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Quantification of the carcinogens 2-amino-3,8-dimethyl- and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in food using a combined assay based on gas chromatography–negative ion mass spectrometry

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ABSTRACT

A gas chromatographic–mass spectrometric assay has been developed for the measurement of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in food. Stable isotope-labelled analogues of MeIQx and PhIP are used as internal standards and the synthesis of deuterated PhIP is described. The mass spectrometer is operated in the electron-capture negative ion chemical ionisation mode and the amines are chromatographed as their di-3,5-bistrifluoromethylbenzyl derivatives. All three compounds can be measured in a single chromatographic run and detection limits of 0.05, 0.1 and 0.2 ng/g for MeIQx, DiMeIQx and PhIP, respectively, in food are obtained. Various home-cooked and commercially prepared foodstuffs were analysed with this assay and several were found to contain measurable amounts of one or more of the three amines. These results are presented and discussed.

INTRODUCTION

There is now considerable experimental and epidemiological evidence to associate dietary factors with a substantial portion of human cancer. During the last decade, more than a dozen fused-ring heterocyclic aromatic amines have been isolated from a variety of cooked foods, especially meat products, and their chemical structures established [1,2]. These compounds are formed during the cooking process and are present at concentrations of ng/g of food. Bacterial mutagenicity assays have shown most of these compounds to be powerful mutagens and several have subsequently been found to be carcinogenic

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in rodents and non-human primates [2]. The presence of these compounds in the diet and thus their potential as human carcinogens has created a requirement for accurate methods of quantification. Only by determining the dose of these compounds in the diet can an assessment of the risk to the human population of ingesting these compounds be made.

Several heterocyclic aromatic amines have been isolated from cooked beef and three of the most important are 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Fig. 1). It has been reported that each of the three compounds is responsible for ~20% of the mutagenic activity found in beef fried at 300°C [1]. We have developed an assay based on capillary column gas chromatography (GC)–negative ion mass spectrometry (MS) for the simultaneous measurement of MeIQx and DiMeIQx in fried beef [3]. The extraction procedure was subsequently modified to allow the detection and measurement of MeIQx in human urine after consumption of a cooked meat meal [4]. The method uses a stable isotope-labelled analogue of MeIQx as an internal standard and the amines are chromatographed as their di-3,5-bistrifluoromethylbenzyl (di-bisTFMB) derivatives. By synthesising a deuterium-labelled analogue of PhIP for use as an internal standard, this assay has now been extended to include the measurement of PhIP and all three amines can be measured in a single chromatographic run. The combined assay was then used to examine a variety of foodstuffs, some commercially prepared and others home-cooked, for the presence of MeIQx, DiMeIQx and PhIP.

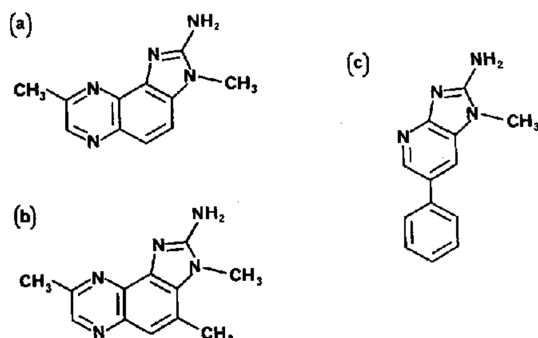

Fig. 1. Chemical structures of (a) MeIQx, (b) DiMeIQx and (c) PhIP.

EXPERIMENTAL

Chemicals

MeIQx, DiMeIQx and PhIP were obtained from Toronto Research Chemicals (Downsview, Canada). Stock solutions of the three compounds in methanol of varying dilutions were prepared and stored at -20°C until required. Chemicals used in the synthesis of [$^{2}H_{5}$]PhIP were purchased from Aldrich (Milwaukee, WI, USA) and [$^{2}H_{6}$]benzene from this source contained 99.5 atom-% deuterium. 3,5-Bistrifluoromethylbenzyl bromide was supplied by Fluorochem (Glossop, UK) while diisopropylethylamine and dodecane were obtained from Sigma (Poole, UK). Acetonitrile, ethyl acetate, methanol, dichloromethane and hexane were all of Analar grade and acetonitrile and ethyl acetate were redistilled before use.

