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Heterocyclic Aromatic Amines in Muscle Foods -

Relevance, Occurrence and Mitigation

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Dissertation Thesis

Heterocyclic Aromatic Amines in Muscle Foods - Relevance, Occurrence and Mitigation

Dissertation presented to Faculty of Nutrition and Food Science to obtain the degree of Doctor of Philosophy (PhD) in Food Consumption and Nutrition Sciences

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Abstract

Heterocyclic aromatic amines (HAs) are considered a dietary risk factor for human cancer. Their capability of formation on muscle foods during ordinary cooking practices (grilling, broiling, barbecuing, roasting, frying, pan-frying), even at low parts-per-billion (ppb), implies frequent exposure by the general public. Over the past 30 years, numerous studies have been stimulated aiming to alleviate human health risk associated with HAs. The three main areas are still a challenge: their occurrence in foods, the strategies to inhibit their formation, and the search for chemopreventive agents. Furthermore, several researchers highlighted an urgent need of studying HAs and other concomitant mutagens at the same time, as polycyclic aromatic hydrocarbons (PAHs).

The occurrence of HAs and PAHs in different cooked muscle foods (beef, salmon, and sardines) and different cooking procedure (barbecuing, grilling and pan-frying) on Portuguese household cooking procedures were evaluated. The samples were analyzed for HAs contents using a reference method, which consists in solid-phase extraction (SPE) and high-performance liquid chromatography-diode array detection/ fluorescence detection (HPLC-DAD/FLD). To PAHs a similar methodology was ascertained, which also consists in SPE and separation and detection by HPLC-FLD.

Concerning the HAs formation in analyzed samples, in general the most abundant HA was PhIP (1.45-33.8 ng/g), followed by AαC (1-19 ng/g) and MeIQx (not detected-9.07ng/g). The HAs, IQ, 4,8-DiMeIQx, MeAαC, Trp-P-1, Trp-P-2 and Glu-P-1 were also formed in some muscle foods and cooking procedures with relative significance. In contrast IQx, MeIQ, 7.8-DiMeIQx, 4,7,8-TriMeIQx, and Glu-P-2 were not detected at all. Thermic HAs were frequently studied, whereas pyrolytic HAs were usually neglected. However, the contribution of AαC is crucial in HAs exposition. In relation to PAHs occurrence in grilled muscle foods, quantitative PAHs profiles were different among salmon> chicken> beef. PAH8 content is significantly correlated with fat content of these muscle foods.

The cooking conditions affect the formation of HAs and PAHs in muscle foods. Efficient strategies on the reduction of HAs in fatty fish were grilling at longer distance from charcoal or substitute the wood charcoal by coconut shell charcoal or use an electrical grill. The continuous barbecuing with the same charcoal contributed to higher formation of HAs and PAHs.

The most promising mitigation strategies on HAs are the addition of antioxidant compounds. The inhibitory effect of antioxidant rich marinades was evaluated in pan-fried beefs. Beer as marinade applied 4 hours prior to cook the beefs, inhibited around 80% of the total HAs formation. Considering the inhibitory effect of beer as the standard, the relative efficiencies of other liquid marinades on the total HAs formation were: 1 to beer, 0.9 to dealcoholized white wine, 0.7 to red

wine, 0.7 to white wine and 0.6 to green tea marinade. A mixture of spices (*herbs*) commonly used as meat flavoring (garlic, ginger, thyme, rosemary and red chili pepper) was also evaluated in combination with the liquid marinades (beverages). Relative efficiencies were: 1.1 to beer with herbs, 0.95 to dealcoholized white wine with herbs, 0.6 to white wine with herbs. Herbs explained around 30% of inhibition of PhIP formation, in contrast alcohol seems to perform a strong influence on PhIP formation. No correlation was observed between antiradical activity of marinades and total or individual HAs formation.

Xanthohumol (XN) is a hop compound contained in beer, described as a "broad-spectrum" chemopreventive. In the present study XN completely prevented the HAs (PhIP and MeIQx) induced DNA strand breaks at nanomolar concentrations, in HepG2 cells. *Q*RT-PCR gene expression analysis of the main enzymes involved in the biotransformation revealed that the protective effect of XN against HAs may be mediated by up-regulation of *UGT1A1* expression.

Beer or their compounds can prevent HAs carcinogenesis, either by inhibiting their formation or also as chemopreventing agent.

Resumo

As aminas aromáticas heterocíclicas (HAs) são consideradas um fator de risco para o desenvolvimento de cancro. A capacidade de formação das HAs em carnes e pescado cozinhados pelos métodos culinários habituais (fritos, assados, grelhados na frigideira, no carvão ou em dispositivo elétrico), mesmo em quantidades muito pequenas (ppb), implicam uma frequente exposição pela população em geral. Nos últimos 30 anos, realizaram-se inúmeros estudos visando a minimização do risco para a saúde associado às HAs. Contudo, as três principais áreas de estudo das HAs ainda são um desafio: a formação em alimentos, as estratégias de inibição da formação, e a procura de compostos quimiopreventivos. Além disso, vários investigadores têm realçado a necessidade do estudo de outros carcinogénios que ocorram em simultâneo com as HAs, como os hidrocarbonetos policíclicos aromáticos (PAHs).

A presença de HAs e PAHs foi avaliada em carnes (bife e frango) e pescado (salmão e sardinha) cozinhados de diferentes formas, refletindo as práticas portuguesas. O conteúdo em HAs nas amostras foi determinado usando um método de referência, que consiste em extração e purificação por extração em fase sólida (SPE) e separação e deteção por cromatografia líquida de alta eficiência acoplada a sistemas de deteção por díodos e fluorescência (HPLC-DAD/FLD). Os PAHs foram analisados através de uma metodologia determinada na presente dissertação, que consiste também em extração e purificação por SPE e separação e deteção por HPLC-FLD.

Relativamente à formação de HAs nas amostras analisadas, em geral, a HA mais abundante foi o PhIP (1,45-33,8 ng/g), seguido de AαC (1-19 ng/g) e MeIQx (não detectado-9,07ng/g). A formação das HAs IQ, 4,8-DiMeIQx, MeAαC, Trp-P-1, Trp-P-2 e Glu-P-1 verificou-se em algumas amostras em função do tipo de músculo ou método culinário. Em contrapartida as HAs IQx, MeIQ, 7.8-DiMeIQx, 4,7,8-TriMeIQx, e Glu-P-2 não foram detetadas em nenhuma das amostras em estudo. Na literatura verifica-se que as HAs térmicas são frequentemente estudadas, enquanto as HAs pirolíticas são normalmente negligenciadas. Contudo, a contribuição do AαC, HA pirolítica, é crucial para a avaliação da exposição às HAs. Em relação aos PAHs, observaram-se diferenças no perfil quantitativo entre as amostras: salmão> frango> bife. Verificou-se que o conteúdo em PAH8 está correlacionado significativamente com o teor de gordura da amostra.

Alterar as condições de confeção pode afetar a formação de HAs e PAHs em carnes e pescado. Relativamente aos peixes gordos grelhados, a redução da formação de HAs verificou-se ao grelhar o peixe a maior distância do carvão, ao substituir o carvão vegetal tradicional por carvão de casca de coco e através do uso do grelhador elétrico. Constatou-se ainda que grelhar em carvão de casca de coco também reduz a formação de PAHs em salmão. O uso contínuo do mesmo carvão na preparação de frango de churrasco contribui para uma maior quantidade de HAs e PAHs neste alimento.

Estratégias promissoras na mitigação de HAs passam pela aplicação de compostos antioxidantes. O potencial efeito inibidor de marinadas ricas em antioxidantes na formação de HAs foi avaliado em bifes cozinhados na frigideira sem adição de gordura. Marinar os bifes com cerveja 4h antes de cozinhar inibiu cerca de 80% da formação de HAs. Considerando o efeito inibidor da cerveja como o efeito padrão, as eficiências relativas dos outros líquidos (bebidas) usados como marinadas, no presente trabalho, foram: 1 para cerveja; 0,9 para o vinho branco desalcoolizado; 0,7 para o vinho tinto; 0,7 para o vinho branco; e 0,6 para o chá verde. Uma mistura de temperos normalmente usados em carne (alho, gengibre, tomilho, alecrim e malagueta), foi aplicada em bifes, juntamente com alguns dos líquidos supra-referidos, 4 horas antes de cozinhar. A eficiência da mistura de temperos (*ervas*) no efeito inibidor relativamente ao padrão foi: 1,1 para a cerveja com *ervas*; 0,95 para o vinho branco desalcoolizado com *ervas*; 0,6 para o vinho branco com *ervas*. Uma redução de cerca de 30% da formação de PhIP pode ser explicada pela adição das *ervas*; pelo contrário, o álcool parece influenciar bastante a formação desta HA. Não se observou uma correlação entre a atividade anti-radicalar das marinadas e a formação individual ou total de HAs.

O xanto-humol (XN) é um flavonoide do lúpulo presente na cerveja que tem sido descrito como um quimiopreventivo de "largo espetro". No presente trabalho verificou-se que o XN, em concentrações nanomolares, preveniu por completo a lesão no DNA induzida pelas HAs (PhIP e MeIQx) em células HepG2. A análise da expressão dos genes das principais enzimas envolvidas na biotrasformação das HAs por *Q*RT-PCR, revelou que o efeito protetor do XN contra as HAs pode ser mediado pelo aumento da expressão do gene da enzima de fase II *UGT1A1*.

A cerveja ou os seus compostos, podem prevenir a carcinogenicidade das HAs, quer através da inibição da sua formação, quer atuando como quimiopreventivos ao modular a biotransformação destas.

Scope and Aims

The current scientific evidence supports that the consumption of high-temperature cooked muscle foods, especially at well-done level, may be associated with an increased risk of several cancers. It has been suggested that heterocyclic aromatic amines (HAs) may play a major role in the etiology of cancers associated to well-done meat intake. HAs are produced under normal household cooking conditions (roasting, grilling, broiling, barbecuing, pan-frying and frying) from foods rich in protein. In addition an association between barbecued, but not fried, meat and stomach cancer suggests that dietary exposure to polycyclic aromatic hydrocarbons (PAHs) may be involved in gastric carcinogenesis. Besides HAs, it also must need to be taking into account the content of PAHs in the evaluation of the hazard of that cooking procedure on muscle food.

On the other hand cooking meat has clear beneficial impact, as the microbial content decreases, the digestibility increases and the flavor and texture improves. As human diets include muscle food, it is impossible to avoid the risk of exposure to the carcinogenic compounds generated from the cooking of protein food. Minimization strategies of their formation are of great importance from the viewpoint of food safety, along with consumers' acceptance.

The risk of exposure to heat generated hazard compounds depends on the kind of diet, eating habits and cooking practices, which often result from given world regions and cultural traditions.

For reliable risk assessments, it is important to determine the levels of HAs, and PAHs if justified, in foods cooked under ordinary conditions. Additionally, accurate analytical methodologies to evaluate these compounds at low levels of parts per billion in complex food matrixes are needed. In spite of their occurrence at low levels in cooked muscle meats, the continuously exposition to these compounds can be detrimental to human health by inducing cancer and the looking for effective protective strategies stills a matter of concern. Since the HAs may play a major role in the etiology of cancers related to well-done meat intake it should be highlighted that antioxidants, especially those that come from dietary sources, are considered the main promising strategies to reduce HAs exposure either by inhibiting their formation or acting as chemopreventive agents.

The work depicted in the present dissertation thesis has the following overall aim:

To determine the occurrence of the main heat generated hazard compounds in muscle foods cooked in various ways and to assess mitigation strategies to reduce the health risk associated.

Accordingly with the main aim of this dissertation and subsequent to bibliographical research it was firstly elaborated one book chapter that was included in the Introduction section of the present thesis. The Introduction briefly describes the State of the Art concerning HAs and PAHs in cooked muscle foods.

The results obtained from experimental work will be presented in three sections according with specific objectives:

Section A. To determine the levels of the main heat generated hazard compounds in barbecued foods and to examine in which way their formation can be reduced.

A1. "Heterocyclic aromatic amines formation in barbecued sardines (*Sardina pilchardus*) and Atlantic salmon (*Salmo salar*)."

- To investigate the formation of thermic and pyrolytic HAs on barbecued fatty fishes in various degrees of doneness and different grilling conditions.
- To compare the influence of charcoal and electric heat on HAs formation in grilled salmon.
- To accurate the grilling conditions which minimize the HAs formation.
- To achieve an indicator (HA) for drastic conditions used on processing foods.

A2. "A comparison of the extraction procedures and quantification methods for the chromatographic determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish."

- To establish a methodology for an accurate determination of priority PAHs in charcoal grilled meat and fish.
- To develop a separation of 16 US-EPA PAHs by high performance liquid chromatography/fluorescence using an acetonitrile free elution.

A3. "Effect of charcoal types and grilling conditions on formation of heterocyclic aromatic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs) in grilled muscle foods."

- To study the influence of charcoal type on HAs and PAHs formation and select the safer charcoal to grill muscle foods.
- To assess whether continuous barbecuing with the same charcoal can influence the HAs and PAHs formation.
- To provide reference HAs and PAHs levels in barbecued muscle foods and to compare the contribution of the different muscle foods to the diet exposure of these hazard compounds.

Section B. To determine the levels of HAs in pan-fried meat and to investigate the potential inhibitory effects of antioxidant rich marinades in their formation.

B1. "Effect of beer/red wine marinades on the formation of heterocyclic aromatic amines in pan-fried beef".

- To provide reference HAs levels in pan-fried beef.
- To compare the effect of beer marinades and red wine marinades in the reduction of thermic and pyrolytic HAs formation in pan-fried beef.
- To evaluate the influence of beer and red wine marinades in pan-fried meat organoleptic characteristics.

B2. "Effect of green tea marinades on the formation of heterocyclic aromatic amines and sensory quality in pan-fried beef".

- To study the effect of a green tea marinade rich in the thermic and pyrolytic HAs formation in pan-fried beef.
- To evaluate the influence of green tea marinade in meat organoleptic characteristics.

B3. "Inhibitory effect of antioxidant rich marinades on the formation of heterocyclic aromatic amines in pan-fried beef".

- To evaluate the antiradical activity of antioxidant rich marinades containing beer and white wine and a mixture of herbs commonly used as meat flavoring.
- To understand the contribution of the antioxidant rich marinades in the HAs inhibition under household cooking conditions.
- To investigate whether the applied herbs together with the alcoholic beverages on pan-fried meat can present adequate sensory characteristics.

Section C. To evaluate the chemopreventive potential of xanthohumol on HAs.

C1. "Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells"

- To evaluate the chemopreventive potential of XN against the most abundant HAs on the diet.
- To shed light the mechanism that account for the antigenotoxic effect.

List of Publications

The work performed during PhD research, which constitutes the present dissertation, has been already submitted to international publication.

Book Chapter:

Viegas, O., Melo, A., Petisca, C., Quelhas, I., Pinho, O., Ferreira, I.M.P.L.V.O., 2009. Modulating effects of red wine and beer on heterocyclic aromatic amines carcinogenesis. In: Mazzei, A., D'Arco, A. (Eds.), Alcoholic Beverage Consumption and Health. Nova Science Publishers, Inc., New York, pp. 137-156.

ISI index publications:

Melo, A., Viegas, O., Petisca, C., Pinho, O., Ferreira, I.M.P.L.V.O., 2008. Effect of beer/red wine marinades on the formation of heterocyclic aromatic amines in pan-fried beef. J. Agric. Food Chem. 56, 10625-10632.

Quelhas, I., Petisca, C., Viegas, O., Melo, A., Pinho, O., Ferreira, I.M.P.L.V.O., 2008. Effect of green tea marinades on the formation of heterocyclic aromatic amines and sensory quality in pan-fried beef. Food Chem. 122, 98–104.

Costa, M., Viegas, O., Melo, A., Petisca, C., Pinho, O., Ferreira, I.M.P.L.V.O., 2009. Heterocyclic aromatic amines formation in barbecued sardines (*Sardina pilchardus*) and Atlantic salmon (*Salmo salar*). J. Agric. Food Chem. 57, 3173-3179.

Viegas, O., Novo, P., Pinho, O., Ferreira, I.M.P.L.V.O., 2012. A comparison of the extraction procedures and quantification methods for the chromatographic determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish. Talanta 88, 677-683.

Viegas, O., Žegura, B., Pezdric, M., Novak, M., Ferreira, I.M.P.L.V.O., Pinho, O., Filipič, M., 2012. Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells. Food Chem. Toxicol. 50, 949-955.

Viegas, O., Novo, P., Pinto, E., Pinho, O., Ferreira, I.M.P.L.V.O., 2012. Effect of charcoal types and grilling conditions on formation of heterocyclic aromatic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs) in grilled muscle foods. Food Chem. Toxicol. *in press*. doi.org/10.1016/j.fct.2012.03.051

Viegas, O., Amaro, L.F., Pinho, O., Ferreira, I.M.P.L.V.O., 2012. Inhibitory effect of antioxidant rich marinades on the formation of heterocyclic aromatic amines in pan-fried beef. Submitted.

Abbreviations

The abbreviations list comprises acronyms from the dissertation thesis, except from the results section. Acronyms also present in *Resumo* have the English denomination followed by the Portuguese denomination.

4,7,8-TriMeIQx	2-amino-3,4,7,8-tetramethylimidazo[4,5-f]-quinoxaline
4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]-quinoxaline
4'-OH-PhIP	2-amino-1-methyl-6-(4′-hydroxyphenyl)-imidazo[4,5-*b*]-pyridine
4-CH_2OH-8-MeIQx	2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]-quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethylimidazo[4,5-*f*]-quinoxaline
7,9-DiMeI*g*Qx	2-amino-1,7,9-trimethylimidazo[4,5-*g*]-quinoxaline
8PN	8-prenylnaringenin
AC(s)	Aminocarboline(s)
AIA(s)	Aminoimidazoazarene(s)
AαC	2-amino-9*H*-pyrido[2,3-*b*]índole
BaA	Benzo[*a*]anthracene
BaP	Benzo[*a*]pyrene
BbF	Benzo[*b*]fluoranthene
BgP	Benzo[*g*,*h*,*i*]perylene
BkF	Benzo[*k*]fluoranthene
C_{18}	octadecyl carbon chain
cDNA	complementary DNA
CE	capillary electrophoresis
Ch	Chrysene
CONTAM Panel	Panel on Contaminants in the Food Chain
COX-1	cyclooxigenase-1
COX-2	cyclooxigenase-2
Cre-P-1	4-amino-1,6-dimethyl-2-methylamino-1*H*,6*H*-pyrrolo-[3,4-*f*]benzimidazole-5,7- dione
CYP	Cytochrome P450
DAD	diode array detection; deteção por díodos

DAD/FLD	tandem diode array and fluorescence detectors
DD	dihydrodiol dehydrogenase
DhA	Dibenzo[*a*,*h*]anthracene
DMIP	2-amino-1,6-dimethylimidazo[4,5-*b*]-pyridine
DNA	deoxyribonucleic acid; ácido desoxirribonucleico
ED	electrochemical detection
EFSA	European Food Safety Authority
EH	epoxide hydrolase
FAO	Food and Agriculture Organization
FLD	fluorescence detection; deteção por fluorescência
GC	gas chromatography
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
GST	glutathione *S*-transferase
HA(s)	heterocyclic aromatic amine(s); amina(s) aromática(s) heterocíclica(s)
Harman	1-methyl-9*H*-pyrido[3,4-*b*]índole
HepG2	human hepatoma cell line
HPLC	high performance liquid chromatography; cromatografia líquida de alta precisão
IARC	International Agency for Research on Cancer
IFP	2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]-pyridine
I*g*Qx	2-amino-1-methylimidazo[4,5-*g*]-quinoxaline
IP	Indeno [1,2,3-*cd*]pyrene
IPCS	International Programme on Chemical Safety
IQ	2-amino-3-methylimidazo[4,5-*f*]-quinoline
IQ[4,5-*b*]	2-amino-1-methylimidazo[4,5-*b*]-quinoline
IQx	2-amino-3-methylimidazo[4,5-*f*]-quinoxaline
IX	isoxanthohumol
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
LLE	liquid-liquid extraction

LOD	limit of detection
LOQ	limit of quantification
Lys-P-1	3,4-cyclopenteno-pyrido[3,2-*a*]carbazole
MeAαC	2-amino-3-methyl-9*H*-pyrido[2,3-*b*]índole
MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]-quinoline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NAT	*N*-acetyltransferase
Norharman	9*H*-pyrido[3,4-*b*]indole
NPD	nitrogen-phosphorus detection
Orn-P-1	4-amino-6-methyl-1*H*-2,5,10,10*b*-tetraazafluoranthene
PAH(s)	polycyclic aromatic hydrocarbon(s); hidrocarboneto(s) aromático(s) policíclico(s)
PAH4	Sum of BaP, Ch, BaA and BbF; conjunto formado por BaP, Ch, BaA and BbF
PAH8	Sum of BaP, Ch, BaA, BbF, BkF, BgP, DhA and IP; conjunto formado por BaP, Ch, BaA, BbF, BkF, BgP, DhA and IP
pH	hydrogen ion potential
Phe-P-1	2-amino-5-phenylpyridine
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine
ppb	parts-per-billion
PRS	propyl sulfonic acid
***Q*RT-PCR**	quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
SCF	Scientific Committee on Food
SPE	solid phase extraction, extracção em fase sólida
SULT	Sulfotransferase
TMIP	2-amino-3,5,6-trimethylimidazo[4,5-*b*]-pyridine
Trp-P-1	3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]índole
Trp-P-2	3-amino-1-methyl-5*H*-pyrido[4,3-*b*]índole
UGT	UDP-glucuronosyltransferase

US-EPA	Environmental Protection Agency of the United States of America
UV	Ultraviolet
vs.	*Versus*
WCRF/AICR	World Cancer Research Fund and the American Inst. For Cancer Research
WHO	World Health Organization
XN	xanthohumol
~	Approximately
=	similar to
>	higher than

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1. Introduction

1.1. Cooked muscle foods and risk of cancer

Epidemiological studies suggest that diet plays an important role in etiology of human cancer (Doll and Peto, 1981). On one hand human diet often contains components that cause DNA damage and are potentially carcinogenic, and on the other hand it contains numerous natural constituents with protective effects against cancer and other mutation-related diseases (Knasmüller et al., 2002; Ferguson et al., 2004).

Of greater concern are the naturally-formed carcinogenic compounds generated during the preparation of food for consumption. Carcinogens produced in cooking have been receiving special attention since 1977, when Sugimura and collaborators have demonstrated that charred parts of broiled fish and meat produce powerful mutagens that cannot be accounted for only by the production of polycyclic aromatic hydrocarbons (PAHs) (Nagao et al., 1977), which were detected in grilled meat in 1963 (Seppilli and Scassellati Sforzolini, 1963). While PAHs were shown to be the major mutagens on a mass basis, the new compounds were found to be by far the most potent mutagens (Gooderham et al., 2001). The new mutagenic compounds were termed heterocyclic aromatic amines (HAs) due to the molecular structure with at least one aromatic ring, one heterocyclic structure and an exocyclic amine function (Cheng et al., 2006).

The current scientific evidence supports that the consumption of fried, grilled or barbecued meat and fish that are well-done or browned may be associated with an increased risk of lung, breast, prostate and colorectal cancers (Rohrmann et al., 2002; Zeng and Lee, 2009). These cancers have been prevalent in Western countries, however they have been increasing in Japan and other countries that are adapting western dietary habits (Sugimura et al., 2004).

Muscle foods, especially red-meat, naturally present haem iron, high-fat and consequent high energy density and cholesterol which are promoters of carcinogenesis (Ferguson, 2010), however the carcinogens produced in high-temperature cooking techniques may explain some of the cancer risk associated to meat and fish. The main food cooking carcinogens in meat and fish are HAs and/or PAHs (Jägerstad and Skog, 2005).

HAs are produced under normal household cooking conditions (roasting, grilling, broiling, barbecuing, and frying) from foods rich in protein, namely in muscle foods. Despite some inconclusive or less convincing epidemiological results (Alaejos et al., 2008a), HAs exposure may play a major role in the etiology of cancers associated to well-done meat intake (Zeng and Lee, 2009). An association between barbecued, but not fried, meat and stomach cancer suggests that dietary exposure to PAH may be involved in gastric carcinogenesis (WCRF/AICR, 1997). To

evaluate the hazard of grilled/barbecued muscle food it is necessary to know both HAs and PAHs content.

Meat and fish are primary sources of essential fat and proteins with high biological level as well as are important sources of various micronutrients. Muscle foods are easily absorbable sources of iron, zinc and selenium. They contain potential anticarcinogens such as the micronutrients selenium, vitamins B6, B12, and D, and the "healthy fats" omega-3 polyunsaturated fatty acids, and conjugated linoleic acid (Mann, 2000; Connor, 2001; Ferguson, 2010).

Cancers associated with high consumption of fried, grilled or barbecued meat and fish may be reduced by the addition of anticarcinogens in the diet, either consumed at the same time, such as dietary fiber that can modify the absorption and excretion of carcinogens or with an adequate fruit and vegetables consumption through the modulation of biotransformation of HAs and PAHs by polyphenols. Furthermore, the addition of other ingredients, especially antioxidant rich, or modification of food preparation methods may reduce the formation of these contaminants (Ferguson, 2010).

1.2. Heterocyclic aromatic amines

In 1977 it was found that particles of smoke, produced by cooking proteinaceous foodstuffs and trapped in glass-fiber filters, contained significant quantities of mutagens (Nagao et al., 1977). Additionally, mutagenic activity was shown for charred parts of broiled fish and meat, and in pyrolysed proteins and amino acids (Sugimura, 1997). More than 25 HAs have been isolated and identified in cooked muscle foods prepared under common household cooking practices (Alaejos and Afonso, 2011). Some HAs have also been identified in tobacco smoke condensate, incineration ash, and diesel-exhaust particles, however the main source of exposure to HAs occurs by the consumption of well-done cooked meats (Turesky and Le Marchand, 2011).

1.2.1. Toxicological classification

Nowadays HAs comprise the class of hazardous chemicals that are receiving heightened attention as a risk factor for human cancer. In 1993, the International Agency for Research on Cancer (IARC, 1993) considered eight of the HAs tested to date (MeIQ, 8-MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2, and Glu-P-1) as *possible human carcinogens* (class 2B) and one (IQ) as a *probable human carcinogen* (class 2A) and has recommended the reduction of human exposure to these compounds. Four prevalent HAs (IQ, MeIQ, MeIQx, and PhIP) are listed in the United States

Department of Health and Human Services´s 12th Report of Carcinogens (2011) as compounds *reasonably anticipated to be a human carcinogen*.

1.2.2. Structure and formation of HAs in muscle foods

HAs normally have planar structures and consist of two to five (generally three) fused aromatic rings with at least one nitrogen atom in the ring structure and with one exocyclic amino group, except in the case of Lys-P-1, harman, and norharman, and up to four methyl groups as substituents (Melo et al., 2008; Alaejos and Afonso, 2011). Table 1 presents chemical and abbreviated names of HAs and Table 2 shows some of their chemical structures.

Temperature has an important influence on the kind of HAs formed. The temperatures that are needed for the formation of significant amounts of aminoimidazoazarenes (AIAs) also designated as IQ-type or thermic HAs, in general range between 150 and 250 ºC. At higher temperatures, above 250 ºC, the aminocarbolines (ACs), also designated non-IQ type or pyrolytic HAs, are formed preferably (Murkovic, 2004; Alaejos and Afonso, 2010). According to the chemical behavior of HAs, they are grouped as polar (AIAs together with the γ –carbolines: Glu-P-1 and Glu-P-2) and nonpolar (all the others) HAs (Alaejos and Afonso, 2011).

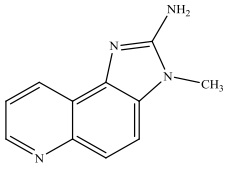
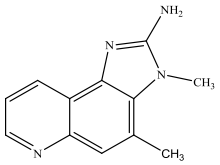
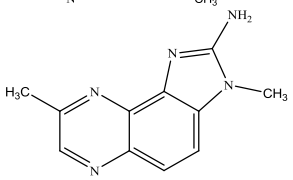
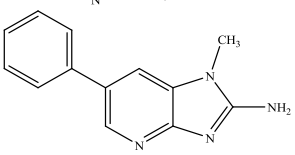
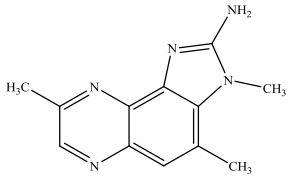
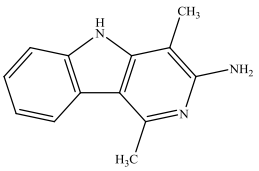
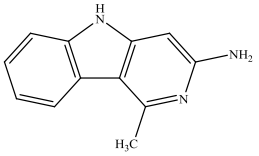
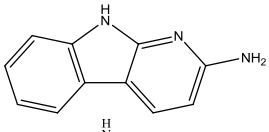
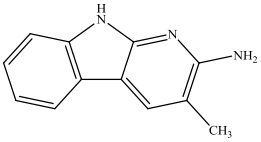
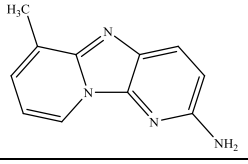
The formation of thermic HAs in cooked or processed meats has been elucidated to be due to the Maillard reaction of sugars/amino acids/ creatin(in)e (Kato et al., 1996, Jägerstad et al., 1998). In spite of thermic HAs to comprise the same 2-aminoimidazole moiety they can be divided in three different groups: quinolines, quinoxalines and pyridines.

