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Screening of Raw Coffee for Thiol Binding Site Precursors Using "In Bean" Model Roasting Experiments

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The purpose of the following study was to investigate the influence of coffee roasting on the thiol-binding activity of coffee beverages, and to investigate the potential of various green bean compounds as precursors of thiol-binding sites by using promising "in bean" model roast experiments. Headspace gas chromatographic analysis on coffee brews incubated in the presence of the roasty−sulfury smelling 2-furfurylthiol for 20 min at 30 °C in septum-closed vessels revealed that the amounts of "free" thiol decreased drastically with increasing the roasting degree of the beans used for preparation of the brews. A half-maximal binding capacity (BC_{50}) of 183 mg of 2-furfurylthiol per liter of standard coffee beverage was determined for a roasted coffee (CTN value of 67), thus demonstrating that enormous amounts of the odor-active thiol are "bound" by the coffee. Furthermore, biomimetic "in bean" precursor experiments have been performed in order to elucidate the precursor for the thiol-binding sites in the raw coffee bean. These experiments opened the possibility of studying coffee model reactions under quasi-natural roasting conditions and undoubtedly identified chlorogenic acids as well as thermal degradation products caffeic acid and quinic acid as important precursors for low-molecular-weight thiol-binding sites. In particular, when roasted in the presence of transition metal ions, chlorogenic acids and even more caffeic acid showed thiol-binding activity which was comparable to the activity measured for the authentic coffee brew.

KEYWORDS: Coffee aroma; 2-furfurylthiol; thiols; chlorogenic acid; coffee beans; bean reconstitutes

INTRODUCTION

In addition to its stimulatory and potential health-promoting effects, the wide popularity of a freshly brewed roast coffee beverage is mainly based on its pleasant, attractive overall aroma. Unfortunately, the freshness of the brew cannot be preserved since the desirable aroma, in particular, the roasty−sulfury note is rather unstable and changes shortly after preparation of the coffee brew (*1*−*4*).

Aimed at characterizing the odor-active compounds involved in that undesirable aroma deterioration on a molecular level, headspace gas chromatography (GC)/olfactometry was successfully used to compare the odorant profile before and after storage of a freshly prepared coffee brew in a Thermos flask (*3*). In particular, the concentration of the key coffee odorant 2-furfurylthiol contributing to the sulfury−roasty odor quality of coffee brew was found to be drastically reduced upon coffee processing or storage (*3*). Comparative aroma dilution analysis revealed that, besides 2-furfurylthiol, also the decrease of 3-methyl-2-butene-1-thiol, 3-mercapto-3-methylbutyl formate, 2-methyl-3-furanthiol, and methanethiol are responsible for the aroma change (*1*−*4*). Such thiols have been also identified to be involved in the aroma changes induced by the instant process (*5*) as well as by sterilization of canned coffee beverages (*6*).

The first systematic studies on the influence of coffee melanoidins on thiol stability revealed that these dark-brown polymers exhibiting molecular weights above 3000 Da are able to effectively bind 2-furfurylthiol (*1*−*3*). Quantitative model studies and spectroscopic data obtained from ^2H NMR and LC−MS experiments using [$^2H_2$]-labeled 2-furfurythiol gave evidence that odor-active thiols are covalently bound to pyrazinium dications, which are known as the oxidation products of protein-bound 1,4-bis-5-amino-5-carboxy-1-pentylpyrazinium radical cations, called "Crosspy" (*7*−*9*), and have been identified as important key intermediates in roasting-induced melanoidin genesis (*2*, *3*). On the basis of model experiments with synthetic reference compounds, these pyrazinium dications were demonstrated to be rapidly attacked by the nucleophilic thiols, such as 2-furfurylthiol, thus forming 2-(2-furyl)methylthio-1,4-dihydropyrazines, bis[2-(2-furyl)methylthio]-1,4-dihydropyrazines, and 2-(2-furyl)methylthiohydroxy-1,4-dihydropyrazines as the primary reaction products (*2*, *3*).

These findings gave the first evidence that the binding of thiols to pyrazinium moieties as being part of the macromolecular coffee melanoidins does contribute to the decrease of the sulfury−roasty odor quality after grinding of roast coffee and, in particular, after preparation of the coffee brew. As recent ultrafiltration experiments demonstrated that about 80% of the coffee components in a roasted coffee brew are still exhibiting molecular weights below 1 kDa, and that less than 15% are

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Table 1. Yields of Ultrafiltration Fractions Isolated from Aqueous Raw Bean Extract (BE) and Amounts Used for Bean Reconstitution Experiments

ultrafiltration fraction	yield BE isolated[a] [%]	amt BE used[b] [g]
LMW-BE (MW < 1 kDa)	97.7	11.76
HMW-BE (MW > 1 kDa)	2.3	0.28

[a] Amounts isolated from 100 g of raw bean solubles (BE). [b] Amount of LMW-BE (expt b) and HMW-BE (expt c), respectively, incorporated into 50 g of raw bean solids (BS).

due to melanoidin-type polymers (*10*), the question arose whether the major low-molecular-weight fraction contains compounds which, due to their quantitative predominance, might also contribute to thiol binding of coffee.

