JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Chemical Interactions between Odor-Active Thiols and Melanoidins Involved in the Aroma Staling of Coffee Beverages

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Comparative aroma dilution analyses of the headspaces of aqueous solutions containing either the total volatiles isolated from a fresh coffee brew, or these volatiles remixed with the melanoidins isolated from coffee brew, revealed a drastic decrease in the concentrations of the odorous thiols 2-furfurylthiol, 3-methyl-2-butenthiol, 3-mercapto-3-methylbutyl formate, 2-methyl-3-furanthiol, and methanethiol when melanoidins were present. Among these thiols, 2-furfurylthiol was affected the most: e.g., its concentration decreased by a factor of 16 upon addition of melanoidins. This was accompanied by a decrease in the overall roasty−sulfury aroma. Quantitations performed by means of stable isotope dilution assays confirmed the rapid loss of all thiols with increasing time while keeping the coffee brew warm in a thermos flask. Using [$^2H_2$]-2-furfurylthiol as an example, [^{2}H]-NMR and LC/MS spectroscopy gave strong evidence that thiols are covalently bound to the coffee melanoidins via Maillard-derived pyrazinium compounds formed as oxidation products of 1,4-bis-(5-amino-5-carboxy-1-pentyl)pyrazinium radical cations (CROSSPY). Using synthetic 1,4-diethyl diquaternary pyrazinium ions and 2-furfurylthiol, it was shown that 2-(2-furyl)methylthio-1,4-dihydro-pyrazines, bis[2-(2-furyl)-methylthio]-1,4-dihydro-pyrazines, and 2-(2-furyl)methylthio-hydroxy-1,4-dihydro-pyrazines were formed as the primary reaction products. Similar results were obtained for models in which either 1,4-diethyl diquaternary pyrazinium ions were substituted by N_α-acetyl-L-lysine/glycolaldehyde, or the 2-furfurylthiol by 2-methyl-3-furanthiol and 3-mercapto-3-methylbutyl formate. On the basis of these results it can be concluded that the CROSSPY-derived pyrazinium intermediates are involved in the rapid covalent binding of odorous thiols to melanoidins, and, consequently, are responsible for the decrease in the sulfury−roasty odor quality observed shortly after preparation of the coffee brew.

KEYWORDS: Melanoidins; flavor binding; thiols; 2-furfurylthiol; coffee flavor; CROSSPY

INTRODUCTION

Besides its stimulatory effect, a freshly prepared coffee brew is appreciated by the consumer due to its pleasant overall aroma. However, this aroma is not stable, and rapidly changes shortly after preparation of the coffee brew. Although numerous studies have been performed to understand the aroma changes occurring during storage of ground coffee powder (*1*, *2*), information available on the aroma staling of coffee beverages is as yet very fragmentary.

Recent investigations in which strategies combining instrumental analyses with human olfactory perception have been applied (e.g., GC/olfactometry) have revealed a rapid decrease in the concentrations of odorous thiols when coffee brews were stored or processed. Such observations were made in the manufacturing of instant coffee (*3*), heat sterilization of coffee beverages (*4*), or keeping a freshly prepared coffee brew warm in a thermos flask (*5*). The results have shown that, in particular, the key coffee odorant 2-furfurylthiol is significantly reduced, causing a strong decrease of the sulfury−roasty odor quality in the overall aroma of the coffee beverages (*5*).

Preliminary systematic studies on the chemical mechanisms behind the aroma change have recently indicated that the macromolecular melanoidins imparting the dark brown color to the coffee beverage are involved in the loss of 2-furfurylthiol (*5*, *6*). It is, however, as yet unclear (i) which compounds, besides 2-furfurylthiol, are influenced by melanoidins, and (ii) whether the odorants are simply degraded, e.g., by oxidation in the presence of melanoidins, or are covalently bound to the colored macromolecules. The following investigation was, therefore, done to (i) characterize the key coffee odorants affected by melanoidins, and (ii) elucidate the chemical mechanisms involved in the aroma change occurring during keeping coffee beverages warm.

MATERIALS AND METHODS

Chemicals. 2-Furfurylthiol, bis-(2-furfuryl)disulfide, methanethiol, and 2-methyl-3-furanthiol were from Aldrich (Steinheim, Germany). 3-Mercapto-3-methylbutyl formate (*7*), 3-methyl-2-buten-1-thiol (*6*),

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10.1021/jf010823n CCC: $22.00
Published on Web 12/07/2001

Table 1. Results of Comparative Aroma Dilution Analyses of the Headspaces of Isolated Coffee Brew Volatiles Incubated Either in the Absence (I) or Presence of Coffee Melanoidins (II)[a]

odorant	aroma quality	rFD factor I	rFD factor II
butane-2,3-dione	buttery	256	128
pentane-2,3-dione	buttery	128	128
3-methylbutanal	malty	64	64
2-methylbutanal	malty	32	64
acetaldehyde	fruity	32	32
methional	potato-like	32	16
2-furfurylthiol	roasty, sulfury	32	2
2-ethyl-3,5-dimethylpyrazine	earthy	32	32
2,3-diethyl-5-methylpyrazine	earthy	32	32
2-methoxyphenol	phenolic	16	32
dimethyl trisulfide	cabbage-like	16	32
2-isobutyl-3-methoxypyrazine	green, earthy	16	16
3-methyl-2-butenthiol	foxy, skunky	8	1
3-mercapto-3-methylbutyl formate	catty	8	2
2-methyl-3-furanthiol	meatlike	4	2
methanethiol	cabbage-like	2	<1

[a] Aqueous solutions (10 mL) containing the total volatile fraction isolated from a freshly prepared coffee brew (10 mL) were incubated for 30 min at 30 °C. Model II contained 125 mg of melanoidins (molecular weight > 3000 Da).

