JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 11653–11660 **11653**

Isolation of High Molecular Weight Components and Contribution to the Protective Activity of Coffee against Lipid Peroxidation in a Rat Liver Microsome System

MARIA DAGLIA,[†] ADELE PAPETTI,[†] CAMILLA ACETI,[†] BARBARA SORDELLI,[†] CESARINA GREGOTTI,[‡] AND GABRIELLA GAZZANI*,[†]

Department of Pharmaceutical Chemistry, School of Pharmacy, University of Pavia, Via Taramelli 12, and Department of Internal Medicine and Therapeutics, School of Medicine, University of Pavia, Piazza Botta 10, 27100 Pavia, Italy

One of the most extensively studied and best-established properties of coffee is its antioxidant activity. We have shown that coffee brew has the ability to inhibit lipid peroxidation completely in a rat liver microsome biological system. The inhibitory activity was mainly due to the high molecular weight (HMW) fraction; this consisted of five components that were isolated, purified, and seen to occur in different amounts in the brew. Each component had different spectra and element compositions, although they all contained nitrogen. HMW, nitrogen content, and brown color enabled three components to be attributed to the melanoidin family; the two nonbrown components could not be considered as melanoidins. Each melanoidin and nonmelanoidin component contributes to a different extent to the protective action exerted by coffee brew. None of the isolated components completely inhibited microsomal lipid peroxidation alone, suggesting that each acts at different sites and/or possesses different mechanisms of action. The protective activity of coffee brew is thus underpinned by the antiradical properties, reducing power, and metal chelating ability of the individual components, each contributing to a different extent.

KEYWORDS: Roasted coffee; antiradical and antioxidant activity; mechanism of action

INTRODUCTION

Epidemiological studies have shown that moderate coffee consumption has positive effects on human health (*1, 2*). Recent research has also documented potential cardiovascular benefits related to NO bioavailability and to low-density lipoprotein oxidation, via antioxidant mechanisms, as well as a blood pressure-lowering action (*3*). Coffee has protective effects against several chronic diseases, including colorectal cancer, type 2 diabetes, and Parkinson's disease (*4, 5*). A number of studies have documented a positive action on liver disorders such as cirrhosis and hepatocellular carcinoma (*6, 7*). Such positive effects may be related to naturally occurring components in green beans or to compounds induced by the roasting process, where the high temperature and the low water activity of coffee beans promote the Maillard reaction, with the formation of hundreds of compounds. Such compounds are responsible for both the organoleptic properties and the biological activities of coffee brew.

Little and largely generic information is available on the chemical structure of the high molecular weight (HMW) components of coffee, which greatly influence biological properties, since these compounds easily decompose and polymerize in the last stage of the Maillard reaction. There is increasing evidence that polysaccharides (galactomannan-like and arabinogalactan-like carbohydrates) (*8*) and protein moieties are incorporated into the polymeric chains as are phenolic compounds linked both covalently (*9*) and noncovalently (*10*). The variety of compounds that can form in relation to several reactants and reaction conditions has led HMW components to be generically indicated as coffee melanoidins (CMs), brown-colored nitrogen-containing anionic polymers (*9*).

One of the most extensively studied and best-established properties of coffee is its antioxidant activity, related to natural phenolic compounds such as chlorogenic acids and to Maillard reaction products like melanoidins, reductones, furan, pyrroles, and maltol (*11, 12*). The antioxidant properties of CMs, which account for up to 25% of coffee brew dry matter (*9, 10*), have been documented in chemical and biological assays. They have high radical scavenging capacity toward 2,2-diphenyl-picryl-hydrazyl radical (DPPH$^{\bullet}$), *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD$^{\bullet+}$), and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic

* To whom correspondence should be addressed. Tel: +39 0382 987 373. Fax: +39 0382 422 975. E-mail: gabriella.gazzani@unipv.it.
[†] Department of Pharmaceutical Chemistry.
[‡] Department of Internal Medicine and Therapeutics.

10.1021/jf802018c CCC: $40.75
Published on Web 11/19/2008

acid) ($ABTS^{\bullet+}$), and medium/low activity toward hydroxyl radicals in the oxygen-radical absorbance capacity (ORAC) assay and in the deoxyribose assay. CMs can also prevent linoleic acid peroxidation in different model systems (*10*) and induce the reduction of ferric ion in the ferric reducing/antioxidant potential (FRAP) assay (*13*−*18*). More recently, CMs have been found to exert a protective action on a human hepatoma HepG2 cell model against oxidative stress induced by *tert*-butylhydroperoxide, modulating reduced GSH concentration, MDA production, and antioxidant enzyme activity (*19, 20*).

In a previous study, we documented the in vitro antiperoxyl radical activity (AA) and ex vivo protective activity (PA) of coffee brew against microsomal lipid peroxidation in rat hepatocytes; these properties are mainly due to the HMW components induced by the Maillard reaction and are influenced by plant species and degree of roasting (*20*). The aims of the present paper are (1) to isolate the individual HMW components occurring in coffee brew responsible for the ex vivo PA showed by coffee, (2) to assess the contribution to the overall activity, and (3) to explore the relevant mechanisms of action of each isolated component.

MATERIALS AND METHODS

Reagents and Chemicals. All chemical reagents were analytical grade; solvents (methanol and formic acid) were high-performance liquid chromatography (HPLC) grade. All were purchased from Sigma-Aldrich (St. Louis, MO).

Coffee Brew Preparation. Green *C. robusta* beans from Java were roasted in a pilot roaster apparatus (STA Impianti S.r.l., Bologna, Italy). The degree of roasting was measured by the weight lost due to vapor formation and cell fragment loss. Weight loss was about 12%, corresponding to a medium degree of roasting. Roasted coffee beans were ground in a laboratory scale mill and sieved through a no. 30 sieve. Coffee extract (CR) was then obtained by the coffee brewing procedure commonly used in Italy. Briefly, 6 g of roasted coffee powder was boiled for 10 min in 100 mL of Millipore grade water (Millipore Corp., Billerica, MA). The extract (100 mL) was filtered through a 0.45 μm Millipore membrane of cellulose acetate/cellulose nitrate mixed esters and subdivided into three aliquots: One was subjected to chemical analysis and pH determination, the second was freeze-dried for dry matter determination, and the third was used in the biological lipid peroxidation assay.

