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QUANTIFICATION OF THE FIVE MOST FREQUENTLY FOUND HETEROCYCLIC AROMATIC AMINES IN MEAT SAMPLES WITH PLANAR CHROMATOGRAPHY

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Introduction

Heterocyclic Aromatic Amines (HAA) are among the most potent mutagenic substances [1]. Around 20 different HAA at low µg/kg level could be identified in food. In complex reactions at high temperatures they are formed of the precursors creatine, creatinine, amino acids and reducing carbohydrates. The concentrations depend amongst others on the way of preparation, heating time and temperature [2]. It could be ascertained that meat samples cooked under normal conditions contained PhIP, MeIQx, 4,8-Di-MeIQx, Norharman and Harman, which are the most widespread HAA in meat.

Objectives

HAA are typically quantified by column chromatography (HPLC, GC) with detection by UV-absorbance, fluorescence or mass spectrometry (MS) [3]. The aim of our research was the development of a new planar chromatographic method to identify and quantify the five most frequently found HAA in complex meat matrix more effectively.

Methodology

Chemicals

The standard substances MeIQx, 4,8-Di-MeIQx and PhIP were purchased from Toronto Research Chemicals, Ontario, Canada, Harman and Norharman from Sigma-Aldrich GmbH, Taufkirchen, Germany. All standards were dissolved in methanol containing small amounts of ammonia. All solvents used were of analytical grade; solvents of the mobile phase were chloroform (Sigma-Aldrich GmbH, Seelze, Germany), diethyl ether (Fluka, Buchs SG, Switzerland) and methanol (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK).

Preparation of meat samples

Beef patties (60 g ± 0.5 g) were purchased from Ranch Master, Wunstorf, Germany. The patties were coated with sunflower oil and put between two parts of tin foil and between two grill plates of a double contact grill (Nevada, Neumärker, Hemer, Germany). At a plate temperature of 230 °C the patties were grilled simultaneously on both sides for six minutes.

Sample Preparation

The extraction of HAA from the meat matrix was performed according to the solid phase extraction method of Gross and Grueter [4] with slight modifications [5].

Analysis of HAA

As stationary phase HPTLC plates silica gel 60 WRF_{254s} (20 x 10 cm) from Merck, Darmstadt, Germany were employed. For sample application, the dissolved substances were sprayed as bands by Automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland). Afterwards, the plates were repeatedly developed in the same direction by the Automated Multiple Development System (AMD 2, CAMAG). The AMD 2 gradient scheme designed to separate the 5 HAA is shown in Table 1. Densitometry is performed via multi-wavelength scan with TLC Scanner 3 (CAMAG) at UV 262 nm, UV 316 nm and UV 366/>400 nm. For data acquisition and processing, winCATS software (CAMAG) was used.

Results & Discussion

After the determination of the most suitable plate material, different parameters of the mobile phase were optimized including solvents and solvent mixtures, the necessary number of gradient steps and migration distances. The gradient scheme in Table 1 shows that a seven-step development was necessary to separate the substances of interest. In step 1 the plate was developed with 100 % diethyl ether up to a migration distance of 30 mm. During the following steps the composition of the solvent mixture changed from 85 % chloroform/15 % methanol continuously to 87 % chloroform/13 % methanol with a migration distance of 60 mm each. Before each gradient step alkaline conditioning of the stationary phase was automatically performed.

After multiple development for about two hours the plate was scanned densitometrically. The optimized separation of the five HAA is shown in Figure 1. Detection was performed via multi-wavelength scan at 316 nm for PhIP (1), 262 nm for MeIQx (2) and 4,8-DiMeIQx (3), and at 366/>400 nm for Norharman (4) and Harman (5).

The conclusive next step was the investigation of the influence of meat matrix on the determination of HAA and the applicability of the developed method. Due to the matrix and the low levels of HAA in meat (µg/kg), accurate analysis is difficult and relatively high standard deviations are common [6].

