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Heterocyclic Aromatic Amine Formation in Barbecued Sardines (*Sardina pilchardus*) and Atlantic Salmon (*Salmo salar*)

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The formation of heterocyclic aromatic amines (HAs) during barbecuing of sardines (*Sardina pilchardus*) and Atlantic salmon (*Salmo salar*) to various degrees of doneness and grilling conditions was evaluated by HPLC−diode array (DAD)/fluorescence (FLD) detection. Additionally, the influences of charcoal and electric heat sources on formation of HAs in grilled salmon were compared. With regard to sardine samples barbecued at 280−300 °C, "rare" samples produced nondetectable amounts of HAs, "medium" sardines presented IQ, MeIQx, PhIP, and AαC at levels of 1.9, 4.4, 3.3, and 2.0 ng/g, respectively, and "well done" sardines presented IQ, MeIQx, Trp-P-1, Trp-P-2, PhIP, AαC, and MeAαC at levels of 0.9, 2.2, 1.8, 8.2, 6.5, 17.7, and 10.6 ng/g, respectively. Different qualitative and quantitative profiles of HAs were observed in sardine and salmon samples cooked under similar conditions of temperature and doneness. Levels of 13.3, 3.5, 1.13, and 3.18 ng/g were obtained, respectively, for PhIP, AαC, MeAαC, and Glu-P-1 in salmon samples barbecued at 280−300 °C. The contents of HAs were significantly higher in these samples than in salmon samples barbecued at 180−200 °C or in the electric device. However, MeIQx content (0.5 ng/g) was lower in salmon samples barbecued at 280−300 °C than in the other samples.

KEYWORDS: Heterocyclic aromatic amines; sardines; salmon; HPLC; charcoal grilling

INTRODUCTION

The consumption of fish provides utilization of proteins of high biological value, certain minerals, and vitamins. Additionally, fish and fish oil are rich sources of omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (*1*). Over the past 20 years, there has been a dramatic increase in the scientific scrutiny of and public interest in fish consumption, omega-3 intake, and impact on personal health (*1*−*9*). Scientific data indicate that the consumption of fish or fish oil containing omega-3 polyunsaturated fatty acids (PUFAs) reduces the risk of coronary heart disease, lowers the incidence of diabetes, and plays a vital role in the development and function of the nervous system (brain) (*8*), photoreception (vision), and the reproductive system (*9*). Sardine (*Sardina pilchardus*) and salmon (*Salmo salar*) are fish species widely consumed and rich in omega-3 PUFAs, providing an adequate amount of these compounds (2.7−7.5 g per meal) (*1, 2, 10*). On the other hand, fish is usually cooked in different ways before consumption, and heat treatment can lead to undesirable modifications, such as the loss of nutritional value and formation of undesirable mutagenic and/or carcinogenic compounds such as heterocyclic aromatic amines (HAs), depending on the cooking method and fish preparation (*11*).

Generally speaking, the types of cooking that involve temperatures of around 100 °C (boiling in water and steaming with or without previous browning) lead to a production of mutagenic agents that is too low to be quantifiable (*12*). However, grilling and barbecuing, the most common methods for preparation of fatty fishes, usually require high temperatures, and HAs are sometimes formed. Several studies show that charcoal-cooked meat presents higher amounts of these compounds (*13*). Fish sample studies are scarce but indicate a similar trend (*14, 15*).

To date, about 20 carcinogenic/mutagenic HAs have been isolated and identified in cooked foods (*16, 17*). The achieved temperature has an important influence on the kind of HAs formed; the temperature that is needed for the formation of significant amounts of "thermic HAs", or IQ type, is between 150 and 250 °C (**Figure 1a**). At higher temperatures, above 300 °C, the "pyrolytic HAs", or non-IQ type, are formed preferably (**Figure 1b**) (*18, 19*). Factors reported to affect the

