Possible Presence of 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline and Other Heterocyclic Amine-Like Mutagens in Roasted Coffee Beans

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Mutagens in regular hot-air-roasted, charcoal-roasted (Sumibiyaki in Japanese), and high-temperature-roasted coffee beans were extracted with methyl alcohol/ammonium hydroxide, partitioned into the acidic water and chloroform after alkalization, and finally purified by blue cotton. They showed positive response to *Salmonella typhimurium* TA98 and TA100 strains with metabolic activation. The mutagens were separated into mutagenic fractions A and B by use of high-performance liquid chromatography. The mutagen in fraction A was suggested to be 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) by cochromatography, ultraviolet absorption spectrum, and activity. The MeIQ contents were estimated to be 0.16 ng/10 g for hot-air-roasted, 0.32 ng/10 g for charcoal-roasted, and 1.5 ng/10 g for high-temperature-roasted coffee beans. The mutagens in fraction B were not identified but suggested to be heterocyclic amine mutagens.

It has been shown that the heating of several foodstuffs produces mutagenic and/or carcinogenic heterocyclic amines. The heating of meats (Sugimura and Nagao, 1982; Sugimura and Sato, 1983), fish meats (Kato et al., 1986; Kikugawa and Kato, 1987; Kikugawa et al., 1986), and soybean proteins (Yoshida et al., 1978) produces heterocyclic amine mutagens. Several heterocyclic amine mutagens have been elucidated so far (Sugimura and Sato, 1983). In light of these findings, it is conceivable that commercial roasted coffee beans prepared by treating coffee beans at an elevated temperature (Clarke, 1967) contain mutagens generated by heating. Several studies have demonstrated that boiling water extracts of roasted coffee beans exhibit significant mutagenicity on *Salmonella typhimurium* TA100 without metabolic activation (Aeschbacher et al., 1980; Aeschbacher and Würzner, 1980; Nagao et al., 1979). This mutagenicity was suggested to be due to methylglyoxal and/or hydrogen peroxide (Fujita et al., 1985), both of which are not heterocyclic amines. Another unknown material(s) exhibiting mutagenicity on *S. typhimurium* TA98 with metabolic activation was found in the volatile fraction of an overheated coffee (Blair and Shibamoto, 1984). In our preliminary test, it has been suggested that compounds that can be adsorbed to blue cotton and are positive in the mutagenesis assay on *S. typhimurium* TA98 with metabolic activation are present in the boiling water extract of roasted coffee beans and also of instant coffee (Kikugawa et al., 1985). We describe here the possible presence of 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ) and the other heterocyclic amine-like mutagenic materials in roasted coffee beans.

MATERIALS AND METHODS

Materials. A brand of coffee beans (Mocha) roasted by hot air in a drum (hot-air-roasted) and five brands of coffee beans roasted on charcoal fire (charcoal-roasted) were obtained at a local market in Tokyo. The hot-air-roasted coffee beans were experimentally heated to reach 400 °C on a pan (high-temperature-roasted). Authentic heterocyclic amine mutagens were gifts of Dr. S. Sato of the National Cancer Center Research Institute, Tokyo, and Dr. H. Hayatsu of Okayama University, Okayama. Blue cotton (Hayatsu et al., 1983) was the product of Funakoshi Chemical Co., Ltd., Tokyo, and was washed with dimethyl sulfoxide and subsequently with a mixture of methyl alcohol/concentrated ammonium hydroxide (50:1) prior to use. Amberlite XAD-2 was washed with acetone for use (Yamasaki and Ames, 1977).

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Analyses. Ultraviolet absorption spectra were measured with a Hitachi 557 double-wavelength double-beam spectrophotometer. High-performance liquid chromatography (HPLC) was performed by use of a Shimadzu LC-2 liquid chromatograph equipped with a YMC S-343 ODS column (2 cm (i.d.) × 25 cm), a YMC A-303 ODS column (4.6 mm (i.d.) × 25 cm) (Yamamura Chemical Laboratories, Ltd., Kyoto), or an Inertsil ODS column (4.6 mm (i.d.) × 25 cm) (Gasukuro Kogyo Inc., Tokyo). The chromatograph was operated with 0.01 M triethylammonium bicarbonate (pH 7.3)/methyl alcohol (2:3) (solvent A), 0.01 M triethylammonium bicarbonate (pH 7.3)/methyl alcohol (1:1) (solvent B), 0.01 M triethylammonium bicarbonate (pH 7.3)/methyl alcohol (3:2) (solvent C), 0.025 M phosphoric acid–sodium hydrogen phosphate (pH 3.0)/acetonitrile (4:1) (solvent D), 0.025 M phosphoric acid–sodium hydrogen phosphate (pH 3.0)/acetonitrile (9:1) (solvent E), or methyl alcohol (solvent F). Ultraviolet-absorbing peaks were detected by use of a Shimadzu SPD-6A UV spectrophotometric detector.

