JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2003,** *51,* 6861−6869 **6861**

Activity-Guided Identification of a Chemopreventive Compound in Coffee Beverage Using in Vitro and in Vivo Techniques

VERONIKA SOMOZA,*,† MICHAEL LINDENMEIER,‡ ELISABETH WENZEL,† OLIVER FRANK,§ HELMUT F. ERBERSDOBLER,# AND THOMAS HOFMANN*,§

Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, 85748 Garching, Germany; Institut für Lebensmittelchemie, Technische Universität München, Lichtenbergstrasse 4, 85748 Garching, Germany; Institut für Lebensmittelchemie, Westfälische Wilhelms-Universität, Corrensstrasse 45, 48149 Münster, Germany; and Institut für Humanernährung und Lebensmittelkunde, Universität Kiel, 24105 Kiel, Germany

The aim of the present study was to apply an activity-guided screening procedure to coffee brew to identify a key chemopreventive compound by means of in vitro antioxidant tests as well as cell culture experiments and to prove the in vivo activity of that compound by an animal feeding experiment. Solvent fractionation, followed by multiple-step ultrafiltration, revealed that the polar coffee compounds with molecular weights below 1 kDa show the major inhibitory effect on the in vitro peroxidation of linoleic acid as well as the predominant chemopreventive enzyme modulating activity on the NADPH−cytochrome *c* reductase (CCR) and glutathione *S*-transferase (GST) in human intestinal Caco-2 cells. To identify the chemical structure of the most active antioxidants and chemopreventive compounds, the polar compounds were further separated by HPLC techniques, followed by the activity-guided screening of the individual HPLC fraction. These experiments demonstrated 5-chlorogenic acid to be the most powerful antioxidant in vitro, whereas, in contrast, chemopreventive effects on the GST activity were found for the *N*-methylpyridinium ion, the structure of which was elucidated by LC-MS and NMR experiments and confirmed by synthesis. The in vivo activities of coffee beverage and *N*-methylpyridinium ions were tested in a 15-day feeding experiment on rats. In the liver, feeding of 4.5% coffee beverage resulted in increases of GST and UDP-GT activities by 24 and 40% compared to animals fed the control diet ($p > 0.05$), respectively. Plasma total antioxidant capacity and plasma tocopherol were elevated in animals fed the coffee beverage and the *N*-methylpyridinium-containing diet. In summary, the results demonstrating a strong in vitro antioxidant activity for coffee were confirmed by the feeding study. Surprisingly, feeding of *N*-methylpyridinium also resulted in an increased total antioxidant capacity in the plasma. The data indicate that the mode of action demonstrated for *N*-methylpyridinium in biological systems is different from that in foods.

KEYWORDS: Coffee; *N*-methylpyridinium; xenobiotic enzymes; chemoprevention; antioxidants; chlorogenic acid; GST; CCR

INTRODUCTION

With worldwide consumption of ∼5 million tons in 2001 (*1*), coffee is one of the most popular beverages in the world. The habitual consumer highly appreciates coffee beverages for their salubrious, desirable aroma as well as their stimulating properties. Besides its well-studied, caffeine-mediated, psychoactive effects (*2*, *3*), coffee is believed to stimulate other organ systems, such as the gastrointestinal tract (*4*).

To date, most of the physiological effects of coffee beverages have been ascribed to their content in caffeine, the diterpenes kahweol and cafestol (*5*), or phenols such as chlorogenic acids (*6*). As phenolic compounds present in green coffee beans and in roast coffee brews have been demonstrated to exert antioxidative effects in in vitro systems (*7*, *8*), these compounds were hypothesized to primarily increase the total antioxidant capacity in human plasma after consumption of coffee beverages (*9*, *10*).

On the other hand, the antioxidant properties of coffee brews in vitro have been shown to depend on roasting conditions applied to the green coffee beans (*11*). During roasting, proteins and carbohydrates undergo complex Maillard-type reactions,

* Authors to whom correspondence should be addressed [(V.S., for physiology) telephone +49-89-1417-0, fax +49-89-1324-8, e-mail veronika.somoza@lrz.tu-muenchen.de; (T.H., for chemistry) telephone +49-251-8333-391, fax +49-251-8333-396, e-mail thomas.hofmann@uni-muenster.de].

† Deutsche Forschungsanstalt für Lebensmittelchemie.

‡ Technische Universität München.

§ Westfälische Wilhelms-Universität.

Universität Kiel.

10.1021/jf034750e CCC: $25.00
Published on Web 10/03/2003

resulting in the formation of chemically heterogeneous, nitrogen-containing, brown compounds called melanoidins (*12*−*14*). Some of these melanoidin structures have been identified to induce aroma staling of freshly prepared coffee beverages (*15*), but structure-specific antioxidative effects of chemically defined roasting products in vivo are still unknown.

Very recently, a melanoidin structure, called pronyl-L-lysine, has been identified as a key antioxidant formed during heat treatment of Maillard-type reaction mixtures and was found in high amounts in thermally treated, dark foods, such as bread crust (*16*). It was also shown that pronyl-L-lysine modulates a chemopreventive phase II enzyme, the glutathione *S*-transferase (GST), in a biological system in such a way as to be interpreted as a functional parameter of antioxidant activity.

Current data suggest that the balance between the phase I carcinogen-activating enzymes and the phase II detoxifying enzymes is critical to determining an individual's risk for cancer. Human deficiencies in phase II enzyme activity, specifically GST, have been identified and associated with increased risk for colon cancer (*17*). On the other hand, induction of GST or other phase II enzymes, for example, the UDP−glucuronyltransferase (UDP-GT), by antioxidants represents a promising strategy for cancer prevention. Although the molecular mechanism by which antioxidants bind to an antioxidant responsive element resulting in the specific, monofunctional induction of phase II enzymes has been intensively studied (*18*), it is still an open question as to whether a compound showing antioxidant activity in vitro may function as a phase II inducer in biological systems. Among all of the biological systems suitable for in vitro studies, the intestinal Caco-2 cell line is widely used to investigate the effects of dietary compounds on phase I/II enzymes as the colon is clearly one of the most likely sites for the development of different types of dietary-induced cancers (*19*, *20*).

The induction of phase II GST and UDP-GT enzymes in vivo has already been demonstrated in animal feeding experiments for green coffee beans, coffee brew, and the diterpenes kahweol and cafestol (*5*, *6*, *21*, *22*). Moreover, decaffeinated coffee brew, like regular coffee brew, also induced GST activity in mice, indicating that this chemopreventive effect is not related to caffeine (*23*). Thus, all of the animal experiments reported so far demonstrate a significant increase in chemopreventive enzyme activities after coffee consumption, but no nonphenolic key compound to which these in vivo effects can be fully ascribed has been identified. Currently unidentified melanoidin-type structures, present, for example, in coffee beverages, are also hypothesized to act as key players for both the antioxidant activity (*8*) and the activation of chemopreventive enzymes (*24*−*26*).

Therefore, the objectives of the present study were to apply the activity-guided screening procedure detailed in **Figure 1** to coffee brew, to identify a key chemopreventive compound by means of in vitro antioxidant tests as well as cell culture experiments, and to prove the in vivo activity of that compound by an animal feeding experiment.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: ethanol, 1-chloro-2,4-dinitrobenzene (Merck, Darmstadt, Germany); fetal bovine serum, L-glutamine, penicillin, streptomycin, porcine trypsin (Sigma, Deisenhofen, Germany); 5-chlorogenic acid, pyridine, iodomethane, iodomethane-d_3 (Aldrich, Deisenhofen, Germany); Trolox (Fluka, Deisenhofen, Germany); cytochrome *c* (Boehringer, Mannheim, Germany). DMSO-d_6 was obtained from Isocom (Landshut, Germany).