The identification of those thiol-binding sites in complex fractions isolated from coffee brew is a very lengthy, challenging procedure. The purpose of the following study was, therefore, first to study the influence of roasting on thiol-binding activity of coffee, and then to investigate the potential of various green bean compounds as precursors of thiol-binding sites by using promising "in bean" model roast experiments.

MATERIALS AND METHODS

Chemicals. L-Arginine was purchased from Serva (Heidelberg, Germany), L-cystine was purchased from Roth (Karlsruhe, Germany), L-histidine and L-lysine were purchased from Sigma-Aldrich (Steinheim, Germany), 4-aminobutyric acid, ascorbic acid, DL-β-aminoisobutyric acid, L-2-aminoadipic acid, L-asparagine, L-citrulline, L-cystathionine, L-phenylalanine, *N*-methyl-L-histidine, *O*-phospho-L-serine, and taurine were purchased from Fluka (Taufkirchen, Germany), and trigonelline was purchased from Extrasynthese (Lyon, France). All other chemicals were obtained from Merck (Darmstadt, Germany).

Extraction of Raw Coffee Beans. Following a procedure reported in the literature (*11*) with some modifications, whole green coffee beans (Arabica, Colombia, 50 kg) were extracted with demineralized hot water at 95 °C (1 × 60 min with 200 L, 3 × 30 min with 200 L). The extracts were combined, and concentrated in a Luwa TFE evaporator at 40 °C and 90 mbar to a total solid content of about 24%. This aqueous raw bean extract (BE) and the fully extracted bean shell (BS) were subsequently freeze-dried and stored in a desiccator at −18 °C.

Ultrafiltration of the Aqueous Raw Bean Extract (BE). The freeze-dried BE (20 g) was dissolved in demineralized water (200 mL) and then fractionated by means of an ultrafiltation cell (Amicon, Witten, Germany) using a YM 1 membrane (Millipore, Bedford, MA) with a cutoff of 1 kDa at a nitrogen pressure of 3 bar. The residue was washed with water (5 × 100 mL) and then carefully removed from the filter. The collected low-molecular-weight filtrates (LMW-BE) and the high-molecular-weight residue (HMW-BE) were freeze-dried, with the yield determined by weight (**Table 1**), and stored at −18 °C.

Analysis of Chemical Composition of Aqueous Raw Bean Extract (BE). For quantification of carbohydrates, an exact amount of fraction BE (5−10 mg) was taken up in hot Millipore water (10 mL), membrane filtered (0.45 μm), and then analyzed by ion chromatography as reported previously (*12*). Quantification of free amino acids was done by means of an amino acid analyzer with ninhydrin detection (*13*). Organic acids were analyzed as *p*-nitrobenzyl esters by HPLC as previously described (*14*). Total chlorogenic acids, caffeine, and trigonelline were quantified by means of reversed-phase HPLC with UV−vis detection (chlorogenic acids at 325 nm, caffeine at 275 nm, and trigonelline at 265 nm) following a procedure described previously (*11*).

The concentrations of iron, copper, manganese, and zinc ions in green coffee beans were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Plasma 40, Perkin-Elmer, Rodgau, Germany) using the standard addition method. Iron, copper, manganese, and zinc nitrate ($Fe(NO_3)_3 \cdot 9H_2O$, $Cu(NO_3)_2 \cdot 3H_2O$, $Mn(NO_3)_2 \cdot 4H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$; 1 g each) were dissolved in nitric acid (1 L, 6%). Aliquots (20, 40, 60, and 100 mL) of this solution were diluted to 100 mL, and each was added to freeze-dried green coffee beans (200 g). After stirring overnight, the beans completely took up the standard solution and were freeze-dried again. A control sample consisting of green coffee beans (200 g) and nitric acid (6%, 100 mL) was treated the same way. An aliquot (3 g) of the freeze-dried samples was kept in concentrated nitric acid (6 mL) overnight and, after addition of another 6 mL of concentrated nitric acid, was treated by microwave digestion (1000 W, 100 bar). The temperature was raised from 20 to 80 °C in 1 h, from 80 to 85 °C in 5 min, from 85 to 90 °C in 10 min, from 90 to 110 °C in 10 min, from 110 to 150 °C in 10 min, from 150 to 200 °C in 9 min, and from 200 to 220 °C in 10 min. The solutions were diluted to 20 mL, and the concentrations of the transition metals were measured by ICP-AES.

Moisture Content of Lyophilized Green Coffee Beans. Raw green coffee beans (50 g) and reconstituted green beans (50g), respectively, were freeze-dried for 48 h. A portion of each sample (3−4 g) was frozen with liquid nitrogen, ground in a batch mill (IKA, Staufen, Germany), and mixed with sand (30 g). The materials were heated in an oven at 103−105 °C until constant weight of the samples was reached. Before weighing, samples were cooled in a desiccator (30 min).