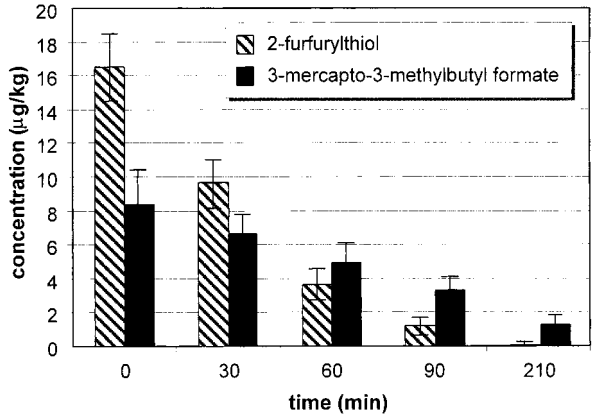

Figure 1. Influence of storage time on the concentrations of 2-furfurylthiol and 3-mercapto-3-methylbutyl formate in an original coffee brew maintained at 80 °C in a thermos flask.

and 1,4-diethyl pyrazinium diquaternary salt (*9*) were synthesized by following procedures reported recently.

Isolation of the Total Volatile Fraction from Coffee Brew. Coffee powder (*Coffea arabica*, var. *caturra*; from Equador; medium roasted, color value 12) was freshly prepared by grinding coffee beans in liquid nitrogen by means of a Waring blender. Hot water (95 °C) was poured on the coffee powder (50 g powder/L of water) in a filter (Kaffee-Filterpapier Nr. 4, Plus Warenhandelsgesellschaft, Hamm, Germany). Using the solvent-assisted flavor extraction (SAFE) device recently developed by Engel et al. (*10*), an aqueous fraction of the volatiles was isolated from the fresh brew (100 mL).

Isolation of Coffee Melanoidins. Freshly ground coffee powder (50 g) was extracted with hot tap water (1 L, 80−90 °C). The aqueous solution was defatted by extraction with dichloromethane and concentrated by freeze-drying (yield: 12.5 g). Aliquots of this material (1.25 g) were redissolved in distilled water (20 mL) and were either fractionated by ultrafiltration (Diaflo YM3 membrane, Amicon, Witten, Germany; molecular weight cutoff of 3000 Da) to obtain melanoidins (0.44 g) after freeze-drying, or were separated by gel permeation chromatography on Sephadex G-25 fine (column, 75 × 5 cm i.d.; Pharmacia, Uppsala, Sweden) affording four fractions (I, 258 mg; II, 221 mg; III, 570 mg; and IV, 141 mg) as recently reported by us (*6*).

Static Headspace Analysis. Aqueous solutions of either the total coffee brew volatiles (10 mL) alone, or mixed with the coffee melanoidins (125 mg; MW > 3000 Da) were pipetted into a septum-sealed vessel (240 mL total volume) and were equilibrated for 30 min at 45 °C. Stepwise decreased headspace volumes (25 to 0.1 mL corresponding to relative flavor dilution factors of 1 to 256) were injected onto an HRGC column (60 m × 0.32 mm i.d., fused silica capillary; RTX-5, methyl polysiloxane-5% phenyl, film thickness, 3 μm) installed in a gas chromatograph type CP 9001 (Chrompack, Frankfurt, Germany) and connected to either a sniffing device or a mass spectrometer (Incos XL, Finnigan, Bremen, Germany). Separation was done by increasing the oven temperature from 30 °C to 230 °C at a rate of 6 °C per min.

Stable Isotope Dilution Analysis. 2-Furfurylthiol, 3-methyl-2-butenethiol, 3-mercapto-3-methylbutyl formate, and bis(2-furfuryl) disulfide were quantified in all samples using $[^2H]_2$-2-furfurylthiol, $[^2H]_8$-3-methyl-2-butenthiol, $[^2H]_6$-3-mercato-3-methylbutyl formate, and $[^2H]_4$-bis(2-furfuryl)disulfide as the internal standards (*11*). Quantification was performed by mass spectrometry using an ion trap detector

Table 2. Relative Amounts of "Free" 2-Furfurylthiol Present in the Headspaces of Aqueous Solutions of 2-Furfurylthiol Stored in the Presence of Different Model Mixtures

2-furfurylthiol stored in the presence of	rel. amount of 2-furfurylthiol[b]
no additive (control)	100
chlorogenic acid (20 mg)	92 (88–94)
thermally pretreated chlorogenic acid (20 mg)[a]	86 (82–90)
albumin/glucose (10 mg each)[a]	58 (55–61)
albumin/glycolaldehyde (10 mg each)[a]	31 (28–34)
N_α-acetyl-L-lysine/glycolaldehyde (10 mg each)[a]	17 (15–19)

[a] Compounds were intimately mixed and dry-heated for 5 min at 230 °C. [b] Relative amount of "free" thiol is given as the mean of triplicates. Ranges of data measured are given in parentheses.

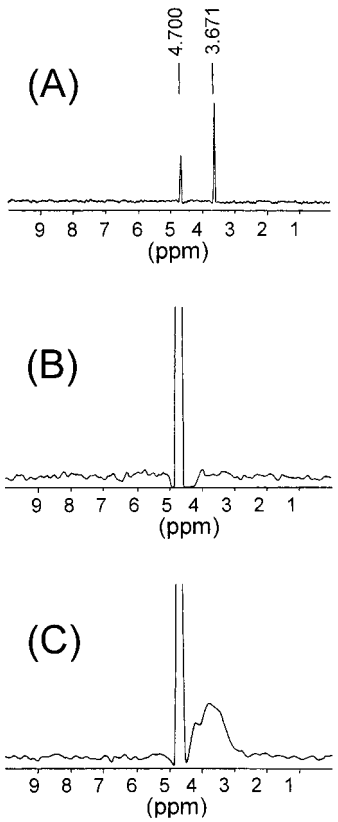

Figure 2. ^2H NMR spectra (500 MHz, H_2O) of (A) $[^2H_2]$-2-furfurylthiol (1 mg/mL); (B) coffee melanoidins (100 mg/mL); and (C) coffee melanoidins (100 mg/mL) after preincubation (90 min, 30 °C) with $[^2H_2]$-2-furfurylthiol (1 mg) and isolation.