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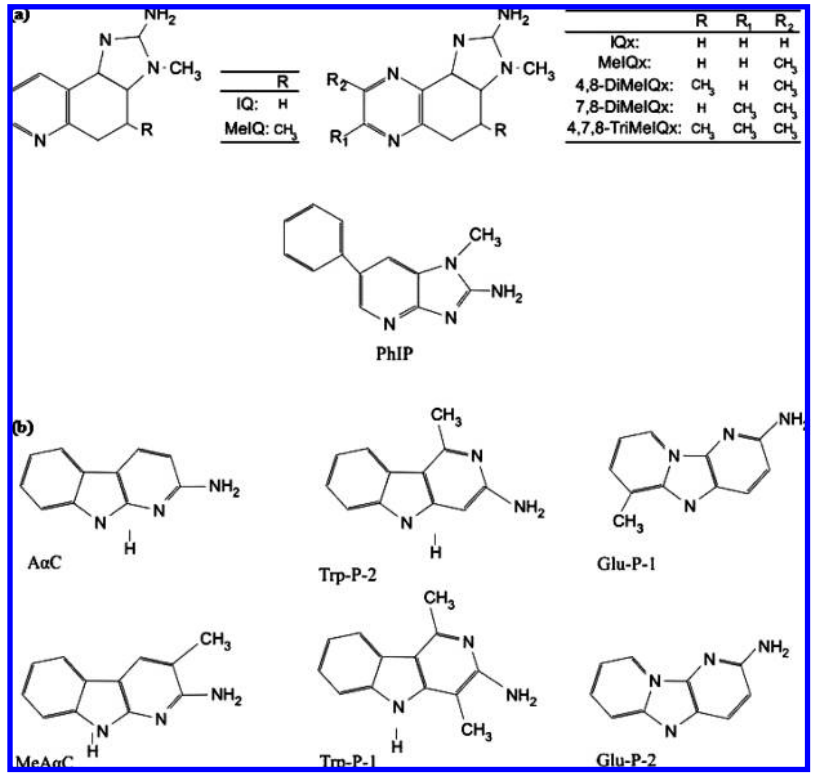

Figure 1. (**a**) Structures of thermic HAs, IQ and IQx types; (**b**) structures of pyrolytic HAs.

formation of HAs in foods include physical factors such as food type, food amount, cooking duration, cooking temperature, cooking equipment and method, pH, water activity, free amino acids, and creatine. In addition, heat and mass transfer, lipid, lipid oxidation, and antioxidants have effects on the concentration of HAs (*15*, *20*, *21*).

As HAs are candidates in the etiology of human cancer, the search for ways to minimize their intake by limiting their occurrence in cooked foods is very important. In the present study, we focused on conditions favoring the formation of thermic and pyrolytic HAs during barbecuing of sardines (*S. pilchardus*) and Atlantic salmon (*S. salar*) to various degrees of doneness and grilling conditions. Additionally, the influences of charcoal and electric heat source on the formation of HAs in grilled salmon were compared. Thus, the main objective of this study was to examine in which way the formation of HAs can be reduced in barbecued fatty fish and which HAs can be better indicators of drastic conditions used. This information are needed to make health hazard assessments. It is hoped that with subsequent consumer education about precautions that are needed during fish barbecuing, exposure of humans to these carcinogens can be reduced, thus making such cooked foods safer for human consumption.

MATERIALS AND METHODS

Materials. Water was distilled and additionally purified with activated carbon. All solutions were passed through a 0.45 μm filter (Milex, Bedford, MA). The methanol, acetonitrile, and dichloromethane were of HPLC grade and were provided by Merck (Darmstadt, Germany). The chemicals used for sample treatment [sodium hydroxide, hydrochloric acid, ammonium acetate, ammonia solution 25% (v/v)] and for mobile phase triethylamine were of analytical grade and were also purchased from Merck. All of the solutions were measured using a combined pH glass electrode connected to a pH-meter (MicropH 2001, Crison, Barcelona, Spain) and passed through a membrane nylon 0.22 μm from Magna before injection into the HPLC system. Heterocyclic amine standards, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (TriMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 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-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), were purchased from Toronto Research Chemicals (North York, ON, Canada). Stock standard solutions of 100 μg/mL in methanol were prepared and used for further dilution.

For the solid-phase extraction, Extrelut reservoirs and Extrelut HM-N diatomaceous earth refill material were obtained from Merck. Bond Elut PRS (500 mg) and end-capped Bond Elut C_{18} (100 and 500 mg) cartridges were from Varian (Harbor City, CA). A Supelco Visiprep and a Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for manipulations with solid-phase extraction cartridges and solvent evaporation, respectively.

A Vortex Mixer VV3 (VWR International, West Chester, PA) and ultrasonic cleaner (FungiLab SA, Barcelona, Spain) were used to homogenize grilled fish.

Preparation of Fish Samples and Grilling Conditions. Fresh sardine samples (15 in total), 18 cm in length and approximately 84.9 $\pm$ 3.1 g in weight, were obtained at the summer station from the coast of Portugal, in the region of Ovar. Samples of fresh salmon were obtained in a fish market of the district of Porto. Initial weights of salmon samples were 986 $\pm$ 34.7 g. Mature salmon has a long body appropriate to be grilled as fillets. Thus, fillets, 3 cm in length, 12 cm in width, and 18 cm in height (six in total), were prepared. Weights of salmon fillets were 272 $\pm$ 9.7 g.

