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Influence of temperature on the formation of heterocyclic aromatic amines in pork steaks

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Abstract: The aim of the present study was to evaluate the effects of grilling temperatures on the formation of heterocyclic aromatic amines (HAAs) in steaks from the pork loin (*longissimus lumborum* muscle). Grilling was carried out on a double hot plate grill set to the usual grilling temperatures of 120 °C to 280 °C and stopped when the internal temperature of 72 °C was reached. Among individual HAAs, the most abundant was 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP), as a maximum of 28.62 ng g^{-1} pork steak. in general, the total HAA levels increased with increasing grilling temperature. Higher HAA levels were observed at 260 °C compared to 240 °C, at 13.97 ng g^{-1}, as a 68.7% increase. The highest total HAA levels were found at 280 °C (29.64 ng g^{-1} grilled pork steak), as a 258.0% increase compared to 240 °C. These data indicate that the formation of potentially carcinogenic HAAs during the grilling of pork steaks can be minimised by the using of lower grilling temperatures (≤ 240 °C).

Keywords: carcinogenic compounds; thermal treatment; pork; grilling temperature; solid-phase extraction

Cooking improves the palatability and digestibility of meat, although this process can also produce heterocyclic aromatic amines (HAAs) which have a high mutagenic and carcinogenic potential (Sanz et al. 2008; Rahman et al. 2014).

HAAs are known to be formed in protein-rich foods during conventional heat treatment processes, such as frying and roasting (Busquets et al. 2004). in most cases, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) and 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx) are the most mass-abundant HAAs produced.

The literature includes several reports on HAA levels in poultry, like mainly chicken (Skog & Solyakov 2002), chicken and duck breast (Liao et al. 2010) and pan-fried chicken breast (Skog & Solyakov 2002), and also in fried ground beef patties (Knize et al. 1994; Balogh et al. 2000) and grilled beef (Szterk 2015). on the other hand, not many studies have been focused on pork, such as fried pork chops and pork steaks, where the influence of meat quality and proteolysis stages on HAA formation has been studied (Polak et al. 2009).

Investigating the thermal treatment temperature and type of meat as important factors in HAA formation, the aim of the present study was to study the effects of different grilling temperatures on the main types of HAAs and their levels in pork steaks. We investigated here pork grilled to an internal temperature of 72 °C, with grilling temperatures from 120 °C to 280 °C, which are suitable from a culinary point of view.

MATERIAL AND METHODS

Material. The pork loin (*longissimus lumborum* muscle) was purchased in a local supermarket. Steaks (2.5 cm thick) were prepared from the large muscle cuts purchased, with all approximately of the same weight (100 g) and size (6 cm × 5 cm). These pork steaks were stored in a refrigerator until they reached the internal temperature of 4 °C, and then they were thermally treated at different grilling temperatures. The rest of the fresh meat (off-cuts) was vacuum packed and stored at –20 °C until analysis for creatine and creatinine.

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The 13 HAA standards used were all obtained from Toronto Research Chemicals (Canada): PhIP, MeIQx, 2-amino-3,4,8-trimethyl-3H-imidazo(4,5-f)quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethyl-3H-imidazo-(4,5-f)quinoxaline(7,8-DiMelQx),dipyrido(1,2-a:3›,2›-d) imidazol-2-amine (Glu-P-2), 1-methyl-9H-pyrido(3,4-b) indole (Harman), 9H-pyrido(3,4-b)indole (Norharman), 2-amino-9H-pyrido(2,3-b)indole (AαC), 3-amino-1,4-dimethyl-5-H-pyrido(4,3-b)indole (Trp-P-1), 3-amino-1-methyl-5-H-pyrido(4,3-b)indole (Trp-P-2), 2-amino-3-methylimidazo(4,5-f)quinoline (IQ), 2-Amino-3,4-dimethyl-3H-imidazo(4,5-f)quinoline (MeIQ), 2-amino-3-methylimidazo(4,5-*f*)quinoxaline (IQx) and 2-amino-3,4,7,8-tetraimethylimidazo(4,5-*f*)quinoxaline (4,7,8-TriMeIQx) (used as an internal standard).

