In Vitro Antioxidant and ex Vivo Protective Activities of Green and Roasted Coffee

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The antioxidant properties of green and roasted coffee, in relation to species (*Coffea arabica* and *Coffea robusta*) and degree of roasting (light, medium, dark), were investigated. These properties were evaluated by determining the reducing substances (RS) of coffee and its antioxidant activity (AA) in vitro (model system β-carotene−linoleic acid) and ex vivo as protective activity (PA) against rat liver cell microsome lipid peroxidation measured as TBA-reacting substances. RS of *C. robusta* samples were found to be significantly higher when compared to those of *C. arabica* samples ($p < 0.001$). AA for green coffee samples were slightly higher than for the corresponding roasted samples while PA was significantly lower in green coffee compared to that of all roasted samples ($p < 0.001$). Extraction with three different organic solvents (ethyl acetate, ethyl ether, and dichloromethane) showed that the most protective compounds are extracted from acidified dark roasted coffee solutions with ethyl acetate. The analysis of acidic extract by gel filtration chromatography (GFC) gave five fractions. Higher molecular mass fractions were found to possess antioxidant activity while the lower molecular mass fractions showed protective activity. The small amounts of these acidic, low molecular mass protective fractions isolated indicate that they contain very strong protective agents.

Keywords: *Coffee; roasted coffee; antioxidants; lipid peroxidation*

INTRODUCTION

Green coffee, roasted coffee, and the beverage prepared from it are very complex mixtures of several hundreds of chemicals which are both naturally occurring and induced by the roasting process. The chemistry and the biological activities of these substances have not yet been completely elucidated. Most literature reports concern caffeine and its pharmacological effects. In recent years the high worldwide consumption of coffee has stimulated research to study other biological activities of green and especially of roasted coffee, used to prepare the different types of coffee brews. During roasting, the beans are heated to 200−240 °C for 10−15 min depending on the degree of roasting required which is generally evaluated by the weight loss of the sample thermally treated. The roasting process gives rise to changes which confer to coffee its pleasant taste and aroma. It leads to profound changes in the chemical composition and biological activities of coffee, according to the transformation of naturally occurring substances in green coffee and the generation of compounds deriving from the Maillard reaction, carbohydrate caramelization, and pyrolysis of organic compounds (Belitz and Grosch, 1999; Giullot et al., 1996).

Recently, green coffee has been shown to possess in vitro antioxidant activity against lipid peroxidation (Kroyer et al., 1989) and antineoplastic activity (Wattenberg and Lam, 1984; Rosenberg, 1990).

Roasted coffee has been found to possess mutagenic activity, probably due to the formation of hydrogen peroxide that has been implicated as a major contributor to coffee genotoxicity in vitro (Miller et al., 1993) and to possess antibacterial activity against a wide range of bacteria. The activity was found to be dependent on the coffee bean species but not on the country of origin, and for the same species on the degree of roasting of the coffee beans (Daglia et al., 1994a). Moreover, coffee was demonstrated to act in vitro as a prooxidant and antioxidant. Its prooxidant activity appears to be attributed to polyphenolic thermal degradation products which reduce atmospheric oxygen in the presence of transition metal catalysis (Turesky et al., 1993). However the same research group reported that roasted coffee can act as a potent antioxidant and inhibit lipid peroxidation in a model system (Stadler et al., 1994).

The aim of this paper was to investigate the antioxidant properties of green and roasted coffee in relation to the species and degree of roasting. Green coffee in fact contains large but variable amounts of chlorogenic, caffeic acids and other polyphenolic compounds depending on its species. Many of the recently studied plant polyphenols were found to act as potent antioxidants (Laughton et al., 1991; Vinson et al., 1998; Velioglu et al., 1998). On the other hand, coffee beverages are commonly prepared with roasted coffee, where most polyphenolic compounds are destroyed (Daglia et al., 1994b) while Maillard reaction products (MRP), with antioxidant properties (Elizalde et al., 1992; Tubaro et al., 1996), are generated. Antioxidant properties of coffee were evaluated, as generally is done for foods, by determining its content of reducing substances (RS) and its antioxidant activity (AA) in a model system based on coupled oxidation of β-carotene and linoleic acid (Taga et al., 1984). Furthermore, given that the ability

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of a compound or a system to act as antioxidant in vitro does not necessarily mean that it can act in the same manner in vivo, the antioxidant properties of coffee were also evaluated as protective activity (PA) on lipid peroxidation of rat liver cell microsomes (Plaa and Hewit, 1992), which is a model system that better simulates the in vivo conditions. Again, we intended to isolate the active components from roasted coffee, to obtain preliminary information about the compounds possessing antioxidant and protective activity.