Preparative Isolation of Chlorogenic Acids from Raw Bean Extract (BE). Following a method reported previously (*15*) with some modifications, an aliquot (40 g) of fraction BE was taken up in water (500 mL, pH 6.0), phosphoric acid (4 mL, 85%) and ammonium sulfate (35 g) were added, and the mixture was kept overnight at 4 °C under an atmosphere of argon. After precipitation of the coffee proteins, the aqueous solution was separated by filtration (595 1/2, Schleicher & Schuell, Dassel, Germany); the aqueous filtrate was extracted with petroleum ether (3 × 150 mL), decaffeinated by extraction with dichloromethane (3 × 200 mL), and finally extracted with ethyl acetate (4 × 200 mL). The combined ethyl acetate fractions were dried over anhydrous Na_2SO_4, filtered, and, finally, freed from solvent in a vacuum to obtain the chlorogenic acid fraction (yield 8.98 g). The presence of the individual chlorogenic acids was checked by HPLC/DAD and HPLC/MS.

Preparation of Coffee Bean Reconstitutes. Soluble coffee compounds, either individually or in combination, were dissolved in water (25 mL) in concentrations corresponding to their natural concentrations in the authentic raw coffee bean (**Table 2**). These solutions were added to aliquots (50 g) of the freeze-dried, water-extracted raw bean shell (BS), slowly stirred overnight at room temperature, and then freeze-dried for 48 h. Using this procedure, the following bean reconstitutes have been prepared by infusing BS with aqueous solutions of the compounds as detailed in **Table 2**: expt a, total bean extract (BE); expt b, LMW-BE (MW < 1 kDa); expt c, HMW-BE (MW > 1 kDa); expt d, a mixture of free amino acids, organic acids, carbohydrates, chlorogenic acids, caffeine, trigonelline, and Fe(III), Cu(II), Mn(II), and Zn(II) ions; expt e, caffeine; expt f, trigonelline; expt g, free amino acids; expt h, sucrose; expt i, a mixture of free amino acids and carbohydrates; expt j, organic acids; expt k, natural chlorogenic acid fraction; expt l, a mixture of the chlorogenic acid fraction and free amino acids; expt m, a mixture of the chlorogenic acid fraction and organic acids; expt n, a mixture of the chlorogenic acid fraction and Fe(III), Cu(II), Mn(II), and Zn(II) ions; expt o, 5-*O*-caffeoylquinic acid; expt p, a mixture of 5-*O*-caffeoylquinic acid and Fe(III), Cu(II), Mn(II), and Zn(II) ions; expt q, a mixture of caffeic acid (1.54 g/50 g of bean solids) and Fe(III), Cu(II), Mn(II), and Zn(II) ions; expt r, a mixture of quinic acid (1.65 g/50 g of bean solids) and Fe(III), Cu(II), Mn(II), and Zn(II) ions.

Roasting of Authentic and Reconstituted Coffee Beans. Prior to roasting, samples were freeze-dried for 48 h to adjust the same humidity of 6.0%. The raw coffee beans (50 g) were roasted at 240 °C for different roasting times (3.0−5.5 min) by means of a Probat BRZ II-type batch roaster (Emmerich, Germany), and the roast degree was characterized by a color test Neuhaus (CTN) value. For precursor experiments, aliquots (50 g) of the fully extracted bean shell (BS) and the various recombined coffee beans (expts a−r), respectively, were roasted for 4.5 min at 240 °C to be comparable with the roast coffee (CTN 67) used as the reference. All the roasted beans and roasted reconstitutes obtained were ground by means of a batch mill (IKA, Staufen, Germany).

Table 2. Amounts of Components in Raw Bean Extract (BE) and Amounts Used for Bean Reconstitution Experiments

components		amount [mg] in BE[a]	amount [mg] used for reconst expt[b]
total chlorogenic acids		4890	3030
caffeine		1160	720
trigonelline		730	450
organic acids	citric acid	850	530
	malic acid	410	250
	quinic acid	250	160
	lactic acid	120	74
	phosphoric acid	100	62
	acetic acid	74	46
	formic acid	12	7.40
	pyroglutamic acid	1.2	0.7
carbohydrates	sucrose	6410	3970
	glucose	68	42
	fructose	60	37
	galactose	10	6
free amino acids	*N*-methyl-L-histidine	0.47	0.29
	L-glutamic acid	95.89	59.49
	L-proline	38.60	23.94
	L-asparagine	29.39	18.23
	4-aminobutyric acid	28.74	17.83
	L-alanine	24.59	15.25
	L-aspartic acid	21.34	13.24
	L-serine	12.94	8.03
	L-phenylalanine	10.46	6.49
	L-valine	6.97	4.32
	L-isoleucine	6.61	4.10
	L-tryptophan	6.04	3.75
	L-leucine	5.52	3.42
	L-lysine	5.34	3.31
	L-tyrosine	5.24	3.25
	L-glutamine	5.13	3.18
	L-arginine	4.67	2.90
	L-2-aminoadipic acid	3.59	2.22
	glycine	3.01	1.87
	DL-β-aminoisobutyric acid	2.87	1.78
	L-threonine	2.70	1.67
	L-histidine	2.49	1.55
	L-cysteine	1.54	0.96
	L-methionine	1.42	0.88
	β-alanine	1.25	0.78
	O-phospho-L-serine	1.02	0.63
	taurine	0.80	0.49
	L-cystathionine	0.43	0.27
	L-citrulline	0.36	0.22
transition metals	Fe(III)[c]	5.38	3.34
	Mn(II)[d]	3.37	1.91
	Cu(II)[e]	3.10	2.11
	Zn(II)[f]	0.50	0.30

[a] Amounts determined in 100 g of raw bean extract (BE). [b] Amounts incorporated into 50 g of raw bean solids (BS). [c] As $FeCl_3$. [d] As $MnCl_2 \cdot 4H_2O$. [e] As $CuSO_4$. [f] As $ZnSO_4 \cdot 7H_2O$.

