ORGANIC COMPOUNDS FORMED IN THERMALLY TREATED HIGH-PROTEIN FOOD PART II: AZAARENES

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SUMMARY

An analytical scheme developed for determination of PAH and aminoazaarenes has been applied to the analysis of aza-PAH formed in thermally treated high-protein food. The clean-up procedure, which was based on tandem solid-phase extraction (SPE) on columns filled with Extrelut diatomaceous earth and with cation exchanger (propylsulphonic acid, PRS), enabled selective isolation of carcinogenic azaarenes (benzo-acridines and dibenzoacridines) from meat samples. Nine meat dishes (beef, pork, and poultry) grilled or prepared according to recipes used in the Upper Silesia region (roasted, fried) were investigated. Identification and quantitative analysis of four individual compounds – benzo(*a*)acridine, benzo(*c*)acridine, dibenzo(*a,c*)acridine and dibenzo(*a,h*)acridine – were achieved by HPLC and GC–MS. The total azaarene content of the meat ranged from 0.79 to 3.30 ng g^{-1} and the value calculated for daily human exposure to these azaarenes did not exceed 0.35 μg day^{-1} person^{-1}.

INTRODUCTION

It has been noticed for many years that the increase in the incidence of malignant diseases, mainly colorectal, hepatic, breast, kidney, and urinary bladder cancer, especially in highly developed countries is closely connected with the diet [1–3]. Polycyclic aromatic hydrocarbons (PAH), among the most strongly carcinogenic xenobiotics ingested by the human organism with its food, have been quite successfully investigated and many reports describe the isolation and determination of PAH in food samples, including thermally treated (fried, roasted, grilled, smoked) high-

protein food [4,5]. Less information is available on basic nitrogen-containing PAH (azaarenes, aza-PAH, *N*-heterocyclic PAH). A significant number of azaarenes, particularly compounds containing four and five rings are highly mutagenic in the Ames test, often more so than their parent hydrocarbons [6,7]. Most metabolites of azaarenes (dihydrodiols and diol-epoxides) are highly carcinogenic in experimental animals [8–11]. Aza-PAH are emitted into the environment during burning of solid and liquid fuels such as coal, crude oil products, or coal tar [12,13] and chemically synthesized azaarenes are used as antioxidants, pesticides, and pharmaceuticals [14]. These anthropogenic azaarenes can enter the environment in different ways. Aza-PAH have been determined in aerosols in urban atmospheres [12,15], in river and lake sediments [16], in sewage sludges [17], and in groundwater [18]. As a result of pyrolysis and incomplete combustion of organic matter containing nitrogen [19], azaarenes have also been identified in thermally–treated meat [20–22].

Procedures similar to those used for PAH can be used for clean-up and separation of aza-PAH from food, although it should be stressed that selective separation of azaarene base fraction from hydrocarbon neutral fraction, a crucial step in the analysis of these compounds, requires use of special methods. The PAH content of the complex matrix of food samples is higher than the azaarene content. Liquid–liquid extraction has been used to separate the azaarene fraction [22,23]. Nowadays, because of better recoveries of the compounds, other methods are applied, including solid-phase extraction [20,21] and ion-exchange chromatography [22,24]. Qualitative and quantitative determinations of azaarenes are, as for PAH, performed by chromatographic methods, i.e. high-performance liquid chromatography (HPLC) [20,21,25] and capillary gas chromatography with flame ionisation detection or mass spectrometry (GC–FID, GC–MS) [21,26].

This paper describes the use of tandem solid-phase extraction, HPLC, and GC–MS, for the first time in Poland, for separation and determination of aza-PAH in meat dishes thermally treated according to domestic conditions.
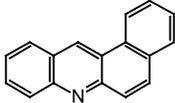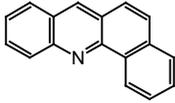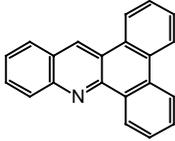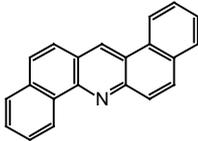

EXPERIMENTAL

Materials, Reagents, Samples

The compounds studied, benzo(*a*)acridine, benzo(*c*)acridine, dibenzo(*a,c*)acridine, and dibenzo(*a,h*)acridine were obtained from Promochem

(Wesel, Germany). The structures of the compounds and the abbreviations used are given in Table I.

