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Determination of less polar heterocyclic aromatic amines in standardised beef extracts and cooked meat consumed in Austria by liquid chromatography and fluorescence detection

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Abstract

An analysis method was developed for the determination of trace levels of less polar heterocyclic aromatic amines (HAs) in food samples. The development started from a frequently used sample pre-treatment scheme which was slightly improved to make it applicable with high-performance liquid chromatography (HPLC) with fluorescence detection. The method was applied for the analysis of a standardised beef extract containing 5–15 ng/g of HAs and the results are compared with those of the other participants in the same European project. In addition, the method was used for the analysis of less polar HAs in cooked meat consumed in Austria.
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Keywords: Food analysis; Heterocyclic aromatic amines

1. Introduction

It is widely recognised that diet is a major life style factor contributing to cancer risk. In 1981, the epidemiologists Richard Doll and Richard Peto estimated that between 10 and 70% of all cancer deaths in the US are potentially avoidable by dietary changes [1]. The general problem situation seemed to be the same in all industrialised countries and it has not changed since then [2,3]. Heterocyclic aromatic amines (HAs) form one class of substances with strong mutagenic activity and therefore also carcinogenic potential. They are not a priori present in foods but are formed during cooking of proteinaceous foods such as meat and fish. It has already been shown that both, the type of meat and the cooking conditions, especially cooking temperature and cooking time, are the main parameters influencing types and amounts of HAs formed [4,5]. Up to now about 20 HAs have been identified in cooked food [6]. All 10 HAs tested so far proved to be carcinogenic in experimental animals with target organs including lung, liver, mammary gland, colon and skin [7,8]. The International Agency for Research on Cancer [9] classified eight of the known HAs (MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2 and Glu-P-1) as possible human carcinogens (class 2B) and IQ as a probable human carcinogen (class 2A).

Accurate exposure data are essential for any attempt to assess the potential health risks of HAs for humans. Although numerous data on the amount of HAs in various cooked food products [10–15], food flavours [16] and soup cubes [17] have been reported in the past, it remains a challenging analytical task to obtain reliable data in the quantitative determination of HAs in complex food matrices at the ng/g level. Elaborate sample clean-up steps have to be carried out before trace amounts of these analytes can be quantified. Two recently published review papers discuss sample preparation methods [18] and chromatographic techniques [19] frequently used for the determination of HAs in food samples.

As one of several European laboratories we took part in an European project focusing on the improvement of analytical

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

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methods for the determination of HAs in food products. One objective of the project was to evaluate precision and accuracy of analytical methods by comparing data obtained by different participating laboratories in the analysis of HAs in standardised beef extracts. A method similar to the method developed by Gross et al. [20] had been proposed [18] for sample pre-treatment. Participants were, however, allowed to modify the sample preparation scheme and to use any determination method available to them [21].

The aim of the present paper is to present our efforts to improve the proposed method for the analysis of less polar HAs and to compare the data obtained by analysing the standardised beef extract with the results obtained by other laboratories.

Since only a limited data on the content of HAs in cooked Austrian foods have been reported up to now [11] the potential health risks for Austrian consumers can not be assessed. In order to enlarge the data base we report some analytical results obtained for traces of less polar HAs in cooked meat consumed in Austria.

2. Experimental

2.1. Chemicals and reagents

HAs were purchased from Toronto Research Chemicals (Toronto, Canada). According to the producers, the chemical purity of these compounds was higher than 99%. Ammonia (32%), dichloromethane, sodium hydroxide, hydrochloric acid, naphthalene and 2-aminofluorene (all of p.A. grade) were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile, both of high-performance liquid chromatography (HPLC) grade, were obtained from Riedel-de Haen (Seelze, Germany). Glacial acetic acid (p.A.) was bought from Fluka (Buchs, Switzerland).

Diatomaceous earth extraction cartridges (Extrelut NT) and refill material were obtained from Merck. Propylsulfonic acid silica (PRS) columns (500 mg) and octadecyl silica (C_{18}) Bond Elut columns (500 mg) were received from Varian (Harbor City, USA).