For the charcoal barbecued fish, a bed of charcoal was prepared and ignited using an appropriate device of 35 cm width, 52 cm length, and 15 cm height. When all flames had subsided, the bed was leveled by raking. The fish were then barbecued over charcoal; different distances from charcoal were assayed. The fish were turned once during grilling at half the total cooking time. No salt or oil was applied to fish before or after grilling. Temperatures were measured by using a digital

thermocouple (part 0560 9260, Testo 926, Lenzkirch, Germany) with a surface probe (0603 1992, Testo 926, Lenzkirch, Germany). All experiments were repeated twice.

Sardines are a small fish size with a flat body, covered with large, reflective, silvery scales; thus, barbecued sardines present high scale surface area/volume ratio. Sardine samples used in this study were weighed before and after the cooking process and barbecued in different controlled conditions. The whole sardine was barbecued. The head, bones, and scales of the cooked sardines were removed; only the muscle was taken for analysis.

Sardine samples were broiled 12−15 cm from the charcoal source. Maximum temperature for grilling reached 280−300 °C. Three sardines were used in each assay, except for blank sardines, in which case only two sardines were barbecued. Duplicate assays were performed:

"Blank" sardines, used in the recovery assays, were barbecued for 3 min on each side with the aim of being used as blank samples, without HAs. Sardine weights before and after cooking were 83.3 ± 1.1 and 74.7 ± 2.3 g, respectively.

Sardines were barbecued for 5 min on each side, to a "rare" condition; the surface presented a silvery aspect, but they were appropriate for intake. These samples were designated sardines A. Sardine weights before and after cooking were 85.5 ± 2.3 and 63.2 ± 2.0 g, respectively.

Sardines were barbecued for 6 min on each side, to a "medium" condition; the surface changed to a golden color. These samples were designated sardines B. Sardine weights before and after cooking were 86.1 ± 3.4 and 58.2 ± 2.1 g, respectively.

Sardines were barbecued for 7 min on each side, classified as "well done"; the surface was dark. These samples were designated sardines C. Sardine weights before and after cooking were 85.3 ± 1.2 and 53.4 ± 2.3 g, respectively.

Other sardine samples, designated sardines D, were broiled 25 cm from the charcoal source for 10 min on each side to a "medium" condition, presenting a golden color on the surface, similar to sardines B. Using this conditions the maximum temperature for grilling was 180−200 °C.

Salmon fillets were submitted to three types of grilling conditions:

Salmon A samples were grilled over charcoal, close to the heat source (12−15 cm). The temperature next to the charcoal was 280−300 °C. To obtain "medium" doneness, corresponding to a homogeneous golden color on the surface, the grid time was 6 min for each side. Salmon fillet weights before and after cooking were 275 ± 24.0 and 174 ± 8.1 g, respectively.

Salmon B samples were grilled over charcoal at a distance of approximately 25 cm. The temperature next to the grid was close to 180−200 °C. To present a similar aspect to the samples grilled next to the charcoal, the grid time was 20 min for each side. Salmon fillet weights before and after cooking were 281 ± 15.5 and 173 ± 22.4 g, respectively.

Salmon C samples were griddled on an electric griddle. The temperature next to the griddle (immediately above the heat source) was 180−200 °C. The electric griddle was preheated, and the fish fillets were griddled without fat or oil. To present a similar aspect to salmon A and B, the griddle time was 11 min per side. Salmon fillet weights before and after cooking were 262 ± 19.8 and 166 ± 11.3 g, respectively.

The external crust and the inner part of the cooked fillets were weighed and separately crushed, and samples were homogenized using a kitchen blender (Moulinex, France) to produce a uniform sample. In the end, the homogenized samples were properly identified and frozen at −20 °C until analyzed for heterocyclic aromatic amines. The fishbones and skin were rejected.

Determination of HAs. Extraction and purification of HAs were performed using the method developed by Gross (*22*) and modified by Galceran et al. (*23*), because this procedure is the reference method in interlaboratory exercises (*24*).