Table I

Structures of azaarenes and detection limits determined by HPLC

Compound	Abbreviation	Structure	Detection limit (ng on column)[a]
Benzo(*a*)acridine	B(a)Ac		0.02
Benzo(*c*)acridine	B(c)Ac		0.02
Dibenzo(*a,c*)acridine	DB(a,c)Ac		0.1
Dibenzo(*a,h*)acridine	DB(a,h)Ac		0.08

[a]Detection limits (based on $S/N = 3$) were determined by use of azaarene standard mixtures loaded directly on to a Supelcosil LC-PAH column by means of a 20-μL loop injector

The mobile phases used were dichloromethane, toluene, methanol, and acetonitrile (J.T. Baker, Groß-Gerau, Germany). Solvents and chemicals were of HPLC grade. All solutions were passed through a 0.45-μm filter (J.T. Baker) before injection into the HPLC or GC–MS systems. Diatomaceous earth extraction columns (Extrelut, 20 mL) and refill material were from Merck (Darmstadt, Germany). Propylsulphonic acid (PRS, 500 mg) SPE columns were from J.T. Baker. These columns were preconditioned with dichloromethane (4 mL).

The meat dishes most commonly eaten in Poland (especially in the Upper Silesia region) were investigated for azaarene content:

- *Well-Done Pan-Fried Pork Chops*. Meat was sliced into portions, coated in eggs and bread crumbs, and fried in margarine for 15 min on each side. The frying temperature was between 190 and 200°C.

- *Medium Pan-Fried Beef Collar*. Meat slices were pounded, covered with smoked bacon, onions, and pickles, and after coating fried in fat preheated to 200°C, without covering, for 20 min. Water was then added and the whole preparation was simmered under cover for 1 h at 90–95°C. The coating was removed before analysis.

- *Very Well Done Grilled Pork Neck (No. 1)*. Meat pieces were grilled for 30 min (15 min each side) on a common garden-type grill fuelled with charcoal.

- *Well Done Roasted ("On Salt") Pork Neck (No. 2)*. Meat (1 kg) was placed on a baking pan previously covered with 1 kg salt and the whole preparation was placed in an electric oven preheated to 220°C. The meat was roasted at 180°C for 3 h.

- *Very Well Done Pan-Fried Beef/Pork Minced Chops*. Minced meat (0.5 kg) was mixed with 1 egg then 2 tablespoons bread crumbs and 1 tablespoon sour cream were added. The 1.5-cm-thick burgers were covered with bread crumbs and fried in margarine for 12 min on each side. After frying the fat was drained off by use of filter paper.

- *Well Done Pan-Fried Turkey Breast (No. 1)*. Meat slices (150–200 g, 1.5 cm thick) were fried in margarine at 190–200°C for 15 min on each side in a Teflon-coated frying pan. After frying the fat was drained off by use of filter paper.

- *Well Done Roasted Turkey Breast (No. 2)*. Meat slices (150–200 g, 1.5 cm thick) were lightly pounded, brushed with vegetable oil, wrapped in aluminium foil and roasted in a gas oven at 160°C for 1 h.

- *Very Well Done Grilled Pork Fillet and Chicken Breast*. Pieces of meat were grilled for 30 min (15 min on each side) on a common garden-type grill fuelled with charcoal.

