JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation, Identification, and Quantification of Roasted Coffee Antibacterial Compounds

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Coffee brew is a widely consumed beverage with multiple biological activities due both to naturally occurring components and to the hundreds of chemicals that are formed during the roasting process. Roasted coffee extract possesses antibacterial activity against a wide range of microorganisms, including *Staphylococcus aureus* and *Streptococcus mutans*, whereas green coffee extract exhibits no such activity. The naturally occurring coffee compounds, such as chlorogenic acids and caffeine, cannot therefore be responsible for the significant antibacterial activity exerted by coffee beverages against both bacteria. The very low minimum inhibitory concentration (MIC) found for standard glyoxal, methylglyoxal, and diacetyl compounds formed during the roasting process points to these α-dicarbonyl compounds as the main agents responsible for the antibacterial activity of brewed coffee against *Sa. aureus* and *St. mutans.* However, their low concentrations determined in the beverage account for only 50% of its antibacterial activity. The addition of caffeine, which has weak intrinsic antibacterial activity, to a mixture of α-dicarbonyl compounds at the concentrations found in coffee demonstrated that caffeine synergistically enhances the antibacterial activity of α-dicarbonyl compounds and that glyoxal, methylglyoxal, and diacetyl in the presence of caffeine account for the whole antibacterial activity of roasted coffee.

KEYWORDS: Roasted coffee; antibacterial activity; *Staphylococcus aureus*; *Streptococcus mutans*; α-dicarbonyl compounds; caffeine

INTRODUCTION

Coffee brew is among the most widely consumed beverages all over the world, in part because of its putative health benefits. Epidemiological studies have shown that regular coffee drinking has favorable effects on health aspects ranging from psychoactive responses to neurological disorders (Alzheimer's and Parkinson's diseases) and metabolic disorders (type 2 diabetes and liver cirrhosis) to liver function (*1*–*4*). A high level of worldwide coffee consumption has stimulated research into the chemical composition and biological activities of green and, especially, roasted coffee. Most investigations have addressed naturally occurring compounds such as caffeine (and its pharmacological activity) and chlorogenic acids. These phenolic acids are found in greater amounts in green beans and may be the main factor responsible for the antioxidant activity of green coffee (*5*, *6*).

In recent years, research interest has been focused on bioactive compounds induced by the Maillard reaction, carbohydrate caramelization, and organic compound pyrolysis during the roasting process. Roasted coffee has been seen to exert both in vitro and ex vivo antioxidant activity (*5*–*8*). Given its significantly decreased chlorogenic acid content (up to 50–90%), the antioxidant properties of roasted coffee are mainly attributable to Maillard reaction products formed during the roasting process, which include both high-molecular mass components (melanoidins) and heterocyclic compounds such as furan, pyrroles, and maltol (*9*).

Roasted coffee has been found to interfere with streptococcal sucrose-independent adsorption to hydroxyapatite beads through the involvement of small molecules naturally occurring in coffee, such as trigonelline, nicotinic and chlorogenic acids, and high-molecular mass compounds that are formed during the roasting process (melanoidins) (*10*). Roasted coffee has also been shown to possess antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria, including *Streptococcus mutans*. Daglia et al. (*11*–*14*) found no detectable antibacterial activity of green coffee and concluded that naturally occurring compounds are not responsible for the antibacterial activity of roasted coffee. Almeida et al. (*15*) showed that roasted coffee is active against several strains of Enterobacteria and that trigonelline, caffeine, and protocatechuic acid are potential

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10.1021/jf0722607 CCC: $37.00
Published on Web 11/15/2007

natural antimicrobial agents against *Salmonella enterica*. In particular, they reported that caffeine may account for up to 50% of the antimicrobial effect of coffee brew against *S. enterica*. Dogazaki et al. (*16*) and Furuhata et al. (*17*) also reported that roasted coffee was active against a strain of *Legionella pneumophila*, a bacterium involved in respiratory diseases, pointing out a role for caffeic, chlorogenic, and protocatechuic acids.