Experimental design. The steaks from the refrigerator (temperature of 4 °C at the geometric centre) were thermally treated on a double hot plate grill (Silex, Germany) to the final internal temperature of 72 °C, when the grilling was stopped. Nine different grilling temperatures were used: 120, 140, 160, 180, 200, 220, 240, 260, 280 °C. The internal temperature of steaks and the contact temperature of grill plates were continuously monitored with a temperature data logger (Testo 177-T4; Testo, Germany), equipped with a thermocouple Type K temperature probe with a flexible measuring tip. The final times of the grilling to the internal temperature of 72 °C ranged from 4.08 min (at 120 °C) to 3.02 min (at 280 °C). After heat treatment the samples were vacuum packed and stored at a temperature of 4 °C until further analysis.

The data are from nine different temperatures in six repetitions, each carried out in two parallel measurements, to give a total of 108 samples.

Methods. The creatine/creatinine levels in the raw pork meat were determined as described by Polak et al. (2009). The HAA levels were determined according to Polak et al. (2009).

The high performance liquid chromatography (HPLC) analysis (1100 system; Agilent, USA) was performed using an analytical Kinetex C18 column (2.6 × 2.1 × 100 mm; Cat. No. 00F-4439-B0; Phenomenex, USA) under reverse phase at 30 °C. The separation was performed at a flow rate of 0.35 mL min^{-1} by gradient elution with 30 mM ammonium formate (Cat. No. 09739; Fluka), pH 3.2, as solvent A, and acetonitrile (Cat. No. 1.00030; Merck, Germany) as solvent B. A mass selective detector (Micromass Quattro micro API; Waters, USA) equipped with electrospray ionisation using a cone voltage of 40 V and a capillary voltage of 3.5 kV was used for positive ionisation of the analytes (electrospray ionisation+ mode). Dry nitrogen was heated to 350 °C and the drying gas flow was 350 L h^{-1}. The cone gas (nitrogen) flow was 50 L h^{-1}.

Statistical analysis. The experimental data were evaluated statistically using the SAS/STAT programme (SAS Software, 1990). The basic statistical parameters were calculated using the "means" procedure and then tested using the "univariate" procedure (SAS Software, 1990). The relationships between the data were analysed by the least-squares method with the general linear model procedure.

The data on the temperature effects of the grilling plates used for grilling, like the levels of HAA precursors, some individual HAAs, and the total HAAs, along with the texture parameters, were analysed using a statistical model for the influence of temperature (i = 9, as 120, 140, 160, 180, 200, 220, 240, 260, 280 °C), and the repeatability (j = 6), and their interactions. The following Model (1) was used:

$$y_{ijk} = \mu + T_i + R_j + T \times R_{ij} + e_{ijk} \quad (1)$$

where:
y – the observed parameter;
μ – the general mean;
T – effect of temperature (i = 9);
R – repeatability (j= 6);
k – parallel (1,2);
e – residual random term with variance σ_e^2

The means for the experimental groups were obtained, and Duncan's tests were used for comparisons at the 5% probability level.

RESULTS AND DISCUSSION

Total and individual heterocyclic aromatic amine levels. The analysis of the pooled data for the total and individual HAAs in the grilled pork steaks across the full range of grilling temperatures provided the basic statistical parameters presented in Table 1. Thirteen different HAAs were identified here, and as can be seen in Table 1, seven were quantified: PhIP, MeIQx, 4,8-DiMeIQx, 7,8-DiMelQx, Glu-P-2, Harman, and Norharman. Among these, Harman and Norharman are not reported to be directly mutagenic, but they are called co-mutagens (Szterk 2015). Polak et al. (2009) evaluated five of these HAAs in pork, i.e. PhIP, MeIQx, DiMeIQx, Harman, and Norharman, with the highest levels found for PhIP and MeIQx. Aaslyng et al. (2013) investigated different barbecued meat samples, when they identified and evaluated only four HAAs, namely

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Table 1. Total and individual HAA levels in grilled pork steaks (combining all grilling temperatures; n = 108)