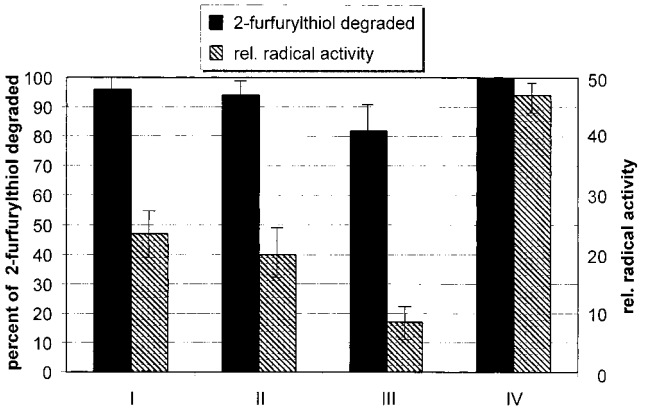

Figure 3. Amounts of 2-furfurylthiol degraded during incubation with melanoidin fractions. Correlation with the relative radical activity.

(ITD 800, Finnigan, Bremen, Germany) running in the chemical ionization mode with methanol as the reactant gas.

Determination of Headspace Concentrations of 2-Furfurylthiol. 2-Furfurylthiol (500 μg) was dissolved in phosphate buffer (10 mL; 0.1 mol/L; pH 6.0) and stored at 30 °C in a septum-sealed vessel (240 mL) either alone, or in the presence of the following compounds or pretreated mixtures: (a) chlorogenic acid (20 mg); (b) heated chlorogenic acid (20 mg; 5 min at 230 °C); (c) a heated mixture of albumin and glucose (10 mg each; 5 min at 230 °C); (d) a heated mixture of glycolaldehyde and albumin (10 mg each; 5 min at 230 °C); (e) a heated mixture of glycolaldehyde and N_α-acetyl-L-lysine (10 mg each; 5 min at 230 °C); or (f) coffee melanoidins (125 mg).

Labeling Experiments. Total melanoidins (100 mg) isolated from a fresh coffee brew by ultrafiltration were dissolved in tap water (10 mL), [$^2H_2$]-2-furfurylthiol (1.0 mg) was added, and the mixture was incubated at 30 °C in a closed vessel. After 90 min, the melanoidins were freed from low-molecular weight compounds by ultrafiltration, The retentate (molecular weight > 3000 Da) obtained was taken up in water (1 mL) and analyzed by [^{2}H] NMR spectroscopy. As the controls, aqueous solutions of either the coffee melanoidins (100 mg) or [$^2H_2$]-2-furfurylthiol (1.0 mg) were analyzed.

Model Reactions. *Diquaternary 1,4-Diethyl Pyrazinium Ions and Thiols.* Diquaternary 1,4-diethyl pyrazinium salt (1.0 mg) was dissolved in tap water (2 mL), and 2-furfurylthiol (500 μg), 3-mercapto-3-methylbutyl formate (500 μg), or 2-methyl-3-furanthiol (500 μg) were added individually. After 30 min at 30 °C, the mixtures were analyzed by LC/MS.

N_α-Acetyl-L-lysine/Glycolaldehyde and 2-Furfurylthiol. A mixture of glycolaldehyde (20 mg), N_α-acetyl-L-lysine (20 mg), and tap water (100 μL) was heated in an open beaker for 5 min at 230 °C to generate the CROSSPY radical (*9*). After cooling, the presence of the radical was checked by application of LC/MS and EPR spectroscopy on an aliquot (10 mg) of the material taken up in water (3 mL). 2-Furfurylthiol (500 μg) was then added, and the solution was incubated for 30 min at 30 °C. The reaction products formed were analyzed by LC/MS spectroscopy and LC/MS^2.

Spectroscopic Measurements. High-resolution gas chromatography/mass spectrometry (GC/MS) was performed using a CP 9001 gas chromatograph (Chrompack, Frankfurt, Germany) equipped with a fused silica capillary CP-WAX 52 CB (25 m × 0.32 mm i.d., 1.2 μm film thickness, Chrompack, Frankfurt, Germany) and coupled with the mass spectrometer Incos XL (Finnigan, Bremen, Germany). Using helium as the carrier gas (2 mL/min), the temperature of the oven was held at 0 °C for 2 min, then raised at a rate of 6 °C/min to 50 °C, held for 2 min, and then raised at a rate of 8 °C/min to 250 °C. Liquid chromatography/mass spectrometry (LC/MS) was performed with an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) operating in the electrospray ionization (ESI) mode, and using a direct injection system. Electron paramagnetic resonance spectroscopy (EPR) and [^{2}H] NMR spectra were recorded by means of an ESP 300 spectrometer (Bruker, Rheinstetten, Germany) or an AMX 500 spectrometer (Bruker, Rheinstetten, Germany), respectively.

RESULTS AND DISCUSSION

To systematically study the influence of melanoidins (MW > 3000 Da) on the aroma-active volatiles of a coffee brew, both fractions were separately isolated from a fresh coffee brew prior to the analytical experiments and then recombined. In an initial study, by application of the comparative aroma dilution analysis, the odor-active compounds in a stored model (30 min; 40 °C) containing only the coffee brew volatiles were compared to those of a second model containing both the volatiles and the melanoidins in their "natural" concentrations. The results