MATERIALS AND METHODS

Coffee Solution Preparation. Three samples of *Coffea arabica* and three samples of *Coffea robusta* coffee beans, from a total of six different sources, were subdivided into four batches (green, light, medium, and dark roasted, prepared in duplicate). The roasted batches were prepared in a pilot roaster apparatus (by courtesy of Dr. Ardino, La casa del Caffè sas, Florence, Italy). The roasting process is controlled by the weight loss, due to the vapor formation and cell fragment (silverskin particles) loss (Belitz and Grosch, 1999). The weight loss was about 9% for light-roasted (L_R) samples (100−180 °C, 19−20 min), 12% for medium-roasted (M_R) samples (100−190 °C, 19−20 min), and 17% for dark-roasted (D_R) samples (100−210 °C, 19−20 min). The coffee beans were ground in a laboratory scale mill and sieved through a no. 30 sieve. Coffee solutions were prepared with the brewed coffee procedure. Six grams of ground green or roasted coffee were boiled for 10 min in 100 mL of distilled water. The solutions were then filtered on Millipore membranes of cellulose acetate/cellulose nitrate mixed esters (0.45 μm), and the pH value of each sample was immediately measured.

In Vitro Antioxidant Assay. The antioxidant activity of coffee solutions based on coupled oxidation of β-carotene and linoleic acid were evaluated following the method of Taga et al. (1984) with some modifications (Gazzani et al., 1998a). β-Carotene (5 mg) (Merck) was dissolved in 50 mL of chloroform solution. A 3 mL aliquot of β-carotene chloroform solution was added to a conical flask along with 40 mg of linoleic acid (Merck) and 400 mg of Tween 20 (Merck). Chloroform was evaporated to dryness under reduced pressure at low temperature (less than 30 °C). Distilled water (100 mL) was added to the dried mixture, and the mixture was shaken. Four aliquots (50 μL), chosen because this was the greatest volume that could allow us to distinguish the antioxidant activity of coffee according to the different degree of roasting of coffee solutions, were added to 5 mL of β-carotene emulsion in test tubes, and the mixture was mixed well (samples). For one sample the absorbance at 470 nm was immediately measured using the spectrophotometer, and for the other samples absorbance was measured after 10, 20, and 30 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). To correct for the influence of the coffee beverage color in calculating β-carotene degradation rate, four aliquots (50 μL) of each sample were added to 5 mL of blank (blank sample). The absorbances of the mixtures for each time point were read with a spectrophotometer, and the absorbance measured was subtracted from that of the corresponding sample. The degradation rate of β-carotene was calculated by first-order kinetics:

$$(\ln(A_0/A_t))/t = \text{degradation rate (dr) of sample} \quad (1)$$

where A_0 = absorbance of the sample − the absorbance of blank sample at time 0 (absorbance was read immediately after the addition of coffee solutions), A_t = absorbance of the sample − absorbance of blank sample at time t, and t = 10 or 20 or 30 min of incubation at 50 °C.

$$(\ln(a_0/a_t))/t = \text{degradation rate (dr) of control sample} \quad (2)$$

where 50 μL of distilled water was added to 5 mL of β-carotene emulsion and treated as the corresponding sample, a_0 = absorbance of the control sample at time 0, and a_t = absorbance of the control sample at time t.