Synthesis of 2-[^{15}N]amino-3,8-dimethyl-[1-^{15}N,2-^{13}C]imidazo[4,5-f]quinoxaline ([^{13}C,$^{15}N_{2}$]MeIQx)

The synthesis of this compound has been described elsewhere [3].

Synthesis of 2-amino-1-methyl-6-pentadeuterophenylimidazo[4,5-b]pyridine ([$^{2}H_{5}$]PhIP)

2-Chloro-5-pentadeuterophenylpyridine (II). Following the diazotization method of Stavenuiter *et al.* [5], 5-amino-2-chloropyridine (I) (1.5 g) was added to potassium acetate (2.14 g), 18-crown-6 (0.48 g), *n*-pentyl nitrite (1.8 g) and [$^{2}H_{6}$]benzene (50 g) in an oven-dried flask and the mixture was heated to reflux with stirring for 3 h. The reaction product was evaporated to dryness and purified by flash chromatography with chloroform to give 2-chloro-5-pentadeuterophenylpyridine (yield 1.25 g, 55%). Mass spectrum (electron impact ionisation), *m/z* (relative intensity): 196(36) $M^{+\cdot}$, 194(100) $M^{+\cdot}$, 159(28), 121 (23).

2-Amino-5-pentadeuterophenylpyridine (III). 2-Chloro-5-pentadeuterophenylpyridine (500 mg) and cupric sulphate (10 mg) in liquid ammonia were heated at 200°C for seven days in a PTFE-lined pressure bomb (Parr Instrument, Moline, IL, USA). After the ammonia had been allowed to evaporate, the residue was dissolved in water and extracted with chloroform. The extract was purified by flash chromatography with chloroform to give 2-amino-5-pentadeuterophenylpyridine (yield 225 mg, 50%). Mass spectrum (electron impact ionisation), *m/z* (relative intensity): 175(100) $M^{+\cdot}$, 149(15), 148(20).

2-Amino-3-bromo-5-pentadeuterophenylpyridine (IV). Bromine (80 μl) in glacial acetic acid (1 ml) was added dropwise to a solution of 2-amino-5-pentadeuterophenylpyridine (200 mg) in glacial acetic acid (10 ml) and the resulting mixture was stirred for 3 h. The acetic acid was removed by evaporation and the residue purified by flash chromatography with chloroform to give 2-amino-3-bromo-5-pentadeuterophenylpyridine (yield 170 mg, 59%). Mass spectrum (electron impact ionisation), *m/z* (relative intensity): 255 (86) $M^{+\cdot}$, 253(100) $M^{+\cdot}$, 119(15).

2-Amino-1-methyl-6-pentadeuterophenylimidazo[4,5-b]pyridine ([$^2H_5$]PhIP) (V). 2-Amino-3-bromo-5-pentadeuterophenylpyridine (150 mg) and cupric sulphate (10 mg) were added to a 40% (w/w) solution of methylamine in water (50 ml) in a PTFE-lined pressure bomb and heated at 200°C for 72 h. After cooling, the aqueous mixture was extracted with chloroform and the organic extract evaporated to dryness. The residue was redissolved in a 25% solution of phosphoric acid (30 ml), the latter placed in a PTFE-lined pressure bomb together with cyanogen bromide (2 g) and the bomb heated at 200°C for 2 h. The contents of the pressure bomb were then poured onto ice and the pH adjusted to 7. The aqueous mixture was extracted with dichloromethane–methanol (75:25, v/v) and the organic extract, after evaporation of the solvent, was purified by flash chromatography with chloroform–methanol (95:5, v/v) to give 2-amino-1-methyl-6-pentadeuterophenylimidazo[4,5-*b*]pyridine (yield 42 mg, 31%). Mass spectrum (electron impact ionisation), *m/z* (relative intensity): 229(100) $M^{+\cdot}$, 228(74), 213(7), 201(12).