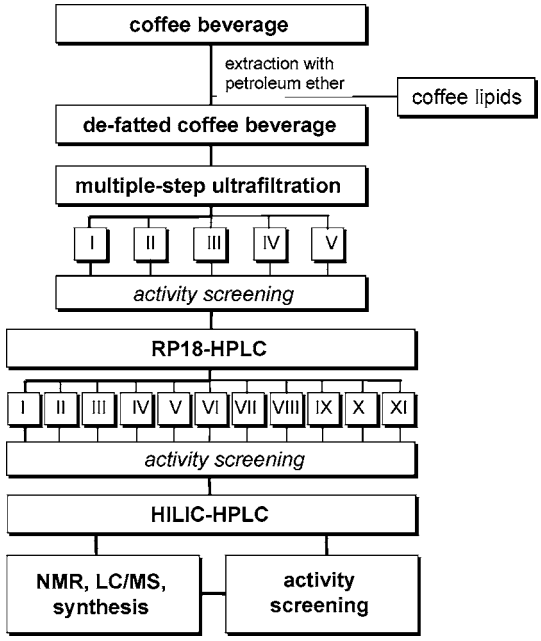

Figure 1. Scheme used for the activity-guided fractionation and identification of a chemopreventive compound in coffee beverage.

Table 1. Dry Weight and in Vitro Antioxidant Activity of Coffee Beverage, Defatted Beverage, and Isolated Lipid Fraction

sample	dry wt (g/L of beverage)	antioxidant activity (mmol of TE equiv)
coffee beverage	13.81	4.02
defatted coffee beverage[a]	13.57	3.93
coffee lipids[a]	0.24	0.38

[a] Fractions were obtained by extracting the coffee beverage with petroleum ether and removing the solvent in vacuo or by freeze-drying.

Synthesis of *N*-Methylpyridinium and *N*-[$^2H_3$]Methylpyridinium Iodide. Following the procedure reported in the literature (*30*), methyl iodide (60 mmol) or [$^3H_2$]methyl iodide (60 mmol) was added dropwise to a solution of pyridine (60 mmol) in acetonitrile (15 mL). The mixture was stirred for 20 min at room temperature and then heated for 6 h at 50 °C. The oily mixture was then poured on ice (200 g), and the precipitate obtained was washed with ice-cooled acetonitrile and filtered. After recrystallization from acetonitrile, the target compound was obtained as colorless crystals (55 mmol; yield = 92%). *N*-Methylpyridinium iodide: LC-MS (APCI), *m*/*z* 94.1 (100; $[M + 1]^+$); UV−vis (water): λ_{max} = 258 nm; ^{1}H NMR (400 MHz; DMSO-d_6 DQF-COSY; arbitrary numbering of the carbon atoms refers to the structure in **Figure 7**) δ 4.38 (s, 3H, H−C(7)), 8.15 (t, 2 × 1H, J = 6.9 Hz, H−C(3), H−C(5)), 8.60 (t, 1H, J = 7.9 Hz, H−C(4)), 9.03 (t, 2 × 1H, J = 6.25 Hz, H−C(2), H−C(6)). *N*-[$^2H_3$]Methylpyridinium iodide: LC-MS (APCI), *m*/*z* 97.1 (100; $[M + 1]^+$); UV−vis (water) λ_{max} = 258 nm; ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.15 (t, 2 × 1H, J = 6.9 Hz, H−C(3), H−C(5)), 8.60 (t; 1H, J = 7.9 Hz, H−C (4)), 9.03 (t, 2 × 1H, J = 6.25 Hz, H−C(2), H−C(6)).

Preparation of Coffee Beverage. Roasted coffee beans (*Coffea arabica*, Colombia, decaffeinated) were ground in an ultra mill (Retsch, Hann, Germany), equipped with a metal sieve (2 mm pore diameter). An aliquot (54 g) of the coffee powder was percolated with hot water (1.1 L) by means of a commercial coffee machine equipped with a cellulose-type coffee filter (no. 4, Melitta, Minden, Germany). The fresh coffee beverage obtained (1.0 L) was rapidly cooled in an ice bath and was then extracted with petroleum ether (3 × 300 mL). The defatted beverage was freeze-dried, the organic layer was freed from solvent in vacuo, and the yields of both fractions were determined by weight (**Table 1**).

Antioxidant Activity of Coffee Fractions and Plasma Samples. The antioxidant activity of coffee fractions and plasma samples was

determined in vitro by measuring their inhibitory effect on linoleic acid peroxidation closely following the procedure reported recently (*16*). For measuring the antioxidant activity in the coffee fractions, an aliquot (60 μL) of a solution of the coffee sample (1 mg/mL) in water/ethanol (50:50; v/v) was used. As for the plasma samples, an aliquot (120 μL) of the plasma sample was diluted with ethanol (30 μL) and then used for antioxidant analysis. The results were related to the absorption of a standard solution of Trolox (1 mmol/L) in water/ethanol (50:50; v/v) and were expressed as Trolox equivalents (TE values). Each of the experiments was performed in triplicate.

Multiple-Step Ultrafiltration. An aliquot (2.5 g) of the freeze-dried, defatted coffee beverage was dissolved in water (200 mL) and fractionated by multiple-step ultrafiltration (Amicon, Witten, Germany), starting with a filter having a cutoff of 100 kDa (Diaflo YM 100), followed by 30 kDa (Diaflo YM 30), 10 kDa (Diaflo YM 10), and, finally, 1 k Da (Diaflo YM 1). The separation was performed under a nitrogen pressure of 1.5 bar for the filter YM 100, or at 4.0 bar for the filters YM 1, YM 10, and YM 30, respectively. The individual fractions I−V were freeze-dried, the residues were weighed, and aliquots were used for measuring the antioxidant activity.

Fractionation of Ultrafiltration Fraction I by RP-HPLC. Aliquots (100 μL) of the low molecular weight ultrafiltration fraction I containing the coffee compounds with molecular weights below 1 kDa were fractionated by semipreparative HPLC using an RP-18 material (Hypersil, 250 × 10 mm; 5 μm; Thermo Hypersil, Kleinsotheim, Germany) as the stationary phase. Chromatography was started with aqueous formic acid (0.1% in water), and the acetonitrile content was increased to 100% within 50 min at a flow rate of 3.0 mL/min. The effluent of each individual fraction was collected from 20 runs and freeze-dried, yielding 11 subfractions (yield in percent): I-1 (35.8), I-2 (14.6), I-3 (5.4), I-4 (4.3), I-5 (4.3), I-6 (3.3), I-7 (11.0), I-8 (4.6), I-9 (5.3), I-10 (6.4), I-11 (5.1). A yield proportional aliquot of each fraction was dissolved in water/ethanol (50:50, v/v; 1 mL), and aliquots (60 μL) were then used to measure the antioxidant activity using the in vitro assay described above. To investigate the effects on chemopreventive enzyme activity, Caco-2 cells were exposed to each fraction in yield proportional concentrations.

Fractionation of Fraction I-1 by Hydrophilic Interaction Liquid Chromatography (HILIC). Fraction I-1 isolated by RP-HPLC was dissolved in an aqueous ammonium formate solution (7.0 mmol/L, pH 5.5), and aliquots (100 μL) were fractionated by HILIC-HPLC using a TSK-Gel Amide-80 column (7.8 × 300 mm; 10 μm; Tosoh Biosep, Stuttgart, Germany), connected to a diode array or an LC-MS. The mobile phase consisted of a mixture A (20:80; v/v) of aqueous ammonium formate solution (7.0 mmol/L, pH 5.5) and acetonitrile and a mixture B (80:20, v/v) of aqueous ammonium formate solution (7.0 mmol/L, pH 5.5) and acetonitrile. Starting with 100% of mixture A, the content of mixture B was increased to 40% within 45 min and then raised to 85% within 5 min (flow = 1.5 mL/min). Monitoring the effluent at 250 nm revealed a major peak at t_R = 25.7 min, which was collected from 15 HPLC runs and then analyzed by LC-MS and ^{1}H NMR spectroscopy. The major compound was identified as *N*-methylpyridinium: LC-MS (APCI), *m/z* 94.1 (100; $[M + 1]^+$); UV−vis (water) λ_{max} = 258 nm; ^{1}H NMR (400 MHz; DMSO-d_6; DQF-COSY; arbitrary numbering of the carbon atoms refers to the structure in **Figure 7**) δ 4.36 (s, 3H, H−C(7)), 8.12 (t, 2 × 1H, J = 6.9 Hz, H−C(3), H−C(5)), 8.58 (t, 1H, J = 7.9 Hz, H−C(4)), 9.00 (t, 2 × 1H, J = 6.25 Hz, H−C(2), H−C(6)).

