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Quantification of Heterocyclic Aromatic Amines in Fried Meat by HPTLC/UV-FLD and HPLC/UV-FLD: A Comparison of Two Methods

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A recently developed HPTLC/UV-FLD method was compared to the routinely used HPLC/UV-FLD method for the quantification of heterocyclic aromatic amines (HAA) formed at trace levels during the heating process of meat. For formation of these process contaminants under normal cooking conditions, beef patties were fried in a double-contact grill at 230 °C for five different frying times and extracted by solid-phase extraction. The HAAs most frequently found, that is, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 9*H*-pyrido[3,4-*b*]indole (norharman), and 1-methyl-9*H*-pyrido[3,4-*b*]indole (harman), were quantified by two chromatographic methods, which were orthogonal to each other (normal versus reversed phase system). Both methods showed a similar performance and good correlation of the results (R^2 between 0.8875 and 0.9751). The comparison of running costs and run time in routine analysis proved HPTLC/UV-FLD to be more economical (factor of 3) and faster (factor of 4) due to its capability of parallel chromatography. The HAA findings calculated by standard addition increased with the heating time from <1 to 33 μg/kg related to 3−6 min of frying time. The precision (RSD) was between 7 and 49% (HPTLC) and between 5 and 38% (HPLC) at these very low HAA levels formed.

KEYWORDS: Food; process contaminants; heterocyclic aromatic amines; HAA; meat; planar chromatography; HPTLC; HPLC; method comparison

INTRODUCTION

Heterocyclic aromatic amines (HAA) are among the most mutagenic and carcinogenic substances (*1*). More than 20 representatives are formed during the heating process of meat products or model systems (*2*). Besides the cocarcinogens norharman and harman, which strengthen the genotoxicity of other HAA (*3, 4*), PhIP, MeIQx, and 4,8-DiMeIQx (**Figure 1**) are the predominant HAA found in fish and meat products that are still palatable under domestic cooking conditions (*5, 6*). Among them, MeIQx is considered to be one of the most mutagenic (*7*); however, PhIP is regarded as the HAA of the most clinical relevance because of its high findings (up to 60% of all HAA formed) depending on the meat and cooking conditions (*8*). The International Agency for Research on Cancer (IARC) classified MeIQx and PhIP as possibly carcinogenic to humans (*9*). As a benchmark for the daily intake of several HAA, the California Environmental Protection Agency defined the No Significant Risk Level (NSRL) for MeIQx at 0.41 μg/day; for PhIP no NSRL exists so far (*10*). For PhIP, the margin of exposure (MOE, ratio between a dose leading to tumors in experimental animals and the human intake) shows a lower risk for human health compared to other food contaminants such as aflatoxin B_1 or ethylcarbamate (*7*). Normally these process contaminants are determined by column chromatography (HPLC, GC) with detection by UV absorbance, fluorescence (FLD), or mass spectrometry (MS) (*11*). Quantification of HAA is difficult due to the low concentrations found in complex meat matrix. Thus, despite optimized extraction/cleanup steps and quantification, high standard deviations are prevalent.

Only two interlaboratory studies have been performed by leading laboratories in HAA analysis in the past. In the first study in 1998 (*12*), eight laboratories used an extraction procedure and a quantification protocol based on that of Gross and Grueter (*13*) with the option to choose the most accurate method available. They all used HPLC as analytical method, with different columns and mobile phase conditions, and UV absorbance, fluorescence, electrochemical, or mass spectrometric detection. In first experiments MeIQx, 4,8-DiMeIQx, and PhIP have been quantified in unknown methanolic standard solutions at high HAA concentrations of about 200 μg/kg (*12*). After elimination of statistical outliers, RSD values of about 20% were

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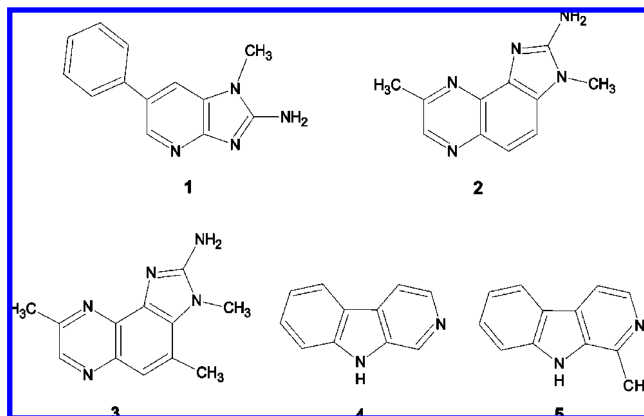


Figure 1. Structure formulas of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP, **1**), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, **2**), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx, **3**), 9*H*-pyrido[3,4-*b*]indole (norharman, **4**), and 1-methyl-9*H*-pyrido[3,4-*b*]indole (harman, **5**).

obtained, and the results (without meat matrix) showed good correlations between the laboratories. In second experiments, HAA were quantified in commercially available beef extract, however, without satisfying results. Major discrepancies not only between laboratories (e.g., PhIP ranged between 0 and 24 μg/kg, MeIQx between 7 and 47 μg/kg, and 4,8-DiMeIQx between 3 and 42 μg/kg) but also within-laboratories and between-days were observed. Thus, the meat matrix inclusive sample preparation turned out to be a crucial factor of influence.

In the second study in 2004 (*14*), in first experiments again, methanolic HAA standard solutions at very high concentrations of 1200 μg/kg were analyzed and the outlier-corrected RSD values were up to 10%. Compared to the previous interlaboratory trial (*12*) the precision was by a factor of 2 better, but at a factor of 6 higher concentrations. In second experiments with beef extract, the extraction and cleanup procedure, recommended by Gross (*15*) with several modifications (*16*), was standardized for all participants; however, different chromatographic conditions of the reversed phase system were allowed. The reversed phase columns used differ in the sorbent (RP8 and RP18, particle size = 3.5−5 μm) and manufacturer. The gradients mainly used were based on acetonitrile or acetonitrile/methanol mixtures with different acid additives and pH values (3.3−5.8). For detection mostly mass spectrometry [ESI-MS (single-quadrupole, ion trap), ESI-MS/MS, and APCI-MS/MS (ion trap, triple-quadrupole)] was employed, but also UV-DAD, UV and fluorescence, and electrochemical detection. As internal standards 4,7,8-TriMeIQx, naphthalene, or isotopically labeled compounds, such as MeIQx-d_3 and PhIP-d_3, were used. The outlier-corrected data sets regarding all 10 HAA were satisfying and showed relative standard deviations (RSD) between-laboratories and within-laboratories ranging from 8 to 24% and from 3 to 38%, respectively, for meat samples spiked at 75 μg/kg, and from 9 to 45% and from 5 to 40%, respectively, for meat samples spiked at 10 μg/kg. However, the number of individual data values, used after statistical tests for the final data calculation, varied from 21 to 40, indicating a considerable, up to 50%, number of outliers. Again, this clearly showed the great effort regarding sample preparation and the immense challenge to find these toxic traces in the very complex meat matrix.