Mutagenicity Test. Mutagenicity was assayed according to the preincubation method of Yahagi et al. (1977) using *S. typhimurium* TA98 and TA100 (Ames et al., 1975). The S9 was prepared from liver microsomes of rats treated with polychlorinated biphenyl. The S9 mix used contained 50 μL of S9. Test samples were dissolved in 100 μL of dimethyl sulfoxide for assay. All the experiments were performed with duplicate plates, and all the data were expressed by subtracting spontaneously formed His^+-revertant colonies. The background numbers of His^+ revertants/plate were 14–24 for TA98 +S9 mix, 16–23 for TA98 −S9 mix, 112–138 for TA100 +S9 mix, and 138–141 for TA100 −S9 mix.

Purification of the Heterocyclic Amine Mutagens in Roasted Coffee Beans. *Extraction and Partition.* Roasted coffee beans (100 g) were ground and shaken in 1000 mL of methyl alcohol/concentrated ammonium hydroxide (100:1) for 1 h twice, and the solid material was filtered off through glass wool. The filtrate was evaporated in vacuo to dryness below 50 °C. The residual material was dissolved in 1000 mL of 0.1 N hydrochloric acid, and the mixture was partitioned by addition of 400 mL of chloroform. After removal of chloroform layer, the same amount of chloroform was added and the mixture similarly treated. The acidic solution was alkalized by addition of 100 mL of 2 N sodium hydroxide, and the mixture was extracted with 200 mL of chloroform four times. The chloroform layer was evaporated in vacuo to dryness.

Adsorption to Blue Cotton. The residue was dissolved in 800 mL of water, and to this was added blue cotton (800 mg). The mixture was shaken at room temperature for 1 h. After removal of the blue cotton, 800 mg of fresh blue cotton was added and the mixture similarly treated. The recovered blue cotton was washed with water, and the mutagens adsorbed were eluted during shaking with 400 mL of methyl alcohol/concentrated ammonium hydroxide (1000:1) for 30 min. This elution was repeated again with a fresh eluent. The combined eluates were evaporated in vacuo to dryness below 50 °C.

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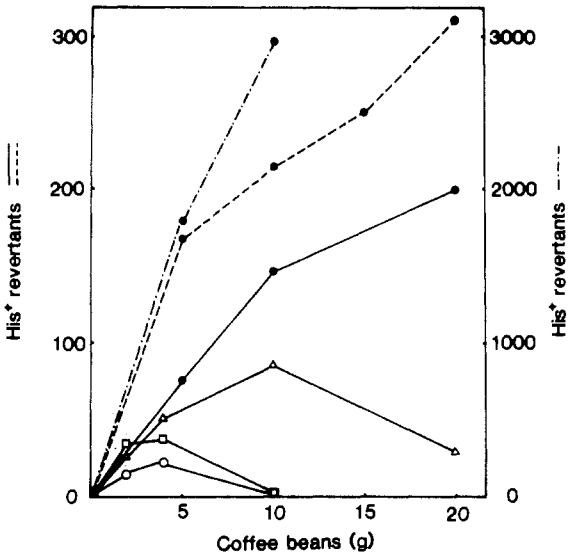

Figure 1. Dose–response curves of the mutagenicity of roasted coffee beans: hot-air-roasted (—); charcoal-roasted (- - -); high-temperature-roasted (-·-). The blue cotton purified mutagens in the boiling water extract (○), the methyl alcohol/ammonium hydroxide (100:1) extract (□), the acidic fraction of the methyl alcohol/ammonium hydroxide extract (△), and the chloroform fraction of the alkalized acidic fraction (●) were tested. For preparation of the boiling water extract, roasted coffee beans (50 g) were extracted with 500 mL of boiling water for 10 min twice and the solid materials were filtered off through glass wool. For preparation of other extracts, see Materials and Methods. The mutagenicity of the samples was assayed with *S. typhimurium* TA98 with S9 mix.

