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Evaluation of a new model system for studying the formation of heterocyclic amines

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Abstract

Heterocyclic amines (HAs) are an important class of food mutagens and carcinogens, which can be found in cooked meat and fish. Increasing heating temperatures and times usually increase mutagenic activity in meat and meat extracts during cooking. We developed a model system, which allows to examine the effects of precursor composition and heating conditions (time and temperature) on the formation of HAs in meat. Homogenized and freeze dried meat samples (beef, pork chops, chicken breast and turkey breast) are heated with diethylene glycol in closed vials under stirring in a thermostated heating block. After an appropriate sample preparation (extraction and clean-up) ten different HAs were measured by HPLC analyses with gradient elution and mass selective detection. The time courses of HA-formation in the different kinds of meat at varying heating temperatures were determined up to heating times of 30 min. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was the most abundant HA in these experiments and reached the highest concentrations in the beef meat samples, as did the other HAs (MeIQ, AαC) at 220 °C in the heating block under stirred conditions. Additionally the influence of the antioxidant TBHQ (*t*-butylhydroquinone) on the formation of HAs in the model system was tested. However TBHQ effected only slight reductions of HA formation in all kinds of meat.

Keywords: Model system; Heterocyclic aromatic amines; 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Food mutagens; Antioxidants

1. Introduction

The high temperature cooking of meats (including grilling and frying) is known to produce heterocyclic amine (HA) compounds that have been shown to be mutagenic and carcinogenic. To date, more than 20 carcinogenic/mutagenic HAs have been isolated and identified in cooked foods [1–3]. One group of these substances is formed by the reaction of creatinine, amino acids and carbohydrates (polar HAs) and another one at higher temperatures through pyrolysis of amino acids (apolar HAs).

Epidemiological evidence appears to imply that consumption of the HAs-containing meat products, in excess, may induce colon and other cancers [2,4,5]. Increasing heating temperatures and times usually increase mutagenic activity in meat and meat extracts. Thus, it is important to study the formation of HAs and develop methods for reduction of these compounds in cooked foods.

The amount and variety of HAs formed in cooked meat products is dependent on many factors, among which processing methods and conditions are the most important [6,7].

To increase our knowledge on the human risk from exposure to HAs, data are being collected on the type and amounts of HAs in common foods and food consumption patterns. It has earlier been shown that, when fried, different sorts of meat form different types and amounts of HAs [7–9]. Thus, there is a need to identify factors that influence the formation of HAs. The most important parameters are cooking conditions, the amounts of the precursors creatine, carbohydrates as well as free amino acids, present in the meat, and the presence of compounds with enhancing or inhibiting effects. Considering the fact that water is evaporating continuously during cooking (and thus the water content in the

Abbreviations: HAs, heterocyclic amines; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline CAS no: 76180-96-6; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline CAS no: 77094-11-2; MeIQx, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline CAS no: 77500-04-0; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline CAS no: 95896-78-9; 4,7,8-TriMeIQx, 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline CAS no: 95896-78-9; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine CAS no: 105650-23-5; DMIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine CAS no: 105650-23-5; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole CAS no: 62450-10-3; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole CAS no: 62450-06-0; AαC, 2-amino-9H-pyrido[2,3-*b*]indole CAS no: 26148-68-5; MeAαC, 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole CAS no: 68806-83-7

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outer part of the meat is decreasing), greater attention should also be paid to the influence of water on the yield of various heterocyclic amines in cooked foods. Model systems are useful tools for studying the influence of different physical and chemical parameters on the formation of HAs [10,11].

The main objective of this study was to examine in which way the formation of HAs can be reduced. In studies involving the frying of meat, many parameters, such as heat and mass transfer, vaporisation of water and crust formation, are difficult to control [12]. For this reason we decided to use homogenized freeze dried fresh meat to have the same chemical composition throughout all experiments carried out. This powder was suspended in diethylene glycol to achieve good heat transfer. Using this experimental set-up meat of different origin (ox, pork, turkey and chicken) was studied.

This model system was also used to study an influence of antioxidants on the formation of HAs by the addition of t-butylhydroquinone (TBHQ), a heat stable antioxidant.