It was postulated and later demonstrated that creatine formed the aminoimidazo moiety of IQ and IQx by cyclization and water elimination, while the Strecker degradation products such as pyridines or pyrazines, formed in the Maillard reaction between amino acids and hexose contributed to the remaining part of the molecule, probably via aldol condensation (Jägerstad et al., 1998; Murkovic, 2004; Cheng et al., 2006) (Figure 1).

Table 1. Chemical names and usual abbreviations of HAs.

Abbreviation	Compound name
AMINOIMIDAZOAZARENES or THERMIC HAs	
Quinolines	
IQ	2-amino-3-methylimidazo[4,5-*f*]-quinoline
MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]-quinoline
IQ[4,5-*b*]	2-amino-1-methylimidazo[4,5-*b*]-quinoline
Quinoxalines	
IQx	2-amino-3-methylimidazo[4,5-*f*]-quinoxaline
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
4,7,8-TriMeIQx	2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]-quinoxaline
4-CH_2OH-8-MeIQx	2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]-quinoxaline
I*g*Qx	2-amino-1-methylimidazo[4,5-*g*]-quinoxaline
7,9-DiMeI*g*Qx	2-amino-1,7,9-trimethylimidazo[4,5-*g*]-quinoxaline
Pyridines	
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine
4'-OH-PhIP	2-amino-1-methyl-6-(4′-hydroxyphenyl)-imidazo[4,5-*b*]-pyridine
DMIP	2-amino-1,6-dimethylimidazo[4,5-*b*]-pyridine
TMIP	2-amino-3,5,6-trimethylimidazo[4,5-*b*]-pyridine
IFP	2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]-pyridine
CARBOLINES or PYROLYTIC HAs	
Pyridoindole	
α- Carbolines	
A*α*C	2-amino-9*H*-pyrido[2,3-*b*]indole
MeA*α*C	2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
γ- Carbolines	
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
ß- Carbolines	
Harman	1-methyl-9*H*-pyrido[3,4-b]indole
Norharman	9*H*-pyrido[3,4-b]indole
Pyridoindole	
δ- Carbolines	
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
Other carbolines	
Phe-P-1	2-amino-5-phenylpyridine
Orn-P-1	4-amino-6-methyl-1*H*-2,5,10,10*b*-tetraazafluoranthene
Lys-P-1	3,4-cyclopenteno-pyrido[3,2-*a*]carbazole
Cre-P-1	4-amino-1,6-dimethyl-2-methylamino-1*H*,6*H*-pyrrolo-[3,4-*f*]benzimidazole-5,7-dione

Table 2. Chemical structure and IARC classification of relevant HAs.

Compound name	Abbreviation	Structure	IARC Group
2-amino-3-methylimidazo[4,5-*f*]quinoline	IQ		2A
2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline	MeIQ		2B
2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline	MeIQx		2B
2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine	PhIP		2B
2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline	4,8-DiMeIQx		–
3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole	Trp-P-1		2B
3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole	Trp-P-2		2B
2-amino-9*H*-pyrido[2,3-*b*]indole	AαC		2B
2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole	MeAαC		2B
2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole	Glu-P-1		2B

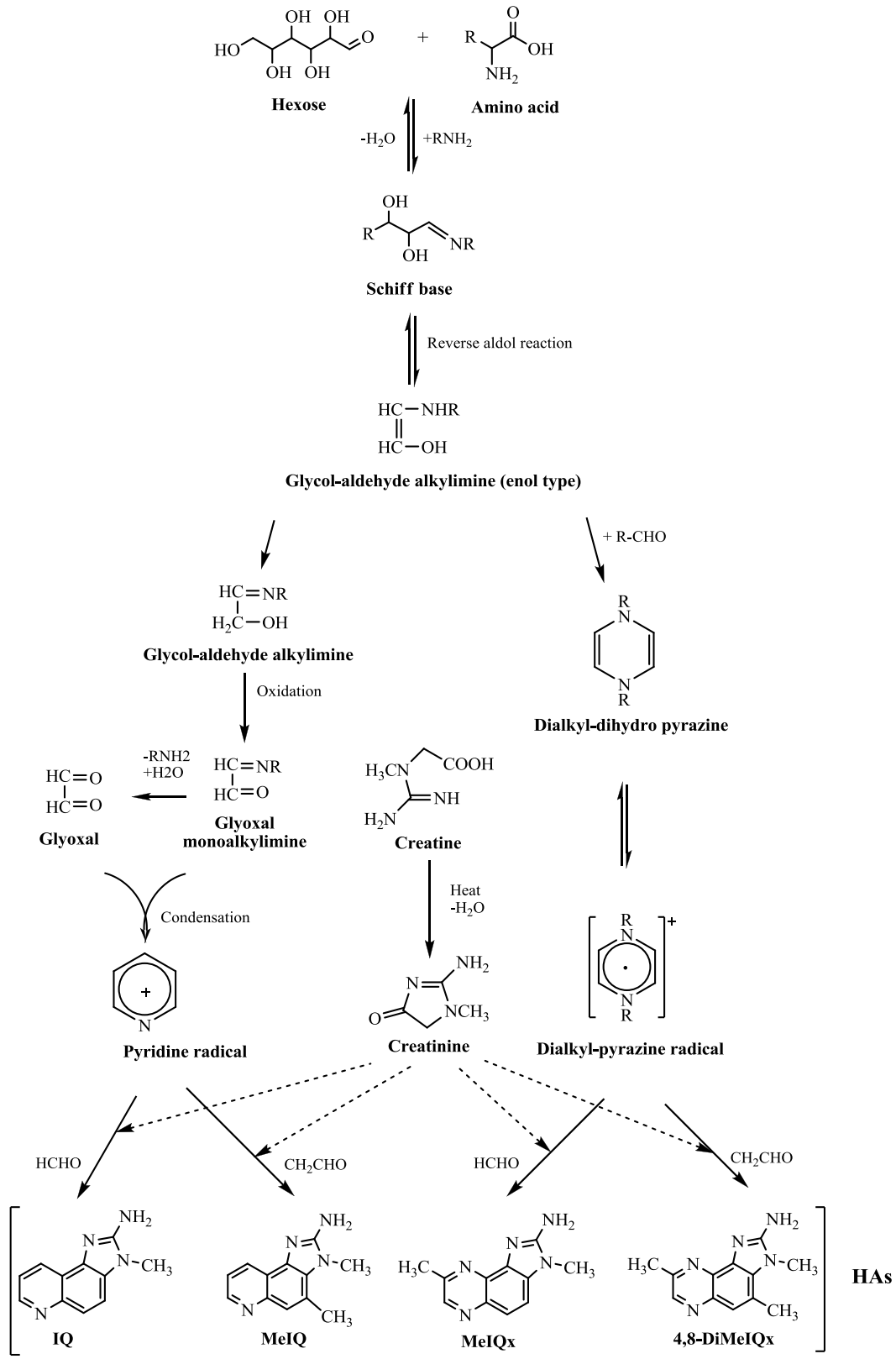


Figure 1. Scheme of HAs formation involving free radical mechanisms (adapted from Jägerstad et al., 1998; Murkovic, 2004; Vitaglione and Fogliano, 2004).

Further studies have supported the hypothesis on the precursors and intermediates, and suggested free radical mechanisms to be involved. It was proposed that alkylpyridine free radicals and creatinine produce IQ and MeIQ, and dialkylpyrazine free radicals and creatinine produce MeIQx and 4,8-DiMeIQx. The initial step in the formation of MeIQx and DiMeIQx was dependent on the kinetics of the Maillard and Strecker reactions, with the formation of pyridine and pyrazine free radicals, and finally the stabilization of the free radicals, giving pyridine and pyrazine derivatives, which react with creatinine (Figure 1). Evidence supporting this pathway was obtained when the heating of creatinine with 2,5-dimethylpyrazine or 2-methylpyridine and acetaldehyde at 130 ºC for 3 h was shown to produce MeIQx and DiMeIQx (Murkovic, 2004).

The formation of the mutagenic non-IQ type HAs occurs under drastic thermal environment. These mutagens were first isolated from smoke condensates collected either from cigarettes or from pyrolysed single amino acids, e.g., tryptophan, glutamic acid, lysine, phenylalanine, ornithine or creatine, or from pyrolysed proteins like casein, albumin, gluten or soybean globulin (Jägerstad et al., 1998). A pathway via free radical reactions, which produce many reactive fragments (Skog et al., 2000) is the most popular hypothesis for their formation. These fragments may then condense to form new structures (Skog et al., 2000). However, due to their much lower occurrence in normally cooked foods and greater difficulty in manipulation of the high temperatures required in experimental set up, relatively little investigation has been carried out to verify the above hypothesis compared with the IQ type (Murkovic, 2004).

1.2.3. Separation and quantification of HAs in muscle foods

The challenge has been to develop rapid analytical methods that isolate and unequivocally identify HAs in complex matrices at the low parts-per-billion (ppb) level (Kataoka and Kijima, 1997; Toribio et al., 2007).

A purification step must be carried out, followed by a separation technique such as liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE). A number of excellent reviews have been compiled over recent years, to describe the extraction, detection, and quantification of HAs and provide a concise appraisal of the relative merits of each approach (Kataoka and Kijima, 1997; Pais and Knize, 2000; Toribio et al., 2000; Skog, 2004; Alaejos et al., 2008b).

Laborious cleanup procedures based on liquid-liquid extraction (LLE) (Tikkanen et al., 1996) extraction with blue cotton (Murkovic et al., 1998), solid-phase extraction (SPE) with disposable columns (Richiling et al., 1997) or tandem extraction procedures consisting of the coupling of LLE and SPE (Gross, 1990; Gross and Grüter, 1992) have been developed.

Usually, the sample is dispersed using different solvents, namely, methanol, acetone, ethyl acetate, hydroalcoholic mixtures, or aqueous, like hydrochloric acid, water, or sodium hydroxide. LLE is the separation method preferred by most of the authors for the first step in the isolation of the analytes from the food matrix. LLE can be achieved by using inert solid materials such as diatomaceous earth and coupled with solid phase extraction (Toribio et al., 2000). The use of diatomaceous earth as solid support for LLE is recommended for samples homogenized in sodium hydroxide solution; the aqueous phase distributes itself in the form of a thin film over the chemically inert matrix and macromolecules like proteins and carbohydrates remain adsorbed on the inert material (Toribio et al., 2000).

The Gross method (Gross, 1990; Gross and Grüter, 1992), which uses the coupling of LLE with diatomaceous earth as solid support and two SPE steps with propylsulfonic acid (PRS) and C_{18}, is the most popular tandem method and can yield two extracts clean enough for the determination of polar and less polar heterocyclic aromatic amines. More recently Toribio et al. (2007) proposed a faster method to extract HAs from meat samples on a single extract.

Identification and quantification of HAs have been commonly carried out by means of chromatographic or related techniques using different detection systems. Essential aspects in the analysis of complex matrices are the unequivocal identification of HAs and the accurate quantitative determination of the HAs. This can be achieved with mass spectrometry (MS) or diode array (DAD) detections (Pais and Knize, 2000).

Liquid chromatography (LC) with different detection systems such as ultraviolet (UV) (Perfetti, 1996, Sinha et al., 1998) electrochemical (ED) (Krach and Sontag, 2000), fluorescence (FLD) (Pais et al., 1999) and MS (Toribio et al., 2002) detections are the most used. GC is avoided because the derivatization step is required. The detection method most commonly used is the DAD (Toribio et al., 2000), which allows on-line identification of the analytes by spectral library matching and has a low cost. The FLD is sometimes used as a complement to DAD, because unavoidable interferences are frequently produced when using UV detection. To enhance the selectivity of the detection, LC-MS/MS using triple quadrupole (Richiling et al., 1997; Stillwell et al., 1997; Guy et al., 2000), ion-trap (Pais et al., 2000; Toribio et al., 2002), or time-of-flight (Barceló-Barrachina et al., 2004) instruments have been used.

In high performance liquid chromatography (HPLC) systems, authors refer that the TSK gel column with a ternary gradient elution system shows the best peak symmetry and separation efficiency. However, good UV spectra were difficult to obtain at pH 3.2 and 3.6. The HPLC at pH 7.0 can be used as an alternative when samples contain multiple interfering peaks such as high temperature meats (Pais and Knize, 2000).

Capillary electrophoresis, either with mass spectrometry (CE-MS) (Zhao et al., 1998), ultra-violet (CE-UV) (Mendonsa and Hurtubise, 1999), or electrochemical detection (CE-ED) (Olsson et al., 1997), has also been proposed, although high detection limits have been obtained.

Gas chromatography with nitrogen-phosphorus selective detection (NPD) (Kataoka and Kijima, 1997) or with MS detection (Casal et al., 2004) has been also used to analyse HAs. However, most of these compounds are polar and non-volatile, and consequently, a derivatization step is needed.

1.2.4. Occurrence of HAs in cooked foods

The first quantitative data on HAs in various meat and fish products, based on chemical analysis, were published in the late 1980s. Earlier literature data on HA levels in foods consist mainly of amounts estimated from the mutagenic activity according to the Ames/*Salmonella* assay (Skog et al., 1998). The concentration of HAs is normally in the low range (ng/g) food composition and cooking methods are known to greatly influence the formation of HAs (Layton et al., 1995; Murkovic, 2007; Alaejos and Afonso, 2011). An overview of the HAs contents of heated foods is given in Table 3.

Table 3. Occurrence of HAs in cooked foods (ng/g) according to references (Layton et al., 1995; Jägerstad et al., 1998; Murkovic, 2007).

	IQ	**MeIQ**	**MeIQx**	**4,8-DiMeIQx**	**PhIP**	**AαC**	**Trp-P-1**	**Trp-P-2**
Red Meat	0-2	0-2	0-10	0-5	0-35	0-20	0-1	0-1
Poultry	0-1	0-1	0-3	0-3	0-10	0-1	0-2	0-1
Fish	0-1	0-0.3	0-2	0-1	0-10	0-10	0-1	nd
Meat extracts, pan residues	0-15	0-6	0-80	0-9	0-10	0-3	0-5	0

nd- not detected

PhIP is the most abundant HA followed by MeIQx (Layton et al., 1995). PhIP is typically found in amounts up to and around 35 ng/g (Skog, 2002). MeIQx is frequently reported in cooked foods, present at levels up to half the amount of PhIP generally up to 10 ng/g. The formation of PhIP was much higher relative to MeIQx especially in the chicken meat, however MeIQx can be present in similar amounts than PhIP in the beef meat (Pais et al, 1999).

The levels of AαC are generally 0–20 ng/g in red meat, but higher amounts of AαC have been reported in grilled, barbecued or high temperature fried red meat and fish, in concentrations comparable with those of PhIP (Jägerstad, et al., 1998; Skog, 2002). Lower frequency and concentrations are seen for 4,8-DiMeIQx. The other HAs are in general reported in considerably lower amounts and found, but also looked for, less frequently. The amounts of HAs change according to meat and fish type are generally higher in cooked meats than in fish and in pure meat than in mixed and ground meat products, e.g. meatballs, hamburgers or sausage (Jägerstad, et al., 1998).

1.2.5. Metabolism of HAs

It is generally accepted that in order a toxicant to exert its toxicity, the prime factor is its ability to overcome biological barrier(s) such as the gastrointestinal barrier and blood-tissue barrier. Most HAs are not mutagenic/carcinogenic in their native form acquiring the capability of forming DNA adducts after metabolic activation. A variety of enzymes are involved in this process. The major activation pathway of HAs occurs through cytochrome P450 1A2 (CYP1A2)-mediated *N*-oxidation, it involves phase I *N*-hydroxylation followed by phase II esterification, both take place at the exocyclic amino group (Cheng et al., 2006; Turesky, 2007).

In human, the expression of CYP1A2 was found to be confined to the liver, and only negligible levels have been detected in extrahepatic tissues (Airoldi et al., 2004). However, the *N*-oxidation reaction of HAs is also catalyzed by cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1) in extrahepatic tissues (Crofts et al., 1998; Turesky, 2007). Extrahepatic expression of CYP1A1 may play an important role in PhIP-related mutagenesis in target tissues such as the colon and breast (Crofts et al., 1998).

N-hydroxylated-HAs themselves can react with DNA, but the subsequent *O*-esterification catalyzed by phase II enzymes, mainly acetyltransferases (NAT) and sulfotransferases (SULT), leads to the formation of *O*-derivatives that are more reactive electrophiles (Airoldi et al., 2004). These metabolites may undergo heterolytic cleavage to generate the highly reactive nitrenium ion, which attacks guanine base at N-2 or C-8 position forming DNA adducts (Turesky, 2002).

Moreover, recent studies showed that to tryptophan pyrolysates (Trp-P-1, Trp-P-2) only conversion by CYP1A2 is required to lead to formation of DNA-reactive metabolites. Quinolines and quinoxalines involve subsequent *O*-acetilation catalyzed by NAT whereas SULT are essential for the activation of PhIP and aminocarbolines (Muckel et al., 2002; Glatt et al., 2004).

Alternatively, other pathways are described as detoxifying through conjugation by sulphation and glucuronidation mechanisms by means of glutathione *S*-transferase (GST) and UDP-

glucuronosyltransferase (UGT), respectively. The formed polar compounds are not reactive and are excreted by urine or bile (Alaejos et al., 2008c).

Comparison between experimental animals and human cytochrome P450 enzymes (CYPs) and phase II enzymes is essential for the extrapolation of animal carcinogenicity data to assess human health risk and consideration of species differences in catalytic activities of these enzymes is important. For example, the low level of CYP1A2 expressed in rat liver combined with the strong differences between rat and human CYP1A2 activities in HA activation suggest that the carcinogenicity data of the rat may underestimate the human health risk of HAs (Turesky, 2005).

Differences in biotransformation capacity are a result of both genetic variation in the expression and the forms of enzymes (polymorphism) as well as dietary constituents that modulate the expression and enzyme activity (MacLeod et al., 2000). More studies are needed concerning chemical analyses of metabolites, as well as design of human intervention trials in which the impact of dietary factors on HAs-induced DNA damage are monitored. Such experimental systems can be complemented by data obtained with advanced molecular techniques, such as gene expression analyses and proteomics.

1.2.6. Mitigation of HAs formation

Formation and amount of HAs depend on several factors such as meat type, cooking methods, temperature and time, the degree of doneness, the concentration of precursors, pH, water activity, presence of carbohydrate, free amino acids and creatine, heat and mass transfer, lipid level, the presence of oxidative or antioxidants used (Alaejos et al., 2011). Cooking time and temperature had the most significant linear effects on the formation of HAs, among the processing variables studied by Dundar et al. (2012).

Use of low pan temperatures to cook meat is probably the most practical and effective method to reduce HAs formation. It is important to frequently turn the meat as it cooks. However, it is critical to attain an internal temperature of 72 °C in the cooked meat to ensure microbial inactivation (Salmon et al., 2000). Since the inner temperature is not a practice in domestic conditions, cooking time is often associated with the degree of doneness. Prolonged cooking time increases the HAs formation, on the other hand at lower temperatures and higher cooking time may reduce their formation (Skog and Jägerstad, 2006). Microwave pretreatment release juice that carry precursors which explain a reduction of the amount of HAs formed during the frying of ground beef (Felton et al., 1994). Coating foods with breadcrumbles prior frying or cooking food with the skin can reduce the HAs formed by acting as an insulating layer (Skog et al., 1997; Gašperlin et al., 2009).

A high cooking loss has been found to be related to the formation of larger amounts of HAs. The increase of water-holding capacity can avoid the cooking water loss that prevents cooking loss and

the extrusion of precursors to the surface of the meat inhibiting the HAs formation. The addition of ingredients with this capacity to the surface, or mixture with the ground meat on hamburgers or by marinating may inhibit the HAs formation (Persson et al., 2003; Skog and Jägerstad, 2006). Salt (Persson et al., 2003), polysaccharides such as starch, gums and dietary fiber (Shin et al., 2003; Persson et al., 2004).

The fat amount can affect the HAs formation, however different behaviors can be observed in each HA dependent on the amount of fat. Fat is a good heat-transferring agent and can shorter the cooking time, however results are conversely concerning fat (Skog and Jägerstad, 2006).

To avoid the dark brown parts of grilled food and refuse the pan-residue as sauces or gravy are minimizing strategies when the food is already cooked (Skog and Jägerstad, 2006).

Addition of ingredients that may react with the precursors/intermediates could also reduce the amounts of HAs. Some studies have shown that the concentrations of HAs can also be reduced by addition of compounds with an antioxidant potential. The addition of natural products containing antioxidants that may act as free radical scavengers, such as polyphenols, the main dietary antioxidants, reduces the amount of HAs in the heat-processed meat and model systems (Vitaglione and Fogliano, 2004). This subject is depicted in the next section (1.2.7.1).

1.2.7. Modulation of HAs effects by antioxidants

On one hand human diet often contains HAs that are potentially carcinogenic, on the other hand it contains numerous natural constituents with protective effects against that. Antioxidants, especially those that come from dietary sources, are considered the main promising strategies to reduce HA exposure either by inhibiting HAs formation or acting on biotransformation as blocking/suppressing agents (Knasmüller et al., 2002; Vitaglione and Fogliano, 2004).

1.2.7.1. Mitigation of HAs formation by antioxidants

Since the mechanism of formation of HAs involves free radicals, it has been proposed that antioxidants should act as inhibitors along the different pathways of the reaction, through radical quenchers and free radical scavengers activity (Kikugawa, 1999).

In the last years several studies have demonstrated that the addition of synthetic and natural antioxidants (pure compounds such as vitamins and polyphenols; or foods either whole or extracts) reduces the mutagenic activity of the cooked products and the amount of HAs formation (Vitaglione and Fogliano, 2004). In most cases the antioxidants effect on HAs were evaluated by mutagenicity using *Salmonella* assay, while only a few studies have measured these effects using chromatographic methods (Damašius et al., 2011).

Dietary phenolics have been receiving great attention. Phenolic compounds from tea (*Camellia sinensis*), namely green tea cathechins have been among the most promising inhibitors on model systems and also on mutagenic activity (Oguri et al., 1998; Weisburger et al., 2002; Cheng et al., 2007a). Phenolic compounds from fruits (Oguri et al., 1998) namely naringenin from citrus fruits (Cheng et al., 2007a) and proanthocyanidins from elderberry and grape seed (Cheng et al., 2007b) shown the same trend. Model systems and synthetic or pure compounds are excellent to understand the effect of antioxidants on HAs formation. On the other hand, studies carried out on meat matrix using whole food or extracts as a source of antioxidants provide conditions as close as possible to practical applications (Damašius et al., 2011).

The application of antioxidants can be performed in several ways, by spreading on the meat surface (Murkovic et al., 1998; Damašius et al., 2011), cooking together with the meat (Melo et al., 2008; Janoszka, 2010), mixing with ground meat (Britt et al., 1998; Balogh et al., 2000; Tsen et al., 2006), or by making marinades (Busquets et al., 2006; Smith et al., 2008).

Antioxidant spreading on the surface usually consists in superficial application of herbs and spices or other seasonings. Murkovic et al. (1998) showed that the individual addition of dried spices (rosemary, thyme and sage) or garlic on the surface of the meat 24h prior to frying resulted in inhibition of HAs formation. Oz and Kaya (2011a; 2011b) reported high inhibitory effect on HAs formation by black pepper in fried high-fat meatballs and by red pepper in fried beef chops. In both cases spices were spread on the meat surface 12h prior to frying. Monti et al. (2001) reported that olive oil inhibited the formation of IQ-type HAs. In a recent study, Lee et al. (2011) described that the application of 2 or 4 g of extra virgin olive oil on beef by spreading prior to pan-frying considerably inhibited both types of HAs formation.

Janoszka (2010) pan-fried pork meat together with onion or garlic and observed inhibitory effect on HAs formation. Melo et al. (2008) also cooked meat together with ingredients commonly used in the Portuguese diet and rich in antioxidants but no effect was observed. Gu et al. (2001) observed that seasoning (soy sauce, sesame oil, sugar, garlic and onion powders applied 4h prior to cooking) had desmutagenic activity when meat was pan-roasted, but increased mutagenic activity was observed when meat was charcoaled with decreased levels of HAs, suggesting that the higher mutagenicity may be due to the presence of other co-mutagens formed in charcoaled meat.

Another way to evaluate the antioxidant effect on HAs formation is through the patties or hamburgers formed mixing ground meat with minced or powered spices or other antioxidants. Puangsombat et al. (2011) observed inhibitory activity on HAs formation of Asian spices in cooked beef patties. Tsen et al. (2006) showed that rosemary derivatives minced into the ground beef reduced both MeIQx and PhIP formation and overall mutagenic activity. Another work (Ahn and Grün, 2005) reported an effective decrease on formation of both polar and apolar HAs by natural

extracts of rosemary, grape seeds and pine bark. It should be highlighted that the evaluation of antioxidant effect against apolar HAs (pyrolytic HAs) was not usual. The effective inhibitory effect of vitamin E in muscle meat was well described (Balogh et al., 2000; Liao et al., 2009, Wong et al., 2012) the same trend was observed to water-soluble vitamins mixed with the beef prior frying.

Marinating meat with several ingredients before grilling or frying is a common way to improve flavor, tenderness, and moistness of the cooked product. Compared with the others antioxidants applications, marinades has the advantage that the cooked food, namely fried or grilled, are not over spiced and the muscle food do not acquire negative sensory properties (Gibis and Weiss, 2010). Since the first report describing that marinades reduced the HAs formation and mutagenicity (Tikkanen et al., 1996) several studies with this subject have been performed. Namely, the Salmon et al. (1997) work that evaluated the effect of a marinade made with a mixture of selected ingredients (containing olive oil, brown sugar, cider vinegar, lemon juice, crushed garlic), inhibition of PhIP and increased of MeIQx were observed.

Different ingredients (spices and liquids), different meat types and several marinades, based on cooking habits and culture (Lan and Chen, 2002; Busquets et al., 2006; Salmon et al., 2006), can be found in literature. Red wine (Busquets et al., 2006), plant extracts or spices (Ahn and Grün, 2005; Gibis and Weiss, 2010), or combined spice mixtures with other antioxidant rich liquids, such as soy sauce, oil or lemon juice (Salmon et al., 1997; 2006; Gibis, 2007; Smith et al., 2008) induces changes in formation of some HAs.

Antioxidants are known to exert anti- and pro-oxidative effects depending on many factors, such as cooking method, cooking conditions, technological factors, and type, combinations and concentrations of antioxidants (Zöchling et al., 2002; Alaejos and Afonso, 2011). Some differences have been found among model systems and real foods (Cheng et al., 2007a), furthermore one antioxidant compound can exert different effects in different HAs (Oguri et al., 1998; Damašius et al., 2011). In spite of the common general mechanism (section 1.2.2), the formation of each HA is explained by specific mechanisms and different precursors which income peculiarities of their molecular structure (Damašius et al., 2011; Dundar et al., 2012). These controversial findings and the search for effective ways to minimize the formation of HAs makes this subject stills a challenge.

1.2.7.2. Modulation of HAs metabolism by antioxidants

Epidemilogical findings suggest that diets rich in fruit and vegetables can protect against cancer. The phytochemicals present in these foods, such as dietary fiber and phenolic compounds may protect from the mutagenic and carcinogenic effects of food-borne carcinogens, as HAs (Schwab et al., 2000; Platt et al., 2010). The mechanisms of protection include inactivation of HAs and their

metabolites by direct binding, inhibition or induction of enzymes involved in activation or detoxifying pathways respectively, and acting on DNA repair mechanisms, tumor promotion and tumor progression (Schwab et al., 2000; Vitaglione and Fogliano, 2004).

Red wine and beer contain a large number of polyphenolic constituents that have been shown to block carcinogenesis and to inhibit the growth of tumors in animals, or in cell culture by modulation of the activity of certain enzymes or the expression of especific genes (Waterhouse, 2002).Thus, moderate consumption of red wine or beer is associated with reduced risk of cancer. An overview of the possible modulation of general metabolism of HAs by some red wine and beer compounds are described in next and summarized in Figure 2.

1.2.7.2.1. Modulation of HAs metabolism by red wine compounds

Several studies have shown that polyphenols from red wine, like resveratrol (Jang et al., 1997), quercetin (Elattar and Virji, 1999), (+)-catechin (Soleas et al., 2001) and gallic acid (Soleas et al., 2002) are potential cancer chemopreventive agents. However, in some cases the protective effects of wine components have not been associated with a specific fraction or compound, so it is not yet clear which compounds present in red wine are correlated with protective activity (He et al., 2008). Resveratrol is a compound found in juice and wine produced from dark-skinned grape cultivars (found at concentration between 1 and 10μM) and reported to have anti-inflammatory and anticarcinogenic activities (Soleas et al., 1997; Piver et al., 2003). Chun et al. (1999) investigated resveratrol anticarcinogenic activities, through the effects on CYPs. Resveratrol exhibited potent inhibition of human CYP1A1 in a dose-dependent manner. However, the inhibition of human CYP1A2 by resveratrol was not so strong. These results suggest that resveratrol, an important non-flavonoid phenolic compound of red wine is a selective human CYP1A1 inhibitor, and inductor of endogenous antioxidants and phase II enzymes such as GSH and GST and may be considered for use as a strong cancer chemopreventive agent in humans (Chun et al., 1999; Dubuisson et al., 2002). Other compounds identified in red wine are described as inhibitiors of CYP1A2 (quercetin and coumarin), CYP1A1 (quercetin and kaempferol) and SULT (quercetin, kaempferol, catechin and myricetin) (Dubuisson et al., 2002; Moon et al., 2006).