HAA	HAA (ng g^{-1} grilled pork steak)			SD	*CV* (%)
	mean	min.	max.		
PhIP	3.30	0	28.62	5.84	176.70
MeIQx	1.10	0.06	4.87	0.82	74.34
4,8-DiMeIQx	0.57	0	6.64	0.53	92.90
7,8-DiMelQx	1.91	0.03	5.02	1.24	64.76
Glu-P-2	2.06	0	9.98	2.36	114.41
Harman	1.74	0	9.14	1.83	104.79
Norharman	1.52	0	23.53	2.78	183.03
Total HAAs	12.20	2.33	59.15	9.59	78.57

SD – standard deviation of three independent experiments; *CV* – coefficient of variation; 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-trimethyl-3H-imidazo(4,5-f)quinoxaline; 7,8-DiMelQx – 2-amino-3,7,8-trimethyl-3H-imidazo(4,5-f)quinoxaline; Glu-P-2 – dipyrido(1,2-a:3',2'-d)imidazol-2-amine; Harman – 1-methyl-9H-pyrido(3,4-b)indole; Norharman – 9H-pyrido(3,4-b) indole; HAA(s) – heterocyclic aromatic amine(s)

PhIP, 4,8-DiMeIQx, Harman, and Norharman. in their pork meat samples, the highest levels of these individual HAAs were revealed for Norharman.

In the present study, the total HAA levels in these grilled pork steaks varied from 2.33 ng g^{-1} to 59.15 ng g^{-1}. These differences in the HAA levels and compositions appear to be related to the different temperatures set for grilling. Besides temperature, the formation of HAAs will depend on the presence of their precursors, which include creatinine and amino acids (Pfau et al. 2006). The precursor levels will vary both between and within the animal species. to confirm the uniformity of these pork steaks used in the present study, creatine levels were also determined. This analysis showed the creatine levels from 9.47 mmol kg^{-1} to 10.09 mmol kg^{-1} pork meat, with no significant differences between repetitions, demonstrating the uniformity of this HAA precursor across the pork steak samples used here.

In general, for these individual HAA levels across the full range of grilling temperatures, the highest mean level was found for PhIP (3.30 ng g^{-1}), and the lowest for 4,8-DiMeIQx (0.57 ng g^{-1}) (Table 1). PhIP and MeIQx are frequently detected in commonly consumed meats, including fish and poultry, with their levels typically ranging from 0.1 ng to hundreds of nanogrammes per gramme of cooked meat (Salmon et al. 2006). The PhIP levels in grilled beef can be as high as 38.0 ng g^{-1}, with similar levels reported during frying processes (Oz & Kaya 2011). Compared to Gibis & Weiss (2015), who studied the formation of different HAAs in grilled pork patties, only the PhIP levels are comparable with the present study, while the levels of MeIQx, 4,8-DiMeIQx, Harman, and Norharman differ. Such variations will be due to different sample preparations (steaks) and different grilling temperatures. Very high HAA levels have been reported in pan residues from frying minute beef (PhIP up to 82.4 ng g^{-1}, MeIQx up to 23.3 ng g^{-1}) (Jagerstad & Skog, 2005). According to Zhang et al. (2013), the levels of three HAAs (PhIP, MeIQx, and 4,8-DiMeIQx) in three different forms of pork (patties, balls, strips) fried at 180 °C for 5 min varied from 0.28 ng g^{-1} to 18.4 ng g^{-1}, among which only 4,8-DiMeIQx levels were comparable with the present study. Zhang et al. (2013) showed that the patties (compared to the balls and strips) had the highest HAA levels, and they suggested that these HAAs are generated at the surface of the pork, and that the patties/steaks have a larger contact surface, which results in more HAAs being formed. This might also be explained by higher temperatures on the surface compared with those on the inside of the fried/grilled patties, as HAAs are mainly generated at high temperatures. However, it is difficult to compare these data here directly with other studies, because the HAA levels can vary widely due to sample preparation and method of thermal treatment. Also, to the best of our knowledge, the literature does not include any studies that have investigated the influence of such a wide temperature range (from 120 °C to 280 °C here) on HAA formation in pork steaks.

