2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline Is Not Detectable in Commercial Instant and Roasted Coffees

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A sensitive method has been developed for the isolation of 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) from instant coffee and lyophilized home-brewed coffee. Sample cleanup was performed in four steps using Amberlite XAD-2, Extrelut/copper phthalocyanine–Sephasorb, Sephasorb HP, and propanesulfonic acid silica/octadecyl silica. The purified extracts were then separated by high-performance liquid chromatography. To validate the procedure, picogram levels of [2-^{14}C]MeIQ were added prior to extraction to the coffee samples; 61% and 49% of the added reference standard could be recovered in chromatographic fractions from purified instant and home-brewed coffee, as measured by liquid scintillation and Ames test mutagenicity (TA98, +S-9). At 600 and 60 ppt spiking levels, clear signals were observed allowing the estimation of the detection limit for MeIQ to be about 5–10 pg/g of coffee beans. On this basis, MeIQ was not detectable in either sample of instant or roasted coffee.

INTRODUCTION

The heating of protein-rich food products may lead to the formation of trace levels of highly mutagenic contaminants, belonging to two groups of compounds, the amino acid pyrolyzates and aminoimidazoazaarenes (AIA) (Sugimura, 1986; Hatch et al., 1984; Felton and Knize, 1990). Most of these chemicals are moderately potent rodent carcinogens (Ohgaki et al., 1986) and, therefore, their role in the development of human cancer is today of major concern (Sugimura, 1990). Formation of AIAs occurs mainly in meat-based food products upon cooking above 100 °C (Knize et al., 1985; Övervik et al., 1984, 1989; Knize et al., 1988). AIAs are derived from the precursors amino acids, carbohydrates, and creatinine, whereas formation of protein pyrolyzates requires more drastic heating conditions (Yamamoto et al., 1978; Tada et al., 1983). Furthermore, weak food-borne mutagenicity as well as antimutagenicity was found to be induced by nonenzymatic browning in heat-processed carbohydrate-rich foods (Pariza et al., 1979; Powrie et al., 1986).

Green coffee beans contain substantial amounts of amino acids and carbohydrates (Trugo, 1985; Macrae, 1985) which might act as precursors of mutagenic compounds formed during roasting. Indeed, coffee, both home-brewed filtered and spray dried, exhibited direct acting mutagenicity (Ames tester strain TA100) which was deactivated in the presence of a microsomal metabolic activation system (S-9) (Nagao et al., 1979; Aeschbacher and Würzner, 1980; Aeschbacher, 1984). This activity originated from the interaction of several labile coffee constituents including aliphatic dicarbonyl compounds, e.g., methylglyoxal (Aeschbacher et al., 1989). A basic fraction of the condensed vapor of overheated coffee brew showed weak activation-dependent mutagenicity (TA98, +S-9) (Blair and Shibamoto, 1984; Sasaki et al., 1987), suggesting that heterocyclic amine-like mutagens might be present. Recently, the presence of 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) (Kikugawa et al., 1989) and other unidentified heterocyclic amine-like mutagens (Kato et al., 1989) has been suggested in the MeOH extract of high-temperature-roasted coffee. MeIQ was first isolated from broiled sardines (Kasai et al., 1980) and later detected in a heated model mixture of creatinine, alanine, and fructose (Grivas et al., 1985). It exhibits strong activity in the Ames test with strain TA98 under conditions of metabolic activation by S-9 (Nagao, 1981).

The purpose of the present study was to verify whether MeIQ could be detected in aqueous coffee extracts, i.e., instant coffee or home-brewed coffee.

MATERIALS AND METHODS

Materials. Chemicals and solvents were of HPLC or analytical grade. Water was from a Milli-Q water purification system (Millipore, Bedford, MA). Pure reference standards of MeIQ, IQ, and [2-^{14}C]MeIQ (40 mCi/mmol) were from Toronto Research Chemicals (Toronto, Canada). The unlabeled material was 98% pure as checked by HPLC and comparison of extinction coefficients with published values (Hatch et al., 1984). [2-^{14}C]-MeIQ was radiochemically pure as confirmed by HPLC with radioactivity detection. Analytical grade Amberlite XAD-2 (Serva, Heidelberg, FRG) of 0.3–1-mm particle size was washed with acetone and MeOH before use. Extrelut 20 and Extrelut 3 extraction cartridges were from Merck AG (Darmstadt, FRG). Sephasorb HP was obtained from Pharmacia-LKB (Uppsala, Sweden). Remazol Turquoise Blue G133 EE AF 505 (Lot 74575) was from Hoechst AG (Frankfurt, FRG). Empty reservoirs (8 mL), Bond-Elut PRS (500 mg), and Bond-Elut C18 (100 mg) cartridges as well as coupling pieces and stopcocks were from Analytichem International (ICT AG, Basel, Switzerland). Ultima-Gold scintillation fluid was from Packard Instruments (Zurich, Switzerland).

