JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Determination of α-Dicarbonyl Compounds by RP-HPLC-DAD in Green and Roasted Coffee

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Glyoxal, methylglyoxal, and diacetyl formed as Maillard reaction products in heat-treated food were determined in coffee extracts (coffee brews) obtained from green beans and beans with different degrees of roast. The compounds have been reported to be mutagenic in vitro and genotoxic in experimental animals in a number of papers. More recently, α-dicarbonyl compounds have been implicated in the glycation process. Our data show that small amounts of glyoxal and methylglyoxal occur naturally in green coffee beans. Their concentrations increase in the early phases of the roasting process and then decline. Conversely, diacetyl is not found in green beans and forms later in the roasting process. Therefore, light and medium roasted coffees had the highest glyoxal and methylglyoxal content, whereas dark roasted coffee contained smaller amounts of glyoxal, methylglyoxal, and diacetyl. For the determination of coffee α-dicarbonyl compounds, a reversed-phase high performance liquid chromatography with a diode array detector (RP-HPLC-DAD) method was devised that involved the elimination of interfering compounds, such as chlorogenic acids, by solid phase extraction (SPE) and their derivatization with 1,2-diaminobenzene to give quinoxaline derivatives. Checks of SPE and derivatization conditions to verify recovery and yield, respectively, resulted in rates of 100%. The results of the validation procedure showed that the proposed method is selective, precise, accurate, and sensitive.

KEYWORDS: Coffee; glyoxal; methylglyoxal; diacetyl; HPLC-DAD; roasting process

INTRODUCTION

Dicarbonyl compounds are reactive intermediates formed during food heat treatment, such as roasting, baking, broiling, and frying. Caramelization and the Maillard reaction are the two main types of reactions that occur in heated foods (*1*). At the chemical level, caramelization involves ring-opening, enolization of reducing sugars, and sugar dehydration, which consists of a sequence of water *β*-eliminations from sugar molecules and formation of α-dicarbonyl compounds. This sequence of reactions is normally a slow process. It becomes significant at high sugar concentrations, in the presence of a catalytic base, and at high temperature. The Maillard reaction consists of three main stages. In the first, an amino acid condenses with a reducing sugar to form the Schiff base, which undergoes cyclization to N-substituted 1-amino-1-deoxy-2-ketose by Amadori rearrangement. Recent studies suggest that α-dicarbonyl compounds are formed from Schiff base. A major pathway of the Maillard reaction leads from the 1,2-eneaminol form of the Amadori compound to a deoxyhexosone intermediate (*2*). A minor pathway involves conversion of 2,3-enediol to the methyl α-dicarbonyl intermediate, which undergoes transformation to aldehydes, ketones, furans, pyrroles, quinolines, and indoles. In foods heated at temperatures much higher than 100 °C in the absence of free water, the process of thermal degradation, called pyrolysis, involves sugars, amino acids, and other organic constituents.

The most extensively studied α-dicarbonyl compounds are methylglyoxal, found in foodstuffs such as bread, soy sauce, instant tea, and roasted coffee, and glyoxal, which has attracted considerable attention as a lipid peroxidation product. Glyoxal can also derive from sugar fragmentation and has been isolated in many foodstuffs such as roasted coffee and cocoa (*3*).

The toxicological profile of α-dicarbonyl compounds, particularly glyoxal and methylglyoxal, is not completely clear. In the 1980s, they came under scrutiny for their possible carcinogenic potential due to their in vitro mutagenic activity (*4*) and genotoxicity in experimental animals (*5–7*). In recent years, the interest has focused on the implications of the glycation process. The formation of α-dicarbonyl compounds is a key step in the Maillard reaction because these intermediates are up to 20000 times more reactive than glucose in glycation reactions (*8*). Advanced glycation end products (AGEs) formed by nonenzy-

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10.1021/jf071917l CCC: $37.00 © 2007 American Chemical Society
Published on Web 10/10/2007

matic glycations and glycoxidations are implicated in the pathophysiological changes associated with a number of diseases, such as diabetes, atherosclerosis, and neurodegenerative disorders (*9–12*). AGEs form in physiological conditions in the presence of α-dicarbonyl compounds derived from a Schiff base without the delay involved in Amadori rearrangements. Methylglyoxal, as an AGE precursor, has been shown to be highly active in glycation even at the low concentrations occurring in physiological systems. The role of dietary glycation products is debated. As considerable amounts of Maillard reaction products are formed in foods due to heat processing, recent papers discuss food-derived AGEs as possible glycotoxins (*13–15*).