This study was performed to isolate and identify the compounds responsible for the antibacterial activity of roasted coffee against *Staphylococcus aureus* and *Streptococcus mutans* observed in previous studies.

MATERIALS AND METHODS

Chemicals. HPLC-grade solvents (methanol, acetic acid, and formic acid), glyoxal, methylglyoxal, diacetyl, quinoxaline, 2-methylquinoxaline, 2,3-dimethylquinoxaline, 5-methylquinoxaline, 1,2-diaminobenzene, 5-*O*-caffeoylquinic acid, caffeine, and 5-hydroxymethylfurfural were purchased from Sigma-Aldrich (St. Louis, MO).

Coffee Beans and Coffee Extract (Coffee Brew) Preparation. Green *Coffea 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 lost due to vapor formation and cell fragment loss. Loss was 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 then subdivided into three aliquots. One was freeze–dried, and the dry matter was dissolved in an appropriate volume of Millipore-grade water for antibacterial activity testing; the second was subjected to dialysis and the third to chemical analysis.

Dialysis. Sequential dialysis was performed using a Spectra/Por Biotech cellulose ester membrane (Spectrum Europe B.V., Breda, The Netherlands) with molecular mass cutoffs of 3500, 1000, and 500 Da. Aliquots (10 mL) of coffee extract were fractionated by dialysis in 1000 mL Millipore-grade water for 6 h at 4 °C. Dialysates and retentates were freeze-dried; dry residues were assessed and then dissolved in 1 mL of Millipore-grade water. Recovered 5-*O*-caffeoylquinic acid (>95%) was used as a standard molecular mass marker. Dialysates and retentates were tested against *Sa. aureus* and *St. mutans*.

Bacterial Strains, Media, and Buffers. The following strains were used: *Sa. aureus* ATCC 25923 and *St. mutans* 9102 (*18*). *St. mutans* was cultured in Todd Hewitt Broth (THB) (Oxoid, Basingstoke, U.K.) at 37 °C and *Sa. aureus* in Tryptone Soya Broth (TBS) (Oxoid) at 37 °C.

Evaluation of the Coffee Brew Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC). Concentrated coffee extract (10×) was filtered through a 0.22 μm Millex GP membrane. MIC and MBC values were determined in Iso-Sensitest broth (ISB) (Oxoid) according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) procedures (*19*). The MIC was the lowest coffee solution concentration (% by volume or milligrams per milliliter) inhibiting observable growth; the MBC (% by volume or milligrams per milliliter) was the lowest concentration resulting in a >99.9% reduction of the initial inoculum (*20*). All experiments were performed in triplicate.

Sample Preparation for RP-HPLC–DAD Analysis. A C_{18} Sep-Pak cartridge (Waters, Milford, MA) was conditioned with methanol (10 mL) and distilled water (2 × 10 mL). Aliquots (2 mL) of coffee extract were passed through the cartridge at a flow rate of ≤2 mL/min. Polar substances, among which were coffee α-dicarbonyl compounds, i.e., glyoxal, methylglyoxal, and diacetyl, were eluted first, with 2 mL of Millipore-grade water (SPE-F1). Less polar substances, including chlorogenic acids, 5-hydroxymethylfurfural, and caffeine, were then eluted with 4 mL of methanol (SPE-F2). To determine the α-dicarbonyl compound content, SPE-F1 was derivatized with 1,2-diaminobenzene (1.0 mg) to obtain quinoxaline derivatives using the method described by de Revel et al. (*21*), with some modifications. The pH of the reaction mixture was adjusted to 8.0 with NaOH (0.5 M), and the mixture was kept at 60 °C for 3 h; after cooling, the solutions were injected into the HPLC system. SPE-F2 (diluted 1:5 with methanol) was directly analyzed by HPLC for the assessment of chlorogenic acids, 5-hydroxymethylfurfural, and caffeine. All experiments were performed in triplicate.