Instrumentation. A Minipulse 2 peristaltic pump (Gilson, Villier-le-Bel, France) or a Supelco Visiprep vacuum manifold (Supelco, Gland, Switzerland) was used for manipulations with solid-phase extraction (SPE) cartridges. HPLC was performed with a Merck L-6200 pump and a Gilson 231-401 automated sample processor and injector (Rheodyne 7010, 50-μL sample loop). On-line detection was performed with a Hewlett-Packard Model 1046A programmable fluorescence detector connected in series with a Hewlett-Packard Model 1040A diode array detector, both driven by a HP9000 Series 300 workstation with Chemstation software. A TSK gel ODS80 (Toyo Soda) 25 cm × 4.6 mm i.d. (5 μm) column equipped with a precolumn of the same stationary phase type was used. LC conditions were as follows: solvent A, 0.01 M triethylamine adjusted with phosphoric acid to pH 3.2; solvent B, acetonitrile. The gradient profile was linear: 0–20 min, 5–25% B in A; 20–30 min, 25–55% B in A; 30–35 min, 55–85% B in A. This program allowed separation of all heterocyclic aromatic amines (Gross, 1990). Fractions were collected into disposable 10-mL polypropylene tubes using a time-programmed Gilson 202 fraction collector. Collected fractions were lyophilized in a Hetovac high-vacuum centrifuge (HETO Lab Equipment, Birkerød, Denmark). An LKB Model 1217

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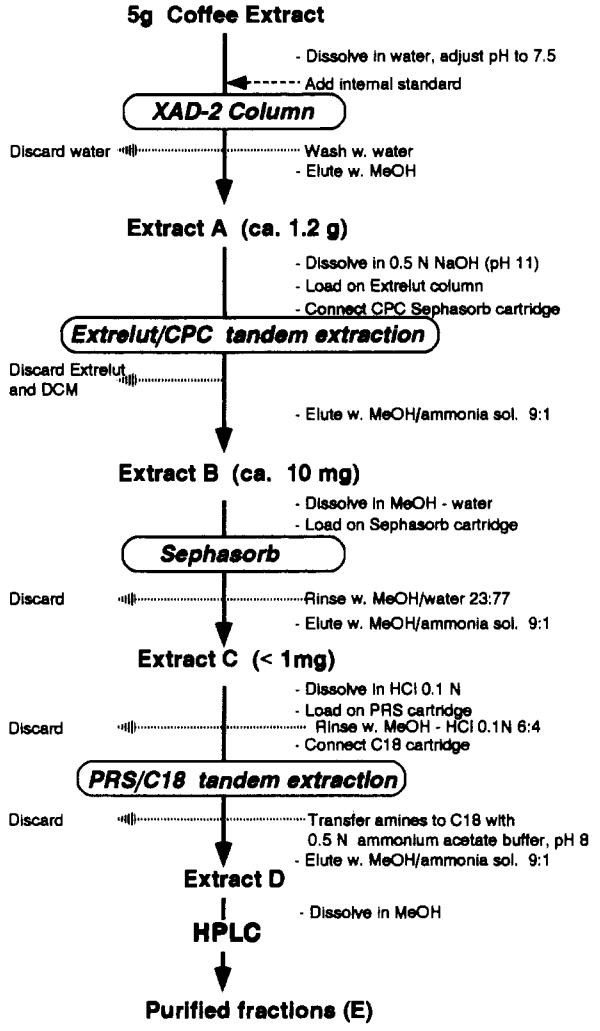
Scheme I. Isolation Procedure III



(Pharmacia-LKB, Bromma, Sweden) scintillation counter was used for radioactivity measurements. A Metrohm (Herisau, Switzerland) Type E604 pH meter was used for adjusting the pH of HPLC mobile phases and sample solutions. Anotop 0.2- or 0.02-μm microfilters (Merck) were used for filtration of HPLC samples stored in 1.1-mL conical borosilicate glass vials (Chromacol gold, Infochroma, Zug, Switzerland).