Stock solutions of HAs were prepared by dissolving 4.0 mg of the substances in 50.0 ml acetonitrile. These solutions were further diluted with acetonitrile/water (50:50, v/v) and stored in flasks covered with aluminium foil at 4 °C.

A standard solution containing Trp-P-1, Trp-P-2, PhIP, AαC and MeAαC [4 ng/100 μl acetonitrile/water (50:50, v/v)] was used as spiking solution. Either 2-aminofluorene [100 ng/100 μl acetonitrile/water (50:50, v/v)] or naphthalene [438.5 ng/100 μl acetonitrile/water (50:50, v/v)] was used as internal standard.

2.2. Samples

Two standardised beef extracts were received from M.T. Galceran (Co-ordinator of the interlaboratory exercise) from the Department of Analytical Chemistry, University of Barcelona. Beef extract 1 was spiked with two less polar (PhIP and AαC) and three polar (IQ, MeIQ and MeIQx) HAs in the concentration range from 35 to 60 ng/g. Beef extract 2 contained five less polar (Trp-P-1, Trp-P-2, PhIP, AαC and MeAαC) and 6 polar (DMIP, IQ, MeIQ, MeIQx, 7,8-DiMeIQx and 4,8-DiMeIQx) HAs in the range from 5 to 15 ng/g.

Most food products were purchased from local stores and home-cooked according to typical Austrian recipes. Table 1 summarises the cooking conditions (cooking method, time and the degree of doneness). The meat was prepared with spices and other typical ingredients. After the cooking process only meat pieces were used for analysis.

Grilled sausage, knuckle of pork and minced meat were bought in a local fast-food shop. Thus, exact cooking conditions were not available but all products were very well-done. One chicken sample from a factory canteen was obtained freeze-dried from M. Murkovic from the Technical University of Graz and stored at −18 °C until analysis.

2.3. HPLC separation and detection

A modular liquid chromatograph consisting of a high pressure gradient pump (Model L-6200, Merck), an autosampler

Table 1
Cooking conditions for home-cooked meat

Type of meat	Pieces	Cooking method	Cooking time (min)	Degree of doneness
Poultry				
Chicken	Cubes, 2 cm × 2 cm	Wok, pan-fried	3	Just until done
Goose	Cubes, 10 cm × 10 cm	Oven-broiled, 200 °C	75	Very well done
Turkey	Slices, 10 cm × 10 cm	Oven-broiled, 180 °C	8	Well done
Venison				
Deer	Cubes, 10 cm × 10 cm	Oven-broiled, 200 °C	60	Very well done
Rabbit	Cubes, 10 cm × 10 cm	Boiled	60	Very well done
Pheasant	Cubes, 1 cm × 1 cm	Boiled	60	Very well done
Fish				
Mackerel	In toto	Oven-broiled, 200 °C	20	Very well done

(Model 2000A, Merck), a column thermostat (Model bfo-04 dt, W.O. electronics, Langenzersdorf, Austria), and a fluorescence detector (Model F-1080, Merck) was used. Peaks were integrated using the D-6000 Chromatography Data Station Software, HPLC Manager Version 2 from Merck.

The injection volume was 5 μl. The analytical column was a LichroCart Superspher 60 RP-select B, 250 mm × 2 mm i.d., 4 μm (Merck). Elution was carried out at 30 °C with a flow rate of 0.25 ml/min applying a gradient program changing the mobile phase composition from 100% mobile phase A to 100% mobile phase B within 40 min. Mobile phase A consisted of acetonitrile/glacial acetic acid/distilled water (30:2:68, v/v/v), pH 5.3 adjusted with concentrated ammonia, mobile phase B of acetonitrile/glacial acetic acid/distilled water (50:2:48, v/v/v), pH 5.3 adjusted with concentrated ammonia. Fluorescence detection was carried out applying the following excitation/emission wavelengths: PhIP, AαC (320/380 nm), MeAαC (335/380 nm), Trp-P-2, Trp-P-1 (263/380 nm), 2-aminofluorene, naphthalene (263/370 nm).