Preparation of Coffee Brews from Roasted Authentic and Reconstituted Coffee Beans. For the preparation of the reference coffee brew, the powder obtained from the authentic roast coffee (54 g) was percolated with hot water (1 L, 88−92 °C) using a drip filter (Kaffeefilterpapier Nr. 4, ALDI Einkauf GmbH & Co. oHG, Essen, Germany). To account for the differences in weight of the individually reconstituted beans, the brews from powdered roasted extracted beans and reconstituted beans were prepared using the following amounts of ground powder for the individual experiments: expt a, 54.0 g/L; expt b, 53.8 g/L; expt c, 43.8 g/L; expt d, 51.9 g/L; expt e, 44.2 g/L; expt f, 43.9 g/L; expt g, 43.7 g/L; expt h, 47.0 g/L; expt i, 47.1 g/L; expt j, 44.5 g/L; expt k, 46.2 g/L; expt l, 46.3 g/L; expt m, 47.2 g/L; expt n, 46.2 g/L; expt o, 46.2 g/L; expt p, 46.2 g/L; expt q, 44.9 g/L; expt r,45.0 g/L. To prepare the brew of the water-soluble green bean extract (BE), an aliquot of BE (10.5 g), matching the amount of this fraction in 54 g of roasted and ground coffee, was percolated with hot water (1 L, 88−92 °C) using a drip filter (Kaffeefilterpapier Nr. 4, ALDI Einkauf GmbH & Co. oHG, Essen, Germany). After preparation, the brews were cooled in an ice bath to room temperature and the pH values were adjusted to the value (pH 5.70) measured for the reference coffee (CTN 67) by adding hydrochloric acid (0.1 mol/L). All coffee brews were used for analysis immediately after preparation.

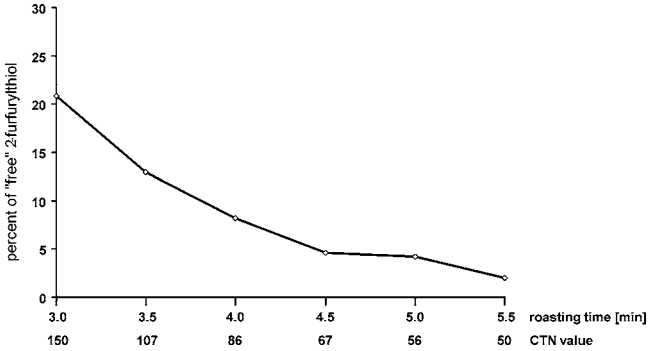

Figure 1. Influence of roasting degree (240 °C) on amount of "free" 2-furfurylthiol in coffee brew. Coffee brew (9 mL) and 2-furfurylthiol in phosphate buffer (500 mg/1 mL) were mixed, incubated for 20 min at 30 °C, and then analyzed by static headspace/HRGC.

Determination of Headspace Concentrations of 2-Furfurylthiol. An aliquot (9 mL) of the individual coffee brews was spiked with an aqueous solution of 2-furfurylthiol (1 mL; 500 μg in 0.1 mol/L phosphate buffer, pH 5.70). The solution was then incubated in a temperature-controlled, septum-sealed vessel (180 mL) at 30 °C. After 10, 20, 30, and 60 min, respectively, an aliquot (2.5 mL) of the headspace was withdrawn by means of a gastight syringe and was analyzed by high-resolution gas chromatography (HRGC). The loss of 2-furfurylthiol induced by the coffee ingredients is given as the percent of the amount of "free" 2-furfurylthiol, which is defined as the amount of the nontrapped thiol.

High-Resolution Gas Chromatography (HRGC). HRGC was performed on a 30 m × 0.32 mm i.d. fused silica capillary (HP-5, methyl polysiloxane−5% phenyl, 0.25 μm, Agilent, Waldbronn, Germany) located in a gas chromatograph Type 8521 (Dani Instruments, Cologno Monzese, Italy) equipped with a flame ionization detector operating at 250 °C and a programmed temperature vaporizer (PTV) injector. Prior to injection, the PTV was cooled to −20 °C with liquid carbon dioxide; after injection the temperature was increased to 180 °C in 0.2 min. Using hydrogen as the carrier gas at a flow rate of 2 mL/min, chromatographic separation was performed by raising the oven temperature from 30 to 60 °C at a rate of 6 °C per min and then to 225 °C at a rate of 25 °C per min.