Preparation of Extraction Cartridges. Disposable cartridges containing copper phthalocyanine (CPC)–Sephasorb or Sephasorb HP were prepared and conditioned as described (Gross, 1990). Bond-Elut PRS cartridges were rinsed with a few milliliters of dichloromethane (DCM). Bond-Elut C18 cartridges were rinsed with 2 mL of methanol (MeOH) followed by 10 mL of water. Extraction cartridges used in this study were discarded after one use.

Coffee samples investigated in this study included a commercial spray-dried coffee and a batch of pure Ivory Coast coffee beans. The latter was roasted for 2 min in a hot air flow at 400 °C.

Extraction Efficiency of Industrial and Home-Brewed Aqueous Coffee Preparations. The extraction yield of commercial spray-dried coffee extract was estimated as about 50%. One hundred grams of roasted Ivory Coast coffee beans was powdered and extracted using 2000 mL of boiling water. The mixture was filtered through a Melita-type paper filter. The resulting coffee brew was allowed to cool and lyophilized, affording 32 g of crude lyophilizate. The procedure was repeated several times, and the resulting lyophilizates were combined.

Isolation Procedure I (Evaluation of XAD-2 Loading Capacity). One-hundred milliliter batches of 0.5–15% w/v aqueous solutions of instant coffee were spiked with 1.25 nCi of [2-^{14}C]MeIQ, and the pH was adjusted to 7.5 using 1 N NaOH. Glass columns (15 × 1.4 cm i.d.) fitted with glass filter frits and stopcocks at the bottom were filled with 3 g of XAD-2 resin, washed with 15 mL of acetone and MeOH, and equilibrated with 50 mL of water. The coffee solution, followed by 50 mL of water were passed slowly through the column. Elution was carried out with 50 mL of MeOH and 50 mL of MeOH–acetone (1:1). The solvent of the eluate was rotary evaporated in vacuo. The residue was dissolved in 2 mL of 0.5 N NaOH and pipetted on an Extrelut 3 cartridge placed on a Visiprep apparatus. Vacuum was applied for rapid dispersion of the extract. DCM was filled into the Extrelut 3 cartridge and, as soon as the solvent front reached the bottom of the cartridge, a conditioned 1.3 mL CPC–Sephasorb gel containing cartridge was connected. DCM (15 mL) was allowed to run through the tandem. After disconnecting, the CPC–Sephasorb cartridge was eluted with 20 mL of MeOH–DCM–concentrated ammonium hydroxide (14:85:1). The solvent was evaporated from the extract and the residue, dissolved in 1 mL of MeOH, used for scintillation counting.

Isolation Procedure II (Evaluation of Extraction pH on Extrelut). Four samples (1–4) of instant coffee, each weighing 3 g, were dissolved each in 15 mL of water. The pH of samples 2, 3, and 4 was adjusted with 2 N NaOH to 7.11, 9.06, and 11.05, respectively; sample 1 was left unmodified (pH 5.08). Each sample solution was pipetted onto an Extrelut 20 cartridge placed on a Visiprep apparatus and adsorbed by applying vacuum. After 5 min, the cartridges were removed from the Visiprep apparatus and mounted on a rack. DCM was filled in the loaded Extrelut 20 cartridges, and conditioned 2.6-mL CPC–Sephasorb gel cartridges were coupled as soon as DCM reached the bottom of Extrelut; 50 mL of DCM was allowed to flow through the tandem. The cartridges were separated, and the CPC–Sephasorb gel was eluted with 20 mL of MeOH–DCM–ammonium hydroxide (14:85:1). The eluate was concentrated to dryness, redissolved in 1 mL of MeOH, and, after filtration through a 0.2-μm microfilter, used for the Ames test.

