ELSEVIER

Journal of Chromatography A, 938 (2001) 155–165

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Clean-up procedures for the analysis of heterocyclic aromatic amines (aminoazaarenes) from heat-treated meat samples

B. Janoszka[a], U. Błaszczyk[b], L. Warzecha[a,*], M. Strózyk[c], A. Damasiewicz-Bodzek[a], D. Bodzek[a]

[a]*Medical University of Silesia, Department of Chemistry, Jordana 19, 41-808 Zabrze, Poland*
[b]*Medical University of Silesia, Department of Biochemistry, Jordana 19, 41-808 Zabrze, Poland*
[c]*Department of Molecular Biology, Centre of Oncology M. Skłodowska-Curie Institute, Wybrzeże Armii Krajowej 15, 44-101 Gliwice, Poland*

Abstract

The purpose of the study was to determine optimum conditions for the isolation and quantitation of five most biologically active aminoazaarenes {2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)}. Some multistep procedures based on ultrasonic extraction, Soxhlet extraction, liquid–liquid extraction and solid-phase extraction (SPE) were tested in order to choose the optimum isolation conditions for aminoazaarenes from fried meat samples spiked with known amounts of standards. According to the tested methods the qualitative–quantitative analysis was performed on the unspiked sample of pork roasted in typical household conditions. The qualitative–quantitative analysis of the aminoazaarenes was performed by a HPLC method. A HPLC Hewlett-Packard HP 1090 liquid chromatograph equipped with a UV diode array detector (DAD) was used. Chemically bonded HPLC columns C_8 and TSK-gel ODS 80-T_M were used under gradient elution conditions. A two-component mixture containing triethylamine–phosphate buffer (pH 3.2 and 3.3) and acetonitrile was used as a mobile phase. The results of the studies showed that a solid-phase extraction procedure using diatomaceous earth (Extrelut, 20 ml), propylsulphonic acid (PRS, 500 mg) and octadecylsilane (C_{18}, 500 mg) columns was the quickest and simplest one. Recoveries of the aminoazaarenes, spiked and isolated from meat samples by the chosen SPE procedure, were as follows: IQ 85%, MeIQ 50%, MeIQx 46%, 4,8-DiMeIQx 62%, PhIP 50%.

Keywords: Meat; Food analysis; Extraction methods; Amines, heterocyclic aromatic; Aminoazaarenes

1. Introduction

In recent years, particular attention has been paid to the new group of potential mutagens and cancerogenes, which can be formed in trace quantities in proteinaceous foods prepared at high temperature (frying, grilling) [1–3]. Heterocyclic aromatic amines (HAAs), described also as aminoazaarenes, can be synthesized in proteinaceous food from free amino acids, creatine or creatinine and carbohydrates [4–8]. These compounds may contain imidazopyridine, imidazoquinoline, imidazoquinoxaline or pyridoindole structure in the molecule.

Analysis of these food compounds at the ppb and ppt levels requires highly sensitive and selective analytical methods to be applied. The major prob-

*Corresponding author.

0021-9673/01/$ – see front matter
PII: S0021-9673(01)01364-4

lems associated with the analysis of aminoazaarenes in food are as follows: many organic compounds can be co-extracted with aminoazaarenes from foods, which can interfere with the subsequent separation and identification of HAAs.

Various extraction and purification methods, including solid-phase extraction, liquid–solid chromatography and solid-phase microextraction, immunoaffinity chromatography have been used to isolate aminoazaarenes from food samples [9–12]. For separation of the aminoazaarenes fraction, liquid chromatography, gas chromatography or capillary electrophoresis have been applied. The main problem in GC analysis is the need to derivatize the HAAs into less polar compounds to improve their volatility as well as the selectivity, sensitivity and separation of these amines. However, incomplete derivatization may lead to non-reproducible results [13,14].

In recent years, the qualitative and quantitative analysis of heterocyclic aromatic amines has been more and more dominated by application of high-performance liquid chromatography (HPLC) procedures using various detection systems: UV, diode array detection (DAD), electrochemical detection (ED), coulometric electrode array detection, mass spectrometry (MS or MS–MS) [15–19].

The purpose of the study was to determine optimum conditions for the isolation of five most biologically active aminoazaarenes (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP) from in-home cooked meat samples. Procedures used by Turesky et al. [20], Gross and co-workers [10,21,22], Hayatsu et al. [23,24], Rivera et al. [25] and Felton et al. [26] were modified by us and applied to determine the selected HAAs. Quantitative analysis was performed by RP-HPLC method with DAD. GC–MS was used for qualitative analysis of aminoazaarenes derivatized to amides.

2. Experimental

2.1. Chemicals

Aminoazaarenes: 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethyl-imidazo[4,5-*f*]-quinoxaline (MeIQx), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Toronto Research Chemicals, Ontario, Canada) were used as standards.

HPLC-grade organic solvents: dichloromethane, *n*-hexane, methanol, acetone, acetonitrile, toluene, ethyl acetate, ammonium hydroxide (POCH, Gliwice, Poland) and water from a simplified water purification system (Millipore Vienna, Austria) were used as the component mobile phases and as extraction solvents. Sodium hydroxide, hydrochloric acid and ammonium acetate (analytical-reagent grade) were purchased from POCH, (Gliwice, Poland). Triethylamine (Fluka, Buchs, Switzerland) and 85% phosphoric acid (Merck, Darmstadt, Germany) were used for buffer preparation. Diatomaceous earth extraction columns (Extrelut, 20 ml) were obtained from Merck. Prophylsulphonic acid (PRS, 500 mg) and octadecylsilane (C_{18}, 500 mg) SPE columns were from J.T. Baker, Gross-Gerau, Germany. PRS-columns were preconditioned with dichloromethane (4 ml) and C_{18}-columns with methanol (10 ml) and water (10 ml). Blue Rayon (Copper phthalocyanine rayon) from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) was used for column chromatography. Amberlite XAD-2 (Supelco, Bellefonte, PA, USA) was used for column chromatography (20 cm×1 cm I.D.). It was preconditioned with acetone, methanol and water. Pentafluoropropionic anhydride (Aldrich, Dorset, UK) was used for derivatization of aminoazaarenes to amides.