Clean-up Procedure

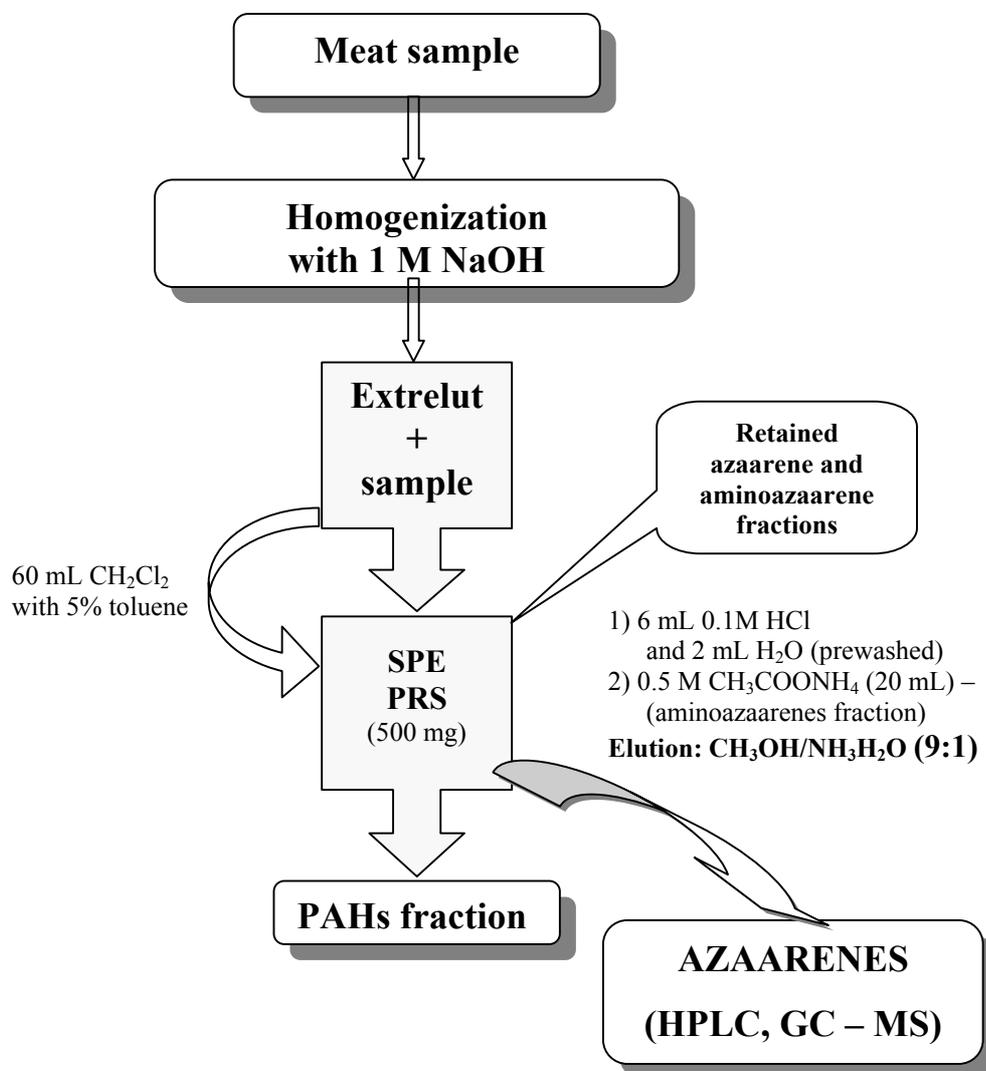The separation scheme (Fig. 1) involved two steps.

Fig. 1

Schematic diagram of clean-up procedure

Step I. Solid-Phase Extraction Using Diatomaceous Earth (Extrelut-20) Columns Coupled with Propylsulphonic acid (PRS) Columns

The meat sample (25 g) was homogenised with aqueous NaOH (1 M, 75 g). From the dense suspension obtained, 20 g (containing 5 g meat) was sampled four times (equivalent to a total of 20 g meat). Cold 1 M NaOH solution (10 mL) and 15 g Extrelut were added to each portion

and, after thorough mixing, each portion was loaded on to a column. After filling, each column was connected to a PRS–SPE column and eluted with dichloromethane. This fraction contains polycyclic aromatic hydrocarbons whereas aminoazaarenes and aza-PAH were adsorbed on the PRS column.

Step II. Desorption of Azaarenes

The PRS columns were rinsed with 0.1 M HCl (6 mL) and 0.5 M ammonium acetate (20 mL) until the aminoazaarene fraction was eluted. These compounds were then adsorbed on a C_{18} column for further clean-up. The azaarene fraction was eluted from the PRS column with 4 mL 9:1 (*v/v*) methanol–aqueous ammonia.