Reverse-Phase High-Performance Liquid Chromatography with Diode Array Detection (RP-HPLC–DAD). All experiments were performed using a 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a gradient quaternary pump, a thermostated column compartment, and a DAD apparatus. The Agilent Chemstation software was used for HPLC system control and data processing. Determinations were carried out using a 250 mm × 4.6 mm, 5 μm C18 Hypersil column (CPS Analitica, Milan, Italy) with a matching Lichrospher 100 RP-18, 5 mm guard column (Merck, Darmstadt, Germany).

Chromatographic Conditions for α-Dicarbonyl Compound Assessment. Chromatographic conditions for gradient elution were as follows: flow rate, 0.6 mL/min; volume injected, 20 μL; column temperature, 20 °C. UV spectra were recorded in the 190–600 nm range, and chromatograms were acquired at 314 nm. Separations were performed using a gradient of increasing methanol concentrations in water acidified (pH 3.00 ± 0.01) with 0.5% acetic acid (v/v) as follows: 40 min linear gradient from 10 to 50% methanol, 10 min linear gradient from 50 to 75% methanol, and 4 min increasing gradient segment to 100% methanol followed by a 4 min isocratic period with 100% methanol. The composition of the mobile phase was taken to the initial condition (10% methanol) in 4 min, and the column was equilibrated for 10 min before the next injection.

Chromatographic Conditions for 5-Hydroxymethylfurfural, Chlorogenic Acid, and Caffeine Assessment. Chromatographic conditions for gradient elution were as follows: flow rate, 1 mL/min; volume injected, 20 μL; column temperature, 20 °C. UV spectra were recorded in the 190–600 nm range, and chromatograms were acquired at 280 nm (5-hydroxymethylfurfural and caffeine) and 324 nm (chlorogenic acids). 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 mobile phase composition was taken to the initial condition (5% methanol) in 5 min, and the column was equilibrated for 5 min before the next injection.

Identification and Quantification of α-Dicarbonyl Compounds, 5-Hydroxymethylfurfural, Chlorogenic Acids, and Caffeine. Retention times and UV spectra were used to identify the quinoxaline derivatives, 5-hydroxymethylfurfural, 5-*O*-caffeoylquinic acid, and caffeine. Stock standard solutions of quinoxaline, 2-methylquinoxaline, 2,3-dimethylquinoxaline, 5-*O*-caffeoylquinic acid, 5-hydroxymethylfurfural, and caffeine were prepared by dissolving carefully weighed amounts of each standard compound in a 50% (v/v) methanol/Millipore-grade water mixture. Solutions were analyzed by RP-HPLC–DAD. Each standard solution was diluted with mobile phase to five final concentrations ranging from 5 to 100 μM for quinoxaline, 2-methylquinoxaline, and 2,3-dimethylquinoxaline, from 10 to 200 μg/mL for 5-hydroxymethylfurfural, and from 50 to 250 mg/mL for 5-*O*-caffeoylquinic acid and caffeine. Each concentration was analyzed in triplicate. Quantification of individual compounds was performed by the external standard method using a five-point regression curve.

Bioactive Roasted Coffee Compound Mixture Preparations. Six aqueous solution mixtures containing glyoxal, methylglyoxal, and diacetyl (mixture 1), 5-*O*-caffeoylquinic acid, 5-hydroxymethylfurfural, and caffeine (mixture 2), glyoxal, methylglyoxal, diacetyl, and 5-*O*-caffeoylquinic acid (mixture 3), glyoxal, methylglyoxal, diacetyl, and 5-hydroxymethylfurfural (mixture 4), glyoxal, methylglyoxal, diacetyl, and caffeine (mixture 5), and glyoxal, methylglyoxal, diacetyl, 5-*O*-caffeoylquinic acid, 5-hydroxymethylfurfural, and caffeine (mixture 6) were prepared at concentrations corresponding to a 10× coffee extract, like those used to evaluate coffee brew MIC and MBC values (**Table**

Table 1. α-Dicarbonyl Compounds, 5-Hydroxymethylfurfural, Caffeine, 5-*O*-Caffeoylquinic Acid, and Total Chlorogenic Acids in Roasted *C. robusta* Extract[a]

compound in SPE-F1	HPLC t_R (min)	content in CR
glyoxal	36.35	4.73 ± 0.14 μg/mL
methylgyoxal	43.52	11.64 ± 0.29 μg/mL
diacetyl	51.22	2.72 ± 0.06 μg/mL

compound in SPE-F2	HPLC t_R (min)	content in CR
5-hydroxymethylfurfural	17.78	180.92 ± 3.61 μg/mL
caffeine	25.94	2.18 ± 0.09 mg/mL
5-*O*-caffeoylquinic acid	25.94	0.62 ± 0.02 mg/mL
chlorogenic acids	–	1.68 ± 0.04 mg/mL

[a] All experiments were performed in triplicate.