Separation by HPLC. The solution of the mutagens in 0.6 mL of methyl alcohol was divided into four portions, and each portion was chromatographed by use of a YMC S-343 ODS column. The chromatograph was operated with solvent A and subsequently with solvent F at a flow rate of 4.0 mL/min. Each fraction was evaporated to dryness.

Purification by XAD-2. The mutagens in HPLC fractions A and B were separately dissolved in 200 mL of water, adsorbed to a XAD-2 resin column (1.0 cm (i.d.) × 20 cm), and eluted successively with 100 mL of methyl alcohol/water (4:1) and 100 mL of methyl alcohol. The eluates from the resin were evaporated to dryness.

Rechromatography of the Mutagens in XAD-2 Fraction AX. The mutagens in fraction AX purified by XAD-2 resin were rechromatographed successively on a YMC A-303 ODS and an Inertsil ODS column. The chromatograph was operated with use of solvents B–E. Each 1.0-mL fraction was evaporated to dryness.

Warning! Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, MeAαC, IQ, MeIQ, MeIQx, 4,8-Me_2IQx, 7,8-Me_2IQx, and PhIP, all of which are carcinogens or mutagens, should be handled with safety cautions.

RESULTS

Commercial regular hot-air-roasted coffee beans (Mocha) were extracted with boiling water for 10 min, and components in the extract that were adsorbable to blue cotton were evaluated for mutagenicity. Dose–response curve of the mutagenicity on *S. typhimurium* TA98 with S9 mix (Figure 1; solid line and open circles) indicated that the number of His^+-revertant colonies was maximum (about 20) at the dose of 4 g of the beans and decreased at the doses larger than 10 g. This decrease was probably due to the bactericidal effect of some components in the boiling water extract that could not be removed by subsequent blue cotton adsorption. When the initial extraction was performed by use of methyl alcohol/ammonium hydroxide (100:1), the numbers of His^+-revertant colonies increased about 2-fold, indicating that the mutagens in the coffee beans were eluted more effectively (Figure 1; solid line and open squares). However, bactericidal components in the extract disturbed the mutagenicity testing. When the mutagens in the extract were subsequently partitioned with acidic water and chloroform, they went into the acidic fraction and the number of His^+-revertant colonies increased to 90 at the dose of 10 g of the beans (Figure 1; solid line and open triangles). When the mutagens in the acidic water were transferred into the chloroform after alkalization, the number slightly increased with doses up to 20 g of beans (Figure 1; solid line and closed circles). The bactericidal components were removed to some extent by these procedures.

Table I. Mutagenicity of the Blue Cotton Purified Extract of Roasted Coffee Beans[a]

	His^+-revertant colonies/10 g beans			
	S. typhimurium TA98		*S. typhimurium* TA100	
coffee bean	+S9 mix	−S9 mix	+S9 mix	−S9 mix
charcoal-roasted	230	6	75	4
high-temp-roasted	2700	27	378	14

[a] Roasted coffee beans were extracted with methyl alcohol/ammonium hydroxide (100:1), subsequently extracted into the acidic solution and into the chloroform after alkalization, and finally purified by blue cotton adsorption. The process was repeated three times, and the numbers of His^+-revertant colonies are expressed as mean values of three different experiments.

Commercial coffee beans roasted by charcoal fire (so-called Sumibiyaki in Japanese) are popular items of roasted coffee beans in Japan. Charcoal-roasted coffee beans are generally made by heating with charcoal fire at the higher temperatures than the regular hot-air-roasted coffee beans. Charcoal-roasted coffee beans were extracted with methyl alcohol/ammonium hydroxide, partitioned into the acidic water, partitioned into the chloroform layer after alkalization, and finally purified by blue cotton adsorption. Dose–response curve of the mutagenicity (Figure 1; dotted line) indicated that the mutagenicity increased with the dose of the beans and it was higher than that of hot-air-roasted coffee beans. The numbers of His^+-revertant colonies at the dose of 10 g of the beans with *S. typhimurium* TA98 and TA100 with and without S9 mix are shown in Table I. The mutagens were positive on both TA98 and TA100 strains with S9 mix. When the mutagenicity of other four brands of charcoal-roasted coffee beans was tested with TA98 strain with S9 mix, the numbers of His^+-revertant colonies were higher (330, 348, 535, 628) than that (230) of the brand tested in Figure 1 and Table I. These results indicate that charcoal-roasted coffee beans contain larger amounts of the mutagens.