Antioxidant activity was expressed as the percent of inhibition relative to the control using the equation:

$$\text{AA\%} = \frac{\text{dr control sample} - \text{dr sample}}{\text{dr control sample}} \times 100 \quad (3)$$

Biological Lipid Peroxidation Assay (Protective Activity, PA%). Liver microsomes were prepared from male Wistar rats weighing 200−250 g following the method of Horie et al. (1988) with some modification (Gazzani et al., 1998b). The microsomal pellets obtained were suspended either in 0.1 M sodium phosphate buffer, pH 7.4 (control sample), or in coffee solutions (sample) to make a total volume of 6 mL, respectively. An aliquot (0.1 mL) of the obtained suspension was immediately removed and used for determination of microsomal proteins (Wang et al., 1996).

The remaining preparation was added to NaCl (1 mL, 140 μM), EDTA (1 mL, 50 μM), and sodium phosphate buffer (1 mL, 0.1 M, pH 7.4) and then subdivided into two aliquots of 4 mL, respectively. All test tubes containing samples were stoppered, and N_2 was bubbled through the solution at 37 °C for 15 min to obtain anaerobic conditions for the following induction of the lipid peroxidation. To one group of samples were then added NADP (0.5 mL, 500 μM), G6P (0.5 mL, 250 μM), and CCl_4/EtOH (20 μL, 50% v/v). An equivalent amount of buffer was instead added to second group. Both samples were placed in a shaking water bath at 37 °C for 30 min, and then the equal volumes of 30% TCA at 0 °C and 0.75% TBA were added (Lowry et al., 1951).

The reaction mixtures were heated in boiling water for 15 min, kept in ice for 5 min, and then centrifuged for 10 min at 3000 rpm to separate corpuscolate particles.

Absorbance of supernatants was read in a spectrophotometer (λ = 545 nm) using the second series of samples treated as above but without coenzymes to bring the spectrophotometer to zero. This was done to correct for interference due to color and TBA reacting substances naturally occurring in coffee solutions.

The protective activity was expressed as the percentage decrease of TBA reacting substances relative to the control using the equation

$$\text{PA\%} = (a - b)/a \times 100 \quad (4)$$

where a is the TBA reacting substances in control sample and b represents the TBA reacting substances in sample.

Reducing Substances (RS%). The reducing substances were determined as described by Daglia et al.,1992, with some modifications. A 1 mL aliquot of coffee solution (diluted 1:250 mL) was placed in a test tube containing 0.4 mL of potassium hexacyanoferrate (III) (0.02 M), 0.05 mL of chloridric acid (0.01M), 0.4 mL of iron (III) chloride (0.02M), and 0.7 mL of distilled water. Samples were mixed well and incubated in the dark for 15 min. Sample absorbance was read in a spectrophotometer at 720 nm. The reducing substances were expressed as the percentage of chlorogenic acid, used as standard.

Organic Extraction. A 50 mL aliquot of each coffee solution, obtained from dark roasted *C. robusta* (Ivory Coast) and *C. arabica* (Brazil) beans as described, was acidified with HCl (10% w/v, pH 2) and extracted with either ethyl acetate, ethyl ether, or dichloromethane. The aqueous phase was adjusted to pH 12 with NaOH (10% w/v) and reextracted with the organic solvents. Acidic and basic organic extracts were evaporated to dryness, the aqueous phases were brought to the initial pH value of the coffee solutions and freeze-dried. The residues were dissolved in 50 mL of distilled water.

Gel Filtration Chromatography of Ethyl Acetate Acidic Extract. After extraction of coffee solution obtained from *C. robusta* Ivory Coast DR, with ethyl acetate in acidic conditions, 2.18% of ground coffee powder was recovered. This fraction was then separated by preparative gel filtration chromatog-

Table 1. pH, Reducing Substances (RS), Antioxidant Activity (AA), Protective Activity (PA), and Their Relative Standard Deviations of *C. robusta* and *C. arabica* of Different Origin