RESULTS AND DISCUSSION

To monitor the influence of the roasting degree on the thiol-binding activity of the corresponding coffee brew, raw coffee beans were roasted in a batch roaster at 240 °C for 3.0−5.5 min, yielding coffees of different roasting degrees from light (CTN 150) to strong roast (CTN 50). After grinding, standardized coffee brews were prepared and immediately used for thiol-binding experiments. To achieve this, the corresponding coffee brews were spiked with 2-furfurylthiol and incubated for 20 min at 30 °C in a septum-closed vessel; then the amount of "free" thiol in the headspace was measured by static headspace gas chromatography. Incubation of an aqueous solution of 2-furfurylthiol in phosphate buffer under comparative conditions did not show any loss of the thiol (control). As given in **Figure 1**, just 21% of the starting amount of 2-furfurylthiol was detectable after incubating the thiol in the presence of the coffee roasted for 3.0 min. Increasing the roasting time induced a further strong decrease of the amount of "free" thiol in the headspace of the corresponding beverage; e.g., the dark roasted coffee evaluated with a CTN value of 67 bound more than 95%

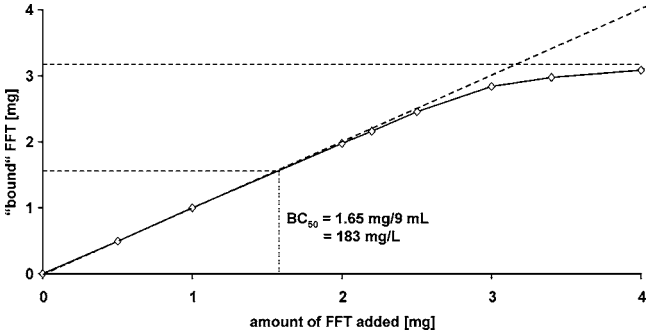

Figure 2. 2-Furfurylthiol binding capacity of brew prepared from beans (CTN 67) roasted for 4.5 min at 240 °C. Coffee brew (9 mL) and 2-furfurylthiol in phosphate buffer (0–4 mg/1 mL) were mixed, incubated for 60 min at 30 °C, and then analyzed by static headspace/HRGC.

of the starting amount of the thiol. Additional increase of the roasting degree had, however, a comparatively small effect on further thiol binding. Therefore, the roasting degree with a CTN value of 67 was chosen as the reference for all the following experiments.

To get first insight into the maximum amounts of 2-furfurylthiol that can be bound by the active binding sites in coffee, the binding capacity of a coffee brew prepared from the reference coffee beans (CTN 67) was analyzed. To achieve this, the coffee brew was spiked with various amounts up to 4.0 mg of 2-furfurylthiol and, after incubation for 60 min at 30 °C, the amount of "free" thiol was determined by headspace gas chromatography. Spiking the coffee with up to 3 mg of 2-furfurylthiol led to a complete loss of "free" thiol (**Figure 2**). With further increase of the starting amount of 2-furfurylthiol, the "bound" thiol approximated a maximum amount of about 3.3 mg/9 mL coffee beverage or about 0.23 mmol/g dry weight, respectively. On the basis of these data, a half-maximal binding capacity (BC_{50}) of 1.65 mg/9 mL coffee brew could be determined, thus indicating that the enormous amount of 183 mg of thiol is necessary to cover 50% of the binding sites in 1 L of coffee beverage (**Figure 2**).

With the aim of characterizing the precursors of thiol-binding sites in raw coffee beans, first the intact raw bean was separated into the aqueous bean extractables (BE), yielding about 19.4 g per 100 g of coffee beans, and the fully extracted bean shell (BS), accounting for 80.6 g per 100 g of coffee beans, by using an exhaustive aqueous extraction procedure similar to those reported recently (*11*). This technology allowed an extraction of the water solubles without any prior grinding of the beans and opened the possibility of using the nonsoluble, intact bean shell as the "matrix" or "architecture" in model roasting experiments using a batch roaster. This allowed us to infuse the freeze-dried bean shell with aqueous solutions of either single coffee compounds or fractions isolated from the raw bean solubles, to roast these reconstituted beans after freeze-drying, and finally to measure the activity of these compounds "in bean" as precursors of thiol-binding sites.

As proof of the principle, a fully reconstituted coffee bean was prepared by reincorporation of the raw bean extractables (BE) in their "natural" concentration into the bean matrix (BS). These reconstituted beans and, in comparison, the authentic raw beans were then roasted for 4.5 min at 240 °C and ground, and aliquots of the brews freshly prepared from both beans were spiked with 2-furfurylthiol (500 μg). After incubation for 10, 20, 30, and 60 min at 30 °C in septum-closed vessels, the amounts of "free" thiol in the headspace above the solutions were measured by static headspace analysis. As the control

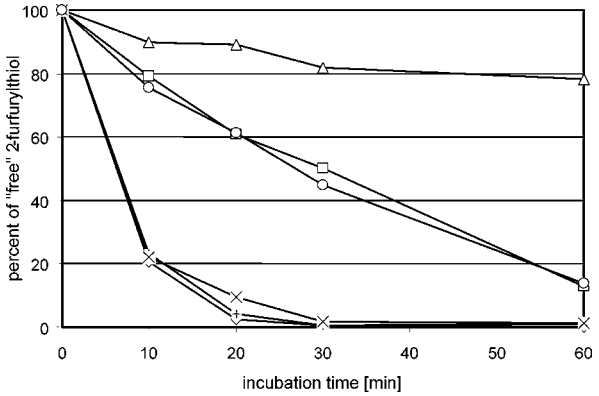

Figure 3. Influence of reincorporation of raw coffee fractions into the bean matrix prior to roasting on thiol-binding activity of the corresponding brew: reference coffee (–◇–); bean solids without any additives (–□–, BS); bean solids spiked with fraction BE (– + –, expt a); bean solids spiked with fraction LMW-BE (–×–, expt b); bean solids spiked with HMW-BE (–○–, expt c). In addition, thiol-binding activity was measured for the fraction BE prior to roasting (–△–).