Various methods have been developed to determine α-dicarbonyl compounds. Methylglyoxal has been determined by spectrometry after chemical derivatization with 2,4-dinitrophenylhydrazine or 4-amino-5-hydrazino-3-mercapto-1,2,4-triazole (*16*). α-Dicarbonyl compounds have also been analyzed by gas chromatography (GC) after derivatization with cysteamine to give 2-acetyl-thiazolidine derivatives (*17*). The best current assays involve derivatization of α-dicarbonyl substances with 1,2-diaminobenzene followed by quantification of the resulting quinoxaline by GC or high performance liquid chromatography (HPLC). Methods for the determination of α-dicarbonyl compounds have been applied to different matrices, such as wine (*18*) and cultured animal cells (*19*). However, only GC has been applied to coffee, probably due to the complexity of its matrix (*20–22*).

The aims of this study were (1) to devise a method of sample preparation allowing simultaneous and accurate determination of glyoxal, methylglyoxal, and diacetyl in coffee brew by reversed-phase high performance liquid chromatography with a diode array detector (RP-HPCL-DAD) (2) to investigate their formation kinetics during the roasting process.

MATERIALS AND METHODS

Chemicals and Standards. HPLC-grade solvents (methanol and acetic acid), glyoxal (MW 58.04 Da), methylglyoxal (MW 72.06 Da), diacetyl (MW 89.09 Da), quinoxaline (MW 130.15 Da), 2-methylquinoxaline (MW 144.18 Da), 2,3-dimethylquinoxaline (MW 158.20 Da), 5-methylquinoxaline, and 1,2-diaminobenzene were purchased from Sigma-Aldrich (St. Louis, MO).

Coffee Sample and Coffee Extract Preparation. A 200 g aliquot of *Coffea robusta* (CR) beans from Java was roasted up to 210 °C in a pilot roaster apparatus (STA Impianti S.r.l., Bologna, Italy) that allowed the collection of small aliquots of coffee beans every 2 min. During roasting, 20 g aliquots of coffee beans were collected every 2 min for 20 min to obtain 10 batches with 10 different degrees of roast (CR2-CR20). On the basis of the weight lost during the roasting process, due to vapor formation and cell fragment loss, CR samples roasted for 10 min (CR10, weight loss 11%), 14 min (CR14, weight loss 14%), and 20 min (CR20, weight loss 20%) are generally considered as light, medium, and dark roasted coffee, respectively. All batches were ground in a laboratory scale mill and passed through a no. 30 sieve. Green and roasted coffee extracts were prepared by boiling 10 g aliquots of coffee powder in 100 mL of Millipore grade water for 10 min; each 100 mL solution was filtered through a cellulose acetate/cellulose nitrate mixed esters membrane (0.45 μm; Millipore Corporation, Billerica, MA).

Solid-Phase Extraction (SPE). 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 glyoxal, methylglyoxal, and diacetyl water solution mixture (30 μM) or of coffee extracts obtained from beans with different degrees of roast were passed through the cartridge at a flow rate of ≤2 mL min^{-1}. Polar substances were eluted first, with 2 mL of Millipore-grade water (SPE-F1). Less polar substances were then eluted of with 4 mL of methanol (SPE-F2). The two SPE fractions were concentrated to dryness by a Rotavapor R-210 rotary evaporator (BÜCHI Italia S.r.l., Milan, Italy), and the residues were dissolved in 2 mL of Millipore-grade water. All experiments were performed in triplicate.

Quinoxaline Derivative Preparation. The method described by de Revel et al., with some modifications (*18*), was used for the preparation of derivatized quinoxaline. 1,2-Diaminobenzene (1 mg) was added to 2 mL of α-dicarbonyl standard aqueous solutions of glyoxal (75.2 μM), methylglyoxal (75.2 μM) or diacetyl (79.3 μM) separately. The derivatization reaction was carried out also on coffee extract (CR14) as well as the SPE-F1 fraction obtained either from the α-dicarbonyl standard mixture (each compound at the same concentrations reported above) or from coffee extracts. The pH was adjusted to 8.0 with NaOH (0.5 M). The reaction mixture was kept at 60 °C for 3 h; after cooling, the solutions were directly injected into the HPLC system.