						AA%					
origin	dr[a]	pH	SD	RS%	SD	10 min	20 min	30 min	SD_{30}	PA%	SD
					C. robusta						
Zaire	G[b]	5.95	0.21	11.22	1.04	89	91	93	1.73	36	6.70
	LR[c]	5.46	0.13	8.90	2.33	79	86	87	3.51	100	0.00
	MR[d]	5.64	0.09	9.98	0.85	77	84	89	1.00	100	0.00
	DR[e]	5.66	0.14	12.16	0.75	81	89	93	3.53	100	0.00
Ivory Coast	G	5.90	0.18	10.30	1.14	80	90	91	1.53	62	8.93
	LR	5.50	0.08	10.33	0.77	64	74	79	7.23	100	0.00
	MR	5.47	0.09	11.35	0.70	78	83	83	2.31	100	0.00
	DR	5.43	0.04	12.04	0.56	75	82	88	4.24	100	0.00
Ecuador	G	5.82	0.11	10.87	1.50	84	91	93	1.15	47	9.00
	LR	5.61	0.09	9.22	0.90	79	86	86	6.03	100	0.00
	MR	5.65	0.18	10.28	0.70	74	84	89	2.51	100	0.00
	DR	5.68	0.13	11.03	0.16	85	90	91	2.08	100	0.00
					C. arabica						
Brazil	G	5.77	0.14	4.45	0.56	84	86	89	7.50	53	8.60
	LR	5.13	0.10	6.68	1.00	62	73	76	6.24	100	0.00
	MR	5.20	0.11	7.70	0.44	75	78	84	5.29	100	0.00
	DR	5.39	0.16	7.80	0.42	72	82	86	2.64	100	0.00
Colombia	G	5.93	0.11	5.68	0.19	82	89	92	2.51	44	7.07
	LR	5.01	0.034	6.80	1.41	70	74	80	12.50	100	0.00
	MR	5.34	0.01	7.30	0.69	69	83	87	3.60	100	0.00
	DR	5.37	0.03	7.36	0.09	75	80	87	1.73	100	0.00
Costa Rica	G	5.89	0.21	5.51	1.61	84	88	92	1.00	24	6.98
	LR	5.03	0.09	7.00	0.06	65	75	82	5.68	100	0.00
	MR	5.04	0.05	7.18	0.18	72	80	85	3.05	100	0.00
	DR	5.29	0.11	7.32	0.04	69	82	86	5.65	100	0.00

[a] Degree of roasting. [b] Green. [c] Light roasted. [d] Medium roasted. [e] Dark roasted.

raphy (GFC) using a Merck Superformance universal glass-cartridge system 300 × 10 mm i.d. column with Toyopearl HW 40 (F) packing (Tosohaas, Tokyo). The system was equipped with a Waters 490E UV−vis detector (Waters Chromatography Division, Milan) and a Hitachi-Merck D 2500 integrator. Mobile phase was Millipore grade distilled water and flow rate was 1 mL/min. UV detection was at 270 nm.

The acidic extract was resolved into five fractions. After evaporation of the mobile phase, each of the five fractions obtained was brought to the correspondent volume of coffee beverage with distilled water and tested for antioxidant (AA) and protective (PA) activity.

Statistical Analysis. Values represent means of five replications. Data were analyzed by analysis of variance (ANOVA) and multifactor analysis of variance (MANOVA) with the statistical package Statgraphics (1991). Means were separated with the confidence interval method (Conf. Int.) at a confidence level of 99%. To provide an exploratory data analysis tool that is useful for studying symmetry, for distributional assumption, and for detecting outliers, the box and whisker plot procedure was used. This is particularly useful for comparing parallel batches of data because this procedure compares the medians, ranges, and extreme values of each group. Each box-and-whisker plot is a five-number statistical summary of a set of univariate observations. The plot divides the data into four areas of equal frequency. The box encloses the middle 50%. The median is drawn as a horizontal line inside the box. Vertical lines, called whiskers, extend from each end of the box. The lower whisker is drawn from the first quartile to the smallest data point within 1.5 interquartile ranges from the first quartile. The other whisker is drawn from the third quartile to the largest data point within 1.5 interquartile ranges from the third quartile. The system plots far outliers; data points that lie more than three interquartile ranges below the first quartile or above the third quartile, are indicated with a plus sign, so they are easier to spot.

RESULTS

Table 1 reports pH, RS%, AA%, and PA% values and relative standard deviations (SD) of all types of brewed coffee examined.

The solutions prepared using green coffee all show higher pH values than those obtained from roasted coffee. When roasted coffee samples are considered, the pH of the beverage increases with increased roasting except in the case of sample of *C. robusta* from Ivory Coast.