Ongoing results from toxicological studies increase the demand for sensitive high-throughput methods and for detailed investigation of food prepared in the household, besides in catering and industrial processing, to enable estimation of the risk associated with the uptake with alimentary HAA (*11*). Recently, a UPLC/MS-MS method (*17*) was demonstrated to allow a higher throughput, based on a 3 min gradient, at excellent detection limits down to the nanograms per kilogram range in lyophilized meat extract and RSDs up to 13.4%. The quantification was performed in the selected reaction monitoring (SRM) mode using one ion transition.

The aims of the following study were to meet the need for a high-throughput method as well, but by the employment of an HPTLC/UV-FLD method (*18*), and additionally to evaluate, by method comparison, the accuracy for quantification of HAA in real beef patties, and not just lyophilized meat extracts, at the low micrograms per kilogram level. Thereby, the focus was laid on the comparison of two orthogonal methods (HPLC and HPTLC) using the same detectors (UV, FLD). The issue was addressed whether a simpler HPTLC method was suitable for lower cost estimation of the risk associated with the HAA uptake of food prepared in the household.

Although these simple detectors might be less selective and less sensitive than MS or MS/MS, and thus the methods being not at all (HPTLC/UV-FLD and HPLC/UV-FLD) comparable with UPLC/ESI-MS-MS, it is important to have further analytical methods for confirmation of the findings, especially such methods that offer a different/orthogonal separation principle such as the normal phase system of HPTLC. Also, so-called state-of-the-art methods face limits when matrix coelution leads to quantification matters in the ESI ion source. Thus, it is essential to present not only the extracted ion chromatogram for one transition but also the respective TIC chromatogram. Only this would give an impression about the interfering/competitive situation in the ion source's gas phase. Furthermore, a sequence of three or more reliable ions and their ratios are judged to be essential for identification (identification points according to 2002/657/EC) even when a single ion/transition is selected for quantification by MS/MS in a food matrix. The consequence for many issues is nowadays the application of comprehensive methods such as LC×LC/MS-MS.

As there is an ongoing compromise between effort and costs, the question arose whether a simple HPTLC method is suitable for lower cost estimation of the risk associated with HAA uptake.

MATERIALS AND METHODS

Materials. PhIP, MeIQx, and 4,8-DiMeIQx were purchased from Toronto Research Chemicals (North York, Canada) and norharman, harman, caffeine, and blue rayon trisulfonated from Sigma-Aldrich (Taufkirchen, Germany). Beef patties were bought from Ranch Master (Wunstorf, Germany) and sunflower oil and aluminum foil from the local market. Ultrapure water (18 μS/cm^2) was produced on site by a Millipore system (Millipore, Schwalbach, Germany). Aqueous ammonia (25 and 28%) acetonitrile and chloroform (both LiChrosolv, 99.8%) were obtained from Fluka (Buchs, Switzerland); hydrochloric acid (HCl), ammonium acetate, sodium hydroxide (NaOH), *n*-hexane (per analysis), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), dichloromethane, toluene, triethylamine, and phosphoric acid were from Merck (Darmstadt, Germany); methanol (gradient grade) was from Fisher Scientific (Loughborough, U.K.); and paraffin subliquidum was from Roth (Karlsruhe, Germany). Diatomaceous earth Isolute and blank cartridges were purchased from Separtis and propanesulfonic acid (PRS) (100 mg) and C 18 Bond Elut cartridges (500 and 100 mg) from Varian (Palo Alto, CA). LiChrospher Si 60 WRF254s 20 cm × 10 cm plates (0.1 mm layers, no. 105647) were obtained from Merck, the TSK-gel ODS-80TM column (5 μm, 250 mm × 4.6 mm i.d) was from Tosoh-Biosep (Stuttgart, Germany), and Supelguard LC-18-DB was from Supelco (Bellefonte, PA).

Stock and Standard Solutions. HAA were dissolved in methanol in the range of about 200 μg/mL. The exact concentration of each HAA stock solution was determined photometrically (HP 8453 spectral

photometer, Hewlett-Packard, Boeblingen, Germany) using the appropriate molar coefficients of extinction (*18, 19*). Additionally for HPLC analysis, a methanolic caffeine standard solution (2.5 μg/mL ultrapure water/methanol 1:1, v/v) was used as internal standard. For spiking meat samples a mixture of 0.20 ng/μL MeIQx, 0.19 ng/μL 4,8-DiMeIQx, 0.39 ng/μL PhIP, 0.09 ng/μL norharman, and 0.09 ng/μL harman was dissolved in methanol containing 1% aqueous ammonia.

For HPLC analysis the same mixture was used as standard solution. Volumina of 10−200 μL of this standard mixture were evaporated to dryness. After redissolving each with 100 μL of internal standard solution and injection of 50 μL aliquots, the calibration range was between 1.95 and 39.1 ng for PhIP, between 1 and 20.2 ng for MeIQx, between 1 and 18.7 ng for 4,8-DiMeIQx, between 0.46 and 9.2 ng for norharman, and between 0.45 and 8.9 ng for harman. The external calibration was checked via control runs (40 μL injections) repeatedly after 16 runs.