Hot-air-roasted coffee beans were experimentally heated to reach 400 °C on a pan. The beans were extracted with methyl alcohol/ammonium hydroxide, partitioned into the acidic water and chloroform after alkalization, and finally purified by blue cotton. Dose–response curve of the mutagenicity on TA98 strain with S9 mix (Figure 1; chain line) indicated that the mutagenicity increased with the dose and it was much higher than those of hot-air- and charcoal-roasted coffee beans. The number of His^+-revertant colonies at the dose of 10 g of the beans was 2700. The dry weight was reduced to 2.2 mg from the initial methyl alcohol/ammonium hydroxide extraction (6330 mg). The profiles of the mutagenic activity due to the beans were similar to those of the activity due to charcoal-roasted coffee beans (Table I).

It was suggested that coffee beans generated mutagens by heating. The mutagens could be extracted from the beans, and they may be basic because they were trans-

Table II. Recovery of the Mutagenicity of Roasted Coffee Beans by HPLC

	His$^+$-revertant colonies/10 g beans, %		
		HPLC[b]	
coffee bean	blue cotton[a]	fraction A	fraction B
hot-air-roasted	120 (100)	66 (55)	30 (25)
charcoal-roasted	217 (100)	127 (59)	77 (35)
high-temp-roasted	2919 (100)	615 (21)	1620 (55)

[a] Roasted coffee beans were extracted with methyl alcohol/ammonium hydroxide (100:1), subsequently extracted into the acidic solution and into the chloroform after alkalization, and finally purified by blue cotton adsorption. [b] The blue cotton purified mutagens were separated by HPLC (Figure 2). Mutagenicity was tested on *S. typhimurium* TA98 with S9 mix.

ferred into acidic water and chloroform after alkalization. Furthermore, the mutagens adsorbed to blue cotton, which is known as an effective adsorbent of heterocyclic amines (Hayatsu et al., 1983). They were positive to TA98 and TA100 strains with S9 mix. Thus, the coffee mutagens detected in the present investigation may be heterocyclic amine mutagens.

The coffee mutagens in hot-air-, charcoal- and high-temperature-roasted beans were subjected to preparative high-performance liquid chromatography (HPLC) with a reversed-phase column using solvents A and F (Figure 2). Most of the mutagenicity of hot-air-roasted coffee beans appeared by elution with solvent A (fraction A) and the rest with solvent F (fraction B) (Figure 2A). Total recovery of the mutagenicity in these fractions was about 80% (Table II). The profile of HPLC pattern of the mutagens from charcoal-roasted coffee beans was similar (Figure 2B). The mutagenicity of fraction A was higher than that of fraction B, and both the mutagenicities of fractions A and B were higher than those of the corresponding fractions of hot-air-roasted coffee beans (Table II). The mutagens from high-temperature-roasted beans showed a similar HPLC pattern (Figure 2C). The mutagenicities of fractions A and B were much higher than those of the corresponding fractions of hot-air- and charcoal-roasted coffee beans (Table II). It is interesting to note that the mutagenicity of fraction B of high-temperature-roasted beans was much higher than that of fraction A.

Isolation and identification of the mutagens in high-temperature-roasted beans were attempted. As shown in Figure 2C, substantial amounts of ultraviolet-absorbing materials were eluted throughout the HPLC fractions. The amount of mutagenic materials relative to that of the ultraviolet-absorbing impurities may be small, and the retention times of the mutagens may be disturbed by these impurities. When fractions A and B were rechromatographed under the same conditions, 92% activity of fraction A was eluted by solvent A and 96% activity of fraction B was eluted only after elution with solvent F. Thus, the mutagens in fractions A and B were essentially different. Most of the known heterocyclic amine mutagens,