Cell Culture Experiments. Caco-2 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DMSZ, Braunschweig, Germany). Caco-2 cells (passages 15−18) were maintained at standard conditions as described earlier (*16*). At confluency, cells were exposed for 72 h to lyophilized preparations of coffee extract, defatted coffee extract, or different fractions prepared by ultrafiltration of HPLC separation. The exposure time of 72 h was chosen according to results from time course experiments comprising 24, 48, 72, and 96 h of exposure. From these experiments, the cell's viability in the presence of coffee preparations was highest after 72 h (data not shown). The concentrations used for the total and for the defatted coffee extract were 0.025, 0.05, and 0.10 mg/100 mL of cell culture medium. Fractions prepared either by ultrafiltration or by HPLC separation were tested at concentrations of 0.05 mg/100 mL of medium. Additionally, HPLC fractions were used at yield proportional concentrations. Chlorogenic acid and *N*-methylpyridinium iodide were dissolved at 0.025 and 0.05 mg/100 mL of medium. After exposure to the different coffee fractions, the cells were harvested, homogenized, and centrifuged according to a standard protocol (*16*). The cytosolic 105000*g* supernatant was used for the analysis of the GST activity (*27*), whereas the NADPH−cytochrome *c* reductase (CCR) activity was analyzed in the remaining pellet (*28*). GST activity was analyzed using 1-chloro-2,4-dinitrobenzene as the substrate, and protein content was measured according to the method of Lowry (*29*).

Data obtained from triplicate experiments are given as means and standard deviations in relation to the basal activity of nonexposed control cells (basal activity analyzed for CCR and GST was 4.15 ± 0.37 nmol of cytochrome c × mg of protein^{-1} × min^{-1} and at 382 ± 37 nmol of CDNB × mg of protein^{-1} × min^{-1}, respectively). Means of each treatment were compared with untreated control cells by Student's t test. The level of significance was set at $p < 0.05$ (*).

Quantification of 1-Methylpyridinium Ions in Coffee Beverage. Following the procedure reported (*31*), an aliquot (350 mg) of the freeze-dried, defatted coffee beverage was suspended in an aqueous hydrochloric acid solution (20 mL, 10 mmol/L) at 70 °C, stirred for 20 min, and filtered in a volumetric flask (25 mL). After cooling to room temperature, the solution was made up to exactly 25 mL with aqueous hydrochloric acid (1 mmol/L). An aliquot (2 mL) was spiked with 1-[$^2H_3$]methylpyridinium iodide (200 μg) and was then diluted 10-fold with an aqueous hydrochloric acid solution (10 mmol/L). An aliquot (2.0 mL) of this solution was centrifuged in an Eppendorf cap (2 mL; Eppendorf, Hamburg, Germany) for 5 min at 8000 rpm, and an aliquot (1 mL) was then applied onto a CBA cartridge (1 g, Mega Bound Elut, Varian, Darmstadt, Germany), which was flushed with methanol (5 mL) and preconditioned with water (5 mL) prior to use. After application of the sample (1 mL), the cartridge was rinsed with water (5 mL), followed by methanol (5 mL). After the cartridge had been flushed with aqueous formic acid (5 mL, 2 mmol/L), the target compound and the labeled standard were eluted and analyzed by LC-MS using the ions *m/z* 94 and 97 for 1-methylpyridinium and the labeled reference, respectively. An MS response factor of 0.99 was calculated from mixtures containing known amounts of the analyte and the labeled standard. As the mean of three analytical measurements, 390 mg/kg of dry weight have been determined in the beverage prepared from the roasted coffee beans investigated.

Quantification of 5-Chlorogenic Acid. Coffee powder (2 g) was filled in an extraction thimble (33 × 94 mm, Schleicher & Schuell, Dassel, Germany) and was then extracted with methanol/water (50:50, v/v; 150 mL) for 90 min using a Soxhlet apparatus. The solution was then quantitatively transferred into a volumetric flask (250 mL). After filtration (0.25 μm), the solution was analyzed by HPLC-DAD. The results of three different analyses were averaged. A solution of 5-chlorogenic acid in methanol was used as external standard for quantification.

Animal Feeding Experiments. Male Wistar rats (Wistar Unilever HsdCpb:WU) were obtained from Harlan-Winkelmann (Borchen, Germany). Adult animals weighing 326 ± 1.04 g were individually housed in metabolic cages and kept under standard conditions (12 h dark/light cycle; air temperature and relative humidity were adjusted at 20 ± 0.8 °C and 55 ± 2%, respectively). The animals were allowed free access to drinking water and were fed daily at 8:00 a.m. with an average of 30 ± 0.01 g of the diet per capita. The experiment comprised three randomized groups (n = 8 rats per group) that were fed either a standard chow (Altromin C1004 protein-free diet containing <1% protein, Altromin, Lage, Germany) supplemented with 2% cellulose (Bilaney Consultants, Düsseldorf, Germany) and 28% casein (New Zealand Milk Products, Rellingen, Germany) (control group) or an experimental diet, additionally supplemented with lyophilized decaffeinated coffee beverage or *N*-methylpyridinium as detailed in **Table 2**. Throughout the feeding period, body weight gain and food intake were calculated daily by weighing the animals and the remaining food, respectively. Feces and urine, collected into 0.3% sodium azide, were sampled daily, weighed, lyophilized, and stored at −80 °C. The

Table 2. Diet Composition (Percent) of the Animal Feeding Study

	control	coffee extract	*N*-methypyridinium
standard chow[a]	70	65.5	70
casein	28	28	28
cellulose	2	2	2
test compound		4.5	0.0328

[a] Standard chow: Altromin C1004 protein free (<1% protein).

experimental protocols and procedures were approved by the Animal Care and Use Committee at the University of Kiel.

After the feeding period of 15 days, the animals were fasted for 24 h and sacrificed by decapitation. Blood was collected into a heparinized tube and immediately centrifuged (3000*g*, 10 min). The resulting plasma and erythrocyte samples were stored at −80 °C. Livers and kidneys were removed rapidly, weighed, washed in ice-cold 0.9% NaCl, and stored at −80 °C. Prior to the analysis of the CCR and GST activities, the livers and kidneys were homogenized and centrifuged according to a standard procedure reported earlier (*26*).

Enzyme Activities. The enzyme activities of the CCR and GST were analyzed according to the procedure reported above for the Caco-2 cells. The enzyme activity of the UDP-GT was analyzed fluorometrically according to the method of Frei et al. (*32*). The data presented are given as percent enzyme activity change related to the mean enzyme activity analyzed for control animals. Liver enzyme activities measured in control animals for CCR, GST, and UDP-GT were 56.6 ± 18.8 nmol of cytochrome *c* × mg of protein^{-1} × min^{-1}, 335 ± 94 nmol of CDNB × mg of protein^{-1} × min^{-1}, and 26.3 ± 8.99 milliunits × mg of protein^{-1}, respectively.