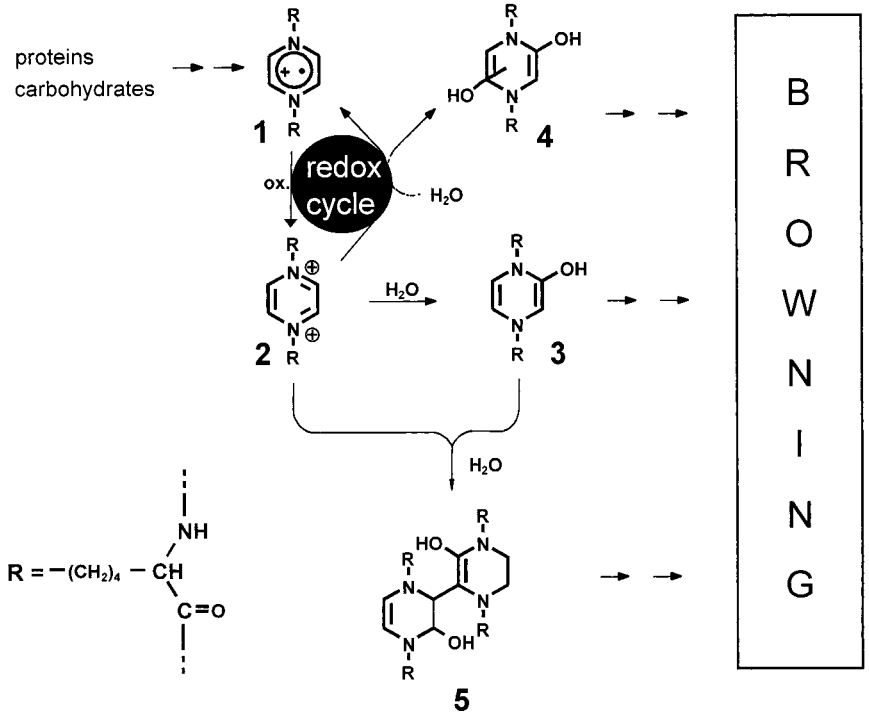

Figure 4. Scheme of melanoidin genesis via the CROSSPY radical (I) and diquaternary pyrazinium ions (II) as the key intermediates.

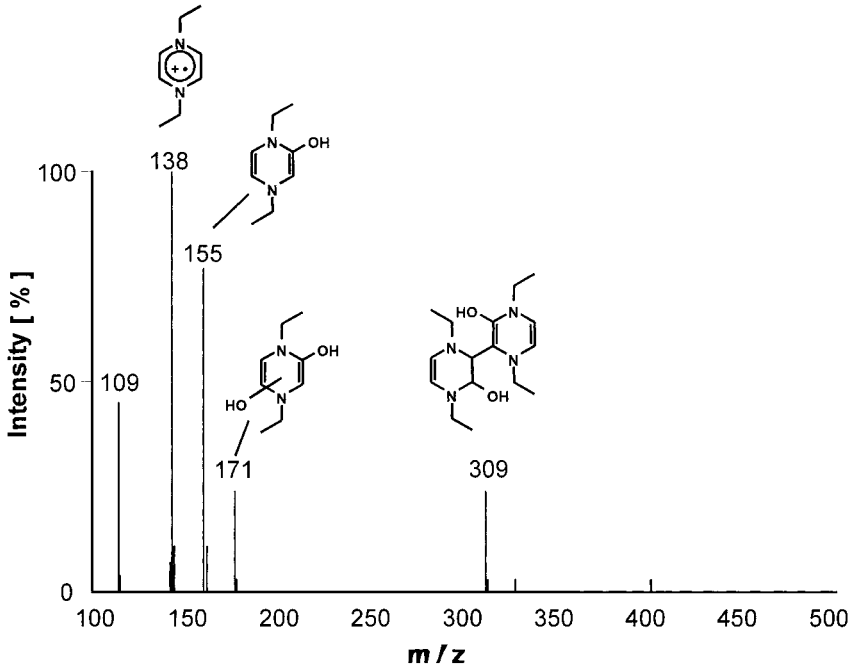

Figure 5. Molecular ions (ESI; *m/z*) of the reaction products formed upon dissolving 1,4-diethyl pyrazinium diquaternary ions in water.

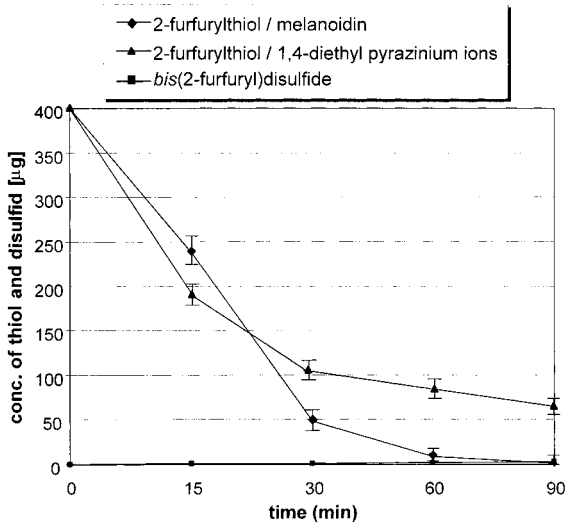

Figure 6. Time course of 2-furfurylthiol binding and disulfide formation (bis(2-furfuryl)disulfide) in the presence of 1,4-diethyl pyrazinium diquaternary ions or melanoidins.

revealed 16 odorants in the volatile fraction of the coffee brew after incubation for 30 min with relative flavor dilution (rFD) factors of 2 to 256, which were calculated from the stepwise reduced headspace volumes used for analysis (I in Table 1). After incubation in the presence of melanoidins (II in Table 1), only 15 odorants were sensorially detected. As indicated by the lower rFD factors, all thiols were drastically decreased when melanoidins were present. The most pronounced effects were measured for 2-furfurylthiol, 3-methyl-2-buten-1-thiol, and 3-mercapto-3-methylbutyl formate, the rFD factors of which were decreased by 16, 8, or 4, respectively (Table 1). Also 2-methyl-3-furanthiol was significantly decreased, and methanethiol could not be detected at all. In contrast, the aroma impacts of odorants belonging to other chemical classes, such as the 2,3-diones, the phenols, or the pyrazines, were not significantly changed upon addition of melanoidins.