2.4. External calibration

The HPLC system was calibrated by injecting seven standard solutions in the concentration range from 4 to 200 ng/ml in acetonitrile/water (50:50, v/v). The analysis function was obtained by linear regression of the ratio of peak areas on standard concentrations.

2.5. Analysis of samples

2.5.1. Sample preparation

Samples were extracted using a sample preparation scheme developed by slightly modifying the procedure given by Toribio et al. [18].

One gram of the beef extract (5 g of the food samples) was dissolved and homogenised in 12 ml (20 ml) of 1 M NaOH using an Ultra Turrax mixer. Then the extract was mixed with 13 g (20 g) of diatomaceous earth (Extrelut) and transferred to an empty glass column (200 mm × 25 mm i.d.). HAs were eluted from Extrelut with 75 ml dichloromethane and adsorbed onto a PRS column which had been preconditioned with dichloromethane (7 ml). Less polar HAs were eluted by washing the column with three different solvents: 6 ml of 0.01 M HCl, 15 ml of MeOH/0.3 M HCl (50:50, v/v) and 2 ml distilled water. The combined fraction containing the less polar amines was neutralised with 2 ml ammonia and diluted with 25 ml water. The extract was passed through a preconditioned (5 ml methanol and 5 ml water) C_{18} column. The HAs retained in the C_{18} column were eluted with 1.5 ml of methanol/ammonia (90:10, v/v). The extract was evaporated to dryness under a stream of nitrogen and redissolved in 100 μl of acetonitrile/water (50:50, v/v) containing 438.5 ng of internal standard (naphthalene). Finally, 5 μl of the extract were injected into the HPLC system.

2.5.2. Standard addition

Two unspiked and four spiked samples were analysed as described above. Samples were spiked with 4, 8, 12 and 16 ng/g of each of the five HAs. Peak area ratios were plotted against the amounts of analytes added. Recoveries were determined by dividing the slope of the linear regression lines for the standard addition by the slope of the linear regression lines of HAs standard solutions.

3. Results and discussion

3.1. Analysis of the standardised beef extracts

Participants of the European project received standardised beef extracts (see Section 2.2). The analysis methods used and the results obtained were collected and evaluated by the project co-ordinator with regard to precision and accuracy. Although a sample preparation procedure was proposed the participants were invited to modify it if required.

3.1.1. Optimisation of the recommended sample pre-treatment method

Our experiments using the sample preparation scheme as proposed resulted in intolerably low recovery values for some of the analytes. It was therefore slightly modified to optimise the recovery values.

The modified sample preparation method developed is outlined in Section 2.5.1. It is very similar to the method proposed in the EU project by Toribio et al. [18], but the elution of the analytes from the PRS cartridge was changed. In the method proposed less polar HAs were eluted from the PRS column with 6 ml of 0.01 M HCl followed by 15 ml of MeOH/0.1 M HCl (60:40, v/v). The recovery of PhIP was, however, very low when we analysed beef extract 1 by applying the proposed procedure. We therefore investigated possibilities to increase the recovery of PhIP from the PRS cartridge by modifying the elution conditions using standard solutions of the five less polar HAs. Various HCl concentrations (0.1, 0.3 M) and various ratios of MeOH and HCl (50:50, v/v; 70:30, v/v) were used. For all five HAs the highest recoveries were obtained with MeOH/0.1 M HCl (50:50, v/v) which is in agreement with the studies of Toribio et al. [18]. When beef extracts were analysed using the modified elution method we succeeded in obtaining high recoveries for Trp-P-2, Trp-P-1, AαC and MeAαC, but the recovery of the relatively polar HA PhIP dropped to below 1% (see Fig. 1). Increasing hydrochloric acid concentration to 0.3 M drastically increased the recovery of PhIP but correlated with a significant decrease of the recovery of the more less polar HAs. Fig. 1, however, also shows that the use of 0.3 M HCl is an unavoidable and acceptable compromise for the analysis of the five analytes selected for this project. The sample pre-treatment scheme originally proposed was therefore slightly modified to include the new PRS column elution conditions and applied for further analysis of beef extracts and food samples.