1). With regard to 5-*O*-caffeoylquinic acid, it was added to each mixture at the total concentration of chlorogenic acids determined in coffee brew. Solutions were analyzed for antibacterial (MIC) and bactericidal (MBC) activity against *Sa. aureus* and *St. mutans.*

Statistical Analysis. The reported data represent the means of the values obtained from the analysis of three coffee extracts or fractions, analyzed in triplicate.

RESULTS AND DISCUSSION

Antibacterial and Bactericidal Activities of Roasted Coffee. Ten times concentrated roasted *C. robusta* extract (CR), i.e., coffee brew (containing 124.6 mg/mL dry matter), was tested for its antibacterial activity toward *Sa. aureus* and *St. mutans*. MICs and MBC values, reported in **Table 2**, showed that CR is active against both bacteria, with lower values for *St. mutans*. The extract obtained from green coffee exhibited no activity. These data agree with our previous results for green and roasted coffee extracts (*14*).

Antibacterial Compound Isolation. The compound(s) responsible for the antibacterial activity detected in roasted coffee was investigated. To isolate the active coffee components and obtain preliminary indications of their molecular mass, CR was dialyzed using membranes with different cutoffs starting from 3500 Da. This procedure enabled the separation of naturally occurring low-molecular mass coffee compounds (e.g., caffeine, chlorogenic acids, and trigonelline) or Maillard reaction-induced low-molecular mass products (e.g., 5-hydroxymethylfurfural and α-dicarbonyl compounds) from the higher-molecular mass components, such as polysaccharides, proteins, and medium- and high-molecular mass melanoidins. Dialysates and retentates were freeze-dried; dry residues were assessed, then dissolved in 1 mL of Millipore-grade water (10× concentration), and tested for antibacterial activity.

Results (**Table 2**) showed that only the dialysate (containing 94.8 mg/mL dry matter), corresponding to 76% of coffee brew dry matter, possesses antibacterial activity and that it has the same MIC values (expressed as % by volume) as CR against the two bacteria. The dialysate was then subjected to sequential dialysis with 1000 and 500 Da membrane cutoffs; dialysates and retentates were tested for antibacterial activity. Both <1000 and <500 Da dialysates, containing 58.1 and 54.1 mg/mL dry matter, respectively, were found to be active (**Table 2**) and to have the same MIC values (% by volume), indicating that the molecular mass of roasted coffee antibacterial compounds is <500 Da. The decreased level of dialysate dry matter explains the decrease in the dialysate MIC when expressed as milligrams per milliliter. Conversely, none of the retentates were seen to be active. These data agree with a previous report in which we attributed the antibacterial activity of coffee to unidentified compounds with a molecular mass of <200 Da (*14*).