2. Materials and methods

2.1. Materials

All chemicals and solvents were of HPLC or analytical grade. Water was distilled twice and additionally purified with activated carbon (Millipore, Bedford, USA). Acetone, acetonitril, ethyl acetate and methanol were purchased from Promochem (Wesel, Germany). Sodium hydroxide, hydrochloric acid, acetic acid and ammonium hydroxide (25%) were purchased from Merck (Darmstadt, Germany) and diethylene glycol (DEG) and TBHQ (97%) from Sigma–Aldrich (Steinheim, Germany). The following compounds were used as reference compounds: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, DMIP, Trp-P-2, Trp-P-1, AαC, MeAαC, 4,7,8-TriMeIQx, and were purchased from Toronto Research Chemicals (Toronto, Canada). Stock standard solutions of 300 $\mu g\,g^{-1}$ in methanol were prepared and used for further dilution. 4,7,8-TriMeIQx was used as internal standard (10 $\mu g\,g^{-1}$ methanolic solution).

Diatomaceous earth extraction cartridges (Extrelut-20) and refill material were provided by Merck (Darmstadt, Germany) and Oasis MCX cartridges (3 cm^3/60 mg) by Waters (Milford, Massachusetts, USA). MCX cartridges were preconditioned with ethyl acetate (2 ml).

Meat samples (beef, pork chops, chicken breast and turkey breast) were purchased from local stores. One kilogram of each raw meat was homogenised using a BÜCHI homogeniser (BÜCHI mixer B400, BÜCHI Labortechnik AG, Flawil, Switzerland), lyophilised and stored in the freezer at −18 °C until use.

2.2. Model system

The applied model system allowed the heating of different kinds of meat under well controlled conditions. A stirring hot plate (Heidolph, MR 3001 K, from Heidolph Instruments GmbH & Co KG, Schwabach, Germany) with a temperature probe (Heidolph, EKT 3001) was equipped with a heating block made of aluminium (dimensions: 158 mm diameter, 60 mm height) with drilled holes of 10 mm diameter and 30 mm deepness for the vials. The maximum temperature of this experimental set-up is 250 °C. Lyophilised samples of raw meat (0.1 g) were heated with diethylene glycol (1 ml) in 4 ml vials at 180 and 220 °C for 0–30 min using the thermostated heating block with electronically controlled temperature and magnetic stirring (500 rpm). For control of the temperature obtained in the vials diethylene glycol was heated and the temperature measured every 30 s. After heating the vials were immediately cooled on ice to terminate the reaction and the samples were prepared for analysis.

In case of experiments with the antioxidant TBHQ, 0.01, 0.05, and 0.1% were added to the vials with the meat samples before heating.

2.3. Extraction of HAs

For extraction and clean up of all 10 HAs studied a modified method originally developed by Gross and Grüter [13] was used. Samples were dissolved in 12 ml 1 M NaOH and the suspension was homogenised by magnetic stirring for 1 h with 500 rpm (Heidolph, MR 3001 K). The alkaline solution was mixed with diatomaceous earth (13 g), and then transferred to empty Extrelut columns. Ethyl acetate was used as the extraction solvent and the eluate was passed through the coupled Oasis MCX cartridges. The MCX cartridges were washed with 0.1 M HCl (2 ml) and MeOH (2 ml). The analytes were eluted with 2 ml MeOH-concentrated ammonia (19/1, v/v). The samples were evaporated to dryness under a stream of nitrogen and the final extracts were dissolved in 100 mg methanol just before measurement.

2.4. Identification and quantification of HAs

The HPLC analyses were performed on a Hewlett Packard HP 1100 MSD (Waldborn, Germany). The analytical column used was a reversed phase material (Semi Micro ODS-80 TS column, 5 μm, 250 mm × 2 mm i.d.) from Tosoh Bioscience GmbH (Stuttgart, Germany). The separation was performed at a flow rate of 0.3 ml min^{-1} by gradient elution with methanol/acetonitril/water/acetic acid (8/14/76/2, v/v/v/v) at pH 5.0 (adjusted with ammonium hydroxide 25%) as solvent A and acetonitril as solvent B. The gradient program was: 0% B, 0–12 min; 0–30% B, 12–20 min; 30% B, 20–35 min and the injection volume was 10 μl (an injection program 9.5 μl sample and 0.5 μl internal standard 4,7,8-TriMeIQx was used). The employed mass selective detector was equipped with an atmospheric pressure ionisation electrospray (API-ES) using a fragmentation voltage of 80 V for positive ionisation of the analytes. Drying nitrogen was heated to 350 °C and the drying gas flow was 10 l min^{-1}.