For HPTLC analysis a standard mixture of 10.3 ng/μL PhIP, 18.7 ng/μL MeIQx, 18.0 ng/μL 4,8-DiMeIQx, 1.9 ng/μL norharman, and 2.2 ng/μL harman was dissolved in methanol containing 1% aqueous ammonia (28%). Depending on the application volume (2−20 μL) calibration ranged between 0.8 and 205.0 ng/band for PhIP, between 1.5 and 373.9 ng/band for MeIQx, between 1.4 and 360.4 ng/band for 4,8-DiMeIQx, between 0.2 and 38.1 ng/band for norharman, and between 0.2 and 43.4 ng/band for harman. Later, the low findings in the micrograms per kilogram range showed that the wide calibration range in HPTLC was not necessary, especially the higher levels.

Frying of Beef Patties. Each beef patty (60 g) was coated with sunflower oil, placed between two parts of aluminum foil and then between the two plates of a double-contact grill (Nevada, Neumärker, Hemer, Germany). At a plate temperature of 230 ± 1 °C the patties were grilled, simultaneously on both sides, for five different cooking times between 3 and 6 min at time intervals of 45 s, that is, 3 min; 3 min, 45 s; 4.5 min; 5 min, 45 s; and 6 min. The samples of each batch were mixed, packed in plastic bags under vacuum, and stored at −18 °C until use.

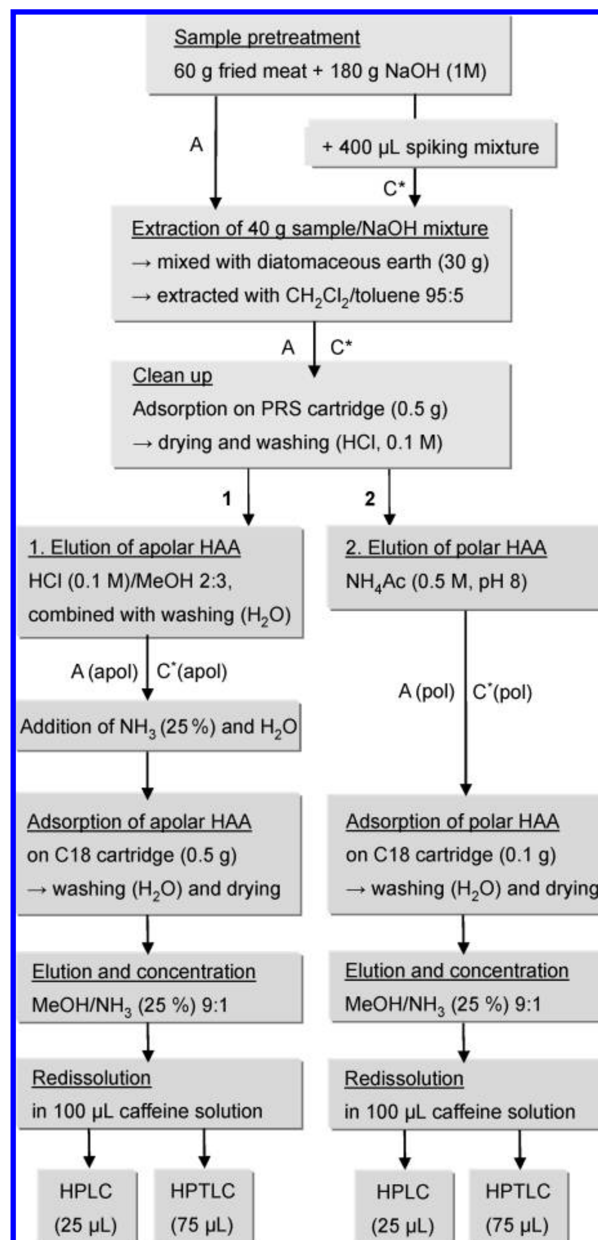
Extraction of HAA from Meat Samples. The sample preparation using blue rayon was performed according to the method of Hayatsu et al. and Kato et al. (*20, 21*) with slight modifications and optimizations.

The sample preparation using solid-phase extraction was performed according to the method of Gross and Grueter with slight modifications (*6*). About 60 g of fried meat and 180 g of sodium hydroxide (1 M) were homogenized with the Ultra Turrax (Janke & Kunkel, Staufen, Germany) for 4 min at high speed and divided into two sample portions of 40 g each (A and C*). For standard addition the sample portion assigned as C* was spiked with 400 μL of HAA spiking mixture (1−4 μg/kg). Each of the two sample portions was mixed with 30 g of diatomaceous earth and filled in blank cartridges. The HAA were extracted with dichloromethane/toluene 95:5 (v/v) at a flow rate of 2 mL/min and directly adsorbed onto preconditioned PRS cartridges containing 0.5 g of sorbent and dried by a slight nitrogen flow.

After washing with 6 mL of hydrochloric acid (0.1 M), the apolar HAA (step 1) were eluted with 15 mL of a mixture of HCl (0.1 M)/methanol 2:3 (v/v) at a flow rate of 2 mL/min. The cartridges were then washed with 6 mL of ultrapure water. Eluate and washing solution were combined, 25 mL of H_2O was added and made alkaline with 0.5 mL of ammonia (25%). The apolar HAA were subsequently adsorbed on preconditioned C18 cartridges containing 0.5 g of sorbent. Elution of the polar HAA from the PRS cartridges (step 2) was performed with 20 mL of ammonium acetate (0.5 M, pH 8). The HAA were subsequently adsorbed on preconditioned C18 cartridges containing 0.1 g of sorbent. After washing with ultrapure water (2 mL) and drying, the apolar and polar HAA were eluted from the C18 cartridges with 1.2 mL of a methanol/ammonia (25%) mixture 9:1 (v/v) into 1.8 mL vials. After drying by a nitrogen flow in an evaporator (Barkley, Germany), the residue was redissolved in 100 μL of caffeine standard solution, which was used as internal standard for correction of the injection volume in HPLC analysis.

Scheme 1 presents an overview of the sample preparation steps for the analysis of a single meat sample (sample A and standard addition C*). Additionally for each sample a 2-fold determination over the whole procedure was performed (named sample B and standard addition D*).