Concerning direct effect of plant phenols present in red wine on HAs biotransformation, ellagic acid, vannilic acid and ferulic acid, promoted a decrease in the mutagenicity of N-hydroxy-Trp-P-2 when a simultaneous treatment with mutagen and phenol was assayed *in vitro* (Arimoto-Kobayashi et al., 1999).

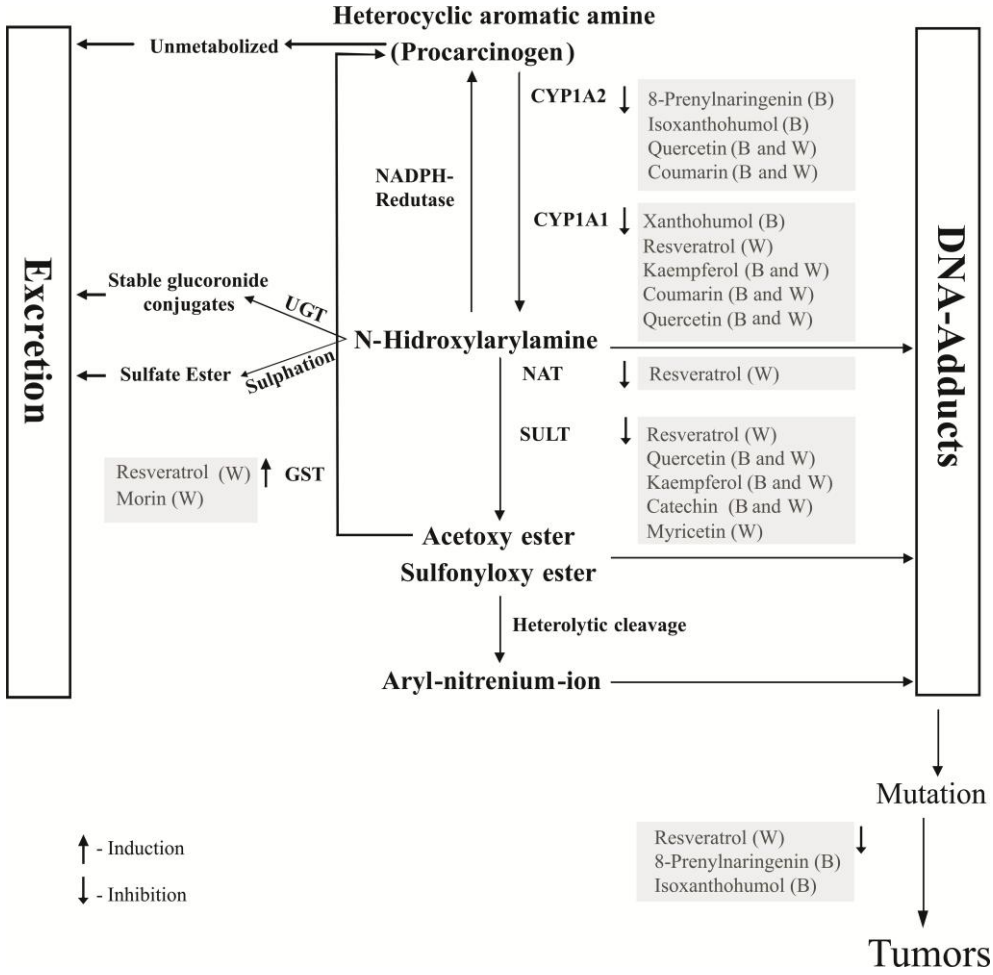


Figure 2. Modulation of general metabolism of HAs by some beer (B) and wine (W) compounds.

The effects of resveratrol on DNA binding via esterification reactions with the activated carcinogenic heterocyclic amine N-hydroxy-PhIP were evaluated by Dubuisson et al. (2002). Using substrate-specific assays, resveratrol inhibited PhIP-DNA adduct formation by *O*-acetyltransferase and sulfotransferase catalysis in human mammary epithelial cell. In addition to resveratol other preventive effects, these results suggest that *O*-acetyltransferases and sulfotransferases may represent antioncogenic targets for resveratrol (Dubuisson et al., 2002).

Boyce et al. (2004) revealed that resveratrol was potent in blocking the mutagenicity of MeIQx in *Salmonella typhimurium* and PhIP in Chinese hamster V79 cells at micromolar concentrations. These results confirm the potent anti-mutagenic activity of resveratrol and support its potential as a chemopreventative.

Recently Platt et al. (2010) showed that red wine strongly inhibited genotoxicity of PhIP, but against IQ genotoxicity it showed weak inhibitory effect. Authors reported that the effect was not by modulation of CYP1A2, but due to an inhibition of the second step of HAs activation.

1.2.7.2.2. Modulation of HAs metabolism by beer compounds

Beer contains many components presenting antimutagenic and anticarcinogenic properties, which have been suggested from *in vitro* and *in vivo* experiments (De Keukeleire et al., 2003). Most of these compounds are derived from hops and are unique in beer. In 1999, Arimoto-Kobayashi et al. (1999) reported antimutagenic affects of beer against several carcinogens. Additionally, phytoestrogenic compounds might exert antiestrogenic action and affect hormone-dependent carcinogenesis *in vivo* (Stevens and Page, 2004).

Xanthohumol (XN), isoxanthohumol (IX), 8-prenylnaringenin (8PN), and nine other prenylflavonoids from hops were shown to strongly inhibit, the cDNA-expressed human CYPs, CYP1A1, CYP1B1, and CYP1A2 (Henderson et al., 2004). The inhibitory effects of hop prenylflavonoids on CYPs may offer an explanation for the reported inhibitory effects of beer on mutagenesis and DNA adduct formation induced by carcinogens (Arimoto-Kobayashi et al., 1999; Henderson et al., 2004). An additional advantage of the hop flavonoids as chemopreventive agents for some types of carcinogens is their ability to induce the phase II detoxifying enzymes, such as, GST (Nozawa et al., 2006). As the ultimate outcome of carcinogenicity is dependent on a balance of bioactivation and detoxification enzymes, compounds which inhibit bioactivation while inducing detoxification could be particularly useful (Henderson et al., 2000).

Additionally, uncontrolled proliferation of tumor cells has been associated with inflammation and increased production of hormone-like mediators, such as, prostaglandins. Food constituents that can interfere with proliferation mechanisms are of great interest as cancer chemopreventive agents due to their long-term exposure.

Gerhäuser et al. (2002) showed that XN can be an effective anti-inflammatory agent by inhibition of endogenous prostaglandin synthesis through inhibition of cyclooxygenase (constitutive COX-1 and inducible COX-2) enzymes. Cyclooxygenase also may contribute to extra-hepatic bioactivation of HAs (Turesky, 2002). Prostaglandins are also known to initiate formation of new blood vessels (angiogenesis), an important event in tumor growth. The effect of 8PN on angiogenesis was studied by Pepper et al. (2004) who demonstrated that 8PN inhibits angiogenesis using an *in vitro* model in which endothelial cells can be induced to invade a three-dimensional collagen gel and form capillary-like tubes.

Several studies, have suggested that beer contains components with effects against mutagens and putative human carcinogens, such as azoxymethane (Hamilton et al., 1987; Nozawa et al., 2004a), dimethylhydrazine (Nelson and Samelson, 1985) and HAs (Arimoto-Kobayashi et al., 1999, 2005, 2006; Miranda et al., 2000; Edenharder et al., 2002; Nozawa et al., 2004b, 2006). The studies that describe the beneficial effects of beer against HAs, include *in vitro* and *in vivo* experiments. The

key for beer antimutagenicity appears to be the inhibition of the metabolic activation through the inhibition of CYPs, in particular CYP1A2 (Arimoto-Kobayashi et al., 1999).

Beer is the most important dietary source of related prenylflavonoids, derived from hops, such as XH, 8PN and IX (Stevens et al., 1999; Stevens and Page, 2004) that have received a lot of attention lately, owing to their inhibitory effects on CYPs which may offer an explanation for the reported inhibitory effects of beer on mutagenesis and DNA adduct formation induced by carcinogens (Henderson et al., 2000). XN, 8PN, IX and nine other prenylflavonoids from hops were shown to strongly inhibit the cDNA-expressed human CYPs, CYP1A1, CYP1B1, and CYP1A2, at low micromolar concentrations. Henderson, et al (2000) presented evidence that two of the prenylated flavanones, 8PN and IX, are the most effective inhibitors of CYP1A2, the major activation pathway of HAs in human, confined to liver, whereas the prenylated chalcone, XN, is the best inhibitor of CYP1A1 and CYP1B1, and CYP1A1 may play the most important route activation in extrahepatic tissues (Crofts et al., 1998; Turesky, 2007). These results were in agreement with those from Gerhäuser et al. (2002), for XN and IX using CYP1A.

A lot of attention is given to XN, because has been characterized as 'broad-spectrum' cancer chemopreventive agent in *in vitro* studies, while 8PN enjoys fame as the most potent phytoestrogens know to date (Stevens et al., 1999; Stevens and Page, 2004). However, IX is the principal beer prenylflavonoid because during the brewing process, XN (the most prevalent in hops), is converted to IX, and 8PN is a minor prenylflavonoid, thus, the major contribution of beer for inhibition of CYP1A2 is mainly from IX. However, a new technology has been developed which allows the production of beers with increased XN levels up to 20 mg/L (Wunderlich et al., 2005) which are already commercially available (www.xan.com).

Miranda et al. (2000) proved that XN, IX, and 8PN are strongly inhibitors of activation of IQ in Ames assay mediated by the recombinant human enzyme CYP1A2, and suggested that these compounds may have potential to act as chemopreventive agents against cancer induced by HAs through the CYP1A2. Plazar et al. (2007; 2008) observed, in the test systems with metabolically active human hepatoma HepG2 cells and precision-cut rat liver slices, XN completely prevented formation of IQ induced DNA damage at concentrations as low as 10 nM. Additionally, several works suggested beer inhibition against genotoxic effects of HAs and reduced risk of carcinogenesis caused by that food borne carcinogen, through *in vivo* experiments in rodents which are summarized in Table 4.

Table 4. Summary of *in vivo* studies that suggested that beer possesses anticancer promotion activity.

HAs	Target organ	Experimental conditions	Evaluated parameters	Ref.
PhIP	Colon	Rats fed with and without a diet containing freeze-dried beer	- Number of aberrant crypt foci (the putative preneoplasic-lesions for colon cancer)	Nozawa et al., 2004b
PhIP	Mammary	Rats fed with a high fat diet with and without freeze-dried beer	- Assay for CYP1A2, GST and DNA adducts - Tumor incidence and tumor multiplicity	Nozawa et al., 2006
MeIQx **Trp-P-2**	Liver and Lungs	Mice fed with and without a beer solution drink/beer mixed in the diet	- Formation of DNA adducts	Arimoto-Kobayashi et al., 2005
PhIP	Colon	Mouse fed with and without a beer solution	- Assay for CYP1A2, CYP1A1 and DNA adducts	Arimoto-Kobayashi et al., 2006

1.3. Polycyclic aromatic hydrocarbons

In 1775 Sir Percival Pott observed an unusually high scrotal cancers incidence in chimney sweeps attributing the cause to the soot. Later, along the 20th century, PAHs were identified as the carcinogenic chemicals compounds present in soot, confirming Pott observations (Šimko, 2002). At the present time PAHs comprise the largest class of known chemical carcinogens. PAHs have been implicated as causative agents of breast, lung, and colon cancers and have been associated with immunossupression, teratogenicity, neuro-, reproductive, and developmental toxicities, and acute or chronic toxicity in other tissues such as bone morrow and intestinal mucosa (IPCS, 1998; Ramesh et al., 2004).

PAHs are continuously produced and released into the atmosphere from natural and mostly from anthropogenic sources. They are primarily formed by incomplete combustion or pyrolysis of organic matter (incomplete burning of coal, oil, gas, wood, garbage or other organic substances), during various industrial processes, and geochemical processes (forest fires, volcanic activities) (EFSA, 2008). PAHs occur in considerable amounts in the environment (air, water, soils) and foods (Wenzl et al., 2006). As PAHs are ubiquitous environmental contaminants, humans are unavoidable exposed to them. In the non-tobacco smoking and non-occupationally-exposure population, diet may contribute to more than 70% of total PAH exposures (Philipps 1999;

European Commission, 2002; Martorell et al., 2010). The multiple sources of human exposure, however, make it difficult to assess the contribution due to food intake.

1.3.1. Toxicological classification

Exposure to PAHs is the major concern for human health. They are toxic; some of them carcinogenic and other non carcinogenic may act as synergists; persistent and bioacumulative compounds (Wenzl et al., 2006). Since they comprise the largest class of known chemical carcinogens it was not surprise their inclusion in the priority pollutant lists of competent organizations. In 1970s the Environmental Protection Agency (EPA) of the United States of America designed 16 PAHs, US-EPA PAHs, as compounds of interest based on their occurrence and carcinogenicity (Wenzl et al., 2006). In the International Programme on Chemical Safety (IPCS, 1998), other 17 PAHs were added to the 16 PAHs listed by US-EPA. Scientific Committee on food (SCF) (European Commission, 2002) concluded that 15 out of the 33 risk assessed PAHs, should be regarded as potentially genotoxic and carcinogenic to humans, and represent a priority group in the assessment of the risk of long-term adverse effects following dietary intake. SCF (European Commission, 2002) suggested Benzo[*a*]pyrene (BaP) as a marker of occurrence of the carcinogenic PAHs in food. However, in a screening of levels of PAHs in foods (European Commission, 2005) it was observed that one third of samples containing carcinogenic PAHs were negative for BaP. The 2002 SCF opinion was revised by EFSA (European Food Safety Authority) Panel on Contaminants in the Food Chain (CONTAM Panel). The CONTAM Panel maintained the prior 15 PAHs and added one 16th analyte to the list as suggested by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA, 2005). From the "15+1" PAHs list EFSA concluded that the eight PAHs (PAH8) - benzo[*a*]anthracene (BaA), chrysene (Ch), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), BaP, indeno[1,2,3-*cd*]pyrene (IP), benzo[*g,h,i*]perylene (BgP), dibenzo[*a,h*]anthracene (DhA) - are currently the possible indicators of the carcinogenic potency of PAHs in food. Considering the frequency of PAH8, the sum of BaA, Ch, BbF, BaP (PAH4) may provide enough information about this subject, so EFSA concluded that both PAH4 and PAH8 are the most suitable indicators of PAHs in food (EFSA, 2008).

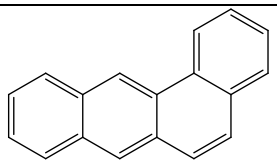
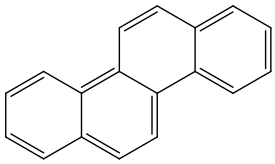
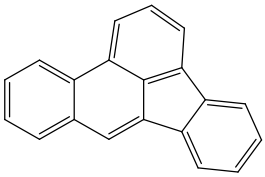
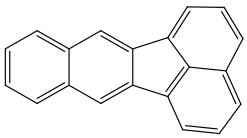
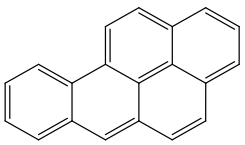
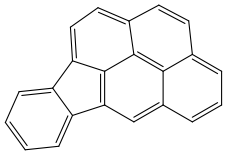
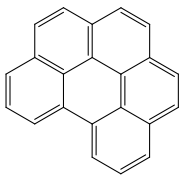
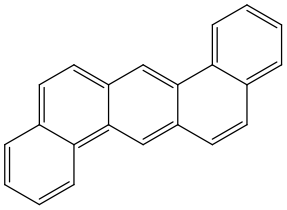
There is no general international agreement on the panel of individual PAHs should be analyzed and PAHs lists released from the different authorities may contain different compounds, however the priority PAHs from UE, PAH8, comprise the same eight carcinogenic PAHs from US-EPA list. In recent revised IARC (2010; 2012) classification, BaP was reclassified as *human carcinogen* and some other PAHs are classified in the other groups of IARC toxicity (Table 5).

1.3.2. Structure and formation of PAHs in muscle foods

PAHs represent a major class of organic compounds, comprising hundreds of compounds, each of them containing two or more fused aromatic rings, without other elements than carbon and hydrogen, in a linear or angular configuration, (Wenzl et al., 2006; EFSA, 2008; Luch and Baird, 2010). PAHs containing two to four fused benzene rings are called "light PAHs" and those containing five or more benzene rings are called "heavy PAHs". Heavy PAHs are more stable and more toxic than light ones (Wenzl et al., 2006). Table 5 presents the compound and abbreviated names, structures and IARC classification of PAH8.

Although the exact mechanism of formation of PAHs is not precisely known, it is generally considered that PAHs are formed by condensation of smaller organic compounds by either pyrolysis or pyrosynthesis. At high temperatures (> 400 ºC), organic compounds are easily fragmented (pyrolysis) and the free radicals produced recombine to form stable polynuclear aromatic compounds (pyrosynthesis). A common reaction may involve Diels – Alder - type rearrangements to yield the PAHs. PAHs formation is only significant at temperatures higher than 400°C and most of the thermal procedures of foods are below that temperature. However, procedures that involve incomplete combustion of woods or charcoal (e.g. smoking, grilling and barbecuing) easily can achieve high temperatures and consequently generate PAHs (Park and Penning, 2009).

Table 5. Compound name and abbreviations, chemical structure and IARC classification of PAH8.

Compound name	Abbreviation	Structure	IARC group
Benzo[*a*]anthracene	BaA		2B
Chrysene	Ch		2B
Benzo[*b*]fluoranthene	BbF		2B
Benzo[*k*]fluoranthene	BkF		2B
Benzo[*a*]pyrene	BaP		1
Indeno [1,2,3-*cd*]pyrene	IP		2B
Benzo[*g*,*h*,*i*]perylene	BgP		3
Dibenzo[*a*,*h*]anthracene	DhA		2B

At least three possible mechanisms exist in the PAHs generation in grilled meat. Although PAHs formation is favored at temperatures between 400-1000ºC, some endogenous formation of PAHs on the surface of the food through the pyrolysis of organic matter such as colestherol, tryglicerides, protein and carbohydrates at temperatures above 200 ºC can also occur. Another mechanism, especially relevant in smoked foods, is the contamination from the smoke generated by the

incomplete combustion of the heat source which can generate PAHs that are brought onto the surface of the food. It has been suggested that low molecular weight PAHs (containing 2-3 aromatic rings) arise from smoke generated during meat grilling as these PAHs are more volatile than high molecular weight PAHs (containing more than 3 aromatic rings) (Chen and Lin, 1997; Ferrarese et al., 2008; Park and Penning, 2009). A third mechanism, accountable to the greatest concentrations of PAHs, has been shown to arise from pyrolysis of fat. When melted fat drips down on the intense heat source generates these compounds that are carried back to the food surface (Farhadian et al., 2010), and fat also induces flashing which increases the temperature near the food (Sinha et al., 1995). The fat content is an important factor affecting the PAHs level in muscle foods (EFSA, 2008; Farhadian et al., 2010).

1.3.3. Separation and quantification of PAHs in muscle foods

PAHs extraction and quantification in charcoal meat and fish samples is difficult because they occur in food at low ppb or lower levels and many other organic components such as proteins, lipids and compounds similar to PAHs that also result from thermal processing, such as heterocyclic aromatic amines that can be co-extracted from the matrix (Pan and Cao, 2010). Additionally, meat and fish contain plenty of lipids, which have similar polarity to PAHs, the variable fat content influences extraction yield, and appropriate conditions for each type of food should be selected.

The most common methods for the isolation of PAHs from foods involve saponification of lipids by methanolic KOH solution followed by extraction procedures to isolate the PAHs-containing fraction. Different procedures are described for PAHs extraction, namely, liquid–liquid, Soxhlet or sonication extraction (Janoszka et al., 2004; Brasseur et al., 2007; Pan and Cao, 2010), solid-phase extraction (SPE) (Rivera et al., 1996; Moret and Conte, 2002; Janoszka et al., 2004; Purcaro et al., 2008; Farhadian et al., 2010), solid-phase microextraction (Vichi et al., 2005; Purcaro et al., 2007a; 2007b;), supercritical-fluid extraction (Lage Yusty and Cortizo Davina, 2005) and microwave-assisted extraction (Akpambang et al., 2009; Purcaro et al., 2009). Owing to the complexity of the matrices these methods, in general, present inconstant recoveries and in some cases interfering peaks in the chromatograms.

High performance liquid chromatography with fluorescence or diode array detection has often been used for quantitative determination of PAHs (Chen et al., 1996; Rivera et al., 1996; Moret and Conte, 2002; Janoszka et al., 2004; Brasseur et al., 2007; Varlet et al., 2007; Purcaro et al., 2008; 2009; Akpambang et al., 2009; Farhadian et al., 2010; 2011; Pan and Cao, 2010). The eluent usually used for chromatographic separation is acetonitrile–water under isocratic conditions (Janoszka et al., 2004) or gradient elution (Akpambang et al., 2009; Purcaro et al., 2009).

Water–methanol–acetonitrile (Brasseur et al., 2007) is also described for PAHs separation by high performance liquid chromatography/ fluorescence detection (HPLC/FLD). Alternative elution solvents can be used to make PAHs analysis less expensive, namely methanol–water, however, in general poor peak resolution is obtained (Pan and Cao, 2010).

External calibration curve method is currently the most popular in the quantitative determination of PAHs in various food matrices. However, analyte losses can occur in the course of sample preparation due to incomplete extraction, and cause underestimated results. At the same time, overestimated results can also be obtained (matrix effect) if the chromatographic separation of PAHs and co-extractive substances is inadequate. The use of the external calibration curve method for the quantitative evaluation of PAHs does not take into account the effect of systematic errors due to either sample preparation or chromatographic separation and detection. The standard addition method has not found use in the determination of PAHs in food matrices. This method implies the use of the analyte as an internal standard, the number of sample preparation operations increase, but the effect of systematic errors decreases, thus it can be a good choice when low levels of analyte are quantified.

1.3.4. Occurrence of PAHs in cooked foods

PAHs occur as contaminants in different food categories, drinking water and beverages. They can reach the food chain by different ways: environmental contamination, through the air (by direct deposition from the atmosphere), soil (by transfer) and/or water (deposition and transfer), e.g. fruits, vegetables and seafood; contamination from packaging materials; and contamination (from wood fumes) and/ or generation (from food components) of PAHs during the thermal processing of foods, that occur in processes of food preparation and manufacture (drying and smoking) and cooking (roasting, baking, grilling and frying) (EFSA, 2008; Plaza-Bolaños et al., 2010).

The highest PAHs concentration are usually found in charcoal grilled foods (such as fatty meat and meat products grilled under prolonged and severe conditions) and contributes significantly to the intake of PAHs if such foods are a large part of the usual diet (Philips, 1999; EFSA, 2008). Considering cooking methods other than grilled the amounts of PAHs produced are usually low. In general PAH levels produced in grilled muscle food increases with increased fat content (kind of food), closeness to the heat source and longer time to reach the required doneness. The heat source and geometry are also important, wood and charcoal grill may arise more PAHs than oven or electric grill, and vertical grills forms very low levels compared with horizontal apparatus (EFSA, 2008).

Kazerouni et al. (2001) screened 200 food items for BaP and found that well and well-done grilled/ barbecued meats exhibit the highest concentration, yielding values around 5 ng/g. In meat samples

(beef, chicken and pork) grilled/ barbecued to medium done and in all oven-broiled, baked, roasted or pan-fried meat samples regardless of doneness level, in general the values were low than 0.2 ng/g. Similarly to HAs (Sinha et al., 1998; Ni et al., 2008), there was a striking difference in the BaP concentrations between whole beef steaks and hamburgers, lower amounts are formed in the ground beef patties.

In many studies concerning cooked muscle foods, the focus has been only in BaP or BaP with few other PAHs. One exception was the study performed by White et al. (2008), which investigated the effects of several cooking techniques on the formation of 28 PAHs, including all the 16 EU PAHs, in foods prepared in the home (256 samples) and cooked retail products (77 samples). BaP concentrations were negligible in the majority of retail products, only three, all beef burgers, contained higher BaP concentrations (8.4, 9.8 and 20.4 ng/g). Concerning home cooking practices, in general there was little evidence of PAH formation during the grilling, frying, and roasting experiments. In contrast, barbecuing (4 and 7 cm of heat source) with charcoal plus wood chips gave the highest BaP levels in each type of food. The maximum BaP concentrations were found in barbecued over charcoal beef burgers and pork sausages, 29.1 and 30.6 ppb, respectively, whereas median concentrations were 12.2 and 1.9 ppb, respectively. The maximum BaP concentrations in other types of barbecued food were considerably lower than for sausages or beef burgers.

Mottier et al. (2000) determined the 16 US-EPA in different barbecued sausages (10 cm of heat source) and the sum of carcinogenic PAHs (PAH8 of EFSA) was higher in barbecued sausage lamb, 1.88 ng/g, and when the same sample was heavily barbecued the sum of PAH8 content was 13.17 ng/g. Authors highlighted that results are in accordance with previous findings, where PAHs formation during charcoal grilling was dependent upon the fat content of the meat, the time of cooking and the temperature. The higher values achieved by White et al. (2008) compared with Mottier et al. (2000) were probably due to the closeness to the heat source.

EFSA (2008) presented mean barbecued meat (39 samples) concentrations of BaP and PAH8 as 1.92 and 7.96 ng/g respectively. Concerning the mean grilled meat samples (53 samples) concentrations of BaP and PAH8 as 0.63 and 3.48 ng/g respectively. According with IARC grilled meat in general was estimated to contain 10.5 ng/g BaP (IARC, 1993).

1.3.5. Metabolism of PAHs

PAHs are not mutagenic/carcinogenic in their native form but acquire the capability of forming DNA adducts after metabolic activation.

Liver is the major organ for PAHs metabolism. However, other organs may play a greater role depending on the site of PAHs entry. In the case of ingestion, gut micro flora and intestinal cytochrome P450 enzymes can contribute to PAHs metabolism (Ramesh et al., 2004).

To form highly reactive intermediates PAHs can undergo at least three major metabolic pathways (Xue and Warschawsky, 2005). The quantitatively most important is the bay region dihydrodiol epoxide pathway, it is catalyzed by CYP enzymes, particularly CYP1A1, 1A2 and 1B1, and microsomal epoxide hydrolase (EH), leading to formation of electrophilic bay-region diol-epoxides capable of binding to DNA. Another pathway, may relevant for certain PAHs, is via radical cation by one-electron oxidation catalyzed by P450 peroxidase, resulting in the formation of unstable DNA-adducts that can spontaneously generates apurinic sites. The third major pathway involves the formation of PAH-*o*-quinones, catalyzed by dihydrodiol dehydrogenases (DD). The *o*-quinones can form both stable and depurinating DNA adducts; furthermore this metabolic pathway generates high levels of reactive oxygen species (ROS) that can also attack the DNA. A minor metabolic activation of PAHs, *O*-sulfonation pathway by SULTs, with formation of reactive benzylic esters from methyl-substituted PAHs has been described as relevant under certain conditions (Xue and Warschawsky, 2005; Luch and Baird, 2010).

Based on their chemical reactivity, PAH metabolites generated during metabolism can undergo enzymatic detoxification by phase-II enzymes. Nucleophilic metabolites regularly undergo conjugation to glucuronic acid or sulfate by UGT or SULT. Electrophilic metabolites mainly generate glutathione conjugates by GST. Concerning ROS induced by PAHs metabolism may undergo neutralization by a great variety of additional protective systems (Luch and Baird, 2010).

The relative contribution of these competing pathways is an unknown but important issue and it apparently depends on the nature of the PAH, level of expression of the activation enzymes, metabolic site, and so forth (Xue and Warschawsky, 2005). The active metabolites predominantly attack DNA through the exocyclic amino groups of guanine and adenine amino groups. The major adduct is formed on the N2 position of desoxyguanosine (EFSA, 2008).

1.3.6. Mitigation of PAHs formation

Following a number of evaluations, the IARC has come to the conclusion that several of these food-borne toxicants present in cooked foods are *possibly or probably* carcinogenic to humans, and recently classified one PAH, BaP, as carcinogenic to humans (IARC, 2012). Although their presence at ng/g levels in cooked foods, the competent authorities in most Western countries recommend minimizing cooking toxicants occurrence (Jägerstad and Skog, 2005). It is therefore recommended minimizing HAs occurrence in foods consumed by humans.

Because exposure is ubiquitous, effective reduction from all sources is not possible. Furthermore, grilled foods are popular both at home and in restaurants, these foods may present a health risk to

the population due to higher concentrations of carcinogens found (Farhadian et al, 2011) and PAHs formation.