The data were acquired in the selected ion mode (*m/z* 163 for DMIP, 184 for AαC, 198 for Trp-P-2 and MeAαC, 199 for IQ, 212 for Trp-P-1, 213 for MeIQ, 214 for MeIQx, 225 for PhIP, 228 for 4,8-DiMeIQx, 242 for TriMeIQx) and calculated in the extract ion mode.

The HAs were quantified using two different calibration curves, 2.5–50 ng g^{-1} of each HA in MeOH and 50–150 ng g^{-1}, respectively, depending on the concentration of the individual HAs. Recovery rates for the different HAs in the model system were determined by the standard addition method. The reaction mixture was spiked with all the analysed compounds at four spiking levels (2.5, 5.0, 7.5, and 10 ng g^{-1} freeze dried meat) by adding different volumes of a methanolic solution of the analytes to the sample after heating the reaction mixture.

3. Results

3.1. HPLC analysis of HAs

The method of sample preparation originally developed by Gross and Grüter [13] used a PRS column for solid phase extraction, which was eluted with two different solvents,

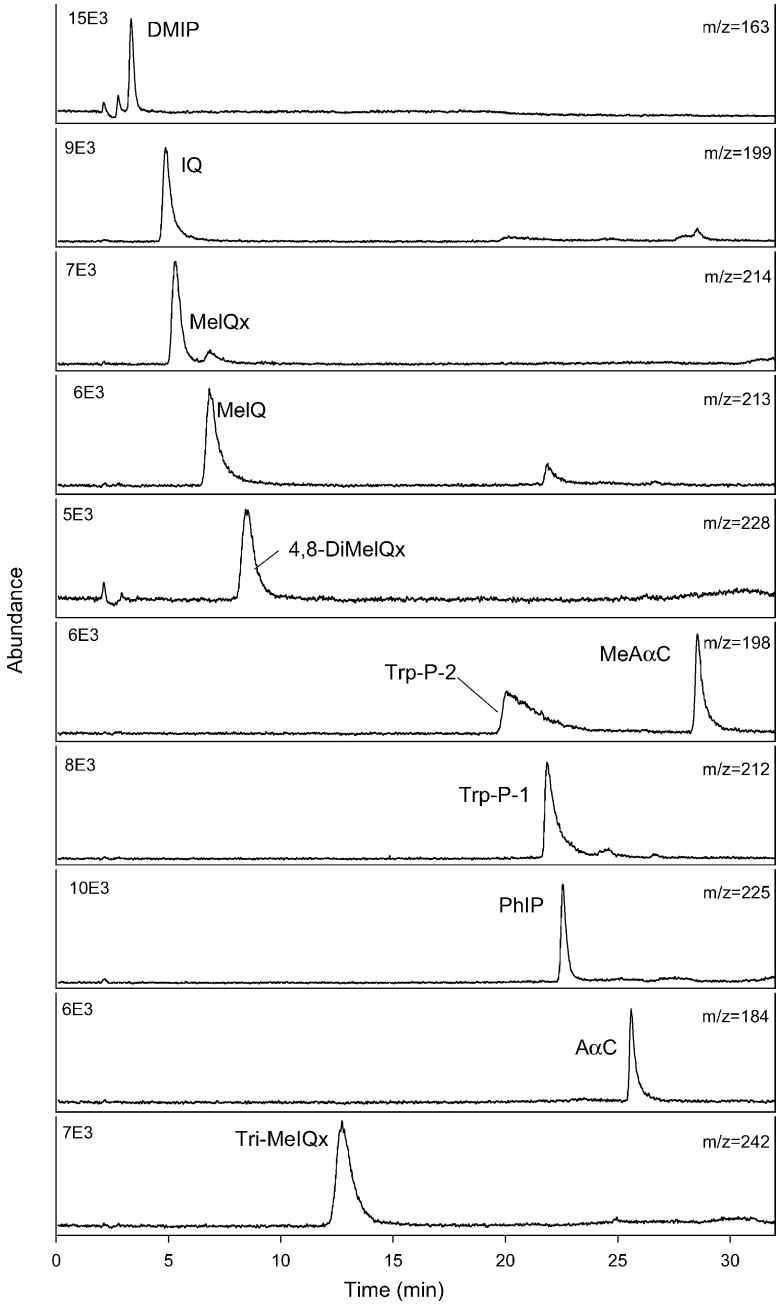

Fig. 1. LC–MS chromatogram of a solution of 10 different HA standards in MeOH (each 200 ng g^{-1}) and TriMeIQx as internal standard.

resulting in two fractions with the polar and the less polar HAs, respectively. Additionally a second clean-up step with a C18 cartridge was necessary. This sample clean-up was simplified, as the Oasis MCX cartridges used allowed the clean up in one step and all the HAs were found in one fraction.

The applied HPLC method provided a good separation of the HAs in question. Fig. 1 shows a LC–MS chromatogram of a solution of 10 different HA standards (each 200 ng g^{-1}) and TriMeIQx as internal standard and Fig. 2 gives a LC–MS chromatogram of a chicken meat sample heated for 2 min in the vial block and spiked with all the standards (200 ng g^{-1} freeze dried meat). Addition of the standards was done directly before the clean up of the samples. The limits of detection (LOD) for standard solutions were calculated with a signal to noise ratio of 3 (S/N = 3) and were in the range of 0.01–0.05 ng HA injected. The limits of quantification (LOQ) for the different HAs were in the range of 0.04–0.18 ng injected amount (S/N = 10).

The recovery of IQ, MeIQx, 4,8-DiMeIQx, PhIP, AαC and MeAαC was between 50 and 60%, while DMIP and Trp-P-1 showed varying recoveries in different meats.