2.2. Meat sampling

The aminoazaarene fraction was separated from the roasted pork in a baking-gas oven at 230°C during 1 h using margarine ‘Planta’. To evaluate the percentage recovery of HAAs separated by the use of several multistep methods as well as to prevent matrix effects on the peak positions in the HPLC and GC–MS chromatograms, spiked and unspiked samples were analysed under the same conditions. According to each clean-up procedure, the sample was separated twice and the quantitative determination results were presented as a mean value. The spiked sample was prepared by addition of 40 ng of five known standards: IQ, MeIQ, MeIQx, 4,8-Di-

MeIQx, PhIP to 1 g of meat at the beginning of the extraction step.

Table 1 presents the formulas, names and abbreviations of the tested aminoazaarenes.

2.3. Clean-up procedures

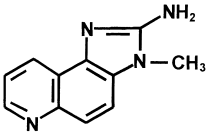
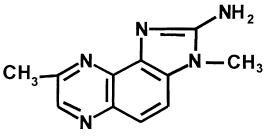
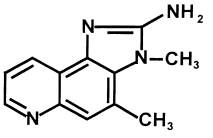
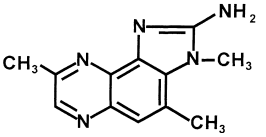
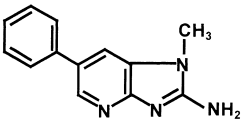
Seven procedures were applied to the separation of the aminoazaarene fraction from spiked and unspiked meat samples. To work out these multistep separation schemes some clean-up procedures used previously by Turesky et al. [20], Gross and co-workers [10,21,22] and Felton et al. [26] were applied after proper modifications, and our own suggestions were introduced as well. Some of the seven suggested schemes resulted from the combination of different steps chosen from the clean-up procedures previously used by the mentioned authors.

2.3.1. Method A

A classical liquid–liquid extraction with the use of methanol was applied to separate the HAA fraction from the meat tested [20,27].

Thirty grams of meat was homogenized with 75 ml of water. After adding to 200 ml methanol the mixture was centrifuged at 10000 g for 10 min to remove precipitated protein. The methanol extract was collected. The precipitate was dissolved in 75 ml water and then added to another 200 ml methanol. The protein was once again removed by centrifugation. The supernatants, containing the aminoazaarenes, were pooled and concentrated by rotary evaporation at 37°C to a final volume of approximately 20 ml. The pH of the solution was adjusted to pH 8.5 with 1 *M* NaOH and then the aminoazaarenes were adsorbed on 7 g Amberlite XAD-2 resin which had been prewashed in sequence with acetone,

Table 1
Structures of the determined compounds

Name	Structure	*M* (g/mol)	Abbreviation
2-Amino-3-methylimidazo[4,5-*f*]quinoline		198	IQ
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline		213	MeIQx
2-Amino-3,4-dimethyloimidazo[4,5-*f*]-quinoline		212	MeIQ
2-Amino-3,4,8-trimethylimidazo[4,5-*f*]-quinoxaline		227	4,8-DiMeIQx
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine		224	PhIP

methanol, water and packed into a glass column (20 cm×1 cm I.D.) [28]. The pork roasted meat solution was passed through the column at a flow-rate of 2 ml/min and the resin was then washed with 70 ml water. The aminoazaarenes were eluted from the resin by successive washing of 70 ml acetone followed by 70 ml methanol. These two organic washes were pooled and rotary evaporated to dryness. The extract was then dissolved in 25 ml water and acidified to pH 2.0 with 1 *M* HCl. Neutral and acidic material was removed by extraction with ethyl acetate (three times with 20 ml). The pH of the aqueous phase was adjusted to 12 with concentrated NaOH. The aminoazaarenes were extracted into ethyl acetate (three times with 20 ml ethyl acetate–water, 2.5:1 v/v). The organic extract was rotary evaporated to dryness. The residue was dissolved in 10 ml water and poured into a glass column (10 cm×1 cm I.D.) which contained 0.5 g packed blue cotton. The solution was passed through the column at a flow-rate of 2 ml/min and then the blue cotton was washed with 25 ml water. The aminoazaarenes were desorbed from the blue cotton by washing the column with 25 ml methanol–ammonium hydroxide (50:1, v/v) [27]. The eluent was evaporated to dryness, next resuspended in acetonitrile and then analysed by HPLC.

2.3.2. Method B

An ultrasonic extraction method with acetone was applied. Thirty grams of meat after homogenisation was extracted in 150 ml acetone for 60 min. The extract was cooled down to −15°C for 18 h, cold dried and washed with a solvent. Further procedures according to Method D, beginning with the clean-up stage by the use of Extrelut, was performed.

2.3.3. Method C

A Soxhlet extraction method was applied, also with the use of acetone. Thirty grams of meat was homogenised with a small amount of solvent. Then it was transported to a Soxhlet apparatus (500 ml flask) and extracted with acetone for 6 h. The acetone extract was later separated according to the same procedure as in Method B.