Dialysis. Dialysis was performed using a Spectra/Por Biotech cellulose ester membrane (Spectrum Europe B.V., Breda, The Netherlands) with a molecular weight cutoff (MWCO) of 3500 Da. Aliquots (10 mL) of coffee brew were fractionated by dialysis in 1000 mL of Millipore grade water for 6 h at 4 °C. Dialysate and retentate were freeze-dried; dry residues were determined and then dissolved in 10 mL of Millipore grade water. Recovered 5-*O*-caffeoylquinic acid (5-*O*-CQA) (>98%) was used as a standard molecular weight (MW) marker. Dialysate and retentate were reconstituted to the initial volume by rotary evaporation of coffee brew and were subjected to pH determination, to the biological lipid peroxidation assay, and to in vitro assays.

Gel Filtration Chromatography (GFC). All experiments were performed using a 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a gradient quaternary pump, and a diode array detector (DAD) system. The Agilent Chemstation software was used for HPLC system control and data processing. A Superformance Universal glass-cartridge system (300 mm × 10 mm) (Merck, Darmstadt, Germany) was used for GFC separation of the coffee brew retentate (MW > 3500 Da). The stationary phase was TSK gel Toyopearl HW-40F (exclusion limits 100−10000 Da). The mobile phase was Millipore grade water at a flow rate of 0.5 mL min^{-1}, with an injected volume of 1 mL. UV spectra were recorded in the 190−600 nm range, and chromatograms were acquired at 280, 324, and 420 nm. GFC fractions were freeze-dried; dry residues were determined, and elemental analysis was carried out. Fractions were reconstituted to the initial volume by rotary evaporation of coffee brew retentate, and pH values were determined. They were then subjected to the biological lipid peroxidation assay and to in vitro assays.

Biological Lipid Peroxidation Assay (Percentage of PA). Liver microsomes were obtained from male Wistar rats weighing 200−250 g, according to a modified version (*21*) of the method of Horie et al. (*22*). Microsomal pellets were suspended in 0.1 M sodium phosphate buffer (PB), pH 7.4 (control), in the coffee solutions (sample), in Trolox C solution (300 μM), and in 5-*O*-CQA solution (1.6 μM) to obtain total volumes of 6 mL. A 0.1 mL aliquot of each suspension was immediately used for determination of microsomal proteins (*23*).

The remaining preparations were added with NaCl (1 mL, 140 μM), ethylenediaminetetraacetic acid (EDTA) (1 mL, 50 μM), and sodium PB (1 mL, 0.1 M, pH 7.4) and then subdivided into two 4 mL aliquots. Test tubes containing samples were stoppered, and N_2 was bubbled through the solutions at 37 °C for 15 min to obtain anaerobic conditions for the subsequent induction of lipid peroxidation. One test tube was added with NADP (0.5 mL, 250 μM) and CCl_4/EtOH (20 μL, 50% v/v) and the other with an equivalent amount of buffer. Both samples were then placed in a shaking water bath at 37 °C for 30 min, and equal volumes of 30% trichloroacetic acid (TCA) at 0 °C and 0.75% thiobarbituric acid (TBA) were added.

The reaction mixtures were heated in boiling water for 15 min, kept in ice for 5 min, and then centrifuged at 3000 rpm for 10 min to separate corpuscolate particles. Supernatant absorbance was read in a spectrophotometer (λ = 545 nm) using the second series of samples without coenzymes, to bring the spectrophotometer to zero and account for any interference from the color and TBA-reacting substances (TBA-RSs) that are naturally found in coffee solutions.

PA was expressed as the percent decrease in TBA-RSs relative to the control solution according to the equation

$$\text{PA } \% = (a - b)/a \times 100$$

where a is TBA-RSs in the control sample and b is TBA-RSs in the sample.

Reverse-Phase (RP) HPLC with DAD Analysis for 5-*O*-CQA and Determination of Chlorogenic Acid Derivatives. Determinations were carried out using a 250 mm × 4.6 mm, 5 μm, C18 Hypersil column (CPS Analitica, Milan, Italy) with matching Lichrospher 100 RP-18, 5 mm guard column (Merck). Chromatographic conditions for gradient elution were as follows: flow rate, 1 mL/min; volume injected, 20 μL; and column temperature, 20 °C. UV spectra were recorded in the 190−600 nm range, and chromatograms were acquired at 324 nm. Separations were performed using a gradient of increasing methanol concentrations in water acidified (pH 3.00 ± 0.01) with 0.1% formic acid (v/v), as follows: 10 min at 5% methanol, 10 min linear gradient from 10 to 30% methanol, 30 min linear gradient from 30 to 45% methanol, and 10 min linear gradient from 45 to 80% methanol. The composition of the mobile phase was brought back to the initial condition (5% methanol) in 5 min, and the column was equilibrated for 5 min before the next injection. The retention time and UV spectrum of the 5-*O*-CQA standard solution were used to identify 5-*O*-CQA and chlorogenic acid derivatives. A stock solution of 5-*O*-CQA standard was prepared by dissolving carefully weighed amounts of the standard compound in 50% (v/v) methanol−Millipore grade water. The standard solution was diluted with the mobile phase to five final concentrations that ranged from 50 to 250 mg/mL. Each concentration was analyzed in triplicate. Quantification of individual compounds was performed by the external standard method using a five-point regression curve.

$DPPH^{\bullet}$ Assay (Percentage of Antiradical Activity, ARA %). ARA was determined using $DPPH^{\bullet}$ as a free radical (*24*). Aliquots (100 μL) of coffee brew retentate, of GFC fraction solutions, or of their diluted solutions (sample) or of KH_2PO_4/NaOH buffer (pH 7.4) (control) were added to 3.9 mL of a DPPH solution containing 6×10^5 mol L^{-1} methanol/KH_2PO_4/NaOH buffer (50:50 v/v). The decrease in absorbance was determined at 515 nm when the reaction reached a steady state (after 20 min).