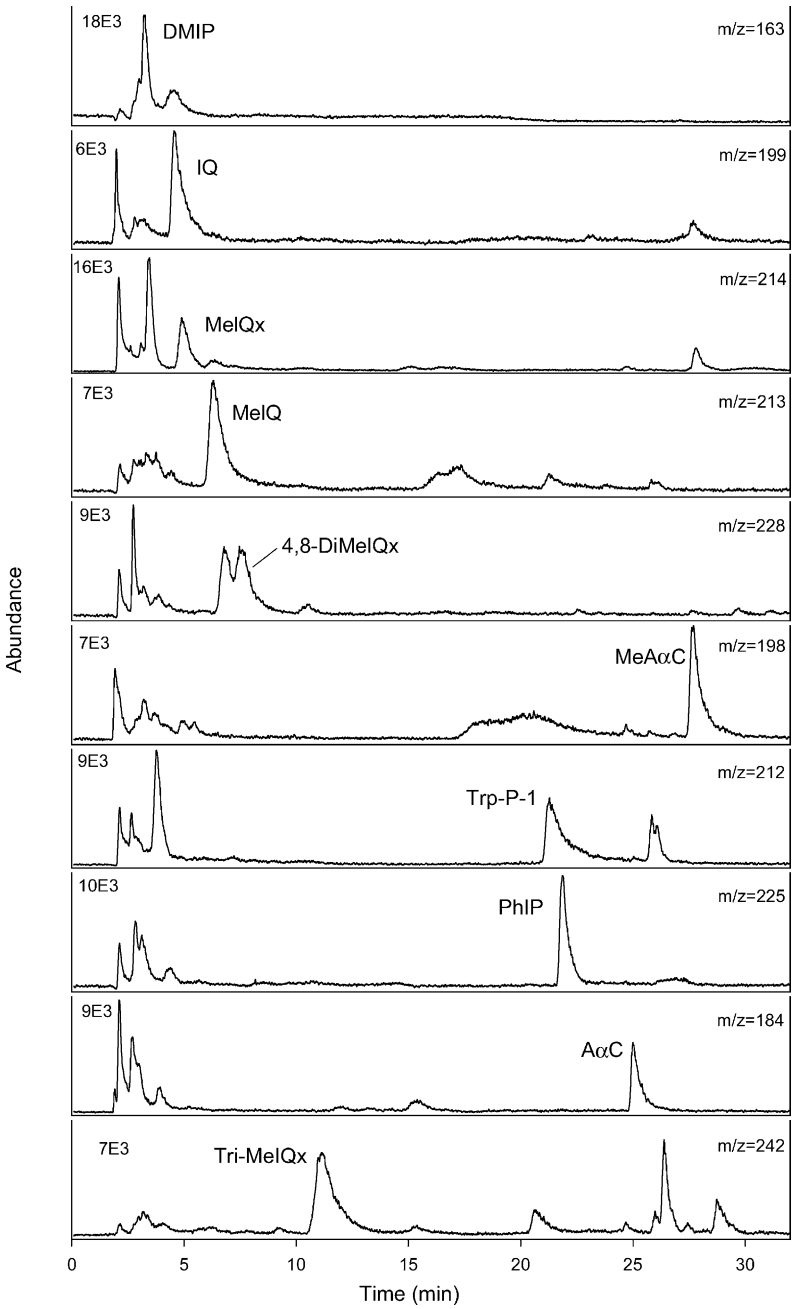

Fig. 2. LC–MS chromatogram of a chicken meat sample heated for 2 min in the vial block, spiked with all the standards (200 ng g^{-1} freeze dried meat) and TriMeIQx as internal standard.

3.2. HAs formation in different kinds of meat in the model system

The loss of weight during lyophilising was around 60% which is comparable with losses during long pan-frying of meat slices. The humidity in freeze dried meat was 8% in beef, 6.0% in chicken, 7.0% in pork and 4.3% in turkey. In comparison, the water content in small slices of chicken breast, pan fried at 200 °C for 25 min was 7.9%. With respect to water content the results can be compared to pan-frying experiments.