2.3.4. Method D

This method was developed by Gross and co-workers [10,21,22] and was used by Rivera et al. [25] among others to separate the aminoazaarene fraction out of the meat extract. In order to determine aminoazaarenes in roasted meat, four portions of the same sample were simultaneously separated and the obtained fractions of HAAs were combined and analysed qualitatively and quantitatively. To do this, 25 g of mince was homogenised for 1 min, with 75 ml of cold 1 *M* NaOH solution. Twenty grams of homogenised substance was taken four times from this dense suspension. Cold 1 *M* NaOH solution (10 ml) and 15 g of loose Extrelut were added to each portion. After thorough mixing it was placed in columns. Aminoazaarenes elution was carried out from the Extrelut column directly onto PRS columns by means of 60 ml of CH_2Cl_2 containing 5% toluene. After drying, the PRS columns were washed with 6 ml of 1 *M* HCl solution, and then with 2 ml of water. Next, C_{18} columns were connected to PRS columns and washed with 20 ml of 0.5 *M* ammonium acetate solution of pH 8. As an effect of the elution the aminoazaarenes moved from PRS columns directly onto C_{18} columns. C_{18} columns were washed with 10 ml water. They were dried under slight vacuum, and next blown through with nitrogen. Aminoazaarenes were eluted with 2 ml of CH_3OH–$NH_3 \cdot H_2O$ (9:1 v/v), the fractions from four separations were joined and after evaporating to dryness they were dissolved in 100 μl acetonitrile for the analysis on HPLC.

2.3.5. Method E

This method is a modified procedure based on the separation methods of Felton et al. [26], Hayatsu et al. [23,24] and Zhang et al. [27]. Thirty grams of chopped meat was homogenised after adding of 150 ml acetone. The obtained homogenised sample was filtered under vacuum through a glass sintered funnel. The solid-phase was placed into the homogeniser again, 150 ml acetone was added, and everything was homogenised and filtered again. This was repeated once more. Acetone extract (3×150= 450 ml) was obtained. The extract was cooled down to −15°C for 18 h to initiate protein precipitation. Next, the extract was cold filtered through Whatman filter No 1. The clear yellow filtrate was thickened to dryness. The obtained acetone extract was dissolved

in 100 ml of 0.01 *M* HCl solution (i.e. three times the initial volume of meat sample) and extracted three times with the use of 30 ml of CH_2Cl_2 each time. Each time, the water phase was separated from the organic phase in a separator, and next, the water and organic phases were joined together. The water layer (about 100 ml) was modified to pH 12 by means of 6 *M* NaOH solution (introduced in drops and mixing). Next, it was extracted again three times by means of 30 ml of CH_2Cl_2 each time. Each time, the water phase was separated from the organic phase in a separator. Then the organic phases were joined (about 90 ml), and the water phases were disregarded.

After CH_2Cl_2 evaporation, the fraction was cleaned with 0.5 g blue cotton (column 10 cm×1 cm), with the rate 2 ml/min. The desorption from the phase was performed with 50 ml of a methanol–25% ammonia (50:1 v/v) solution. After evaporation, the eluate was dissolved in 200 μl acetonitrile and analysed by HPLC.

2.3.6. Method F

Combination of Methods B, D and E is a modification of procedures used by Felton et al. [26], Gross and co-workers [10,21,22] and Rivera et al. [25]. Thirty grams of meat was homogenised and extracted ultrasonically in 150 ml of acetone for 60 min. The extract was cooled down to −15°C for 18 h, cold dried and washed. The obtained acetone extract was dissolved in 100 ml of 0.01 *M* HCl solution (i.e. three times the initial volume of meat sample) and extracted three times with 30 ml of CH_2Cl_2 each time. Each time the water phase was separated from the organic phase in a separator, and the water phases and organic phases were joined together.

The water layer (about 100 ml) was modified to pH 12 by means of 6 *M* NaOH solution (introduce by drops and mix), next it was extracted again by means of three portions of CH_2Cl_2 with the use of 30 ml of CH_2Cl_2 each time. Each time the water phase was separated from the organic phase in a separator. Then the organic phase was joined (about 90 ml), and the water phase was disregarded. After CH_2Cl_2 evaporation, we received a fraction which was subsequently treated as in Method D.

2.3.7. Method G

Combination of Methods C, D and E. This is a modification of procedures used by Felton et al. [26], Gross and co-workers [10,21,22] and Rivera et al. [25]. Thirty grams of meat was homogenised with a small amount of acetone. Then it was transported to Soxhlet apparatus (500 ml flask) and extracted with acetone for 6 h. Next, it was dried by means of Na_2SO_4 (anhydrous). The acetone extract was later separated according to the same procedure as in Method F.

2.4. HPLC

HPLC analyses of aminoazaarenes were performed using a Hewlett–Packard HP 1090 chromatograph equipped with a DAD system and a 100-μl loop (for extracts isolated using Methods A, B, C, E, F and G) or a 20-μl (for Method D) loop injector. Two HPLC analytical systems were used. In the first one we used Synchropak RP-8 chemically bonded C_8 column (25 cm×4.6 mm I.D., Hewlett-Packard). The elution was performed with a mixture of 5% acetonitrile and 95% triethylamine–phosphate buffer, pH 3.2 under isocratic conditions. Such conditions were used for the analysis of the aminoazaarene fraction isolated by the use of Methods A–G, except for Method D. Alternatively, another analytical system included TSK gel ODS 80-T_M column (5 μm particle size), 250×4.6 mm I.D. (TosoHaas, Stuttgart, Germany) and a mixture of 5% acetonitrile and 95% triethylamine-phosphate buffer, pH 3.3 as a mobile phase. The separations were performed with the following gradient elution programme: the mixture described above was initially used for 2 min., then it linearly increased to 25% acetonitrile within 20 min, then to 55% acetonitrile within 10 min and remained at 55% acetonitrile for 10 min. The optimum HPLC conditions were selected as the result of our earlier work described in [29].