The RS of green coffee solutions differed according to the species. Even after roasting, *C. arabica* beverages showed much lower values than *C. robusta* beverages. The coffee solutions of the same species, even from different countries, showed very close values of RS. In *C. arabica* beverages the RS increased with the degree of roasting (green, light, medium, dark), while in *C. robusta* beverages a light roasting process induced a decrease in the RS values which then increased following severe roasting conditions so that dark roasted coffee always showed higher values than green coffee. Analysis of variance always showed significant differences between *C. arabica* and *C. robusta* RS values ($p < 0.001$).

The in vitro antioxidant activity (AA) showed very similar profiles and values during the roasting process for the two species considered. All green coffee solutions showed an immediate, strong activity which nevertheless increased with time of reaction. At the end of the monitoring period (30 min), all the green coffee solutions decreased the lipid peroxidation rate in a model system to at least 90%. When considered in terms of the degree of roasting, AA values were higher for green coffee, decreased slightly with light roasting, and then increased with stronger roasting although they never exceeded the AA values of green coffee.

Conversely, the biological assay (PA) showed that green coffee scarcely protected microsomial lipid from peroxidation. The protective activity increased strongly with thermal treatment so that all types of roasted coffee completely inhibited microsomial lipid peroxidation.

Diluted coffee beverages were also analyzed. It was

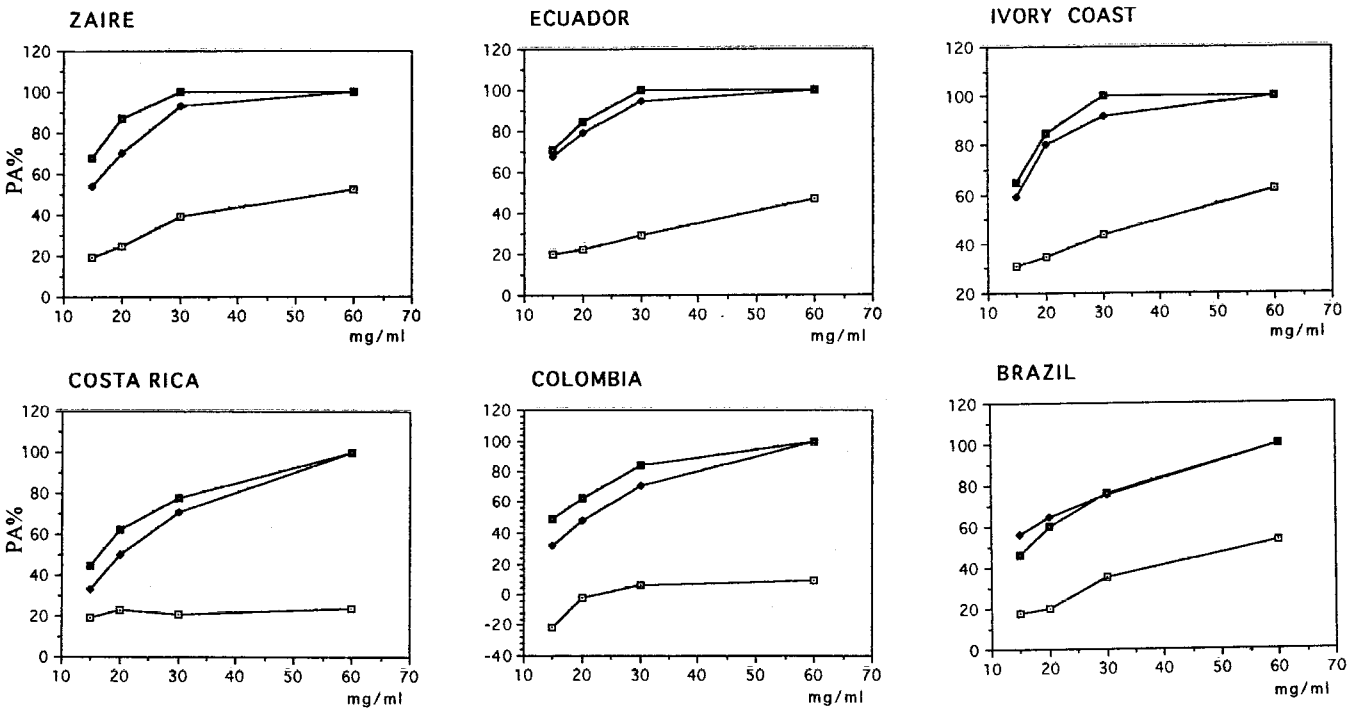

Figure 1. Protective activity (PA %) versus concentration of coffee solutions (mg ground coffee powder/ mL coffee solution) at the different degrees of roasting. —□— = green coffee; —◆— = light roasted coffee; solid square (with white center dot) = dark roasted coffee. Values represent means of four independent experiments.