HPLC Analysis. All experiments were performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a gradient quaternary pump, a thermostatted column compartment, and a DAD. The Agilent Chemstation software was used for control of the HPLC system and data processing. Quinoxaline derivatives were separated by a C18 Hypersilcolumn 250 × 4.6 mm, 5 μm (CPS Analitica, Milan, Italy) with matching Lichrospher 100 RP-18, 5 mm guard column (Merck, Darmstadt, Germany).

Chromatographic conditions for gradient elution were as follows: flow rate, 0.6 mL min^{-1}; 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, 4 min increasing gradient segment to 100% methanol followed by a 4 min isocratic with 100% methanol. The mobile phase composition was taken to the initial condition in 4 min, and the column was equilibrated 10 min before the next injection.

Identification and Quantification of Quinoxaline Derivatives. Retention times and UV spectra were used to identify the quinoxaline derivative peaks. Stock standard solutions of quinoxaline (Q), 2-methylquinoxaline (2MQ), and 2,3-dimethylquinoxaline (2,3DMQ) were prepared by dissolving carefully weighed amounts of each standard compound in 50% (v/v) methanol–Millipore-grade water. Solutions were analyzed by RP-HPLC-DAD. Each standard solution was diluted with mobile phase to six final concentrations ranging from 7.5 to 150 μM for Q and 2MQ, and 5–100 μM for 2,3DMQ. Each concentration was analyzed in triplicate. Quantification of individual compounds was performed by the external standard method using a six-point regression curve. The peak area was plotted against concentration, and least-squares regression analysis was used to fit lines to the data. As the molar ratio between α-dicarbonyl compounds and the respective quinoxaline derivatives is 1:1, the amount of each α-dicarbonyl compound was expressed as μmol L^{-1} (μM). In **Figure 2**, the concentration of α-dicarbonyl compounds is expressed as mg/100 g of coffee powder.

RESULTS AND DISCUSSION

Isolation and Derivatization of Coffee α-Dicarbonyl Compounds. Derivatization of α-dicarbonyl compounds occurring in coffee solutions was performed using 1,2-diaminobenzene (pH 8.0) for 3 h at 60 °C, as described by de Revel et al. (*18*) for the determination of α-dicarbonyl compounds in red and white wines. To verify the yield of the reaction, aqueous solutions of standard glyoxal, methylglyoxal, and diacetyl were derivatized as described and analyzed by RP-HPLC-DAD after addition of 5-methylquinoxaline (5MQ), used as internal standard. Standard Q, 2MQ, 2,3DMQ, and 5MQ solutions at the same concentrations were then analyzed. The ratios of the peak areas of standard quinoxaline derivatives (Q, 2MQ, and 2,3DMQ) or α-dicarbonyl compounds derivatized with 1,2-diaminobenzene to the 5MQ peak area showed that under our experimental conditions, the yield of the reaction was 99.8–