All the studied fractions were passed through a 0.45-μm filter (Bakerbond, Darmstad, Germany) before injection onto the HPLC system.

All separations were carried out at 40°C using a 1-ml/min flow-rate. The UV detection of aminoazaarenes was conducted at 254, 274 and 315 nm. Quantitative determination was performed by using an external calibration curve method.

2.5. GC–MS

GC–MS was used to confirm the results of the HAA fraction separation from the investigated meat sample. A mass spectrometer (QP 2000-Shimadzu) connected with a gas chromatograph (GC-14) was used. The samples were analysed by 2 μl splitless injection onto a 25 m×0.2 mm (film thickness 0.25 μm) fused-silica capillary column HP Ultra 1. Conditions for the analysis of amides of aminoazaarenes were as follows: electron impact (EI) 70 eV; helium flow-rate 1 ml/min; temperatures: injector 270°C, interface 280°C, ion source 250°C; GC temperature programme: 60°C heating at 4°C/min to 280°C (held for 20 min).

Aminoazaarenes were derivatized to amides by acylation with pentafluoropropionic anhydride (PFPA). This reaction was conducted according to the procedure described by Campbell et al. [30].

3. Results and discussion

The usefulness of the investigated multistep procedures for aminoazaarenes isolation from meat samples has been assessed. Until now, the authors of many articles have usually used one method for aminoazaarene fraction separation, e.g. liquid–solid extraction, or liquid–liquid extraction or solid-phase extraction (SPE) only [10,20–22,25,26,28].

The aim of our tests was to select optimum conditions for the separation and determination of aminoazaarenes from the pork sample roasted in the way which is traditional for Polish cuisine. Meat dishes usually have a lot of fat, proteins and pigments comparing to lyophilised meat extract (commercial meat extract) often used for elaborate aminoazaarenes separation methods. And because of that the application of the procedures used previously [31] for aminoazaarenes determination had to be modified with some additional elements. The schemes that had been worked out and tested for the purpose of the present paper resulted from the combination of different procedures used by the authors mentioned above [10,20–22,26], but these procedures were additionally modified by the size of SPE columns and eluent volumes or by introduction of the Soxhlet extraction or the ultrasonic extraction. Methods of this type have been used so far only to isolate polycyclic aromatic hydrocarbons (PAHs) from food samples [32,33] with the use of methanol, cyclohexane or dichloromethane. In this work we used acetone for HAA extraction, having in mind the results achieved by Felton et al. [26,28] among others, who successfully applied this solvent for HAA separation from food by liquid–liquid extraction.

Analytical schemes differ from one another in particular separation steps. And thus, the first step, whose purpose was to isolate from the protein matrix a concentrate of organic compounds that might have formed in the result of meat heat treatment, consisted of liquid–solid extraction with column ion-exchange chromatography (Method A), Soxhlet extraction or ultrasonic extraction (Methods B and C), solid-phase extraction (Method D), liquid–liquid extraction (Method E), Soxhlet or ultrasonic extraction combined with liquid–liquid extraction (Methods F and G). The second step of clean-up procedures included a selective isolation of the aminoazaarene fraction. To achieve the isolation in Methods A, E, F and G, a two-step liquid–liquid extraction with the use of organic solvents and acid–base system was applied, while in Methods B–D a solid-phase extraction with cation-exchange phase was applied.

The aim of the third step was to clean the aminoazaarene fraction up by a column chromatography with a blue-cotton stationary phase, in which heterocyclic nitrogen compounds analogous to other mutagens/carcinogens containing polycyclic planar molecular structures form bonds with copper–phthalocyanin complexes [23,24] (Methods A, E) or by a solid-phase extraction with chemically bonded phase C_{18} (Methods B, C, D, F, G).

The methods were assessed on the basis of the results obtained from the separation and quantitative–qualitative analysis of the meat sample roasted in household conditions. At the same time some aminoazaarene determinations were carried out in an unspiked meat sample and a spiked sample with a known number of standards.

The reproducibility of the HPLC methods in qualitative determinations was assessed based on RSD values determined from the capacity factor k' [34] values obtained for mixtures of standards. The capacity factors range was from 0.58 to 3.94 with