found that PA was dose dependent (Figure 1). *C. robusta* dark roasted coffee beverages reached PA = 100% when prepared with about 30 mg/mL of ground coffee.

The butylhydroxytoluol (BHT) (0.5 mg/mL), assayed under identical test conditions in previous research, yielded $AA\%_{30min}$ = 90% (Gazzani et al., 1998a) and PA% = 57% (Gazzani et al., 1998b).

Multifactor analysis of variance was used to analyze the simultaneous influence of coffee species and degree of roasting on RS, AA, and PA values (Figure 2). As regards the classification factor coffee species, the results showed that only RS is invariably affected (Figure 2a). When the classification factor is degree of roasting, for RS no significant differences were found. Conversely, the AA values for green coffee were significantly higher than those for light and medium roasted coffees and PA values for green coffee were significantly lower than values for all roasted coffees (Figure 2b).

The acidic and basic extracts and the residual aqueous phases obtained after extraction with the different organic solvents were submitted to biological assays; protective activity comparable with those of the coffee solutions were found only in the case of ethyl acetate extracts at acidic pH. Thus, ethyl acetate was shown to be the best solvent for extraction of active components. (Table 2). The yellow-brown ethyl acetate acidic extract obtained from *C. robusta* from Ivory Coast coffee (DR) was submitted to gel filtration chromatography (Figure 3) and yielded five fractions. The dry residues, the molecular masses, and AA% and PA% values for each fraction are shown in Table 3. As regards AA%, the GFC_1 and GFC_2 higher molecular mass components were found to be active, while GFC_4 and GFC_5 lower molecular mass components were found to possess protective activity (Table 3). The fact that protective activity was evident in both fractions, GFC_4 and GFC_5, may be because of their incomplete resolution in the actual experimental conditions used.

Table 2. Dry Residues, % of Coffee Powder, and PA% of Dark Roasted Coffee Fractions Obtained by Acidic and Basic Organic Extraction with Different Organic Solvents

sample	organic extraction	fraction	mg/mL[a]	% coffee powder	PA %
C.r. Ivory Coast (DR)	ethyl acetate	A[b]	16.45	27.41	100
		B[c]	1.31	2.18	100
		C[d]	0.21	0.35	0
		D[e]	14.82	24.71	31
	ethyl ether	B	0.91	1.51	50
		C	0.11	0.18	0
		D	15.12	25.18	34
	dichloro-methane	B	0.56	0.75	35
		C	0.15	0.25	0
		C	15.77	26.28	61
C.a. Brazil (DR)	ethyl acetate	A	15.12	25.21	100
		B	1.21	2.02	100
		C	0.17	0.28	0
		D	14.02	23.36	27
	ethyl ether	B	0.86	1.43	47
		C	0.09	0.15	0
		D	14.18	23.63	30
	dichloro-methane	B	0.43	0.71	31
		C	0.15	0.25	0
		D	14.21	23.68	58

[a] mg dry residue/mL coffee solution. [b] Coffee solution. [c] Acidic extract. [d] Basic extract. [e] Aqueous phase.