Isolation Procedure III. Five grams of lyophilized coffee was dissolved in 100 mL of water and the pH of the solution adjusted to 7.5. [2-^{14}C]MeIQ (0, 0.12, or 1.25 nCi) was added to the sample solution. This was filtered at a flow rate of about 5 mL/min through a glass column (30 cm × 4 cm i.d.) filled with 30 g of XAD-2 resin previously washed with 150 mL of MeOH and 150 mL of acetone and equilibrated with 500 mL of water (Bjeldanes et al., 1982); 200 mL of water was then passed through the column at 10 mL/min. The column was then eluted with 100 mL of MeOH followed by 50 mL of MeOH–acetone mixture (1:1) at 10 mL/min. Used XAD-2 resin was discarded. The eluate was concentrated to dryness in vacuo using a rotary evaporator and weighed (extract A). The residue was dissolved in 15 mL of 0.5 N aqueous sodium hydroxide solution and pipetted on top of an Extrelut 20 cartridge positioned on a Visiprep device. The extract solution was rapidly dispersed on the Extrelut column by use of vacuum. Tandem extraction was done as above with 40 mL of DCM using a 2.6-mL CPC–Sephasorb cartridge. The DCM extract was discarded and the CPC–Sephasorb cartridge eluted using 20 mL of MeOH–DCM–concentrated ammonium hydroxide (14:85:1). This eluate was concentrated to dryness and weighed (extract B). The residue was dissolved in 100 μL of MeOH, diluted with 2 mL of water, and pipetted on a conditioned cartridge filled with Sephasorb HP gel. The sample was allowed to penetrate the sorbent bed under slight positive pressure. The transfer procedure was repeated with another portion of 100 μL of MeOH and 2 mL of water; 6 mL of 23% v/v MeOH in water was then pumped through the cartridge at a flow rate of 0.4 mL/min. The cartridge was eluted using 5 mL of MeOH containing 10% v/v concentrated ammonia and the eluate concentrated to dryness (extract C). The residue was dissolved in 200 μL of MeOH, and 3 mL of 0.1 N aqueous hydrochloric acid was added. This solution was pipetted on top of a conditioned PRS cartridge positioned on the Visiprep and slowly drawn through the cartridge with vacuum. The cartridge was washed at 1 mL/min with 35 mL of MeOH containing 40% 0.1 N aqueous hydrochloric acid followed by 10 mL of water. A conditioned C18 cartridge was coupled to the PRS cartridge, and 20 mL of 0.5 N ammonium acetate buffer of pH 8 was pumped at 1 mL/min through the tandem. The C18 cartridge was removed, the buffer salt washed out using 10 mL of water, and the purified extract eluted with 5 mL of MeOH

Table I. Validation of Extraction Efficiency

extraction step[a]	mass enrichment	recovery of [2-^{14}C]MeIQ, % scintillation counting	recovery of [2-^{14}C]MeIQ, % mutagenicity	intrinsic mutagenicity (TA98 revertants/g equiv, +S-9)
A	HC[b] 3 IC[b] 4	*c*	*d*	*d*
B	HC 170 IC 200	IC 82 ± 2 (n = 2)	*f*	IC 248 HC 340
C	*e*	IC 72 ± 3 (n = 2)	*f*	IC 136 HC 302
D	*e*	IC 64 (n = 1)	*f*	*g*
E	*e*	HC[h] 49 ± 2 (n = 2) IC[h] 61 ± 5 (n = 2)	HC[h] 53 ■ 7 (n = 2) IC[h] 65 ■ 6 (n = 2)	

[a] See Scheme I. [b] HC, home-brew, filtered coffee; IC, instant coffee. [c] Not measurable due to strong coloration of sample. [d] Not measurable due to bactericidal effect. [e] Not determined due to low amounts of residues (<1 mg). [f] Not determined. [g] No intrinsic activity recovered. [h] The recovery calculation includes the fractions marked by hatching in Figure 4.