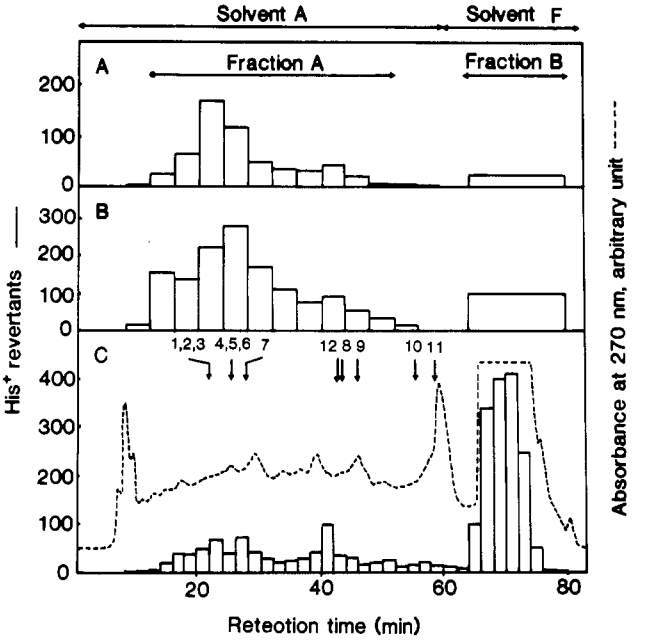

Figure 2. HPLC of the mutagens in roasted coffee beans: hot-air-roasted (A); charcoal-roasted (B); high-temperature-roasted (C). Roasted coffee beans were extracted with methyl alcohol/ammonium hydroxide (100:1), subsequently extracted into the acidic solution and into the chloroform after alkalization, and finally purified by blue cotton adsorption. The mutagens in 50 g of hot-air-roasted coffee beans (A), 100 g of charcoal-roasted coffee beans (B), and 10 g of high-temperature-roasted coffee beans (C) were separated by HPLC on a YMC S-343 ODS column using solvents A and F. The mutagenicity of each fraction was assayed with *S. typhimurium* TA98 with S9 mix. Ultraviolet-adsorbing peaks were detected at 270 nm. Authentic heterocyclic amine mutagens: **1**, IQ; **2**, MeIQx; **3**, Glu-P-2; **4**, 7,8-Me_2IQx; **5**, 4,8-Me_2IQx; **6**, MeIQ; **7**, Glu-P-1; **8**, AαC; **9**, Trp-P-2; **10**, Trp-P-1; **11**, MeAαC; **12**, PhIP.

IQ (**1**), MeIQx (**2**), Glu-P-2 (**3**), 7,8-Me_2IQx (**4**), 4,8-Me_2IQx (**5**), MeIQ (**6**), Glu-P-1 (**7**), AαC (**8**), Trp-P-2 (**9**), Trp-P-1 (**10**), MeAαC (**11**), and PhIP (**12**) were eluted with solvent A, and none of these authentic mutagens were eluted with solvent F. Thus, the mutagens in fraction B may not be these known heterocyclic amines. In order to remove ultraviolet-absorbing impurities, fractions A and B were separately passed through a XAD-2 column. Most of the ultraviolet-absorbing impurities were removed by the column, and more than 88% of the mutagenicity was recovered in fractions AX and BX, respectively (Table III).

HPLC of fraction AX on an analytical reversed-phase column by elution with acidic solvent (solvent D) revealed a major mutagenic fraction AX_1 (recovery 74%) at a retention time of 7 min (Figure 3A). A large amount of ultraviolet-absorbing impurities was removed from the active fraction. The retention time of fraction AX_1 was the same as those of authentic mutagens **1**–**7**. HPLC of fraction AX_1 by elution with neutral solvent (solvent B)

Table III. Recovery of the Mutagenicity and Removal of Ultraviolet-Absorbing Impurities by Passing through a XAD-2 Column of HPLC Fractions A and B of High-Temperature-Roasted Coffee Beans[a]

	before XAD-2 column		after XAD-2 column with			
			methyl alcohol/water		methyl alcohol	
	His$^+$-revertant colonies	abs unit at 270 nm	His$^+$-revertant colonies	abs unit at 270 nm	His$^+$-revertant colonies	abs unit at 270 nm
HPLC fraction A	615	9.52	570	2.00	61	2.97
HPLC fraction B	1487	31.36	1319	6.83	223	11.82

[a] HPLC fractions A and B from 10 g of high-temperature-roasted coffee beans (Table II) were adsorbed to a XAD-2 column as described in Materials and Methods. The column was eluted successively with methyl alcohol/water (4:1) and methyl alcohol. Mutagenicity was tested on *S. typhimurium* TA98 with S9 mix.