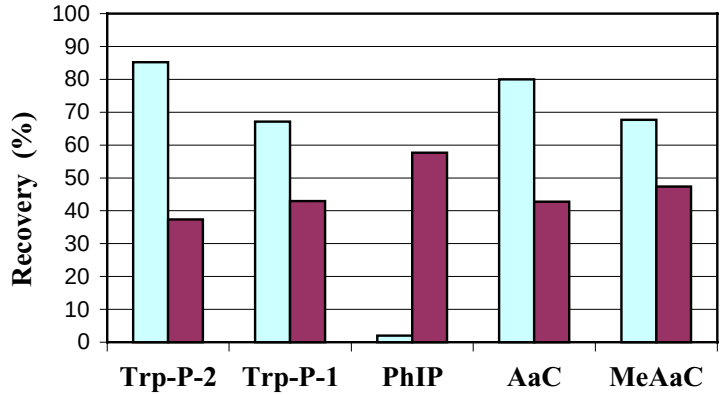

Fig. 1. Influence of hydrochloric acid concentration on the recovery of less polar heterocyclic aromatic amines in the beef extract 1 with a constant ratio of methanol/hydrochloric acid (50:50, v/v). White bar: 0.1 M HCl; black bar: 0.3 M HCl.

3.1.2. Selection of an internal standard

The analytical method was calibrated using the standard addition method. In order to allow peak identification by relative retention times and to correct for injection errors caused by the autosampler after evaporating the extracts to dryness the residues were redissolved in mobile phase containing an internal standard. 2-Aminofluorene, which has already successfully been used by other researchers [22], was added in the first experiments carried out with beef extract 1 containing PhIP, AαC, IQ, MeIQ and MeIQx in the concentration range from 35 to 60 ng/g. Fig. 2 shows that spiking the beef extract with HAs led to a decrease of the peak area of 2-aminofluorene. This phenomenon was caused by a quench effect of the unrealistically high amounts of HAs spiked to the beef extract 1.

Usually, the concentration of HAs in food samples is very low. Therefore, the standard addition method can be carried out by adding only low amounts of HAs. However, to exclude any disturbance we looked for another internal standard. More than 20 fluorescent compounds were tested but found unsuitable since they were not separated from the analytes under the chromatographic conditions applied. Finally, it was observed that naphthalene, which could be separated from the signals of all the other analytes was not effected by quenching and it was therefore used as internal standard in all further experiments.

3.1.3. Quantification of Trp-P-1

When the beef extract 2 was analysed using sample pre-treatment and separation as described in the experimental section interfering matrix peaks made it impossible to quantify Trp-P-1 at the excitation/emission wavelength given. The problem could, however, easily be solved by changing the excitation and emission wavelengths applied for generating the Trp-P-1 fluorescence signal. The interfering matrix peak detected at 263/380 nm disappeared when