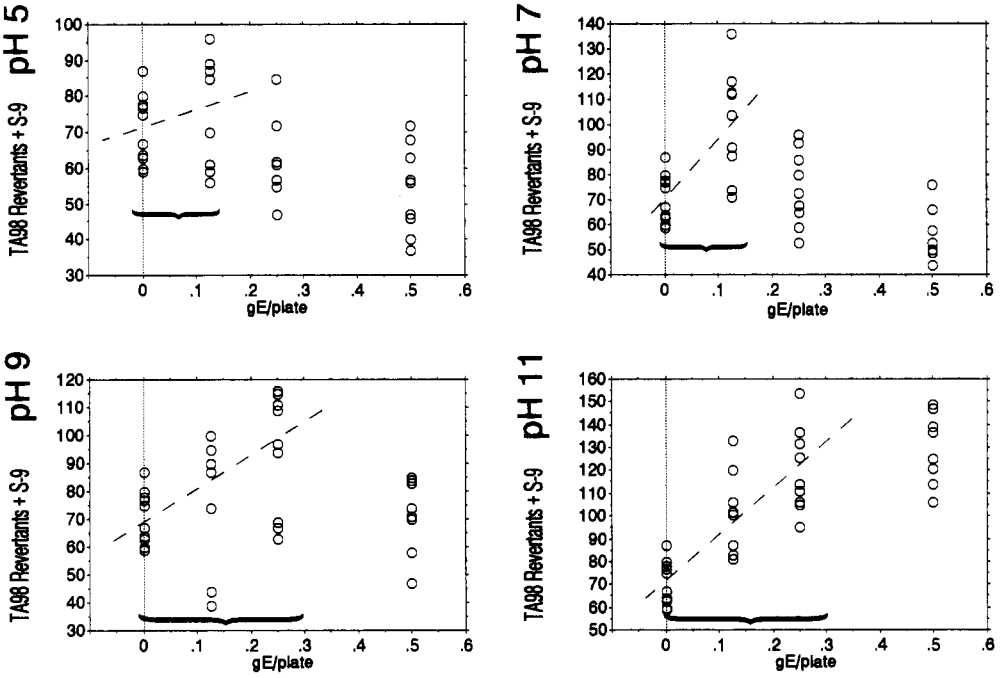


Figure 1. Influence of sample pH on linearity of Ames test results. Samples of 3 g of instant coffee were extracted at pH 5, 7, 9, or 11 on a Extrelut/CPC–Sephasorb tandem as described under Materials and Methods (isolation procedure II). The purified extracts were tested in the Ames test at 0.125, 0.25, and 0.5 g equiv/plate. Linear regression was calculated including the nontoxic portion of the dose–response graphs as indicated by braces on the panels. The mutagenicities were 104, 134, 248, and 222 TA98 revertants/g equiv at pH 5, 7, 9, and 11, respectively. The linearity was best at pH 11 (r^2 = 0.765). At 0.5 g equiv/plate mutagenicity was suppressed by bactericidal effects at each pH value.

containing 10% concentrated ammonia. After evaporation of the solvent on a rotary evaporator in vacuo, the residue was redissolved in MeOH and filtered through a 0.02-μm microfilter into a 1.1-mL vial. The solution was concentrated under a stream of nitrogen and stored at −25 °C (extract D). Before injection, the residue was dissolved in 25 μL of MeOH.

HPLC Separation of Purified Extracts. The HPLC column was washed with MeOH containing 10% acetonitrile at 0.2 mL/min for 16 h. A complete blank run using the gradient profile specified above was then made to equilibrate the column. In a second blank run fractions were collected at the following time intervals: 0–10 min, 1 fraction; 10–25 min, 30 s per fraction (30 fractions); 25–35 min, 1 fraction. The purified extract was separated using the same gradient profile and fractionation scheme. In some cases (see Results), aliquots of 10% v/v of each fraction were used for liquid scintillation counting. The fractions were evaporated using a high-vacuum centrifuge and the residues submitted to the Ames test.

Mutagenicity Test Procedure. The Ames test was performed using the *Salmonella typhimurium* TA98 tester strain including metabolic activation by Aroclor-induced S-9 liver homogenate following the standardized direct plating procedure (Maron and Ames, 1983). Partially purified extracts after stage B or C of the extraction scheme were tested at three concentrations in triplicate together with blank solvent controls for measuring spontaneous reversions and a solution of 0.95 ng/μL IQ as a positive control. Completely purified chromatographic fractions (stage E) from duplicate runs were tested each on single plates. In this case, spontaneous reversions were determined from blank chromatographic runs as explained above. Revertant colonies were counted using a Fisher Count-All 880 instrument. Mutagenic activity of partially purified extracts was calculated by linear regression analysis and expressed as the slope of the linear part of the dose–response relation.

The solution of the radiolabeled [2-^{14}C]MeIQ standard (12.5 nCi/mL) produced 403 revertants/10 μL [573 TA98 revertants/ng (+S-9)] in the Ames test (r^2 = 0.985).