Various barbecuing practices have demonstrated that certain home cooking practices may lead to PAHs levels that are more than one magnitude higher compared to PAH concentrations in foods prepared with proper practices (EFSA, 2008).

Formation of PAHs during cooking of food can be reduced if excess fat is trimmed from meat and meat is cooked at lower temperatures, or short time, or selecting an appropriate distant from charcoal avoiding the contact with the heat source (Lijinsky and Ross, 1967). To minimize PAHs formation techniques that avoid the pyrolysis of fat that drops into the flames should be preferred to grilling, namely electric grills with the heat source above the food, or other grills of appropriate design as a vertical apparatus (Saint-Aubert et al., 1992) or having a water-filled pan underneath the electric resistance (Lintas et al. 1979). Charcoal grilling should be carried out over the embers, and avoid the direct contact of meat with the cooking flame or grill at high temperatures should be avoided (Chen and Lin, 1997).

Recent studies have been emerging with new mitigation strategies concerning PAHs formation in grilled foods. Farhadian et al. (2011) showed that the applied preheating (steam and microwave) and wrapping treatments (aluminium and banana leaf) to the samples (beef and chicken) strongly reduces the PAHs levels in the charcoal-grilled meat. Janoszka (2011) observed that onion (30/100 g of meat) caused an average decrease of 60% of the total content of PAHs in pan fried pork meat and garlic (15/100 g of meat) lowered the concentration of 54%. Author justified the observed effect with the presence of many compounds with antioxidant activity which may scavenge free radicals involved in PAHs formation.

2. Results

2.1. Section A

To determine the levels of the main heat generated hazard compounds in barbecued foods and to examine in which way their formation can be reduced.

2.1.1. Heterocyclic aromatic amines formation in barbecued sardines (*Sardina pilchardus*) and Atlantic salmon (*Salmo salar*) (**A1)**

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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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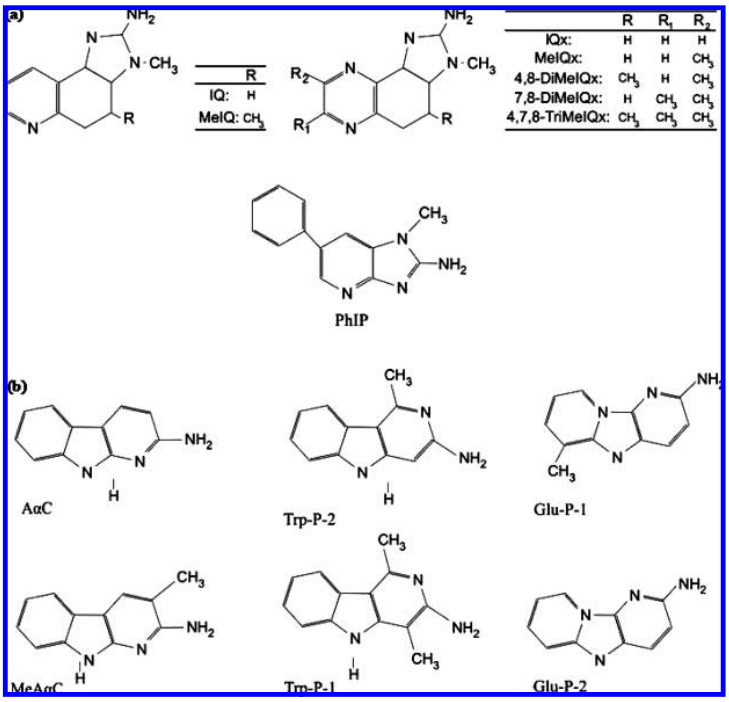

Figure 1. (**a**) Structures of thermic HAs, IQ and IQx types; (**b**) structures of pyrolytic HAs.

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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2.1.2. A comparison of the extraction procedures and quantification methods for the chromatographic determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish

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A comparison of the extraction procedures and quantification methods for the chromatographic determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish

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ABSTRACT

A method for analysis of 15 PAHs in charcoal-grilled meat/fish was established by high performance liquid chromatography and fluorescence detection. Gradient elution was performed with methanol/water/ethyl acetate. Maxima excitation and emission wavelengths were selected for each PAH. Retention times were very stable with coefficients of variation below 0.24% within analytical day and below 0.60% across analytical days. Two different methods of cleanup and pre-concentration steps were compared. Solvent extraction assisted by sonication carried out with *n*-hexane on 2 g of lyophilized meat or 1 g of lyophilized fish allowed to obtain high sensitivity, reproducibility and better extraction efficiency. Limits of quantification (LOQs, $s/n = 10$) were lower than 0.01 ng/g of meat wet weight and lower than 0.02 ng/g of fish wet weight for all PAHs (except for Na, Fl and IP that were lower than 0.1 ng/g). Two different quantification methods were compared. Standard addition method compensated PAHs losses due to incomplete extraction and it is recommended for analyses of grilled meat and fish samples that usually contain very low amounts of the eight high molecular weight PAHs (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA).

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are primary formed during incomplete burning (pyrolysis) of carbon-containing materials, such as oil, wood, garbage or coal. Environmental contamination and food processing are the main reasons for the presence of PAHs in foodstuff [1,2]. In mammalian cells PAHs undergo metabolic activation to diol epoxides that bind covalently to cellular macromolecules, including DNA, thereby causing errors in DNA replication and mutations that start the carcinogenic process [3].

The US Environmental Protection Agency (US-EPA) proposed to use a selection of 16 PAHs which are frequently found in environmental monitoring samples, namely, naphthalene (Na), acenaphthene (Ac), acenaphthylene (Ace), fluorene (F), anthracene (A), phenanthrene (Pa), fluoranthene (Fl), pyrene (P), benzo[a]anthracene (BaA), chrysene (Ch), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DhA), benzo[g,h,i]perylene (BgP), indeno[1,2,3-cd]pyrene (IP) [4]. In 2005 EU recommended monitorization of 15 priority PAHs including eight high molecular weight from the US-EPA list (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA). BaP was chosen as a marker of the occurrence and carcinogenic potency of the entire class of carcinogenic and genotoxic PAHs [5]. EFSA CONTAM Panel (2008) concluded that BaP is not a suitable indicator for the occurrence of PAHs in food and that eight high molecular weight PAHs (PAH8) are the most suitable indicators of PAHs in food [6]. Recently, Commission Regulation (EU) n° 836/2011 of 19 August 2011 established analytical performance criteria for BaP, BaA, BbF and Ch in relevant food matrices [836/2011] [7].

The consumption of smoked meat or fish has been associated to high incidence of stomach cancer in some population that consume such products with high frequency [8]. The amount of PAHs in smoked meat and fish have been extensively reported [9–11]. However, information concerning grilled foods is scarce.

When food, particularly, meat and fish, are cooked over an open flame, PAHs are formed [12,13]. If the meat/fish is in direct contact with flame, pyrolysis of the fats from the meat/fish generates PAHs that can become deposited on its surface. Even if not in direct contact, fat dripping on to the flame or hot coals generates these compounds that are carried back on the surface of the meat/fish [13]. The presence of PAHs in charcoal grilled meat/fish should be a matter of concern and alert to consumers, because even if present in low levels, the intake of this type of food can be quite frequent and

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representing a high portion (~120 g or more per meal). However, PAHs extraction and quantification in charcoal grilled meat and fish is difficult because they occur in food at ppb or lower levels and many other organic components such as proteins, lipids and compounds similar to PAHs that also result from thermal processing, such as heterocyclic aromatic amines that can be co-extracted from the matrix [14]. Additionally, meat and fish contain plenty of lipids, which have similar polarity to PAHs, the variable fat content influences extraction yield, and appropriate conditions for each type of food should be selected.

The most common methods for the isolation of PAHs from foods involve saponification of lipids by methanolic KOH solution followed by extraction procedures to isolate the PAHs-containing fraction. Different procedures are described for PAHs extraction, namely, liquid–liquid, Soxhlet or sonication extraction [14–16], solid-phase extraction (SPE) [13,15,17–19], solid-phase microextraction [20–22], supercritical-fluid extraction [23] and microwave-assisted extraction [24,25]. Owing to the complexity of the matrices these methods, in general, present inconstant recoveries and in some cases interfering peaks in the chromatograms.

High performance liquid chromatography with fluorescence or UV diode array detection has often been used for quantitative determination of PAHs [13–19,23–29]. The eluent usually used for chromatographic separation is acetonitrile–water under isocratic conditions [15] or gradient elution [24,25]. Water–methanol–acetonitrile [16] is also described for PAHs separation by high performance liquid chromatography/fluorescence detection (HPLC/FLD). Alternative elution solvents can be used to make PAHs analysis less expensive, namely methanol–water, however, in general poor peak resolution is obtained [14].

External calibration curve method is currently the most popular in the quantitative determination of PAHs in various food matrices [12–30]. However, analyte losses can occur in the course of sample preparation due to incomplete extraction, and cause underestimated results. At the same time, overestimated results can also be obtained (matrix effect) if the chromatographic separation of PAHs and co-extractive substances is inadequate. The use of the external calibration curve method for the quantitative evaluation of PAHs does not take into account the effect of systematic errors due to either sample preparation or chromatographic separation and detection. The standard addition method has not found use in the determination of PAHs in food matrices. This method implies the use of the analyte as an internal standard, the number of sample preparation operations increase, but the effect of systematic errors decreases, thus it can be a good choice when low levels of analyte are quantified.

The aim of this study was to compare two extraction procedures and two quantification methods for the accurate determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish by HPLC/FLD using an acetonitrile free eluent. Fifteen EPA-priority PAHs were selected as they include the eight PAHs previously indicated as the most suitable indicators of the presence of carcinogenic and genotoxic PAHs in foodstuffs [6]. Two different extraction and clean-up approaches were adapted from methods described in the literature [15,24]. The first approach was based on saponification, followed by extraction on diatomaceous earth and use of tandem solid-phase extraction with propylsulphonic acid (PRS) and silica (SiO_2) SPE columns as stationary phase [15]. The second approach was based on extraction of PAHs from lyophilized meat/fish with *n*-hexane and clean-up with SiO_2 cartridges [24]. The extraction procedure that presented best sensitivity, precision and accuracy was used for comparison between quantification by external calibration curve method and standard addition method.

2. Materials and methods

2.1. Reagents and standards

All the solvents used were of HPLC grade (Sigma, St Louis, MO, USA). Water was purified with a Milli-Q System (Millipore, Bedford, MA, USA). The standard PAHs mixture in 1 ml of acetonitrile (Supelco, Bellefone, PA, USA) consisted of: 10 μg/ml of Na, Ac, Ace, F, A, Pa, Fl, P, BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA (47940-U, Supelco, Bellefonte, PA, USA). The glassware, mostly amber, was carefully washed and rinsed with distilled solvent (acetone and hexane) before use.

2.2. Apparatus

An ultrasonic bath (FungiLab SA, Barcelona, Spain) was used to carry out the extraction step. Separation and quantification of PAHs was performed by HPLC/FLD. The chromatographic analysis was carried out in an analytical HPLC unit (Jasco, Japan) equipped with one Jasco PU-1580 HPLC pump, a MD 910 and a type 7125 Rheodyne injector equipped with a 20 μL loop. The column was a C18 reversed phase: Supelcosil™ LC-PAH (25 cm length; 4.6 mm internal diameter; 5 μm particle size) (Supelco, Bellefonte, PA, USA), thermostated at 32.0 ± 0.2 °C. The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used. The mobile phase was as follows: solvent A: 75% methanol (in water); solvent B: 100% methanol, solvent C: 100% ethyl acetate with a flow rate 1 ml/min. The linear gradient program was: 0–18 min, 0–80% B in A; 18–19 min, 80–100% B in A; 19–20 min, 100–90% B in C; 20–28.5 min, 90–82% B in C; 28.5–37.5 min, 82–80% B in C; 37.5–40 min, 80–100% B in C, 40–45 min 100–0% B in A, rinsing and re-equilibration of column to the initial conditions. Excitation/emission wavelengths selected were 276/330 nm for Na, Ac and F; 250/336 nm for Pa; 250/402 nm for A; 270/460 nm for Fl; 270/390 nm for P, BaA and Ch; 260/430 nm for BbF; 290/410 nm for BkF, BaP, DhA, and BgP; 290/470 nm for IP.

2.3. Samples

The meat samples used in this study were obtained from the *Longissimus dorsi* muscle of middle-aged bovine carcasses. The meat was obtained from a major butchery in Porto, Portugal. The beef samples six steaks with 1.5 cm of thick, and weighing about 253 g (±49.4 g) were chilled for 24 h in a cooling room (5 ± 1 °C). Following the chilling process, all trimmable fat and connective tissue (epimysium) were removed from the *Longissimus dorsi* muscle.

Samples of fresh salmon were obtained in a fish market in the same city. Three fillets of salmon with 2 cm of thick and weighting 236.6 (±50.65 g) were prepared.

For preparation of charcoal barbecued meat and 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 meat and fish were then barbecued over charcoal samples close to the heat source (10–12 cm). The meat and fish were turned once during grilling at half the total cooking time. No salt or oil was applied to meat and fish before or after grilling. Temperatures were measured by using a digital thermocouple (0560 9260, Testo 926, Lenzkirch, Germany) with a surface probe (0603 1992, Testo 926, Lenzkirch, Germany). The temperature near to the charcoal was 280–300 °C. To obtain "well-done" doneness, the grilling time was 9 min for beef, and 15 min for salmon. After cooking, the three samples of each kind of muscle food were mixed all together in a grinder, obtaining a representative and homogeneous amount of sample.

2.4. Sample pre-treatment, extraction and clean-up

Two different extraction approaches were adapted from methods described in the literature. The first approach (extraction method 1) for extraction and clean up procedures was based on the method described by Janoszka, et al. [15] for identification and quantitative analysis of five PAHs in meat. Meat samples were extracted by saponification, followed by extraction on diatomaceous earth and use of SPE columns filled with PRS. The eluted PAHs fraction was cleaned by SPE SiO_2 column, packed manually. Briefly, each meat/fish sample (5 g) was homogenized for 1 min shaking with a vortex shaker (Vortex mixer, EU-plug, VWR International, Darmstadt, Germany), in 25 ml of cold NaOH solution (1 M). Each sample was mixed with Extrelut refill material, diatomaceous earth (17 g) and the mixture obtained was placed in a 20 ml Extrelut column (Extrelut®, Merck, Darmstadt, Germany). Then the column was connected to a 500 mg PRS SPE column (Bond Elut PRS, 500 mg, 3 ml from Agilent Technologies, USA), where the PAHs fraction was retained, and eluted with a 95:5 (v/v) dichloromethane/toluene (60 ml). The dichloromethane extract was evaporated to dryness with a rotatory evaporator (Rotavapor Büchi RE-111, coupled with a water bath Büchi 461, BÜCHI, Flawil, Switzerland) at 40 °C and the residue was dissolved in *n*-hexane (1 ml), the residue was then placed on the top of the column which was manually packed with deactivated silica gel (10 g) and preconditioned by using *n*-hexane (25 ml). The column was eluted with 60:40 (v/v) *n*-hexane/dichloromethane (60 ml) to collect the PAHs fraction. The solvent was evaporated by rotatory evaporator and the PAHs residue was dissolved in acetonitrile (100 μl) and injected into the HPLC/FLD.

The second approach (extraction method 2) for extraction and clean up procedures was based on the method of Moret and Conte [18], applied for quantification of 15 PAHs in vegetable oils [18] and smoked meat [24]. A representative amount of meat sample was freeze-dried with a freeze dryer (Cryodos-90, from Telstar®, Terrassa, Spain) and reduced to a fine powder with a knife mill (Grindomix GM 200, Retsch, Hann, Germany). The lyophilization step has the advantage to eliminate water facilitating extraction of PAHs with *n*-hexane (24). Briefly, 2 g of meat lyophilized were weighed into a flask, added of 20 ml of *n*-hexane and sonicated for 1 h at room temperature. After this period the sample was filtered on paper, the solvent was evaporated in a round flask and taken near to the dryness with a rotatory evaporator at room temperature. The *n*-hexane residue was quantitatively transferred with 3 ml of *n*-hexane into a 5 g silica cartridge (Mega BE-Si, 5 g, 20 ml, from Agilent Technologies, USA) (previously washed with 20 ml of dichloromethane, dried completely by means of vacuum, and conditioned with 20 ml of *n*-hexane), and eluted through the column with 17 ml of a mixture of *n*-hexane/dichloromethane (70:30) (v/v). The first 8 ml of eluate were discharged and the following fraction, containing the PAHs was collected in a vial. The flow rate was adjusted at about 1 drop per second.

The collected fraction was evaporated to dryness under nitrogen stream at room temperature, in order to minimize volatile losses. The residue was dissolved in 100 μl of acetonitrile and injected into the HPLC/FLD.

2.5. Analytical performances

The validation of HPLC/FLD method for quantification of PAHs in barbequed meat and fish was accomplished by testing the linearity, the detection limit, the precision (repeatability and reproducibility) and the accuracy.

The linearity of the method was checked through the calibration curves, which were calculated for each PAH and obtained by linear regression of the peak area versus concentration of each PAH in the injected solution. The detection limit values (LODs) were based on a signal-to-noise ratio of 3:1, and the quantification limits (LOQs) were established as the amount of analyte that produces a signal-to-noise of 10:1.

One standard solution containing the mixture of PAHs was analyzed daily, repeating the analysis over three days. The repeatability was calculated as the RSD of peak areas and retention times across days. The reproducibility was studied by running three consecutive replications of the same mixture and calculating the RSD for peak areas and retention times.

The precision of the two extraction approaches was evaluated by estimating the standard deviation of three different extractions of the same meat sample. Recovery studies were carried out to determine the accuracy of the two extraction procedures. The extraction procedure that presented best precision and accuracy was further improved to guarantee reliable quantification of PAHs even if present in trace amounts.

The identities of the compounds were established by comparing the retention times of the peaks with those obtained from a standard mixture of PAHs and from spiked samples analyzed under the same conditions. Quantification of PAHs in meat and fish samples was performed by external calibration curve method and by standard addition method (using two fortified levels 20 and 40 ng of PAHs for fish samples and 10–20 ng/g for meat samples and unspiked samples).

2.6. Statistics

The averages of triplicate analysis were calculated for each PAH. The results were statistically analyzed by analysis of variance. Differences (*t*-test) were considered significant for $p < 0.05$. Statistical analyses were all performed with SPSS for Windows version 18 (SPSS Inc, Chicago, IL).

3. Results and discussion

3.1. Validation of HPLC/FLD method for PAHs separation

The mobile phase containing three solvents: 75% methanol (in water), 100% methanol, and 100% ethyl acetate was selected as an alternative elution, less expensive than acetonitrile for PHAs elution. The addition of ethyl acetate improved resolution between DhA and BgP. The gradient conditions described in Section 2.2 allowed resolving correctly all target compounds as shown in Fig. 1.

Excitation and emission wavelengths were selected from literature in order to choose the most appropriate excitation and emission wavelengths for each PAH under study, creating a new program to obtain higher sensitivity [19,24,27].

To evaluate the analytical performance of the HPLC-FLD method, calibration curve parameters, limits of detection, limits of quantification, repeatability or run-to-run precision and reproducibility or day-to-day precision were determined. Results are summarized in Table 1. Calibration curves were constructed by injecting in duplicate 7 diluted standard solutions (in the range 0.2–500 ng/ml) and plotting the mean peak area against PAHs standard concentration. For all peaks, there was a tight relationship between the amounts of each PAH and the detector response as indicated by *r* values that exceeded 0.99. Limits of detection ranged between 0.07 and 0.47 ng/ml and limits of quantification ranged between 0.22 and 1.44 ng/ml. Results indicate that the precision (repeatability and reproducibility) was good and comply with the requirements of the criteria for the chromatographic separation found in the European guidelines (Commission Regulation (EU) No 836/2011) [7]. The RSD values for retention times (RT) were below 0.24% within analytical day (repeatability) and below 0.60% across

Table 1
Parameters of regression equations for calibration curves, limit of detection (LOD), limit of quantification (LOQ), repeatability and reproducibility for PAHs under study.

PAHs	Slope[a] (area count/ng)	Intercept[a] (area count)	Regression coefficient	LOD (ng/ml)	LOQ (ng/ml)	Repeatability ($n=3$)		Reproducibility ($n=6$)	
						RT (RSD%)	Area (RSD%)	RT (RSD%)	Area (RSD%)
Na	412.8	2162	0.9985	0.22	0.73	0.24	0.75	1.24	5.54
Ac	1119	5997	0.9982	0.07	0.22	0.15	0.60	1.42	4.88
F	1176	6249	0.9984	0.13	0.37	0.15	0.95	1.42	8.05
Pa	1376	3170	0.999	0.13	0.37	0.13	0.57	1.33	4.07
A	7324	45493	0.9977	0.07	0.22	0.12	0.50	0.36	6.98
Fl	845.1	5245	0.9977	0.46	1.43	0.12	0.43	1.07	5.37
P	3229	17908	0.998	0.07	0.22	0.12	0.39	1.99	8.17
BaA	4556	12762	0.997	0.07	0.22	0.07	0.33	0.44	5.15
Ch	2284	6235	0.997	0.12	0.37	0.06	0.34	0.24	5.10
BbF	2159	1989	0.998	0.07	0.22	0.08	0.34	0.62	7.76
BkF	13352	21301	0.997	0.07	0.22	0.09	0.34	0.59	6.35
BaP	7298	-23575	0.996	0.07	0.22	0.10	0.35	3.36	6.91
DhA	2593	730	0.998	0.07	0.22	0.12	0.34	3.79	7.75
BgP	2029	1876	0.997	0.07	0.22	0.11	0.30	3.44	8.15
IP	400.4	-25.7	0.997	0.47	1.44	0.14	0.29	2.16	4.49

[a] Seven points were considered for the regression. Each point represents the average of two injections of each standard solution.

analytical days (reproducibility). Values of RSD for peak areas were below 3.79% within day and below 8.17% across days. All RSD values were similar to those reported in literature for within- and between-days variation [15].

3.2. Improvement of extraction procedure for meat/fish

The precision (RSD%) and recoveries (%) data obtained for charcoal-grilled meat samples were determined by two different extraction procedures (extraction method 1 and extraction method 2). The recoveries were calculated (in triplicate) by comparing the difference between spiked and unspiked meat samples with two levels of PAHs added (Table 2). The RSD% of triplicate extractions was evaluated. As can be seen in Table 2, the recoveries for meat samples ranged from 15.37 to 145% and from 16.1 to 82.12%, respectively for extraction method 1 and 2. Several authors also describe great variation on recovery percentages for analyses of PAHs in thermally treated meat [14,15,23,27]. Pan and Cao describe recoveries ranging between 68.5 and 102.8% [14] for the same PAHs using saponification followed by solid phase extraction, Purcaro et al. [25] reported recoveries that ranged between 9.7 and 102.5% for the analyses of the same PAHs using microwave-assisted extraction.

Method 1 uses 5 g of wet meat samples and involves several steps of extraction and clean up, finally, the residue is dissolved in 100 μl of acetonitrile, whereas method 2 uses 2 g of lyophilized meat sample (corresponding to 4 g of wet sample used in method 1) and few purification steps, only extraction with *n*-hexane and purification with silica column, finally, the residue is dissolved in 100 μl of acetonitrile. Method 2 with the referred modifications

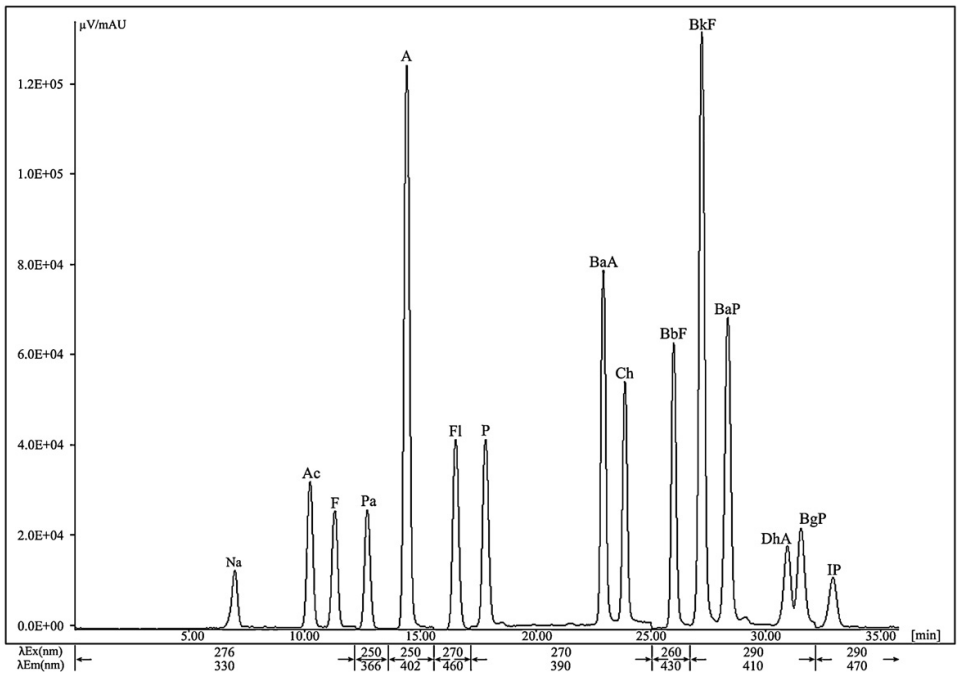

Fig. 1. HPLC chromatogram of standard solution (500 ng/ml). For conditions, see text.

Table 2
Recovery and precision of PAHs, obtained for charcoal-grilled meat samples, determined by two different extraction procedures.

PAHs	Extraction method 1						Extraction method 2					
	Initial content (ng/g)[a]	RSD (%)	Addition (ng/g)[a]	Measured content (ng/g)[a]	RSD (%)	Recovery (%)	Initial content (ng/g)[a]	RSD (%)	Addition (ng/g)[b]	Measured content (ng/g)[a]	RSD (%)	Recovery (%)
Na	1.91	30.79	8	3.23	58.22	16.5	2.90	26.76	10	3.58	57.06	16.12
			16	5.01	36.52	19.37			20	5.64	76.21	16.30
Ac	8.67	19.29	8	10.82	14.65	26.87	7.44	16.25	10	9.99	13.64	49.08
			16	14.56	15.32	35.7			20	15.18	12.02	74.63
F	5.28	2.71	8	8.97	10.91	46.2	5.94	1.77	10	8.89	7.95	56.81
			16	13.42	5.29	50.87			20	14.03	3.19	77.86
Pa	37.92	12.05	8	49.3	4.12	142	31.50	10.25	10	39.44	3.11	79.40
			16	61.21	10.39	145			20	47.41	8.34	79.55
A	0.91	9.89	8	4.99	11.51	51.00	2,13	10.02	10	5.68	6.21	68.42
			16	9.54	12.41	53.93			20	9,89	9.49	74.77
Fl	1.31	3.55	8	6.4	6.22	51.12	4.06	12.15	10	7.83	4.01	72.67
			16	10.0	8.99	54.31			20	12.58	7.22	82.12
P	15.43	16.09	8	22.9	5.27	93.37	18.24	6.69	10	25.39	4.38	71.50
			16	31.5	10.35	100.4			20	33.98	5.70	78.71
BaA	n.q.	–	8	3.99	11.23	49.87	0.51	14.64	10	4.01	5.98	67.66
			16	7.54	8.54	47.13			20	8.32	7.96	75.32
Ch	n.q.	–	8	3.41	10.23	42.62	1.04	13.78	10	4.77	5.56	72.00
			16	8.43	17.72	52.68			20	8.84	7.42	75.19
BbF	n.q.	–	8	4.46	12.91	55.75	0.37	12.74	10	3.48	4.97	59.98
			16	8.1	12.96	50.62			20	7.28	8.96	66.61
BkF	0.16	5.39	8	3.71	12.12	44.4	0.16	9.49	10	6.44	6.38	62.79
			16	9.4	14.72	57.75			20	13.49	8.72	66.67
BaP	2.64	7.20	8	5.28	1.69	33.2	0.84	9.28	10	6.84	6.69	59.60
			16	10.6	11.12	49.7			20	14.06	9.11	66.12
DhA	n.q.	–	8	3.14	8.56	39.2	0.21	15.19	10	6.56	10.93	63.50
			16	7.88	15.22	49.25			20	13.94	19.99	68.62
BgP	n.q.	–	8	3.51	6.38	43.87	0.52	5.05	10	6.53	6.38	60.08
			16	8.02	12.39	50.12			20	14.01	11.19	67.49
IP	n.q.	–	8	7.1	5.99	88.7	0.75	17.28	10	6.88	5.49	61.30
			16	13.5	11.27	84.37			20	12.96	7.47	61.05

[a] Analyte concentration expressed as ng/g of wet weight.
[b] Analyte concentration expressed as ng/g of dry weight (1 g of dry weight corresponds to approximately 2 g of wet weight, thus, the added amount corresponds to 5 and 10 ng/g of wet weight).

proved to be more sensible since all PAHs under study were quantified in the charcoal-grilled meat sample, only 8 analytes were quantified with method 1, additionally, in general, higher RSD and lower recoveries were obtained with method 1. Thus, the method 2 with modifications was selected for further studies and its application extended for quantification of PAHs in the analysis of fatty fish.