Preparation of food samples for extraction

Cooked meat products. A portion of cooked meat was coarsely minced in a domestic food processor. To a sample of the chopped tissue (2 g) was added [^{13}C,$^{15}N_2$]MeIQx (10 ng) in methanol (200 μl) and [$^2H_5$]PhIP (50 ng) in methanol (100 μl), the mixture then being homogenised in 0.25 *M* HCl (10 ml) with a Polytron tissue homogeniser (Kinematica, Lucerne, Switzerland). After centrifugation (1500 *g* for 10 min), an aliquot of the acidic aqueous supernatant (5 ml) was transferred to a clean, screw-capped glass tube.

Powdered and liquid foodstuffs. A sample of foodstuff (0.5 g) was weighed out in a screw-capped glass tube. 0.5 *M* Hydrochloric acid (4 ml) was pipetted into the tube and the mixture shaken vigorously until all solid material had dissolved. [^{13}C,$^{15}N_2$]MeIQx (5 ng) in methanol (100 μl) and [$^2H_5$]PhIP (25 ng) in methanol (50 μl) were added and the tube contents mixed thoroughly by manual inversion.

Extraction of food samples

Dichloromethane (5 ml) was added to each glass tube containing acidic aqueous solution. The tube contents were mixed by manual inversion and then separated by centrifugation (1200 *g* for 5 min). The upper aqueous layer was transferred to a clean tube while the lower organic layer was discarded. This washing procedure was repeated twice more, then 2 *M* sodium carbonate solution (4 ml) was added to the aqueous phase and the alkaline product extracted with ethyl acetate (2 × 5 ml). The combined organic extract was evaporated to dryness under nitrogen and the residue transferred to a half-dram glass vial with methanol (2 × 0.75 ml). Samples were stored at −20°C prior to derivatisation.

Standards

Six standards containing [^{13}C,$^{15}N_2$]MeIQx (5 ng), [$^2H_5$]PhIP (25 ng) and various amounts of MeIQx (0–4 ng), DiMeIQx (0–4 ng) and PhIP (0–20 ng) in methanol (150 μl) were prepared.

These solutions were stored in half-dram glass vials at −20°C prior to derivatisation.

Derivatisation procedure

Methanol present in standards and food extracts was removed by evaporation under nitrogen. To each vial was added a 5% solution of 3,5-bistrifluoromethylbenzyl bromide in acetonitrile (80 μl) and diisopropylethylamine (20 μl). The reaction mixture was left at room temperature overnight and then evaporated to dryness under nitrogen. To the residue was added 0.1 *M* hydrochloric acid (200 μl) and hexane (750 μl). The vial contents were vortex-mixed, centrifuged (600 *g* for 5 min) and the upper organic layer discarded. This washing procedure with hexane was repeated, then 0.5 *M* sodium carbonate solution (100 μl) was added to the aqueous phase and the alkaline product extracted with ethyl acetate (2 × 750 μl). The combined organic extract in a half-dram glass vial was evaporated to dryness under nitrogen and the residue reconstituted in dodecane (50 μl for standards and 20 μl for food extracts). Aliquots of 2 μl were injected into the gas chromatograph–mass spectrometer.

Gas chromatography–mass spectrometry

A Finnigan-MAT 4500 combined gas chromatograph–quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA, USA) was used. The gas chromatograph was equipped with a 15 m × 0.25 mm I.D. DB5 J&W fused-silica capillary column (0.25 μm film thickness) which was routed through the separator oven (maintained at 290°C) directly into the mass spectrometer ion source. Helium was used as carrier gas at a head pressure of 69 kPa. The gas chromatograph was fitted with a Grob-type capillary injector operated in the splitless mode and maintained at a temperature of 270°C. The gas chromatograph oven temperature was held at 200°C for 1 min, then raised to 320°C at 20°C min^{-1} and held at 320°C for a further 1 min. Under these conditions, the retention times of the di-bisTFMB derivatives of MeIQx and [^{13}C,^{15}N$_2$]MeIQx were 5.65–5.70 min, of DiMeIQx 5.90 min and of PhIP and [^{2}H$_5$]PhIP 6.65 min. The mass spectrometer was operated in the negative ion chemical ionisation mode with an electron energy of 100 eV. Ammonia gas was admitted to an indicated ion source pressure of 53 Pa and the indicated ion source temperature was maintained at 150°C. The mass spectrometer was tuned to monitor negative ions at *m/z* 438 (MeIQx), *m/z* 441 ([^{13}C,^{15}N$_2$]MeIQx), *m/z* 449 (PhIP), *m/z* 452 (DiMeIQx) and *m/z* 454 ([^{2}H$_5$]PhIP) and data acquisition and reduction were performed by an INCOS data system using IDOS 2 software.