RESULTS

The purification of coffee extracts was performed in five steps, as summarized in Scheme I (see also Materials

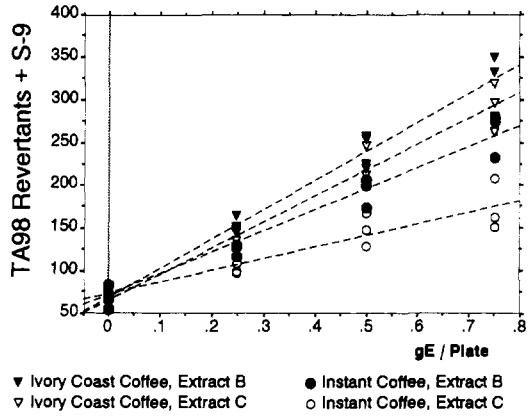


Figure 2. Preextraction on XAD-2 improves linearity of Ames test results. Coffee extracts were fractionated either by XAD-2 and Extrelut/CPC–Sephasorb (extract B, solid symbols) or by XAD-2, Extrelut/CPC–Sephasorb, and Sephasorb (extract C, open symbols) according to isolation procedure III (see Materials and Methods). The extracts were tested at 0.25, 0.5, and 0.75 g equiv/plate. Better linearity up to the highest dose was observed in both XAD-2-pretreated extracts.

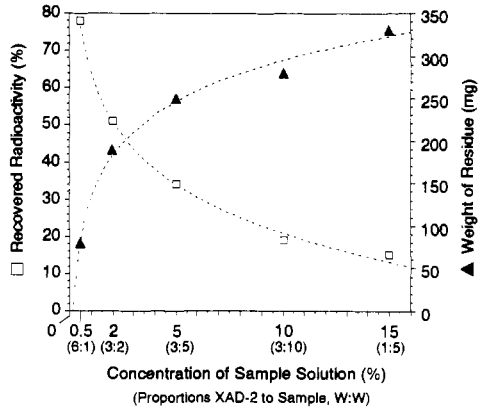


Figure 3. Loading capacity of XAD-2 and Extrelut/CPC–Sephasorb. Aqueous solutions of instant coffee (0.5–15 g/100 mL) were spiked with [2-^{14}C]MeIQ and extracted on 3 g of XAD-2 (isolation procedure I, see Materials and Methods). Scintillation was not measurable in these extracts due to strong chemiluminescence; hence, they were further purified on Extrelut/CPC–Sephasorb. The recovery was found to be a function of the sample concentration. A rapid decrease in recovery of labeled MeIQ at ratios of sample load to XAD-2 amount in excess of 1:6 (concentration of coffee 0.5%) is easily deducible from the graph. The exponential function fitted to the measured data allowed the prediction that as much as twice the quantity of XAD-2 would be theoretically necessary to increase recovery by 10%. Hence, the 1:6 ratio of sample to resin was used for the subsequent extractions.

and Methods). The validation of extraction efficiency for MeIQ included measurement of recovered radioactivity and/or mutagenic activity after [2-^{14}C]MeIQ standard addition (spiking). Recovery of intrinsic mutagenicity in unspiked samples was also monitored (see Table I).

A major problem was to remove bactericidal compounds from both coffee samples to get a linear dose–response relationship in the Ames test. Therefore, intrinsic mutagenic activity could only be detected after extraction stage B. Preliminary experiments with instant coffee extracted on Extrelut/CPC–Sephasorb without previous treatment through XAD-2 showed that the dose–response relationship in the Ames test was closest to linearity at extraction pH 11. This is documented in Figure 1. However, toxic effects were also seen at the highest dose levels of the pH 11 extract. On the other hand, XAD-2 extraction alone led to extracts with even stronger bacterial toxicity (no data shown), but the combination of both the XAD-2 and the Extrelut/CPC tandem extraction proved to be useful for significant improvement of the linearity of dose–response relationships (see Figure 2). The recovery of MeIQ from XAD-2 was strongly dependent on the column loading as determined in a series of small scale extractions with spiked samples (see Figure 3).