Quantification of Tocopherol in Plasma Samples. Concentrations of tocopherol equivalents in plasma were determined by HPLC with UV−vis detection according to a method reported in the literature (*33*). Plasma samples (100 μL) were mixed with a mixture (40:10, v/v) of *n*-hexane and ethanol and centrifuged (3000*g*, 5 min). The *n*-hexane phase was then removed in vacuo, and the residue obtained was dissolved in a mixture (85:15, v/v; 700 μL) of methanol and dichloromethane. α- and γ-tocopherol were separated isocratically using methanol/dichloromethane (85:15, v/v) as mobile phase (flow rate = 0.8 mL/min) and a SpheriGROM ODS-1.5 μm analytical column (250 mm length, 4.6 mm internal diameter, 5 μm particle size, Grom, Herrenberg, Germany). Detection of α- and γ-tocopherols was performed by UV−vis (λ = 295 nm). Both tocopherol isomers were quantified by external standard calibration. Tocopherol equivalents were calculated according to the method of Jacob and Elmadfa (*33*), taking into account that the bioactivity of γ-tocopherol in vivo is ∼25% that of α-tocopherol (milligrams of α-tocopherol + milligrams of γ-tocopherol × 0.25 = milligrams of tocopherol equivalents).

Statistical Analysis. Statistical analyses were performed using Student's *t* test at a level of significance of $p < 0.05$.

Measurement of the CIE-Lab Color Space. Urine color was measured in CIE Lab space (*34*) by reflection spectrometry using a spectrocolor pen (Dr. Lange, Berlin, Germany). Data are reported as *L**, uniform lightness, and the chromaticity coordinates *a** (+red to −green) and *b** (+yellow to −blue). The results are given as triplicates. To exclude the confounding factor of urine dilution, urine samples were standardized for their creatinine content prior to analysis. Urine creatinine content was determined by using the Jaffé method (*35*).

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Bio-Tek Instruments, Eching, Germany) consisted of two pumps (type 422), a gradient mixer (M 800), a Rheodyne injector (100 μL loop), and a diode array detector (DAD type 540+) monitoring the effluent in a wavelength range between 210 and 500 nm. Separations were performed on a stainless steel column packed with RP-18 (C-18 Nucleosil 300 nm, 5 μm, Macherey Nagel, Düren, Germany) either in an analytical (4.6 × 240 mm, 1.6 mL/min) or in an semipreparative scale (10 × 250 mm, 3.0 mL/min). For the separation of the ultrafiltration fraction <1 kDa, a gradient was used starting with 100% aqueous trifluoroacetic acid (0.1% TFA in water), followed by increasing the methanol content to 100% within 60 min (flow = 1.6 mL/min). For the chlorogenic acid quantification, a gradient was used starting with a mixture (10:90, v/v) of acetonitrile and an aqueous phosphoric acid solution (1% phosphoric acid) and stepwise increasing the acetonitrile content to 20% within 20 min, then to 30% within 5 min, to 40% within 10 min, and, finally, to 40% within 5 min (flow = 1.0 mL/min).

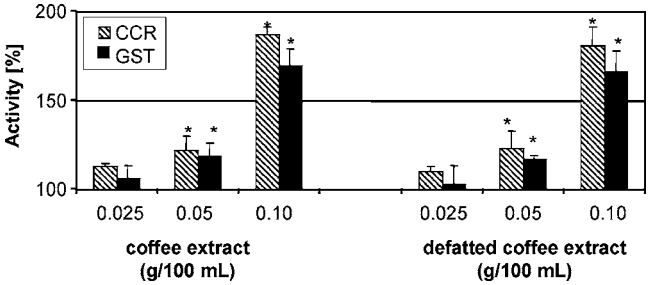

Figure 2. Effect of total and defatted coffee extract on the enzyme activity of NADPH–cytochrome *c* reductase (CCR) and glutathione *S*-transferase (GST) in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 0.025, 0.5, or 1.0 g of the individual fraction per 100 mL of cell culture medium for 72 h. Analyses were performed in triplicate, and the data obtained are given as means and standard deviations. Asterisks (∗) indicate that the enzyme activity of cells exposed to coffee fractions was significantly different from that of nonexposed control cells ($p < 0.05$).

Liquid Chromatography—Mass Spectrometry (LC-MS). An analytical HPLC column (Grom-Sil 80 SCX; 5 μm, 50 × 2 mm i.d., Grom) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI). After injection of the sample (50 μL), analysis was performed using isocratic conditions at a flow rate of 0.3 mL/min and using methanol/water (1:1, v/v) containing a final concentration of 50 mM ammonium acetate.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR spectroscopy was performed on an AMX 400 spectrometer (Bruker, Rheinstetten, Germany).

RESULTS AND DISCUSSION

Aimed at locating antioxidant and chemopreventive compounds in coffee beverage, a coffee brew was freshly prepared and fractionated using an activity-guided approach as depicted in **Figure 1**. In a first step, the coffee beverage, for which a dry weight of 13.81 g/L was determined after lyophilization, was defatted by triple extraction with petroleum ether. After the aqueous phase obtained had been freeze-dried and the solvent removed from the organic layer in vacuo, the yields of both fractions were determined by weight, yielding 13.57 g of hydrophilic coffee constituents and 0.24 g of coffee lipids (**Table 1**).

Antioxidant Activity of Coffee Fractions. To investigate the antioxidant activity of these solvent fractions, their efficacy in inhibiting the peroxidation of linoleic acid was measured in vitro using an antioxidant assay recently successfully applied to the antioxidants in bread crust (*16*). Using Trolox as the reference for a highly active antioxidant, the antioxidative potential was calculated as Trolox equivalents (TE values). As given in **Table 1,** the antioxidative potential of the total coffee beverage was estimated with a TE value of 4.02, closely followed by the defatted beverage, which showed a TE value of 3.93. In comparison, the coffee lipids showed a 10 times lower activity in inhibiting linoleic acid autoxidation; a TE value of only 0.38 has been determined (**Table 1**).

To investigate whether the defatted coffee beverage showed also chemopreventive activity in human intestinal cells, Caco-2 cells were exposed to lyophilized preparations of either the total or the defatted coffee beverage (**Figure 2**). Compared with nonexposed control cells, exposure to both coffee beverages

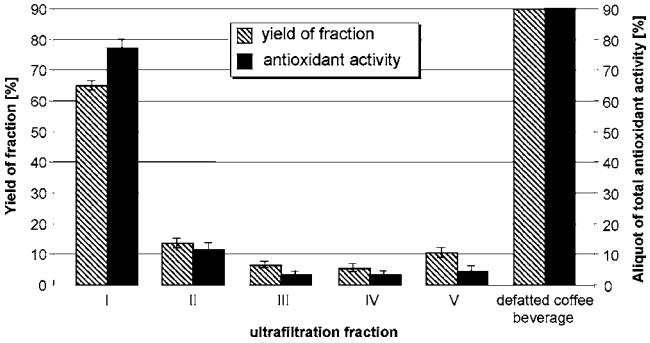

Figure 3. Yields and in vitro antioxidant activity of molecular weight fractions isolated from a freshly prepared coffee beverage by means of ultrafiltration. The molecular weight ranges of the different fractions obtained were <1 kDa (fraction I), 1–10 kDa (fraction II), 10–30 kDa (fraction III), 30–100 kDa (fraction IV), and >100 kDa (faction V). Experiments were performed in replicates. Error bars represent the range of data obtained for the individual measurements.

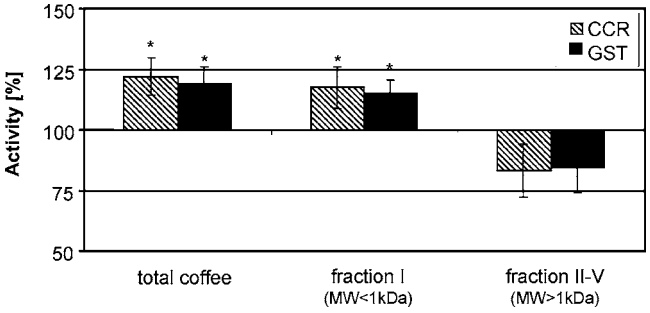

Figure 4. Effect of total coffee extract and two fractions isolated by ultrafiltration, <1 and >1 kDa, on the enzyme activity of CCR and GST in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 0.5 g of the individual fraction per 100 mL of cell culture medium for 72 h. Analyses were performed in triplicate, and the data obtained are given as means and standard deviations. Asterisks (*) indicate that the enzyme activity of cells exposed to coffee fractions was significantly different from that of nonexposed control cells ($p < 0.05$).