(0 min), the amount of the headspace of a solution of 2-furfurylthiol (500 μg) in phosphate buffer was determined by headspace/HRGC. The results, shown in **Figure 3**, revealed a rapid decrease of the concentrations of the "free" thiol for both solutions. For example, 80% of the 2-furfurylthiol was "lost" after 10 min and after 30 min the thiol was nearly absent in the headspace. These data clearly showed that the time-dependent progression of thiol binding in the brews made from reconstituted coffee (**Figure 3**, expt a) matched that of the reference bean rather well, thus demonstrating that the soluble components can be successfully reincorporated into the bean matrix without any loss of activity.

To investigate the precursor potential of the nonsoluble bean matrix (BS) for the generation of thiol-binding sites, just the bean solids were roasted under comparative conditions, and the thiol-binding activity of the brew prepared thereof was determined as reported above. The headspace analysis clearly showed that, compared to either the reference coffee or the fully reconstituted coffee, the brew prepared from the roasted bean solids exhibited a drastically lower activity in binding the 2-furfurylthiol (**Figure 3**); e.g., after 10 or 30 min, respectively, about 80 or 52% of the "free" thiol was still detectable. In conclusion, the main precursors for thiol-binding sites have to be present in the water-extractable fraction BE rather than in the bean solids.

To investigate the influence of roasting on the thiol-binding activity of the water-soluble fraction BE, an aqueous brew was prepared from the nonroasted fraction BE, 2-furfurylthiol (500 μg) was added, and after incubation for 10, 20, 30, and 60 min at 30 °C, the amount of "free" thiol was measured by static headspace analysis. As given in **Figure 3**, the amount of thiol in the headspace did not change drastically; e.g., after incubation for 60 min about 80% of the thiol was still detectable. These data clearly confirmed that roasting is essential to convert raw bean precursors into highly active thiol-binding sites.

With the aim of investigating the influence of the molecular weight of the water solubles on their precursor potential, the bean extractables (BE) were fractionated by means of ultrafiltration using a cutoff of 1 kDa into a low-molecular-weight fraction (LMW-BE, MW < 1 kDa) with a yield of 97.7% and a high-molecular-weight fraction (HMW-BE, MW > 1 kDa) accounting for 2.3% of the fraction BE (**Table 1**). Both fractions were then separately reincorporated into the bean matrix (BS),

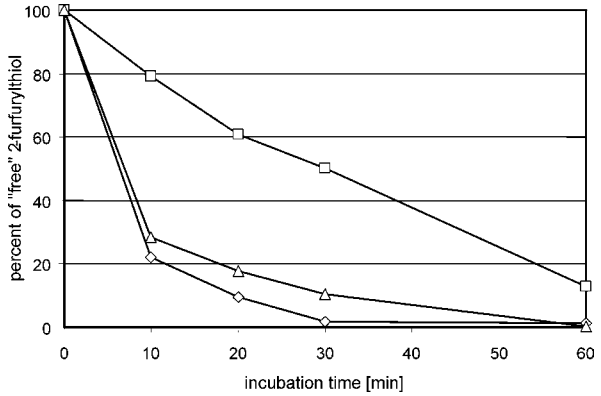

Figure 4. Influence of spiking bean solids (BS) with an artificial cocktail of raw been components on thiol-binding activity of corresponding roast coffee beverage: bean solids spiked with an artificial cocktail of the constituents given in **Table 2** (–△–, expt d); bean solids spiked with fraction LMW-BE (–◇–, expt b); bean solids without additives (–□–).

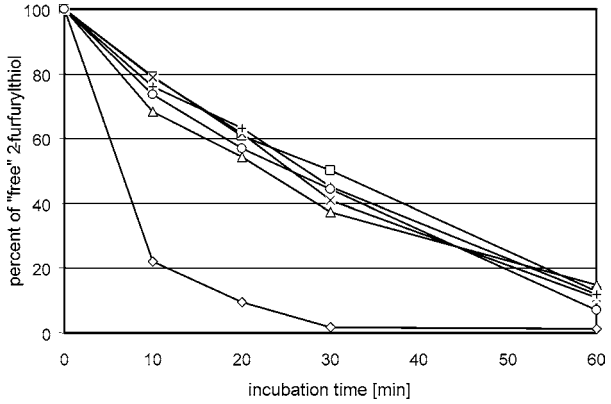

Figure 5. Influence of individual nitrogen-containing compounds on thiol-binding activity of roast coffee brews: bean solids spiked with fraction LMW-BE (–◇–, expt b); bean solids without additives (–□–); bean solids spiked with caffeine (–△–, expt e); bean solids spiked with trigonelline (–×–, expt f); bean solids spiked with free amino acids (– + –, expt g); bean solids spiked with free amino acids and carbohydrates (–○–, expt i).