RESULTS AND DISCUSSION

The method developed in this laboratory for the isolation of MeIQx and DiMeIQx from meat uses solvent extraction together with manipulation of pH [3]. A stable isotope-labelled analogue of MeIQx ([^{13}C,^{15}N$_2$]MeIQx) is added as an internal standard for both compounds prior to extraction. The amines are then converted to their di-bisTFMB derivatives before analysis by capillary column GC–negative ion MS. The di-bisTFMB derivatives of MeIQx, DiMeIQx and [^{13}C,^{15}N$_2$]MeIQx have good GC properties and, when the mass spectrometer is operated in the electron-capture negative ion chemical ionisation (ECNICI) mode, give mass spectra which contain intense, high-mass ions suitable for selected ion monitoring (SIM).

PhIP has the same aminoimidazole ring system that is present in the chemical structures of MeIQx and DiMeIQx (Fig. 1) and so PhIP should have a similar pK_a and derivatise in the same way as the other two amines. Using the same extraction and derivatisation procedures for the three compounds would have the advantage of allowing all three amines to be analysed in a single chromatographic run. However, while it is possible to use [^{13}C,^{15}N$_2$]MeIQx as a common internal standard for the measurement of MeIQx and DiMeIQx [3], initial experiments showed that this compound was not a suitable internal standard for the analysis of PhIP and so a structural analogue of PhIP labelled with five deuterium atoms ([^{2}H$_5$]PhIP) was synthesised. The reaction scheme used for the preparation of the deut-

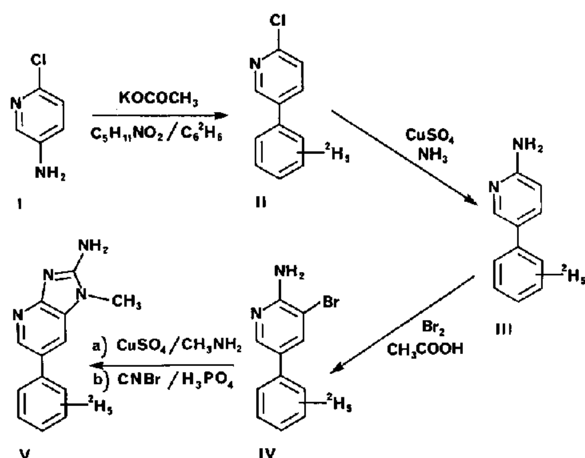

Fig. 2. Synthetic route for the preparation of [$^2H_5$]PhIP.

erated compound (Fig. 2) is based on a synthetic route for the preparation of PhIP which has been published elsewhere [6].

PhIP and [$^2H_5$]PhIP form di-bisTFMB derivatives under the experimental conditions described above and the negative ion mass spectra of these derivatives are analogous to those of the di-bisTFMB derivatives of MeIQx, DiMeIQx and [^{13}C,$^{15}N_2$]MeIQx described elsewhere [3]. The mass spectra of the PhIP and [$^2H_5$]PhIP derivatives (Fig. 3) contain small [M − 1] ions at *m/z* 675 and 680, respectively, which constitute less than 1% of the total ion current. The most abundant ions in the mass spectra (*m/z* 449 and 454) correspond to loss of a bisTFMB group from the respective molecular ions. The di-bisTFMB derivatives of PhIP and [$^2H_5$]PhIP afford the same good limits of detection for the parent compounds as has been obtained with MeIQx and DiMeIQx. When the mass spectrometer was set to monitor ions *m/z* 449 and 454, amounts of derivative equivalent to 1 pg of the parent amine could be detected.