The percent scavenger activity (ARA %) against $DPPH^{\bullet}$ was calculated according to the equation

$$\text{ARA } \% = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (1)$$

The scavenger activity of Trolox C methanol solution, assayed at a final concentration of 250 μM, was also determined.

Linoleic Acid-β-Carotene Assay (Percentage of AA). The antioxidant activities of the coffee brew retentate and of GFC fraction solutions were determined according to a modified version (*21*) of the method of Taga et al. (*25*), which is based on the coupled oxidation of linoleic acid and β-carotene. In brief, 5 mg of β-carotene was dissolved in 50 mL of chloroform solution. A 3 mL aliquot of the solution was added to a conical flask along with 40 mg of linoleic acid and 400 mg of Tween 20. The chloroform was evaporated until dry under reduced pressure at low temperature (<30 °C). Millipore grade water (100 mL) was added to the dried residue and shaken. Two aliquots (100 μL) of each coffee solution were added to 5 mL of β-carotene emulsion in test tubes and thoroughly mixed (samples). In preliminary tests, the addition of coffee solutions produced no significant change in pH. In one sample, the absorbance was measured immediately with the spectrophotometer set at 470 nm; in the other sample, it was measured after 5, 10, and 20 min of incubation in a water bath at 50 °C. Each sample was read against an emulsion prepared as described but without β-carotene (blank). An aliquot of each coffee solution was added to 5 mL of blank (blank sample) to account for the influence of the color of the coffee solution on the calculation of the degradation rate (dr) of β-carotene. The mixtures were read spectrophotometrically, and the absorbance was subtracted from that of the corresponding sample. The dr of β-carotene was calculated assuming first-order kinetics:

$$[\ln(A_0/A_t)]/t = \text{dr of sample} \quad (2)$$

where A_0 = absorbance of the sample − absorbance of the blank sample at time 0 (read immediately after addition of the coffee solutions), A_t = absorbance of the sample − absorbance of the blank sample at time t (t = 5, 10, and 20 min of incubation in a water bath at 50 °C).

$$[\ln(a_0/a_t)]/t = \text{dr of control sample} \quad (3)$$

where 100 μL of Millipore grade water was added to 5 mL of β-carotene emulsion and treated as the corresponding sample; a_0 = absorbance of the sample − absorbance of the blank sample at time 0; and a_t = absorbance of the sample − absorbance of the blank sample at time t.

AA was expressed as percentage of inhibition relative to the control according to the equation:

$$\text{AA } \% = \frac{\text{dr}_{\text{control sample}} - \text{dr}_{\text{sample}}}{\text{dr}_{\text{control sample}}} \times 100$$

Trolox C solution (200 μM) was also assayed for AA.

Determination of Reducing Activity (RA). The reducing power of coffee brew retentate and of the GFC fraction solutions was determined according to Daglia et al. (*21*). The reducing substances were expressed as the concentration (μg/mL) of an ascorbic acid solution, used as standard, having the same reducing power.

Determination of Percentage of Iron Chelating Activity (ICA %). The percent chelating activity of the coffee brew retentate and GFC fraction solutions on Fe^{2+} was measured according to Yen et al. (*26*), by adding 50 μL of $FeCl_2 \cdot 4H_2O$ (2.0 mM) to 200 μL of the retentate and of each GFC solution. The reaction was initiated by the addition of 200 μL of ferrozine (5 mM) and finally brought to 1500 μL with methanol. Reaction mixtures were read spectrophotometrically at 562 nm after reaching equilibrium (10 min). EDTA was used as the chelating standard compound (positive control); samples containing neither coffee solution nor EDTA were used as negative controls.

Total Polyphenol Content. Total GFC fraction polyphenol content was determined with the Folin−Ciocalteu reagent. In brief, 500 μL of Folin−Ciocalteu reagent was added to 100 μL of each GFC fraction, mixed, and added with 2000 μL of a 15% Na_2CO_3 solution and Millipore grade water to a volume 10 mL. After mixing and waiting 2 h, the mixtures were read spectrophotometrically at 750 nm. Chlorogenic acid was used as the phenolic standard compound (*16*). Values are means of four independent experiments.

Table 1. Dry Matter Content of Coffee Brew, Retentate (MW > 3500 Da), and Dialysate (MW < 3500 Da), pH Values, and Hydroxycinnamic Acid Derivative Content

sample	dry matter (mg/mL)	pH	hydroxycinnamic acid derivative (mg/mL)
coffee brew	12.46 ± 0.11	5.88 ± 0.13	1.68 ± 0.04
dialysate	9.47 ± 0.17	5.93 ± 0.46	1.64 ± 0.09
retentate	2.96 ± 0.03	5.76 ± 0.31	0.03 ± 0.00

Amino Acid Analysis. Amino acid analysis of the GFC fractions was performed with an HP Amino Quant system series II 1090 L connected to an HP Kayak P III 500 MHz elaborator. The weight GFC fractions were freeze-dried in sterile vials and then submitted to hydrochloric acid hydrolysis by HCl 6 M in an inert atmosphere (under N_2). The hydrolysis was performed for 24 h at 110 °C; the materials obtained were resuspended in 100 mM HCl and analyzed. The amino acids were derivatized with *o*-phthalaldehyde (OPA) and fluorenylmethyl chloroformate (FMOC) reagents, which created a molecule-absorbing light radiation at 338 and 262 nm, respectively. The amino acids were then separated in RP-HPLC and identified according to a chromatogram obtained using standard amino acids. The C18 column was equilibrated by two mobile phases: the mobile phase for channel A was a 20 mM sodium acetate buffer containing 0.018% (v/v) triethylamine (TEA), adjusted to pH 7.2 and added with 0.3% (v/v) tetrahydrofuran (THF); the mobile phase for channel B was 20% 100 mM sodium acetate buffer, adjusted to pH 7.2, 40% acetonitrile, and 40% methanol.