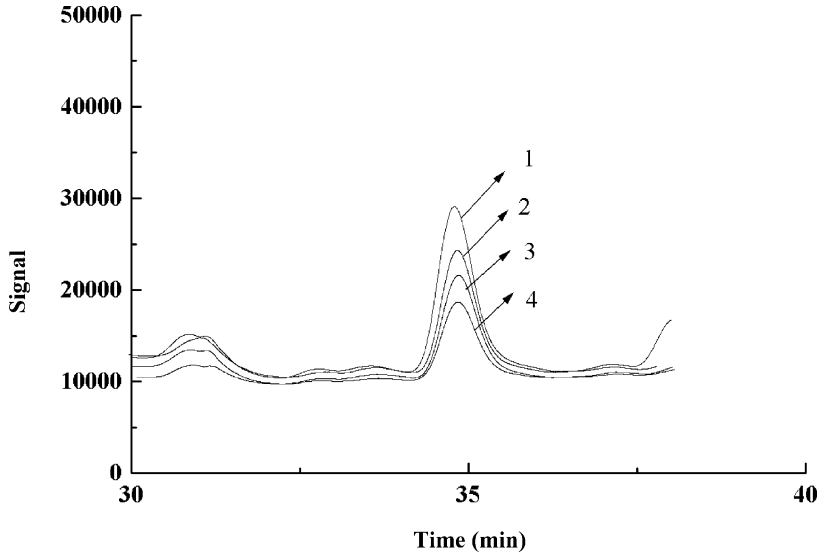

Fig. 2. Quench effect of added HAs on the internal standard 2-aminofluorene. 1: beef extract 1; 2, 3 and 4: addition of 80, 120 and 160 ng HAs/g, respectively.

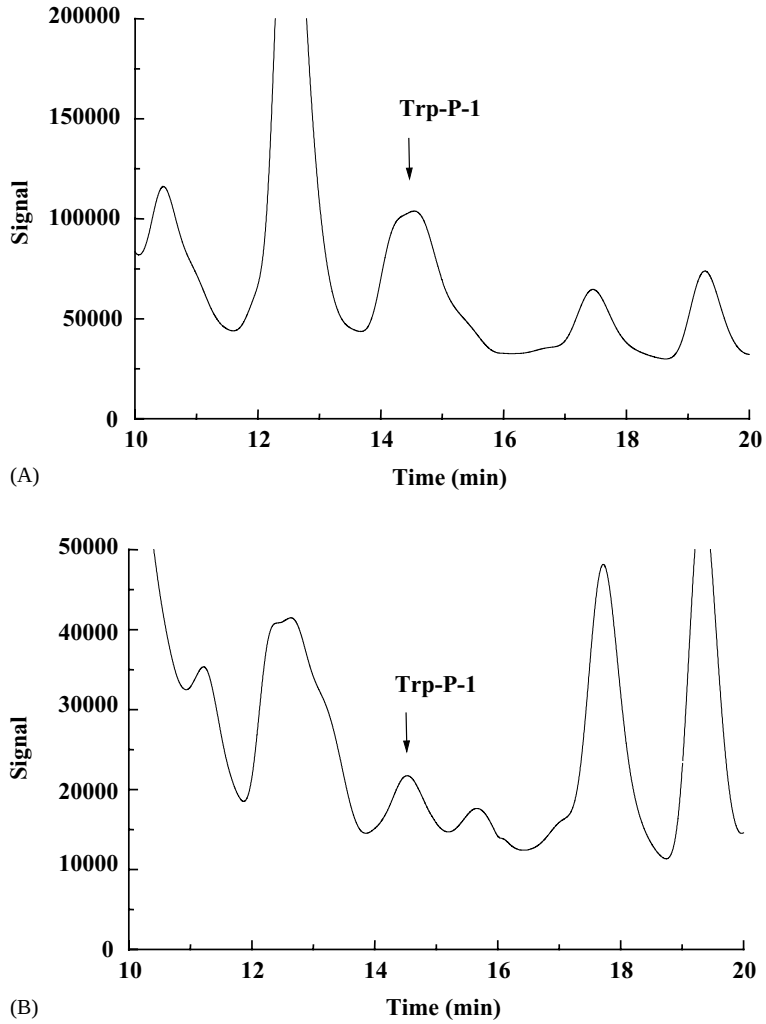

Fig. 3. Increasing detection selectivity for Trp-P-1 in beef extract 2 at different excitation/emission wavelengths: (A) 263/380 nm, (B) 320/410 nm.

the wavelengths were changed to 320/410 nm (see Fig. 3). The increased detection selectivity made it possible to quantify Trp-P-1, but it was obtained by a decrease in sensitivity.