According to the method, a 5 g sample of grilled fish was homogenized in 20 mL of 1 M NaOH with sonication (10 min), and the suspension was then shaken for 1 h using a vortex mixer. The alkaline solution was mixed with Extrelut refill material (16 g) and was used to fill an empty Extrelut column. After being preconditioned with 7 mL of dichloromethane, an Isolute PRS column was coupled online to the Extrelut column. To extract the analytes from diatomaceous earth, 75 mL of dichloromethane was passed through the tandem. The washing solutions arising from the PRS cartridge, which consisted of 6 mL of 0.01 M HCl, 15 mL of MeOH, 0.1 M HCl (6:4, v/v), and 2 mL of water, were collected for the analysis of the PhIP and less polar compounds (AαC, MeAαC, Trp-P-1, Trp-P-2). After their organic solvent content had been lowered by adding 25 mL of water, the acidic washing solutions were neutralized with 500 μL of ammonia solution. The resulting solution was passed through a C18 cartridge (500 mg), previously conditioned with 5 mL of MeOH and 5 mL of water, and less polar HAs were concentrated. Finally, the C18 cartridge was rinsed with 5 mL of water and the sorbed HAs were eluted using 1.4 mL of methanol/ammonia solution (9:1, v/v). On the other hand, a 100 mg Bond Elut C18 cartridge was conditioned with 5 mL of MeOH and 5 mL of water and was then coupled online with the PRS cartridge. After that, the most polar amines (Glu-P-1, Glu-P-2, IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP) were eluted from the cationic exchanger with 20 mL of 0.5 M ammonium acetate at pH 8.5. Finally, the C18 cartridge containing the most polar analytes was rinsed with 5 mL of water, and the sorbed HAs were eluted using 0.8 mL of methanol/ammonia solution (9:1, v/v). The extracts containing either the most or least polar analytes were gently evaporated under a stream of nitrogen, and the analytes were redissolved in 80 μL of methanol.

Identification and Quantification of HAs. Separation and quantification of HAs were performed by liquid chromatography with diode array and fluorescence detection (HPLC-DAD/FLD). Diode array detection was set at 263 nm and the fluorescence detector at excitation 307 nm and emission 370 nm. Quantification of PhIP, MeAαC, and AαC was based on fluorescence peak area. The chromatographic analysis was carried out in an analytical HPLC unit (Jasco, Japan) equipped with one Jasco PU-1580 HPLC pump, a MD 910 multiwavelength detector, and a type 7125 Rheodyne injector with a 20 μL loop. The column was a TSK gel ODS80 (Toyo Soda) (5 μm; 250 mm length; 4.6 mm internal diameter). Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used. The mobile phase was as follows: solvent A, 0.01 M triethylamine adjusted with phosphoric acid to pH 3.2; solvent B, same as A but adjusted to pH 3.6; solvent C, acetonitrile. The linear gradient program was as follows: 0−10 min, 5−15% C in A; 10−10.1 min, exchange of A with B; 10.1−20 min, 15−25% C in B; 20−30 min, 25−55% C in B; 30−55 min, column rinse and re-equilibration. Separations were carried out at ambient temperature.

Peak identification in food samples was carried out by comparing retention times and spectra of unknown peaks with reference standards, as well as cochromatography with added standards and peak purity.

The detection limits (LOD) were calculated as the concentration corresponding to 3 times the background noise of the blank. A standard addition method was used for the quantification of HAs using two fortified levels (around 5−20 ng/g) and two nonspiked samples. Addition of the standards was done directly before the cleanup of the samples.

Statistical Analysis. In the present study, a completely randomized design was employed (two replicates), and results were analyzed using SPSS for Windows, v. 16 (SPSS, Chicago IL). Comparison of mean values was made using the Duncan test.

RESULTS AND DISCUSSION

HPLC-DAD/FLD for Quantification of HAs in Fish Samples. The presence of 14 heterocyclic aromatic amines that have commonly been studied and reported in the literature was investigated. HPLC with UV and fluorescence detection proved to be a convenient method of analyzing HAs in fish samples. Identification of HAs was feasible using their typical UV spectra even at low nanogram per gram levels.

Detection limits (LODs), based on a signal-to-noise ratio of 3:1, were determined in fish extracts by fortifying blank samples at very low levels. Quantification limits (LOQs) were established

Table 1. Extraction Efficiency of HAs in Fish Samples[a]

	added amount (ng/g)	quantified amount (ng/g)	recovery (% ± SD)	fish extract LOD (ng/g)	fish extract LOQ (ng/g)
		Thermic HAs			
Glu-P-2	10.0	6.02	60 ± 0.04	1.00	3.3
Glu-P-1	10.0	6.04	60 ± 0.03	1.00	3.3
IQ	5.0	3.10	62 ± 0.02	0.50	1.7
IQx	5.0	3.42	68 ± 0.06	0.80	2.6
MeIQ	5.0	2.55	51 ± 0.04	0.26	0.86
MeIQx	5.0	3.25	65 ± 0.05	0.80	2.6
7,8-DiMeiQx	5.0	3.30	66 ± 0.04	0.20	0.60
4,8-DiMeIQx	5.0	3.35	67 ± 0.06	0.20	0.60
TriMeIQx	5.0	3.15	63 ± 0.06	0.50	1.7
		Pyrolytic HAs			
Trp-P-2	10.0	2.30	23 ± 0.01	0.80	2.6
Trp-P-1	5.0	1.55	23 ± 0.02	0.20	0.8
PhIP	10.0	2.30	31 ± 0.06	0.5	1.5
AαC	10.0	2.50	25 ± 0.01	0.20	0.8
MeAαC	10.0	3.20	32 ± 0.01	0.20	0.8