Analysis of Ionically Bound Phenolic Compounds. GFC fractions were analyzed for ionically bound phenolic compounds using the method of Delgado-Andrade et al., with some modifications (*17*). In brief, each GFC fraction solution was added with 10 mL of 2 M NaCl, incubated overnight, and subjected to dialysis (through a 1000 Da MWCO membrane) in 1000 mL of Millipore grade water for 6 h at 4 °C. Dialysate and retentate were freeze-dried, dissolved in 10 mL of Millipore grade water, and analyzed by RP-HPLC-DAD to determine 5-*O*-CQA and chlorogenic acid derivatives, as described above.

RESULTS AND DISCUSSION

Coffee brew was fractionated by dialysis, a common membrane procedure applied to isolate CMs, through a membrane with a nominal MWCO of 3500 Da. Whole coffee brew, dialysate, and retentate were freeze-dried; dry residues were weighed and then brought back to the initial volume. After pH determination, solutions were subjected to RP-HPLC-DAD analysis for hydroxycinnamic acid derivative content. Dry matter, pH, and total hydroxycinnamic acid isomer content (expressed as 5-*O*-CQA) of coffee brew, dialysate, and retentate are reported in **Table 1**. They demonstrate that the chlorogenic acid content of roasted coffee is in line with the literature and that these low molecular weight (LMW) components (<3500 Da) were efficiently separated in the dialysate. The HMW fraction (>3500 Da) isolated in the retentate accounted for 24% of dry matter and showed acidic feature, also in line with the literature (*9, 10, 15*).

The antioxidant ability of coffee brew and of the HMW and LMW fractions was evaluated ex vivo in a biological system consisting of rat hepatocyte microsomal membranes, where oxidative damage was induced by CCl_4 (**Figure 1**). Antioxidant activity, expressed as PA % against lipid peroxidation, was evaluated as the percent decrease in the TBA-RSs formed in samples as compared with controls. The addition of coffee brew and of the retentate at the same concentration found in coffee brew to the microsome system produced no TBA-RSs, demonstrating their ability to inhibit lipid peroxidation completely (PA = 100%), even when induced by CCl_4. Trolox C solutions used as standards failed to prevent lipid peroxidation completely

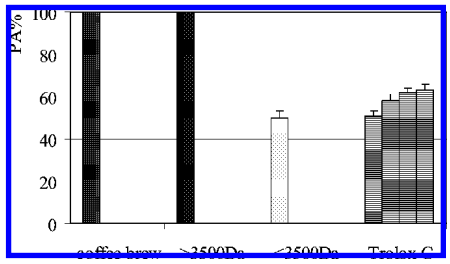

Figure 1. PA % of coffee brew, retentate (>3500 Da), dialysate (<3500 Da), Trolox C solution (100, 200, 250, and 300 μM), and 5-*O*-CQA standard solution (1.60 μM). Values are means of four independent experiments.

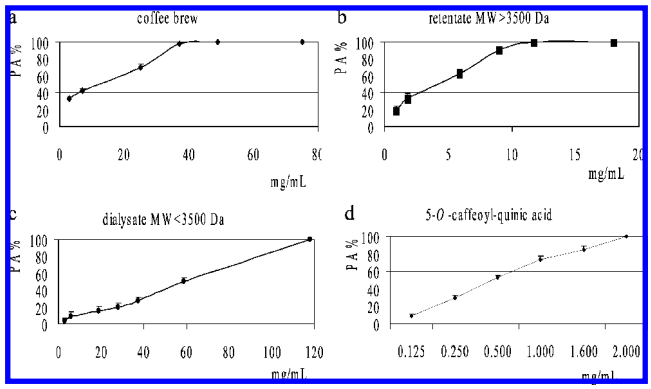

Figure 2. Percentage of protective (PA %) vs concentration (mg dry matter/mL) of coffee brew (**a**), retentate MW > 3500 Da (**b**), dialysate MW < 3500 Da (**c**), and 5-*O*-CQA standard solution (**d**). Values are means of four independent experiments.

(PA = 63%), even at very high concentrations (250−300 μM). The dialysate was less active (PA = 52%). Because the dialysate contains all of the hydroxycinnamic acid derivatives of coffee brew, we tested a 5-*O*-CQA standard solution at the overall concentration of hydroxycinnamic acid isomers occurring in the dialysate and in coffee brew (**Table 1**). Its activity was greater (PA = 72%) than that of the dialysate, suggesting that the other LMW compounds of coffee do not contribute to the dialysate's antioxidant activity and that they interfere with the activity of chlorogenic acid derivatives or else that 5-*O*-CQA is more active than the chlorogenic isomers occurring in coffee. Nevertheless, at higher concentrations, both the dialysate and the 5-*O*-CQA standard solution reached PA = 100%, showing a dose−response relation (**Figure 2c**,**d**). A dose−response relationship was seen for coffee brew and the retentate only with highly diluted solutions (**Figure 2a**,**b**), confirming that the PA of coffee brew is mainly due to the HMW fraction and that the content in antioxidant components of both brew and retentate actually exceeds the requirements for total protection against lipid peroxidation.

The HMW fraction was further studied. It was resolved by the GFC technique using TSK gel as column material and by recording chromatograms at three different wavelengths (280, 324, and 420 nm). The 280 and 324 nm GFC chromatograms exhibited five peaks (**Figure 3a**,**b**), whereas the one obtained at 420 nm (**Figure 3c**) displayed three peaks, indicating that only three GFC fractions (GFC1, GFC2, and GFC4) are brown-colored. Fractions were further purified by running a new GFC cycle using the same procedure. Analysis of the UV−Vis spectra obtained at the beginning, at the maximum, and at the end of the chromatographic peak of each fraction confirmed the purity of GFC fractions. The first fraction had the same retention time as blue dextran, used as a MW standard to evaluate dead volume, and consisted of an unretained compound. The other fractions had higher retention times. However, such increased retention times merely reflect a lower MW of the components, because interactions between analytes and column material may result in interference with the elution order. Besides, the unknown chemical structure of HMW components makes it impossible to select standards to determine the MW of our substances (*15*).