In Figure 2 an overlay of two meat sample tracks is shown. One of them (blue analog curve) is a sample track spiked with standard solutions of Norharman and Harman, and the other (red analog curve) shows the track of a pure meat sample. The identity was confirmed by comparing these peaks with reference substances concerning retention time and spectral identity. As example, in Figure 3 a comparison

of the absorption spectra of MeIQx standard (1) and the corresponding peak from a meat sample (2) is shown. However, the complex meat matrix and the low levels of HAA in meat make a final identification unsure. In this context, i.e. for confirmation of positive results, the application of HPTLC-MS by a new online extractor is under investigation [7].
The quantification was carried out by external calibration with reference substances simultaneously on the same HPTLC plate and under idem chromatographic conditions. As example, the calibration curve of MeIQx which has a low relative standard deviation of ± 1.10 % is shown in Figure 4. The absolute amount of MeIQx on the plate was determined to be 12.7 ng in the pure and 33.9 ng in the spiked sample. With a recovery rate of 44 % the concentration of MeIQx in the meat sample was calculated to be 0.72 µg/kg. Thus besides screening, the presented method is suitable for quantification.

Modern planar chromatography shows a variety of advantages. The method is considered as very fast because of the possibility to applicate up to 20 tracks on one plate and to perform a simultaneous development. Ten tracks were needed for a five-point calibration (two tracks per concentration level for a repeat determination) whereas the residual ten tracks can be used for samples.

Before HPLC analysis, it is necessary to separate HAA from meat samples in polar and apolar fractions, because two different HPLC gradients for the fractions are necessary. Thus, one meat sample in duplicate analysis (= two pure and two spiked samples = 8 fractions) can be determined on one HPTLC plate. Comparing the retention time of about two hours in planar chromatography with the time in HPLC analysis (one hour per one fraction), it becomes obvious that planar chromatographic separation is four times faster than HPLC. In further studies it will be clarified whether it is also indispensable to divide the sample into two fractions for analysis by planar chromatography.
Furthermore, the new method is cost effective: only 60 mL solvent per plate are needed, less than 10 mL per fraction. The costs for HPTLC plates are less than 5 Euro (6 US$) per plate.

Conclusions

In spite of the complex meat matrix and difficult determination involved, the planar chromatographic quantification of the five most widespread HAA is possible. The presented method is not only suitable for screening but even adequate for quantitative trace analysis. Further research will focus the validation of the developed method as well as the comparison between planar chromatography and the commonly used HPLC method. In the last few decades a crucial disadvantage of planar chromatography compared to HPLC and GC was the lack of coupling possibilities with MS as a sensitive and selective detector. To hyphenate both methods a new extraction device was developed by Luftmann [7]. The investigation of the appliance of this new device for HAA analysis will be the aim of further studies.

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Abbreviations

MeIQx: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4,8-Di-MeIQx: 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
Norharman: 9*H*-pyrido[3,4-*b*]indole
Harman: 1-methyl-9*H*-pyrido[4,3-*b*]indole

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Tables and Figures

Table 1: AMD-gradient scheme with solvent compositions and migration distances to separate the five most widespread HAA.

step number	chloroform [%]	methanol [%]	diethyl ether [%]	migration distance [mm]
1	0.0	0.0	100.0	30.0
2	85.0	15.0	0.0	60.0
3	85.4	14.6	0.0	60.0
4	85.8	14.2	0.0	60.0
5	86.2	13.8	0.0	60.0
6	86.6	13.4	0.0	60.0
7	87.0	13.0	0.0	60.0