3.1.4. Evaluation of results

Fig. 4 shows the chromatograms of the unspiked beef extract 2 (A) and beef extract 2 spiked with 4 ng/g of each of the five HAs (B). Table 2 summarises mean concentrations, confidence intervals and variation coefficients obtained in repeated analysis of the beef extract. Quantification of all five less polar HAs was possible without any problems. Table 2 also lists the mean of the means obtained in the analysis of beef extract 2 by all participants in the European project, together with their confidence intervals and coefficients of variation [21]. The data show that for Trp-P-2, Trp-P-1, PhIP and AαC the mean values obtained for the beef extract 2 by

Table 2
Results of the analysis of less polar heterocyclic aromatic amines in standardised beef extract 2

HA	Beef extract 2							
	Mean (ng/g)	n_1	CI (ng/g)	CV (%)	Mean of all means (ng/g)	n_2	CI (ng/g)	CV (%)
Trp-P-2	10.8	6	1.8	15.9	9.2	5	1.4	12.4
Trp-P-1	6.6	6	0.7	9.6	7.2	6	0.9	11.7
PhIP	13.0	6	2.1	15.6	9.6	5	3.6	40.6
AαC	11.6	6	1.2	10.0	8.6	5	3.2	30.2
MeAαC	12.1	6	1.4	11.4	8.5[a]	4	1.2	8.7

CI: confidence interval; CV: variation coefficient; n_1: number of determinations; n_2: number of participants.
[a] After removal of two values one of them being our value, the other still higher.

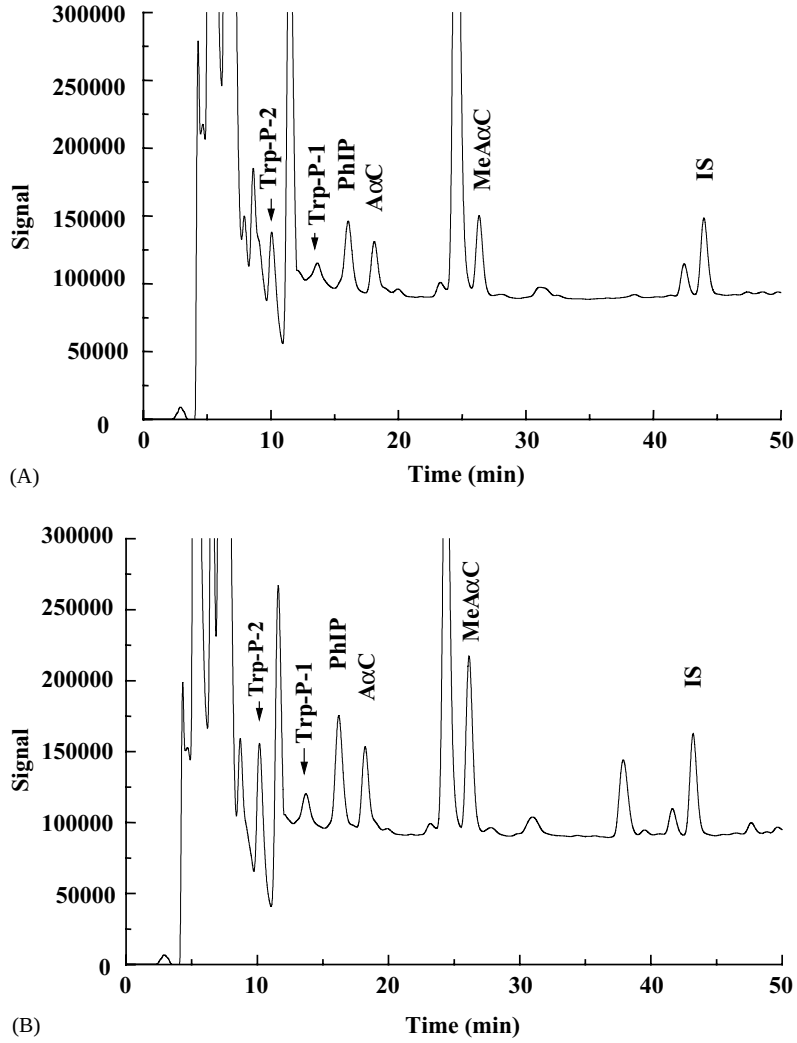

Fig. 4. (A) Chromatogram of the unspiked beef extract 2. (B) Chromatogram of the beef extract 2 spiked with 4 ng/g of each of the five less polar HAs.