The dry matter and pH values of twice-purified GFC fractions showed that the five fractions occurred in different amounts in the HMW fraction and that they had different acidic features. Again, elemental analysis showed a different element composition, even though all fractions contained nitrogen (**Table 2**). Spectral data were collected to obtain preliminary information about the chemical features of the GFC fraction (**Figure 4**). All GFC fractions in acidic media showed at least two absorption maxima—at 270−280 and at 324 nm, respectively—that are also known to occur in the spectra of hydroxycinnamic derivatives. Because the absorption maximum of the latter derivatives recorded at 324 nm shifted to 380 nm in alkaline conditions, the behavior of each HMW GFC fraction was also analyzed in an alkaline medium. The shift lends further support to the presence of hydroxycinnamic derivatives in each of them.

The occurrence of phenolic compounds in HMW molecules can be confirmed using various procedures. The Folin−Ciocalteu method, or alkaline fusion followed by GC analysis (*8*), allow us to determine total polyphenols, while salt treatment followed by HPLC analysis highlights only ionically bound phenols (*9*). We were able to apply the Folin−Ciocalteu method because of the low tyrosine content determined in the GFC fractions by amino acid analysis (**Table 3**). Phenolic groups, expressed as chlorogenic acid, used as the phenolic standard compound, were found in all GFC fractions (retentate, 11.27 μg/mL; GFC1, 1.86 μg/mL; GFC2, 2.65 μg/mL; GFC3, 2.32 μg/mL; GFC4, 1.66 μg/mL; and GFC5, 0.62 μg/mL) with GFC2 showing the highest content. Nevertheless, phenolic compounds were not detected by RP-HPLC-DAD in the isolated GFC fractions after treatment with NaCl (*9*), suggesting that polyphenols are covalently bound (data not shown). Thus, their HMW, nitrogen content, and brown color confirmed GFC1, GFC2, and GFC4 to be melanoidins, while GFC3 and GFC5, which showed no absorbance at 420 nm, cannot be considered as melanoidins.

All GFC fractions tested for ex vivo ARA at the same concentration as in coffee brew exhibited PA (**Figure 5**); PA

Table 2. Dry Matter, pH, and Element Analysis of GFC Fractions

GFC fraction	dry matter (mg/mL)	pH	element analysis		
			C %	H %	N %
GFC1	0.35 ± 0.03	6.18 ± 0.10	44.67 ± 0.27	6.92 ± 0.16	14.48 ± 0.09
GFC2	1.22 ± 0.09	5.54 ± 0.11	39.02 ± 0.15	5.66 ± 0.18	14.81 ± 0.10
GFC3	0.05 ± 0.02	5.96 ± 0.16	38.01 ± 0.29	5.77 ± 0.12	10.45 ± 0.27
GFC4	0.30 ± 0.06	6.83 ± 0.21	42.89 ± 0.27	5.44 ± 0.13	11.35 ± 0.26
GFC5	0.29 ± 0.05	6.22 ± 0.16	60.17 ± 0.34	15.79 ± 0.21	7.59 ± 0.15

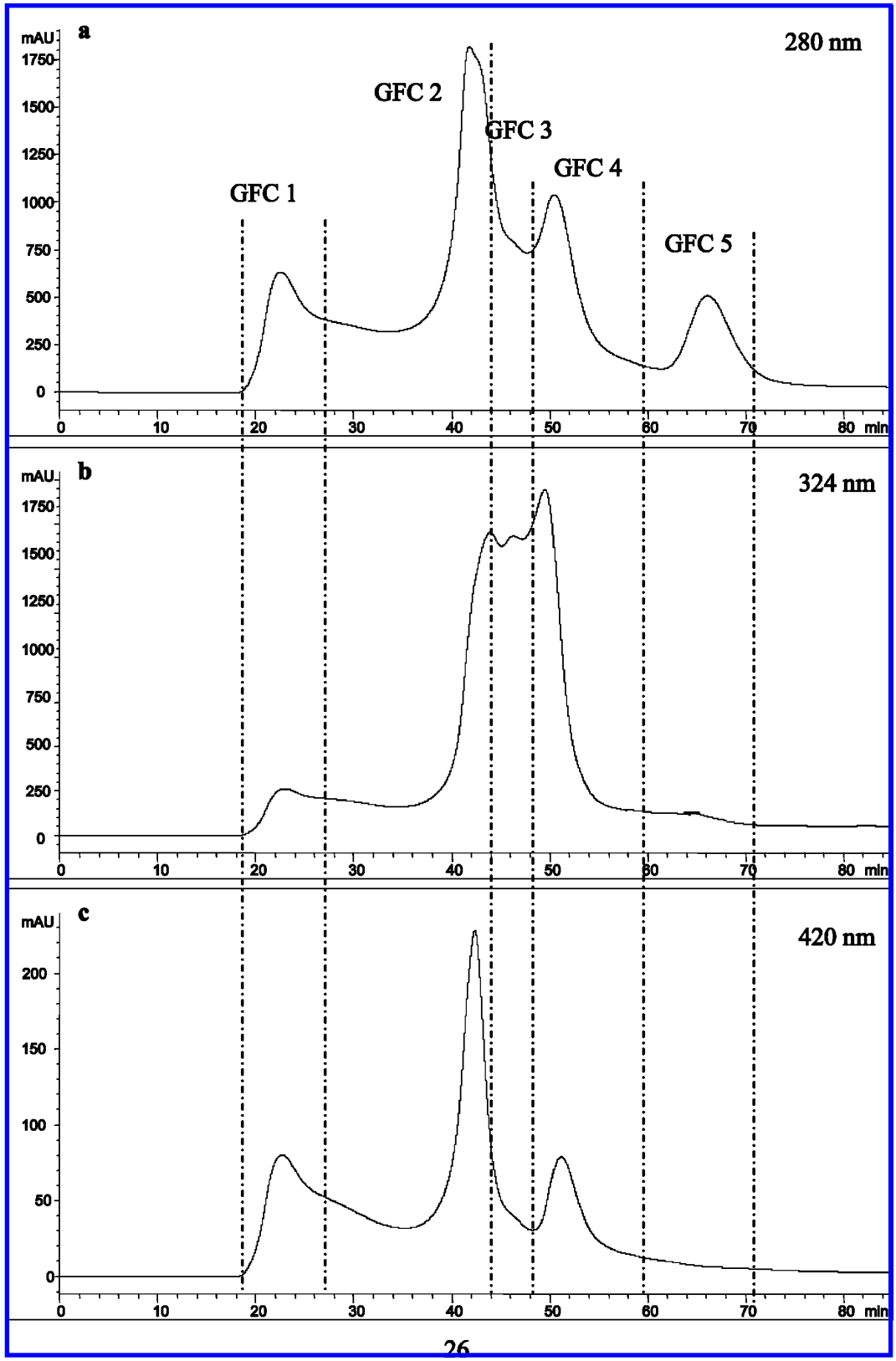

Figure 3. Gel filtration chromatograms of coffee brew retentate (MW > 3500 Da) measured at 280 (**a**), 324 (**b**), and 420 nm (**c**). Operative conditions are reported in the Materials and Methods.