[a] Two "blank" sardines, barbecued for 3 min on each side, were used in the recovery assays, LODs, and LOQs. PhIP, MeAαC, Trp-P-1, and AαC were quantified by fluorescence detection. The other HAs were quantified by diode array detection.

Table 2. Formation of HAs in Barbecued Sardines (Expressed in Nanograms per Gram)[a]

HA	sardines A	sardines B	sardines C	sardines D
IQ	nd a	1.9 ± 0.6 b	0.9 ± 0.3 b	nd a
MeIQx	nd a	4.4 ± 1.2 b	2.2 ± 0.9 c	nd a
Trp-P-2	nd a	nd a	8.2 ± 1.1 b	nd a
Trp-P-1	nd a	nd a	1.8 ± 0.5 b	nd a
PhIP	nd a	3.3 ± 1.0 b	6.5 ± 1.3 b	nd a
AαC	nd a	2.0 ± 0.9 b	17.7 ± 2.3 c	nd a
MeAαC	nd a	nd a	10.6 ± 1.4 b	nd a

[a] Sardine samples A−C were broiled 12−15 cm from the charcoal source. Maximum temperature for grilling reached 280−300 °C: sardines A, barbecued for 5 min on each side, to a "rare" condition, the surface presented a silvery aspect; sardines B, barbecued for 6 min on each side, to a "medium" condition, the surface changed to a golden color; sardines C, barbecued for 7 min on each side, classified as "well done", the surface was dark. Sardines D were broiled 25 cm from the charcoal source (maximum temperature for grilling was 180−200 °C) for 10 min on each side to a "medium" condition, presenting a golden color on the surface, similar to sardines B. Letters a−c indicate significant differences at $p < 0.05$ in the Duncan test. Three sardines were used in each assay. Results obtained by fluorescence for PhIP, Trp-P-1, MeAαC, and AαC and diodes for the remaining HAs. Only HAs detected in sardine samples are given. [b] nd, not detectable.

as the amount of analyte that produces a signal-to-noise of 10:1 (see **Table 1**). A detection limit of about 1 ng/g in purified extracts was obtained, and for fluorescent HAs this limit was slightly lower. Limits of detection in the sample were consistent with those reported by other authors for fish samples (*15*).

Blank samples (without HAs) were also used in recovery assays. The results of average recoveries, LODs, and LOQs are presented in **Table 1**. Messner and Murkovic (*25*) found similar recoveries of IQ, MeIQx, 4,8-DiMeIQx, PhIP, AαC, and MeAαC. The low recovery values obtained indicate that the standard addition method is the most appropriate for quantification of HAs.

Formation of HAs during Barbecuing of Sardines. Data from the quantitative HPLC analysis of HAs, expressed as nanograms per gram of barbecued sardines, are presented in **Table 2**. Concerning sardine samples broiled 12−15 cm from the charcoal source at 280−300 °C, "rare" samples (sardines A) produced nondetectable amounts of HAs; "medium" and "well done" sardines (sardines B and C, respectively) presented different qualitative and quantitative profiles of HAs. Thermic amines are the most abundant in sardines B, whereas pyrolytic amines are most abundant in sardines C. The amines IQx, MeIQ, 4.8-DiMeIQx, 7.8-DiMeIQx, 4,7,8-TriMeIQx, Glu-P-1, and Glu-P-2 were not detected in "medium" (sardines B) and "well done" sardines (sardines C). Additionally, MeAαC was not detected in sardines B. The cooking time of sardines was decisive in the formation of HAs.

With respect to thermic HAs, usually formed at lower temperatures by Maillard reactions that involve creatin(in)e and reduced sugars, only two HAs (MeIQx and IQ) were quantified in sardines B and C, but "medium" sardines presented higher levels of these HAs. Thermic HAs are the most studied amines, because they are the HAs most often found in processed foods. In 1981, Kasai et al. (*26*) isolated IQ (described by the same authors as a potent mutagen) from an extract of sardines grilled in domestic conditions. Later, Yamaizumi et al. (*27*), quantified IQ and MeIQ in grilled sardines by LC-MS using deuterated standards. These authors report levels of 4.9 and 16.6 ng/ g, respectively. The presence of MeIQx was not mentioned by these authors, although this amine is often measured in other species of fish (**Table 3**) and was quantified in sardines B and C. **Table 3** summarizes studies from the literature concerning the content of thermic HAs in sardines and other types of fish. As shown, different qualitative and quantitative profiles regarding the composition of HAs in fish samples are described. However, it is worth noting that it is difficult to compare results obtained by various researchers because they depend heavily on sample purification process as well as detection method used in HPLC.