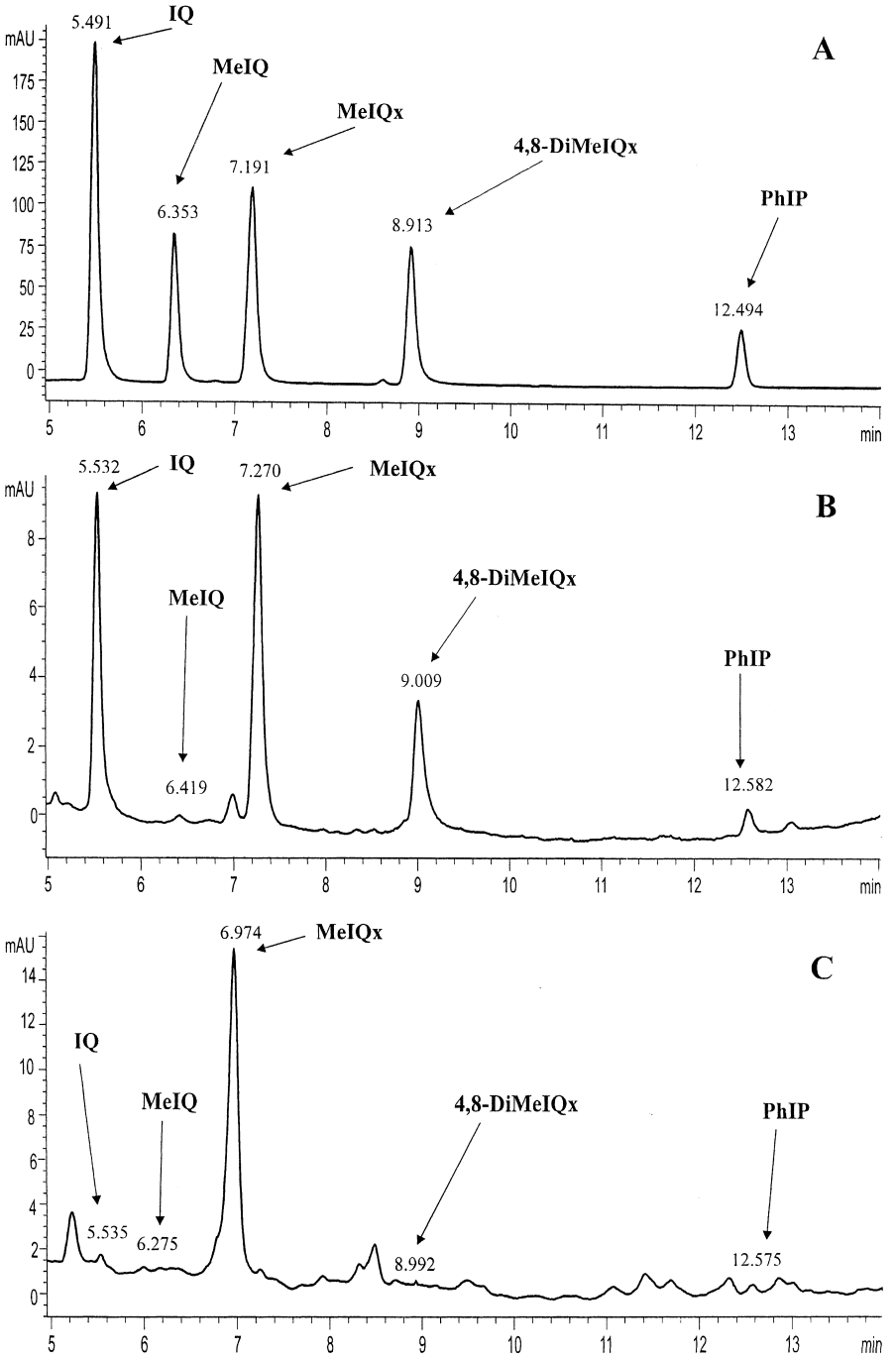


Fig. 1. HPLC chromatograms (column: TSK–gel ODS-80-T_M) of aminoazaarenes: (A) Standard mixture; (B) fraction separated from spiked meat sample; (C) fraction separated from unspiked meat sample using Method D.

RSD ($n=5$) values from 1.0 to 2.5%. Detection limits of the five aminoazaarenes analysed by HPLC were from 2.0 mg to 5.0 mg.

HPLC chromatograms of HAA standard mixtures and the aminoazaarenes fraction isolated from a spiked and an unspiked meat sample by Method D are presented in Fig. 1.

HPLC chromatograms of aminoazaarenes fractions separated according to chosen methods (Methods A, B, C and E) are presented in Figs. 2, 3, 4 and 5. Some aminoazaarenes in fraction separated by Methods A, B and C and Methods E, F and G were not identified possibly due to a sample matrix effect

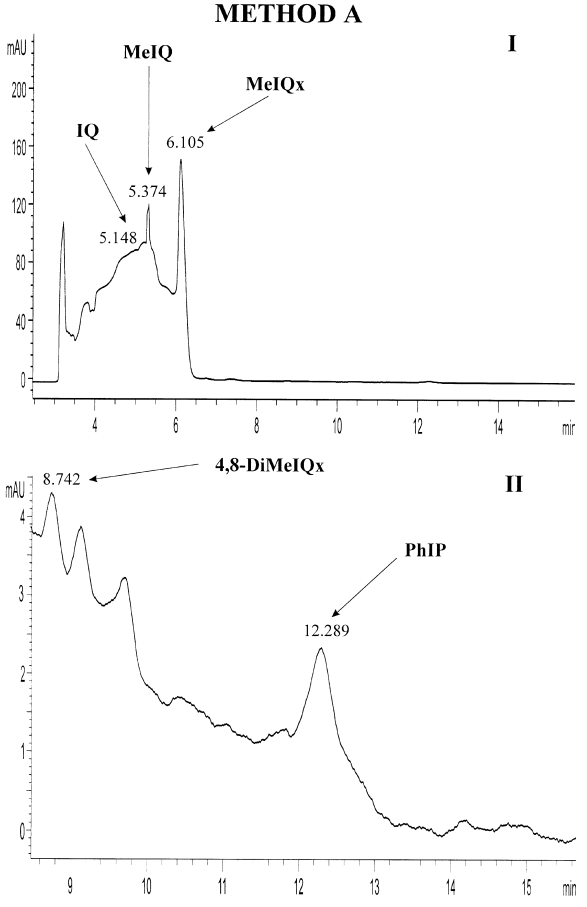


Fig. 2. HPLC chromatogram (column: Synchropak RP-8) of aminoazaarenes fraction isolated from unspiked meat sample using Method A. (I) Total range of retention time; 2.5–16 min. (II) Retention time range: 8–16 min.