was highest for the GFC4 and GFC2 melanoidin fractions (accounting for 15.8 and 54.7% of HMW fraction dry matter, respectively) and for the GFC5 nonmelanoidin fraction (13.6% of HMW fraction dry matter), indicating that the chromophore group is not critical for antioxidant activity. Further testing documented a dose−response relationship when fractions were diluted, while higher concentrations (up to three times the concentration occurring in the HMW fraction) failed to enhance PA (**Figure 5**). When GFC fractions were pooled to reconstitute the whole retentate, PA was again 100%, excluding a loss of PA of the HMW components caused by GFC separation and suggesting that each HMW component contributes to achieve complete inhibition of the lipid peroxidation in the microsome system. The mechanism of action of each isolated GFC fraction

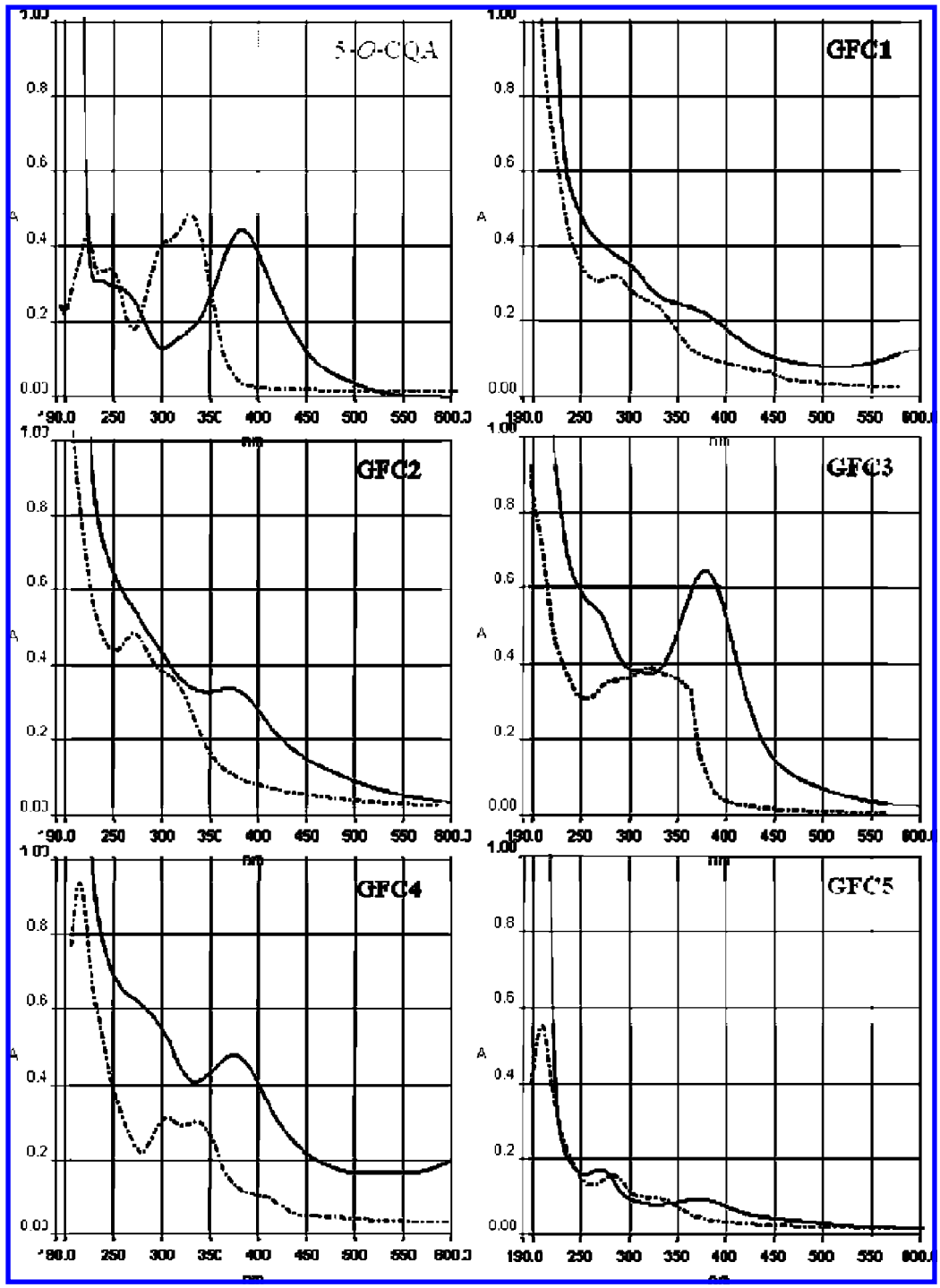

Figure 4. UV−Vis spectra of 5-*O*-CQA and GFC fractions obtained from coffee brew retentate in acidic media (dashed line) and alkali media (solid line).

was thus investigated using different chemical systems capable of highlighting different properties, such as radical scavenging capacity, reducing power, and metal ion chelating activity (*27, 28*).