Scheme 1. Flow Diagram of the Standardized Extraction and Cleanup Protocol



Thus, for a single meat sample four sample portions (A, B, C*, D*) were extracted and eluted, polar and apolar analytes each, leading to eight sample eluates (A_{apolar}, A_{polar}, B_{apolar}, B_{polar}, C^*_{apolar}, C^*_{polar}, D^*_{apolar}, D^*_{polar}). Aliquots of 25 μL of all eight sample eluates of a single sample analysis were subjected to HPLC/UV-FLD, and each residual portion (evaporated to dryness and taken up in 100 μL of methanol containing 1% aqueous ammonia) was subjected to HPTLC/UV-FLD.

This protocol was repeated twice for each of the five heating times. Thus, six sample preparations were performed over three different days for each heating time. By the mathematical recombination of the samples calculated by standard addition, for example, A combined with D* instead of C* and B with C* instead of D*, 12 values were obtained for evaluation of each heating time.

HPTLC/UV-FLD. On the LiChrospher Si 60 WRF254s plate 20 tracks with a track distance of 8.9 mm were applied, for example, 16 tracks for meat samples and 4 tracks for four-point calibration. Samples (residual volume of 100 μL) were sprayed on as 8 mm bands by the Automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland). The application volumes of the standard solution ranged from 2 to 20 μL. After sample application, the bands were dried for 3 min in a stream of warm air.

Chromatographic separation was performed in an Automatic Developing Chamber (ADC 2, CAMAG). Before the separation, the chamber was automatically saturated for 20 min with aqueous ammonia (25%)/ultrapure water 1:4 (v/v), and simultaneously the plate activity was adjusted to 34% relative humidity with a saturated aqueous $MgCl_2$ solution. Then the layer was automatically hung in the almost saturated chamber and preconditioned for 15 min. Chromatography was performed at room temperature up to a migration distance (MD) of 60 mm with methanol/chloroform, 1:9 (v/v). Migration time was 30 min. Automated plate drying for 2 min followed.

For fluorescence enhancement the layer was dipped in a mixture of paraffin/*n*-hexane 1:1 (v/v) with the Chromatogram Immersion Device III (CAMAG) for 3 s at 30 mm/s and dried for 2 min. Densitometry was performed by multiwavelength scanning with the TLC Scanner 3 (CAMAG) at UV 262 for absorbance measurement and at UV 313/ >340 nm and 366/>400 nm for fluorescence detection (FLD). The measurement slit was 6 × 0.30 mm and the scanning speed 20 mm/s. Quantification was performed via peak area by standard addition.

HPLC/UV-FLD. Twenty-five microliters of each sample fraction was diluted in gold-coated vials (to reduce glass wall adsorption) with 75 μL of the caffeine standard solution and 40 μL was injected for HPLC analysis. A previously described HPLC method (*15*) was modified and used as follows: HPLC analysis was performed with a Gynkotek HPLC system (Germering, Germany) consisting of an M480 pump, a Gina 50 autosampler, a DG 1310 S degasser, an RF 1002 fluorescence detector, and a UVD 320 diode array detector. Gynkosoft chromatography data system version 5.50 was used for data acquisition and processing.

Chromatography was performed on a TSK-gel ODS-80TM column with a Supelguard LC-18-DB guard column. The mobile phase consisted of eluent A (triethylamine phosphate buffer, 0.01 M, pH 3.2), eluent B (triethylamine phosphate buffer, 0.01 M, pH 3.6), and eluent C (acetonitrile). The gradient program started at 82% A, 10% B, and 8% C and ended at 15% A, 10% B, and 75% C (*6*). The HPLC separation took 52 min at a flow rate of 1 mL/min and 25 °C, followed by equilibration for 3 min. UV detection was performed at 258 nm with 3D-field for spectra plots (200−360 nm), and fluorescence detection (low sensitivity) at 300/440 nm (14−24 min, norharman, harman) and 306/371 nm (24−26 min, PhIP). Quantification was performed via peak area by external calibration (norharman, harman) or standard addition (PhIP, MeIQx, 4,8-DiMeIQx).

RESULTS AND DISCUSSION

This method comparison differs from the previously described interlaboratory studies that used model systems or lyophilized beef extract (*12, 14*) inasmuch as beef burgers were fried with respect to domestic cooking conditions, that is, at 230 °C for 3−6 min, and the five heating times were correlated to the findings. Hence, this setup allowed exemplarily the estimation of real findings in the context of risk assessment.

HAA as process contaminants are always formed during grilling and frying. Thus, it is impossible to grill beef patties without generating HAA formation. Cooking in water or microwave heating are cooking methods with low HAA formation, but cooked meat and grilled meat are definitely not comparable from the point of coextracting matrix. Thus, during validation of the HPTLC method, LOD/LOQ have been determined without matrix and, for quantification of HAA in grilled matrices, the employment of the standard addition method was investigated and confirmed by HPTLC/MS (*18, 22*). Hence, the standard addition method was used for the reliable quantification of process contaminants in the following method comparison.

Sample Preparation and Recovery. HAA analysis is time-consuming—it requires trace analysis in a heavy meat matrix—and a careful sample preparation is *conditio sine qua non*. On the other hand, sample preparation is the most extensive step in HAA analysis. Thus, the primary intention was to simplify the extraction procedure by the application of the selective blue rayon technique (*4, 21, 23–25*). Hayatsu et al. (*23*) reported that the planar aromatic ring system of the blue pigment copper phthalocyanine trisulfonate, which was linked to polymeric carbohydrates such as cotton or rayon, selectively interacted with planar molecules. HAA recoveries from fried meat samples were reported to be between 45 and 75% for IQ, MeIQ, MeIQx, 4,8-DiMeIQx, and PhIP (*25*). Initial investigations in this study showed that the extraction of the planar HAA molecules from the meat matrix was very selective, resulting in a low matrix content of the extracts but, on the other hand, inadequate recoveries (RSD ≤ 15%). Insufficient amounts of blue rayon for a quantitative HAA adsorption or an incomplete elution of the adsorbed HAA from the dye might be reasons. Further extensive work would have been necessary for optimization of the blue rayon technique; however, the focus of this work was laid on the comparison of two analytical methods.