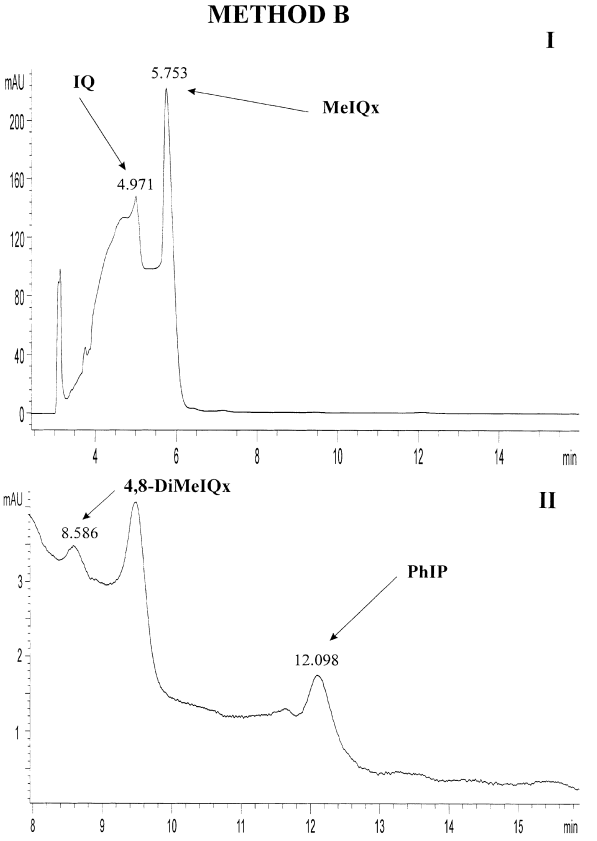


Fig. 3. HPLC chromatogram (column: Synchropak RP-8) of aminoazaarenes fraction isolated from unspiked meat sample using Method B. (I) Total range of retention time: 2.5–16 min; (II) Retention time range: 8–16 min.

resulting from a worse HAA fraction cleaning-up than in Method D.

Confirmation of peak identity for the HAA fraction separated from meat sample was performed by GC–MS analysis. The aminoazaarenes were analysed as pentafluoropropyl amide derivatives. Fig. 6 presents an example of total ion chromatogram and characteristic mass chromatograms for the amide derivatives of IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP identified in meat. A molecular ion peak $M^+ = [(M_{HAA}-M_H)+M$ of the COC_2F_5 group] was observed for each of the derivatives. The abundant fragment (base peak) in the amides mass spectra was [$M^+ - M$ of the C_2F_5 group]. All the aminoazaarenes mentioned above were identified in the spiked sam-

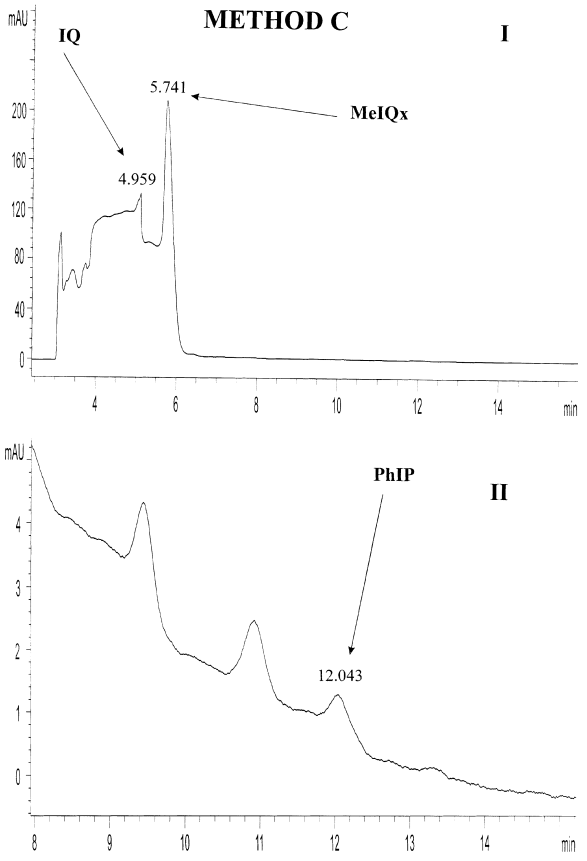


Fig. 4. HPLC chromatogram (column: Synchropak RP-8) of aminoazaarenes fraction isolated from unspiked meat sample using Method C. (I) Total range of retention time: 2.5–16 min. (II) Retention time range: 8–16 min.

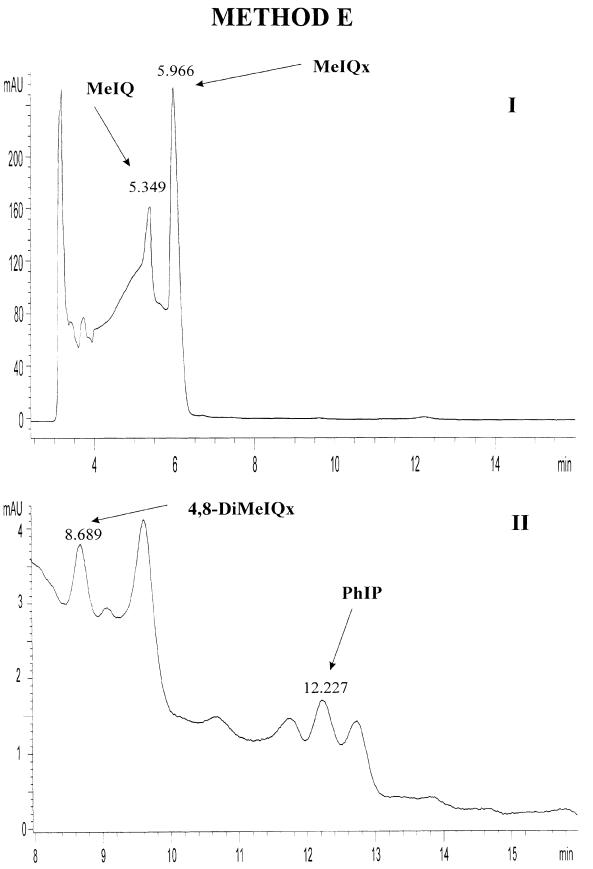


Fig. 5. HPLC chromatogram (column: Synchropak RP-8) of aminoazaarenes fraction isolated from unspiked meat sample using Method E. (I) Total range of retention time: 2.5–16 min; (II) Retention time range: 8–16 min.

ples. It proves the effectiveness of the procedures applied for the isolation of HAAs from food samples.