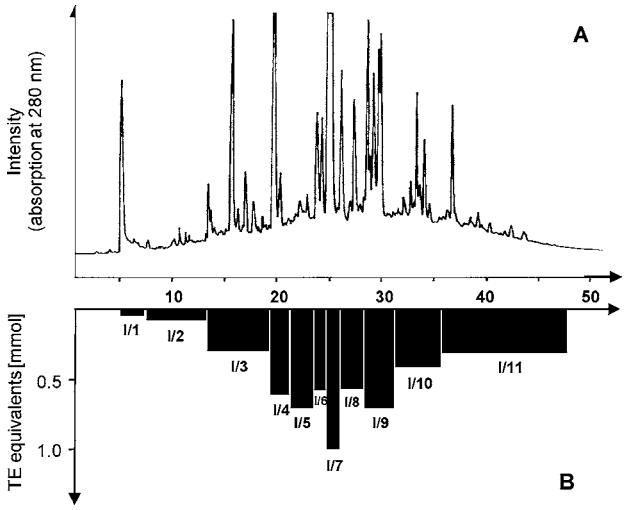

Figure 5. RP-HPLC chromatogram (upper part) and in vitro antioxidative potential of subfractions (lower part) obtained from the low molecular weight fraction I isolated from the coffee beverage.

resulted in a dose-dependent activation of phase I CCR and phase II GST activities. Similar to the results shown for the TE values, the effects observed for the defatted and the total coffee beverage were comparable, indicating that coffee lipids play a minor role in stimulating CCR and GST activities.

To gain further insights into the molecular weight of the coffee compounds causing the high chemopreventive activity in the hydrophilic fraction, the defatted coffee beverage was further fractionated by means of multistep ultrafiltration. Using membrane filters with stepwise decreasing molecular weight cutoffs, five fractions were obtained after freeze-drying containing water-soluble coffee compounds with molecular weights of <1 kDa (fraction I), 1−10 kDa (fraction II), 10−30 kDa (fraction III), 30−100 kDa (fraction IV), and >100 kDa (fraction V). The highest yield was determined for the low molecular weight compounds; for example, nearly 65% of the hydrophilic coffee compounds were found to have molecular weights below 1 kDa (**Figure 3**). Compounds showing molecular weights between 1 and 10 kDa were present in yields of 12%, whereas the yields of all of the other fractions were below 10%. In contrast to the distribution of the yields of the individual fractions, a significant increase in browning intensity was observed from the low molecular weight fraction I to the high molecular weight fraction V. To rate these fractions for their antioxidant activity and to establish their contribution to the overall activity for the total coffee beverage, the inhibitory effect of these individual fractions on the peroxidation of linoleic acid was measured by means of the in vitro antioxidant assay. By far the highest activity was found for the low molecular weight fraction I, for example, 78% of the overall antioxidant activity of the total coffee beverage was found in the low molecular weight fraction. Fraction II containing hydrophilic compounds with molecular weights between 1 and 10 kDa covered ~10% of the activity of the total defatted coffee, whereas the antioxidant activity of all the other fractions accounted for <5% (**Figure 3**). On the basis of these data, it can be concluded that, in particular, the hydrophilic, low molecular weight compounds are responsible for the antioxidant potential of the freshly brewed coffee beverage.

To check whether the low molecular weight fraction I, showing the highest antioxidant activity in vitro, may also act as an active compound in human intestinal cells, Caco-2 cells were exposed either to the lyophilized total coffee beverage, to fraction I containing the low molecular weight compounds with MW <1 kDa, or to a recombinate containing the high molecular weight fractions II−V in their authentic concentrations (**Figure 4**). After 72 h of exposure to the fraction below 1 kDa, both CCR and GST enzyme activities were increased as compared to nonexposed control cells, whereas exposure to the molecular weight compounds >1 kDa resulted in decreased enzyme activities. From these results, it is clear that the key chemopreventive compounds in coffee beverage show molecular weights below 1 kDa. Therefore, the following experimental step aimed to identify the major antioxidant and the most chemopreventive active compound in coffee beverage.

Identification of a Chemopreventive Compound. To locate the key player responsible for the phase I/II modulating activity of the low molecular weight coffee constituents, ultrafiltration fraction I was subfractionated by semipreparative HPLC using reverse phase material as the stationary phase, and the effluent was monitored using either a diode array detector or an LC-MS. The effluent was separated into 11 fractions (fractions I-1 to I-11, labeled in **Figure 5B**), which were separately collected in glass vials and freeze-dried. Aliquots of the residues obtained from the individual HPLC fractions were then used to determine their antioxidant activities by measuring their inhibitory effect on linoleic acid peroxidation (**Figure 5B**) as well as to determine their phase I/II modulating activity in Caco-2 cells. The in vitro antioxidant assay revealed the highest antioxidant activity for

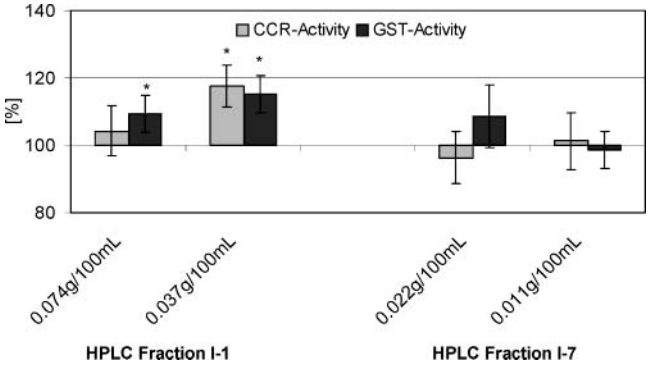

Figure 6. Effect of coffee extract fractions obtained by ultrafiltration and subsequent HPLC separation, fractions I-1 and I-7, on the enzyme activity of CCR and GST in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to yield proportional amounts of the individual fraction/compound per 100 mL of cell culture medium for 72 h. Analyses were performed in triplicate, and the data obtained are given as means and standard deviations. Asterisks (∗) indicate that the enzyme activity of cells exposed to coffee fractions was significantly different from that of nonexposed control cells ($p < 0.05$).

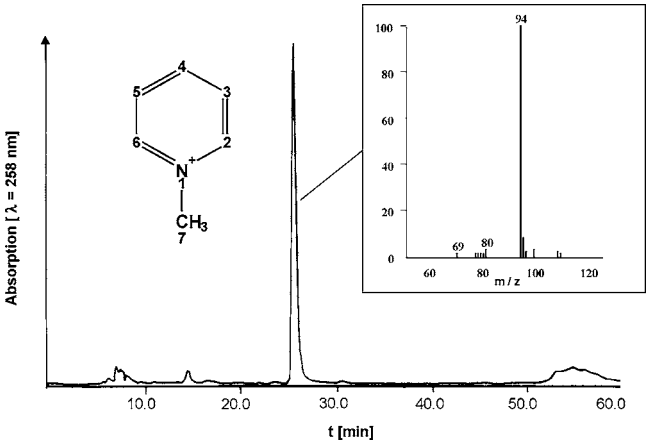

Figure 7. HILIC-HPLC separation of the RP-HPLC fraction I-1; LC-MS spectrum and chemical structure of the major compound.

fraction I-7 with a TE value of nearly 1.0. All of the other HPLC fractions were evaluated with significantly lower TE values. In particular, fractions I and II did not show any significant activity in inhibiting linoleic acid peroxidation in vitro (**Figure 5**). By comparison of the UV−vis absorption spectra as well as the LC-MS data with those observed for a reference compound, the compound evoking the antioxidant activity of fraction I-7 was unequivocally identified as 5-chlorogenic acid.