The higher contents of IQ and MeIQx in sardines B when compared with sardines C were only statistically significant for MeIQx and can be the result of degradation of thermic HAs with the increasing heating time. Chiu and Chen (*28*) studied the stability of HAs during heating and found that the degradation losses of HA standards increased with both increasing temperature and heating time, and the degradation rate of each HA fits a first-order model. Arvidsson et al. (*29*) examined the formation and stability of thermic 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 HAs were susceptible to significant degradation at 225 °C.

PhIP results from the condensation of alcoholic phenylacetaldehyde (which is formed by thermal degradation of phenylalanine). Sardines C presented significantly higher levels of this HA than sardines B. Other authors found PhIP in fried cod and fried herring (**Table 3**).

With regard to pyrolytic amines, the γ-carbolines—Trp-P-2 and Trp-P-1—and the α-carbolines—AαC and MeAαC—were found in sardines C, but only AαC was found in sardines B. As already mentioned, these amines are usually formed above 300 °C, through pyrolysis of amino acids such as tryptophan, but some authors detected these HAs in processed foods using domestic conditions. For example, Trp-P-1 and Trp-P-2 were found in foods cooked at temperatures close to 225 °C (between 0.5 and 7.4 ng/g) and in meat sauce obtained at temperatures below 200 °C (*30*). In 1980, these HAs were quantified in grilled sardines by Yamaizumi et al. (*27*), and contents close to 13 ng/g were described. These levels are higher than those found in sardines B (1.8 and 8.2 ng/g, respectively, for Trp-P-1 and Trp-P-2).

The levels of AαC and MeAαC in sardines C were 17.7 and 10.6 ng/g, respectively. These HAs are usually described as less abundant in grilled and fried meat or fish and have not received significant attention from the scientific community. However,

Table 3. Levels of Thermic HAs Found in Some Processed Fish

type of fish	food processing	detection method	HA (concn)	source
sardine	grilled	LC-MS, deuterated standards	IQ (4.9 ng/g) MeIQ (16.6 ng/g)	Yamaizumi et al., 1986 (*27*)
eel	oven cook	HPLC-UV	MeIQx (1.1 ng/g) 7,8-DiMeIQx (5.3 ng/g)	Lee and Tsai, 1991 (*36*)
cod	fried	HPLC-UV	IQ (0.16 ng/g) MeIQ (0.03 ng/g) MeIQx (6.44 ng/g) 4,8-DiMeIQx (0.10 ng/g) PhIP (69.2 ng/g)	Wakabayashi et al., 1993 (*37*)
herring	fried	HPLC-UV	MeIQx (0.2 ng/g) PhIP (0.07−0.3 ng/g)	Skog et al., 1997 (*30*)
brown trout	grilled	HPLC-UV/DAD	IQ (0.12 ng/g) 4,8-DiMeIQx (0.02 ng/g)	Oz et al., 2007 (*15*)
rainbow trout			4,8-DiMeIQx (0.02 ng/g)	

Table 4. Levels of HAs in Grilled Salmon

	salmon A		salmon B		salmon C	
HA	ng/g of salmon crust	ng/g of cooked salmon fillet	ng/g of salmon crust	ng/g of cooked salmon fillet	ng/g of salmon crust	ng/g of cooked salmon fillet
Glu-P-1	6.6 ± 4.6	3.18 ± 2.25 a	2.5 ± 1.5	1.0 ± 0.8 ab	nd[b]	nd b
MeIQx	1.0 ± 0.7	0.5 ± 0.35 a	3.3 ± 1.2	1.3 ± 0.8 b	1.7 ± 0.091	0.86 ± 0.09 b
PhIP	28.9 ± 10	13.0 ± 3.3 a	10.6 ± 4.9	4.3 ± 2.0 b	5.0 ± 0.59	2.6 ± 0.42 b
AαC	8.9 ± 4.8	3.5 ± 2.4 a	0.93 ± 0.52	0.37 ± 0.22 b	3.9 ± 2.2	1.95 ± 0.1 b
MeAαC	2.7 ± 2.0	1.13 ± 0.8 a	nd	nd b	nd	nd b