To further confirm this decrease in thiol concentration, the amounts of 2-furfurylthiol and 3-mercapto-3-methylbutyl formate were quantified in coffee brews kept warm in a thermos flask for 0, 30, 60, 90, and 210 min. As given in Figure 1, the freshly prepared coffee beverage contained about 16.0 or 8.2 μg of FFT or MMBF, respectively. Keeping the brew warm then led to a drastic decrease in the concentrations of both thiols. After 60 min the 2-furfurylthiol concentration decreased by a factor of more than four compared to that of the fresh coffee brew. Extending the storage time to 210 min finally resulted in a complete loss of 2-furfurylthiol, and only small amounts of 3-mercapto-3-methylbutyl formate were detectable (Figure 1). The data are well in line with the results of the comparative aroma dilution analysis (cf. Table 1) and clearly demonstrate that the decrease of the sulfury−roasty odor quality observed during storage of coffee brews is mainly due to the loss of odor-active thiols.

To investigate whether thiols may be covalently bound to the macromolecular browning products, melanoidins were stored for 90 min at 30 °C in the presence of [$^2H_2$]-2-furfurylthiol ([$^2H_2$]-FFT) for 90 min at 30 °C, freed again from low-molecular compounds by ultrafiltration, and analyzed by [^{2}H] NMR spectroscopy.

In a control experiment, [$^2H_2$]-2-furfurylthiol dissolved in tap water showed two resonance signals, one at 3.67 ppm corresponding to the deuterated methylene group, and another at 4.70 ppm corresponding to the natural [^{2}H] abundance in tap water (Figure 2A). [^{2}H] NMR of the total coffee melanoidins did not show any signals besides the natural [^{2}H] abundance of the solvent water (Figure 2B). Coffee melanoidins, however, which had been preincubated with [$^2H_2$]-2-furfurylthiol, showed an additional strong resonance between 3.0 and 4.2 ppm, which is in the range expected for the deuterated methylene group of bound 2-furfurylthiol (Figure 2C). The strong line broadening observed is typical for compounds which are restricted in molecular rotation due to covalent linking to macromolecules, and furthermore, indicates that, as the result of dipolar interactions, each spin in the 2-furfurylthio-moiety is present indifferent magnetic enviroments in the melanoidins (*12*). These data clearly support the idea that the odor-active thiols do bind to the coffee melanoidins while the beverage is stored.

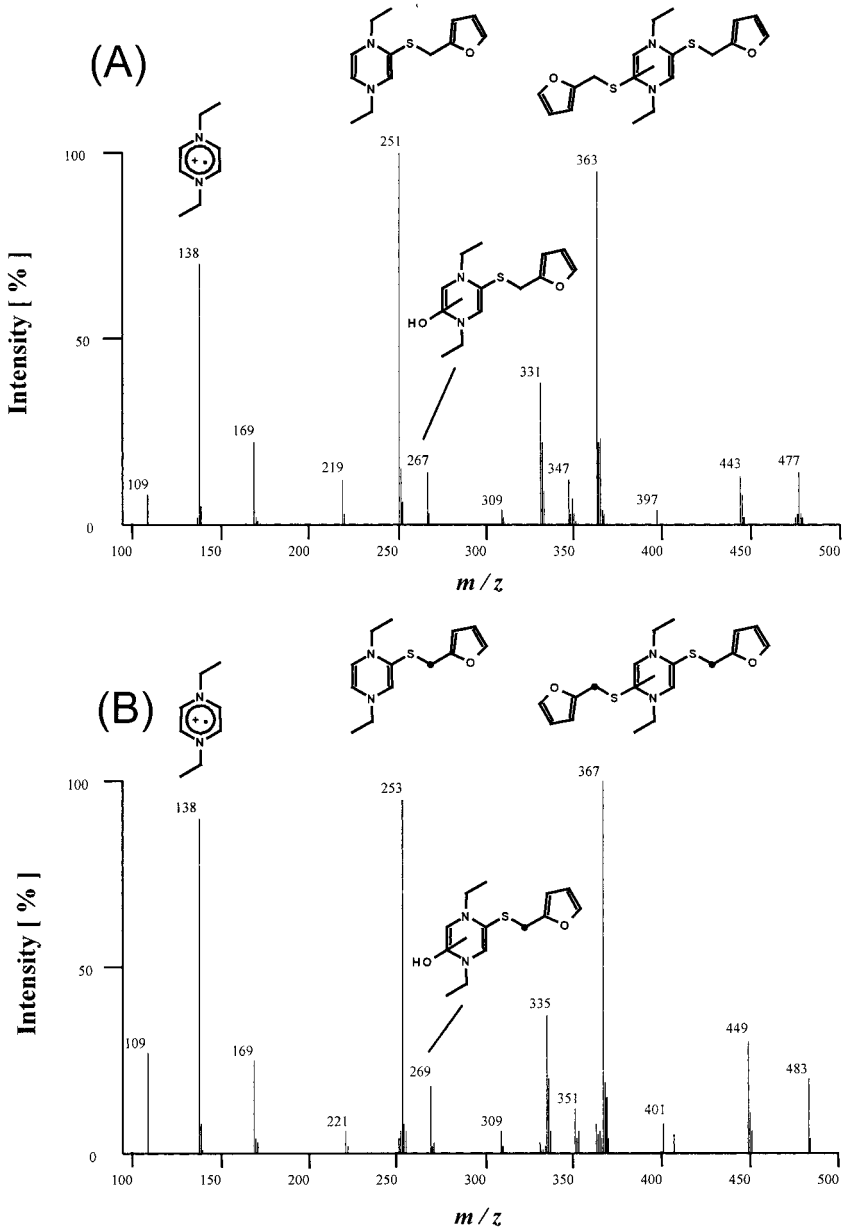

Figure 7. LC/MS spectra (ESI; *m*/*z*) of the reaction products formed in an aqueous solution of 1,4-diethyl pyrazinium diquaternary ions, and 2-furfurylthiol (A) or [$^2H_2$]-2-furfurylthiol (B).

Because recent studies had clearly shown that the addition of the reducing agent dithioerythritol was not able to regenerate major amounts of the "free" thiol from a melanoidin, which had been "loaded" with 2-furfurylthiol (*6*), it can be speculated that the thiols do not bind via disulfide bonds of, e.g. cysteinyl residues, to the coffee melanoidins. Obviously, the thiols preferably react with other reactive sites present in the macromolecules.