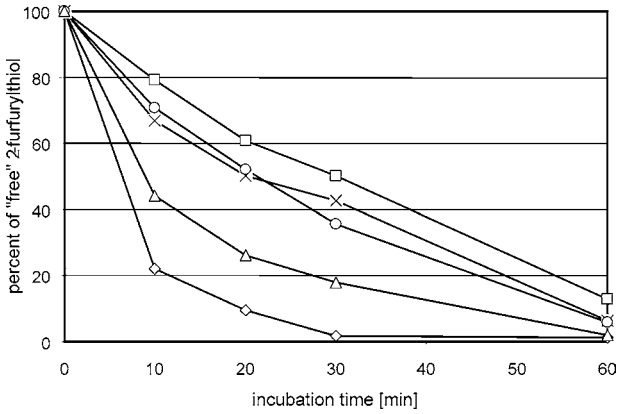

Figure 6. Influence of individual oxygen-containing precursors on thiol-binding activity of roast coffee brews: bean solids spiked with fraction LMW-BE (–◇–, expt b); bean solids without additives (–□–); bean solids spiked with sucrose (–×–, expt h); bean solids spiked with organic acids (–○–, expt j); bean solids spiked with the natural chlorogenic acid fraction (–△–, expt k).

and, after roasting, both brews were analyzed for their activity in binding 2-furfurylthiol as given above. The brew prepared from the roasted bean matrix infused with the fraction LMW-BE showed nearly identical thiol-binding activity as the reference coffee bean (**Figure 3**, expt b). In contrast, the bean solids supplemented with the fraction HMW-BE (**Figure 3**, expt c) showed a similar time-dependent course of thiol degradation as already measured for the brew prepared just from roasted bean solids. As these data showed that mainly the low-molecular-weight water solubles in raw coffee generate the thiol-binding sites upon roasting, the results of the following experiments with partially reconstituted beans are related to the bean matrix supplemented with the LMW-BE fraction as the reference.

To investigate the compounds present in the fraction LMW-BE, the chemical composition of this fraction should be analyzed next. To achieve this, total chlorogenic acids, caffeine, trigonelline, organic acids, mono- and disaccharides, free amino acids, and transition metals have been identified and quantified in the aqueous bean extract following analytical procedures reported in the literature. On the basis of the quantitative data obtained (**Table 2**), an artificial bean reconstitute was prepared by incorporating an aqueous cocktail of all the components given in **Table 2** into the bean matrix. After freeze-drying and roasting, an aqueous brew was prepared, 2-furfurylthiol (500 μg) was added, and after incubation for 10, 20, 30, and 60 min at 30 °C, the amount of "free" thiol in the headspace was measured by static headspace analysis (**Figure 4**, expt d). The data clearly showed that the thiol-binding activity of the roasted coffee solids containing the mixture of all the major and minor compounds given in **Table 2** in their "natural" concentrations is very close to the activity measured for the brew prepared from the roasted matrix spiked with the fraction LMW-BE. In consequence, the main precursors of thiol-binding sites in coffee have to be among the components summarized in **Table 2**.

To further narrow the number of potential precursors, the bean solids (BS) were spiked individually with "natural" amounts of caffeine (expt e), trigonelline (expt f), a mixture of free amino acids (expt g), sucrose (expt h), a combination of free amino acids and sucrose (expt i), a mixture of organic acids (expt j), and the chlorogenic acid fraction isolated from raw beans (expt k), respectively, and finally were freeze-dried and roasted. The brews prepared from these partial reconstitutes were then used for measuring the 2-furfurylthiol binding activity as already detailed above. As outlined in **Figures 5** and **6**, with the exception of the chlorogenic acid fraction, none of these compounds incorporated into the bean solids prior to roasting induced a remarkable increase of the thiol-binding activity compared to that of the brew prepared from the roasted bean matrix alone. In consequence, caffeine, trigonelline, amino acids, sucrose, and organic acids could be excluded as the key precursors for thiol-binding sites. In contrast, the roasted bean solids supplemented with the natural chlorogenic acid fraction showed strong thiol-binding activity (**Figure 6**, expt k). The presence of this fraction in the coffee bean induced a much faster progression of the thiol binding compared to the brew prepared from the bean solids only. Although the chlorogenic acids were found as the only organic bean ingredients exhibiting a remarkable effect on the binding of 2-furfurylthiol, the brew prepared thereof just showed part of the thiol-binding activity measured for the brew obtained from the roasted, LMW-BE infused bean matrix (**Figure 6**).

To evaluate whether the precursor activity of the chlorogenic acids might be further enhanced in the presence of other bean components, the natural chlorogenic acid fraction was mixed with either the amino acids, the organic acids, or the transition metal ions Fe(III), Cu(II), Mn(II), and Zn(II) present in the

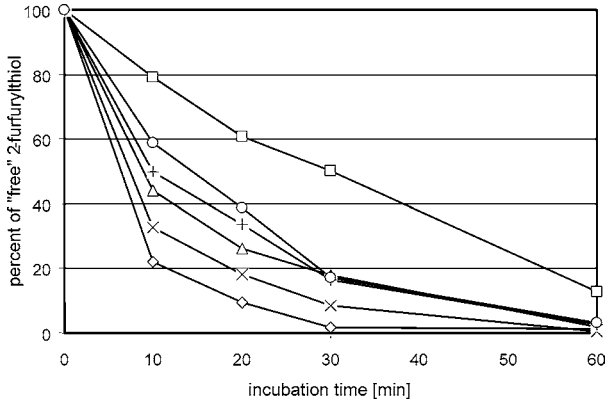

Figure 7. Influence of additional compounds on thiol-binding activity induced by chlorogenic acid fraction upon roasting: bean solids spiked with fraction LMW-BE (–◇–, expt b); bean solids without additives (–□–); bean solids spiked with the chlorogenic acid fraction (–△–, expt k); bean solids spiked with the chlorogenic acid fraction and amino acids (–○–, exp. l); bean solids spiked with the chlorogenic acid fraction and organic acids (–+–, expt m); bean solids spiked with the chlorogenic acid fraction and transition metal ions (–×–, expt n).