[a] All salmon samples presented "medium" doneness, corresponding to a golden color on the surface: salmon A, grilled for 6 min on each side over charcoal, 12−15 cm from heat source at a temperature of 280−300 °C; salmon B, grilled for 20 min on each side over charcoal, 25 cm from heat source at a temperature of 180−200 °C; salmon C, grilled for 11 min on each side on an electrical griddle at a temperature of 180−200 °C. The external crust and the inner part of the cooked fillets were weighed and separately analyzed. Cooked salmon fillet includes external crust and the inner part. Letters a and b following entries indicate significant differences at $p < 0.05$ in the Duncan test. Two fillets were analyzed in duplicate in each assay. Results obtained by fluorescence for PhIP, Trp-P-1, MeAαC, and AαC and diodes for the remaining HAs. Only HAs detected in salmon samples are given. [b] nd, not detectable.

Knize et al. (*13*), when studying the formation of HAs during the cooking of proteinaceous foods, noted the presence of a high content of AαC in charcoal-grilled hamburguers, whereas in other forms of grilled hamburgers this HA was not detected. With respect to the content of pyrolytic HAs in barbecued fish, studies are scarce (*14*).

Concerning sardines B and D, barbecued to a similar "medium" doneness but using different grilling conditions, respectively at 12−15 cm from the heat source (280−300 °C) and at 25 cm from the heat source (180−200 °C), significant differences were observed in the HA content. No HAs were detected in sardines D; however, sardines B presented IQ, MeIQx, PhIP, and AαC at levels of 1.8, 4.4, 3.3, and 2.0 ng/g, respectively. Thus, the barbecuing conditions influenced significantly the formation of HAs. For a similar degree of doneness the distance from the heat source and consequently the cooking temperature influenced significantly HAs content.

Influence of Grilling Conditions on HAs Contents of Salmon Fillets. Data from HAs on grilled salmon are summarized in **Table 4**. No HAs were detected in the inner part of grilled fillets; thus, only results from crust and cooked salmon fillet are presented. A similar qualitative profile of HAs was observed in the crust of all salmon samples, and consequently in the whole cooked fillets, but quantitative differences were observed according to the cooking conditions used. The sum of top and lower crust represented around 50% of the whole fillet; thus, HAs occur in double amounts in the crust when compared with whole salmon fillet. PhIP, AαC, and MeIQx were detected in salmon A−C, but different levels of these HAs were observed. Additionally, salmon A samples, grilled close to the charcoal, presented Glu-P-1 and MeAαC; however, these amines were low or negligible in the salmon grilled far from the heat source (salmon B) and using the electric griddle (salmon C). The amines Trp-P-1, Trp-P-2, 4,8-DiMeIQx, 7,8-DiMeIQx, 4,7,8-TriMeIQx, IQx, IQ, and MeIQ were not found in grilled salmon samples.

According to the results summarized in **Table 4**, obtained for a similar degree of doness (medium condition), the levels of PhIP, AαC, MeAαC, and Glu-P-1 formed were significantly higher in salmon A samples barbecued near the heat source (at 280−300 °C), but no significant differences were found between levels of HAs in salmon B and C. MeIQx content was lower in salmon A than in salmon B and C.

Charcoal burning is, by itself, responsible for the formation of HAs and can increase the level of these compounds in grilled food in this way. In 1977, Nagao et al. (*31*) analyzed the particles trapped in a filter glass through which had passed the smoke released during the grilling of sardines. The Ames test with *Salmonella typhimurium* indicated that the mutagenic potential of these particles was much higher than the expected mutagenic potential for the polycyclic aromatic hydrocarbons (PAHs) and other compounds found in tobacco smoke. This does assume that the smoke from charcoal combustion can contain HAs and increases the possibility of assessing these substances in grilled foods, especially, if they are grilled near the heat source.

HAs can be emitted into the atmosphere through the burning of a wide variety of materials such as vegetation, wood, oil, and other organic substances. Kataoka et al. (*32*) determined HAs in smoke resulting from the combustion of different samples, including cigarettes, splinters of wood, and rubber. Additionally, the natural fish juices that are released during the

grilling and fall from the fish fillet into the charcoal can suffer pyrolysis and lead to the formation of HAs, which, being dragged by the smoke, are deposited on the surface of the food (*13*).

The most abundant HA was PhIP, reaching 13.0 ng/g of salmon grilled near the charcoal (salmon A). A significant decrease of this content was observed in salmon B (4.3 ng/g) and salmon C (2.6 ng/g). Skog et al. (*33*) reported that the formation of PhIP was favored by dry conditions, whereas the formation of MeIQx was favored by wet conditions. The crust formed in the salmon fillet surface, where the heat source is in direct contact with the muscle without protection from the scales or skin, can justify the dry conditions that favor the formation of PhIP.