Antibacterial Compound Identification. Since green coffee did not exhibit detectable antibacterial activity, the active compound(s) must arise during heat treatment, thus ruling out caffeine and other natural components. This conclusion was further supported by the fact that the concentrations of most natural compounds found in green coffee decreased in roasted coffee. We thus turned to α-dicarbonyl compounds and 5-hydroxymethylfurfural, the presence of which in roasted coffee has been reported in a number of papers (*22–24*). α-Dicarbonyl compounds are produced by the multiple-fragmentation reaction of sugar moiety; 5-hydroxymethylfurfural is formed by different pathways, mainly via dehydration of hexoses either in the presence of amines (Maillard reaction) or in their absence (charbohydrate caramelization), giving 3-deoxyhexuloses that can further react to give 5-hydroxymethylfurfural (*25*). The tests carried out on standard glyoxal, methylglyoxal, diacetyl, and 5-hydroxymethylfurfural aqueous solutions showed that all α-dicarbonyl compounds possessed strong antibacterial activity, particularly against *St. mutans*, whereas 5-hydroxymethylfurfural exhibited weak antibacterial activity against both bacteria; *Sa. aureus* MBC values could not be defined (**Table 3**). Standard 5-*O*-caffeoylquinic acid and caffeine aqueous solutions were also tested. 5-*O*-Caffeoylquinic acid exhibited weak antibacterial activity against both bacteria and no bactericidal activity against *Sa. aureus* (MBC > 50%), whereas caffeine exhibited low activity against *St. mutans* (high MIC and MBC) and no activity against *Sa. aureus* (MIC and MBC > 50%) (**Table 3**).

The tested compounds and the total concentration of the compounds of the broad chlorogenic acid family occurring in coffee brew were then determined in CR. This analysis required a preliminary step. The more polar compounds (glyoxal, methylglyoxal, and diacetyl) were separated from the less polar compounds (5-hydroxymethylfurfural, chlorogenic acids, and caffeine) by solid-phase extraction (SPE) using a C_{18}-SPE cartridge. Glyoxal, methylglyoxal, and diacetyl were recovered with water (SPE-F1), whereas elution of the less water soluble 5-hydroxymethylfurfural, chlorogenic acids, and caffeine (SPE-F2) required methanol.

Assessment of α-dicarbonyl compounds by RP-HPLC–DAD required the formation of quinoxaline derivatives (*21*). SPE-F1 (**Figures 1** and **2B**) was derivatized using 1,2-diaminobenzene (pH 8.0) for 3 h at 60 °C after the addition of 5-methylquinoxaline, used as the internal standard (**Figure 2C**). Derivatives were identified by comparison of their retention times (**Figure 2A**) and UV spectra with those of the standard compounds. Quantitative analysis of α-dicarbonyl compounds by the external standard method yielded the CR glyoxal, methylglyoxal, and diacetyl concentrations reported in **Table 1**.

5-Hydroxymethylfurfural, chlorogenic acids, and caffeine were analyzed by RP-HPLC–DAD by direct injection of diluted SPE-F2 (**Figure 2C,D**) and were identified by comparing their retention times (**Figure 2A,B**) and UV spectra with those of the standard compounds. The other chlorogenic acids (*26*) occurring in roasted coffee (such as 5-*O*-caffeoylquinic acid isomers, feruoylquinic acids, and di- and tricaffeoyl/feruoylquinic acids) were identified by their characteristic UV spectra (λ_{max} = 324 nm) and quantified by the external standard method. Their content was expressed as 5-*O*-caffeoylquinic acid (**Table 1**).

To establish their actual role in roasted coffee antibacterial activity, all the coffee components considered were tested in different mixtures at the concentrations found in concentrated

Table 2. Antibacterial (MIC) and Bactericidal (MBC) Activities of Roasted *C. robusta* Extract (CR)[a]

	Sa. aureus ATCC 25923				*St. mutans* 9102			
	MIC		MBC		MIC		MBC	
coffee sample	% v/v	mg/mL	% v/v	mg/mL	% v/v	mg/mL	% v/v	mg/mL
coffee extract	20 ± 2	24.9 ± 2.4	30 ± 2	37.2 ± 2.5	10 ± 1	12.4 ± 1.2	20 ± 2	24.9 ± 2.5
>3500 Da dialysate	20 ± 1	18.8 ± 2.3	nd[b]	–	10 ± 1	9.4 ± 0.9	nd[b]	–
<3500 Da retentate	>50	–			>50	–		
>1000 Da dialysate	20 ± 2	11.6 ± 1.2	nd[b]	–	10 ± 1	5.8 ± 0.6	nd[b]	–
<1000 Da retentate	>50	–			>50	–		
>500 Da dialysate	20 ± 1	10.8 ± 0.5	nd[b]	–	10 ± 1	5.4 ± 0.5	nd[b]	–
<500 Da retentate	>50	–			>50	–		

[a] MIC values of CR dialysis fractions (dialysate and retentate) were obtained with different cutoff membranes against *Sa. aureus* and *St. mutans*. All experiments were performed in triplicate. [b] Not determined.