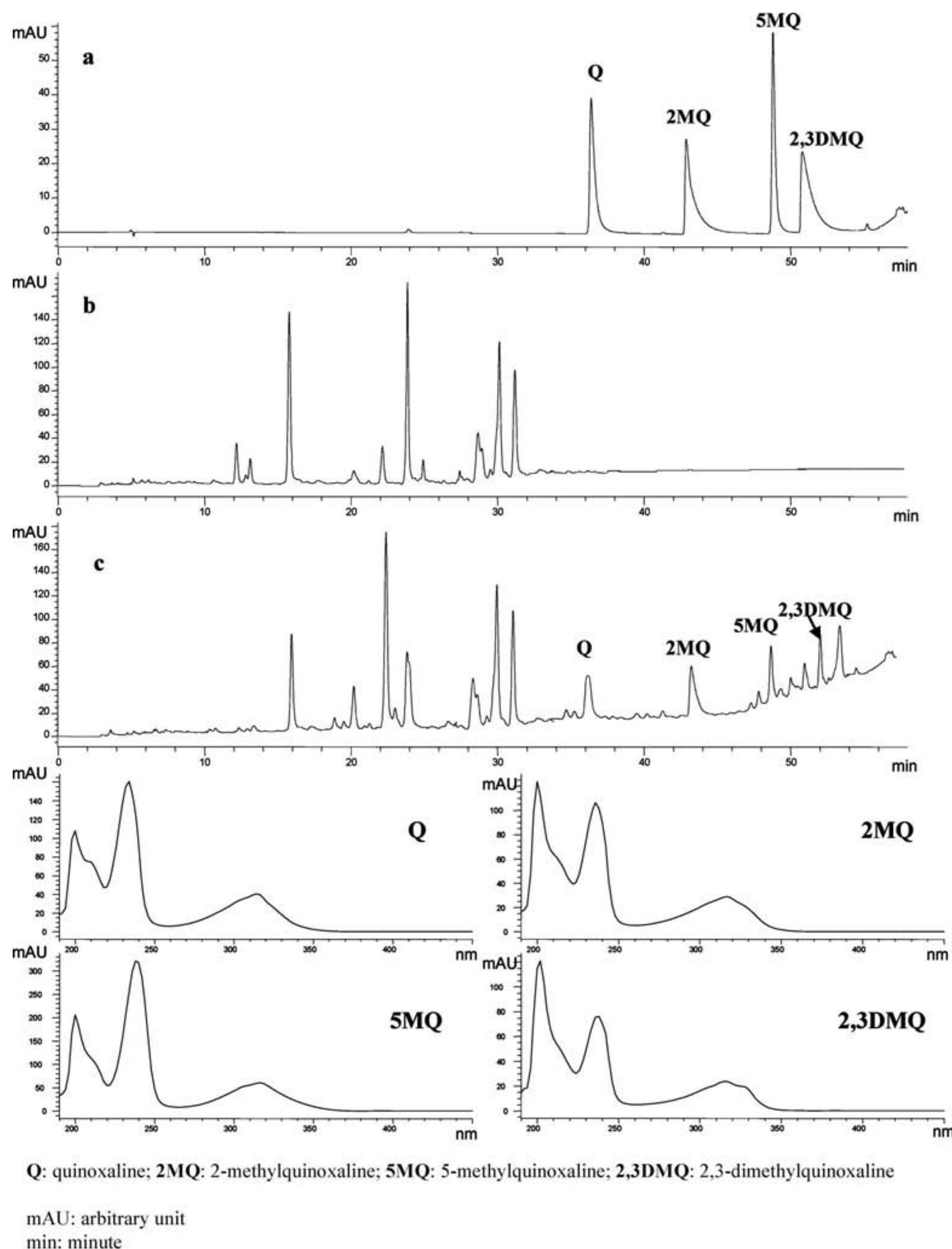

Figure 1. (**a**) RP-HPLC-DAD chromatograms: standard quinoxaline derivatives detected at 314 nm; (**b**) SPE-F1 obtained from CR14 roasted coffee before derivatization with 1,2-diaminobenzene; (**c**) SPE-F1 obtained from CR14 roasted coffee after derivatization with 1,2-diaminobenzene.

Table 1. Yields of Reaction of α-Dicarbonyl Compounds Derivatized with 1,2-Diaminobenzene[a]

		standard		derivatized	
compound	concentration [μM]	peak area[c]	ratio[d]	peak area[c]	ratio[e]
quinoxaline	75.2	928.861	0.981	926.735	0.979
2-methylquinoxaline	75.2	941.621	0.994	942.421	0.995
2,3-dimethylquinoxaline	79.3	1044.12	1.102	1043.281	1.102
5-methylquinoxaline[b]	75.2	947.219		946.898	

[a] All experiments were performed in triplicate. [b] Internal standard. [c] Expressed as arbitrary units. [d] Ratio of standard quinoxaline derivatives to 5MQ peak area. [e] Ratio of derivatized α-dicarbonyl compounds to 5MQ peak area.

100.1% for the considered α-dicarbonyl compounds, in line with the results reported by de Revel et al. (*18*) (**Table 1**).