Heterocyclic aromatic amine levels according to grilling temperature. The data on the total and individual HAA levels according to the different grilling temperatures are shown in Table 2. These pork steaks were

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Table 2. Total and individual HAA levels in grilled pork steaks according to grilling temperature (mean ± SD, n = 12)

Grilling temperature (°C)	Individual HAA (ng g^{-1} grilled pork steak)							Total HAAs (ng g^{-1} grilled pork steak)
	PhIP	MeIQx	4,8-DiMeIQx	7,8-DiMeIQx	Glu-P-2	Harman	Norharman	
120	0.15 ± 0.13^{e}	0.58 ± 0.31^{de}	0.45 ± 0.33^{de}	2.29 ± 1.22^{ab}	0.54 ± 0.28^{d}	2.10 ± 2.10^{a}	0.82 ± 0.90^{b}	6.93 ± 2.76^{d}
140	0.14 ± 0.14^{e}	1.67 ± 1.07^{a}	0.74 ± 0.53^{bc}	2.60 ± 0.77^{a}	2.54 ± 2.36^{ab}	0.88 ± 0.80^{a}	0.25 ± 0.44^{b}	8.81 ± 2.92^{cd}
160	0.21 ± 0.20^{e}	1.15 ± 0.25^{bc}	0.52 ± 0.42^{cde}	2.09 ± 1.23^{bc}	1.74 ± 2.17^{bc}	1.64 ± 2.26^{a}	1.06 ± 2.14^{b}	8.41 ± 5.16^{d}
180	0.55 ± 0.48^{de}	1.29 ± 0.31^{ab}	0.56 ± 0.59^{bcd}	1.33 ± 1.02^{e}	2.45 ± 2.27^{ab}	1.05 ± 0.65^{a}	1.01 ± 1.21^{b}	8.24 ± 4.11^{d}
200	2.12 ± 0.83^{c}	1.43 ± 0.37^{ab}	1.10 ± 0.79^{a}	1.95 ± 1.65^{bc}	2.81 ± 3.30^{ab}	1.80 ± 1.78^{a}	0.82 ± 1.01^{b}	12.03 ± 6.68^{bc}
220	1.59 ± 0.39^{cd}	1.18 ± 0.26^{bc}	0.77 ± 0.53^{b}	1.86 ± 1.33^{bcd}	2.14 ± 1.83^{abc}	1.72 ± 1.37^{a}	1.30 ± 1.02^{b}	10.56 ± 4.94^{cd}
240	2.04 ± 0.42^{c}	0.28 ± 0.21^{e}	0.30 ± 0.36^{e}	1.46 ± 1.07^{de}	1.32 ± 1.21^{cd}	1.57 ± 1.47^{a}	1.31 ± 0.95^{b}	8.28 ± 3.62^{d}
260	3.39 ± 1.21^{b}	1.62 ± 1.45^{ab}	0.47 ± 0.42^{de}	1.70 ± 0.99^{cde}	3.15 ± 3.06^{a}	1.91 ± 2.68^{a}	1.73 ± 0.76^{b}	13.97 ± 5.12^{b}
280	17.17 ± 5.82^{a}	0.81 ± 0.90^{cd}	0.29 ± 0.29^{e}	1.82 ± 1.43^{bcd}	2.11 ± 2.79^{abc}	2.66 ± 2.01^{a}	4.79 ± 6.41^{a}	29.64 ± 14.54^{a}
SD	0.97	0.78	0.87	0.91	0.87	0.65	0.79	0.91
p_T	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	0.115	≤ 0.001	≤ 0.001
p_R	0.035	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	0.001	≤ 0.001	≤ 0.001
$p_{T×R}$	≤ 0.0001	0.003	≤ 0.001	≤ 0.001	≤ 0.001	0.096	≤ 0.001	≤ 0.001

SD – standard deviation of three independent experiments; for other abbreviations see Table 1; p_T – influence of grilling temperature; p_R – influence of repeatability; $p_{T×R}$ – influence of the interaction of grilling temperature and repeatability; data with different superscript letters within columns (^{a,b,c,d}) are significantly different ($P < 0.05$; significance of differences between grilling temperatures); not statistically significant: $P > 0.05$; statistically significant: $P < 0.05$; highly statistically significant: $P < 0.001$

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grilled to an internal temperature of 72 °C. All of the data here were calculated according to the mass of each steak (expressed as ng g^{-1} grilled pork steak), which was nominally 100 g. Comparing these data with the unadjusted data (ng/grilled pork steak; data not shown), it can be concluded that the variation in the mass of the pork steaks did not significantly affect the HAA levels, although the corrected values show less scatter.