Several experiments were performed to optimize meat extraction conditions using method 2, for this purpose the *n*-hexane residue was quantitatively transferred with 3 ml of *n*-hexane into a silica cartridge (previously washed with 20 ml of dichloromethane, dried completely by means of vacuum, and conditioned with 20 ml of *n*-hexane), and eluted through the column with 17 ml of a mixture of *n*-hexane/dichloromethane (70:30). Analyses of PAHs, in fortified meat samples, were performed in the eluate collected in fractions of 2 ml. These fractions of 2 ml were evaporated to dryness under nitrogen stream. The residues were dissolved in 100 μl of acetonitrile and injected into the HPLC/FLD to investigate the presence of PAHs. No PAHs were detected on the first 4 fractions of 2 ml of eluate, corresponding to the first 8 ml that are discharged. As expected the following 4 fractions of 2 ml contained PAHs, the same occurred in the next 2 ml of eluate, but no PAHs were detected in the next 2 ml of eluate, indicating that the most appropriate volume of eluate to guarantee that all PAHs were recovered was 10 ml after discharge of the first 8 ml. Limits of detection (LODs, $s/n = 3$) using 2 g of meat sample were lower than 0.003 ng/g wet weight for all PAHs except for Na, Fl, and IP (that were lower than 0.01 ng/g). Limits of quantification (LOQs, $s/n = 10$) using 2 g of meat sample were lower than 0.01 ng/g wet weight for all PAHs except for Na, Fl, and IP (that were lower than 0.04 ng/g).

A similar procedure was performed in fortified salmon samples, however, for this purpose only 1 g of lyophilized salmon was used, since 2 g of sample contained very high level of fat and exceeded the capacity of silica column of retaining fat [18,31]. No PAHs were detected on the first 4 fractions of 2 ml of eluate, corresponding to the first 8 ml that should be discharged. The following 6 fractions of 2 ml contained PAHs, but no PAHs were detected in the next 2 ml of eluate, indicating that the most appropriate volume of eluate to guarantee that all PAHs were recovered was 12 ml. Limits of detection using 1 g of salmon sample were lower than 0.006 ng/g wet weight for all PAHs except for Na, Fl, and IP (that were lower than 0.02 ng/g). Limits of quantification using 1 g of fish sample were lower than 0.02 ng/g wet weight for all PAHs except for Na, Fl and IP (lower than 0.1 ng/g). The LODs and LOQs values obtained for meat and fish samples were lower than those referred by Commission Regulation (EU) No 836/2011 (LOD < 0.3 μg/kg; LOQ < 0.9 μg/kg) [7].

3.3. Analysis of PAHs by standard addition method meat/fish

Two methods of quantitative analysis were comparatively evaluated using meat and fish samples: external calibration curve method and standard addition method using two fortified levels (20 and 40 ng/g of PAHs for fish samples and 10–20 ng/g for meat samples). Typical chromatograms obtained for an unspiked and two levels spiked meat sample are shown in Fig. 2. Table 3 summarizes the results obtained. The PAHs concentrations obtained by the two quantification methods are significantly different ($p < 0.05$ running a *t*-test, except for BgP and DhA in salmon samples). Ratio between the concentration determined by the different methods of

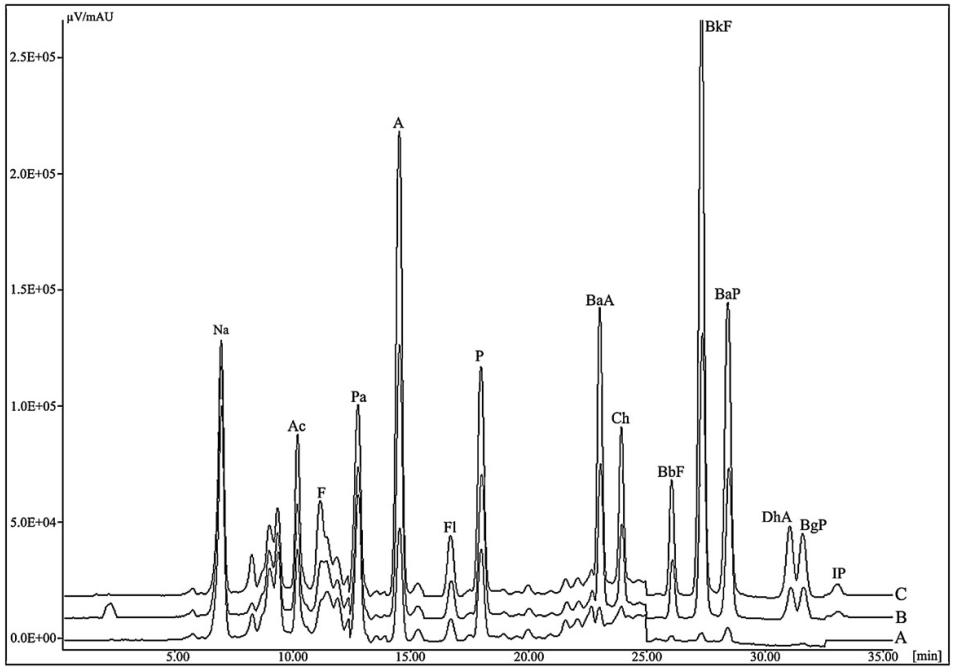

Fig. 2. Typical chromatograms obtained for an unspiked (A) and two levels (10–20 ng/g) spiked meat sample (B and C, respectively). For conditions, see text.

Table 3
Comparison of PAHs concentrations in meat and fish samples determined by external calibration curve method and by standard addition method (triplicate analyses were performed using the extraction method 2).

PAHs	Beef samples						Salmon samples					
	External calibration curve method (A)		Standard addition method (B)		*t*-test *p* value[b]	Ratio between the conc. determined by different methods of quantitative analyses (B)/(A)	External calibration curve method (A)		Standard addition method (B)		*t*-test *p* value[b]	Ratio between the conc. determined by different methods of quantitative analyses (B)/(A)
	Mean conc. (ng/g)[a]	±Standard deviation	Mean conc. (ng/g) [a]	±Standard deviation			Mean conc. (ng/g)[a]	±Standard deviation	Mean conc. (ng/g) [a]	±Standard deviation		
Na	3.75	0.73	5.81	0.62	0.009	1.54	24.76	1.34	36.06	1.98	0.000	1.45
Ac	6.83	0.53	10.94	1.28	0.001	1.60	4.59	0.60	5.06	0.40	0.000	1.10
F	6.09	0.13	7.25	0.08	0.001	1.19	7.04	0.04	9.37	0.83	0.002	1.33
Pa	15.76	0.50	27.09	0.76	0.000	1.71	28.42	0.17	57.01	1.34	0.001	2.00
A	2.02	0.10	3.14	0.11	0.000	1.56	3.81	0.03	6.49	0.90	0.000	1.70
Fl	3.80	0.23	5.39	0.36	0.002	1.41	11.47	0.71	14.95	1.20	0.000	1.30
P	4.18	0.08	5.98	0.57	0.000	1.43	9.05	0.64	13.65	1.70	0.022	1.51
BaA	0.41	0.08	0.68	0.04	0.018	1.64	2.69	0.22	3.98	0.37	0.041	1.48
Ch	0.86	0.15	1.37	0.04	0.002	1.59	4.02	0.19	7.04	0.20	0.012	1.75
BbF	0.32	0.04	0.48	0.06	0.040	1.51	2.14	0.03	3.72	0.37	0.031	1.74
BkF	0.07	0.01	0.14	0.05	0.037	2.02	0.36	0.01	0.80	0.09	0.000	2.21
BaP	0.39	0.04	0.60	0.05	0.007	1.55	1.66	0.07	2.85	0.06	0.001	1.70
DhA	0.11	0.01	0.22	0.02	0.002	2.12	0.59	0.10	0.59	0.04	0.538	1.00
BgP	0.25	0.02	0.43	0.10	0.075	1.71	0.97	0.19	1.47	0.26	0.426	1.51
IP	0.33	0.05	0.50	0.08	0.016	1.51	1.27	0.02	2.06	0.23	0.008	1.63

[a] Mean concentration of triplicate analyses expressed as ng/g of wet weight.
[b] *t*-test, $p < 0.05$ indicate significant differences.

quantitative analyses: standard addition method (B)/external calibration curve method (A) ranged between 1.19–2.12 and 1.00–2.21, respectively for beef and salmon samples, indicating that standard addition method compensates PAHs losses at different stages of sample preparation, and decreases the contribution of systematic errors and improves the accuracy of the results [32]. Therefore it can be recommended for analyses of PAHs in grilled meat and fish samples that contain variable amounts of PAHs. The eight high molecular weight PAHs (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA) present at traces levels could be quantified.

4. Conclusions

This work describes optimization of a methodology for determination of 15 PAHs (including the 8 high molecular weight EU

priority PAHs) in charcoal grilled foods (extraction, HPLC conditions and quantification method).

The analytical strategy consisted in extraction using sonication followed by purification on SPE, based on an extraction procedure for oils (rich in fat) and smoked foods (with high levels of PAHs per gram) with appropriate modifications for the matrix under study. Standard addition method was used to measure at trace levels these analytes with high sensitivity and specificity. Application on meat and fish samples permitted to prove its suitability and to collect data on PAHs contamination profile in this type of foods. The LODs and LOQs values obtained for meat and fish samples were lower than those referred by new European Legislation.

Time consumption and, consequently, the cost of routine analyses with the use of standard addition method increased as compared with the external calibration curve method. This is due to the replications of sample preparation and chromatographic analyses. However, the proposed HPLC method is less expensive than others that require acetonitrile as the eluent. Additionally, the extraction procedure is not very laborious and uses only one silica cartridge per extraction (three for each analysis).

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2.1.3. Effect of charcoal types and grilling conditions on formation of heterocyclic aromatic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs) in grilled muscle foods (**A3**)

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Effect of charcoal types and grilling conditions on formation of heterocyclic aromatic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs) in grilled muscle foods

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ABSTRACT

Grilling muscle foods involves high temperatures that lead to production of cooking toxicants, such as heterocyclic aromatic amines (HAs) and polycyclic aromatic hydrocarbons (PAHs). To obtain realistic exposure levels of these two groups of mutagens analyses of the same samples using similar separation/detection techniques were performed. HAs and PAHs were quantified in well-done meat and fish samples grilled with wood and coconut shell charcoal at 200 °C. Quantitative HAs and PAHs profiles were different for beef and salmon using the same type of charcoal. Higher levels of HAs and PAHs were found in salmon samples. No significant differences were observed for HAs and PAHs in beef samples grilled with both charcoal types, whereas salmon grilled with coconut shell charcoal presented significantly lower amounts of HAs and PAHs than salmon grilled with usual wood charcoal. Continuous barbecuing with the same charcoal shown that combustion of fat that dropped along the grilling period contributed to higher formation of HAs and PAHs. Special attention must be given to the intake of barbecued foods since high amounts of HAs and PAHs can be taken in a single meal.

1. Introduction

Diet contains various carcinogens: naturally occurring chemicals, synthetic compounds and compounds produced during cooking (Doll and Peto, 1981; Domingo, 2011; Nagao and Sugimura, 1993). The cooking toxicants have been receiving special attention in the last decades concerning their formation, occurrence, mitigation, and the impact of these substances on human health (Jägerstad and Skog, 2005; Skog et al., 1998). The risk of exposure to these compounds depends on the kind of diet, eating habits and cooking practices, which often result from regional traditions (Kobayashi et al., 2002; Melo et al., 2008; Gasperlin et al., 2009).

Cooking toxicants (HAs) and (PAHs) were significantly produced through meat grilling process at high temperature (Ferguson, 2010; Jägerstad and Skog, 2005).

Since their discovery 30 years ago, more than 25 HAs have been isolated and identified in cooked foods (Alaejos and Afonso, 2011; Murkovic, 2007). They can be divided in two main families: amino-imidazo-azaarenes or "thermic HAs" and amino-carbolines or "pyrolytic HAs". Formation of thermic HAs is the result of complex reactions that involve creatine/creatinine, free amino acids and sugars through the Maillard reaction at temperatures between 150 and 250 °C (Jägerstad et al., 1998; Nagao et al., 1977). Concerning pyrolytic HAs, a pathway for the formation was not so clear than thermic HAs, however it was suggested that may be produced from pyrolysis of proteins or amino acids heated at higher temperatures (>250 °C), and are not dependent of creatine (Matsumoto et al., 1981).

PAHs can be formed from a variety of combustion and pyrolysis processes and thus their natural or anthropogenic sources are numerous, however food seems to be the major route of exposition. High PAHs concentration in food is usually found in charcoal grilled/barbecued foods through the pyrolysis of fat and smoke from heat source (EFSA, 2008; Phillips, 1999). PAHs comprise fused aromatic rings, those containing two to four benzene rings are called "light PAHs", and those containing more than four, more stable and toxic, are called "heavy PAHs". The EU selected the sum of eight of the 15 priority PAHs as the most suitable indicators of carcinogenic PAHs in food, this PAH8 are the eight high molecular weight/carcinogenic from US-EPA PAHs list (Wenzel et al., 2006).

Competent authorities in most Western countries recommended minimizing the occurrence of cooking toxicants (Jägerstad and Skog, 2005). The IARC (1993) considered eight of the HAs tested to date, including the most abundant 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Viegas et al., 2012a), as *possible human carcinogens* (*Group 2B*) and one, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) as a *probable human carcinogens* (*Group*

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2A), and recommends a reduced exposure to these compounds. Concerning PAHs, recently IARC (2010) reviewed PAHs carcinogenicity, and classified benzo[*a*]pyrene (B*a*P) as *carcinogenic to humans* (*Group 1*), and some of other PAHs as *probably carcinogenic* or *possibly carcinogenic*.

Several researchers have highlighted an urgent need of to study more than one group of mutagens at the same time, to obtain more realistic exposure levels (Ferguson, 2010; Jägerstad and Skog, 2005; Sugimura, 2000). Furthermore, these compounds occur in mixtures, information about combined effect of compounds from the same group or interactions with carcinogens from different groups, but concomitant, especially if some metabolizing pathways are common, as in the case of HAs and PAHs (Dumont et al., 2010; Tarantini et al., 2011), need to be taken into account in considering their risk.

Grilled foods are increasingly popular both at home and in restaurants; however the higher levels of cooking carcinogens, make these foods a risk to the population (Farhadian et al., 2011). Thus, adequate information about exposition to both cooking toxicants, and mitigation strategies in this type of foods is a matter of concern.

The main objective of this work was to study the influence of charcoal type on HAs and PAHs formation and select the safer charcoal to grill muscle foods. With this propose HAs and PAHs were quantified in meat and fish samples grilled with two different types of charcoal under standard temperature conditions. Another goal of this work was evaluate if the continuous barbecuing with the same charcoal influences the HAs and PAHs formation. Contribution of barbecued muscle foods to the diet exposure of these hazard compounds will also be highlighted.

2. Materials and methods

2.1. Reagents and standards

All the solvents used were of HPLC grade (Merck, Darmstadt, Germany). Water was purified with a Milli-Q System (Millipore, Bedford, MA, USA). The chemicals used for HAs extraction [sodium hydroxide, hydrochloric acid, ammonium acetate, ammonia solution 25% (v/v)] and mobile phase triethylamine were of analytical grade and were purchased from Merck.

Concerning HAs standards, all individual, IQ, MeIQx, PhIP, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9H-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 2-amino-6-dimethylpyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1), were purchased from Toronto Research Chemicals (Toronto North York, ON, Canada). Stock standard solutions of 100 μg/mL in methanol were prepared and used for further dilution.

The standard PAHs mixture was purchased from Supelco (Bellefonte, PA, USA) and consisted of: 10 μg/ml of naphthalene (Na), acenaphthene (Ac), acenaphthylene (Ace), fluorene (F), anthracene (A), phenanthrene (Pa), fluoranthene (Fl), pyrene (P), benzo[*a*]anthracene (BaA), chrysene (Ch), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), BaP, indeno [1,2,3-*cd*]pyrene (IP), benzo[*g,h,i*]perylene (BgP), dibenzo[*a,h*]anthracene (DhA) (16 US-EPA PAHs) in 1 ml of acetonitrile.

A combined pH glass electrode connected to a pH-meter (MicropH 2001, Crison, Barcelona, Spain) and a Magna membrane nylon 0.22 μm were used.

2.2. Samples and grilling conditions

2.2.1. Effect of two different types of charcoal

Beef and salmon samples were used to evaluate the effect of two different types of charcoal in HAs and PAHs formation. The beef samples used in this study were obtained from the *Longissimus dorsi* muscle of middle-aged bovine carcasses. The meat was obtained from a major butchery in Porto, Portugal. The beef samples (six steaks with 2.5 cm of thick, and weighing 399.4 g (±22.4 g) were chilled overnight in a cooling room (5 ± 1 °C). Following the chilling process, all trimmable fat and connective tissue (epimysium) were removed from the *Longissimus dorsi* muscle.

Samples of fresh salmon from Atlantic were obtained in a fish market in the same city. Six fillets of salmon with 2 cm of thick and weighting 216.2 (±19.4 g) were prepared.

Two similar garden-type grills (35 cm width, 52 cm length, and 15 cm height) were fuelled with two different types of charcoal: the traditional wood charcoal and another called "ecological charcoal" from 100% coconut shell. Aiming to keep the temperature next to the grid at 200 °C the distance to the heat source was selected depending on the type of charcoal. Temperature was measured by using a digital thermocouple with a surface probe (Testo 926, Lenzkirch, Germany). For wood charcoal the samples were grilled at 18 cm distance to the heat source, and for coconut shells charcoal the samples were grilled closer to the heat source, 8 cm. The grilling time was 18 min for beef and 23 min for salmon until well-done cooked, golden color for salmon and moderately browned for beef. Samples were turned once during grilling at half the total cooking time. Internal temperature reached the minimum 75 °C in all our experiments. No salt or oil was applied to the samples before or after grilling.

2.2.2. Effect of continuous barbecuing with the same charcoal

Barbecued chicken samples were collected to evaluate if the continuous barbecuing with the same charcoal influences the HAs and PAHs formation. Chicken samples were collected in one of the traditional restaurant for chicken charcoal grilled (*Frango de Churrasco*), at lunch time, in Porto city. Raw chickens were open in the breast and cooked during 30 min and turned randomly during cooking period. Salt or other seasonings were not added. The "*churrasco* grill" (horizontal apparatus) was 92 cm width, 190 cm length, and 62 cm height. Operators ignited the fire and when all flames had subsided, the grilling procedure starts. Temperature next to the grid ranged between 230–300 °C (measured by using a digital thermocouple with a surface probe). The final product was well-done cooked, with a white color in the inner and a golden color in the skin (little beat charred).

Chicken collection was performed in two different periods of barbecuing; three samples were collected in each period. The first at the beginning of charcoal combustion when all flames had subsided, corresponding to the first chickens grilled in the charcoal, and the second period (one and a half hour later), was the last chickens grilled before the addition of new charcoal to the processing bed. Between the two periods of collection several chickens were cooked and commercialized.

2.2.3. Samples treatment after cooking procedures

Samples were weighed in all steps (uncooked, after grill, edible and no edible parts. Non-edible parts were removed, such as remaining fat or connective tissue in beef, bones and skin in fish, and in chicken samples, thighs were selected for further analysis and bones were removed.

Each sample was mixed in a kitchen blander (Moulinex, France) to produce a uniform sample. In the end, the homogenized samples were properly identified and frozen at −20 °C until the analysis of contaminants. Samples for PAHs analysis were protected from light and plastic adsorption, wrapping in aluminum foil before placing it in plastic containers (EC, 2011).

2.3. Analysis of HAs

Extraction and purification of HAs were performed using the reference method in interlaboratorial exercises (Santos et al., 2004), developed by Gross (1990) and further modified by Galceran et al. (1996).

Separation and quantification of HAs were performed by liquid chromatography with diode array fluorescence detection (HPLC-DAD/FLD) according by Gross (1990). HAs separation was carried out in an analytical HPLC unit equipped with one HPLC pump PU-1580, a fluorescence detector Jasco FP-920 coupled to a Multi-wavelength detector MD 910 and an auto sampler AS-950 equipped with a 20 μL loop (all from Jasco, Japan). The column was a TSK gel ODS80 (Toyo Soda, Japan) (5 μm; 250 mm length; 4.6 mm internal diameter). The Borwin PDA Controller Software was also used.

Three solvents were used for mobile phase: 0.01 M triethylamine adjusted with phosphoric acid to pH 3.2 (A), 0.01 M triethylamine adjusted with phosphoric acid to pH 3.6 (B) and acetonitrile (C) with a flow rate 1 ml/min. The linear gradient program was: 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 room temperature. DAD was set at 263 nm and FLD at excitation 307 nm and emission 370 nm.

Peak identification in food samples was carried out by comparing retention times and spectra of unknown peaks with reference standards, as well as co-chromatography with added standards and peak purity. Quantification of PhIP, MeAαC, and AαC was based on fluorescence peak area. Standard addition method was used for quantification of HAs using the non-spiked sample and two fortified levels (25 and 50 ng of thermic HAs; 50 and 100 ng of pyrolytic HAs and PhIP) before extraction procedure.

2.4. Analysis of PAHs

Extraction and clean up procedures were performed according by Viegas et al. (2012b) for grilled muscle foods, based on the method of Moret and Conte, applied for vegetable oils (Moret and Conte, 2002) and smoked meat (Purcaro et al., 2009).

The glassware, mostly amber, was carefully washed and rinsed with distilled solvent (acetone and hexane) before use (EC, 2011).

Separation and quantification of PAHs were performed by liquid chromatography with fluorescence detection (HPLC-FLD) according with Viegas et al. (2012b) conditions.

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PAHs separation was carried out using a HPLC unit equipped with one HPLC pump PU-1580, a fluorescence detector Jasco FP-920 and an auto sampler AS-950 equipped with a 20 μL loop (all from Jasco, Japan). The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was used.

The column was a C18 reversed phase: Supelcosil™ LC-PAH (25 cm length; 4.6 mm internal diameter; 5 μm particle size) (Supelco, Bellefonte, PA, USA), thermostated at 32.0 ± 0.2 °C. The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used. Three solvents were used for mobile phase: 75% methanol in water (A), methanol (B) and ethyl acetate (C) with a flow rate 1 ml /min. The linear gradient program was: 0–18 min, 0–80% B in A; 18–19 min, 80–100% B in A; 19–20 min, 100–90% B in C; 20–28,5 min, 90–82% B in C; 28,5–37,5 min, 82–80% B in C; 37,5–40 min, 80–100% B in C, 40–45 min 100–0% B in A, rinsing and re-equilibration of column to the initial conditions. Excitation/emission wavelengths selected were 276/330 nm for Na, Ac and F; 250/336 nm for Pa; 250/402 nm for A; 270/460 nm for Fl; 270/390 nm for P, BaA and Ch; 260/430 nm for BbF; 290/410 nm for BkF, BaP, DhA, and BgP; 290/470 nm for IP.

Peak identification in food samples was carried out based on retention times of standard solution peaks. Standard addition method was used for quantification of PAHs using the non-spiked sample and two fortified levels (20 and 40 ng).

2.5. Statistics

The averages of triplicate analysis were calculated for each HAs and PAHs. The results were statistically analyzed by analysis of variance. Differences (*t*-test) were considered significant for $p < 0.05$. Statistical analyses were all performed with SPSS for Windows version 18 (SPSS Inc, Chicago, IL).

3. Results and discussion

3.1. Effect of two different types of charcoal

Beef and salmon grilled samples presented similar color and average weight loss ranged between 39% and 40%, either in beef and salmon samples, independently of charcoal type, (results not shown). This weight loss is in agreement with literature for charcoal grilled samples at well-done level. Iwasaki et al. (2010) reported 48% weight loss in Brazilian barbecued beef. Costa et al. (2009) observed 37–38% weight losses for barbecued sardine and salmon samples.

The decrease of food weight during cooking, is generally attributed to water from evaporative loss and neglecting other food components (i.e. proteins, lipids) from dripping loss (Goñi and Salvadori, 2010). Dripping contain moisture, fat, protein and ash were all lost to the drippings (Murphy et al., 1975). During cooking, higher fat samples tend to lose large amounts of fat whilst low fat products lost proportionally more water and less fat (Sheard et al., 1998).

The formation of nine HAs and fifteen PAHs in grilled samples is presented in Table 1. Concerning HAs IQ, 4,8-DiMeIQx, Glu-P-1 and Trp-P-2 were not detected. All PAHs were quantified at least in one type of sample. Quantitative HAs and PAHs profiles were different for beef and salmon samples using the same type of charcoal. Although some exceptions, in general way higher levels of HAs and PAHs were found in salmon samples. Turesky et al. (2005) and Costa et al. (2009) observed formation of thermic HAs and amino-α-carbolines in barbecued beef and salmon samples in same order than our samples. Knize et al. (1997) described that flames produces AαC in beef grilled over open flames, from meat juices and carried up with smoke, however, beef samples containing 30% of fat content presented lower AαC than samples with 15% of fat, opposite behavior was observed for PhIP and MeIQx. The same was observed in our work for lean beef and salmon. Accord-

Table 1
HAs and PAHs content on beef and salmon samples grilled with different types of charcoal.

	Beef samples		Salmon samples	
	Wood charcoal	Coconut charcoal	Wood charcoal	Coconut charcoal
	Mean conc. (ng/g) ± Standard deviation	Mean conc. (ng/g) ± Standard deviation	Mean conc. (ng/g) ± Standard deviation	Mean conc. (ng/g) ± Standard deviation
Thermic HAs				
IQ	nd	nd	nd	nd
MeIQx	1.63 ± 0.57	0.69 ± 0.40	4.01 ± 1.63*	0.99 ± 0.11
4.8-DiMeIQx	nd	nd	nd	nd
PhIP	1.45 ± 1.11+	0.91 ± 0.25+	7.76 ± 2.04*	3.03 ± 0.94
Pyrolytic HAs				
Glu-P-1	nd	nd	nd	nd
Trp-P-1	0.75 ± 0.29	1.17 ± 0.66	1.43 ± 0.6	0.90 ± 0.05
Trp-P-2	nd	nd	nd	nd
AαC	1.44 ± 0.87+	1.54 ± 0.59+	0.38 ± 0.24	0.62 ± 0.43
MeAαC	0.49 ± 0.34	0.52 ± 0.24	0.45 ± 0.11	0.50 ± 0.27
Light PAHs				
Na	14.11 ± 6.91+	12.20 ± 4.65+	5.42 ± 1.26	4.74 ± 1.59
Ac	9.75 ± 3.34	4.88 ± 1.54	13.76 ± 7.70	8.15 ± 2.90
F	5.24 ± 1.95+	3.20 ± 1.59+	14.56 ± 6.85	6.77 ± 2.32
Pa	7.74 ± 3.42+	7.75 ± 1.98+	49.29 ± 5.86*	22.47 ± 12.38
A	0.64 ± 0.09 *+	1.19 ± 0.26+	15.01 ± 1.88*	3.26 ± 0.53
Heavy PAHs				
Fl	2.79 ± 1.26+	4.29 ± 0.48+	32.79 ± 6.75*	9.35 ± 3.27
P	3.69 ± 1.87+	4.94 ± 0.39	26.14 ± 7.37*	7.74 ± 2.40
BaA	0.39 ± 0.17*+	1.38 ± 0.44	7.82 ± 1.89*	1.82 ± 0.36
Ch	0.50 ± 0.10*+	0.80 ± 0.20+	20.60 ± 0.75*	3.57 ± 0.30
BbF	1.03 ± 0.25+	0.81 ± 0.17+	4.87 ± 0.44*	1.51 ± 0.13
BkF	0.25 ± 0.20+	0.28 ± 0.22	1.17 ± 0.09*	0.25 ± 0.31
BaP	0.41 ± 0.09+	0.50 ± 0.28+	4.72 ± 0.78*	1.36 ± 0.19
DhA	nd+	nd+	1.22 ± 0.22*	0.31 ± 0.01
BgP	0.64 ± 0.18+	0.63 ± 0.38	1.74 ± 0.24*	0.70 ± 0.43
IP	nd+	nd	1.18 ± 0.12*	nd

(*) *t*-test, $p < 0.05$ indicate significant differences between the same type of sample grilled with different charcoal. (+) *t*-test, $p < 0.05$ indicate significant differences between different type of sample grilled with the same charcoal, namely: wood charcoal beef vs wood charcoal salmon; coconut charcoal beef vs coconut charcoal salmon. (nd) no detected.

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ing to *Portuguese Food Composition Table*, the amount of fat on beef and salmon was 5% and 20%, respectively (INSA, 2006). Concerning PAHs, salmon presented higher content, except Na that was higher in beef than in salmon probably due to difficulties in the quantification, since it is an instable compound and losses are frequent (Viegas et al., 2012b). Since beef and salmon were grilled in the same way, the higher amount of PAHs in salmon may be a function of their fat content (Alomirah et al., 2011). Farhadian et al. (2011) highlighted Na, Pa, Fl and P as the predominant PAHs in food, whereas Martorell et al. (2010) reported similar predominance in their food screening and found lower concentrations of DhA, IP, BkF. These results were in agreement with those obtained in the present work. EFSA (2008) presented mean barbecued meat concentrations of B(a)P and PAH8 (sum of BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA) as 1.92 and 7.96 ng/g, respectively. In beef samples these values were lower (0.41 and 3.22 ng/g, respectively), however, the salmon samples exhibited notable higher levels (4.74 and 43.22 ng/g) than the average reported.