Model Reactions on Potential Binding Sites. To investigate the role of chlorogenic acid moieties present in coffee melanoidins, an aqueous solution of 2-furfurylthiol was incubated for 30 min at 30 °C either in the presence of free chlorogenic acid, or in the presence of chlorogenic acid, which had been preheated for 5 min at 230 °C to simulate roasting conditions. After incubation with 2-furfurylthiol, the amounts of the thiol remaining were analyzed. Neither untreated, nor preheated, chlorogenic acid showed strong binding activity, as the losses in both models were below 20% (Table 2).

To study the role of Maillard-derived reaction products in thiol binding, a dry-heated albumin/glucose mixture was stored together with 2-furfurylthiol. The results showed that the thiol concentration was decreased by a factor of nearly two (Table 2), thus demonstrating that Maillard-derived reaction products might play a role in thiol binding. Another experiment using a thermally processed model of albumin and glycolaldehyde led to a more pronounced effect, because the 2-furfurylthiol concentration was reduced to below 30% (Table 2). Because the amino groups of protein-bound lysine are known as primary targets of Maillard reactions involving proteins, albumin was substituted by N_α-acetyl-L-lysine. The dark brown material formed upon roasting was most effective in thiol binding, because only 17% of the "free" 2-furfurylthiol was left in the headspace of the model reaction mixture (Table 2).

It is well documented in the literature that free radicals are present in roasted coffee (*13*−*19*), and that 1,4-bis-(5-amino-5-carboxy-1-pentyl)pyrazinium radical cations (CROSSPY),

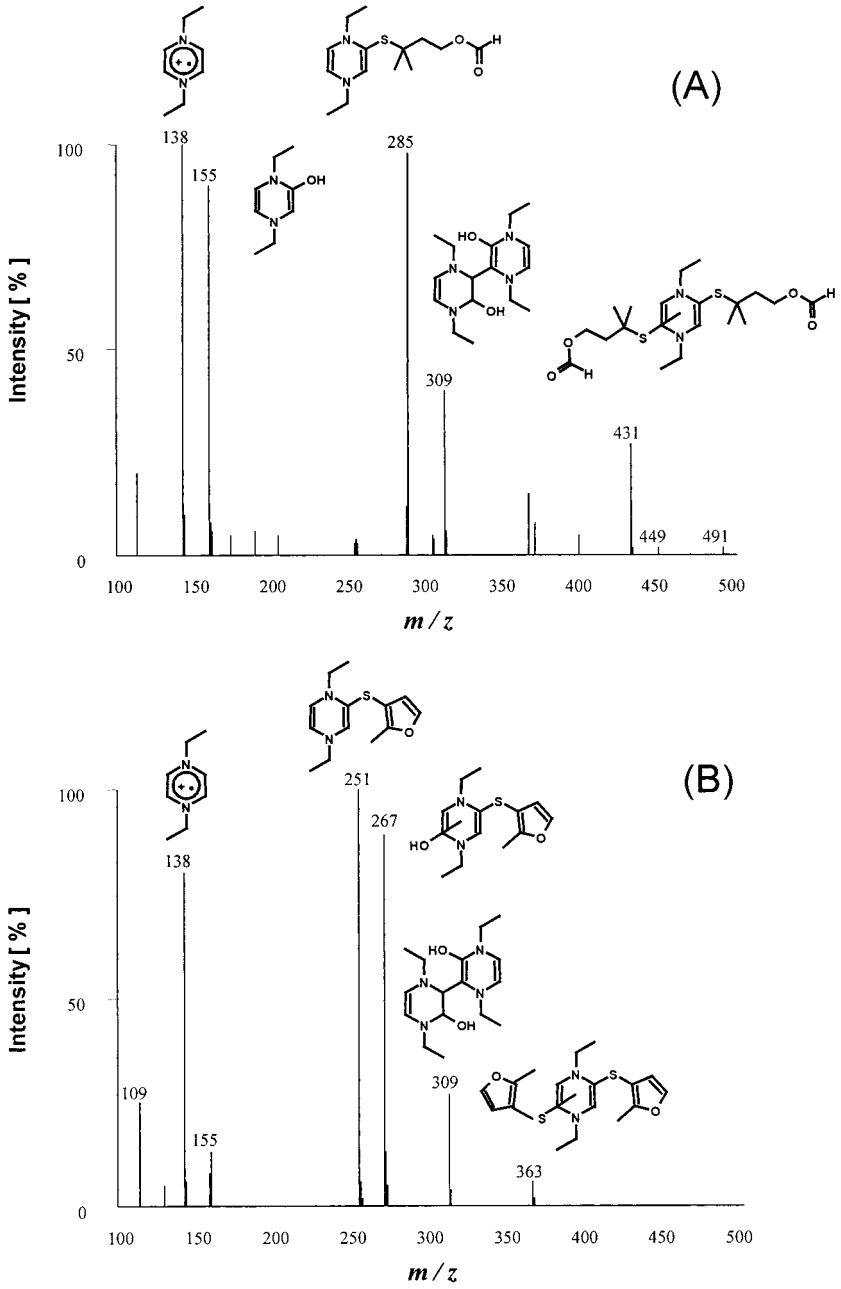

Figure 8. LC/MS spectra (ESI; *m*/*z*) of the reaction products formed in an aqueous solution of 1,4-diethyl pyrazinium diquaternary ions, and (A) 3-mercapto-3-methylbutyl formate or (B) 2-methyl-3-furanthiol.

which are formed from protein-bound lysine side chains and glycolaldehyde, contribute to melanoidin genesis during coffee roasting (*18*, *19*). The following experiments were performed in order to gain more detailed insights into the role of radicals as potential thiol binding sites. To achieve this, coffee melanoidins were separated into four fractions of decreasing molecular weight by gel permeation chromatography (*6*), and the fractions obtained were investigated for their thiol binding as well as for their radical activities. Incubation of fractions I to IV in the presence of 2-furfurylthiol resulted in a complete loss of 2-furfurylthiol after 30 min at 30 °C (IV; Figure 3). Fractions I and II showed somewhat lower activities, whereas fraction III was least effective in 2-furfurylthiol binding. Analysis of the fractions by means of EPR spectroscopy revealed that the radical activities run in parallel with the thiol binding activity, e.g., fraction IV showed the most pronounced effect in thiol binding and exhibited the highest radical activity, whereas fraction III had the lowest potential in thiol binding and the lowest radical activity (Figure 3).