To investigate whether fraction I-7 showing the highest activity in inhibiting linoleic acid peroxidation in vitro exhibits also chemopreventive effects, Caco-2 cells were exposed to these 11 HPLC fractions at yield proportional concentrations for 72 h (data not shown). Surprisingly, by far the strongest effects on the enzyme activities of CCR and GST were found for fraction I-1. For comparison, the results of these measurements on fraction I-1 showing the highest activity in the cell culture experiment and on fraction I-7 showing the highest in vitro antioxidant activity are given in **Figure 6**). In contrast to the results obtained for fraction I-1, no statistical differences compared to nonexposed control cells were demonstrated for fraction I-7, which contained chlorogenic acid.

On the basis of the data obtained from this cell culture experiment, it may be concluded that the strongly hydrophilic fraction I-1, which is not retained on RP-18 material, contains a compound with strong GST-inducing activity. The following experiments were, therefore, aimed at locating this potentially chemopreventive agent in the polar HPLC fraction I-1. To achieve this, the polar compounds present in fraction I-1 were further separated by semipreparative hydrophilic liquid interaction chromatography (*36*) coupled to an LC-MS or a diode array detector monitoring the effluent at 258 nm. A major peak was eluted after 25.7 min, exhibiting a molecular weight of 94 Da and an absorption maximum at 258 nm (**Figure 7**). After that fraction had been collected from 15 HPLC runs, the compound isolated was analyzed by ^{1}H NMR spectroscopy. The signal resonating at 4.36 ppm and integrating for three protons indicated the presence of a strongly activated methyl group in the molecule. In addition, the resonance signals observed at 8.12, 8.58, and 9.00 ppm and integrating for five methine protons suggested the presence of an aromatic ring in the molecule. On the basis of these spectroscopic data, the structure of the target compound in coffee beverage was suggested as the *N*-methylpyridinium ion (**Figure 7**), which was recently identified as a trigonelline degradation product in hydrochloric acid extracts of roast coffee powders (*30*). The identification of this *N*-methylpyridinium ion was further confirmed by comparison of the chromatographic (HILIC-HPLC) and spectroscopic data with those obtained for the synthetic reference compound prepared by N-methylation of pyridine as reported recently (*31*). Quantification of *N*-methylpyridinium ions in the coffee beverage by means of a stable isotope dilution analysis revealed a concentration of 390 mg/kg of dry weight, corresponding to 5.39 mg/L of the standard coffee beverage.

In Vitro Antioxidant Activity and Phase I/II Enzyme Modulating Activity of Chlorogenic Acid and *N*-Methylpyridinium. To compare the antioxidant activity of *N*-methylpyridinium with that of chlorogenic acid and to determine their individual contributions to the overall antioxidant activity of the coffee beverage, aqueous solutions containing *N*-methylpyridinium (5.39 mg/L) or 5-chlorogenic acid (0.69 g/L) at the concentration at which they are present in the authentic coffee brew were used to measure their inhibitory activity on lipid peroxidation in vitro. The results revealed that only chlorogenic acid exhibited significant antioxidant activity in vitro. With a TE value of 0.46, the 5-chlorogenic acid accounted for ∼10% of the overall antioxidant activity of the coffee. In contrast, 1-methylpyridinium did not show any effect in inhibiting linoleic acid peroxidation (data not shown).

To test whether *N*-methylpyridinium is the active compound of fraction I-1, which is responsible for the phase I/II modulating activity in vitro, Caco-2 cells were exposed to *N*-methylpyridinium. Compared with nonexposed control cells, exposure of 0.025 g of *N*-methylpyridinium per 100 mL of cell culture medium resulted in an increased GST activity by 22.4 ± 3.79%, whereas the CCR activity decreased by 24.2 ± 6.67% (**Figure 8**). Exposure of Caco-2 cells to chlorogenic acid did not significantly affect the enzyme activity of CCR and GST.

Taken together, the results obtained from studies on intestinal Caco-2 cells demonstrate that *N*-methylpyridinium is a key compound present in coffee beverage that induces the chemopreventive GST activity in vitro. To verify this in vitro effect for an in vivo system, an animal feeding experiment on rats was performed.

In Vivo Antioxidant Activity and Phase I/II Enzyme Modulating Activity of Coffee Beverage and *N*-Methylpyridinium. The lyophilized coffee beverage prepared for the in vitro studies and *N*-methylpyridinium iodide were administered to male Wistar rats for 15 days. The diet composition of the

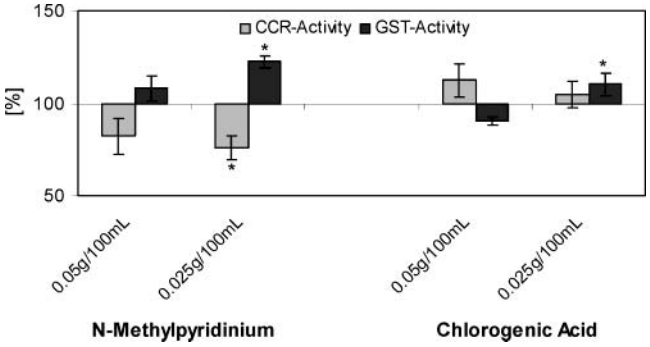

Figure 8. Effect of synthetic *N*-methylpyridinium and 5-chlorogenic acid on the enzyme activity of CCR and GST in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 0.025 and 0.5 g of the individual compound per 100 mL of cell culture medium for 72 h. Analyses were performed in triplicate, and the data obtained are given as means and standard deviations. Asterisks (∗) indicate that the enzyme activity of cells exposed to coffee fractions was significantly different from that of nonexposed control cells ($p < 0.05$).

experimental groups, control, coffee beverage, and *N*-methylpyridinium iodide, is given in **Table 2**. The administered dose of 4.5 wt % lyophilized coffee beverage is in the same range as reported by Hasegawa et al. (*37*) and Iba et al. (*38*), who also fed freeze-dried coffee brews to rats. The administered dose of *N*-methylpyridinium was basically calculated on the basis of its authentic content present in the coffee beverage, which was multiplied by a factor of 10 to initially investigate whether this compound exhibits a physiological effect in vivo. For chemopreventive compounds not having been investigated before, a higher dose is recommended to initially test enzyme modulating effects in vivo (*26*). With an average body weight of 350 ± 15 g and a daily intake of 23.5 ± 1.41 g of diet, the administered dose of lyophilized coffee beverage was ∼3.0 g per kg of body weight and day. The daily dose for *N*-methylpyridinium was calculated on the basis of an average body weight of 351 ± 19 g and a daily intake of 23.4 ± 2.47 g of diet, giving 22 mg of *N*-methylpyridinium per kg of body weight. The dietary intake and the body weight gain throughout the feeding period were not different among the experimental groups (data not shown). A higher content of nondigestible or less digestible browning products present in the coffee beverage diet resulted in slightly increased feces excretion (+ 14% vs controls, $p > 0.05$) and in increased cecum weights (+49% vs controls, $p < 0.05$) in animals of the coffee beverage group. Increased cecum weights, in particular, point to an altered microbial activity in the cecum, which was also observed by visually elevated internal gas production leading to substantial cecum inflation.

Urine excretion did not differ among the experimental groups, but urine color in animals fed the light brown coffee beverage diet differed in brightness and color from that of control animals (**Table 3**). Surprisingly, urine chromaticity coordinates *a** and *b** in animals fed the colorless *N*-methypyridinium diet were also different from that of control rats. These changes in urine color clearly indicate that either compounds present in the coffee beverage or the *N*-methylpyridinium, respectively, or their metabolites, produced by intestinal microbial activity, must have been absorbed.