No significant differences were observed for HAs and PAHs in beef samples grilled with wood charcoal and coconut charcoal, except for A, BaA, Ch, whereas salmon grilled with wood charcoal presented significantly higher amounts of both HAs and PAHs. Incomplete charcoal pyrolysis is, by itself, responsible for the formation of HAs and PAHs contaminating grilled food in this way. The heat in the surface of muscle foods generates these contaminants by direct pyrolysis of food nutrients. Additionally, the natural meat and fish juices that are released during the grilling and fall from the muscle food into the charcoal can suffer pyrolysis and lead to the formation of HAs and PAHs, which, being dragged by the smoke, are deposited on the surface of the food (Costa et al., 2009; Hassan et al., 2010). Fat dripping from salmon samples grilled in wood charcoal lead to flame formation, increasing release of smoke that carries HAs and PAHs, and increasing the temperature near the surface during the flashing (Sinha et al., 1995). Coconut charcoal is labeled as flameless and smokeless charcoal, this justifies lower amounts of HAs and PAHs in salmon samples grilled with this type of charcoal which may be attributable to the way charcoal coconut absorb fat that drips from the cooking food. It should be pointed that both toxicants exhibit the same profiles in both types of charcoal. Salmon samples grilled in coconut charcoal presented B(a)P and sum of PAH8 markedly lower, near the average reported by EFSA (2008) to barbecued foods. Concerning beef samples all fat trimming and connective tissue were removed before grilling, thus the amount of fat that drop was very low, and no flashing were observed, which justify that no significant differences were observed between samples from both types of charcoal.

3.2. Effect of continuous barbecuing with the same charcoal

All barbecued chicken samples presented an average weight loss (39%) similar to that observed for beef and salmon samples. This weight loss in agreement with literature, for example, Sinha et al. (1995) described 42% of cooking losses for barbecued chicken.

Table 2 presents HAs and PAHs content on chicken samples collected in two different periods of barbecuing. The first chickens grilled in the charcoal (first period), contained PhIP, 4,8-DiMeIQx, Trp-P-1, AαC, MeAαC, and all PAHs except DhA. PhIP formation was higher than other HAs. According to literature PhIP seems to be formed easily in chicken (Sinha et al., 1995). MeIQx and 4,8-DiMeIQx are frequent but in lower amounts (Skog and Solyakov, 2002; Puangsombat et al., 2012). The occurrence of these three HAs in thighs of chicken is consistent with the results from Pais et al. (1999), PhIP (8.0 ng/g) > 4,8-DiMeIQx (0.05 ng/g), furthermore they evaluated the occurrence of HAs in drippings, where they found HAs formation much higher than in sample themselves. Information about pyrolytic HAs in chicken samples is scarce.

Table 2
HAs and PAHs content on chicken samples from two different periods of barbecuing.

	Chicken samples	
	From 1st period	From 2nd period
	Mean conc. (ng/g) ± Standard deviation	Mean conc. (ng/g) ± Standard deviation
Thermic HAs		
IQ	nd	nd
MeIQx	nd*	2.86 ± 0.64
4.8-DiMeIQx	3.15 ± 2.06	0.74 ± 0.17
PhIP	8.75 ± 2.99*	15.22 ± 2.83
Pyrolytic HAs		
Glu-P-1	nd	nd
Trp-P-1	3.25 ± 1.80*	6.88 ± 2.34
Trp-P-2	nd	nd
AαC	1.23 ± 0.43	1.77 ± 0.93
MeAαC	1.25 ± 0.76	2.05 ± 1.33
Light PAHs		
Na	2.79 ± 0.25	2.78 ± 0.48
Ac	2.07 ± 0.14*	1.20 ± 0.10
F	1.46 ± 0.20	0.77 ± 0.22
Pa	27.86 ± 1.20	25.59 ± 2.34
A	3.32 ± 0.37	1.91 ± 0.22
Heavy PAHs		
Fl	9.85 ± 0.39	22.10 ± 4.30
P	17.10 ± 3.39*	65.67 ± 10.95
BaA	3.50 ± 0.64	8.29 ± 1.35
Ch	5.26 ± 0.50	9.98 ± 2.06
BbF	6.28 ± 1.32*	18.83 ± 1.40
BkF	0.84 ± 0.11*	1.63 ± 0.11
BaP	3.14 ± 0.28*	8.73 ± 0.26
DhA	nd*	1.58 ± 0.15
BgP	2.65 ± 0.24*	8.69 ± 0.87
IP	3.30 ± 0.10	3.67 ± 0.33

1st Period: first chickens grilled in the charcoal; 2nd Period last chickens grilled before the addition of new charcoal to the processing bed. (*) *t*-test, $p < 0.05$ indicate significant differences.

However, high frequency and amounts of MeAαC were found in charcoal grilled chicken from Korean restaurants (Jo et al., 2008). Liao et al. (2010) observed formation of thermic HAs and amino-α-carbolines in barbecued chicken in similar order of magnitude. Concerning PAHs, Pa, Fl and P were the predominant PAHs, whereas the lowest concentrations were those of DhA, BkF. Alomirah et al. (2011) reported that charcoal grilled chicken contains 1.32/20.3 ng/g of B(a)P/PAH8, whereas Badry (2010) results for chicken grilled on direct flame butane gas were 5.3/25.4 ng/g of B(a)P/PAH8. The chicken samples grilled in the first period exhibit an average of 3.14 and 24.97 ng/g of B(a)P and PAH8, respectively. Chicken samples from second period exhibit approximately 2.5-fold the PAH8 value from first period samples. The value found of B(a)P to barbecued chicken was below of the maximum acceptable (EC, 2006) for smoked meat (5 ng/g), however the chicken samples collected later exceeded this value.

In general the contents of HAs increased in last chickens grilled before the addition of new charcoal (second period) differences were significant for MeIQx, PhIP and Trp-P-1. Concerning PAHs, all heavy PAHs (Fl, P, BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA) increased in samples from the second period. Combustion of fat that dropped along the grilling period, since between the two periods of collection several chickens were cooked and commercialized, contributes to higher formation of HAs and PAHs. Light PAHs are more instable and volatilize immediately, thus no increase was observed after continuous barbecuing, whereas heavy PAHs are more stable (Wenzel et al., 2006) and may accumulate in charcoal, being released later and then deposited on the surface of the later grilled chickens.

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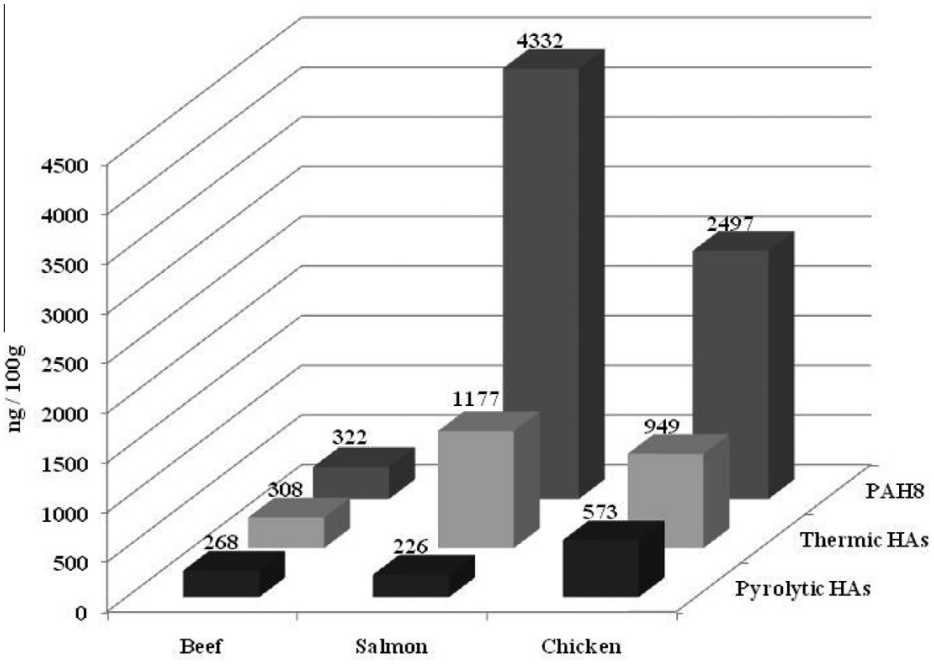

Fig. 1. Intake of thermic and pyrolytic HAs and PAH8 (ng per 100 g of barbecued muscle food).

3.3. Intake of hazard heating compounds from barbecued muscle foods

Intake of hazard heating compounds from barbecued muscle foods was calculated on the basis of consumption of 100 g of grilled muscle food, both thermic and pyrolytic HAs and PAH8 were selected to performe that evaluation. Results are presented in Fig. 1. Similar contents of pyrolytic HAs were formed in beef and salmon, whereas chicken contain twice of this amount. Concerning thermic HAs very high concentration was observed in salmon and chicken samples, beef contained low levels of this HAs. Salmon and chicken contribute to total HAs exposure with similar amount around 1500 ng/100 g. Salmon exhibited the highest amount of PAHs, followed by chicken (more a less half amount) and much lower amount was quantified in beef. PAH8 content is significantly correlated ($p < 0.05$) with fat content (from INSA, 2006) of these muscle food.

Evaluation of daily human exposure of HAs, usually account only with thermic HAs (Warzecha et al., 2004), or only the most abundant HAs are considered (Wakabayashi et al., 1993). Additionally, in some cases barbecued foods are not considered in the cooking method (Layton et al., 1995). Evaluation of pyrolytic HAs in food samples have been neglected, probably due to their lower mutagenicity, however, recent studies describe similar carcinogenicity when compared with thermic ones (Frederiksen, 2005; Skog et al., 1998). Consequently, more studies are needed related to intake of pyrolytic HAs. Pyrolytic HAs were described as presenting similar risk of cancer than thermic HAs (IARC, 1993) thus; these HAs should be accounted to total HAs (Fig. 1). European Prospective Investigation into Cancer and Nutrition reported an average HAs intake of 106 ng/day (Rohrmann and Becker, 2002), whereas an USA case (colorectal cancer) control study reported a daily intake of 364 ng for cases and 261 ng for controls (Nowell et al., 2002). Special attention must be given to the intake of barbecued foods since high amounts of HAs can be taken in a single meal. Prevalence of charcoal grilling is quite variable in different populations (Keating and Bogen, 2004).

Plaza-Bolaños et al. (2010) reported that in general, PAHs content in fish samples were considerably higher than those found in meat, and in EFSA report (2008) the subgroup of smoked fish had higher B(a)P and PAH8 content than smoked meat. The same trend was observed in our study to barbecued samples.

EFSA (2008) reported exposure of 279 ng/day of PAH8 from meat and meat products on basis in the average consumption across Europe (132 g/day) and the occurrence data on PAHs concentrations in this food group. Considering these consumption, the intake per day of PAH8, from barbecued beef, salmon and chicken samples was extremely higher (Fig. 1). If barbecued chicken or salmon are consumed in one meal, theoretically the PAH8 intake will exceed the overall dietary exposure of high consumers across Europe (range: 1415–2136 ng/day) estimated by EFSA (2008). However, in EFSA report (2008) it was highlighted that the consumption of certain barbecued foods, fatty meat, may lead to an exposure to PAHs that considerably exceeds the above estimated.

4. Conclusions

Special attention must be given to the intake of barbecued foods since high amounts of HAs and PAHs can be taken in a single meal. Coconut shell charcoal have been labeled as an ecological option comparing with wood charcoal, and the results of this work indicate that it is also a safer charcoal to human health, considering the reduction of HAs and PAHs formation in muscle foods, especially in fatty ones. Considering the continuous barbecuing with the same charcoal it was observed that the combustion of fat that dropped along the grilling period contributes to higher formation of HAs and PAHs.

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2.2. Section B

To determine the levels of HAs in pan-fried meat and to investigate the potential inhibitory effects of antioxidant rich marinades in their formation.

2.2.1. Effect of beer/red wine marinades on the formation of heterocyclic aromatic amines in pan-fried beef (**B1)**

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Effect of Beer/Red Wine Marinades on the Formation of Heterocyclic Aromatic Amines in Pan-Fried Beef

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The effect of beer or red wine marinades on the reduction of heterocyclic aromatic amines (HAs) formation in pan-fried beef was compared. The cooking experiments were performed under well-controlled temperature and time conditions. The samples were analyzed for HAs contents using solid-phase extraction and high-performance liquid chromatography−diode array detection/fluorescence detection. Unmarinated samples cooked in similar conditions provided reference HAs levels. Marinating with beer or with red wine resulted in decreased levels of HAs. The amount of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline reduced significantly, respectively, around 88 and 40% after 6 h of marinating with beer or with wine. High variations were observed for reductions of AαC, ranging between 7 and 77%. Only beer marinade significantly reduced the levels of 4,8-DiMeIQx at 1, 2, and 4 h of marinating. Multivariate statistical treatment of results indicated that beer can be more efficient on the reduction of some HAs formation. In addition, results from descriptive sensory analysis of unmarinated and 2 h marinated beef samples, tested for by two trained sensory panels, pointed to beer marinade as the most adequate for maintaining the usual overall appearance and quality of the pan-fried steaks.

KEYWORDS: Heterocyclic aromatic amines; antioxidants; beer; red wine; meat; marinades; sensory analysis

INTRODUCTION

More than 20 mutagenic/carcinogenic heterocyclic aromatic amines (HAs) have been isolated and identified in cooked foods. These substances are found particular in the crusts of fried, broiled, and cooked meat and fish. The International Agency for Research on Cancer (IARC) has classified several HAs as possible or probable carcinogens and has recommended reducing human exposure to these compounds (*1*).

There are two classes of HAs, aminoimidazole-azaarenes (AIAs) and amino-carbolines (ACs). AIAs formation is the result of complex reactions that involve creatine, free amino acids, and carbohydrates through the Maillard reaction (MR). The development of MR also occurs through a free radical mechanism, which has been shown to play an important role in the formation of AIAs (*2*−*5*). ACs are produced from pyrolysis of proteins or amino acids, such as tryptophan and glutamic acid, heated at high temperature (>250 °C) (*6*). The most popular hypothesis for the formation of ACs under such drastic thermal environment has been a pathway via free radical reactions; however, relatively little investigation has been carried out to verify the above hypothesis as compared with the AIAs (*7*).

The presence of HAs in foods depends on many factors such as type of meat, cooking method, water transport, time, and temperature (*3, 8*). The presence of precursors, enhancers and inhibitors, lipids, antioxidants, and the water content can also influence the formation of HAs (*9*). Meat marinating with several ingredients is used for improvement of flavor, tenderness, and moistness of the cooked product; additionally, they affect the formation of HAs. Earlier works studied the effect of marinating using mixtures of culinary ingredients, and changes in the formation of some HAs were observed (*10, 11*).

Some studies have shown that the concentrations of HAs can also be reduced by addition of compounds with an antioxidant potential. The addition of natural products containing antioxidants that may act as free radical scavengers, such as polyphenols, the main dietary antioxidants, reduces the amount of HAs in the heat-processed meat and model systems. Among these additives, the effect of tea (*12, 13*), red wine (*14*), olive oil (*15*), garlic (*9, 16*), and others has been demonstrated. The hypothesis for their action has been that these inhibitors act against the free radicals generated during HAs formation, preventing the mutagens formation through radical quenchers

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and free radical scavengers activity. However, antioxidants are known to exert both anti- and pro-oxidative effects depending on their concentrations and interactions with other food components during cooking (*7*). Marinating in red wine may reduce the formation of some HAs in chicken meat (*14*), but the influence on red meat, where higher levels of 2-amino-9H-pyrido[2,3-*b*]indole (AαC) may be formed, and the effect of other commonly used beverages rich in antioxidants, such as beer, remain to be clarified. Additionally, practical aspects must be considered, such as the extent to which a particular modulator might affect the appearance or sensory quality of the food.

Evaluation of influence of marinades on cooked meat organoleptic characteristcs is performed by sensory analysis, using in general descriptive analysis (profiling) (*9*). The consistency of results obtained from different sensory panels is an issue that has been frequently addressed, and the need to demonstrate the reliability of sensory panel results has been brought to the forefront of discussions, because even highly trained panels on a product can be subject to the occasional inconsistency. Thus, with the aim of ensuring consistent sensory work, the use of more than one panel is recommended (*17*).

The objective of this study was to compare the effect of beer marinades and red wine marinades in the reduction of AIAs and ACs formation in pan-fried beef. Unmarinated samples cooked in similar conditions provided reference HA levels. In addition, the influence of beer and red wine marinade in meat organoleptic characteristics was evaluated. Thus, the beef samples were tested for descriptive sensory analysis by two trained sensory panels.

MATERIALS AND METHODS

Materials. The compounds studied were 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-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), AαC, 2-amino-3-methyl-9H-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), purchased from Toronto Research Chemicals (North York Ontario, Canada). Stock standard solutions of 100 μg/mL in methanol were prepared and used for further dilution.

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, and 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 nylon membrane, 0.22 μm, from Magna before injection into the HPLC system.

Extrelut reservoirs and Extrelut HM-N diatomaceous earth refill material were obtained from Merck (Darmstadt, Germany). Bond Elut PRS (500 mg) and endcapped Bond Elut C_{18} (100 and 500 mg) cartridges were from Varian (Harbor City, United States). 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, United States) and ultrassonic cleaner (FungiLab SA, Barcelona, Spain) were used to homogenize cooked meat.

Preparation of Beef Samples and Pan Frying Conditions. The meat samples used in this research were obtained from the *Longissimus dorsi* muscle of middle-aged bovine carcasses. The meat was obtained from a major butchery in Porto, Portugal. The beef sample was chilled for 24 h in a cooling room (5 ± 1 °C). Following the chilling process, all trimmable fat and connective tissue (epimysium) were removed from the *Longissimus dorsi* muscle. Steaks (0.8−1.0 cm thick) were cut manually to pieces of similar dimensions weighing about 90−100 g each. Two different marinades were tested as follows: pilsner beer (5.4% alcohol, made from water, malt, unmalted cereals, and hops) and red wine (13% alcohol, from Douro valley region, produced with Tinta Roriz, Touriga Nacional, and Touriga Franca grape varieties).

Twenty beef samples were used for determination of HAs, divided by the two marinades (eights beef samples for each marinade) and control samples (four beef samples were not marinated). Samples were marinated during 1, 2, 4, and 6 h at 18 °C, using 350 mL of wine or beer; afterward, they were removed from the marinade and were then dried lightly and pan fried. For each condition studied, two beef steaks were marinated and cooked independently. Control beef samples were treated identically to the test samples, except that they were not marinated. Average cooking losses of around 48−50 and 52−55% were observed for unmarinated and marinated samples, respectively. Meat was weighed before and after cooking to calculate the percent loss of weight with cooking.

Beef samples were fried in a Teflon-coated pan 4 min on each side, without adding oil. The heat source was a gas cooker, and the temperature on the surface of the meat was monitored continuously during cooking with a meat thermometer; it ranged from 180 to 200 °C. The steaks were cut up using a knife, ground with a food blender, and stored at −20 °C until analysis. Samples were codified as follows: 1W, 2W, 4W, and 6W, respectively, for 1, 2, 4, and 6 h wine marinades, and 1B, 2B, 4B, and 6B, respectively, for 1, 2, 4, and 6 h beer marinades (two steaks each). CS was for control beef samples.

A total of 270 beef samples were used for sensory tests, including 108 for training and 162 for evaluation sessions. Beef samples were marinated in three different plastic containers, one with pilsner beer and another with red wine, so that all of the steaks could be covered completely by the respective marinade at 18 °C. Control steaks were not marinated. Meat was pan-fried at the same conditions used for determination of HAs.

Determination of HAs. Extraction and purification of HAs were performed according to the method developed by Gross (*18*) and modified by Galceran et al. (*19*), since this procedure is the reference method in interlaboratorial exercises (*20*). Sample preparation was as follows. A 5 g sample of fried beef 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, and Trp-P-2). After their organic solvent content was 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, and 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 the least polar analytes were gently evaporated under a stream of nitrogen, and the analytes were redissolved in 80 μL of methanol.

A standard addition method was used for quantification of HAs using two fortified levels (around 5−20 ng g^{-1}) and two nonspiked samples.

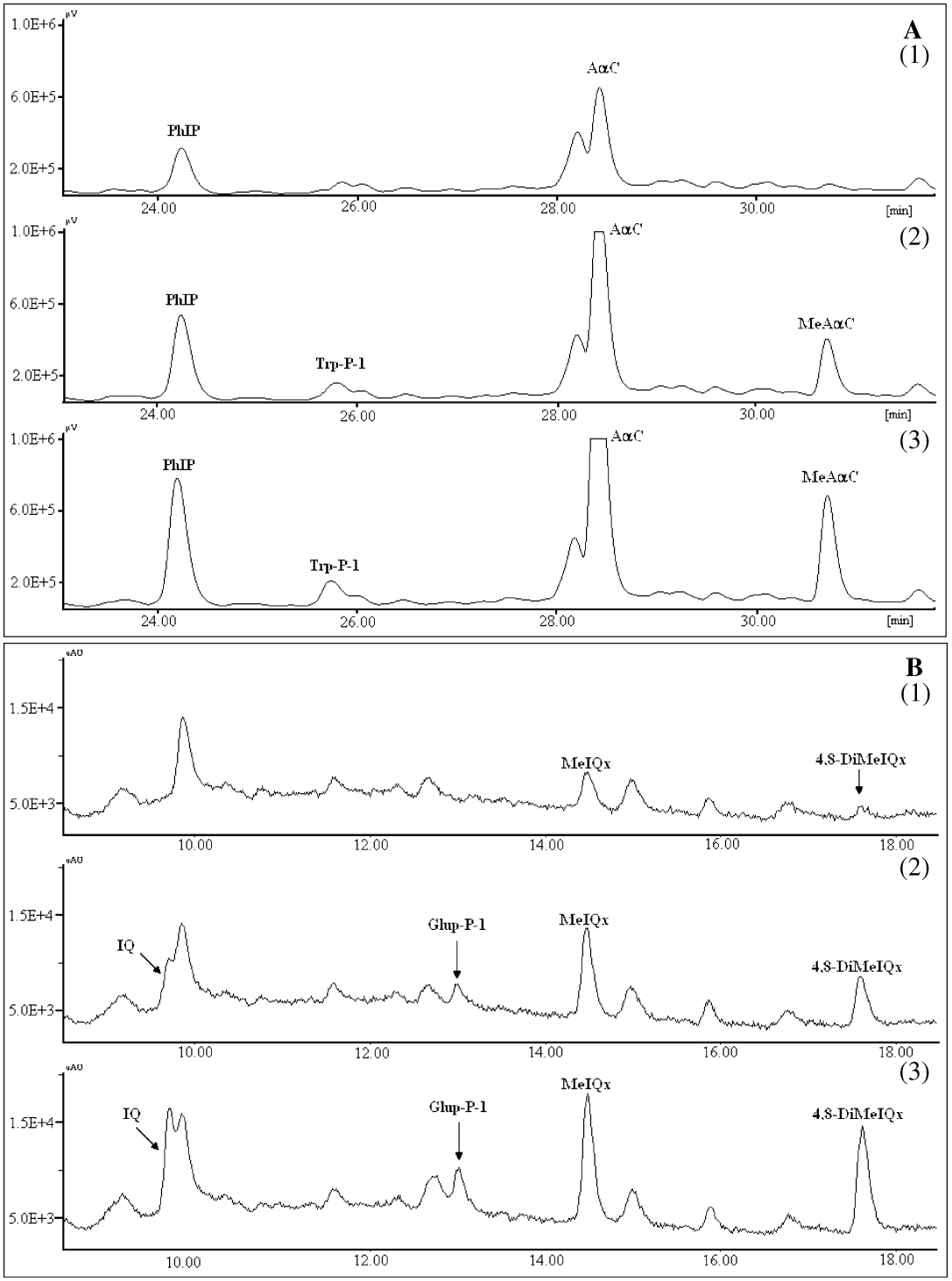

Figure 1. HPLC chromatograms of HAs meat extract. (**A**) Less polar HAs with FLD detector at an excitation of 307 nm and an emission of 370 nm. (**B**) Most polar HAs with DAD at 263 nm. Parts: 1, unspiked; 2, spiked with 5 ng/g for IQ, MeIQx, 4,8-DiMeIQx, and Trp-P-1 and with 10 ng/g for Glu-P-1, PhIP, AαC, and MeAαC; and 3, spiked with 10 ng/g for IQ, MeIQx, 4,8-DiMeIQx, and Trp-P-1 and with 20 ng/g for Glup-P1, PhIP, AαC, and MeAαC.

Separation and quantification of HAs were performed by liquid chromatography with diode array and fluorescence detection (HPLC-DAD/FLD). DAD was set at 263 nm, and the fluorescence detector was set at an excitation of 307 nm and an emission of 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). The 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 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. Triplicate analyses were performed, and the data were subjected to two-way analysis of variance (ANOVA) and principal component analysis using SPSS for Windows, ver. 16 (SPSS, Chicago, IL).

Sensory Tests. Descriptive analysis was conducted by two trained panels (27 members) to evaluate the intensities of the sensory characteristics of the pan-fried samples. After cooking, the samples were served hot to the two sensory panels. Analysis included the evaluation of strange odor, acid, bitter, juiciness, odor intensity, meat odor, red/brown color, overall appearance, wine aroma, beer aroma, adstringency, strange aroma, residual aroma, and overall quality. The sensory evaluation was conducted using a 1−7 scale, with 1 representing the lowest intensity and 7 representing the highest intensity for all attributes. Similar performance was obtained for the two panels (*21*).

The two sensory panels were master students of two different faculties from University of Porto that had sensory analysis in their curriculum and expressed an interest and disposition to undertake the work. Panelists were trained using marinated and unmarinated beef samples in four 1 h sessions for term optimization and calibration for accuracy in interpretation and repeatability. The two panels were trained separately. In session 1, panelists tasted control beef samples with specific highlighted appearance, flavor, and texture attributes. Panelists were invited to generate terms to describe personal observations. In session 2, redundant descriptive terms were removed, and samples exhibiting specific attributes were tasted to include on ballots. Session 3 was designed to establish ballot anchors where all attributes and their synonyms were fitted on an unstructured scale (seven points). To assist panelists, terms were used to describe each attribute at low intensity (score 1) and high intensity (score 7). In session 4, the ballots were tested by panelists in individual booths with unknown representative samples. Collected data were analyzed by ANOVA, and panelist deviations were assessed to determine where additional training was needed. A Learning Management System (WebCT vista, United States) was used for data acquisition during training and evaluation sessions (*21*).

In evaluation sessions, samples, including control and marinated samples, were labeled with random three-digit codes. In each session, panelists received a maximum of five samples to evaluate. The experimental samples were served to panelists in random order, and two evaluation sessions were performed for each panel.

RESULTS AND DISCUSION

HPLC/DAD/FLD for Quantification of HAs. *Quality Control of the Analysis.* The coefficient of variation of the intraday variability for retention times of 14 standards ranged between 0.12 and 1.84%, while the interday variability ranged between 0.33 and 2.75%. For concentration, the coefficient of variation of the intraday variability ranged between 0.33 and 2.75%, while the interday variability ranged between 3.12 and 17.61%. Similar values were obtained by other authors (*22, 23*).

The standard addition calibration curves were within the linearity range of the method. **Figure 1** shows chromatograms of fractions 1 and 2 for spiked and unspiked HAs extracts by DAD and by fluorescence.

The detection limits of 14 HAs standards expressed as ng per injection and based on a signal-to-noise of three were as follows: Glu-P-2 (0.4 ng), IQ (0.2 ng), IQx (0.2 ng), MeIQ (0.2 ng), MeIQx (0.06 ng), 4,8-DiMeIQx (0.06 ng), 7,8 DiMeIQx (0.06 ng), TriMeIQx (0.2 ng), Glu-P-1 (0.4 ng), Trp-P-2 (0.2 ng), PhIP (0.02 ng), Trp-P-1 (0.06 ng), AαC (0.02 ng), and MeAαC (0.02 ng). In food samples, the quantification limits, established as the amount of analyte that produces a signal-to-noise of 10:1, were 5 ng/g for Glu-P-2, 3.3 ng/g for Glu-P-1, 1.7 ng/g for IQ and TriMeIQx, 2.6 ng/g for IQx, MeIQx, and Trp-P-2, 0.86 ng/g for MeIQ, 0.56 ng/g for 4,8-DiMeIQx, 7,8 DiMeIQx, and Trp-P-1, and 0.25 ng/g for PhIP, AαC, and MeAαC.