For the initial phase of the investigation, a coffee solution obtained from beans roasted for 14 min (CR14; medium degree of roast) was subjected to derivatization. α-Dicarbonyl compounds were then analyzed by RP-HPLC-DAD by direct injection of the reaction mixture using gradient elution. Various compositions of the acetic acid/water–methanol eluting mixture were tested to resolve the derivatized α-dicarbonyl compounds from the other coffee components. Satisfactory resolution was achieved for derivatized 2MQ and 2,3DMQ. These quinoxaline derivatives were identified by comparing their retention times

Table 2. Recovery Rates of Standard α-Dicarbonyl Compounds after SPE[a]

compound	concentration [μM]	standard		derivatized SPE-F1	
		peak area[c]	ratio[d]	peak area[c]	ratio[e]
quinoxaline	75.2	928.861	0.980	924.735	0.974
2-methylquinoxaline	75.2	941.621	0.994	938.421	0.989
2,3-dimethylquinoxaline	79.3	1044.12	1.100	1053.281	1.110
5-methylquinoxaline[b]	75.2	947.219		948.898	

[a] All experiments were performed in triplicate. [b] Internal standard. [c] Expressed as arbitrary units. [d] Ratio of standard quinoxaline derivatives to 5MQ peak area. [e] Ratio of derivatized α-dicarbonyl compounds (obtained from SPE-F1) to 5MQ peak area.

Table 3. Calibration Data and Limit of Quantification (LOQ) and Detection (LOD) of α-Dicarbonyl Compounds

compound	linear range (μmol/L)	regression equation	correlation coefficient	LOQ (μmol/L)	LOD (μmol/L)
quinoxaline	7.0–150.0	$y^a = 12.63x^b - 44.58$	0.9989	0.18	0.05
2-methylquinoxaline	7.0–150.0	$y = 12.49x - 34.96$	0.9974	0.20	0.05
2,3-dimethylquinoxaline	5.0–100.0	$y = 13.38x - 16.46$	0.9999	0.06	0.02

[a] *y* is the peak area in arbitrary units. [b] *x* is the concentration of quinoxaline derivatives (μM).

Table 4. Method Precision for the Determination of α-Dicarbonyl Compounds in Coffee Extract (CR14) SPE-F1 Derivatized with 1,2-Diaminobenzene[a]

compound	mean content (μM) ± SD			RSD (%) intra-assay (day 1)	RSD (%) interassay
	day 1	day 2	day 3		
glyoxal	55.80 ± 2.02	54.28 ± 1.99	52.80 ± 1.56	3.62	3.41
2-methylglyoxal	80.66 ± 1.68	78.66 ± 1.54	80.39 ± 1.67	2.08	2.04
diacetyl	17.55 ± 0.47	17.06 ± 0.58	17.52 ± 0.46	2.67	2.89

[a] All experiments were performed nine times.

Table 5. Recovery Rates of the Method for α-Dicarbonyl Compound Determination in Coffee Extracts SPE-F1

compound	amount added (μg/L)			amount found[a] (μg/L)			% recovery ($n = 3$)		
quinoxaline	2.10	10.00	18.00	2.16	10.02	18.32	102.9	100.2	101.8
2-methylquinoxaline	1.50	10.00	20.50	1.46	10.21	20.77	97.33	102.1	101.3
2,3-dimethylquinoxaline	1.50	7.50	15.00	1.51	7.68	15.21	100.7	102.4	101.4

[a] Values represent the mean of three determinations.

and UV spectra with those of the standard compounds. The determination of derivatized glyoxal (Q) failed due to overlapping peaks. Q was coeluted with a coffee compound whose spectrum showed it to belong to the chlorogenic acid family. Changes in chromatographic conditions never resulted in peak separation.

To eliminate the interfering compounds, coffee extracts were subjected to SPE before derivatization using a C18-SPE cartridge. Glyoxal, methylglyoxal, and diacetyl were recovered with water (SPE-F1), whereas elution of the less water soluble chlorogenic acids (SPE-F2) required methanol.

Freed of interfering components (**Figure 1b**), α-dicarbonyl compounds could then be derivatized and analyzed simultaneously by RP-HPLC-DAD. In fact, the derivatized SPE-F1 (**Figure 1c**) exhibited three peaks with the same retention times and UV spectra as the standard Q, 2MQ, and 2,3DMQ chromatograms (**Figure 1a**). The standard glyoxal, methylglyoxal, and diacetyl mixture was subjected to SPE to check that all α-dicarbonyl compounds had been recovered by the SPE procedure. SPE-F1 given by the standard mixture was derivatized with 1,2-diaminobenzene after adding 5MQ, and analyzed by HPLC. The ratios between the peak areas (**Table 2**) of the standard Q, 2MQ, and 2,3DMQ or α-dicarbonyl derivatized with 1,2-diaminobenzene obtained from SPE-F1 to 5MQ peak area at the same concentrations showed that recovery after SPE was 99.4–100.9% for the considered α-dicarbonyl compounds.