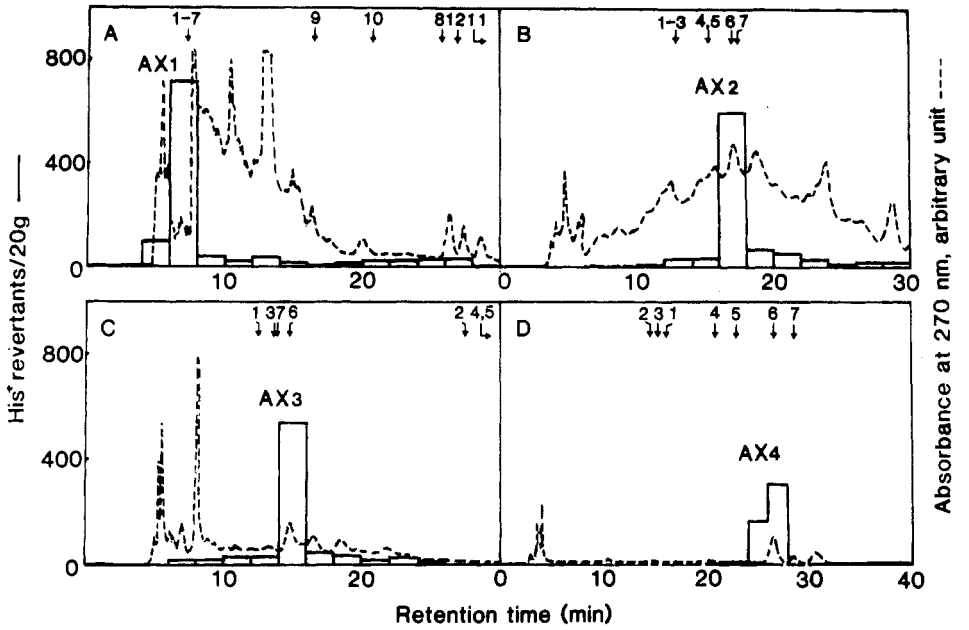

Figure 3. Rechromatography of XAD-2 fraction AX of high-temperature-roasted coffee beans. A: Fraction AX purified by XAD-2 adsorption (20-g equivalent amount of coffee beans) (Table III) was loaded onto a YMC A-303 ODS column and eluted with solvent D at a flow rate of 0.5 mL/min. The recovery of the mutagenic activity in fraction AX_1 was 74%. B: Fraction AX_1 was loaded onto an Inertsil ODS column and eluted with solvent B at a flow rate of 0.5 mL/min. The recovery of the mutagenic activity in fraction AX_2 was 72%. C: Fraction AX_2 was loaded onto the same column and eluted with solvent E at a flow rate of 0.5 mL/min. The recovery of the mutagenic activity in fraction AX_3 was 87%. D: Fraction AX_3 was loaded onto the same column and eluted with solvent C at a flow rate of 0.7 mL/min. The recovery of the mutagenic activity in fraction AX_4 was 91%. The mutagenicity of each 1-mL fraction was measured with *S. typhimurium* TA98 with S9 mix. Ultraviolet-absorbing peaks were detected at 270 nm. The numbering of authentic mutagens is the same as in Figure 2.

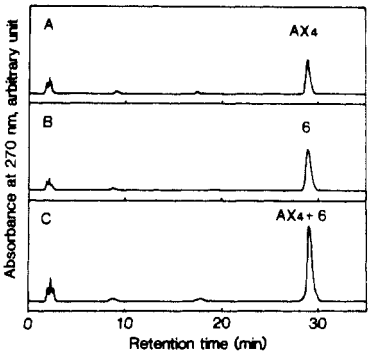

Figure 4. Cochromatography of HPLC fraction AX_4 of high-temperature-roasted coffee beans and MeIQ (**6**). HPLC fraction AX_4 of high-temperature-roasted coffee beans (Figure 3D) and authentic MeIQ (**6**) were cochromatographed on an Inertsil ODS column by elution with solvent C at a flow rate of 0.7 mL/min. The peaks were detected at 270 nm.