Concentration of HAs in Control Samples. As a result of previous studies, it was decided to pan fry the meat for 4 min on each side at a measured surface temperature of 180−200 °C, which gave well-done medium brown products with good organoleptic properties (*8*). HAs were analyzed in unmarinated pan-fried meat. PhIP, MeIQx, 4,8-DiMeIQx, and AαC were identified in all samples, at concentrations above the limit of quantification. The concentrations of HAs in pan-fried unmarinated meat samples, expressed on a cooked steak basis, were as follows: PhIP, 33.8 ± 5.5 ng/g; AαC, 19 ± 2.5 ng/g; MeIQx, 3.6 ± 0.5 ng/g; and 4,8-DiMeIQx, 1.3 ± 0.7 ng/g. Trp-P-1, Trp-P-2, and MeAαC were identified but were below quantification limits. The other seven HAs were not detected.

The HAs most frequently found in pan-fried beef are PhIP, MeIQx, 4,8-DiMeIQx, and AαC (*24*). These HAs were quantified in unmarinated samples in levels within the ranges described in selected typical literature data on HAs in common cooked beef. Layton (*25*) reviewed the literature to research concentrations of the principle HAs identified in cooked food; the resultant database contains 261 records categorized by food item, cooking method and conditions, and the HAs detected. Levels of 39, 5.9, and 1.8 ng/g are described, respectively, for PhIP, MeIQx, and DiMeIQx in broiled and fried beefsteak. More recently, Murkovic (*26*) has summarized some of the literature levels of HAs: PhIP levels in red meat are found typically in amounts up to around 35 ng/g, AαC range between 0 and 20 ng/g, MeIQx range between 0 and 10 ng/g, and 4,8-DiMeIQx range between 0 and 5 ng/g. Lower levels of Trp-P-1 and Trp-P-2 are described, between 0 and 1 ng/g. A loss weight between 40−50% is generally referred. Other authors describe similar levels of HAs for beef samples classified as very well done samples (*27*); in this case, the degree of doneness is defined by the internal temperature of samples and not by the weight loss. Sinha (*28*) mentions similar levels for very well done beefsteak with 35% weight loss during cooking at an internal temperature of 93 °C. Other authors refer to lower levels of HAs at similar cooking conditions (*29, 30*) that can be result from different sample dimensions. Several reports have indicated the weight loss may result in increased transport of water-soluble precursors to the surface where the reactions occur (*3*).

It was decided to use as a control unmarinated samples, since this type of sample is usually used by consumers. Additionally, Busquets et al. (*14*) studied the physical effect due to marinating media and analyzed meat samples marinated (30 min, 3 h, and 24 h) prior to cooking in an ethanol/water mixture, with similar alcoholic composition as wine, and no reduction of HAs content was observed as a result of liquid media.

Effect of Marinade Time and Type on the formation of HAs. Pilsner beer and red wine from North of Portugal were used to marinate beef samples to study the effect on HAs reduction. Beer and red wine are rich sources of polyphenols (*31*), from malt and grapes, respectively. Its total content of polyphenols and antioxidant activity has been extensively studied in the past few years. Red wine presents considerably higher polyphenol contents and antioxidant activity when compared with beer (*31*). Marinating with beer or wine can affect the formation of HAs. **Figure 2** displays the concentrations of HAs formed in the unmarinated and different marinated pan-fried meat. Error bars indicate the standard deviation obtained in the quantification of HAs. The same four HAs were quantified in unmarinated

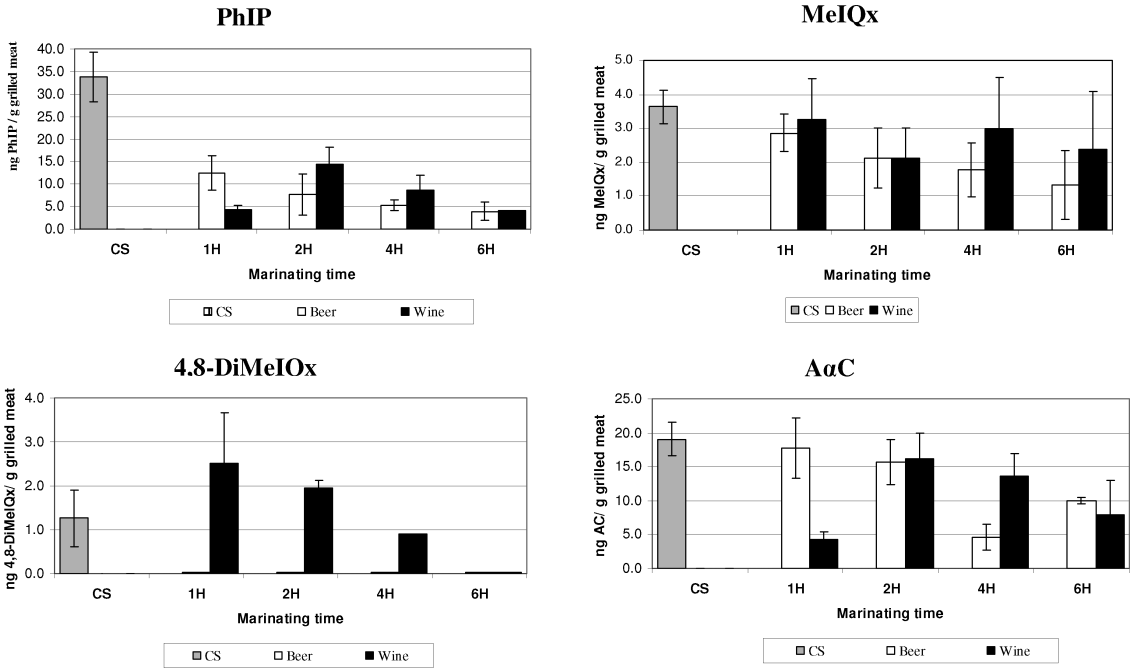

Figure 2. Effect of marinating media and marinating time on HAs formation. Error bars indicate the standard deviation obtained in the quantification of HAs.

and marinated meat. As compared with the unmarinated samples, marinating in beer or in wine resulted in decreased levels of HAs, except for 4,8-DiMeIQx at 1 and 2 h of marinating with wine.

Beer and wine marinades reduced significantly the amount of PhIP ($p < 0.05$), and no significant differences were observed between levels of PhIP of meat marinated with beer and with wine (Tukey test), and the reduction of PhIP levels in meat marinated with the increase of marinating time was not significant ($p = 0.204$) (**Figure 2**).

The reducing effect (88% after 6 h) of wine or beer marinating on the formation of PhIP, the most abundant HAs, was very important. A pronounced reduction of PhIP (83−88%) was also found by Busquets et al. (*14*), using three different types of red wine in fried chicken presenting a high content of PhIP.

In animal studies, an evaluation of the antigenotoxic potential of beer components against carcinogens contained in the human diet, namely, PhIP, was determined (*32, 33*). The results of this study showed that beer components act in a protective capacity against the genotoxic effects of heterocyclic amines in vivo. However, no studies were found concerning the effect of beer marinating on the reduction of PhIP formation.

Beer and wine marinades significantly reduced the amount of MeIQx ($p < 0.05$) after 2, 4, and 6 h of marinating, and higher reduction was observed for beer marinades after 6 h (mean value 44%) when compared with wine marinades (mean value 33%); however, differences between beer and wine reduction were not statistically significant (Tukey test). The reduction of MeIQx levels in meat marinated with the increase of marinating time was not significant ($p = 0.113$).

The reducing effect of wine or beer marinating on the formation of MeIQx was not as remarkable as that observed for PhIP. However, it should be highlighted since the effect of antioxidants in MeIQx formation is still controversial. According to studies for the evaluation of inhibitory effects of antioxidants on the formation of heterocyclic amines, some antioxidants suppressed MeIQx formation, whereas some others promoted MeIQx formation (*34*). An increase of MeIQx formation in pan-fried marinated meat was observed by Busquets et al. (*14*) and by Salmon et al. (*35*) using a different types of marinades. No studies were found concerning the effect of beer marinating on the reduction of MeIQx formation.

The different carbohydrate content of red wine and pilsner beer may also influence the formation of HAs such as MeIQx. The residual sugar content in red wine is generally less than 1.5 g/L, and the polysaccharide level is negligible (*36*), whereas pilsner beer presents around 3.65 g/L of sugars including maltotriose and 24 g/L of dextrines (*37*). It is suggested that maltodextrines can contribute to enhanced water retention due to their ability to imbibe water and, thus, reduce HAs formation (*3*). However, control steaks had one of the lower cooking losses in contrast to marinated samples, and similar results were obtained by Smith (*38*) using commercial marinade packets in grilled steaks.

Concerning 4,8-DiMeIQx, significant differences were observed between levels obtained for meat marinated with beer, wine, and control samples ($p < 0.05$). However, no significant differences were found between control and wine marinade samples (Tukey test). Only beer marinade reduced significantly the levels of 4,8-DiMeIQx at 1, 2, and 4 h of marinating. Wine marinade after 1 and 2 h increased 4,8-DiMeIQx content, but this increase was not significant; after 6 h of wine marinade, the levels of 4,8-DiMeIQx in meat samples were similar to those obtained with beer marinade and near detection limit of the method. The results obtained for wine marinades are in agreement with those found in another study, where inhibition of 4,8-DiMeIQx, up to 87%, was achieved after marinating chicken with wines for long marinating times (*14*).

AαC is present in higher levels in red meat then in other types of meat, such as chicken fillets (*3, 26*), which contain typically between 0 and 1 ng/g; therefore, it was possible to observe the effect of beer and wine marinades on AαC formation, because relatively little investigation has been carried out to verify the hypothesis for the formation of AαC

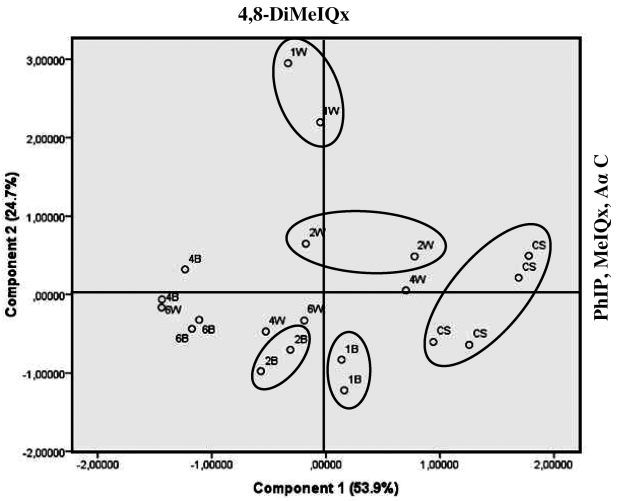

Figure 3. Two-dimensional plot representing the PCA of data from HAs. CS, control samples; 1B, pan-fried steak samples marinated with beer for 1 h; 2B, pan-fried steak samples marinated with beer for 2 h; 4B, pan-fried steak samples marinated with beer for 4 h; 6B, pan-fried steak samples marinated with beer for 6 h; 1W, pan-fried steak samples marinated with wine for 1 h; 2W, pan-fried steak samples marinated with wine for 2 h; 4W, pan-fried steak samples marinated with wine for 4 h; and 6W, pan-fried steak samples marinated with wine for 6 h.

through a pathway via free radical reactions. The two marinades reduced significantly the amount of AαC ($p < 0.05$); no significant differences were observed between levels of AαC of meat marinated with beer and with wine (Tukey test), but significant differences were observed concerning levels of AαC in meat marinated at different time ($p = 0.007$). High variations were observed for reductions of AαC, ranging between 7 and 77%. Person correlation indicates a negative correlation between beer marinating time and concentration of AαC ($p = -0.659$, significant at 0.05 levels); a similar result was not observed for wine marinade. No studies were found concerning the effect of polyphenols on inhibition of AαC formation on meat; however, according to studies of effects of vitamin C, α-tocopherol, and BHT (butylated hydroxytoluene) on the formation of heterocyclic amines in fried fish, inhibition or enhancement of AαC levels depended on the type of antioxidant and concentration (*39*).

The compounds Trp-P-1, Trp-P-2, and MeAαC were identified only in concentrations near the detection limit of the analytical method, so that no statistical evaluation was possible. Principal component analysis (PCA) was performed using HAs levels as variables to reduce the dimensionality of the data and pinpoint the most important effects of marinade time and type on the formation HAs. The results of PCA are depicted on a two-dimensional plot (**Figure 3**)—which is able to explain 78.6% of the total variance. Component 1 explains 53.9% of the variance in the data, and the positive segment of the plot for this component is closely related to the levels of PhIP, MeIQx, and AαC. Component 2 explained 24.7% of the variance in the data; this dimension is positively related to levels of 4,8-DiMeIQx. In this figure, the HAs needed for the definition of these components are shown on the axis edges, indicating the direction in which their levels increase.

Control samples presented high levels of PhIP, MeIQx, and AαC, whereas marinated samples were positioned in different segments of the plot (**Figure 3**). Beer-marinated samples during

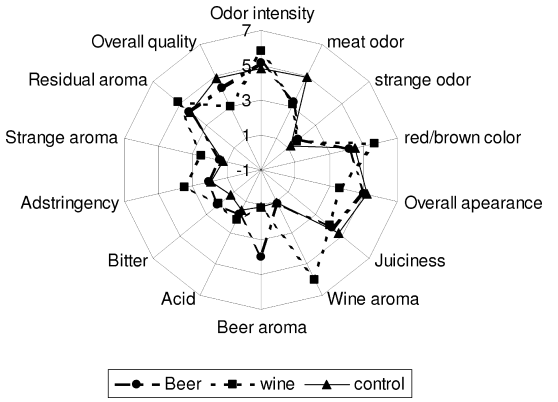

Figure 4. Mean results obtained by the two panels for the 14 sensory attributes assessed in control and beer- and wine-marinated (2 h) pan-fried meat samples cooked according to the description in the Materials and Methods.

1 and 2 h differed from wine-marinated samples, with similar marinating time. For longer marinating times, differences between wine- and beer-marinated samples were lower.

Effect of Beer and Wine Marinades on Sensory Characteristics of Pan-Fried Meat. The increasing requirement for sensory laboratories to show that the results that they provide are repeatable by other laboratories lead to use two different sensory panels. Results obtained by the two panels during training sessions were compared, and after session 4, no significant differences were observed concerning the results. Additionally, the overall conclusions from both analyses were very similar.

Sensory analysis was performed on control pan-fried steaks and on steaks marinated 2 h on wine and beer, because steaks marinated in wine during 4 and 6 h presented very unpleasant wine aromas, strong red color, and poor overall quality, and steaks marinated during 1 h presented higher levels of HAs.

ANOVA performed using the sensory attribute scores was indicative of significant differences ($p < 0.05$) in some of the attributes considered for control samples (unmarinated) and beer- and wine-marinated samples (2 h). In general, data within each attribute were symmetric and mesocurtic. The aforementioned analysis of variance indicated that no significant differences were observed for strange odor, acid, bitter, and juiciness; however, significant differences were noted for all other attributes (odor intensity, meat odor, red/brown color, overall appearance, wine aroma, beer aroma, adstringency, strange aroma, residual aroma, and overall quality). The mean results obtained by the two panels for the 14 sensory attributes assessed in control and beer- and wine-marinated pan-fried meat samples are presented in **Figure 4**.

Control pan-fried steaks presented higher meat odor and higher overall quality when compared with beer- and wine-marinated pan-fried steaks. No significant differences ($p < 0.05$) were observed between beer-marinated pan-fried steaks and control samples concerning red/brown color, overall appearance and quality, adstringency, and strange aroma. Beer-marinated steaks presented a significantly different beer aroma ($p > 0.05$). With respect to wine-marinated pan-fried steaks and control samples, significant differences (Tukey test) were observed for red/brown color, wine aroma, adstringency, and residual aroma. High scores were observed for this attributes. Scores of overall appearance and quality of wine-marinated steaks ($p > 0.05$) were lower when compared with those of beer-marinated steaks and control samples.

In conclusion, our data clearly show that both types of HAs, AIAs, and ACs are affected by beer and red wine marinades.

However, beer marinades can be more efficient on the reduction of some HAs, such as 4,8-DiMeIQx and MeIQx. Additionally, beer marinade has not influenced the usual overall appearance and quality of the pan-fried steaks.

ABBREVIATIONS USED

HAs, heterocyclic aromatic amines; AIAs, aminoimidazole-azaarenes; ACs, amino-carbolines; MR, Maillard reaction; 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-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]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-9*H*-pyrido[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; IARC, International Agency for Research on Cancer; BHT, butylated hydroxytoluene.

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2.2.2. Effect of green tea marinades on the formation of heterocyclic aromatic amines and sensory quality in pan-fried beef (**B2**)

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Effect of green tea marinades on the formation of heterocyclic aromatic amines and sensory quality of pan-fried beef

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ABSTRACT

The effect of a green tea marinade on the formation of heterocyclic aromatic amines (HAs) was examined in pan-fried beef cooked at 180–200 °C for 4 min each side. Different marinating times were assayed and unmarinated samples cooked in similar conditions provided reference HAs levels. A green tea marinade catechin rich was prepared taking 1 g of green tea and infusing with 125 ml of boiling tap water during 10 min. Four HAs were quantified in unmarinated and marinated meat samples during 1, 2, 4 and 6 h at 5 °C. Compared with the unmarinated samples, marinating in green tea resulted in a significant decrease ($p < 0.05$) of levels of PhIP and AαC. Person correlation indicated that the reduction of the levels of these HAs in meat marinated with the increase of marinating time was significant (respectively, $r = -0.799$, $p < 0.0001$; $r = -0.631$, $p < 0.05$). No reduction was observed for 4,8-DiMeIQx and MeIQx.

The beef samples were tested for descriptive sensory analysis by two trained sensory panels to evaluate the influence of the green tea marinade in meat organoleptic characteristics. Analysis of results obtained through the *t*-test, revealed no significant differences ($p < 0.05$) between control samples and tea marinated samples for all the thirteen attributes evaluated.

1. Introduction

Heterocyclic aromatic amines (HAs) are formed in cooked meats through pyrolysis reactions of different amino acids in the presence or absence of creatine/creatinine and sugars. They can be divided in two classes, aminoimidazole-azaarenes (AIAs) and amino-carbolines (ACs). AIAs formation is the result of complex reactions that involve creatine, free amino acids and carbohydrates through the Maillard Reaction (Arvidsson, Van Boekel, Skog, Solyakov, & Jägerstad, 1999; Jägerstad, Skog, Arvidsson, & Solyakov, 1998; Nagao, Honda, Seino, Yahagi, & Sugimura, 1977; Pearson, Chen, Gray, & Aust, 1992; Skog, Johansson, & Jägerstad, 1998). ACs are produced from pyrolysis of proteins or amino acids heated at high temperature (>250 °C) (Matsumoto, Yoshida, & Tomita, 1981).

HAs are described as genotoxic carcinogens associated with important types of human cancer in meat-eating populations, such as cancer of breast, colon or pancreas (Layton et al., 1995). It has also been shown that the amount of HAs formed in meats depends on meat type, muscle quality, cooking temperature, and cooking time (Janoszka, Błaszczyk, Damasiewicz-Bodzek, & Sajewicz, 2009; Jautz, Gibis, & Morlock, 2008; Melo, Viegas, Eça et al., 2008; Polak, Dosler, Zlender, & Gasperlin, 2009).

Several studies indicate that AIAs formation can be reduced by addition of compounds with an antioxidant potential. Thus, the addition of natural products containing antioxidants that may act as free radical scavengers, such as polyphenols from cherry, spices, natural extracts and fresh virgin olive oil reduces the amount of AIAs in the heat-processed meat (Ahn & Grun, 2005; Busquets, Puignou, Galceran, & Skog, 2006; Gibis, 2007; Melo, Viegas, Petisca, Pinho, & Ferreira, 2008; Murkovic, Steinberger, & Pfannhauser,1998; Oguri, Suda, Totsuka, Sugimura, & Wakabayashi, 1998; Persson, Graziani, Ferracane, Foligliano, & Skog, 2003; Vitaglione & Fogliano, 2004).

Studies of the effect on ACs formation are scarce. The formation of AαC in ground beef was reduced by the addition of 0.5% and 1.0% oleoresin rosemary (Herbalox®) (Ahn & Grun, 2005). Furthermore, meat marinating with alcoholic beverages, such as, beer and red wine can reduce significantly the formation of ACs and AIAs (Melo, Viegas, Petisca et al., 2008). Marinating implies preincubation with a fluid of some sort to impart flavour prior to cooking. Marinating prior to cooking is one such method of preparation, used frequently. Meat is marinated for a variety of reasons, including improvement of flavour, tenderness and moistness of the cooked product, additionally; they affect the formation of HAs (Melo, Viegas, Petisca et al., 2008).

Tea, the extract of *Camellia sinensis*, is consumed usually as water extract; it is rich in antioxidants and can be an alternative

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to marinate with alcoholic beverages, although the use of tea to marinate meat is not usual in Western countries. The composition of tea varies with species, season, age of the leaf (plucking position), climate, and horticultural practices. Green tea is prepared by steaming fresh leaves to heat inactivate oxidative enzymes, and then dried. It is chemically characterised by the presence of large amounts of polyphenolic compounds known as catechins, which may account for up 30% of the dry weight (Lin, Tsai, Tasy, & Lin, 2003). The major tea catechins are (−)-epigallocatechin 3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), (−)-epicatechin 3-gallate (ECG), and (+)-catechin (C). Weisburger et al. (2002) found that a purified fraction of green tea (Polyphenon 60®) used to marinate hamburger patty, decreased the formation of mutagens in a dose-related fashion. Cheng, Chen, and Wang (2007) studied the inhibitory activities of dietary phenolic compounds, including powders of catechins, in the formation of AIAs in both chemical model system and beef patties, suggesting its potential for practical application in daily cuisine. Thus, tea products represent another approach to lower the formation of HAs. However, no studies were found concerning the effect of green tea prepared household conditions on the formation of AIAs and ACs and sensory quality of cooked beef.

The objective of this study was to evaluate the effect of a green tea marinade rich in catechins in the AIAs and ACs formation in pan-fried beef, different marinating times were assayed and unmarinated samples cooked in similar conditions provided reference HA levels. The most appropriate water temperature and brewing time to prepare a green tea marinade catechin rich and using household conditions were selected. In addition, the influence of this green tea marinade in meat organoleptic characteristics was evaluated. Thus, the beef samples were tested for descriptive sensory analysis by two trained sensory panels.

2. Materials and methods

2.1. Chemicals

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-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino -1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9H-pyrido-[2,3-*b*]indole (AαC) 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), were purchased from Toronto Research Chemicals, (North York Ontario, Canada). Stock standard solutions of 100 μg/ml in methanol were prepared and used for further dilution.

Catechins, including (−)-catechin (C, >98%), (−)-epicatechin (EC, >98%), (−)-gallocatechin (GC, >98%), (−)-epigallocatechin (EGC), (−)-catechin gallate (CG, >98%), (−)-gallocatechin gallate (GCG, >98%), (−)-epicatechin gallate (ECG, >98%) and (−)-epigallocatechin gallate (EGCG, >98%) for HPLC references were provided by Sigma Chemical Co. (St. Louis, MO, USA). Ultrapure water was from a Seralpur PRO 90 CN and Seradest LFM 20 purification system. All solutions were passed through a 0.45 μm filter (Milex, MA, USA). The methanol, acetonitrile and dichloromethane HPLC grade were provided by Merck (Darmstadt, Germany). The chemicals used for sample treatment (sodium hydroxide, hydrochloric acid, ammonium acetate, ammonia solution 25% (v/v)) and triethylamine were of analytical grade and were also purchased from Merck. All 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.

2.2. Preparation of tea marinades, beef samples and grilling conditions

Green tea (Gorreana, Azores) infusions were prepared according to the conventional tea brewing method: taking 2 g of green tea and infusing with 250 ml (a cup) of hot tap water (1 g/125 ml). Different household conditions of brewing temperature and time were tested; namely, initial water temperature of 100, 90 and 80 °C and catechins extraction during 5 and 10 min.

The meat samples used in this study were obtained from the *Longissimus dorsi* muscle of middle-aged bovine carcasses. The meat was obtained from a major butchery in Porto, Portugal. The beef sample was chilled for 24 h in a cooling room (5 ± 1 °C). Following the chilling process, all trimmable fat and connective tissue (epimysium) were removed from the *Longissimus dorsi* muscle. Steaks (0.8–1.0 cm thick) were cut manually to pieces of similar dimensions weighing about 90–100 g each.

Ten beef samples were used for determination of HAs, divided by marinade (eight samples) and control (two samples, not marinated). Samples were marinated during 1, 2, 4 and 6 h at 5 °C, afterwards; they were removed from the marinade and were then, dried lightly, and grilled. For each condition studied, two beef steaks were marinated and cooked independently. Control beef samples were treated identically to the test samples, except that they were not marinated. It was decided to use as control unmarinated samples, since this type of sample is usually used by consumers.

Meat was weight before and after cooking to calculate the percent loss of weight with cooking. Average cooking losses of around 39–40% and 45–49% were observed for unmarinated and marinated samples, respectively. Two samples were independently processed for each treatment and duplicate analyses were performed.

Beef samples were grilled in a Teflon-coated pan 4 min on each side, without adding oil. The heat source was a gas cooker and the temperature on the surface of the meat was monitored continuously during cooking with a meat thermometer, it ranged from 180 to 200 °C. The steaks were cut up using a knife, ground with a food blender and stored at −20 °C until analysis. Samples were codified as follows: 1, 2, 4 and 6 h, respectively for 1, 2, 4 and 6 h of marinade (two steaks each analysed in duplicate); CS for control beef samples.

A total of 200 beef samples were used for sensory tests, including 90 for training and 110 for evaluation sessions. Beef samples were marinated in a plastic container with of green tea marinade, so that all the steaks could be covered completely by the marinade at 5 °C. Control steaks were not marinated. Meat was grilled at the same conditions used for determination of HAs.

2.3. Quantification of catechins

A simple and precise HPLC/DAD procedure was used to determine the composition of catechins in green tea extracts. The chromatographic analysis was carried out in an analytical HPLC unit (Jasco) equipped with two Jasco PU-2080 HPLC pump, a Column Heater – Model 7981 – Jones Chromatography, a MD-2010 Plus Multiwavelength detector and a Jasco As-950 intelligent sampler. The column was a reversed-phase Waters Spherisorb® C18 5 μm ODS2. The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used. The HPLC was carried out according to Liang, Liang, Dong, and Lu (2007). Gradient elution

was carried out with a mixture of two solvents and a flow of 1 ml/min. Solvent A consisted of acetonitrile/acetic acid/water (6:1:193, v:v:v) and solvent B consisted of acetonitrile/acetic acid/water (60:1:139, v:v:v) changing from 100% (v) solvent A to 100% (v) solvent B by linear gradient during first 40 min, returning to initial conditions. Detection was performed at 280 nm. A working standard solution containing 2.5 μg/ml of CG, 5 μg/ml of EC, GCG, 10 μg/ml of C, 25 μg/ml of ECG, 40 μg/ml of GC, 60 μg/ml of EGCG and EGC in deionised water was prepared and used for calibration curves. Quantification was performed by external standard method.

2.4. Extraction, identification and quantification of HAs

Extraction and purification of HAs was performed using the method developed by Gross (1990) and modified by Galceran, Pais, and Puignou (1996), since this procedure is the reference method in interlaboratorial exercises (Santos et al., 2004).

According to the method, a 5 g sample of pan-fried meat was homogenised in 20 ml 1 M NaOH with sonication (10 min), and the suspension was then shaken for 1 h using a Vortex Mixer. The alkaline mixture was mixed with Extrelut refill material (16 g) and was used to fill an empty Extrelut column. After being preconditioned with 7 ml dichloromethane, an Isolute PRS column was coupled on-line to the Extrelut column. To extract the analytes from diatomaceous earth, 75 ml of dichloromethane were passed through the tandem. The washing solutions arising from the PRS cartridge, which consisted of 6 ml 0.01 M HCl, 15 ml 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 lowering their organic solvent content by adding 25 ml of water, the acidic washing solutions were neutralised with 500 μl ammonia solution. The resulting solution was passed through a C18 cartridge (500 mg), previously conditioned with 5 ml MeOH and 5 ml water, and less polar HAs were concentrated. Finally, the C18 cartridge was rinsed with 5 ml 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 MeOH and 5 ml water, and was then coupled on-line 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 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.

Separation and quantification of HAs were performed by liquid chromatography with diode array and fluorescence detection (HPLC-DAD/FLD) (Melo, Viegas, Petisca et al., 2008). Diode Array detection was set at 263 nm and 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). The 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 programme was: 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 co-chromatography with added standards and peak purity.

The detection limits (LOD) were calculated as the concentration corresponding to three times the background noise of the blank. Standard addition method was used for quantification of HAs using two fortified levels (around 5–20 ng g^{-1}) and two non-spiked samples. Addition of the standards was carried out directly before the clean up of the samples.

2.5. Sensory tests

The assessors in the Faculty of Pharmacy and in the Faculty of Nutrition and Food Science were students of the respective Faculties. Selection criteria were availability for the assessments, interest to participate in the study, the absence of aversions, allergies or intolerance against beef and green tea and normal perception abilities. None of the panellists had a specific training in sensory evaluation of meat, nor previous experience with descriptive sensory analysis. Descriptive sensory analysis was conducted by two trained panels (consisting of 27 members) to assess the influence of green tea in the characteristics of the pan-fried samples. Fifteen persons (11 females, 4 males) contributed to the experiments in the Faculty of Pharmacy, and 16 (14 females, 2 males) contributed to the experiments in the Faculty of Nutrition and Food Science. The study was conducted with 2 h marinades.