Method Validation. Coffee α-dicarbonyl compounds were quantified by RP-HPLC-DAD using the external standard method with six-point regression curve, by plotting the concentration of standard quinoxaline derivatives (μM) as a function of peak area detected at 314 nm, corresponding to their UV absorption maxima. Regression equations and correlation coefficients, reported in **Table 3**, were calculated by the least squares method. The limit of detection (LOD), calculated as the amount of analyte required to obtain a signal-to-noise ratio of 3, was 0.05 μmol L^{-1}, for Q and 2MQ and 0.02 μmol L^{-1}, for 2,3DMQ. The limit of quantification (LOQ), that is, the lowest concentration required to yield a signal-to-noise ratio of 10, was 0.20, 0.18, and 0.06 μmol L^{-1} for Q, 2MQ, and 2,3DMQ, respectively (**Table 3**).

Method repeatability (intra- and interassay) was evaluated by analyzing the CR14 derivatized coffee SPE-F1 solution nine times in three days (**Table 4**). The inter- and intraassay relative standard deviation (RSD) of the α-dicarbonyl compounds was consistently $<3.6\%$.

The accuracy of the HPLC method was determined by recovery tests performed by adding three different concentrations of standard quinoxaline derivative solutions to coffee SPE-F1 before derivatization. Spiked samples were then subjected to the entire procedure. The results showed recovery rates between 97 and 103% (**Table 5**).

Formation Kinetics of α-Dicarbonyl Compounds during the Roasting Process. Green and roasted coffee extracts obtained from beans with different degrees of roasting were analyzed by RP-HPLC-DAD to verify α-dicarbonyl compounds content in relation to degree of coffee bean roasting. The results (**Figure 2**) showed that very small amounts (about 1 mg/100 g coffee powder) of glyoxal and methylglyoxal occurred in green

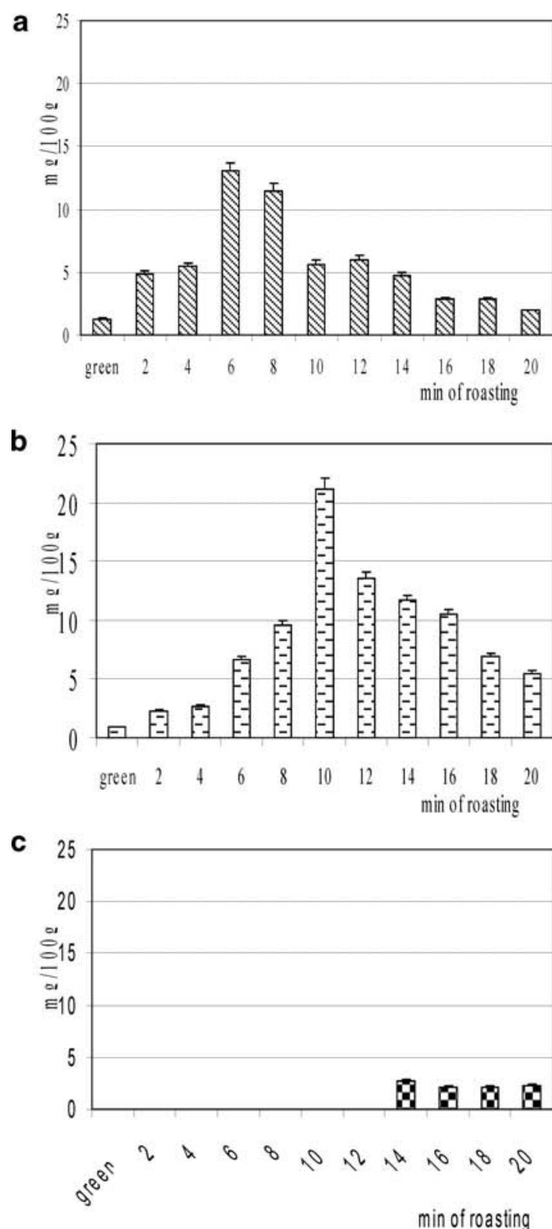

Figure 2. Quantification of (**a**) glyoxal, (**b**) methylglyoxal, and (**c**) diacetyl in green coffee beans and in beans with different degrees of roast (2–20 min).