our method with fluorescence detection fell within the confidence limits of the mean of the means. However, determination of MeAαC resulted in a higher mean value than the means obtained by other participants. Based on the results of a Nalimov *t*-test the evaluating team regarded our value as an outlier and removed it from the data set.

In general, the reproducibility we found was far below or in the order of the interlaboratory variation coefficients of all labs. The data allow the conclusion that the slightly improved sample pre-treatment method in combination with HPLC and fluorescence detection leads to results which are statistically comparable to the results of the other participating laboratories which all used much more cost intensive mass spectrometric methods for detection.

3.2. Analysis of cooked meat in the Austrian diet

The analysis method developed was applied to determine less polar HAs in typical Austrian food products. Some of them were home-cooked according to Austrian recipes (see Table 1 for cooking conditions), some were cooked in a local fast-food shop and one chicken sample was prepared in a factory canteen. In none of the samples matrix interferences made quantification of the analytes impossible. In Fig. 5 representative chromatograms of an unspiked deer extract (A) and the deer extract spiked with 4 ng/g of each of the five HAs (B) are shown. Concentrations of HAs in the analysed food samples are summarised in Table 3.

Several less polar HAs could be determined in each of these food products. The results confirm the well-known fact that the type of meat, the type of cooking procedure and the applied temperature are the main variables influencing the amount of HAs formed. PhIP, the most abundant heterocyclic amine, was found in all 12 meat samples in the range from 0.1 ng/g (fleshy part of the knuckle of pork) to 11.1 ng/g (chicken cooked in a factory canteen). Concentrations of PhIP $\geq$ 1 ng/g were determined in the chicken cooked in the wok, the fleshy part of the goose, the rabbit, the fish, the pan residue, the crust of the knuckle of pork and