The radical scavenging capacity of the whole HMW fraction and the GFC fractions was tested against two different radicals: DPPH$^{\bullet}$, a stable nitrogen synthetic radical, and ROO$^{\bullet}$, a low-reactive radical. The tests with DPPH$^{\bullet}$ (**Table 4**) showed that all fractions possess radical scavenging ability. The slightly greater activity of the GFC4 fraction as compared with the whole retentate suggested that interactions among the different fractions produce a negative synergism when all are present at the same time. The AA of samples was tested in the linoleic acid−β-carotene system by evaluating β-carotene discoloration. Its 11 pairs of double bonds make β-carotene extremely susceptible to free radical-mediated oxidation, leading to easy discoloration by peroxyl radical (*28*). The results (**Table 4**) showed that the retentate possessed a consistently strong scavenging activity. Each GFC fraction showed less activity than the whole retentate; the GFC4 fraction exhibited the highest values in this system, too.

The metal chelating activity of the retentate and its GFC fractions was determined on ferrous ion because of its catalytic action in lipid peroxidation. The tests showed that the retentate ICA (87%) was similar to that of 4 mg/mL EDTA solution, used as the standard, and higher than that of each of its

Table 3. Amino Acid Composition of GFC Fractions

amino acid (% w/w)[a]	GFC1	GFC2	GFC3	GFC4	GFC5
Tyr	0.380	1.900	0.008	0.080	0.240
Asx	1.570	5.500	0.060	54.350	41.526
Glx	6.250	24.090	0.240	0.000	0.000
Gly	0.680	2.550	0.045	18.870	13.790
Thr	0.500	1.780	0.037	17.030	24.400
Ala	0.760	2.630	0.048	21.760	19.980
Arg	0.150	0.550	0.019	10.120	11.150
Val	0.890	3.110	0.053	27.020	25.890
Met	0.020	0.000	0.000	1.180	0.000
Phe	0.440	1.970	0.030	20.140	25.130
Ile	0.650	2.570	0.049	25.940	23.240
N total%	4.06	4.47	1.45	0.90	0.88

[a] Standard deviation: 0.005−0.030.

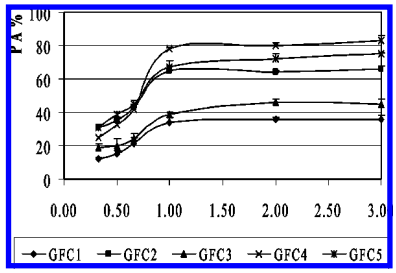

Figure 5. PA % vs concentration (×3.00, ×2.00, ×1.00, ×0.66, ×0.50, and ×0.33) of GFC fractions obtained from coffee brew retentate (MW > 3500 Da). Values are means of four independent experiments.

Table 4. ARA %, AA %, ICA %, and RA of Retentate (MW > 3500 Da) and GFC Fractions

		AA %				
coffee fraction	ARA %	5 min	10 min	20 min	ICA %	RA[a]
retentate	71 ± 3	79 ± 4	90 ± 3	93 ± 1	98 ± 4	258 ± 9
GFC1	25 ± 4	34 ± 2	48 ± 4	56 ± 3	31 ± 2	75 ± 1
GFC2	60 ± 5	9 ± 3	45 ± 2	56 ± 1	38 ± 1	100 ± 8
GFC3	54 ± 2	21 ± 4	36 ± 3	57 ± 3	19 ± 1	137 ± 6
GFC4	74 ± 3	48 ± 5	65 ± 3	75 ± 3	29 ± 2	149 ± 6
GFC5	45 ± 2	7 ± 2	37 ± 1	54 ± 2	39 ± 2	49 ± 3
Trolox	74 ± 2[b]			85 ± 5[c]		

[a] The reducing substances were expressed as the concentration (μg/mL) of an ascorbic acid solution, used as standard. [b] The Trolox C solution concentration was 250 μM. [c] The Trolox C solution concentration was 200 μM.

components (**Table 4**); in fact, with 33% of whole retentate ICA the GFC2 fraction was the most active, closely followed by GCF1.

The retentate and the GFC fractions were also tested for their reducing power with the method based on the reduction of iron(III) chloride to iron(II) chloride, indicated by formation of a blue complex with potassium hexacyanoferrate(III). Reducing power was expressed as the concentration (μg/mL) of ascorbic acid, used as the standard, showing the same reducing power on samples. Because the test involved the use of iron ions and all samples showed chelating activity, the assay was carried out at a lower sample concentration than that yielding ICA (1:10). The whole retentate was shown to have the highest RA and the GFC4 fraction to be again the most active, followed by GFC3 (**Table 4**).

In conclusion, coffee brew prepared using *C. robusta* beans with a medium degree of roasting has the ability to inhibit lipid peroxidation completely in a biological system consisting of rat liver microsomes, even when peroxidation is induced by radicals. Coffee's AA was mainly due to the HMW fraction, consisting of five components. Such components, isolated and purified, were shown to occur in different amounts in the brew and to have different element compositions and spectra. Their HMW, nitrogen content, and brown color enabled three of the compounds to be attributed to the melanoidin family, while the two nonbrown components could not be considered as melanoidins. However, all isolated HMW components contained covalently bound phenolic residues in their polymeric molecules, which probably contribute to the ARA of coffee brew. Each melanoidin and nonmelanoidin HMW isolated component participates to a different extent in the PA exerted by coffee brew. The most active components were found to be the medium and, especially, the lower MW melanoidins, obtained by the retentate (MW > 3500 Da), this latter component exhibited the highest activity also in most of the chemical assays used to test the mechanism of action. In such chemical systems, all of the components resulted to be active but with very different extents, so giving an explanation of the findings showing that none of the isolated components, when alone, was able to inhibit microsomal lipid peroxidation completely in the biological system, even when tested at higher concentrations than occur in coffee brew.

ACKNOWLEDGMENT

The amino acid analysis was performed at the Centro Grandi Strumenti of Pavia University by Dott. Patrizia Arcidiaco.