The carbolines MeAαC and AαC were quantified in samples grilled near the charcoal (salmon A) (1.13 and 3.5 ng/g, respectively). Levels of AαC were 0.37 and 1.95 ng/g of cooked salmon fillet, respectively, in salmon B and C. MeAαC was not detected in these samples. The levels of Glu-P-1, another pyrolytic amine, were 3.18 ng/g of salmon grilled near the charcoal (salmon A) and 1 ng/g of salmon grilled at 25 cm from the grid (salmon B). This HA was not detected in salmon C.

Salmon A presented significantly lower levels of MeIQx (0.5 ng/g of cooked salmon fillet) when compared with salmon B (1.3 ng/g) and C (0.86 ng/g). The higher content of MeIQx in salmon B and C when compared with salmon A can be the result of degradation of thermic HAs with the increasing temperature or lower formation in the dry conditions of the crust (*33*). These results are in good agreement with those of Gross and Gruter (*14*). These authors present the levels of PhIP, AαC, and MeIQx in salmon, cooked in different ways. The barbecued salmon samples were submitted to a temperature of 270 °C during 4, 6, 9, and 12 min. The levels of PhIP ranged between 2 and 73 ng/g (for 4 and 12 min barbecuing times, respectively), the levels of AαC ranged between 2.8 and 109 ng/g, and the levels of MeIQx were always below 1 ng/g. Salmon samples grilled in a pan (200 °C) presented lower levels of PhIP and AαC when compared with the levels achieved in the barbecued salmon with similar grilling times: PhIP, 1.7−14 ng/g; AαC, nd−8 ng/g. In contrast, levels of MeIQx were higher in salmon grilled using a pan than in barbecued salmon, ranging, in the former case, between 1.4 and 3.1 ng/g (levels for 3 and 12 min of grilling, respectively). These results point out the influence of time, temperature, and grilling method in the formation of HAs. Moreover, the three HAs (PhIP, AαC, and MeIQx) detected by Gross and Gruter (*14*) in salmon samples were the same that were quantified in all of the salmon samples of the present study. However, in general, these authors reported higher levels of HAs, which must be related to the thickness of the salmon samples, a parameter that has not been defined by the authors.

In conclusion, qualitative differences of salmon and sardine HAs were observed in samples cooked under similar conditions of temperature and doneness. Krone, Yeh, and Iwaoka (*34*) have shown that the major mutagens in fried and canned salmon are different from those in fried ground beef. Pais et al. (*21*) explained that differences in HA patterns result from significant differences in the amino acid composition of different types of meat. Fish muscle studies are scarce, and in most cases they were carried out only on thermic amines. However, the results from this work indicate that such differences also exist between sardine and salmon muscles; for example, IQ was not detected in salmon samples and Glu-P-1 was not detected in sardine samples.

The results obtained in barbecued sardines and salmon indicated that the pyrolytic HA AαC can be a good indicator of excessive temperature or time during cooking of these two fishes. Recent studies on the carcinogenicity of pyrolytic HAs emphasize the need for more information about their presence in food (*35*), especially in the case of barbecued food, where high temperatures are easily reached. α-Carbolines present relatively low mutagenic potential; however, their potential carcinogenicity can vary from moderate to high and can be responsible for the formation of a large amount of DNA adducts in rat liver cells (*35*). Moreover, the same studies have shown that the process of detoxification and excretion of α-carbolines has little expression; consequently, a large amount of amines is activated in vivo (*35*). Nevertheless, more studies are needed related to metabolism, bioactivation, and intake of pyrolytic amines, in particular, through the development of human biomarkers for these amines.

Precautions must be taken into account when barbecuing fish such as keeping the muscle away from the charcoal heat or using electrical griddle equipment to reduce the formation of HAs. The fish scales and "skin" can also act as a protective layer in preventing the formation of HAs.

ABBREVIATIONS USED

HAs, heterocyclic aromatic amines; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; IQx, 2-amino-3- methylimidazo[4,5-*f*]-quinoxaline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; 7,8-DiMeIQx, 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline; TriMeIQx, 2-amino-3,4,7,8-tetramethylimidazo[4,5]-quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazol[4,5]-pyridine; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole; MeAαC, 2-amino-3-methyl[2,3-*b*]-indole; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole; HPLC-DAD/FLD, high-performance liquid chromatography diode array and fluorescence detection; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PAHs, polycyclic aromatic hydrocarbons; PUFAs, polyunsaturated fatty acids.

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