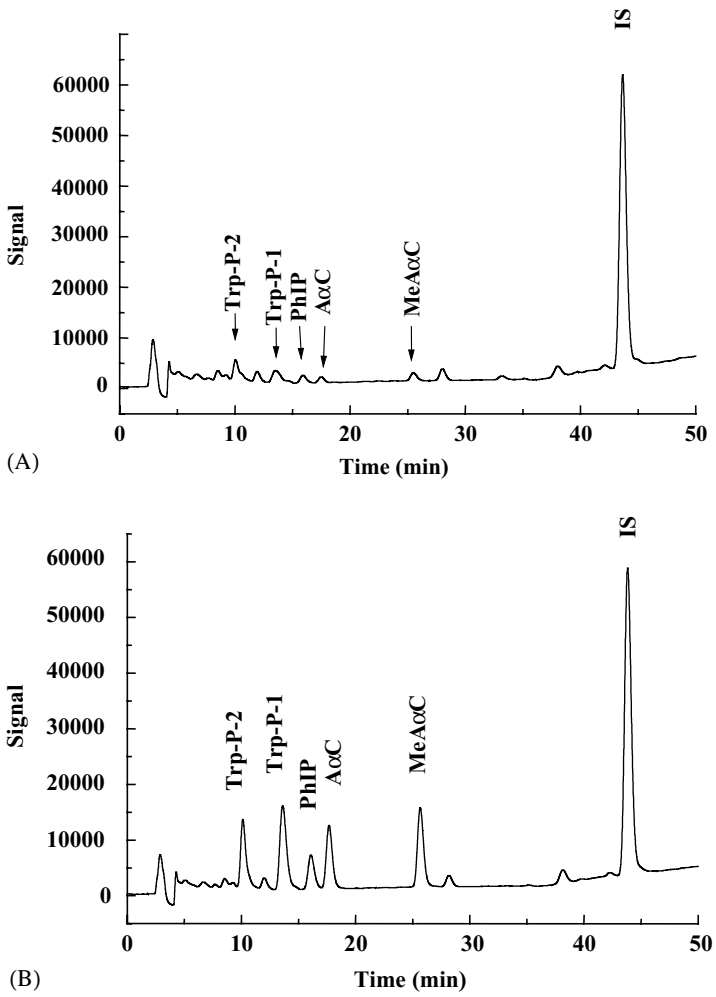

Fig. 5. (A) Chromatogram of an unspiked deer extract. (B) Chromatogram of the deer extract spiked with 4 ng/g of each of the five less polar HAs.

Table 3
Concentration of less polar heterocyclic aromatic amines (ng/g) in typical Austrian meat and fish

Sample	Trp-P-2 (ng/g)	Trp-P-1 (ng/g)	PhIP (ng/g)	AαC (ng/g)	MeAαC (ng/g)
Home cooked					
Chicken wok	0.1	0.1	1.3	ND	ND
Turkey	0.2	0.1	0.8	ND	0.05
Goose—fleshy part	0.1	0.2	1.0	0.1	0.1
Goose—crust	ND	0.2	0.9	0.04	0.1
Pheasant	0.6	ND	0.6	ND	0.3
Deer	0.2	0.1	0.2	0.04	0.03
Rabbit	0.4	0.3	1.4	0.2	0.1
Fish	0.1	ND	1.7	0.1	0.1
Pan residue	0.3	0.1	1.7	0.1	0.2
Fast-food					
Grilled sausage	1.2	1.2	0.8	ND	ND
Knuckle of pork—crust	3.1	1.2	1.5	ND	ND
Knuckle of pork—fleshy part	0.9	0.9	0.1	ND	ND
Minced meat	0.3	0.03	2.0	0.4	ND
Canteen					
Chicken	0.2	0.04	11.1	0.1	0.05

The data given are the mean values of two determinations. ND: not detected.

the minced meat. The highest concentrations of Trp-P-2 and Trp-P-1 were found in the grilled sausage and the crust of the knuckle of pork. In all samples concentrations of AαC and MeAαC were below 0.5 ng/g.

The recovery of HAs is greatly dependent on the sample matrix. Relatively high recoveries were obtained for the analysis of grilled sausage (>80%) and minced meat (>75%), whereas analysis of goose and rabbit resulted in lower recoveries of about 40%. Recovery of PhIP was generally lower than that of the other HAs which is in agreement with the results from Zimmerli et al. [14]. For all five HAs the limit of detection (S/N = 3) was about 0.02 ng/g and the limit of quantitation (S/N = 10) about 0.05 ng/g.

4. Conclusion

This study has shown that a slightly modified form of the procedure recommended by Toribio et al. [18] is applicable for the identification and quantification of less polar HAs in meat samples. The extraction efficiency of the analytes from real samples, however, shows large matrix to matrix variations. It is therefore necessary to use the standard addition method to quantify HAs. Fluorescence detection offers low detection limits in the 0.02 ng/g range and the possibility to adjust the procedure for the analysis of less polar HAs in complex food matrices by optimal choice of excitation and emission wavelength without intolerably large losses in sensitivity.

The results reported add new data to the very limited amount published for the content of less polar heterocyclic amines Trp-P-1, Trp-P-2, PhIP, AαC and MeAαC in food. They show that low concentrations of the five less polar HAs are present in most of the food tested. Their carcinogenic and mutagenic potential justifies future studies with the aim to improve existing methods for the determination of the less polar HAs and explore the possibilities offered by fluorescence detection to optimise the method to make them applicable in a variety of matrices.

Acknowledgements

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