15**)			
ion	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [^{13}C]-ALA	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	BREB[b] + [$^{13}C_5$]-ARA	RGB + [^{13}C]-ALA
M	150	69	58	22	122	76	94	96
M + 1	151	2	2	44	123	1	1	1
M + 2	152	6	6	34	124	7	0	3
M + 3	153	9	11	0	125	0	0	0
M + 4	154	6	4	0	126	13	0	0
M + 5	155	7	14	0	127	0	0	0
M + 6	156	0	1	0	128	3	5	0
M + 7	157	1	3	0				
M + 8	158	0	1	0				
M + 9	159	0	0	0				

[a] Sucrose (SUC), arabinose (ARA), alanine (ALA). [b] Biomimetic recombinate omitted in free sugars.

alkylpyrazine contents (**Table 6**), thus indicating that they are mainly formed from bound sugars (or other carbonyl sources) and that their generation involves competition between bound and free sugars for the water-extractable nitrogen source.

Labeled isotopomers of sucrose (with fully labeled fructose moiety), arabinose, and alanine were incorporated into the sugar-free biomimetic recombinates as well as spiked into green beans to investigate the formation mechanisms of aroma-relevant alkylpyrazines. **Table 8** shows the isotope ratios of compounds **9**−**11** and **13**−**15**, based on the molecular ions as generated by electron impact ionization. Comparison of pyrazines **9** and **10** with respect to their labeling distribution revealed noticeable differences in the experiment with labeled sucrose. Pyrazine **9** resulted in higher yields of $[M + 3]^+$ (*m/z* 139) and to a lesser extent also in some $[M + 6]^+$ ions (*m/z* 142), indicating the reaction route through methylglyoxal (2-oxopropanal) and acetaldehyde as intermediates. Low et al. (*19*) showed that this pathway favors the formation of pyrazine **9**, rather than pyrazine **10**. This is also reflected in their different behavior in the omission and spiking experiments: $[M + 3]^+$ isotopomers (13%) were also found for pyrazine **10**, but in addition remarkable yields of $[M + 1]^+$ (5%, *m/z* 137) and $[M + 4]^+$ (10%, *m/z* 140) ions occurred. These findings indicate that probably another pathway occurs in the formation of pyrazine **10** involving Maillard fragments other than methylglyoxal and acetaldehyde. The molecular mass 137 ($[M + 1]^+$) probably stems from the incorporation of a labeled formaldehyde residue, whereas the relatively high yield of *m/z* 140 ($[M + 4]^+$) must originate from an intact C_4 fructose moiety. The experiment performed with fully labeled arabinose revealed similar trends and thus underscores the diversity in formation mechanism between pyrazines **9** and **10**.

A similar pathway was proposed for the unsaturated analogue 2-ethenyl-3,5-dimethylpyrazine (pyrazine **13**) as for pyrazine **10** (*18*), with 2-(1-hydroxyethyl)-3,5-dimethyldihydropyrazine as intermediate compound. The only difference is that a vinyl group is formed in pyrazine **13** by dehydration and oxidation of the above intermediate. However, addition of labeled sucrose to BREB resulted in considerably different labeling distribution patterns of pyrazine **13** as compared to pyrazine **10**. Pyrazine **13** has been efficiently labeled and resulted in high amounts of $[M + 1]^+$ with 17% and $[M + 3]^+$ ions with 37% yield; hence, still another formation pathway may exist for this compound. Hofmann and Schieberle (*10*) proposed a hypothetical formation pathway, which involves the condensation of two amino carbonyl compounds formed from the α-diketones 1-desoxypentosone and 2-oxopropanal through transamination to the dihydropyrazine intermediate. In a final step, elimination of two molecules of water leads to pyrazine **13**. This pathway is also supported by our results. As formation of 1-deoxypentosone is not favored from the labeled sucrose, predominantly labeled 2-oxopropanal may

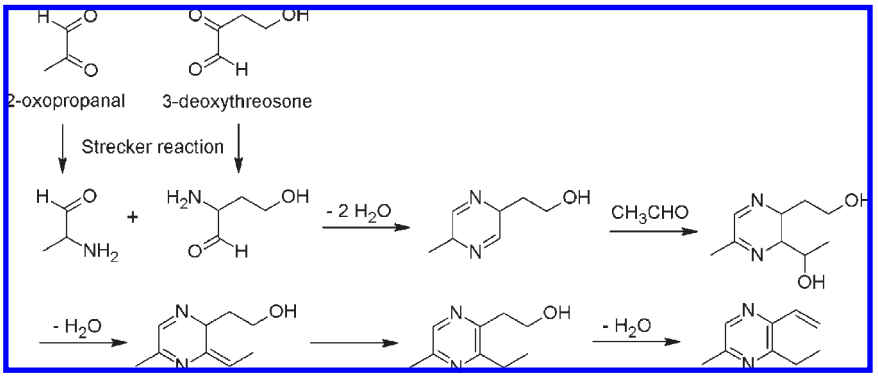

Figure 1. Hypothetical formation of 2-ethenyl-3-ethyl-5-methylpyrazine.