Table 3. Antibacterial (MIC) and Bactericidal (MBC) Activities of Bioactive Coffee Components against *Sa. aureus*and *St. mutans*[a]

	Sa. aureus ATCC 25923				*St. mutans* 9102			
	MIC		MBC		MIC		MBC	
coffee compound	% v/v	μg/mL	% v/v	μg/mL	% v/v	μg/mL	% v/v	μg/mL
glyoxal	15 ± 2	88.3 ± 11.8	30 ± 2	176.7 ± 11.8	2 ± 0.2	11.8 ± 1.2	2 ± 0.2	11.8 ± 1.2
methylglyoxal	15 ± 1	110.2 ± 5.9	40 ± 3	294.0 ± 22.1	2 ± 0.2	14.7 ± 1.5	2 ± 0.2	14.7 ± 1.5
diacetyl	10 ± 1	114.2 ± 11.4	10 ± 2	114.2 ± 11.4	3 ± 0.2	34.3 ± 2.3	6 ± 0.3	68.5 ± 3.4

	Sa. aureus ATCC 25923				*St. mutans* 9102			
	MIC		MBC		MIC		MBC	
coffee compound	% v/v	mg/mL	% v/v	mg/mL	% v/v	mg/mL	% v/v	mg/mL
5-hydroxymethylfurfural	30 ± 2	8.4 ± 0.6	>50	–	10 ± 1	2.8 ± 0.3	20 ± 1	5.6 ± 0.3
5-*O*-caffeoylquinic acid	35 ± 2	6.3 ± 0.4	>50	–	15 ± 3	2.7 ± 0.5	20 ± 3	3.6 ± 0.5
caffeine	>50	–	>50	–	20 ± 1	5.0 ± 0.2	50 ± 3	12.5 ± 0.7

[a] All experiments were performed in triplicate.

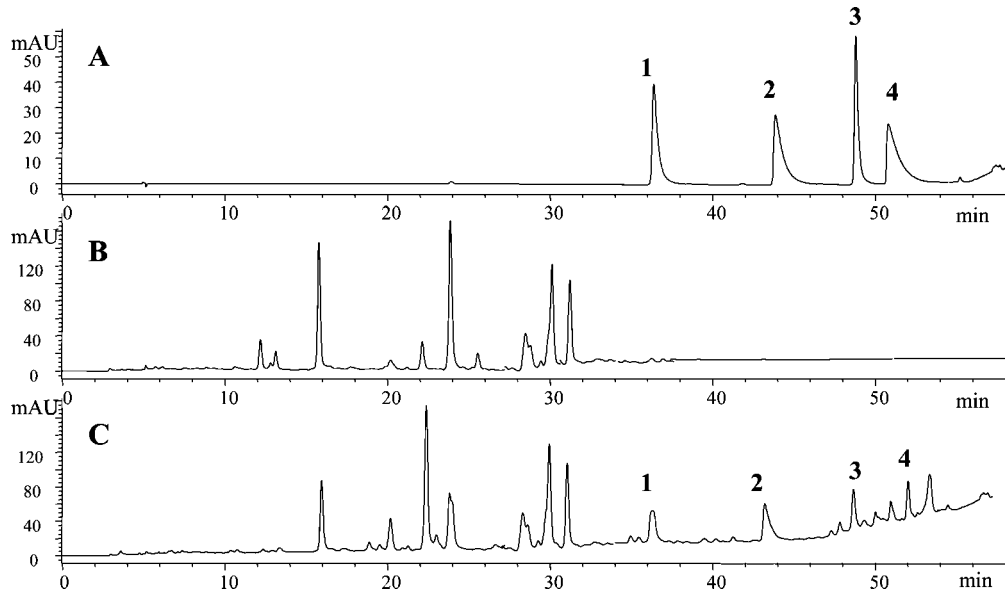

Figure 1. RP-HPLC–DAD chromatograms. (**A**) Standard quinoxaline derivative solution detected at 314 nm and SPE-F1 results obtained from roasted coffee CR (**B**) before and (**C**) after derivatization with 1,2-diaminobenzene: (1) quinoxaline, (2) 2-methylquinoxaline, (3) 5-methylquinoxaline, and (4) 2,3-dimethylquinoxaline.