Thus, the routinely used solid-phase extraction protocol based on that of Gross and Grueter with slight modifications was used as sample preparation procedure for both methods (*6*). For the method comparison the sample preparation protocol was standardized and even identical for both analytical methods besides the last step of taking up the residue (**Scheme 1**). In a previous interlaboratory test (*12*) the minimal within-laboratory recovery rates were set to 70% for MeIQx and 4,8-Di-MeIQx and 40% for PhIP. However, the recovery rates obtained in practice are often lower caused by the generally practiced incomplete extraction (*13*), which was stopped after a defined extraction volume of dichloromethane/toluene 95:5, v/v. Balogh et al. determined recoveries between 31 and 92% depending on the HAA (*26*) and Skog et al., between 20 and 72% (*27*). In this study the mean recoveries obtained by both chromatographic methods ranged between 22 and 91% and were in a similar range as the results in the literature.

Findings. Substance identification in the meat extract was clearly confirmed by the standard addition method. If relevant, additionally HPTLC/MS can be employed for confirmation (*18, 28*).

Increase with Cooking Time at Very Low Micrograms per Kilogram Concentrations. An overview of the PhIP, MeIQx, 4,8-DiMeIQx, norharman, and harman findings in meat, obtained for different cooking times, by both HPLC and HPTLC, is given in **Table 1**. It is obvious that both HPLC and HPTLC findings increased with prolonged cooking times. The concentrations found were in the very low micrograms per kilogram range and by a factor of up to 10 lower as compared to the previous interlaboratory study, which analyzed concentrations at 10, 50, and 75 μg/kg (*14*). The correlation between HAA formation and cooking time (*5, 26, 29*) as well as cooking temperature (*26, 30, 31*)was in accordance with former investigations. The formation of PhIP, which was reported to increase significantly with a longer heating period (*26*), was confirmed by HPTLC and, to a minor extent, by HPLC.

Besides the parameters temperature and heating time, HAA formation is also influenced by several parameters such as the type of meat (beef, pork, chicken) (*32–34*), its intrinsic ingredients (amino acids, reducing sugars, creatine, creatinine, fat, antioxidants) (*2*), and the cooking procedure (grilling, pan-frying, microwave heating, oven-roasting, oven heating, broiling) (*3, 5, 26, 35*) as well as the frying fat used (*36*).

Among the many reports on HAA findings for different foods and cooking conditions, only a few studies (*3, 26, 29, 37*) focused on similar cooking conditions of meat material such as minced meat, beef patties, or ground beef. These findings could probably be matched with our findings, and a comparison is

Table 1. Overview of the Findings (± Standard Deviations and Relative Standard Deviation, RSD) of MeIQx, 4,8-DiMeIQx, PhIP, Norharman, and Harman Obtained by HPLC and HPTLC

		cooking time														
		3 min			3 min, 45 s			4 min, 30 s			5 min, 15 s			6 min		
substance	method	*c* (μg/kg)	RSD (%)	*n*	*c* (μg/kg)	RSD (%)	*n*	*c* (μg/kg)	RSD (%)	*n*	*c* (μg/kg)	RSD (%)	*n*	*c* (μg/kg)	RSD (%)	*n*
PhIP	HPLC	0.2 ± 0.02	12.5	12	0.5 ± 0.1	22.4	12	0.9 ± 0.1	10.2	8	1.9 ± 0.2	11.4	12	3.6 ± 0.8	21.3	12
	HPTLC	3.2 ± 1.5	46.0	8	6.8 ± 1.3	18.5	12	9.9 ± 2.4	24.4	8	25.0 ± 4.1	16.4	10	33.1 ± 9.7	29.3	10
MeIQx	HPLC	1.0 ± 0.4	38.1	12	1.6 ± 0.3	19.1	12	2.0 ± 0.3	17.0	8	3.8 ± 0.7	18.6	12	4.8 ± 1.2	25.1	12
	HPTLC	1.7 ± 0.5	27.5	12	2.4 ± 0.4	15.4	12	1.7 ± 0.3	17.2	8	5.6 ± 1.3	22.3	12	4.8 ± 0.8	15.5	12
4,8-DiMeIQx	HPLC	nd		12	0.3 ± 0.1	24.4	12	0.4 ± 0.1	15.5	8	0.9 ± 0.2	24.0	12	1.3 ± 0.1	9.2	12
	HPTLC	1.1 ± 0.5	48.6	12	1.2 ± 0.5	39.9	12	1.4 ± 0.3	19.1	8	2.8 ± 0.7	24.9	12	3.0 ± 0.3	11.3	12
norharman	HPLC	2.1 ± 0.3	15.0	12	3.4 ± 0.5	14.6	12	5.0 ± 0.3	5.5	8	7.7 ± 0.4	5.6	12	10.4 ± 0.9	8.2	12
	HPTLC	3.7 ± 0.9	24.5	12	5.3 ± 1.0	19.1	12	6.8 ± 0.7	9.8	8	10.7 ± 0.8	7.4	6	11.3 ± 3.3	29.4	12
harman	HPLC	1.5 ± 0.2	10.5	12	2.3 ± 0.3	11.4	12	3.5 ± 0.4	10.1	8	6.0 ± 0.3	4.9	12	8.9 ± 1.0	11.5	12
	HPTLC	1.4 ± 0.2	16.3	12	3.1 ± 0.6	18.4	12	3.1 ± 0.4	14.2	8	8.7 ± 2.3	27.0	6	10.3 ± 2.7	25.9	12

Table 2. Literature Data of Typical HAA Findings in Pan-Fried, Minced Meat, Beef Patties, or Ground Beef Prepared at Temperatures and Cooking Times Comparable to the Conditions Chosen in This Study

cooking temperature (°C)	cooking time (min)	PhIP	MeIQx	4,8-DiMeIQx	norharman	harman	refs
230	2−10	1.3 ± 0.7	0.4 ± 0.1	0.15 ± 0.1			Knize et al. (*29*)
		32.0 ± 10.0	7.3 ± 2.7	1.6 ± 0.5			
225	5	1.1	2.2	0.8			Skog et al. (*37*)
	6	13.3 ± 6.0	3.5 ± 1.0	3.0 ± 1.5			Balogh et al. (*26*)
175−200		0.6 ± 0.02	0.7 ± 0.1	<0.1	0.8 ± 0.1	1.9 ± 0.6	Busquets et al. (*3*)
230	3−6	0.2 ± 0.02	1.0 ± 0.4	nd	2.1 ± 0.3	1.5 ± 0.2	this study, HPLC
		3.6 ± 0.8	4.8 ± 1.2	1.3 ± 0.1	10.4 ± 0.9	8.9 ± 1.0	
		3.2 ± 1.5	1.7 ± 0.5	1.1 ± 0.5	3.7 ± 0.9	1.4 ± 0.2	this study, HPTLC
		33.1 ± 9.7	5.6 ± 1.3	3.0 ± 0.3	11.3 ± 3.3	10.3 ± 2.7	

given in **Table 2**. PhIP, the findings for which differed most in the four studies, was obviously more challenging to analyze. Comparison of the MeIQx, 4,8-DiMeIQx, and PhIP findings (from <1 to 33 μg/kg, **Table 1**) with those results in the literature clearly showed that the values were in the same, very low micrograms per kilogram range.