Means of chemopreventive phase I/II enzyme activities in the liver and the kidneys as well as organ weights adjusted to body weight of animals fed one of the experimental diets did not differ statistically from those analyzed for animals fed the control diet (**Table 4**). However, in the liver, feeding of 4.5% coffee beverage resulted in an increase of phase II GST and UDP-GT activities by 24 and 40% compared to animals fed the control diet, respectively. Liver samples isolated from animals on the *N*-methylpyridinium diet even showed an elevated UDP-GT activity of 65% ($p = 0.08$). Plasma total antioxidant capacity, calculated on the basis of TE values, and plasma tocopherol equivalents were elevated in animals fed the coffee beverage and the *N*-methylpyridinium-containing diet (**Figure 9**). For the coffee beverage, the results demonstrating a strong antioxidant activity in vitro (**Figure 3**) were confirmed by the feeding study. Suprisingly, feeding of the *N*-methylpyridinium diet also resulted in an increased total antioxidant capacity in the plasma, although *N*-methylpyridinium did not show any antioxidant activity in vitro. The data indicate that the mode of action demonstrated for *N*-methylpyridinium in biological systems is different from that in foods. Conceivably, *N*-methylpyridinium iodide either is metabolized into a biologically active compound by intestinal microorganisms or exerts specific in vivo effects by binding to cell surface receptors. Any biochemical reaction of *N*-methylpyridinium seems to be questionable because of its chemically almost inert structure. The difference in urine color as well as the slightly increased phase II GST and UDP-GT activities in rat liver and the increased total antioxidant capacity in rat plasma support the hypothesis that the intake of *N*-methylpyridinium ions present in coffee beverage exerts biological effects in vivo.

A recently published study on pyridine demonstrated that not pyridine but rather chemically unidentified metabolites of pyridine formed by hepatic cells are physiologically active in inducing phase I CYP1A1 isoenzymes (*39*). These results suggest that *N*-methylpyridinium ions, and presumably other alkylpyridinium ion as well, enhance the expression and activity of chemopreventive phase I/II enzymes. This hypothesis is also supported by data obtained with other structurally related pyridine derivatives, such as picolines, which are also present in coffee beverages and are known to induce cytochrome P450 (*40*) and GST (*41*) isoenzymes.

Although it is still an open question as to whether *N*-methylpyridinium or its metabolites induce chemopreventive enzyme activities in vivo, the results of this study strongly

Table 3. Feces Excretion, Cecum Weights (in Percent of Body Weight), Urine Excretion, and Urine Color in Rats Fed Coffee Extract or *N*-Methylpyridinium for 15 Days

				urine color		
	feces (g/day)	cecum (%)	urine (g/day)	*a** value[b] (%)	*b** value[b] (%)	*L** value[c] (%)
control	7.49 ± 0.90	0.43 ± 0.04	15.7 ± 2.31	100 ± 10.2	100 ± 18.7	100 ± 9.7
coffee beverage	8.51 ± 0.38	0.64 ± 0.08[a]	19.9 ± 2.21	155 ± 17.5[a]	326 ± 33.4[a]	73.7 ± 11.6[a]
N-methylpyridinium	7.50 ± 0.72	0.48 ± 0.06	16.1 ± 3.54	166 ± 22.0[a]	75.4 ± 59.4	98.3 ± 14.8

[a] Student's *t* test: $p < 0.05$ vs control. [b] Urine chromaticity coordinates related to urine samples of controls after adjustment for creatinine. [c] Urine color brightness related to urine samples of controls after adjustment for creatinine.

Table 4. Liver and Kidney Enzyme Activity of the NADPH–Cytochrome *c* Reductase (CCR), the Glutathione *S*-Transferase (GST), and the UDP–Glucuronyl-transferase (UDP-GT) in Rats Fed Coffee Extract or *N*-Methylpyridinium for 15 Days[a]

	CCR[b] (%)	GST[b] (%)	UDP-GT[b] (%)
	Liver		
coffee beverage	93.1 ± 18.8	124 ± 16.9	140 ± 32.4
N-methylpyridinium	105 ± 20.3	103 ± 24.9	165 ± 46.7
	Kidney		
coffee beverage	108 ± 16.1	109 ± 12.9	nd
N-methylpyridinium	96.6 ± 10.8	115 ± 17.6	nd

[a] No statistical differences vs means of controls [b] Relative enzyme activity related to that of control animals. nd, not determined.

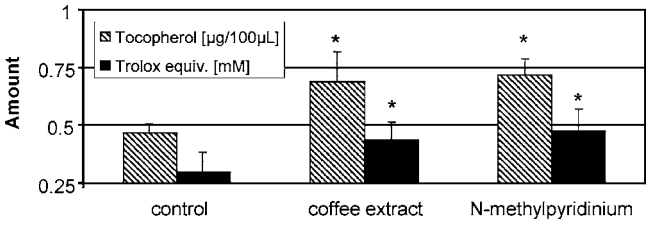

Figure 9. Content of α-tocopherol and Trolox equivalents in plasma of rats fed coffee extract or *N*-methylpyridinium for 15 days.

support the induction of chemopreventive phase II enzymes by dietary coffee beverages containing *N*-methylpyridinium. The molecular mechanism for this induction is hypothesized to be based on kinase-mediated reactions rather than binding to the arylhydrocarbon receptor (AhR) which is the main receptor involved in the phase I/II enzyme induction (*39*). Coffee beverage, in particular, has been recently demonstrated to induce MAP-kinase activation in two different cell lines, intestinal Caco-2 and renal HEK-293 cells (*42*). Further studies will be necessary to investigate the biochemical pathways by which either coffee-derived *N*-methylpyridinium or its metabolites induce chemopreventive enzymes in vivo.

ACKNOWLEDGMENT

We thank Kay Augustin and Dörthe Grothe for skillful technical assistance.