The use of the heating block allowed a good control of physical parameters. The initial temperature of the heating block was kept constant during the experiments at 180 or 220 °C. The diethylene glycol dispersions were heated starting from room temperature. They reached the final temperature within 2 min, irrespective of the type or amount of sample added to the glycol.

The amounts of some HAs formed during the heating experiments in the heating block at 180 and 220 °C for 10 and 30 min are listed in Table 1. As can be seen in Fig. 2 (m/z = 198, RT ~ 20 min) Trp-P-2 could not be evaluated due to possible interferences with the matrix. Fig. 3A and B show the time courses of the formation of PhIP in the different kinds of meat under the specified conditions. The

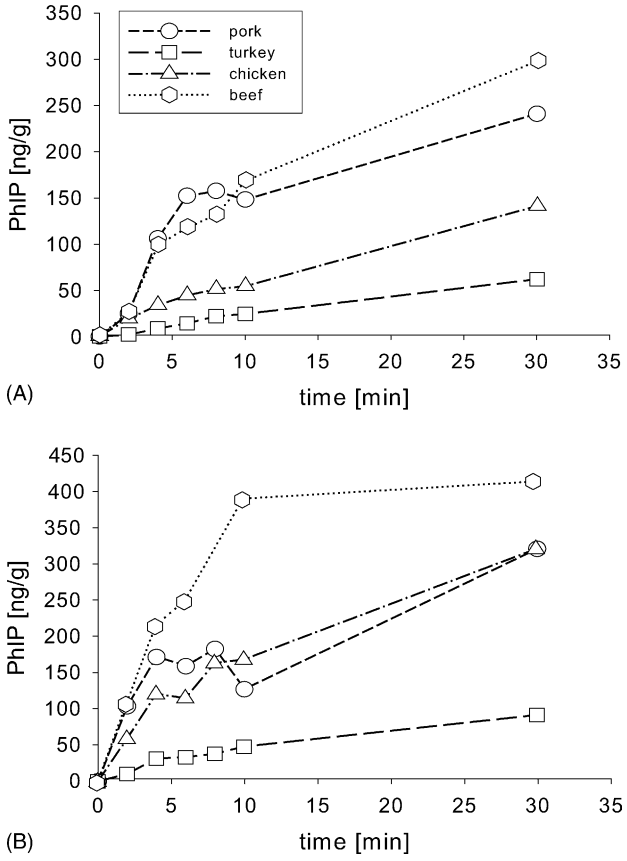

Fig. 3. PhIP concentration in lyophilised meat samples after heating to different temperatures in the model system. (A) 180 °C and (B) 220 °C.