DISCUSSION

The significant differences in RS values consistently found between *C. arabica* and *C. robusta* samples are probably due to their different content of polyphenol compounds, particularly chlorogenic acids, and because there is a different percentage decrease in the content of polyphenol compounds occurring after roasting (Daglia et al., 1994b; Trugo et al., 1984a,b). The increase in the RS values following severe roasting conditions is probably due to the formation of low molecular weight thermolysis products (Belits and Grosch, 1999). Although the RS data do not show any relationship

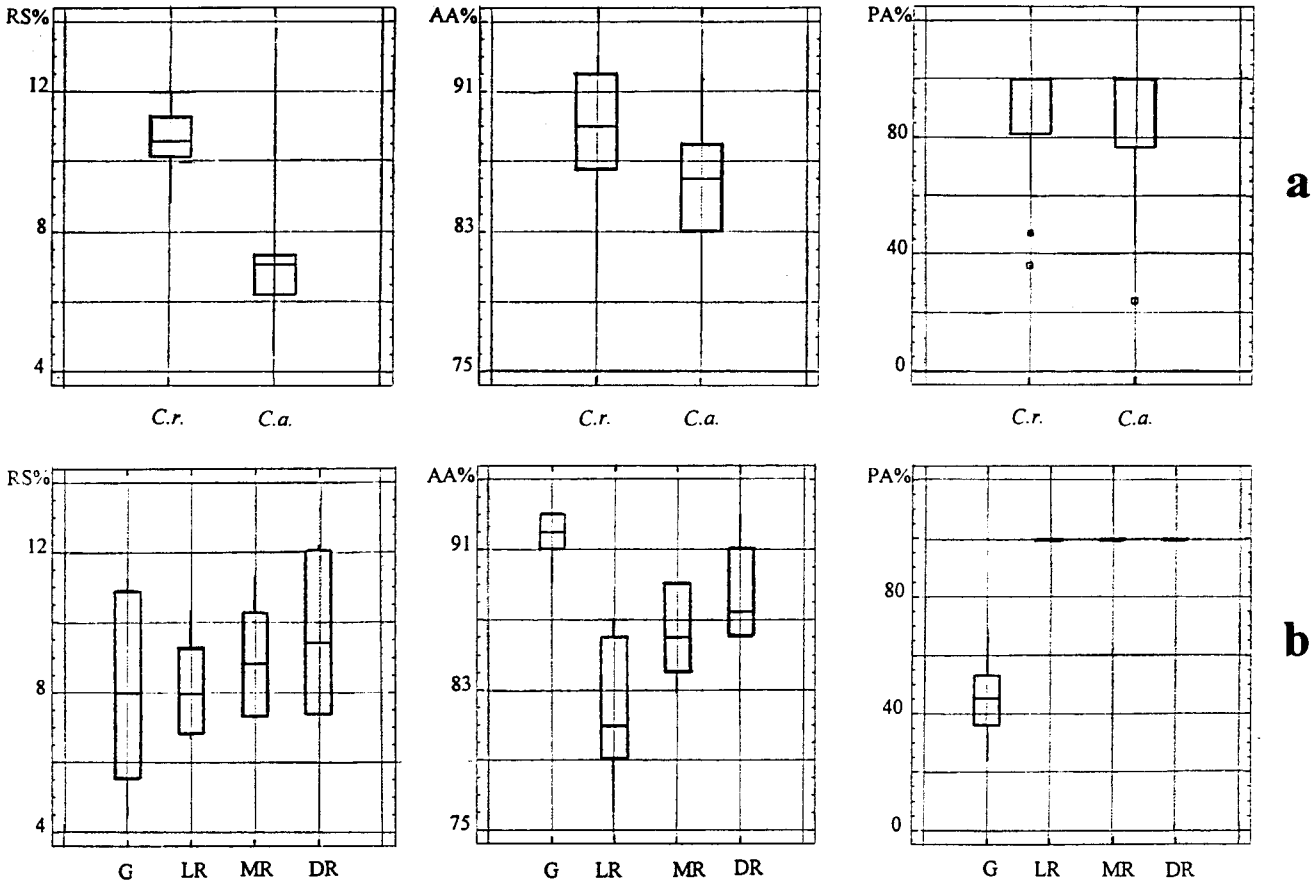

Figure 2. Multifactor analysis of variance of RS, AA, and PA obtained from the coffee solutions. (a) Classification factor = species: *C.a.* = *Coffea arabica*, *C.r.* = *Coffea robusta*. (b) Classification factor = degree of roasting: G = green, LR = light roasted, MR = medium roasted, and DR = dark roasted coffee.