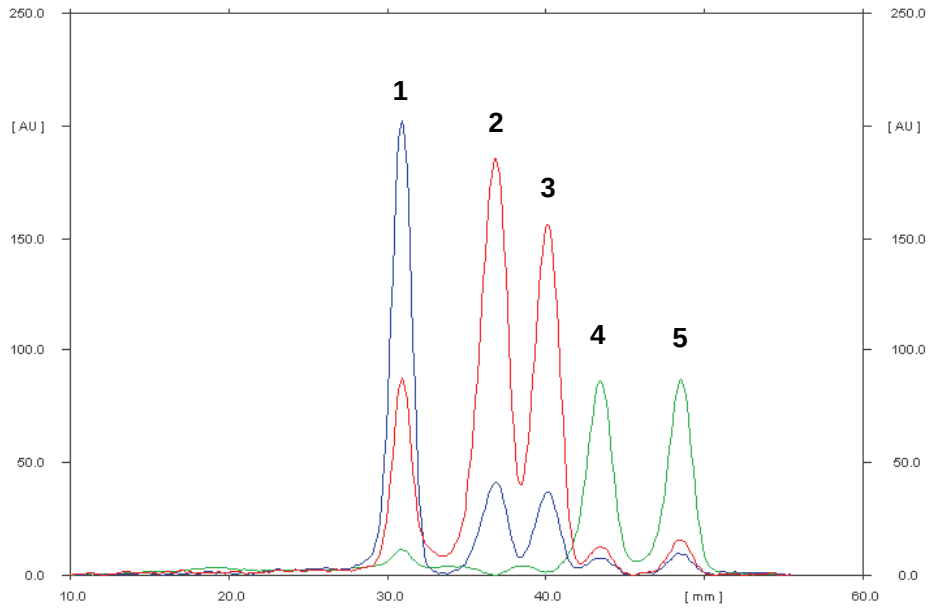

Figure 1: Optimized separation of the five HAA; detection via multi-wavelength scan was performed at 316 nm for PhIP (1), 262 nm for MeIQx (2) and 4,8-DiMeIQx (3), and at 366/>400 nm for Norharman (4) and Harman (5).

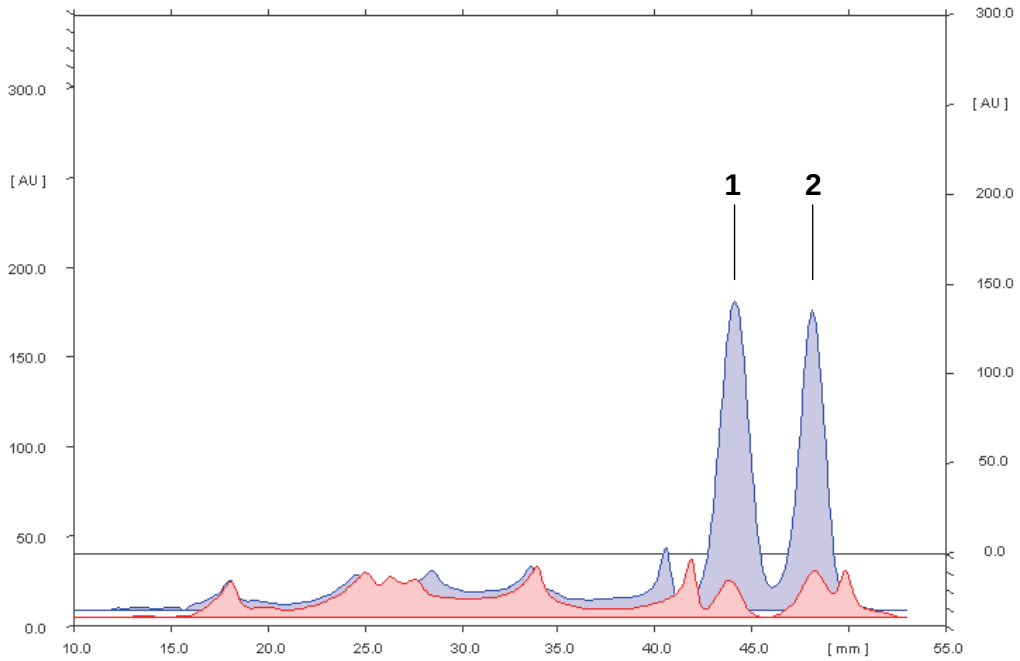

Figure 2: Track overlay of meat sample (red curve) and meat sample spiked (blue curve) with standard solutions of Norharman (1) and Harman (2).