Table 1
Concentrations of HAs (ng g^{-1}) in different kinds of meat after heating in the heating block (a) 180 °C, 10 min; (b) 180 °C, 30 min; (c) 220 °C, 10 min; (d) 220 °C, 30 min

HA	Chicken	Beef	Pork	Turkey
(a)				
DMIP	8.5	32	13	12
IQ	3.0	84	20	–
MeIQx	–	18	11	–
4,8-DiMeIQx	–	3	34	–
Trp-P-1	0.7	14	9	6
PhIP	76	170	150	24
AαC	–	–	1.4	8
MeAαC	1.2	0.3	1.4	11
(b)				
DMIP	9.7	19	10	–
IQ		68	73	–
MeIQx	17	14	3	37
4,8-DiMeIQx	14	95	67	10
Trp-P-1	–	4	11	32
PhIP	140	300	240	49
AαC	–	–	5.5	10
MeAαC	–	–	8.7	–
(c)				
DMIP	12	19	5	6
IQ	23	94	27	14
MeIQx	–	30	3	50
4,8-DiMeIQx	39	5	60	8
Trp-P-1	23	36	87	23
PhIP	170	390	130	40
AαC	58	28	71	6
MeAαC	–	–	63	9
(d)				
DMIP	15	40	58	3
IQ	48	92	57	73
MeIQx	5.2	–	2	79
4,8-DiMeIQx	2.2	28	51	34
Trp-P-1	220	150	190	56
PhIP	320	420	320	92
AαC	31	62	26	16
MeAαC	1.7	6.4	1.7	13

initial concentration of the HAs was not determined prior to lyophilisation. The highest amounts of all HAs were found in the samples heated at 220 °C in the heating block. PhIP was the most abundant HA in all kinds of meat under these conditions. The highest amounts of PhIP were found in beef meat (420 ng g^{-1} at 220 °C) followed by chicken and pork (around 350 ng g^{-1}). In turkey meat only small amounts of PhIP were formed (up to 90 ng g^{-1}). There were also found high concentrations of MeIQ in all kinds of meat, and notably amounts of Trp-P-1, DMIP, 4,8-DiMeIQx and AαC at 220 °C especially at long heating times. Except for AαC and DMIP these findings were not confirmed by LC/MS/MS measurements by the group of M.T. Galceran, where MeIQ did not appear in chromatograms acquired in the MS/MS mode, and Trp-P-1 and 4,8-DiMeIQx were below their limit of detection. The other HAs (IQ, MeIQx, MeAαC) were not found in all samples and were mostly below the limit of quantification. Since the high values of

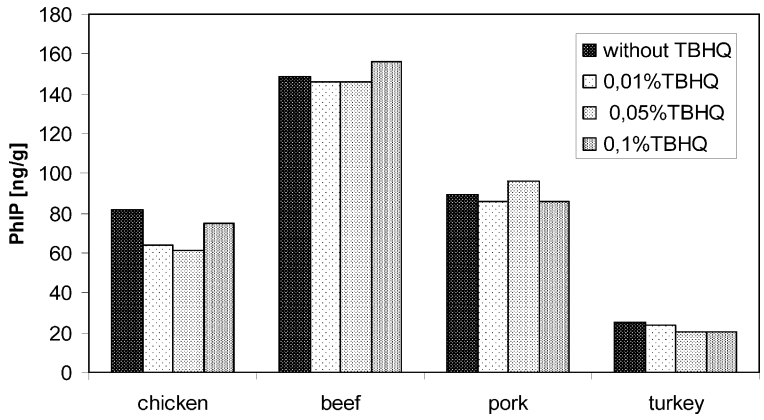

Fig. 4. PhIP concentration in lyophilised meat samples after heating 10 min at 180 °C in the heating block with varying additions of TBHQ.

MeIQ were not verified by MS/MS experiments they are not mentioned in Table 1.