Table 9. Percentage Labeling Distribution of 2,3-Butanedione, 2,3-Pentanedione, and 4-Hydroxy-2,5-dimethyl-3[2*H*]-furanone Generated from Isotope-Labeled Precursors[a] upon Coffee Roasting

	2,3-butanedione (**6**)			2,3-pentanedione (**7**)			4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (**8**)		
ion	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	RGB + [^{13}C]-ALA	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	RGB + [^{13}C]-ALA	*m/z*	BREB[b] + [$^{13}C_6$]-SUC	RGB + [^{13}C]-ALA
M	86	96	99	100	83	95	128	83	99
M + 1	87	1	1	101	0	5	129	0	1
M + 2	88	1	0	102	2	0	130	0	0
M + 3	89	1	0	103	4	0	131	1	0
M + 4	90	1	0	104	1	0	132	0	0
M + 5				105	10	0	133	0	0
M + 6							134	16	0

[a] Sucrose (SUC), arabinose (ARA), alanine (ALA). [b] Biomimetic recombinate omitted in free sugars.

contribute to the labeling of the pyrazine, leading to the $[M + 3]^+$ isotopomer (37%, *m/z* 137). However, considerable amounts of $[M + 1]^+$, probably due to the incorporation of formaldehyde, were also detected, suggesting another formation pathway. Spiking with labeled arabinose corroborated the hypothesis suggested above. In addition to the molecular masses at *m/z* 135 and 137, also $^{13}C_7$ and $^{13}C_8$ isotopomers were found (8 and 6%, respectively) that derive from the reaction of labeled C_2/C_3 and C_5 moieties. In the presence of excess amounts of alanine, a further pathway has to be considered, because the spiking of green coffee with labeled alanine led to efficient incorporation of the labeling (28% $[M + 1]^+$) into pyrazine **13**.

In parallel to pyrazine **13**, the formation of 2-ethenyl-3-ethyl-5-methylpyrazine (pyrazine **14**) was studied. In the biomimetic model system with labeled sucrose, the isotopomers $[M + 3]^+$ and $[M + 4]^+$ were predominantly formed at yields of 22% (*m/z* 151) and 14% (*m/z* 152), respectively. Moreover, some single (*m/z* 149), 6-fold (*m/z* 154), and 7-fold (*m/z* 155) isotopomers were determined. The result of this mechanistic experiment suggests that pyrazine **14** is formed through a combination of C_3 and C_4 sugar moieties. In addition, spiking of green coffee with labeled alanine showed high levels of incorporation (49%; *m/z* 149). On the basis of these results, a mechanism is proposed whereby C_3 and C_4 moieties build the skeleton pyrazine structure, followed by the addition of a C_2 group from alanine. The mechanism shown in **Figure 1** suggests 2-oxopropanal and 3-deoxythreosone as key intermediates. The latter can be formed by transformation of a hexose (fructose or glucose) into erythrulose via β-dicarbonyl cleavage of the transient intermediates 1-deoxyhexo-2,3-diulose and 1-deoxyhexo-2,4-diulose followed by a dehydration step (not shown; (*31*)). Both 3-deoxythreosone and 2-oxopropanal are then transaminated by the Strecker reaction, resulting in 2-amino-4-hydroxy-1-butanal and 2-aminopropanal, respectively. The C_3 and C_4-amino compounds condense to form the dihydropyrazine derivative. Addition of acetaldehyde (Strecker aldehyde of alanine) to the ring followed by subsequent dehydration reactions is supposed to give rise to pyrazine **14**.

Spiking with labeled sugars induced a dispersed labeling pattern for pyrazine **11** with $[M + 2]^+$, $[M + 3]^+$, $[M + 4]^+$, and $[M + 5]^+$ molecular ions (**Table 8**). The various sugar cleavage fragments such as 2-oxobutanal (an aldol reaction product from acetaldehyde and glycolaldehyde) and 2-oxopropanal involved in the proposed pathway can explain such a scattered distribution pattern (*3*, *19*).

2-Acetylpyrazine is different from the alkylpyrazines not only with regard to its structure but also in terms of its odor character (roasty, popcorn-like) as well as formation pathway. Scarpellino and Soukup (*32*) proposed a formation pathway from sucrose via the sugar fragments glyoxal (C2) and the *C*-methyltriose reductone (C4). In contrast to the formation of alkylpyrazines, glyoxal does not undergo transamination by the Strecker reaction, but addition of two molecules of ammonia. Spiking of exhausted beans with biomimetic recombinate and labeled sucrose supported this pathway, as exclusively $[M + 2]^+$, $[M + 4]^+$, and $[M + 6]^+$ isotopomers were found.