coffee. Their content increased in the early phases of roasting; diacetyl was not found in green beans and was detected only after 14 min of roasting. Glyoxal content increased in the first 6 min of roasting, peaking at 13.07 ± 0.39 mg/100 g, then slowly decreased to 1.93 ± 0.05 at 20 min. Methylglyoxal exhibited a similar behavior; its concentration (21.19 ± 0.42 mg/100 g) peaked at 10 min and subsequently declined until the end of the roasting process. The maximum concentration of methylglyoxal was about twice that of glyoxal. The formation kinetics of diacetyl was different; the compound was found at a very low concentrations in roasted coffee, peaking between 14 and 16 min then remaining stable until the end of roasting (peak concentration 2.28 ± 0.07 mg/100 g).

Recovery tests were carried out by adding the standard solutions to coffee SPE-F1 before derivatization. Spiked samples were then subjected to the entire procedure.

In conclusion, glyoxal, methylglyoxal, and diacetyl, bioactive compounds formed as Maillard reaction products in heat-treated food, were determined in coffee extracts (coffee brews) obtained from green and roasted coffee beans with different degrees of roast.

Our data show that small amounts of glyoxal and methylglyoxal occur naturally in green coffee beans, probably as secondary lipid peroxidation products. Their concentrations increase in the early phases of the roasting process (after 6–10 min) and then decline. Conversely, diacetyl is not found in green coffee beans and forms later in the roasting process (14 min). Therefore, light and medium roasted coffees had the highest glyoxal and methylglyoxal content, whereas dark roasted coffee contained smaller amounts of glyoxal, methylglyoxal, and diacetyl. The reduction in their concentrations in the final phase of the roasting process may be ascribed to their involvement in the advanced stages of Maillard reaction, which result in brown pigments (melanoidins) formed by self- and cross-condensation of the aldehydes and ketones forming as fragmentation products (*1*). Because of the high reactivity of α-dicarbonyl compounds, which have been implicated in the glycation process, it would be interesting to monitor their formation during the roasting process, even though host defense mechanisms probably protect humans efficiently against their action, since no adverse effect of regular coffee drinking has been observed in several epidemiological investigations of healthy individuals (*23, 24*).

In this study, α-dicarbonyl compounds were simultaneously detected as quinoxaline derivatives using RP-HPLC-DAD. The applied method previously used to determine dicarbonyl compounds in wine, required some modifications and a specific sample preparation. Because of the very complex matrix of coffee extract (coffee brew), interfering compounds such as chlorogenic derivatives had to be eliminated before the derivatization reaction and elution conditions had to be optimized. Checks of SPE and derivatization conditions to verify recovery and yield, respectively, resulted in rates close to 100%. The results of the validation procedure showed that the proposed method is selective, precise, accurate, and sensitive.

The findings reported here demonstrate that coffee α-dicarbonyl compound content depends on roasting conditions as well as ex vivo antiperoxyl radical activity (*25*) and the antiadhesive effects of *Streptococcus mutans* on hydroxyapatite beads (*21*), previously investigated by our group. On the whole, these results should encourage roasters to select roasting conditions that while maintaining brew taste and aroma can at the same time enhance their safety and healthiness.

ABBREVIATIONS USED

Q, quinoxaline; 2MQ, 2-methylquinoxaline; 2,3DMQ, 2,3-dimethylquinoxaline; 5MQ, 5-methylquinoxaline; CR, *C. robusta* coffee sample; CR14, *C. robusta* coffee sample roasted for 14 min.

ACKNOWLEDGMENT

The work was supported by a grant from MIUR 2005 to G.G. and took place within activities of the Interdepartmental Center of Food Quality and Safety (CISQUA). We thank MOKA SIR'S s.r.l. (Pavia, Italy) for providing the coffee beans and the pilot roasting apparatus.

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Received for review June 28, 2007. Revised manuscript received August 10, 2007. Accepted August 15, 2007.

JF071917L