To evaluate the percentage recovery of azaarenes and to prevent matrix effects affecting peak positions in HPLC and GC–MS chromatograms, spiked and unspiked samples were analysed under the same conditions. Spiked samples were prepared by adding 200 ng of each of four standards (B(a)Ac, B(c)Ac, DB(a,c)Ac, and DB(a,h)Ac) to 5 g minced meat samples at the beginning of the homogenisation and extraction step.

High-Performance Liquid Chromatography and Gas Chromatography–Mass Spectrometry

Because of the very complex composition of the fractions investigated and the need to separate isomeric azaarenes, HPLC analysis was performed on three chromatographic columns and only results obtained at the highest selectivity of a given column and under optimum elution conditions were taken into consideration. The systems used for HPLC analysis are listed in Table II.

Qualitative analysis of aza-PAH was based on comparison of retention factors (*k*) calculated for standard azaarenes with values for appropriate components identified in spiked and unspiked meat samples. Quantitative determination was performed by use of an external calibration curve method. Determination coefficients (r^2) for azaarene standard curves were: Nucleosil 100 C_{18} column, B(a)Ac – 0.989, B(c)Ac – 0.985, DB(a,c)Ac and DB(a,h)Ac – 0.983; Chrom Spher PAH column, B(a)Ac – 0.979, B(c)Ac – 0.961, DB(a,c)Ac – 0.980, DB(a,h)Ac – 0.934; Supelcosil LC-PAH column, B(a)Ac – 0.990, B(c)Ac – 0.990, DB(a,c)Ac – 0.975, DB(a,h)Ac – 0.982. When azaarenes were detected by HPLC their presence was confirmed by GC–MS analysis of standards and of the azaarene fractions isolated from spiked and unspiked meat samples. The conditions used for analysis are presented in Table II.

Table II

Conditions used for HPLC and GC–MS analysis of azaarenes

Method	Apparatus	Detection	Column	Separation conditions
HPLC	Knauer (Berlin, Germany) liquid chromatograph Shimadzu (Kyoto, Japan) LC-9A liquid chromatograph	Fluorescence detection, $\lambda_{ex} = 360$ nm, $\lambda_{em} = 460$ nm (Shimadzu RF-10 Axl)	1. Chrom Spher PAH (5 μm particle size, 250 mm × 4.6 mm i.d., Varian) 2. Nucleosil 100 C_{18} (5 μm particle size, 250 mm × 4.6 mm i.d., Knauer) 3. Supelcosil LC-PAH (5 μm particle size, 250 mm × 4.6 mm i.d., Supelco) with Supelguard precolumn (5 μm, 4 mm i.d., Hypersil ODS), $T = 30°C$	Acetonitrile–water, 84:16 (v/v) under isocratic conditions (flow rate 2.5 mL min^{-1})
GC–MS	Shimadzu QP-2000 mass spectrometer connected to a GC-14 gas chromatograph	Electron-impact, electron energy 70 eV, 250°C	25 m × 0.2 mm i.d. × 0.25 μm Ultra 1, fused-silica capillary column (Hewlett Packard)	Injection splitless, 2 μL, 280°C, interface temp. 280°C Temp. programme from 120°C (7 min) at 20° min^{-1} to 200°C then at 3° min^{-1} to 280°C (25 min); carrier gas helium, flow rate 1 mL min^{-1}

RESULTS AND DISCUSSION

From the data presented in Table III it is apparent that the aza-PAH determined were dominated by four-ring compounds, i.e. benzo(*a*)-acridine and benzo(*c*)acridine, which were found in almost all meat dishes (the exceptions being pork neck no. 1 and pork fillet). Their concentrations were not high – from 0.15 to 0.75 ng g^{-1} and from 0.04 to 0.88 ng g^{-1}, respectively, and were much lower than the PAH content of the thermally treated meat samples [4,5].