LITERATURE CITED

(1) Higdon, J. V.; Frei, B. Coffee and health: A review of recent human research. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 101–123.
(2) Dorea, J. G.; da Costa, T. H. M. Is coffee a functional food? *Br. J. Nutr.* **2005**, *93*, 773–782.
(3) Bonita, J. S.; Mandarano, M.; Donna, S.; Vinson, J. Coffee and cardiovascular disease: In vitro, cellular, animal, and human studies. *Pharmacol. Res.* **2007**, *55*, 187–198.
(4) Ranheim, T.; Halvorsen, B. Coffee consumption and human health—Beneficial or detrimental? Mechanism for effects of coffee consumption on different risk factors for cardiovascular disease and type 2 diabetes mellitus. *Mol. Nutr. Food Res.* **2005**, *49*, 274–284.
(5) Ascherio, A.; Zhang, S. M.; Hernan, M. A.; Kawachi, I.; Colditz, G. A.; Speizer, F. E.; Willett, W. C. Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. *Ann. Neurol.* **2001**, *50*, 56–63.
(6) Tverdal, A.; Skurtveit, S. Coffee intake and mortality from liver cirrhosis. *Ann. Epidemiol.* **2003**, *13*, 419–423.
(7) Porta, M.; Vioque, J.; Ayude, D.; Alguacil, J.; Jarod, M.; Ruiz, L.; Murillol, J. A. Coffee drinking: the rationale for treating it as potential effect modifier of carcinogenic exposure. *Eur. J. Clin. Nutr.* **2004**, *18*, 289–298.
(8) Bekedam, E. K.; Loots, M. J.; Schols, H. A.; van Boekel, M A. J. S.; Smit, G. Roasting effects on the formation mechanism of coffee brew melanoidins. *J. Agric. Food Chem.* **2008**, *56*, 7138–7145.
(9) Nunes, F. M.; Coimbra, M. A. Melanoidins from coffee infusion. Fractionation, chemical characterization, and effect of the degree of roast. *J. Agric. Food Chem.* **2007**, *55*, 3967–3977.
(10) Morales, F. J.; Babbel, M. B. Melanoidins exert a weak antiradical activity in water fluids. *J. Agric. Food Chem.* **2002**, *50*, 4657–4661.
(11) Fuster, M. D.; Mitchell, A. E.; Ochi, H.; Shibamoto, T. Antioxidative activities of heterocyclic compounds formed in brewed coffee. *J. Agric. Food Chem.* **2000**, *48*, 5600–5603.
(12) Yanagimoto, K.; Ochi, H.; Lee, K. G.; Shibamoto, T. Antioxidative activities of fractions obtained from brew coffee. *J. Agric. Food Chem.* **2004**, *52*, 592–596.

(13) Daglia, M.; Racchi, M.; Papetti, A.; Lanni, C.; Govoni, S.; Gazzani, G. In vitro and ex vivo antihydroxyl radical activity of green and roasted coffee. *J. Agric. Food Chem.* **2004**, *52*, 1700–1704.

(14) Delgado-Andrade, C.; Rufian-Henares, J. A.; Morales, F. Assessing the antioxidant activity of melanoidins from coffee brew by different antioxidant methods. *J. Agric. Food Chem.* **2005**, *53*, 7832–7836.

(15) Borrelli, R. C.; Visconti, A.; Mennella, C.; Anese, M.; Fogliano, V. Chemical characterization and antioxidant properties of coffee melanoidins. *J. Agric. Food Chem.* **2002**, *50*, 6527–6533.

(16) Bekedam, E. K.; Schols, H. A.; van Boekel, M. A. J. S. High molecular weight melanoidins from coffee brew. *J. Agric. Food Chem.* **2006**, *54*, 7658–7666.

(17) Delgado-Andrade, C.; Morales, F. J. Unravelling the contribution of melanoidins to the antioxidant activity of coffee brew. *J. Agric. Food Chem.* **2005**, *53*, 1403–1407.

(18) Nicoli, M. C.; Anese, M.; Marzocco, L.; Lerici, C. R. Antioxidant properties of coffee brews in relation to the roasting degree. *Lebensm.-Wiss. Technol.* **1997**, *30*, 292–297.

(19) Delgado-Andrade, C.; Rufian-Henares, J. A.; Bravo, L.; Morales, F. J. Effect of coffee melanoidin on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. *Mol. Nutr. Food Res.* **2007**, *51*, 536–545.

(20) Rufian-Henares, J. A.; Morales, F. J. Effect of in vitro enzymatic digestion on antioxidant activity of coffee melanoidins and fractions. *J. Agric. Food Chem.* **2007**, *55*, 10016–10021.

(21) Daglia, M.; Papetti, A.; Gregotti, C.; Bertè, F.; Gazzani, G. In vitro antioxidant and ex vivo protective activities of green and roasted coffee. *J. Agric. Food Chem.* **2000**, *48*, 1449–1454.

(22) Horie, T.; Murayama, T.; Mishima, T.; Itoh, F.; Minamide, Y.; Fuwa, T.; Awazu, S. Identified diallyl polysulfides from an aged garlic extract which protects the membranes from lipid peroxidation. *Planta Med.* **1989**, *55*, 506–508.

(23) Lowry, O. H.; Rosenbrough, N. J.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(24) Papetti, A.; Daglia, M.; Gazzani, G. Anti- and pro-oxidant water solubile activity of Cichorium genus vegetables and effect of thermal treatment. *J. Agric. Food Chem.* **2002**, *50*, 4696–4704.

(25) Taga, L. C.; Miller, E. E.; Pratt, D. E. Chia seeds as a source of natural lipid oxidant. *J. Am. Oil Chem. Soc.* **1984**, *61*, 928–931.

(26) Yen, W. J.; Wang, B. S.; Chang, L. W.; Duh, P. D. Antioxidant properties of roasted coffee residues. *J. Agric. Food Chem.* **2005**, *53*, 2658–2663.

(27) Aruoma, O. I. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat. Res.* **2003**, *523*−*524*, 9–20.

(28) Wen, X.; Takenaka, M.; Murata, M.; Homma, S. Antioxidative activity of zinc-chelating substance in coffee. *Biosci., Biotechnol., Biochem.* **2004**, *68*, 2313–2318.

Received for review July 2, 2008. Revised manuscript received October 20, 2008. Accepted October 25, 2008. The research was funded by profits from Educazione Continua in Medicina (ECM) conferences.

JF802018C