3.3. Experiments with TBHQ

In experiments with the antioxidant, 0.01, 0.05 and 0.1% of TBHQ were added to the lyophilised meat samples before heating. Fig. 4 shows the PhIP concentration in the samples after heating 10 min at 180 °C in the heating block. A slight decrease of PhIP from 82 to around 60 ng g^{-1} was measured in chicken meat with 0.01 and 0.05% of TBHQ, but no influence on the formation of HAs was seen in the other kinds of meat at any TBHQ concentration added. At a heating time of 30 min the PhIP concentrations were generally higher in all kinds of meat, but the longer heating time with TBHQ did not influence the HA concentrations either (data not shown).

4. Discussion

Many previous studies have shown that cooking conditions are of crucial importance to the formation of heterocyclic aromatic amines. For this model system homogenised and lyophilised meat was used to start from a matrix of constant composition for all the experiments carried out. The heating of lyophilised meat allows a good control of physical parameters during the heating and provides an excellent basis for a comparison of the formation of HAs unaffected by temperature profiles that normally occur during heating of meat. This apparatus could also be used for similar experiments with standards of HAs to obtain valuable data on the thermal stability of HAs.

The use of Oasis MCX cartridges for sample purification had some advantages compared with the method originally developed by Gross and Grüter [13]. This method allowed the sample clean up in one step and all the HAs were found in only one fraction.

For the quantification of the HAs two different calibration curves were applied: from 2.5 to 50 ng g^{-1} of each HA in MeOH and from 50 to 150 ng g^{-1}. This was necessary, because the two described ranges showed different slopes and the concentrations of individual HAs increased very much during heating.

Generally, experiments in the heating block showed at least 10-fold higher HA concentrations than found in pan-fried meat samples [14–16]. Compared to the PhIP concentration of 300–350 ng g^{-1} in chicken meat in the model system Persson et al. [14] measured PhIP concentrations of 0.7 ng g^{-1} at 175 °C, 10 ng g^{-1} at 200 °C and up to 30 ng g^{-1} at 225 °C in chicken breasts in pan-frying experiments. This was in agreement with previous studies: not detectable to 10 ng g^{-1} levels of PhIP for chicken breasts fried for 3 min at 150–225 °C [15] and 0.5–20 ng g^{-1} for chicken breasts fried for 15–25 min at 160–220 °C [16]. Sinha et al. [17] reported considerably higher amounts of PhIP, 12–70 ng g^{-1} in chicken fillets fried for 14–36 min at about 200 °C, whereas no PhIP was found in another study [18] on chicken breasts fried for 12 min at 190 °C. Pais et al. [11] examined six different meat samples including a cod fish and found the highest amounts of PhIP in chicken breast (38 ± 15 ng g^{-1}) while the values for the other meats were between 1 and 8 ng g^{-1}. The explanation of the different results could be the different cooking temperatures and times as well as the efficiency of heat transfer. In our experiments the temperature was constant throughout the whole experiment and homogeneously distributed in the sample because of mixing the suspension intensively. By this procedure temperature gradients were eliminated that normally occur when meat is fried in a pan due to the not homogeneous meat surface pan contact and water evaporation leading to local overheating or not reaching so high temperatures at other spots.

Another reason for the higher HA concentrations in the model system was the fact that the model system allowed the measurement of the HA formation in the meat itself and the dripping in one step. Different studies showed that the formation of HAs in the meat drippings is generally comparable to or even higher compared to the meats themselves during frying or oven roasting [7,11,15,19,20]. For

example, Pais et al. [11] analysed also the meat drippings of the six different meat samples including a cod fish and found that the formation of HAs in the meat drippings corresponding to beef, chicken thigh, turkey breast, pork, and fish was generally much higher than in the meats themselves, only the concentration of HAs formed in the chicken breast meat drippings was lower than in the meat itself. The samples in our model system did not have any losses of HAs in drippings, since the experiments were carried out with a closed system.

Concerning other polar HAs the experiments in the heating block showed high concentrations of MeIQ in all kinds of meat especially at 220 °C in stirred samples. These high concentrations of MeIQ were not confirmed by LC/MS/MS measurements by the group of M. T. Galceran, where MeIQ did not appear in chromatograms acquired in the MS/MS mode.