Table 2 contains the results of aminoazaarenes determination for 1 g of meat and percentage recovery of standards used for meat spiking.

The results of aminoazaarenes determination were compared by seven methods. Recovery values ranged from 38% (for MIQx, Method A) to 85% (for IQ, Method D). The biggest recovery was obtained for IQ (over 60%), the smallest was for MeIQx (max. 46%). The difference in standard recovery from the samples analysed by different methods (Methods A–G) can reach up to 25% (in the case of IQ), but in the case of other HAAs the difference is not bigger than 5%. No fundamental differences were found in HAA content when clean-up procedures of Soxhlet or ultrasonic extractions with acetone (Methods B and C, Methods F and G) were used on the first stage; it suggests the possibility of their alternative application.

Method D, in which multistep solid-phase extraction was applied, appeared to be a separation method for which the smallest standard losses were found. It should be stressed that alkaline hydrolysis by 1 *M* NaOH was performed on the meat samples analysed according to this procedure. It allowed, unlike to other methods, to obtain a hydrolysis substance without fat, which might influence the

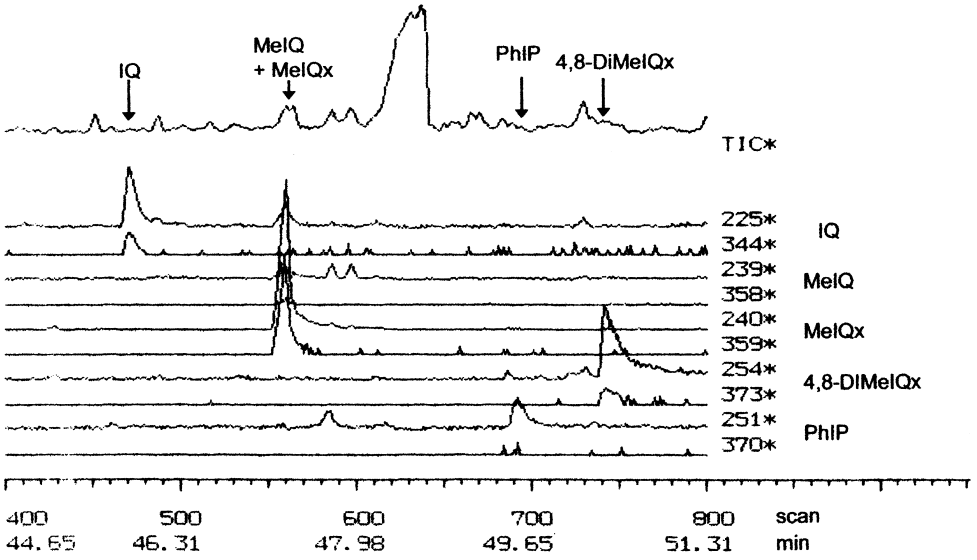


Fig. 6. GC–MS chromatograms (total ion and mass) of aminoazaarenes fraction isolated from spiked meat sample by Method D.

subsequent steps in aminoazaarene fraction separation. Contrary to other procedures, Method D was based only on solid-phase extraction (Extrelut, PRS, C_{18}), i.e. it did not require big volumes of solvents, so it was very fast and ecological. Moreover, particular stages of isolation and determination of the HAA fraction in this clean-up procedures are carried out in on-line system. Thanks to that some indirect stages such as phase separation after liquid–liquid extraction, evaporation and solution of indirect fractions resulting in additional losses can be omitted. Good reproducibility (day-to-day) with low relative standard deviations (RSD between 7 and 12%) were obtained, showing the suitability of the Method D for the analysis of HAAs in meat samples.

In the tested pork meat the concentration of HAAs was from about 0.30 ng IQ in 1 g of meat to about 5.5 ng/g MeIQx. The results are in Table 2. HAA concentrations determined in the fractions isolated according to the procedures A–G are comparable. The values differ from one another by maximum of 20%.

Methods F and G are multistep with liquid–liquid extraction combined with SPE, and this fact is probably the reason of losses and lower (of 20–30%) concentrations of HAAs in comparison with the

Table 2
Quantitative HPLC analysis data of aminoazaarenes identified in roasted pork sample and percentage recovery of aminoazaarenes standards separated from spiked samples using seven methods

Standards	Methods											
	A		B		C		D		E		F	G
	Amount (ng/g)	Recovery (%)	Amount (ng/g)	Recovery (%)	Amount (ng/g)	Recovery (%)	Amount[a] (ng/g)	Recovery (%)	Amount (ng/g)	Recovery (%)	Amount (ng/g)	Amount (ng/g)
IQ	0.35	60	0.36	63	0.35	60	0.50 (7)	85	nd	nd	0.29	0.30
MeIQ	1.58	45	nd		nd		1.72 (8)	50	1.60	48	nd	nd
MeIQx	4.14	38	5.42	40	5.55	41	6.23 (12)	46	5.91	45	3.38	nd
4,8-DiMeIQx	3.44	75	1.95	58	nd		2.09 (6)	62	2.00	60	1.50	1.82
PhIP	1.10	45	1.14	43	1.11	42	1.32 (10)	50	1.28	49	0.95	0.80

[a] The values in parentheses are RSD values, expressed as percentages and were obtained from replicate analyses ($n=4$) carried out at 4 days to determine the RSD for the best method (Method D).

values determined in the fractions isolated by other procedures.