Standard curves for the measurement of MeIQx, DiMeIQx and PhIP were prepared. Six solutions containing [^{13}C,$^{15}N_2$]MeIQx (5 ng), [$^2H_5$]PhIP (25 ng) and various amounts of MeIQx (0–4 ng), DiMeIQx (0–4 ng) and PhIP (0–20 ng) in small volumes of methanol were evaporated to dryness and derivatised. These were then analysed by GC–MS with SIM of negative ions *m/z* 438 (MeIQx), *m/z* 441 ([^{13}C,$^{15}N_2$]MeIQx), *m/z* 452 (DiMeIQx), *m/z* 449 (PhIP) and *m/z* 454 ([$^2H_5$]PhIP). The similar retention times of the derivatives of the three amines and two internal standards mean that all five compounds can be conveniently monitored

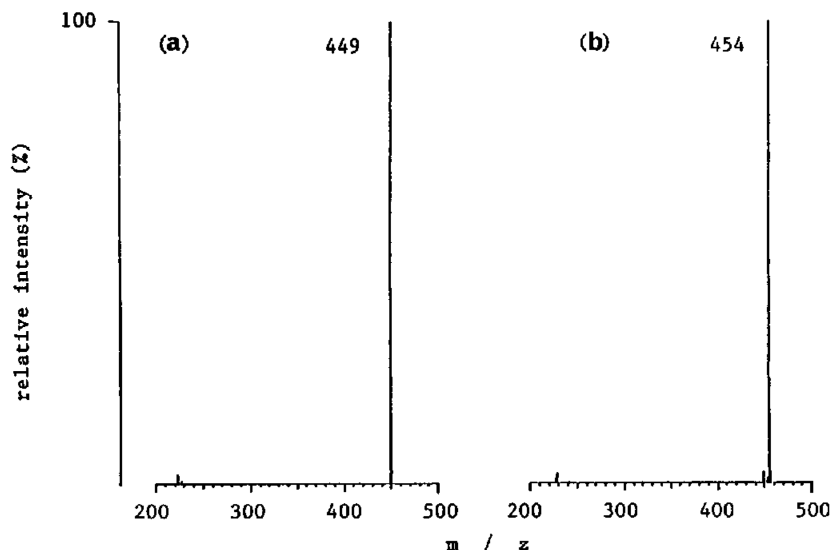

Fig. 3. Negative ion mass spectra of the di-bisTFMB derivatives of (a) PhIP and (b) [$^2H_5$]PhIP.