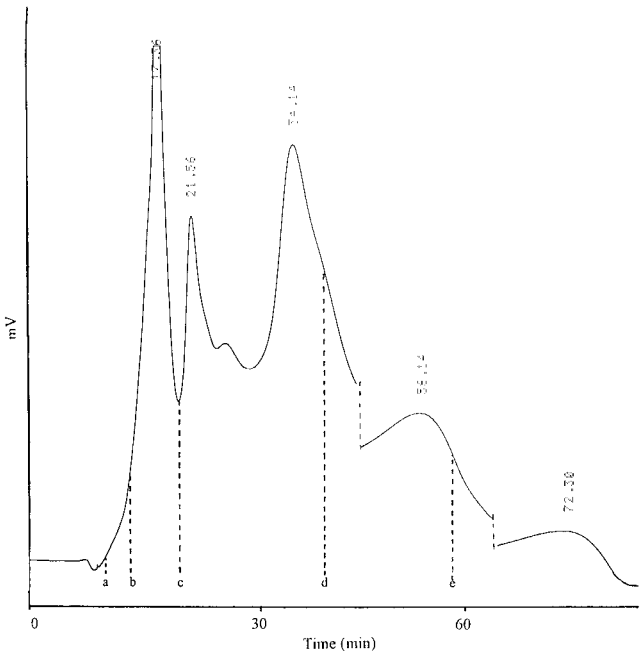

Figure 3. GPC of coffee extract. Operative conditions as described in the text. (a) Dextran T10 (MW = 10000); (b) polystyrene (MW = 5970); (c) melizitose (MW = 594); (d) chlorogenic acid (MW = 354); (e) caffeine (MW = 194).

between AA and PA for the different coffee species, they provide a useful predictive and easily determinable index regarding the species of unknown coffee beans or powders.

Table 3. Dry Residue, Molecular Masses, Antioxidant Activity (AA%), and Protective Activity (PA%) of Coffee Fractions Obtained by GPC of Acidic Extract

sample	mg/mL[a]	molecular mass	AA%[b]	PA%
acidic extract	1.31	-	96	100
GFC_1	0.50	MW < 6000	74	0
GFC_2	0.16	MW < 550	37	0
GFC_3	0.13	350 < MW < 550	0	0
GFC_4	0.23	200 < MW < 350	0	53
GFC_5	0.29	MW < 200	0	85

[a] mg dry residue/mL brewed coffee. [b] Measured at the end of the monitoring period (30 min).

The behavior of AA depending on the degree of roasting can probably be attributed to the loss of polyphenolic compounds (powerful in vitro antioxidants) occurring in green coffee during light roasting and to the successive formation of other antioxidant compounds such as Maillard reaction products (MRP) or pyrolysis products (less active ex vivo), when more severe thermal conditions are applied.

The antioxidant compounds naturally occurring in green coffee were found to be very active in the chemical test. These compounds were found to be far less active in biological systems, where the compounds generated during roasting were found to be highly protective. The obtained results showed that an in vitro active antioxidant may be inactive in the ex vivo test and vice versa. Interestingly, Zeyuan et al. (1998) found similar contradictory results in the case of green and black tea.

The AA and PA of the acidic extracts and of the residual aqueous phases indicate that there are several antioxidant compounds in coffee with different polar and

hydrophilic features. The more hydrophilic compounds remained in the beverage and were shown to be very poorly protective, while the more acidic fractions was shown to be highly active in the biological assay. The GFC analysis of the acidic fraction showed that only the lower molecular mass components, occurring in dark roasted coffee in low amount, can act ex vivo. These results and the finding that coffee beverages maintained high protective activity even when diluted, demonstrates that roasted coffee protective antioxidants are highly efficient even at very low concentrations, i.e., lower than those consumed daily by a modest coffee drinker.

On the other hand our findings confirm that, because of the number of factors that can influence the antioxidant activity of the compounds, this activity should be assessed in the closest possible conditions to those occurring where they are required. In fact, it is apparent that on the basis of the results of chemical tests only, it may be rash to infer antioxidant activity in biological systems (Trugo et al., 1984b; Gazzani et al., 1998a,b).

Investigations are currently under way in our laboratory to isolate the compounds which showed ex vivo protective activity.

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