Typical chromatograms obtained from HPLC of azaarenes are given in Fig. 2. It was found that azaarenes containing five rings, i.e. dibenzo-(*a,c*)acridine and dibenzo(*a,h*)acridine, could also be formed during thermal treatment of meat dishes, but in significantly lower quantities – in most samples their content was close to the detection limit. For such samples

Table III

Azaarene content of the meat samples (ng g^{-1} cooked meat)[a] and daily human exposure (μg day^{-1} person^{-1})[b]

Meat type[c]	B(a)Ac	B(c)Ac	DB(a,c)Ac	DB(a,h)Ac	Total	Daily human exposure
Pork chop[d]	0.20	0.04	0.78	n.q.	1.02	0.10
Beef collar[d]	0.15	0.07	0.57	n.q.	0.79	0.08
Pork neck (no 1)	n.a.	n.a.	n.a.	n.a.	–	–
Pork neck (no 2)[d]	0.21	0.11	0.76	n.q.	1.08	0.11
Beef/pork minced chop[e]	0.67	0.88	n.d.	n.d.	1.55	0.16
Turkey breast (no. 1)[f]	0.75 (10.7)[g]	0.14 (6.5)	n.q. (10.3)	n.q. (10.3)	0.89	0.09
Turkey breast (no. 2)[f]	0.24	0.67	n.q.	n.q.	0.91	0.09
Pork fillet[e]	n.d.	n.d.	2.20	1.10	3.30	0.33
Chicken breast	n.d.	0.06	0.53	0.25	0.84	0.08
Mean recovery (%)	67.0	40.0	82.0	41.0		

[a]Corrected for recovery
[b]Calculated on the basis of azaarene content and daily consumption of 100 g meat
[c]Cooking methods and amount of cooking are given in the text
[d]Azaarenes separated by use of Chrom Spher PAH (Varian) column
[e]Azaarenes separated by use of Supelcosil LC-PAH (Supelco) column
[f]Azaarenes separated by use of Nucleosil 100 C$_{18}$ (Knauer) column
[g]The values in parentheses are *RSD* values (%) obtained from replicate day-to-day analyses of spiked samples ($n = 6$ for turkey breast no. 1)
n.a. – not analysed; n.d. – not detected; n.q. – analyte near its detection limit, i.e. detected in the background but not quantified

the abbreviation 'n.q.' was used in Table III. The highest total concentration of aza-PAH was found in grilled pork fillet (3.30 ng g^{-1}) and in beef/pork minced chops (1.55 ng g^{-1}). It was shown that the strongly carcinogenic five-ring-azaarenes DB(a,c)Ac and DB(a,h)Ac predominate in grilled meat dishes, e.g. in pork fillet and chicken breast. Values calculated for daily human exposure to the four azaarenes determined are low and do not exceed 0.35 μg day^{-1} person^{-1}.

Results from GC–MS identification of azaarenes are presented in Table IV and typical mass spectra of individual azaarenes determined in meat dishes are shown in Figs 3 and 4. For most meat dishes GC–MS analysis confirmed the presence of isomeric benzoacridines but did not give positive results for isomeric dibenzoacridines. This might be because of high detection limits (compared with HPLC) for these compounds, i.e.
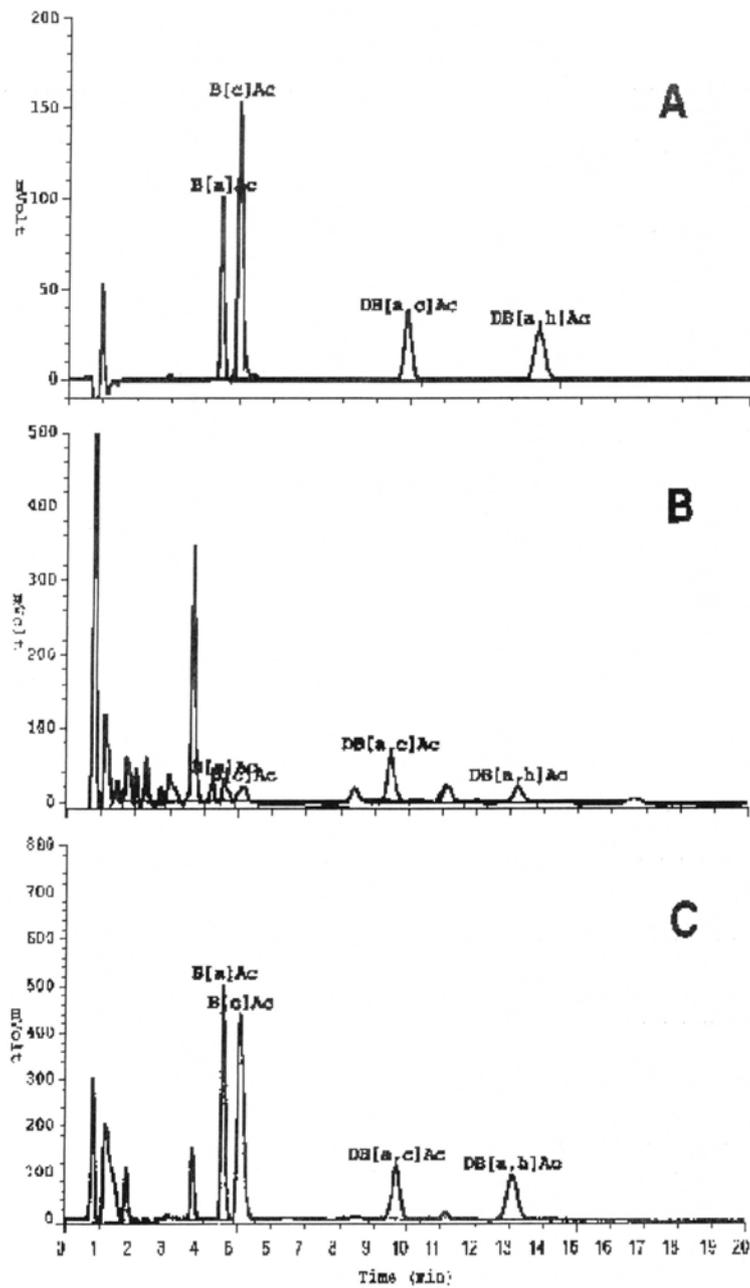
Fig. 2