4. Conclusions

On the basis of the results of the HPLC determinations of aminoazaarenes in spiked and unspiked meat samples, from which the HAA fraction was isolated in several different ways, Method D with a three-step solid-phase extraction with the use of Extrelux-20, PRS and C_{18} columns, where alkaline hydrolysis had been previously performed on the samples, was found to be the most effective for meat samples (fast and without application of big volumes of solvents). The HAA recovery for this procedure was in the range of 46–85%.

Concentrations of aminoazaarenes from in home cooked roasted pork samples determined in the fractions separated by several different methods were comparable and ranged from 0.5 to several ng/g of meat, while MeIQx concentration was the highest (6.23 ng/g).

Acknowledgements

The study was implemented as part of project No 4P05D 015 18 financed in 2000-2002 by the Polish Committee for Scientific Research.

References

[1] T. Sugimura, Mutat.Res. 150 (1985) 33.
[2] T. Sugimura, M. Nagao, K. Wakabayashi, J. Natl. Cancer Inst. 86 (1994) 2.
[3] K. Augustsson, K. Skog, M. Jägerstadt, G. Steineck, Carcinogenesis 18 (1997) 1931.
[4] K. Kukigawa, Cancer Lett. 143 (1999) 123.
[5] M. Jägerstad, K. Skog, S. Grivas, K. Olsson, Mutat. Res. 259 (1991) 219.
[6] K. Skog, M.G. Knize, M. Jägerstad, J.S. Felton, Mutat. Res. 268 (1992) 191.
[7] T. Kato, T. Harashima, N. Moriya, K. Kikugawa, K. Hiramoto, Carcinogenesis 17 (1996) 2469.
[8] P. Pais, C.P. Salmon, M.G. Knize, J.S. Felton, J. Ag. Food Chem. 47 (1999) 1098.
[9] F. Toribio, L. Puignou, M.T. Galceran, J. Chromatogr. A 836 (1999) 223.
[10] G.A. Gross, A. Grüter, J. Chromatogr. 592 (1992) 271.
[11] H. Kataoka, J. Pawliszyn, Chromatographia 50 (1999) 532.
[12] F. Toribo, E. Moyano, I. Puignou, M.T. Galceran, J. Chromatogr. A 880 (2000) 101.
[13] H. Kataoka, K. Kijima, J. Chromatogr. A 767 (1997) 187.
[14] K. Skog, A. Solyakov, P. Arvidsson, M. Jägerstad, J. Chromatogr. A 803 (1998) 227.
[15] P. Pais, M. Knize, J. Chromatogr. B 747 (2000) 139.
[16] M.G. Knize, C.P. Salmon, E. Hopmans, J.S. Felton, J. Chromatogr. A 803 (1997) 179.
[17] F. Toribo, E. Moyano, L. Puignou, M.T. Galceran, J. Chromatogr. A 869 (2000) 307.
[18] P.A. Guy, E. Gremaud, J. Richoz, R.J. Turesky, J. Chromatogr. A 883 (2000) 89.
[19] C. Krach, G. Sontag, Anal. Chim. Acta 417 (2000) 77.
[20] R.J. Turesky, H. Bur, T. Huynh-Ba, H.U. Aeschbacher, H. Milon, Food Chem. Toxicol. 26 (1988) 501.
[21] G.A. Gross, G. Philippossian, H.U. Aeschbacher, Carcinogenesis 10 (1989) 1175.
[22] G.A. Gross, Carcinogenesis 11 (1990) 1597.
[23] H. Hayatsu, T. Hayatsu, S. Arimoto, H. Sakamoto, Anal. Biochem. 235 (1996) 185.
[24] H. Hayatsu, J. Chromatogr. 597 (1992) 37.
[25] L. Rivera, M.J.C. Curto, P. Pais, M.T. Galceran, L. Puignou, J. Chromatogr. A 731 (1996) 85.
[26] J.S. Felton, S. Healy, D. Stuermer, C. Berry, H. Timourian, F.T. Hatch, Mut. Res. 88 (1981) 33.
[27] X.M. Zhang, K. Wakabayashi, Z.C. Liu, T. Sugimura, M. Nagao, Mut.Res. 88 (1988) 33.
[28] J.S. Felton, M.G. Knize, C. Wood, B.J. Wuebbles, S.K. Healey, D.H. Stuermer, L.F. Bjeldanes, B.J. Kimbles, F.T. Hatch, Carcinogenesis 5 (1984) 95.
[29] L. Warzecha, M. Strózyk, B. Janoszka, U. Blaszczyk, D. Bodzek, Chemia Analityczna (Warsaw) (2001) in press.
[30] R.M. Campbell, M.L. Lee, Anal. Chem. 56 (1984) 1026.
[31] L. Warzecha, B. Janoszka, M. Strózyk, Acta Chromatogr. 9 (1999) 176.
[32] B.H. Chen, C.Y. Wang, C.P. Chiu, J. Agric. Food Chem. 44 (1996) 2244.
[33] D.H. Phillips, Mutat. Res. 443 (1999) 139.
[34] L.R. Snyder, J.L. Glajch, J.L. Kirkland, Practical HPLC Method Development, in: Wiley, Chichester, New York, 1988, p. 16.