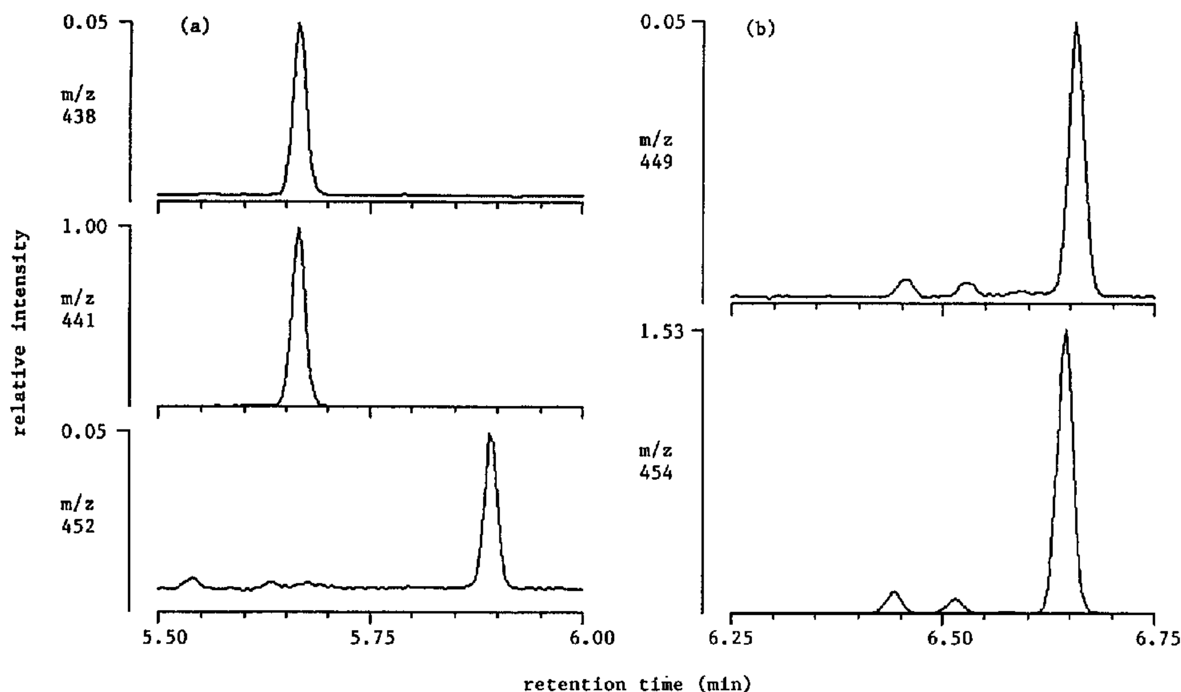

Fig. 4. SIM traces of a derivatised standard containing [^{13}C,^{15}N$_2$]MeIQx (5 ng), [^{2}H$_5$]PhIP (25 ng), MeIQx (0.2 ng), DiMeIQx (0.2 ng) and PhIP (1 ng). Retention times of the di-bisTFMB derivatives of MeIQx (*m/z* 438) and [^{13}C,^{15}N$_2$]MeIQx (*m/z* 441) are 5.65 min, of DiMeIQx (*m/z* 452) 5.90 min and of PhIP (*m/z* 449) and [^{2}H$_5$]PhIP (*m/z* 454) 6.65 min.

in a single chromatographic run (Fig. 4) and so the three standard curves were constructed simultaneously. We have previously shown that the unextracted standard curves for MeIQx and DiMeIQx over the range 0–4 ng are linear with intercepts on the ordinate close to the origin [3]. A similar result was obtained for PhIP over the concentration range 0–20 ng (y = 0.026x + 0.003, r = 0.998).

The extraction procedure used originally for the isolation of MeIQx and DiMeIQx from cooked meat [3] was then applied to the analysis of PhIP. To an acid extract of meat tissue or an acidic solution of foodstuff were added [^{13}C,^{15}N$_2$]MeIQx and [^{2}H$_5$]PhIP in methanol. Following three washes with dichloromethane to remove oil and fats, the aqueous phase was made alkaline and the amines were extracted with ethyl acetate. This sequence, together with a pH-dependent wash and extraction step in the derivatisation procedure, was the only sample purification used and, in almost all cases, the SIM traces obtained were free of chromatographic interference. Recoveries of MeIQx and PhIP through the extraction procedure can be estimated by comparing internal standard peak areas in extracted samples with those in unextracted standards. When this was done, values of ~40% for MeIQx and ~30% for PhIP were obtained. The methanol mixtures of the three amines and two internal standards used in preparing the unextracted standard curves were taken through the extraction procedure prior to derivatisation and SIM analysis. The slopes of the extracted standard curves obtained for MeIQx, DiMeIQx and PhIP were found not to be significantly different from the unextracted standard curves and so routine analysis of the three amines was made by reference to the latter. The accuracy and precision for measurement of MeIQx (2 ng/g food)

TABLE I

AMOUNTS OF MeIQx, DiMeIQx AND PhIP IN VARIOUS FOODS

Results are the means of duplicate analyses of single food samples except for fried beef patties, which are the means (± S.E.M.) of measurements made on 33 meat samples cooked at different times [9]. Int = interference in the chromatogram. Limits of detection of the assay are 0.05, 0.1 and 0.2 ng/g for MeIQx, DiMeIQx and PhIP in food, respectively.

Food	MeIQx (ng/g)	DiMeIQx (ng/g)	PhIP (ng/g)
Fried beef patties	2.2 ± 0.2	0.7 ± 0.1	16.4 ± 2.1
Fried steak (medium rare)	0.5	0.1	0.6
Fried fatty bacon	1.2	0.3	2.7
Fried lean bacon	0.9	0.2	1.6
Barbecued pork	0.4	0.1	4.2
Barbecued chicken	0.3	0.1	Int
Beef stock-cube	0.6	0.3	0.3
Food grade beef extract	0.6	<0.1	<0.2
Condensed consommé	0.1	<0.1	<0.2

were 2.06 ng/g ± 1.5% (mean ± R.S.D., n = 6), for DiMeIQx (1 ng/g food) 0.92 ng/g ± 4.1% (mean ± R.S.D., n = 6) and for PhIP (10 ng/g food) 10.5 ng/g ± 2.6% (mean ± R.S.D., n = 6). The limits of detection of the assay, set in the absence of any chromatographic interference at a signal-to-noise ratio of 5, were 0.05, 0.1 and 0.2 ng/g for MeIQx, DiMeIQx and PhIP in food, respectively.