Formation of Diketones and 4-Hydroxy-2,5-dimethyl-3[2*H*]-furanone. Milo et al. (*25*) showed in biomimetic experiments that diketones are reduced by >50% in roasted exhausted green beans. This result could be confirmed in our study. In particular, the content of 2,3-pentanedione (**7**) was highly reduced, that is, by 83%, as shown in **Table 5**. Yaylayan (*5*) suggested on the basis of model studies with labeled precursors that the major part of 2,3-pentanedione (90%) is formed by recombination of a C2−C3 moiety of L-alanine and a C_3 carbon unit from D-glucose. Hence, to investigate the nature of the precursors involved, either free amino acids or free sugars were omitted in the roasting experiments based on biomimetic recombinates. Surprisingly, quantitative analysis revealed that free amino acids (sample 5) did not influence the content of α-diketones (**Table 5**). Furthermore, spiking of green coffee with an excess of L-alanine resulted in only

small increases of these compounds (7 and 17%, respectively). In contrast to the free amino acids, free sugars were found as a limiting factor in the formation of both α-diketones. Their omission resulted in considerable reduction of α-diketones (sample 6): 2,3-butanedione (**6**) decreased by about 60% and 2,3-pentanedione (**7**) by 91%. On the other hand, significantly higher amounts of diketone **7** were found in coffee spiked with sucrose (+62%), whereas diketone **6** increased by only 12% (**Table 6**, sample 7).

Incorporation of labeled precursors underscored the role of free sugars in diketone formation. Whereas spiking of green coffee with an excess of L-[3-^{13}C]-alanine (0.48 g/150 g beans) resulted in only 5% $^{13}C_1$-labeled 2,3-pentanedione, considerable amounts of fully labeled diketone **7** (10%, m/z 105) and to a lesser extent partially labeled $[M + 2]^+$ (2%) and $[M + 3]^+$ (4%) isotopomers were found in the presence of [U-$^{13}C_6$fructose]-sucrose (**Table 9**). Unlike diketone **7**, only trace amounts of labeled diketone **6** isotopomers were obtained. Spiking of green coffee with an excess amount of cysteine led to an unexpected result showing a strong increase of diketone **7** by 88%, but not of diketone **6**. These data indicate different formation pathways leading to diketones **6** and **7**, which mainly depends on sugars as a direct source of C units. The N pool seems to have an indirect role by affecting the overall turnover of sugar degradation. Cysteine seems to reduce the complexity of chemical reactions, thus leading to fewer competing mechanisms allowing more 2,3-pentanedione (**7**) to be formed.

Similar to the diketones, the free sugars play also a major role in the formation of 4-hydroxy-2,5-dimethyl-3[2*H*]-furanone (**8**). Indeed, the omission of sugars in sample 6 (**Table 5**) resulted in significantly decreased concentrations of odorant **8** (−55%). However, the omission of the amino acids promoted the generation of this furanone derivative, because the concentration increased significantly by 62%. This is probably due to fewer degradation reactions of odorant **8** that are favored by N compounds. Schieberle (*33*) showed by CAMOLA studies that under roasting conditions odorant **8** is solely formed via the intact C6-glucose skeleton. As shown by the spiking experiment with labeled sucrose in the present study, this is probably also valid under coffee roasting conditions. The use of [$^{13}C_6$-Fru−$^{12}C_6$-Glu]−sucrose led to the exclusive generation of fully labeled molecules (m/z 134, 16% yield) and fully unlabeled molecules. As shown before, free sugars (mainly sucrose) contribute to about half of the amount of odorant **8** in roasted coffee. Our data also indicate that fructose is a much more efficient precursor as compared to the glucose because only one-sixth of the total sucrose amount was added as labeled isotopomer, hence yielding 16% labeled odorant **8**. Sample 10 shows very high levels of odorant **8**, which is not surprising as rhamnose is known as its direct precursor (*10*, *34*, *35*).

In conclusion, this study revealed that the in-bean approach is a powerful tool for the elucidation and verification of formation pathways of aroma compounds upon coffee roasting. It was demonstrated that combinations of omission, spiking, and mechanistic experiments under real food matrix conditions are very useful in providing further and more precise insights into Maillard-type reactions and formation mechanisms. The formation pathways of several key aroma compounds, such as alkylpyrazines and carbonyl compounds, proposed by model systems could be largely confirmed by in-bean coffee roasting experiments. However, the results clearly indicated that, due to the great diversity of precursors and other co-reaction agents present in the green bean, competing and even completely different pathways take place in the formation of flavor compounds, as demonstrated for FFT and vinyl-substituted pyrazines. The in-bean experiments represent a promising new avenue to understand the mechanisms leading to various types of coffee aromas. The biomimetic reconstitution of the water-extractable fraction remains a challenge, and the incorporation efficiency of different precursor classes is yet not sufficiently known. However, the in-bean experiment represents the best approach proposed so far for the systematic study of green coffee aroma precursors and formation reactions occurring during coffee roasting.

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