Correlation between the Two Methods. The concentrations of MeIQx, 4,8-DiMeIQx, norharman, and harman obtained by HPTLC were in a range similar to that obtained by HPLC. The HAA correlations of both methods were in a good agreement (R^2 between 0.8875 and 0.9751, **Figure 2**). With regard to PhIP, the HPTLC results were by a factor of 5−10 higher than those obtained by HPLC (**Figure 2E**) and might imply matrix coelution, which impairs especially at the very low concentrations found. Differences can be observed only if results obtained by different methods are compared. However, such comparisons are rarely presented. The question arose whether this difference regarding the PhIP results was tolerable. Comparison with the literature showed that other laboratories have the same dispersion. The only two interlaboratory studies (*12, 14*) have variations in their results as well. Also, in former studies PhIP was the most difficult substance to quantify due to low recovery rates and the high impact of matrix interferences (*26, 38*). Especially PhIP undergoes specified degradation reactions depending on the matrix or pH (*39*) and shows a significant loss during storage at elevated temperatures (*12*).

Typical HPTLC/HPLC chromatograms of standard mixes, spiked and unspiked meat samples, are shown in **Figures 3** and **4**. Proper peak assignment was guaranteed by the standard addition procedure applied. Despite optimized extraction and cleanup procedures, residual matrix in the apolar fraction hampered quantification of PhIP, norharman, and harman at the very low concentrations in beef patties (**Figures 3B**,**C** and **4B**). Thus, a minimization of the matrix effects for HAA quantification at the very low micrograms per kilogram levels is desirable for both chromatographic methods, but it is again a question of compromise between effort and costs.

Precision. In this study the precisions were obtained without any outlier correction at a factor of up to 10 reduced HAA concentrations as compared to the previous interlaboratory study, which analyzed concentrations at 10, 50, and 75 μg/kg (*14*). This means the whole data set was used besides some disturbances during the extensive sample preparation. Over all frying times the HAA concentrations (<1−33 μg/kg) showed a precision (RSD) between 4.9 and 38.1% for HPLC ($n = 8-12$) and between 7.4 and 48.6% for HPTLC ($n = 6-12$). The higher variations of the HAA findings at very low concentrations (0.2−3.7 μg/kg for the 3 min heating time) can be deduced from the increased impact of the meat matrix. Indeed, only the highest frying time (6 min) formed HAA concentrations (in the range of 1.3−33.1 μg/kg depending on the HAA) allowing a comparison with the existing literature. The precisions (RSD) of the findings in beef burgers fried for 6 min were between 11.3 and 29.4% for HPTLC ($n = 10-12$) and between 8.2 and 25.1% for HPLC ($n = 12$) and thus highly satisfying if compared to the literature.