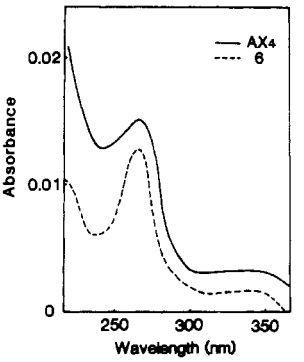

Figure 5. Ultraviolet absorption spectrum of HPLC fraction AX_4 of high-temperature-roasted coffee beans. HPLC fraction AX_4 of high-temperature-roasted coffee beans (Figure 3D) and authentic MeIQ (**6**) were dissolved in methyl alcohol for measurement of the spectrum.

gave a single mutagen fraction AX_2 (recovery 72%) at a retention time of ≈19 min (Figure 3B). Extensive amounts of ultraviolet-absorbing impurities were removed. The retention time of fraction AX_2 coincided with that of authentic **6** and **7**. HPLC of fraction AX_2 by elution with acidic solvent (solvent E) gave a single mutagenic fraction AX_3 (recovery 87%) at a retention time of ≈15 min corresponding to that of **6** and **7** (Figure 3C), which appeared as a sharp ultraviolet-absorbing peak. HPLC of fraction AX_3 by elution with neutral solvent (solvent C) gave a single and sharp ultraviolet-absorbing fraction AX_4 (recovery 91%) at a retention time of 29 min.

Purified mutagenic fraction AX_4, which showed a single ultraviolet-absorbing peak, coincided with that of authentic MeIQ (**6**) (Figure 4A,B), was cochromatographed with authentic MeIQ (**6**) by elution with solvent C (Figure 4C). Combination of fraction AX_4 and MeIQ (**6**) at the same dose showed a single ultraviolet-absorbing peak. These

Table IV. Mutagenicity of HPLC Fraction AX_4 of High-Temperature-Roasted Coffee Beans

	His$^+$-revertant colonies/abs unit at 270 nm: 5×10^{-4}			
	S. typhimurium TA98		*S. typhimurium* TA100	
	+S9 mix	−S9 mix	+S9 mix	−S9 mix
HPLC fraction AX_4 (Figure 3D)	728	4	38	0
MeIQ (**6**)	880	0	15	0

chromatographic results suggested that the major mutagen in HPLC fraction A in Figure 2 was MeIQ. The ultraviolet absorption spectrum of mutagenic fraction AX_4 in methyl alcohol showed an absorption maximum at 268 nm and a shoulder at around 350 nm, which was similar to that of authentic MeIQ (Figure 5).

Mutagenicity of fraction AX_4 on *S. typhimurium* TA98 and TA100 with and without S9 mix (Table IV) showed

Table V. MeIQ Content in Roasted Coffee Beans

coffee bean	MeIQ,[a] ng/10 g coffee beans
hot-air-roasted	0.16
charcoal-roasted	0.32
high-temp-roasted	1.5

[a] The content of MeIQ was determined by the mutagenicity of HPLC fraction A (Table II) and the number of His^+-revertant colonies (409 000/μg) of MeIQ (6) with *S. typhimurium* TA98 with S9 mix.

that it gave positive response toward both strains with S9 mix. The potencies and profiles of fraction AX_4 were similar to those of authentic MeIQ.

As described above, the major mutagen in the initial HPLC fraction A was suggested to be MeIQ. The MeIQ contents in roasted coffee beans were estimated by the mutagenic activities of fraction A in the initial HPLC analysis (Table II) and the mutagenic activity of MeIQ (Table V). These values are minimum ones, because the extraction efficiency in the treatment of roasted coffee beans is not known and possible loss during the purification steps is not taken into account.

DISCUSSION

In previous papers (Aeschbacher et al., 1980; Aeschbacher and Würzner, 1980; Nagao et al., 1979), it has been shown that the mutagenicity present in brewed coffee can be demonstrated on *S. typhimurium* TA100 without S9 mix. The number of His^+-revertant colonies for 30 mg of freezed-dried brewed coffee was about 200, and this mutagenicity was abolished by addition of S9 mix (Aeschbacher et al., 1980; Aeschbacher and Würzner, 1980; Friederich et al., 1985; Nagao et al., 1979). Kasai et al. (1982) suggested that part of the mutagenicity was due to methylglyoxal present in coffee, and Fujita et al. (1985) suggested that most of the mutagenicity could be explained by methylglyoxal and hydrogen peroxide in coffee, since hydrogen peroxide potentiated the activity of methylglyoxal.