Chromatograms obtained from HPLC of the azaarenes: A. standard mixture; B. fraction isolated from unspiked well-done roasted ('on salt') pork neck (no. 2); C. fraction isolated from spiked well-done roasted ('on salt') pork neck (no. 2)

Table IV

Results from GC–MS identification of azaarenes in the meat samples investigated

| Meat type	B(c)Ac; t_R = 23.76 min	B(a)Ac; t_R = 24.36 min	DB(a,c)Ac; t_R = 38.76 min	DB(a,h)Ac; t_R = 39.0 min
Pork chop	–	–	–	–
Beef collar	+ (229, 215, 201)[a]	–	–	–
Pork neck (no. 1)	+ (229)	+ (229, 215, 201)	–	–
Pork neck (no. 2)	+/– (229)	–	–	–
Beef/pork minced chop	+ (229, 201, 129)	+ (229, 201)	–	–
Turkey breast (no. 1)	+ (229, 215, 201)	+ (229)	–	–
Turkey breast (no. 2)	–	–	–	–
Pork fillet	+/– (229, 201)	+/– (229)	–	–
Chicken breast	+ (229, 201)	–	–	–
Detection limit (ng)[b]	3.0	4.0	6.0	5.0

[a]Characteristic ions for azaarenes: B(a)Ac and B(c)Ac: 229 (molecular ion M^+ and base peak), 201, and 215
[b]Amounts of azaarenes standards introduced on to the column
'+' denotes mass spectrum (at appropriate t_R) contains intense peaks corresponding to azaarene fragments
'+/–' denotes mass spectrum contains peaks characteristic of fragment ions originating from azaarenes and other peaks that might originate from fragmentation of other compounds
'–' denotes no peaks corresponding to the investigated azaarenes
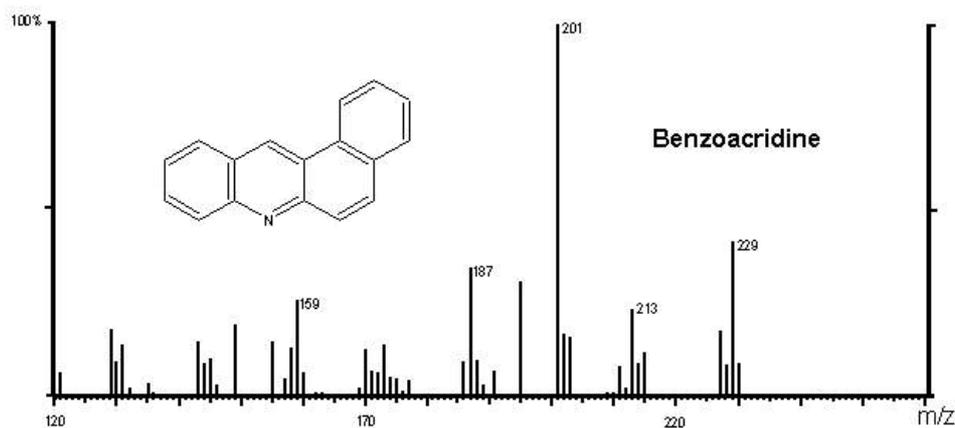
Fig. 3