It should be noted that nine grilling temperatures were used compared to the study of Polak et al. (2009), where the HAA levels in grilled pork steaks were determined only at 220 °C. Oz et al. (2016) investigated the effects of three different temperatures of a hot plate (150 , 200 , 250 °C) on HAA formation in beef chops. Their samples were cooked for 9 min with turning each minute. in the present study, a double hot plate grill was used, and the internal temperatures of the steaks were measured, and consequently the times of grilling varied.

The data obtained in the present study showed that at grilling temperatures between 120 °C and 240 °C the total HAAs formed were from 6.93 ng g^{-1} and 8.28 ng g^{-1} grilled pork steak, respectively. Arvidsson et al. (1997) used a model system to show that the formation of HAAs is functionally dependent on temperature and time of thermal treatment, whereby at 200 °C the HAA levels rapidly increased. in contrast, in the present study, the HAA levels rapidly increased at 260 °C, when 13.97 ng g^{-1} of total HAAs were formed, i.e. by 68.7% more compared to 240 °C. There was then a further increase in the total HAA levels at 280 °C, when 29.64 ng g^{-1} pork steak were formed (increase by 257.97% compared to 240 °C) (Table 2).

At this point, it is important to highlight the relationship between grilling temperature and duration of HAA formation. From a health as pect, to achieve the desired internal temperature of a steak, it is preferable to use lower temperature and longer time of thermal treatment. The results confirmed our working hypothesis that the most HAAs were formed at the highest grilling temperatures. Skog et al. (1997) reported that PhIP was the most abundant HAA and was identified in 73 of their 84 meat product samples. The highest PhIP levels (32.0 ng g^{-1}) were observed for the pan residue from pork fillets after cooking at 225 °C. For the pork fillets themselves at 225 °C, the highest HAA levels were found for PhIP, MeIQx, and DiMeIQx, at a total of 21.3 ng g^{-1}. at 225 °C and 250 °C, HAAs have been reported to be degraded or to react with other compounds (Jackson & Hargraves 1995; Arvidsson et al. 1997). Degradation of HAAs can also occur simultaneously to their formation when frying at temperatures from 130 °C to 225 °C, depending on the heat treatment time (Arvidsson et al. 1997; Randel et al. 2007). The stability against degradation is the highest for beta-carbolines and the lowest for PhIP (Chiu et al. 1998). The data obtained in the present study confirm the hypothesis above because there were no statistical differences in PhIP levels across the temperature range from 120 °C to 180 °C.

Temperature effects on PhIP formation in grilled pork steaks. Figure 1 shows the effects of grilling temperature on PhIP levels in these grilled pork steaks. For the low measured PhIP levels up to 240 °C, there were no significant differences until their large increases that followed to 280 °C. Polak et al. (2009) showed the PhIP levels in grilled pork steaks (1.84 ng g^{-1}) were lower than those of MeIQx (2.45 ng g^{-1}), although Skog et al. (1997) reported that PhIP was not detectable until the temperature of 225 °C (13.4 ng g^{-1}).