In contrast to experiments with whole meat chops [21] very small amounts of MeIQx and other polar heterocyclic amines were detected. This could be due to the fact that in our model system freeze dried meat was used, which lost around 60% of weight during lyophilising. The loss of weight was comparable with losses during long pan-frying of meat slices. With respect to water content the results can be compared with HA formation in the crust or in dry-heating experiments. Skog et al. [12] reported that the formation of TMIP, IFP and PhIP was favoured by dry conditions, while the formation of MeIQx was favoured by wet conditions, which means that the presence of water clearly affects HA formation. The cooking temperature and rate of drip loss have considerable impact on crust formation during pan-frying. These parameters also greatly affected the amount of PhIP formed, a high temperature and high rate of drip loss are most favourable for the formation of PhIP [14]. Also in meat juice the highest levels of PhIP and IFP were detected in meat juice from chicken breast when dry-heated [22].

In case of high PhIP concentrations at a heating time of 10 min (beef meat, 220 °C) the amount of PhIP increased only slightly during the next 20 min of heating. This fact might be due to the instability of PhIP during heating [10,23]. Chiu and Chen [23] studied the stability of HAs during heating and found that the degradation losses of all the 15 HA standards increased both with increasing temperature and heating time, and the degradation rate of each HA fits a first-order model. PhIP is the least stable during heating, while IQx is the most stable. Arvidsson et al. [10] examined the formation and stability of polar HAs by heating the precursors creatinine, glucose and amino acids at 150 and 225 °C for 0.5–120 min. The stability study showed that PhIP was most susceptible to degradation at 225 °C, followed by 7,8-DiMeIQx, 4,8-DiMeIQx and IQx.

Experiments with the antioxidant TBHQ showed only some slight decreases of PhIP in chicken meat samples after heating 10 min at 180 °C in the heating block. TBHQ is known to resist high temperatures. However, no effects on the formation of HAs in the other meat could be observed in the model system. One reason might be that the TBHQ concentrations were too low to show any effects or the temperature was too high and TBHQ was destroyed. Some other groups reported various effects of antioxidants on HA formation [24–27]. Tai et al. [24] studied the effects of various antioxidants on the HA formation in fried fish fibre and found concentration-dependent effects. The addition of a high level of butylated hydroxytoluene (1.5 g) reduced the HA formation by 30% and also vitamin C created an inhibitory effect at high concentrations. A reverse trend occurred for α-tocopherol, where a low amount reduced HAs formation more effectively than a high amount of α-tocopherol.

Previous works by other authors have shown that a significant reduction of such formation of HAs during cooking could be achieved by marinating chickens, prior to cooking, with various marinades mainly consisting of rapeseed oil, spices, soy sauce, sugar, vinegar, prepared mustard, olive oil butylated hydroxyanisole and sodium benzoate [25,28]. The results, however, have been found to be inconsistent in some cases and the mechanism of reduction in HA formation following marinating the meats prior to cooking is not clearly understood. Therefore, further research along these lines is highly desirable. Murkovic et al. [26] reported that the application of spices (rosemary, thyme and garlic) to the surface of meat resulted in significantly lower amounts of PhIP. This could have happened because of the decreasing temperature of the surface and in the inside of the meat. An other study showed that Monascus red and flavours of thyme, marjoram and rosemary increased PhIP in a model system with phenylalanine and creatinine, independent of their pro- or antioxidative properties [27]. Research should be continued on different ways (e. g. adding antioxidants or other safe additives) of inhibiting the formation of HAs during high temperature cooking in the model system.

5. Conclusion

The influence of temperature on the formation of HAs could be shown by using this model system. The model system is simple and allows to obtain reproducible data on concentration changes induced by heating. Meat samples standardized by homogenisation and lyophilisation are heated in a heating block applying preselected temperature gradients. This method also allows to collect data on the influence of food additives on the formation of HAs during thermal food processing. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was the most abundant HA in these experiments and reached the highest concentrations in the beef meat samples at 220 °C in the heating block under stirred conditions. The antioxidant TBHQ (*t*-butylhydroquinone) led to slight reductions of HA formation in all kinds of meat.

In studies involving the frying of meat, many parameters, such as heat and mass transfer, vaporisation of water and

crust formation, are difficult to control. The model system used here, with well-controlled and reproducible reaction conditions, provides an excellent basis for studies on the effects of water, various precursors, and other enhancing or inhibiting compounds, on the formation of heterocyclic amines.

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