10× CR (**Table 4**). Analysis of the α-dicarbonyl compound mixture showed that together they account for about 50% of the antibacterial activity of roasted coffee, the MIC values of the α-dicarbonyl compound mixture being about twice the CR MIC values. In contrast, analysis of the mixture of naturally occurring compounds revealed no activity. The addition of 5-*O*-caffeoylquinic acid (mixture 2) or 5-hydroxymethylfurfural (mixture 3) to the α-dicarbonyl compound mixture failed to affect MIC values, indicating that chlorogenic acids and 5-hydroxymethylfurfural at the concentrations found in roasted coffee do not influence its antibacterial activity. Conversely, the presence of caffeine (mixture 4) in the previously tested α-dicarbonyl compound mixture considerably reduced the α-dicarbonyl compound mixture MIC values, which were close to the CR MIC values. This indicates a synergistic effect of caffeine with respect to α-dicarbonyl compound activity. Therefore, in the

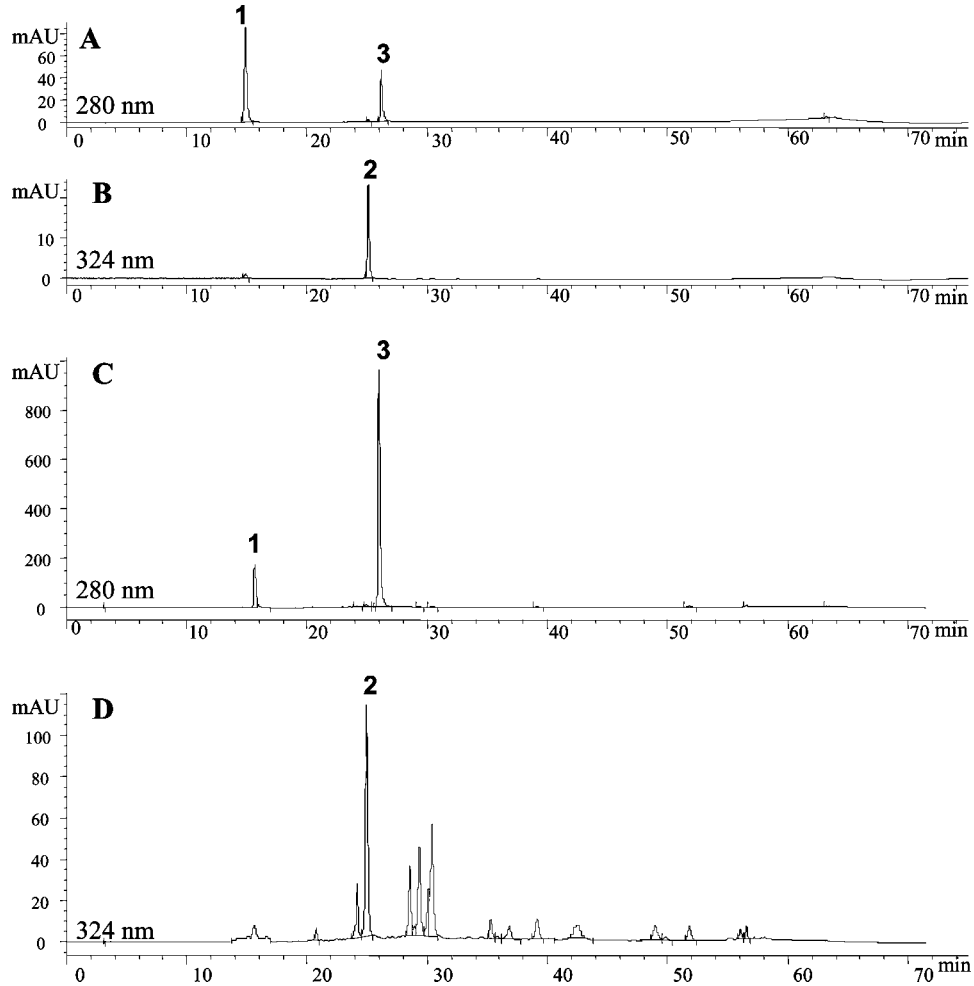

Figure 2. RP-HPLC–DAD chromatograms. Standard 5-hydroxymethylfurfural, 5-*O*-caffeoylquinic acid, and caffeine solution detected at (**A**) 280 and (**B**) 324 nm. SPE-F2 results obtained from roasted coffee CR detected at (**C**) 280 and (**D**) 324 nm: (1) 5-hydroxymethylfurfural, (2) caffeine, and (3) 5-*O*-caffeoylquinic acid.

Table 4. Antibacterial (MIC) and Bactericidal (MBC) Activities of Standard Bioactive Coffee Component Mixtures at the Same Concentrations Found in CR (10× concentrated) against *Sa. aureus* and *St. mutans*[a]

	Sa. aureus ATCC 25923		*St. mutans* 9102	
coffee compound mixture	MIC % v/v	MBC % v/v	MIC % v/v	MBC % v/v
mixture 1	50 ± 2	>50	20 ± 1	40 ± 1
mixture 2	>50	>50	>50	>50
mixture 3	50 ± 2	>50	20 ± 1	40 ± 3
mixture 4	50 ± 3	>50	20 ± 2	40 ± 2
mixture 5	30 ± 1	50 ± 2	9 ± 1	30 ± 2
mixture 6	40 ± 2	50 ± 2	9 ± 1	20 ± 3

[a] All experiments were performed in triplicate.