Role of the CROSSPY Radical. 1,4-bis-(5-Amino-5-carboxy-1-pentyl)pyrazinium radical cations, named CROSSPY (**1** in Figure 4), were recently identified by us in melanoidins isolated from a freshly prepared coffee brew (*18*, *19*). These radical cations, found to be involved in a redox cycle of reaction intermediates, are oxidized into the corresponding diquaternary pyrazinium ions (**2** in Figure 4) when dissolved in water (*18*, *19*). These ions subsequently form 2-hydroxy-1,4-dihydropyrazines (**3** in Figure 4) upon hydration, and regenerate the CROSSPY radicals upon a redox reaction with **3**. Because of their activity in browning development, the mono- and bis-hydroxylated dihydropyrazines (**3** and **4** in Figure 4) were recently proposed as penultimate monomers involved in melanoidin genesis (*19*), e.g. by oligomerization reactions via the dimer **5** (Figure 4).

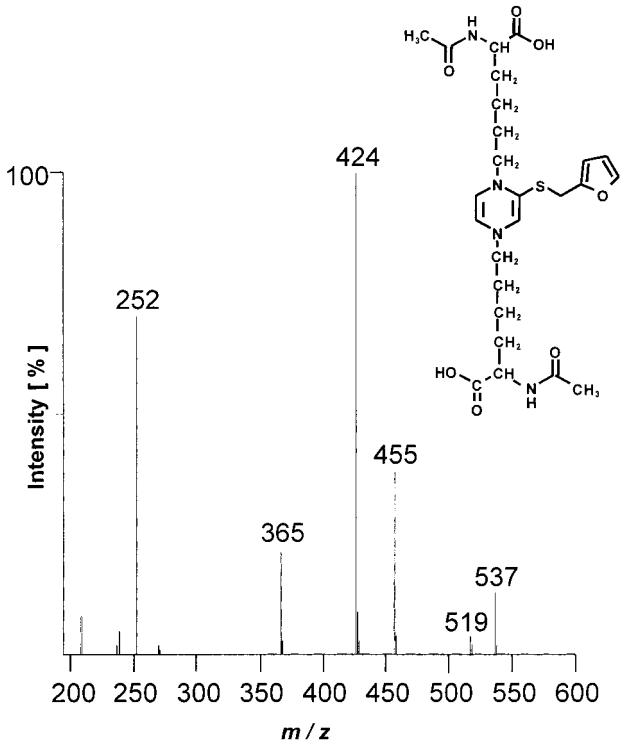

Figure 9. Mass spectrum (ESI) obtained for the main reaction product formed in a thermally pretreated N_α-acetyl-L-lysine/glycolaldehyde mixture after incubation (30 min at 30 °C) with 2-furfurylthiol.

This redox cycle can be modeled with diquaternary 1,4-diethyl pyrazinium ions as a suitable template to mimic the reactions of lysine-bound pyrazinium derivatives (*9*, *19*). An LC/MS spectroscopy characterization of the reaction products formed upon dissolving the diquaternary pyridinium ions in water (Figure 5) revealed that all reaction intermediates proposed in Figure 4 are generated, namely the CROSSPY-type radical cation (*m*/*z* 138), the 2-hydroxy-1,4-diethyl-1,4-dihydropyrazine (*m*/*z* 155), the dihydroxy-1,4-diethyl-1,4-dihydropyrazine (*m*/*z* 171), and the bis-hydroxy dimer (*m*/*z* 309).

To study possible reactions between odor-active thiols and these CROSSPY-associated intermediates, an aqueous solution of pyrazinium diquaternary ions was incubated in the presence of 2-furfurylthiol at 30 °C. Quantitation of the concentrations of 2-furfurylthiol revealed a rapid decrease induced by either the addition of the diquaternary pyrazinium ions, or the coffee melanoidins (Figure 6). Both the pyrazinium-derived intermediates as well as the coffee melanoidins showed similar kinetics of 2-furfurylthiol degradation.

Although about 400 or 330 μg of 2-furfurylthiol was bound to the coffee melanoidins or the pyrazinium-derived intermediates, respectively, less than 6 μg of the corresponding bis(2-furfuryl) disulfide was generated. This result clearly demonstrates that neither the solution containing the diquaternary pyrazinium ions nor the coffee melanoidins are able to oxidize the thiol into its disulfide.

In a further experiment, an aqueous solution of diquaternary pyrazinium ions was spiked with 2-furfurylthiol in equimolar amount, and after 10 min at 30 °C the reaction products formed were analyzed by LC/MS (Figure 7A). The mass spectrum showed a base ion at *m*/*z* 251 (100%), which, on the basis of its LC/MS^2 spectrum (data not given), was proposed to be the 2-(2-furyl)methylthio-1,4-dihydropyrazine. In addition, LC/MS^2 gave evidence that the quasi-molecular ions at *m*/*z* 363 and 267 corresponded to bis[2-(2-furyl)methylthio]-1,4-dihydropyrazine and 2-(2-furyl)methylthio-hydroxy-1,4-dihydro-pyrazine, respectively (A in Figure 7). To further confirm these assumptions, the experiment was repeated with [$^2H_2$]-2-furfurylthiol (B in Figure 7). A comparison of the LC/MS spectrum obtained for the labeled thiol (B in Figure 7) with that observed in the nonlabeled experiment (A in Figure 7) revealed an isotopic shift of two units, e.g., for the ion at *m*/*z* 251 to *m*/*z* 253, thus confirming the incorporation of two deuterium atoms. An isotopic shift of two and four units was also observed for the ions at *m*/*z* 267 (to *m*/*z* 269) and *m*/*z* 363 (to *m*/*z* 367), respectively, being well in line with the structures proposed for 2-(2-furyl)methylthio-hydroxy-1,4-dihydropyrazine and bis[2-(2-furyl)methylthio]-1,4-dihydro-pyrazine (Figure 7).