The mutagens found in the present investigation are apparently different from the previously suggested mutagen. Like the known heterocyclic amine mutagens, the mutagens are soluble in acidic water and transferred into chloroform after alkalization. They are adsorbed to blue cotton, which is known as an effective adsorbent of the known heterocyclic amines (Hayatsu et al., 1983). The mutagens gave positive response to *S. typhimurium* TA98 and TA100 strains with metabolic activation. They do not show mutagenicity in either of these strains without metabolic activation. These properties for mutagenicity are also similar to those of the known heterocyclic amines (Sugimura and Nagao, 1982; Sugimura and Sato, 1983). Thus, the mutagens found in the present experiments seemed to be heterocyclic amine mutagens produced during roasting of coffee beans at an elevated temperature. Blair and Shibamoto (1984) have demonstrated that the volatile fraction of overheated coffee contains mutagens positive to *S. typhimurium* TA98 with metabolic activation, which may be the same as found in the present investigation.

Throughout the present investigation, purification and detection of the mutagens needed much effort since the extracts of coffee beans contained a large amount of bactericidal components and ultraviolet-absorbing impurities. The most successful method for purifying the mutagens was as follows: extraction with methyl alcohol/ammonium hydroxide, partition in acidic water and chloroform, partition in alkaline water and chloroform, blue cotton adsorption of the basic fraction, and finally HPLC fractionation.

While the mutagenicity of commercial regular hot-air-roasted coffee beans was low, commercial charcoal-roasted coffee beans, which are popular items in Japan, showed higher mutagenicity. High-temperature-roasted coffee beans experimentally prepared showed much higher mutagenicity than hot-air- and charcoal-roasted coffee beans. The mutagens were separated into mutagenic fractions A and B by use of HPLC. The mutagen in fraction A was purified by successive XAD-2 column chromatography and HPLC, and it was suggested to be 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) by cochromatography, ultraviolet absorption spectrum, and mutagenic activity. The mutagens in fraction B were not identified but were suggested to be heterocyclic amine mutagens.

It has been shown that MeIQ is present in broiled sun-dried sardine (Kasai et al., 1980a,b; Sugimura and Sato, 1983), beef extract (Hargraves and Pariza, 1983), and broiled fish meats (Yamaizumi et al., 1986). This experiment is the first for detection of MeIQ in processed vegetable foods. Generation of MeIQ in roasted coffee beans may be due to the heating of several components in coffee beans. Carginogenicity of MeIQ has been studied, and it induces tumors in forestomach and liver in mice (Sugimura, 1985).

The significance of the presence of the heterocyclic amine mutagens in roasted coffee beans must await further investigation. These mutagens could be eluted only by methyl alcohol/ammonium hydroxide and hardly eluted by boiling water. The amount of these mutagens in regular hot-air-roasted coffee beans was relatively low. Therefore, it looks unlikely that the presence of these mutagens in roasted coffee beans raises a question of the safety of coffee brew usually consumed.

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ABBREVIATIONS

Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 4,8-Me_2IQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; 7,8-Me_2IQx, 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

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Biochemical and Morphological Characteristics in Maturing Achenes from Purple-Hulled and Oilseed Sunflower Cultivars

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The blackbird feeding preference for sunflower oilseed variety Jacques Discovery (JD) over purple-hulled Neagra de Cluj (NdC) has been attributed to anthocyanin. To test this hypothesis, we compared biochemical and morphological properties in the achene over the maturation period. Comparisons included total phenols, anthocyanin, tannin, fat/oil, protein, nonstructural sugars, and phytomelanin contents, as well as mass properties and moisture content. Only three differences were observed: (1) Hull mass was significantly higher for NdC than JD. (2) Anthocyanins were synthesized by NdC only. (3) JD was higher in oil content. Each of these factors may play a role in reduced preferences exhibited by birds for NdC.

Bird-resistant traits in ripening agricultural crops usually involve a combination of morphological and biochemical characteristics (Bullard and York, 1984). In developing bird-tolerant cultivars the two modes of protection are usually pursued simultaneously, resulting in cultivars that contain traits of both. This has been a productive approach in sorghum (Voight, 1966) and corn (Dolbeer et al., 1982) and is the one being followed by plant geneticists working on bird-resistant sunflower varieties (Fox and Linz, 1983; Foley and Hanzel, 1986). Morphological features thought to increase resistance include concave heads, long bracts, heads that face the ground, head-to-stem distances exceeding 15 cm, and seeds with tough fibrous hulls (Parfitt, 1984). Biochemical features thought to increase resistance include chlorogenic acid (Harada, 1977) and anthocyanins in purple-hulled varieties (Dolbeer et al., 1986; Fox and Linz, 1983; Mason et al., 1986). Chlo-

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