A variety of foodstuffs were analysed for the presence of MeIQx, DiMeIQx and PhIP using the combined assay described above and several were found to contain detectable amounts of one or more of the three amines (Table I). Highest concentrations were found in minced beef steak which had been formed into patties (~100 g) and cooked on a griddle hotplate, without added fat or oil, for 10–15 min at 200–250°C until well browned. These levels of amines in fried beef patties are similar to those found by other workers using different analytical techniques [1,7,8] and are reported in detail elsewhere [9]. The other foodstuffs containing measurable amounts of amines were all fried or barbecued meats (Fig. 5) or commercially prepared cooked meat extracts (Fig. 6). However, we were unable to detect MeIQx, DiMeIQx and PhIP in other home-cooked meat dishes such as beef casserole, beef chili, beef bolognaise, chicken casserole and commercially prepared products such as chicken stock-cube, game soup, condensed oxtail soup and oxtail soup powder. Vegetable-based foodstuffs such as edible vegetable extract, gravy granules and soy sauce also gave a negative result.

These measurements support the suggestion that it is the cooking of muscle tissue from animals by frying or broiling at relatively high temperatures (>200°C) that gives rise to the formation of these compounds [10–13]. The physical contact of meat with a hot metal surface therefore appears to be of major importance in this synthesis although oven roasting and baking, which cook mainly by heat convection, have been reported to produce low to intermediate levels of mutagenicity in meat [13]. However, when we analysed roast beef, roast chicken, roast pork and baked pork sausage, we were unable to detect any MeIQx, DiMeIQx and PhIP in these products and it is possible that the mutagenicity found by other workers is due to one or more of the other heterocyclic amines that have been identified in cooked food.