Fractionation on Sephasorb (extraction stage C) removed much brown coextracted material from stage B. The intrinsic mutagenicity of instant coffee decreased by as much as 45% in this step but with the roasted coffee sample by only 11% (see Table I). For instant coffee, this was significantly more than the loss in recovery of MeIQ from spiked samples. After step C, extracts were still slightly yellow; therefore, a fourth fractionation was performed using a propanesulfonic acid–silica cation exchange cartridge. All colored matter was removed from the sample after this fourth step, and the recovery of radiolabeled MeIQ was good (see Table I). However, no intrinsic mutagenic activity was recoverable either in the extracts or in the washings. At this point, the conclusion was reached that probably our coffee samples did not contain any MeIQ. However, an additional purification was performed to demonstrate the absence of artifacts in the radioactivity recovered from spiked samples and to estimate the detection limit of the method. Extracts of both coffee types were prepared in duplicate at two spiking levels and with unspiked samples. The risk of column contamination by irreversibly retained mutagenic material was virtually eliminated by performing a standardized column cleaning procedure before each series of analyses, as explained under Materials and Methods, and by mutagenicity testing of fractions from blank chromatographic runs. Fractions from extract separations were analyzed by the Ames test (strain TA98, +S-9), and aliquots were measured by liquid scintillation (see Figure 4). Radioactivity and the mutagenic activity from the spiked samples coeluted at the same retention time as MeIQ, therefore confirming the absence of artifacts.

The intensity of the Ames test response in the extracts showed a linear correlation to the spiking level, and the "noise" level in these "mutagrams" was low (Figure 4). It amounted to 14 ± 2 TA98 revertants in both coffee extracts when expressed as the standard deviation of revertant counts in inactive fractions from a several minute wide chromatogram fraction excluding the MeIQ peak. At 60 ppt spiking level 300 ± 17 TA98 revertants were observed. However, MeIQ was not detected in either unspiked homebrewed or instant coffee since in the chromatograms of these coffees no signals exceeding the noise level were visible. On the basis of these data we concluded that MeIQ, if present in the samples, was below an approximate detection limit of 5–10 ppt.

DISCUSSION

The present extraction scheme is based on the CPC and PRS tandem extraction methods which have been successfully applied to the quantification of nanogram per gram levels of many relevant mutagenic heterocyclic aromatic amines formed in heated meat-containing food products (Gross, 1990). An initial XAD-2 purification step was introduced here to reduce the amounts of bactericidal compounds present in the sample. The three consecutive steps (B, C, and D) were a sequential application of the original CPC extraction, followed by the PRS extraction method (Gross, 1990). The extraction efficiency of this multistage procedure was validated through recovery experiments with radiolabeled standard.