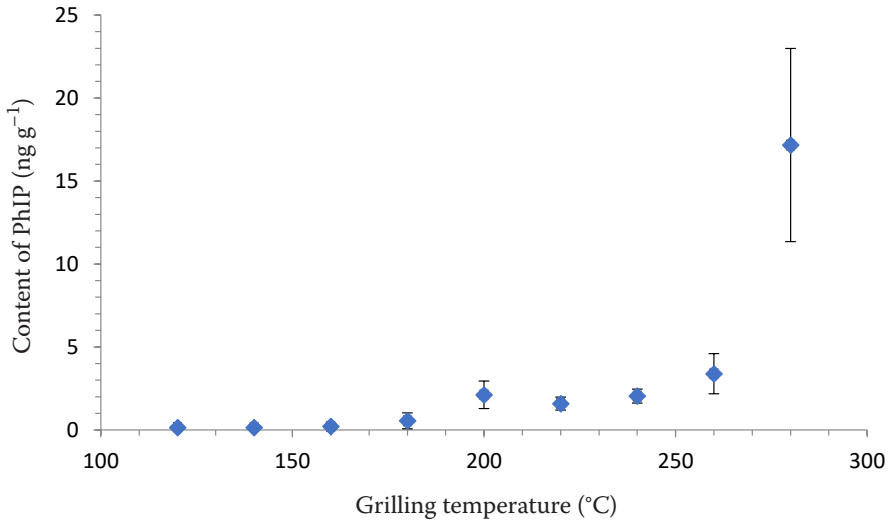

Figure 1. Influence of grilling temperature on the formation of PhIP in grilled pork steaks

PhIP – 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine

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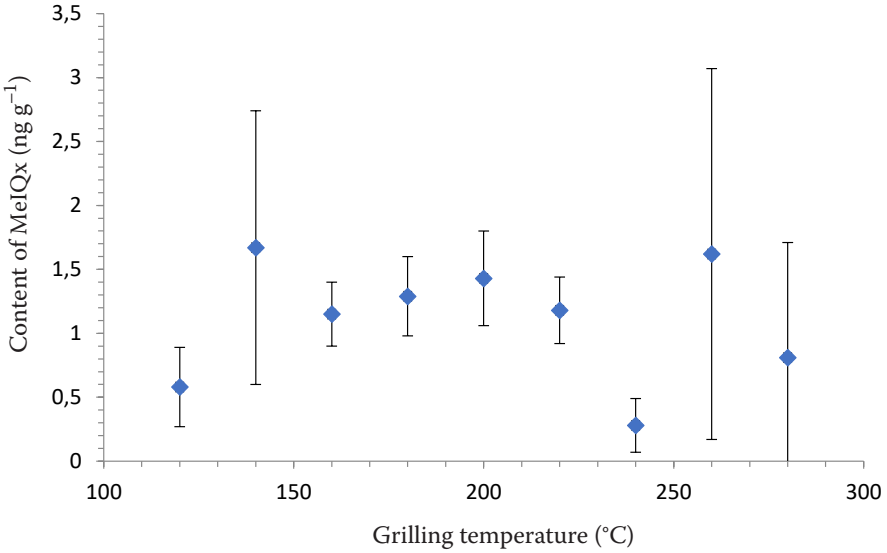

Figure 2. Influence of grilling temperature on the formation of MeIQx in grilled pork steaks

MeIQx – 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline

Temperature effects on MeIQx formation in grilled pork steaks. Figure 2 shows the effects of grilling temperature on MeIQx levels in these grilled pork steaks. The highest concentration of MeIQx was reached at 140 °C (1.67 ng g^{-1}). Between 160 °C and 220 °C, the MeIQx levels were lower, with the lowest levels seen at 240 °C (0.28 ng g^{-1}). This can be explained by degradation of MeIQx at these higher temperatures. The MeIQx levels increased at 260 °C again, which was probably the result of their formation in the deeper layers of the grilled pork steaks. According to Skog et al. (1997), MeIQx levels increase proportionally with increasing temperature, especially between 175 °C and 225 °C. If MeIQx levels from our study are compared with those of PhIP, it can be seen that up to 180 °C, MeIQx levels were higher, but at the higher temperatures this relationship was inverted.

During grilling at higher temperatures, the water content in the steaks will decrease, and consequently there will be drier conditions for MeIQx formation. at the highest MeIQx levels, the standard deviations were greater.

CONCLUSION

In the present study we quantified the levels of seven HAAs in grilled pork steaks according to their dependence on the different grilling temperatures (120–280 °C). in general, the data indicate that the total HAA levels increased with increasing temperature of grilling, and especially at the highest temperatures of 260 °C and 280 °C. Considering that PhIP and MeIQx appear most often in foods, we defined their course of formation according to the different grilling temperatures. at lower temperatures there were higher levels of MeIQx compared to PhIP, while with increasing temperature this ratio was inverted. Considering these data overall, to reduce the exposure to HAAs, it is better to grill pork steaks at lower temperatures for longer times, because at > 240 °C there were large and significant increases in HAA formation.

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