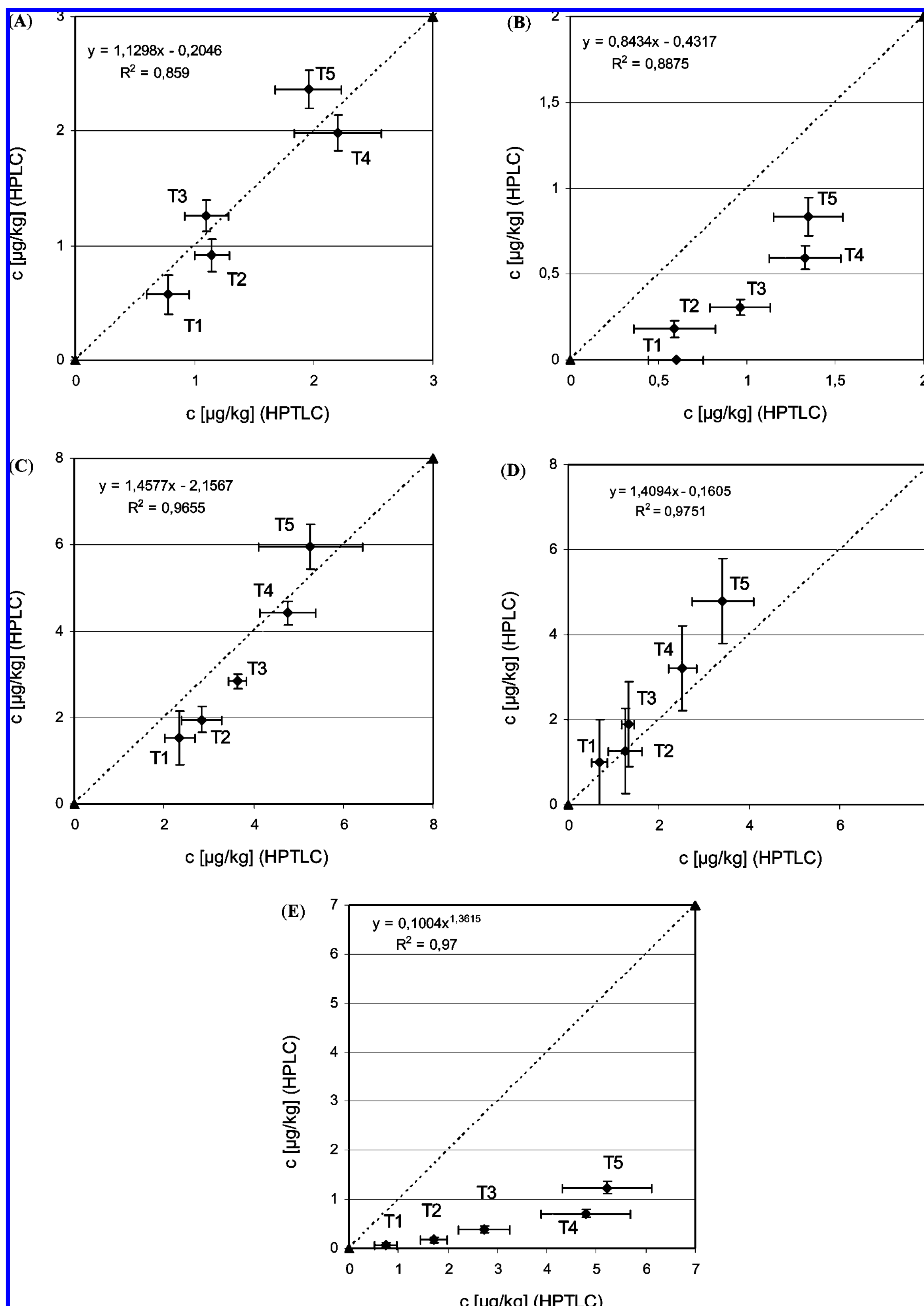


Figure 2. HPLC/HPTLC correlation for HAA findings at five different cooking times (T1−T5, 3 min; 3 min, 45 s; 4 min, 30 s; 5 min, 15 s; 6 min): MeIQx (**A**), 4,8-DiMeIQx (**B**), norharman (**C**), harman (**D**), and PhIP (**E**).

In the 2004 interlaboratory test (*14*) the between-laboratory precision (RSD, $n = 24-40$) for MeIQx was 17.4%, that for 4,8-DiMeIQx 30.0%, and that for PhIP 40.6%. The high RSD for between-laboratory precision resulted from the different extraction steps and cleanup procedures as well as from allowed variations in chromatography and detection. The within-laboratory precision (RSD, $n = 24-40$) ranged from 7.7 to 27.8% for MeIQx, from 5.2 to 36.3% for 4,8-DiMeIQx, and from 10.3 to 27.8% for PhIP, all at 10 μg/kg concentrations in beef extract.

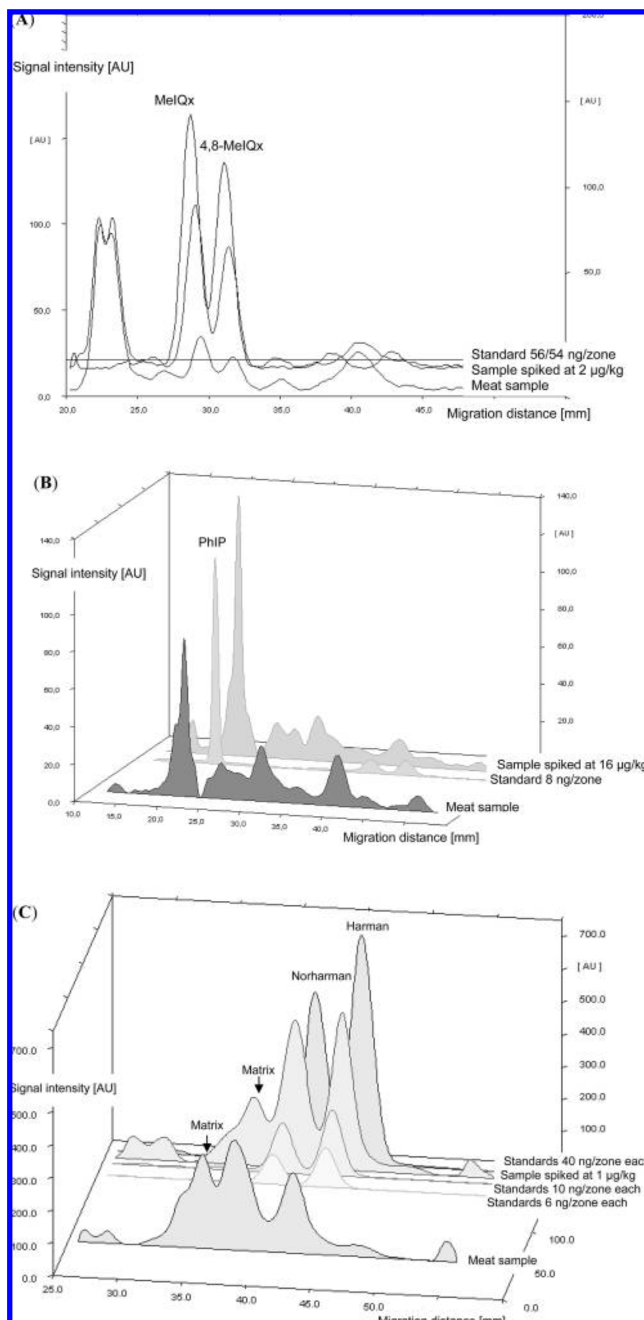


Figure 3. Track overlays of typical HPTLC chromatograms for quantification of MeIQx and 4,8-DiMeIQx (polar fraction, UV detection at 262 nm) (**A**), PhIP (apolar fraction, fluorescence measurement: 313/>340 nm) (**B**), and norhaman and harman (fluorescence measurement: 366/>400 nm) (**C**).