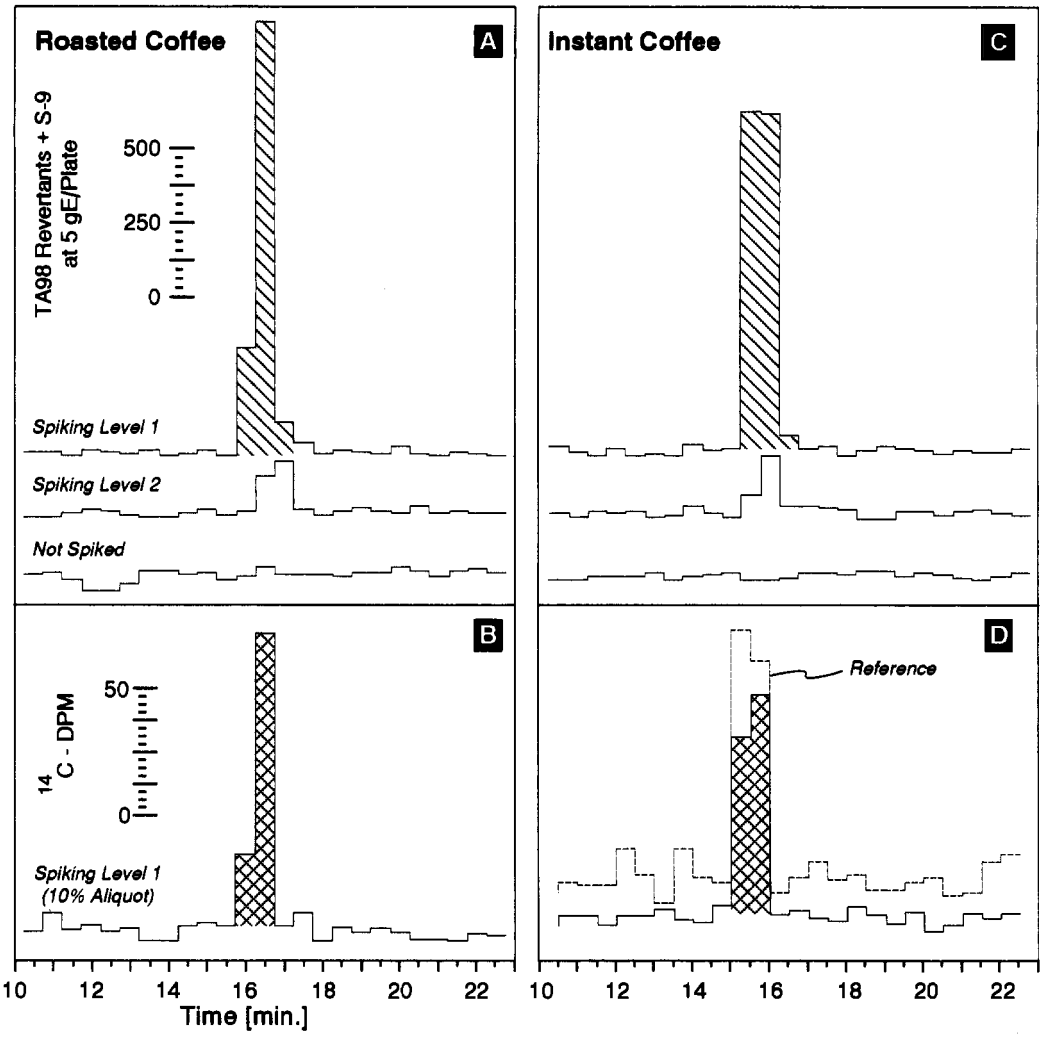


Figure 4. HPLC fractionation of purified coffee extracts. Panels A and C show mutagenicity profiles of coffee extracts at various spiking levels. Level 1 means 0.4 and 0.6 ppb of [2-^{14}C]MeIQ spiked in home-brewed (A) and instant coffee (C), respectively, as calculated with respect to the initial amount of roasted coffee beans. Level 2 was 10-fold lower than level 1. Each line represents the mean result of two determinations. Spontaneous reversions were counted separately for each extract series from residues of a blank fractionation (see Materials and Methods) and subtracted. Panels B and D show radiochromatograms of coffee extracts (solid lines) obtained by counting 10% aliquots of fractions from spiking level 1. The lower right panel includes a radiochromatogram of [2-^{14}C]-MeIQ standard solution (dashed line). Hatched and cross-hatched areas were used for recovery calculations (see Table I).

This procedure avoided potential problems of cross-contamination since only disposable materials were used for the sample preparation prior to HPLC. Special attention was given to the column cleaning procedure before HPLC fractionation, bearing in mind that polar basic compounds such as MeIQ may show strong interactions with residual silanol groups on octadecylsilane phases (Cox and Stout, 1987; Marko et al., 1990). This was considered especially important since the specific mutagenic potency of MeIQ was very high, i.e., nearly 600 revertants/ng. Therefore, traces of this substance could slowly accumulate on the HPLC column and contaminate extracts due to a leaching effect. This risk was eliminated in our experimental setup by counting the spontaneous reversion rates in fractions of blank chromatographic runs prior to analysis.

We investigated two very different types of coffee: a brew from instant coffee, which is usually drunk as such, and a brew from coffee beans, which were intentionally overroasted to amplify the potential formation of mutagens. From our careful analysis MeIQ was undetectable in both of our coffee samples. If present, it was below the estimated detection limit of about 5–10 ppt. Kikugawa and co-workers (Kikugawa et al., 1989) estimated the level of MeIQ in three different coffee types to be as high as 16, 32, and 150 ppt. However, MeIQ was isolated from a methanolic coffee extract of the most heavily roasted sample only. Levels of MeIQ in other samples were estimated by analogy, neglecting thereby that roasting temperature and time might influence the mutagen formation quantitatively and qualitatively. Comparison with our data is further complicated because Kikugawa and co-workers did not estimate losses in recovery, which obviously occur during a multistep purification procedure.

In conclusion, the presence of the mutagen MeIQ in home-brewed as well as instant coffee was not confirmed. If present, it is at levels below picograms per gram. The identification of the weak activation-dependent mutagenic activity found in basic extracts of roasted coffee and instant coffee is not feasible at present and, therefore, must await further investigation.

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