To confirm the binding potential of the pyrazinium derivatives, or the CROSSPY radical, respectively, the experiments were repeated by substituting 2-furfurylthiol by either 3-mercapto-3-methylbutyl formate or 2-methyl-3-furanthiol. The mass spectrum of the solution containing the 3-mercapto-3-methylbutyl formate exhibited a molecular ion at *m*/*z* 285 (100%), most likely corresponding to the thioether of 3-mercapto-3-methyl-

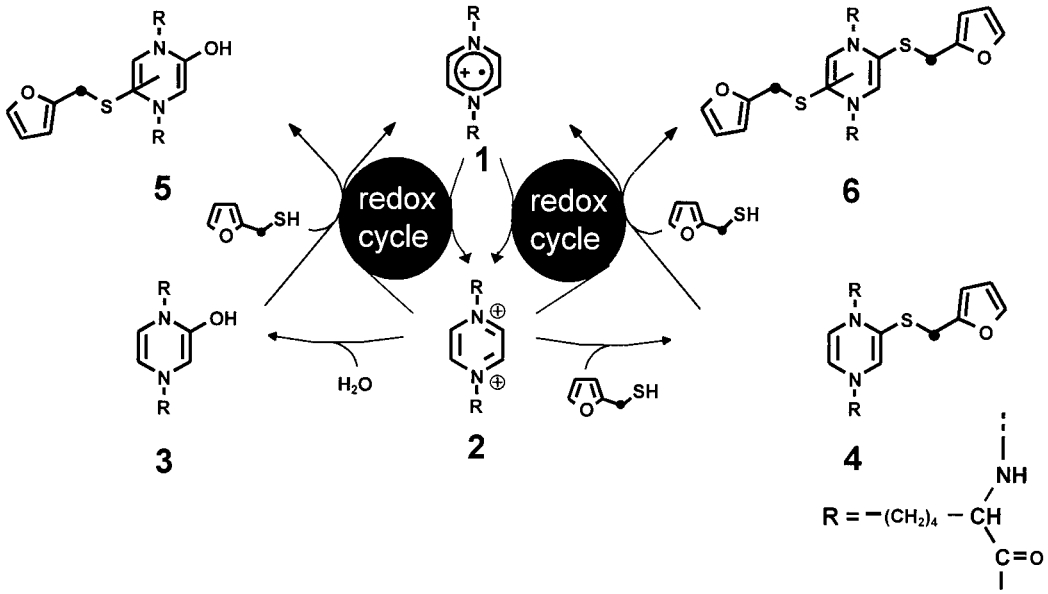

Figure 10. Reaction scheme explaining the covalent binding of [$^2H_2$]-2-furfurylthiol to CROSSPY-related reaction intermediates.

butyl formate and 1,4-dihydropyrazine (A in Figure 8). In addition, LC/MS^2 gave evidence that the ion at *m*/*z* 431 corresponds to the bis-thioether adduct (A in Figure 8). The corresponding experiment with 2-methyl-3-furanthiol, led to the identification of the expected thioether derivatives (B in Figure 8), thus demonstrating that the covalent binding of thiols is a general reaction type leading to the loss of odor-active thiols.

To simulate the situation in food more closely, the CROSSPY radical was generated from N_α-acetyl-L-lysine and glycolaldehyde prior to the addition of 2-furfurylthiol in an additional experiment. The solution containing CROSSPY and its corresponding oxidation products (Figure 4) was incubated in the presence of 2-furfurylthiol for 30 min at 30 °C, and then analyzed by LC/MS spectroscopy. The mass spectrum obtained showed a quasi molecular ion at *m*/*z* 537, consistent with the structure of 2-(2-furyl)methylthio-1,4-bis-(5-acetamino-5-carboxy-1-pentyl)-1,4-dihydropyrazine given in Figure 9. Fragmentation of that ion gave the MS^2 spectrum (ESI) displayed in Figure 9. Loss of 113 or 82 amu, respectively, leads to the base ion at *m*/*z* 424 or 455, most likely corresponding to the cleavage of the 2-furfurylthio- or the 2-furylmethyl-group, respectively. The data clearly corroborated the results obtained for the model experiments performed with the diquaternary pyrazinium ions, and imply that odor-active thiols are covalently linked to pyrazinium moieties in coffee melanodins.

Taking all these data into account, the reaction pathways displayed in Figure 10 can be proposed for the binding of thiols to CROSSPY-related reaction intermediates. Oxidation of CROSSPY (**1**) leads to diquaternary pyrazinium ions (**2**), which, in the absence of thiols, react with water to form the 2-hydroxy-1,4-dihydropyrazine (**3**) as recently reported (*9*, *18*, *19*). In the presence of the more nucleophilic thiols such as 2-furfurylthiol, thioethers such as 2-(2-furyl)methylthio-1,4-dihydropyrazine (**4**) can be formed. Redox reactions of these intermediates involving the diquaternary ions then might form the bis substituted 2-(2-furyl)methylthio-hydroxy-1,4-dihydropyrazine (**5**) and bis[2-(2-furyl)methylthio]-1,4-dihydropyrazine (**6**), respectively, in reaction with a second molecule of the thiol.

On the basis of these results it can be concluded that the CROSSPY-derived pyrazinium intermediates are involved in the rapid covalent binding of odorous thiols to melanoidins, and are responsible for the decrease in the sulfury−roasty odor quality detected shortly after preparation of the coffee brew. Studies on how the activity of these binding sites might be influenced, e.g., by blocking the active binding sites or varying the roasting process of the green coffee beans, are ongoing and will help to find possible means to increase the aroma shelf life of coffee beverages.

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Received for review June 25, 2001. Revised manuscript received October 11, 2001. Accepted October 16, 2001.

JF010823N