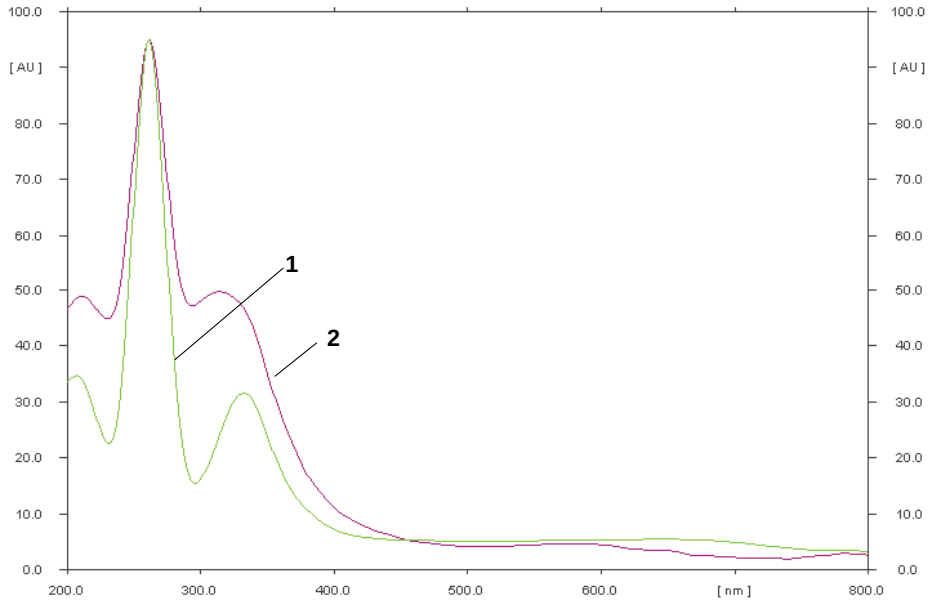

Figure 3: Comparison of UV/VIS absorbance spectra of MeIQx in standard solution (1) and in meat sample (2).

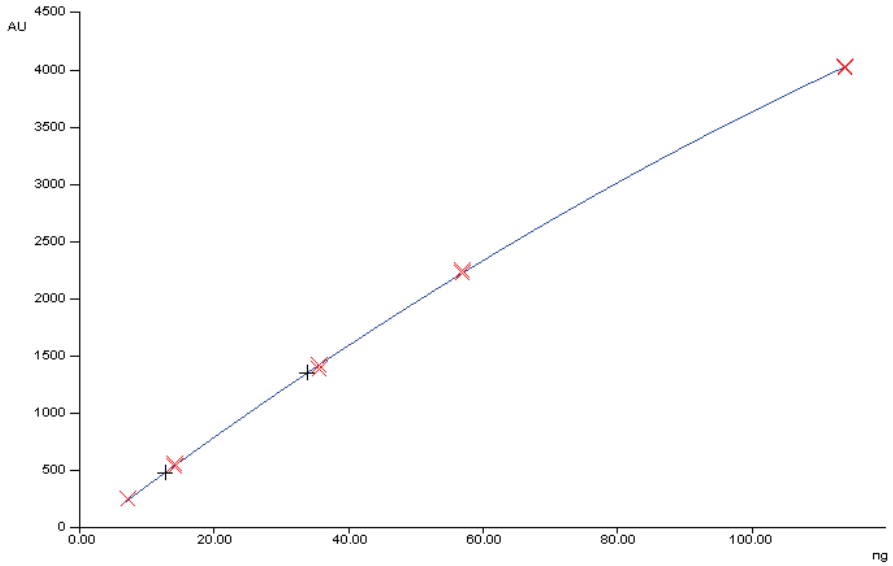

Figure 4: Polynomial calibration curve of MeIQx calculated via peak area (y = – 0.076 x^2+ 44.705 x- 75.741 , r = 0.9992, sdv = ± 1.10 %).