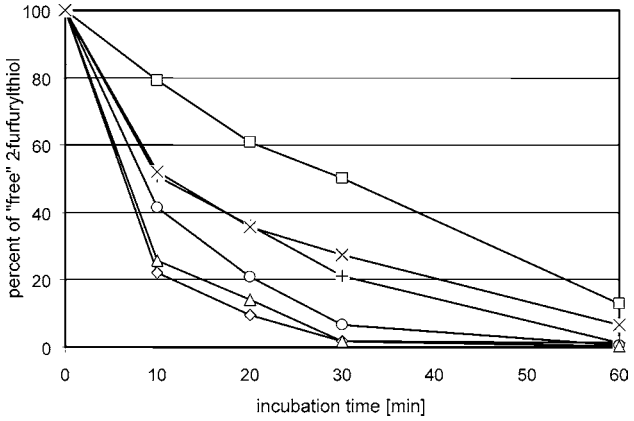

Figure 8. Influence of 5-*O*-caffeoylquinic acid, caffeic acid, and quinic acid on roasting-induced thiol-binding activity: bean solids spiked with fraction LMW-BE (–◇–, expt b); bean solids without additive (–□–); bean solids spiked with 5-caffeoylquinic acid (–+–, expt o); bean solids spiked with 5-*O*-caffeoylquinic acid and transition metals (–○–, expt p); bean solids spiked with caffeic acid and transition metals (–△–, expt q); bean solids spiked with quinic acid and transition metals (–×–, expt r).

aqueous bean extract (BE) prior to incorporation into the bean matrix and roasting (**Table 2**). Measurement of the thiol-binding activity of the corresponding coffee brews revealed that the presence of amino acids (**Figure 7**, expt l) and organic acids (**Figure 7**, expt m), respectively, led to a small decrease in 2-furfurylthiol binding compared to the brew prepared from roasted bean solids spiked just with the chlorogenic acid fraction (**Figure 7**). In contrast, the presence of the transition metal ions Fe(III), Cu(II), Mn(II), and Zn(II) further increased the thermally inducable thiol-binding activity of the chlorogenic acid fraction (**Figure 7**, expt n).

As 5-*O*-caffeoylquinic acid accounts for the major part of the isolated chlorogenic acid fraction (*16*, *17*), its precursor activity was studied next. The thiol-binding activity of the brew made of the roasted bean matrix spiked with 5-*O*-caffeoylquinic acid was slightly weaker (**Figure 8**, expt o), but comparable to the activity measured for the beans infused with the total chlorogenic acid fraction (cf. **Figure 6**, expt k). The brew prepared from the roasted bean matrix spiked with a mixture of 5-*O*-caffeoylquinic acid and the transition metals showed an even faster thiol binding (**Figure 8**, expt p). These data clearly demonstrated chlorogenic acids and, in particular, 5-caffeoylquinic acid as well as transition metal ions as the key precursors in thiol binding site formation upon roasting. As 5-*O*-caffeoylquinic acid is made up of one molecule of caffeic acid esterified with one molecule of quinic acid, additional beans were prepared containing either the caffeic acid or the quinic acid, both in the presence of transition metals, to investigate which part of 5-*O*-caffeoylquinic acid is essential for its precursor activity in thiol binding. Both caffeic acid (**Figure 8**, expt q) and quinic acid (**Figure 8**, expt r) led to an increased thiol-binding activity compared to the brew prepared just from the bean solids (control). Although the quinic acid showed significant precursor activity for thiol binding, the effect of caffeic acid was noticeably stronger than that of the quinic acid, even exceeding the activity of 5-*O*-caffeoylquinic acid, and was comparable to that observed for the fraction LMW-BE.

In summary, the use of biomimetic "in bean" experiments, which allow studying model reactions under quasi-natural roasting conditions, undoubtedly identified for the first time chlorogenic acids as well as thermal degradation products caffeic acid and quinic acid as important precursors for the transition metal catalyzed generation of highly active, low-molecular-weight thiol-binding sites upon coffee roasting. The identification of the thiol-binding sites as well as the structure determination of the thiol adducts formed is currently in progress and will be published elsewhere.

ABBREVIATIONS USED

BC_{50}, half-maximal binding capacity; BE, aqueous raw bean extract; BS, water-extracted bean shell; CTN value, color test Neuhaus value; LMW-BE, low-molecular-weight fraction of BE (MW < 1 kDa); HMW-BE, high-molecular-weight fraction of BE (MW > 1 kDa).

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