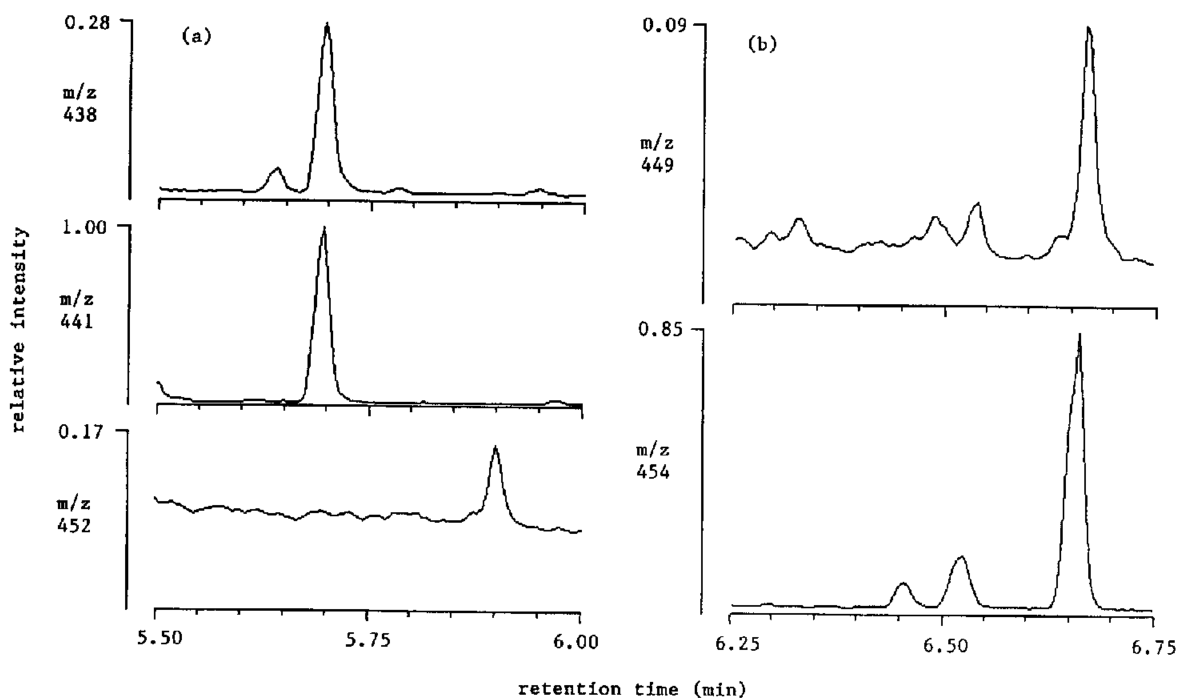

Fig. 5. SIM traces for the analysis of (a) MeIQx and DiMeIQx and (b) PhIP in fried fatty bacon. Retention times of the di-bisTFMB derivatives of MeIQx (*m/z* 438) and [$^{13}C,^{15}N_2$]MeIQx (*m/z* 441) are 5.70 min, of DiMeIQx (*m/z* 452) 5.90 min and of PhIP (*m/z* 449) and [$^2H_5$]PhIP (*m/z* 454) 6.65 min.

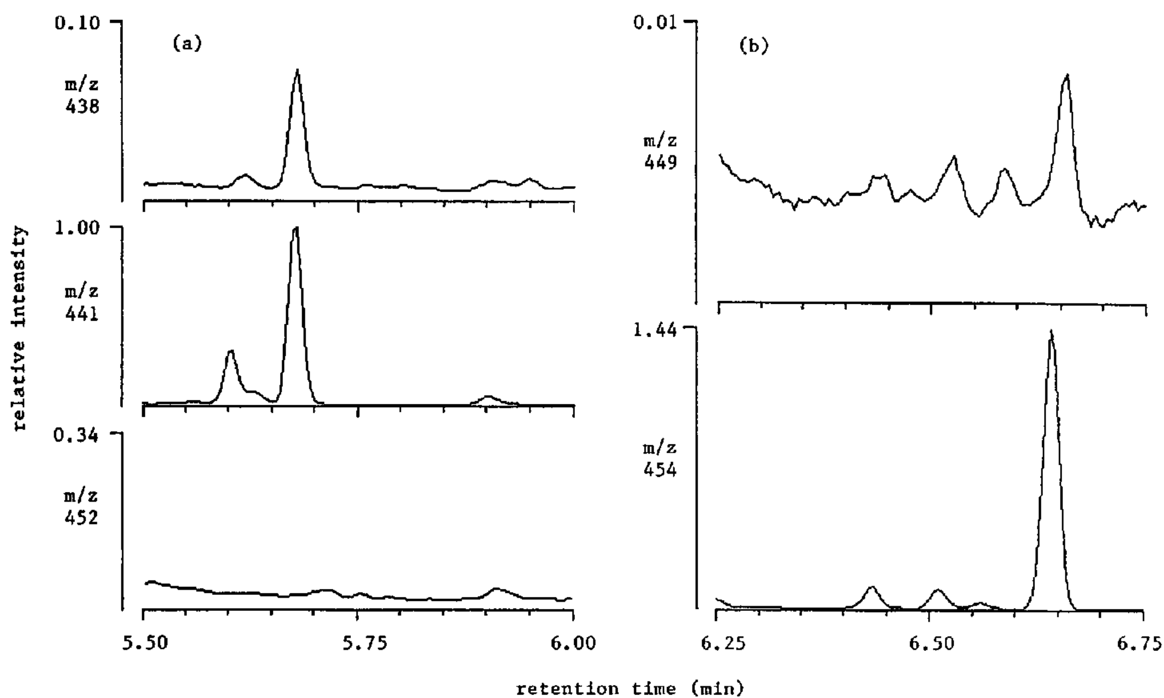

Fig. 6. SIM traces for the analysis of (a) MeIQx and DiMeIQx and (b) PhIP in a beef stock cube. Retention times of the di-bisTFMB derivatives of MeIQx (*m/z* 438) and [$^{13}C,^{15}N_2$]MeIQx (*m/z* 441) are 5.70 min, of DiMeIQx (*m/z* 452) 5.90 min and of PhIP (*m/z* 449) and [$^2H_5$]PhIP (*m/z* 454) 6.65 min.

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