Mass spectrum of benzo(*a*)acridine identified in azaarene fraction isolated from well-done pan-fried turkey breast (no. 1)
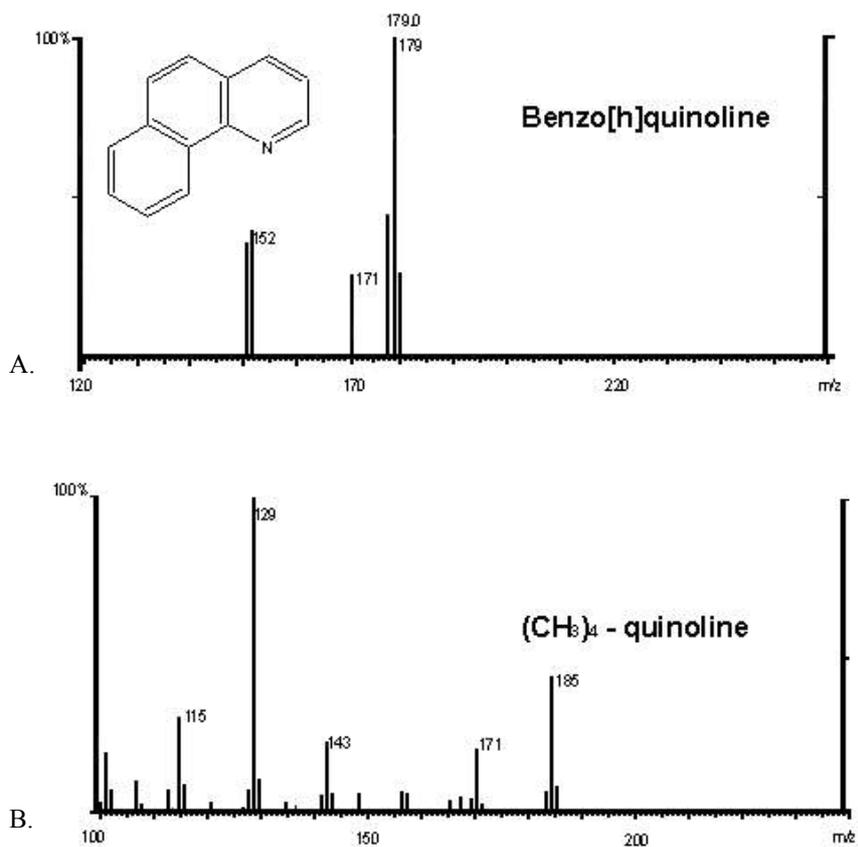
Fig. 4

Mass spectra of azaarenes identified in meat samples: A. benzo(*h*)quinoline in very well-done grilled chicken breast; B. tetramethylquinoline in medium pan-fried beef collar

6.0 ng for DB(a,c)Ac and 5.0 ng for DB(a,h)Ac. It was also found that in addition to the azaarenes determined quantitatively other azaarenes, e.g. isomers of benzoquinoline or acridine and their alkyl (methyl, ethyl) derivatives (Fig. 4), were also present in the samples investigated.

CONCLUSIONS

Solid-phase extraction using coupled columns packed with different adsorbents enables isolation of the azaarenes which can be formed in meat during heat processing. Carcinogenic five-ring dibenzoacridines are

mainly formed by grilling the meat. The amounts of individual azaarenes (benzoacridines and dibenzoacridines) identified in meat dishes were relatively small and generally did not exceed 1.0 ng g^{-1} meat, so daily human exposure to azaarenes was below 0.1 μg day^{-1} person^{-1}.

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