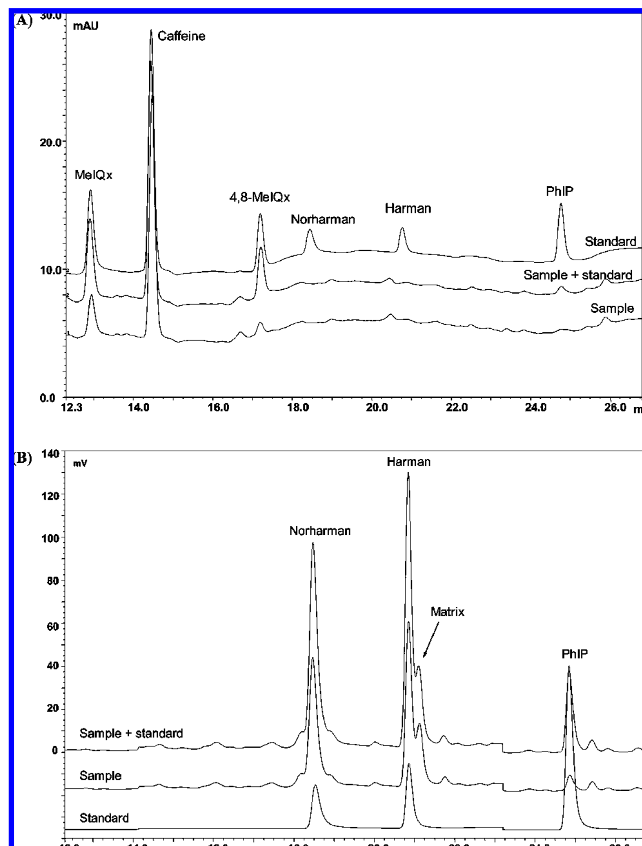


Figure 4. Track overlays of typical HPLC chromatograms for quantification of MeIQx and 4,8-DiMeIQx (polar fraction, UV detection at 258 nm) (**A**), norharman and harman (fluorescence detection, excitation/emission at 300/440 nm, 14−24 min), and PhIP (fluorescence detection, excitation/emission at 306/371 nm, 24−26 min) (**B**).

In another study design comparable to our experiments (frying of beef patties at 230 °C for 2−10 min) the within-laboratory precision (RSD, $n = 4$) was determined to be 25.0−57.1% for MeIQx, 31.3−66.7% for 4,8-DiMeIQx, and 7.7−60.3% for PhIP (*29*). Studies from Balogh et al. with ground beef patties fried at 225 °C for 6 min showed a precision (RSD, $n = 6$) of 28.6% for MeIQx, 50.0% for 4,8-DiMeIQx, and 45.1% for PhIP (*26*). Knize et al. reported for PhIP, MeIQx, and 4,8-DiMeIQx similar standard deviations (RSD) ranging from 22 to 37% (*40*). All in all, the generally high RSD for within-laboratory precision can be inferred from the challenging analysis of the formed HAA traces in the very complex meat matrix.

Comparison of Running Costs and Analysis Time. In the literature a comparison of the costs and analysis time is rarely given. Hence, these parameters were added to the comparison of the findings. The cost comparison was restricted on the running costs due to the study design using the same meat material, the same sample preparation, but two different chromatographic methods. The sample preparation procedure was the same for both methods and thus not considered for calculation. The maintenance costs of both methods were comparably low because no mass spectrometer was used and were expected to be about the same.

For risk assessment a high sample throughput is evident because for a single meat sample eight chromatographic runs are performed. Therefore, the comparison of running costs and time for both methods (**Table 3**) was calculated for the determination of two meat samples enabling a maximal loading of one HPTLC plate. Generally, the stationary phase costs are much less in HPTLC than in HPLC. However, in this case the costs regarding the stationary phases of both methods are almost in the same range because extra-clean, 100 μm LiChrospher plates were used in HPTLC, which were by a factor of 3 more expensive than regular HPTLC plates. The costs for the mobile phase differ because HPTLC analysis is performed simultaneously for 20 runs. All in all, the costs for HPTLC analysis of two meat samples are by a factor of 3 lower than the costs for HPLC analysis, although the column lifetime was calculated for 2200 HPLC runs, changing the precolumn after 100 runs. In HPTLC analysis (offline), extra time is needed for sample application, fluorescence enhancement, and densitometry. However, due to simultaneous analysis of 20 runs, the HPTLC method is 4 times faster than HPLC regarding the analysis of two meat samples. Hence, the comparison of costs and time for analysis clearly demonstrated the profit by HPTLC.

Table 3. Cost/Time Comparison between HPLC and HPTLC for the Determination of Two Meat Samples[a] Inclusive Calibration Standards

calculation	HPLC	HPTLC
mobile phase, incl plate activation/conditioning (euro)	4.93	0.33
stationary phase, incl precolumn (euro)	7.02	4.00
disposal (euro)	0.65	0.03
running costs (euro)	12.59	4.36 → factor 3 less expensive
application/injection (h)	1.0	3.0
chromatography/gradient time (h)	15.6	1.1
detection, incl fluorescence enhancement (h)		0.2
total time (h)	16.6	4.3 → factor 4 faster
all steps	online	offline, but fully automated
personnel time (stand-by work)	→ none	→ 5 min

[a] One sample as 2-fold determination over the whole system (= 2 runs), plus two standard additions (runs × 2), unpolar and polar extract of each sample (runs × 2) → all in all 8 runs for one sample meaning 16 runs for two samples. The sample preparation is the same for both methods and hence not considered in this calculation.

On the other hand, a comparison of the personnel time showed the advantage of HPLC analysis. As an online method, HPLC requires no additional personnel time. The personnel time involved due to the offline mode of HPTLC is generally overestimated. In HPTLC all steps (sample application, chromatographic separation, and densitometry) are fully automated, and just 5 min of extra time was needed for the plate transfer between the equipment.

In conclusion, planar chromatography can be used in a very simple mode; however, when automated instrumentation is employed for each single step, the method can also be used for reliable quantification. This demonstrated the prior method validation (*18*) and the current method comparison, which showed comparable results although different chromatographic systems were employed (normal phase HPTLC versus reversed phase HPLC). The concentrations of the five HAA obtained by HPTLC were in a similar range as such obtained by the HPLC, and highly satisfying correlations of both methods (R^2 between 0.8875 and 0.9751) were obtained with regard to these findings at the very low micrograms per kilogram level in the challenging meat matrix. Moreover, HPLC and HPTLC findings increased with prolonged cooking times. This correlation between increasing HAA formation and prolonged cooking time was in accordance with the literature. Finally, the cost and time comparison showed that HPTLC is 4 times faster and 3 times less expensive than the HPLC reference method. We conclude that HPTLC can be employed and recommended for lower cost estimation of the risk associated with the uptake with alimentary HAA, which was exemplarily proven for domestic cooking of beef patties.

ABBREVIATIONS USED

HAA, heterocyclic aromatic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; norharman, 9*H*-pyrido[3,4-*b*]indole; harman, 1-methyl-9*H*-pyrido[3,4-*b*]indole; LOD, limit of detection; LOQ, limit of quantification.

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