presence of α-dicarbonyl compounds, caffeine, which has weak intrinsic antibacterial activity, is capable of enhancing their total activity and appears to be responsible for approximately half of the antibacterial activity of roasted coffee. The synergistic activity of caffeine is observed even when a lower concentration of caffeine (5 mg/mL) is added to α-dicarbonyl compound mixture, corresponding to approximately the residue mean caffeine content occurring in decaffeinated coffee (10× concentrated) (*27*). This findings justify the results obtained by our group in a previous investigation (data not shown) that indicated that decaffeinated coffee brew exhibited antibacterial activity not significantly different from that in coffee brew.

In conclusion, naturally occurring coffee compounds do not explain the antibacterial activity of coffee brew. Therefore, α-dicarbonyl compounds, formed during the roasting process, were investigated. Glyoxal, methylglyoxal, and diacetyl have so far been studied largely for their cytotoxic, mutagenic, and genotoxic activities, whereas to the best of our knowledge, their antibacterial activity has never been investigated. The very low MIC values of standard glyoxal, methylglyoxal, and diacetyl point to these α-dicarbonyl compounds as the main agents responsible for the beverage's antibacterial activity. Nevertheless, their low concentrations in roasted coffee do not account for all of the beverage's antibacterial activity. However, their MICs are close to coffee brew MIC values when the synthetic mixture of α-dicarbonyl compounds added with caffeine is tested at the same concentrations found in coffee. These findings indicate that caffeine can synergistically enhance α-dicarbonyl compound activity and that glyoxal, methylglyoxal, and diacetyl in the presence of caffeine account for the whole antibacterial activity of roasted coffee.

ABBREVIATIONS USED

MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; SPE, solid-phase extraction; THB, Todd Hewitt broth; ISB, Iso-Sensitest broth; CR, *C. robusta* coffee extract.

ACKNOWLEDGMENT

We thank MOKA SIR'S srl (Pavia, Italy) for providing the coffee samples.

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Received for review July 27, 2007. Revised manuscript received September 17, 2007. Accepted September 24, 2007. The work was supported by a grant from MIUR 2005 to G.G. and took place within the Interdepartmental Center of Food Quality and Safety (CISQUA).

JF0722607