LITERATURE CITED

(1) USDA (U.S. Department of Agriculture), Horticultural and Tropical Products Division. Circular Series 2002; FTROP 4-02.
(2) Fredholm, B. B.; Battig, K.; Holmen, J.; Nehlig, A.; Zvartau, E. E. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* **1999**, *51*, 83−133.
(3) Parliament, T. H.; Ho, C. T.; Schieberle, P. *Caffeinated Beverages, Health Benefits, Physiological Effects, and Chemistry*; American Chemical Society: Washington, DC, 2000.
(4) Boekema, P. J.; Lo, B.; Samsom, M.; van Berge Henegouwen; G. P., Smout; A. J. P. G. Coffee and gastrointestinal functions: A review. *Scand. J. Gastroenterol.* **1999**, *34* S230, 35−39.
(5) Lam, L. K.; Sparnins, V. L.; Wattenberg, L. W. Effects of derivatives of kahweol and cafestol on the activity of glutathione *S*-transferase in mice. *J. Med. Chem.* **1987**, *30*, 1399−403.
(6) Kitts, D. D.; Wijewickreme, A. N. Effect of dietary caffeic and chlorogenic acids on in vivo xenobiotic enzyme systems. *Plant Foods Hum. Nutr.* **1994**, *45*, 287−98.
(7) Roginsky, V.; Barsukova, T. Chain-breaking antioxidant capability of some beverages as determined by the clark electrode technique. *J. Med. Food* **2001**, *4*, 219−229.
(8) Borelli, R. C.; Visconti, A.; Menella, C.; Anese, M.; Fogliano, V. Chemical characterization and antioxidant properties of coffee melanoidins. *J. Agric. Food Chem.* **2002**, *50*, 6527−6533.
(9) Natella, F.; Nardini, M.; Giannetti, I.; Dattilo, C.; Scaccini, C. Coffee drinking influences plasma antioxidant capacity in humans. *J. Agric. Food Chem.* **2002**, *50*, 6211−6216.
(10) Esposito, F.; Morisco, F.; Verde, V.; Ritieni, A.; Alezio, A.; Caporaso, N.; Fogliano, V. Moderate coffee consumption increases plasma glutathione but not homocysteine in healthy subjects. *Aliment. Pharmacol. Ther.* **2003**, *17*, 595−601.
(11) Nicoli, M. C.; Anese, M.; Manzocco, L.; Lerici, C. R. Antioxidant properties of coffee brews in relation to the roasting degree. *Lebensm. Wiss. Technol.* **1997**, *30*, 292−298.
(12) Ledl, F.; Schleicher, E. New aspects of the Maillard reaction in foods and in human body. *Angew. Chem., Int.* **1990**, *29*, 565−594.
(13) Hofmann, T.; Bors, W.; Stettmaier, K. Radical assisted melanoidin formation during thermal processing of food as well as under physiological conditions. *J. Agric. Food Chem.* **1999**, *47*, 391−396.
(14) Hofmann, T. Structure, color and formation of low and high molecular weight products formed by food-related Maillard-type reactions. In *Chemistry and Physiology of Food Colors*; Ames, J., Hofmann, T., Eds.; ACS Symposium Series 775; American Chemical Society: Washington, DC, 2001; pp 134−151.
(15) Hofmann, T.; Schieberle, P. Chemical interactions between odor-active thiols and melanoidins involved in the aroma staling of coffee beverages. *J. Agric. Food Chem.* **2002**, *50*, 319−326.
(16) Lindenmeier, M.; Faist, V.; Hofmann, T. Pronylation of proteins—a novel protein modification in bread crust showing in vitro antioxidative and phase I/II-enzyme modulating activity. *J. Agric. Food Chem.* **2002**, *50*, 6997−7006.
(17) Wilkinson, J.; Clapper, M. L. Detoxication enzymes and chemoprevention. *Proc. Soc. Exp. Biol. Med.* **1997**, *216*, 192−200.
(18) Prochaska, H. J.; Talalay, P. Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res.* **1988**, *48*, 4776−4782.
(19) Peters, W. H. N.; Roelofs, H. M. J. Time-dependent activity and expression of glutathione *S*-transferases in the human colon adenocarcinoma cell line Caco-2. *Biochem. J.* **1989**, *264*, 613−616.
(20) Schmiedlin-Ren, P.; Thummel, K. E.; Fisher, J. M.; Paine, M. F.; Lown, K. S.; Watkins, P. B. Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1α,25-dihydroxyvitamin D3. *Mol. Pharmacol.* **1997**, *51*, 741−754.
(21) Lam, L. K.; Sparnins, V. L.; Wattenberg, L. W. Isolation and identification of kahweol palmitate and cafestol palmitate as active constituents of green coffee beans that enhance glutathione *S*-transferase activity in the mouse. *Cancer Res.* **1982**, *42*, 1193−1198.
(22) Huber, W. W.; Prustomersky, S.; Delbanco, E., Uhl, M.; Scharf, G.; Turesky, R. J.; Their, R.; Schulte-Hermann, R. Enhancement of the chemoprotective enzymes glucuronosyl transferase and glutathione transferase in specific organs of the rat by the coffee components kahweol and cafestol. *Arch. Toxicol.* **2002**, *76*, 209−217.
(23) Abraham, S. K.; Singh, S. P. Anti-genotoxicity and glutathione *S*-transferase activity in mice pretreated with caffeinated and decaffeinated coffee. *Food Chem. Toxicol.* **1999**, *37*, 733−739.
(24) Faist, V.; Erbersdobler, H. F. Metabolic transit and in vivo effects of melanoidins and precursor compounds deriving from the Maillard reaction. *Ann. Nutr. Metab.* **2001**, *45*, 1−12.
(25) Faist, V.; Lindenmeier, M.; Geisler, C.; Erbersdobler, H. F.; Hofmann, T. Influence of molecular weight fractions isolated from roasted malt on the enzyme activities of NADPH−cytochrome *c*−reductase and glutathione-*S*-transferase in Caco-2 Cells. *J. Agric. Food Chem.* **2002**, *50*, 602−606.
(26) Wenzel, E.; Tasto, S.; Erbersdobler, H. F.; Faist, V. Effect of heat treated proteins on selected parameters of the biotransformation system in the rat. *Ann. Nutr. Metab.* **2002**, *46*, 9−16.

(27) Habig, W. H.; Pabst, M. J.; Jakoby, W. B. Glutathione *S*-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **1974**, *249*, 7130−7139.
(28) Masters, B. S. S.; Williams, C. H.; Konnin, H. The preparation and properties of microsomal TPNH cytochrome *c* reductase from pig liver. In *Methods in Enzymology*; Eastabroook, R. W., Pullman, M. E., Eds.; Academic Press: New York, 1967; Vol. 10.
(29) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(30) Stadler, R. H.; Varga, N.; Hau, J.; Vera, F. A.; Welti, D. H. Alkylpyridiniums. 1. Formation in model systems via thermal degradation of trigonelline. *J. Agric. Food Chem.* **2002**, *50*, 1192−1199.
(31) Stadler, R. H.; Varga, N.; Milo, C.; Schilter, B.; Vera, F. A.; Welti, D. H. Alkylpyridiniums. 2. Isolation and quantification in roasted and ground coffees. *J. Agric. Food Chem.* **2002**, *50*, 1200−1206.
(32) Frei, J.; Schmid, E.; Birchmeier, H. *Methoden der enzymatischen Analyse-Bd. 1, 2. Aufl*; Bergmeyer, H. U., Ed.; Verlag Chemie: Weinheim, Germany, 1970.
(33) Jakob, E.; Elmadfa, I. Rapid HPLC assay for the assessment of vitamin K1, A, E and β-carotene status in children (7−19 years). *Int. J. Vit. Nutr. Res.* **1995**, *65*, 31−35.
(34) Hunt, R. G. W. *Measuring Colour*, 2nd ed.; Ellis Horwood: London, U.K., 1995.
(35) Siedel, J.; Möllering, H.; Ziegenhorn, J. Sensitive color reagent for the determination of creatinine. *Clin. Chem.* **1984**, *30*, 968.
(36) Strege, M. A. Hydrophilic interaction chromatography−Electrospray mass spectrometry analysis of polar compounds for natural product drug discovery. *Anal. Chem.* **1998**, *70*, 2439−2445.
(37) Hasegawa, R.; Ogiso, T.; Imaida, K.; Shirai, T.; Ito, N. Analysis of the potential +carcinogenicity of coffee and its related compounds in a medium-term liver bioassay of rats. *Food Chem Toxicol.* **1995**, *33*, 15−20.
(38) Turesky, R. J.; Richoz, J.; Constable, A.; Curtis, K. D.; Dingley, K. H.; Turteltaub, K. W. The effects of coffee on enzymes involved in metabolism of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in rats. *Chem.−Biol. Interact.* **2003**, *145*, 251−65.
(39) Iba, M. M.; Nguyen, T.; Fung, J. CYP1A1 induction by pyridine and its metabolites in HepG2 cells. *Arch. Biochem. Biophys.* **2002**, *404*, 326−3341.
(40) Murray, M.; Sefton, R. M.; Martini, R.; Butler, A. M. Induction of cytochromes P450 B2 and 2E1 in rat liver by isomeric picoline *N*-oxides. *Toxicol. Lett*. **1997**, *93*, 195−203.
(41) Dierckx, P. J. The influence of picolines on glutathione transferase activity and subunit composition in human liver derived HepG2 cells. *Biochem. Pharmacol.* **1994**, *48*, 1976−1978.
(42) Zill, H.; Bek, S.; Hofmann, T.; Huber, J.; Frank, O.; Lindenmeier, M.; Weigle, B.; Erbersdobler, H. F.; Scheidler, S.; Busch, A. E.; Faist, V. RAGE-mediated MAPK activation by food-derived AGE and non-AGE products. *Biochem. Biophys. Res. Commun.* **2003**, *300*, 311−315.

Received for review July 10, 2003. Revised manuscript received August 27, 2003. Accepted August 31, 2003. This research project was supported in part by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn, Germany) the AiF and the Ministry of Economics and Technology (Project AIF-FV 12403 N). We thank the European Commission for supporting the research within the framework of the European Union COST Action 919 "Melanoidins in Food and Health".

JF034750E