After grill the samples, they were served hot to the two sensory panels. The analysis included an evaluation of the strange odour, acid, bitter, juiciness, door intensity, meat odour, red/brown colour, overall appearance, green tea aroma, adstringency, strange aroma, residual aroma and overall quality. The sensory evaluation, for any of the attributes, was held in an unstructured scale of 1–7 points, where 1 represents the lowest intensity and 7 to greater intensity. Similar performance was obtained for the two panels as described by Ferreira, Pinho, Amaral, and Martins (2008).

The two sensory panels of analysis were trained separately using tea marinated beef samples and unmarinated samples. The training took place in four sessions of 1 h in order to get repeatability in the results. In the first session, panellists tested control beef samples with specific highlighted appearance, flavour and texture attributes (without marinade) and were asked to express terms that describe their personal observations.

In the second session, the redundant descriptive terms were eliminated and the tea marinated beef samples were tested to include new attributes. In the third session all the attributes selected were used in an unstructured scale of 1–7. At the fourth session the different attributes were evaluated individually by panellists in unknown representative samples. Data were gathered and analysed by analysis of variance (ANOVA), and panellist deviations were assessed to determine where additional training was needed.

A Learning Management System (WebCT vista, USA) was used for data acquisition during training and evaluation sessions (Ferreira et al., 2008).

In evaluation sessions, samples, including control samples and marinated in tea, were marked with random three-digit codes. The experimental samples were served to panellists in random order, two evaluation sessions were performed for each panel.

3. Results and discussion

3.1. Selection of temperature and time conditions for a catechin rich green tea infusion

Infusions of green tea brewed with tap water were cooled to ambient temperature immediately and the contents of catechins in the infusions were compared. The results are given in Table 1. These show that, by using 100 °C tap water, and 10 min brewing time the content of EGCG, EGC, EC, ECG and GC increased significantly when compared with other conditions. Thus, these conditions were selected.

3.2. Effect of green tea marinade on the reduction of HAs-formation

The effect of green tea marinade on the formation of HAs was examined. The identities of amines in the samples were confirmed by matching their retention times and on-line UV spectral matching to a spectral library made from pure standards. The match factor typically observed was 95% or greater. The extraction recoveries varied with the compound. Average recoveries varied from 27% to 50.6% for the IQx compounds, 60% for PhIP, 31.6% for Glu-P-1 and for the pyridoindoles the recoveries varied from 41.2% to 60.3% (Melo, Viegas, Eça et al., 2008). The recovery values were comparable to those obtained in previous studies (Galceran et al., 1996; Santos et al., 2004). The low extraction efficiencies generally found in the analysis of the cooked meat samples may be due to macrocomponents of the matrix, such as lipids. These substances are not only capable of interacting with the analytes but can also modify the selectivity of the different extraction and purification steps. The standard addition method was used to overcome this problem; calibration and analysis were performed within the sample matrix itself, the extrapolation of the standard addition line to give an estimate of the analyte concentration in a sample. The standard addition calibration curves were within the linearity range of the method.

Table 1
Catechins in green tea infusions brewed with tap water at different temperatures and time, the results are expressed in as mean values and standard deviation of mg/g of green tea ($n = 3$).

Temp. (°C)	Time (min)	EGC	GC	EGCG	GCG	EC	C	ECG	CG
100	5	19.5 ± 0.02	5.3 ± 0.06	24.9 ± 0.04	2.7 ± 0.02	3.4 ± 0.02	2.2 ± 0.02	6.5 ± 0.03	1.1 ± 0.01
	10	27.3 ± 0.1	7.9 ± 0.01	32.4 ± 0.03	3. 8 ± 0.01	4.3 ± 0.02	3.0 ± 0.003	8.4 ± 0.01	1.7 ± 0.01
90	5	8.8 ± 0.01	1.9 ± 0.01	10.1 ± 0.30	0.7 ± 0.004	2.0 ± 0.02	1.0 ± 0.005	3.4 ± 0.03	0.5 ± 0.003
	10	13.4 ± 0.02	2.4 ± 0.03	15.0 ± 0.09	1.0 ± 0.007	2.9 ± 0.01	1.3 ± 0.005	4.7 ± 0.03	0.3 ± 0.001
80	5	8.8 ± 0.02	1.6 ± 0.04	9.6 ± 0.21	0.5 ± 0.002	1.1 ± 0.01	0.8 ± 0.000	2.4 ± 0.03	0.1 ± 0.000
	10	11.1 ± 0.3	2.5 ± 0.02	11.6 ± 0.01	0.6 ± 0.005	1.4 ± 0.01	0.9 ± 0.001	3.0 ± 0.03	0.2 ± 0.001

Abbreviations: EGC, epigallocatechin; GC, gallocatechin; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; EC, epicatechin; C, catechin; ECG, epicatechin gallate; CG, catechin gallate.

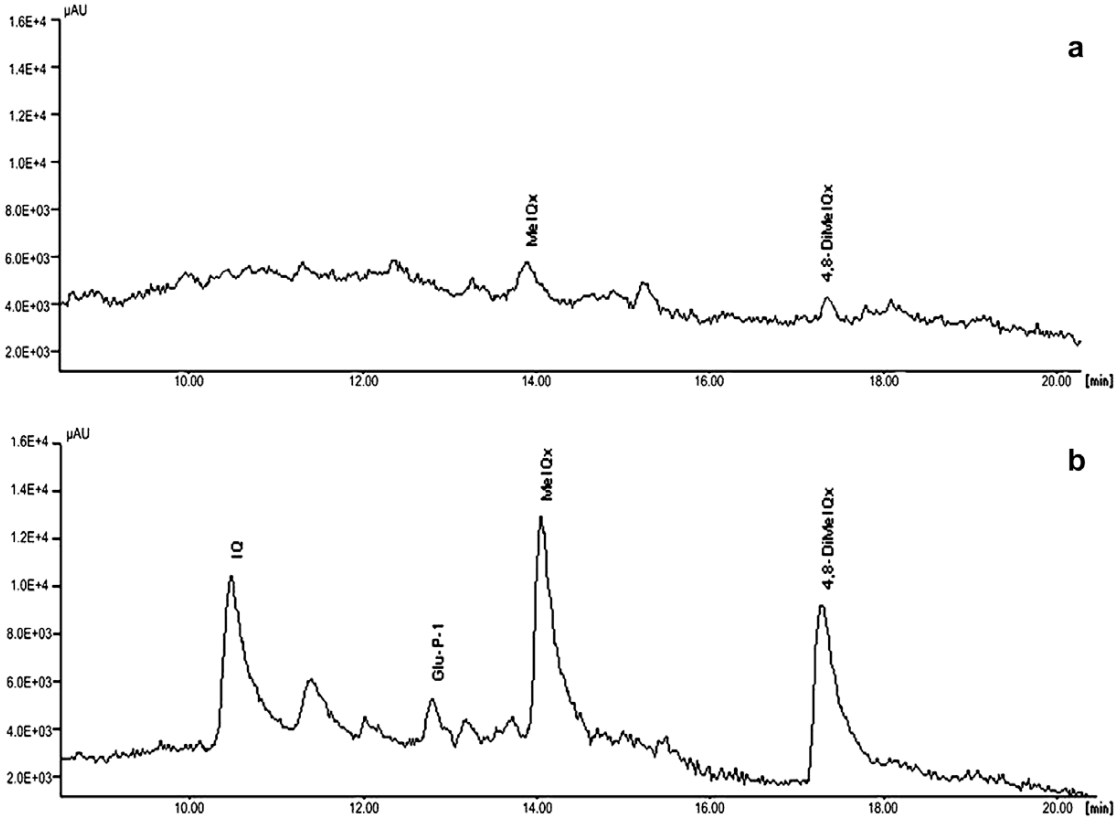

Fig. 1. Chromatograms of polar HAs (2nd fraction) derived from an extract of meat (grilled during 4 min on each side). Detection by DAD: (a) extract without added standard (b) extract fortified (with the addition of 10 µl of standard).

The coefficient of variation of the intraday variability of concentration ranged between 0.33% and 2.75% (n = 6), while the interday variability ranged between 3.12% and 17.61% (n = 10).

Fig. 1a and b shows typical chromatograms of the polar HAs (thermic HAs, second fraction of the extraction process) of control meat by DAD detection, (Fig. 1a) extract without added standard, (Fig. 1b) extract fortified (with the addition of 10 μl of standard). Fig. 2a and b shows typical chromatograms of less polar HAs (pyrolytic HAs, first fraction extracted) of control meat by FLD detection, (Fig. 1a) extract not fortified, (Fig. 1b) extract fortified (with the addition of 10 μl of standard).

The detection limits of 14 HAs standards expressed as ng per injection and based on a signal-to-noise of three were: Glu-P-2 (0.4 ng), IQ (0.2 ng), IQx (0.2 ng), MeIQ (0.2 ng), MeIQx (0.06 ng), 4,8-DiMeIQx (0.06 ng), 7,8 DiMeIQx (0.06 ng), TriMeIQx (0.2 ng), Glu-P-1 (0.4 ng), Trp-P-2 (0.2 ng), PhIP (0.02 ng), Trp-P-1 (0.06 ng), AαC (0.02 ng), MeAαC (0.02 ng) Melo, Viegas, Petisca et al. (2008).

Green tea brewed in the conditions selected above was used to marinate beef samples for studying the effect on HAs-formation at 180–200 °C in during 4 min on each side. Table 2 displays the concentrations of HAs formed in the unmarinated and tea marinated grilled meat. The same four HAs were quantified in unmarinated and marinated meat samples during 1, 2, 4 and 6 h at 5 °C, these are the HAs most frequently found in pan-fried beef (Jautz et al., 2008). These HAs were quantified in unmarinated samples in levels within the ranges described in selected typical literature data on HAs in common cooked beef (Layton et al., 1995; Murkovic, 2007) has summarised some of the literature levels of HAs: PhIP levels in red meat are found typically in amounts up to around 35 ng/g, AαC range between 0 and 20 ng/g, MeIQx range between 0 and 10 ng/g, and 4,8-DiMeIQx range between 0 and 5 ng/g. Lower levels of Trp-P-1 and Trp-P-2 are described, between 0 and 1 ng/g. A loss weight between 40 and 50% is generally referred. Other authors refer to lower levels of HAs at similar cooking conditions (Knize et al., 1998; Toribio, Busquets, Puignou, & Galceran, 2007) that can result from different sample dimensions.

Compared with the unmarinated samples, marinating in green tea resulted in decreased levels of PhIP and AαC, no reduction was observed for 4,8-DiMeIQx and MeIQx. The compounds

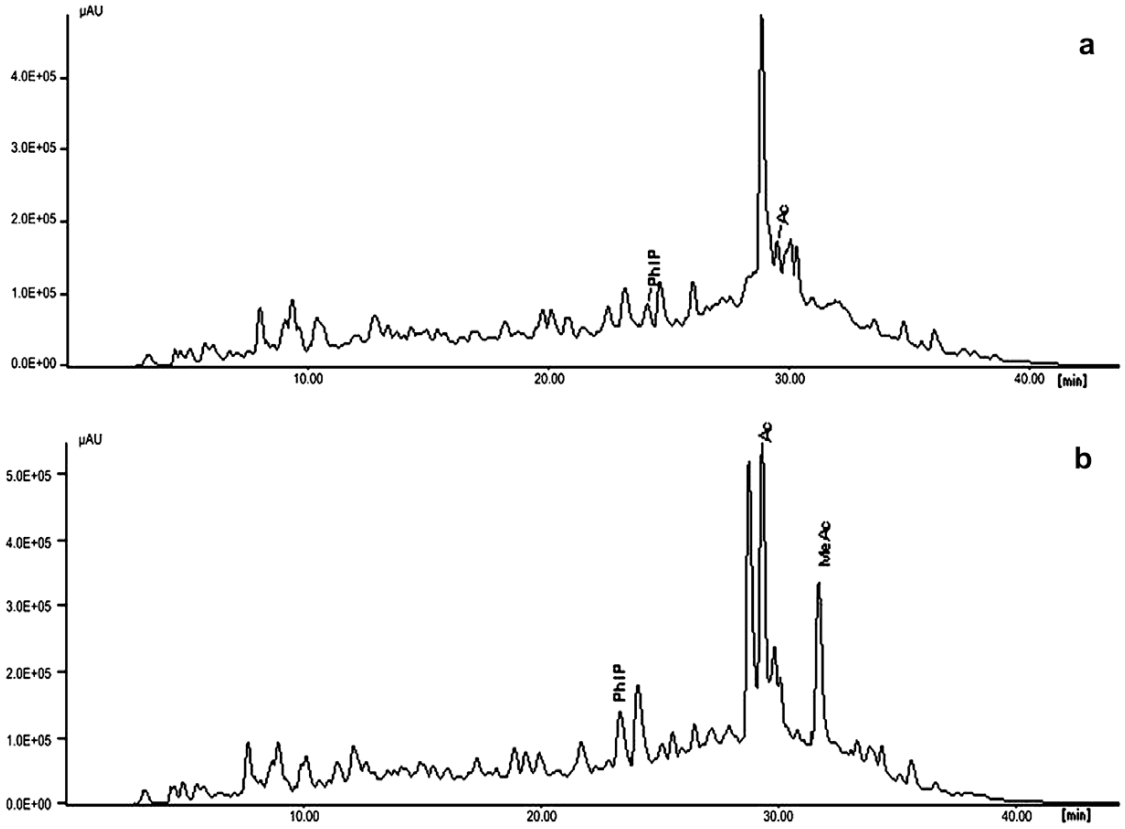

Fig. 2. Chromatograms of less polar HAs (1st fraction) derived from an extract of grilled meat (grilled during 4 min on each side). Detection by fluorescence: (a) extract not fortified (b) extract fortified (with the addition of 10 μl of standard).

Table 2
Effect of green tea marinating time on HAs-formation. Results are expressed as mean values and standard deviation of ng/g grilled meat (n = 4).

HAs	CS	1 h	2 h	4 h	6 h
MeIQx	4.1 ± 0.2	4.1 ± 0.05	3.6 ± 0.2	3.7 ± 0.3	4.9 ± 0.2
PhIP	33.8 ± 5.3	20.9 ± 2.3	24.1 ± 5.8	16.9 ± 5.8	8.8 ± 2.3
AαC	14.7 ± 6.1	3.3 ± 0.05	6.0 ± 2.5	5.6 ± 1.1	2.2 ± 1.0
4,8-DiMeIQx	1.3 ± 0.7	1.7 ± 0.2	1.3 ± 0.6	2.4 ± 0.2	2.01 ± 0.3

Abbreviations: HAs, heterocyclic aromatic amines; CS, unmarinated control samples; 1, 2, 4 and 6 h, 1, 2, 4 and 6 h marinating time, respectively.

Trp-P-1, Trp-P-2 and MeAαC were identified only in concentrations near the detection limit of the analytical method, so that no statistical evaluation was possible. Based on the design of this study it was not possible to rule out that parts of the reducing effect of green tea marinating on HAs-formation during pan-frying could be due to the fact that the control sample was not marinated. However, Busquets et al. (2006) studied the physical effect due to marinating media and analysed meat samples marinated (30 min, 3 h and 24 h) prior to cooking in blank marinade, and no reduction of HAs content was observed as a result of liquid media.

Green tea marinade reduced significantly the amount of PhIP ($p < 0.05$), and Person correlation indicated that the reduction of PhIP levels in meat marinated with the increase of marinating time was highly significant ($r = -0.799$, $p < 0.0001$). The reducing effect (75% after 6 h) of green tea marinating on the formation of PhIP, the most abundant HAs was very important. A reduction of HAs was found by Weisburger, Nagao, Wakabayashi, and Oguri (1994), using the polyphenols theaflavine gallate (TFG, black tea) and EGCG (green tea) on the formation of PhIP in model systems. Solutions of TFG and EGCG lower the formation of PhIP by 62–85% during 1 h heating at 160 °C of creatinine, phenylalanine and glucose. In the latter systems, teas were not, or less effective then the solutions of TFG and EGCG. Recently, (Cheng et al., 2007) evaluated the effects of ECG on PhIP formation in beef patties, 0.1% of powdered compound was mixed with 30 g of ground beef, and it was formed into a disk shape (6.2 cm × 1.2 cm), pan-fried at 200 °C, 3 min each side. 0.1% ECG lowered the formation of PhIP by 30%. A pronounced reduction of PhIP (83–88%) was also found using different types of marinades, namely, by Melo, Viegas, Petisca et al. (2008) using pan-fried meat marinated with beer or with wine, and by Busquets et al. (2006), using three different types of red wine in fried chicken presenting a high content of PhIP. Additionally, Busquets et al. (2006) studied the physical effect due to a marinating media and analysed meat samples marinated (30 min, 3 and 24 h) prior to cooking in a blank marinade, and no reduction of HAs content in chicken meat was observed as a result of blank marinade. Nevertheless, it is recognised from the literature that moisture content may be important factor for the yield of PhIP formation (Skog, Solyakov, & Jagerstad, 2000) reported that the formation of PhIP in model systems was favored by dry conditions.

AαC is present in higher levels in red meat then in other types of meat, such as chicken fillets, which contain typically between 0 and 1 ng/g; therefore, it was possible to observe the effect of green tea marinades on AαC formation, since relatively little investigation has been carried out to verify the hypothesis for the formation of AαC through a pathway via free radical reactions. The tea marinade reduced significantly the amount of AαC ($p < 0.05$), the second most abundant HAs. The reducing effect (85% after 6 h) of green tea marinating on the formation of AαC was more pronounced then the reduction observed by Melo, Viegas, Eça et al. (2008) and Melo, Viegas, Petisca et al. (2008) using pan-fried meat marinated with beer (32% after 6 h) or with wine (47% after 6 h). Person correlation indicate a negative correlation between green tea marinating time and concentration of AαC ($r = -0.631$, significant at 0.05 levels). Skog et al. (2000) studied the effects of heating conditions on the formation of heterocyclic amines with reference to amino-carbolines in a meat juice model system reported that the formation of AαC in model systems was favored by dry conditions. Ahn and Grun (2005) describe that the formation of AαC was significantly retarded in beef patties by the addition of 0.5% and 1.0% oleoresin rosemary (Herbalox®).

No reduction was observed for 4,8-DiMeIQx and MeIQx contents using green tea marinades. The results obtained by Cheng et al. (2007) in beef patties added of 0.1% of ECG powder showed an inhibition of 45% of formation of 4,8-DiMeIQx and MeIQx. However, the conditions used by authors do not reflect usual household cooking, high levels of these amines were obtained owing to larger surface area-to-mass ratio of the patties and the use of catechin powder of mixed with meat results in higher amounts of catechins then those present in green tea marinades. The effect of antioxidants on the generation of HAs has been reported to depend on the type, concentrations, and synergistic effects of pro- and antioxidants, leading to an enhancement or a reduction of HA generation. For example, Vitaglione, Monti, Ambrosino, Skog, and Fogliano (2002) observed that the rate of inhibition or promotion of the quinoxalines in different model systems was not always correlated with the antioxidant concentrations. This fact shows that both anti- and pro-oxidative effects can be exhibited by antioxidants, which caused different rates of formation of HAs (Oguri et al., 1998).

Concerning the total content of heterocyclic amines in unmarinated and green tea marinated samples, comparison was performed with results obtained by the same research group Melo, Viegas, Petisca et al. (2008) using the same conditions and pilsen beer and red wine marinades (see Fig. 3). Total content of HAs at six hours marinating time was similar for green tea, pilson beer and red wine marinades, indicating a reduction of total HAs-formation around 70%.

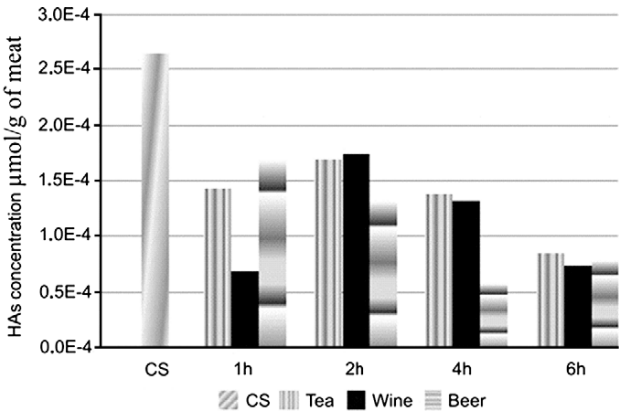

Fig. 3. Total content of heterocyclic aromatic amines in control (unmarinated) and green tea marinated samples (expressed as µmol/g of meat), comparison with mean results obtained by Melo, Viegas, Petisca et al. (2008) using similar conditions and pilsen beer and red wine marinades.

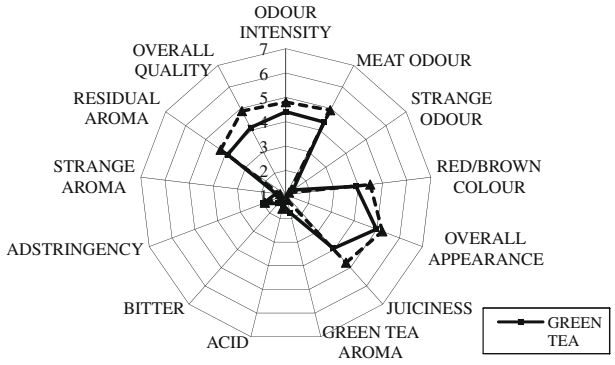

Fig. 4. Mean results obtained by the two panels for the 13 sensory attributes assessed in control and green tea marinated (2 h) pan-fried meat samples cooked according to the description in Section 2.

3.3. Effect of green tea marinades on sensory characteristics of pan-fried meat

The descriptive sensory analysis showed that the marinade of green tea (2 h) does not contributed to improve sensory characteristics. The attributes "odour intensity," "meat odour", "red/brown colour", "juiciness", "overall quality" and "residual taste" in green tea marinated samples have lower scores than the control meat samples (Fig. 4). However, analysis of results obtained through the *t*-test, revealed no significant differences ($p < 0.05$) between control samples and tea marinated samples for all attributes.

4. Conclusions

Previous studies frequently attributed their findings for the inhibitory effects of catechins on HA formation through free radical scavenging/antioxidant activities in both model and meat systems. However, the present work is the first report applied to real household conditions for used of green tea and meat. Also, addition of the green tea marinade slightly changes the gustatory perception of pan-fried meat (sensory panel observation) and therefore, should be readily accepted by the public. Since the catechins are natural products present in green tea consumed world-wide without any human disease risk, this procedure might well be introduced in the future in the cooking of meat practices, especially, for children and consumers that do not use alcoholic marinades owing to medical requirements, food allergies or religious practices.

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2.2.3. Inhibitory effect of antioxidant rich marinades on the formation of heterocyclic aromatic amines in pan-fried beef (**B3**)

Inhibitory effect of antioxidant rich marinades on the formation of Heterocyclic Aromatic Amines in pan-fried beef

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Abstract

The inhibitory effect of antioxidant rich marinades containing beer and white wine and a mixture of herbs commonly used as meat flavoring (garlic, ginger, thyme, rosemary, red chili pepper) on the formation of Heterocyclic Aromatic Amines (HAs) in pan-fried beef was studied. Antiradical activity was evaluated by DPPH assay, wine with herbs possessed the highest scavenging activity (68.3%), followed by wine (60,7%), beer and herbs (34.4%), and beer (25.9%). Antiradical activity of dealcoholized wine with herbs (52.9%) and dealcoholized wine (37.8%) was also evaluated to understand the influence of alcohol in inhibition of HAs. All the six marinades under study exhibited a reduction in total HAs formation keeping meat with good overall sensory quality. Beer marinades were more efficient than white wine marinades and the addition of herbs provided a superior inhibitory effect, reducing around 90% of HAs. No correlation was observed between antiradical activity of marinades and total or individual HAs formation. Herbs explained around 30% of inhibition of PhIP formation, whereas alcohol increased PhIP formation.

Keywords – heterocyclic aromatic amines, herbs, beer, white wine, antiradical activity, sensory analyses

INTRODUCTION

Cooking meat has clear beneficial impact, as the microbial content decreases, the digestibility increases and the flavor and texture improves. However, compounds naturally present can react and under household conditions generate carcinogens, such as Heterocyclic Aromatic Amines (HAs) that are considered important food mutagens/carcinogens.[1]

Several epidemiological studies have shown strong association between intakes of HAs and risk of important types of human cancer in meat-eating populations, such as cancer of breast, colon or pancreas.[2] The International Agency for Research on Cancer (IARC) classified several HAs as probable and possible human carcinogens.[3] When basic human diets include meat it is impossible to avoid the risk of exposure to this group of genotoxic compounds.

HAs can be divided in two classes: aminoimidazoazarenes (AIAs) or thermic HAs with a common structure 2-aminoimidazole moiety and aminocarbolines (ACs) or pyrolitic HAs with 2-aminopyridine as a common structure. HAs formation is the result of complex reactions between creatine, free amino acids and sugars through the Maillard reaction.[4] The amount of HAs formed in meats depends on meat type, muscle quality, namely, pH, water activity, free amino acids and creatine, and cooking conditions including temperature, time and equipment used.[4-6] Over years both HAs formation pathway and their minimization strategies have been investigated. [4, 7-10]

In the last years special attention was given to antioxidant compounds that contribute to inhibition of HAs formation and/or mutagenicity in model systems[11-12] and in real foods.[13-14] Both meats pre-treated or cooked together with sauces and spices, naturally rich in phenolics, are highlight due their antioxidant capability towards free radicals,[15] and provide easy-to-use tools to reduce the HA dietary intake.[16] For example, addition of olive oil,[17-19] tomato,[19] garlic,[20,21] rosemary, thyme, sage, and brine,[14] studied individually, were found to reduce the formation of some HAs in meat. Additionally, marinating meat before cooking with red wine,[8,22] beer,[8] or green tea,[9] can be an effective strategy for reduction of levels of HAs. Moreover, meat marinating with several ingredients is a common practice in several countries for improvement of flavor and tenderness of the cooked product. This pretreatment has the advantage that the cooked meat is not overly spiced and do not develop negative sensory characteristics as only the surface is treated.

Controversial findings on the effect of antioxidant capacity of phenolic compounds or food-extracts and HAs formation have been described.[11, 12, 23] Additionally, information about the effect of mixtures containing antioxidant rich ingredients, in conditions resembling household reality, is still a challenge for researchers. The ability of marinades containing alcoholic beverages and a mixture of aromatic herbs to minimize the formation of HAs is an important issue for further studies in this area. This study aims to understand the contribution of antioxidant rich marinades containing beer and white wine and a mixture of herbs commonly used as meat flavoring (garlic, ginger, thyme,

rosemary, red chili pepper) in the HAs inhibition under household cooking conditions. In addition, meat samples must present adequate sensory characteristics, thus, cooked samples were tested for pleasant flavor by a sensory panel.

MATERIALS AND METHODS

Reagents and standards.

HAs standards, all individual, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 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-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9H-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 2-amino-6-dimethylpyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), were purchased from Toronto Research Chemicals (Toronto North York, ON, Canada). Stock standard solutions of 100 μg/ ml in methanol were prepared and used for further dilution.

The chemicals used for meat 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 (Darmstadt, Germany). Acetonitrile, methanol, and dichloromethane were of HPLC grade (Merck).

The chemicals used for DPPH assay, ethanol and ethyl acetate of analytical grade were from Merck, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO).

Water was purified with a Milli-Q System (Millipore, Bedford, MA, USA). For solutions a combined pH glass electrode connected to a pH-meter (MicropH 2001, Crison, Barcelona, Spain) and a Magna membrane nylon 0.22 μm were used.

Marinated meat samples and cooking conditions.

Preparation of marinades.

Three different liquid marinades were tested: pilsner beer (5.2% alcohol, made from water, malt, unmalted cereals, and hops), white wine (13.5% alcohol, produced from Moscatel Galego, Viosinho, Arinto, and Fernão Fires varieties located from Douro valley region) and dealcoholized white wine (using the same white wine dried on rotary evaporator at 40 ºC (Rotavapor RE 111 equipped with a 461 water bath and Vac V-500 vacuum pump, all from Büchi®, Switzerland) and reconstituted with still water. The herbs selected, were purchased in a local grocery. Preliminary sensory texts were conducted to adjust herbs amounts and liquid marinade with good balancing flavors. The following amounts were selected: 100 ml of marinade contain 2.8 g of ginger

(*Zingiber officinale*), 2.9 g of garlic (*Allium sativum*), 0.4 g of rosemary (*Rosmarinus officiallis*), 0.25 g of thyme (*Thymus vulgaris*) and 0.1 g of red chili pepper (*Capsicum annumm*). The marinades were prepared immediately before use. Meat samples were marinated during 4 hours using beer alone (B), beer with herbs (BH), wine alone (W), wine with herbs (WH) and dealcoholized wine (DW) alone and with herbs (DWH). Pan-fried beef samples were coded as beer beef (BB), Beer + Herbs Beef (BHB), Wine Beef (WB), Wine+Herbs Beef (WHB), Dealcoholized Wine Beef (DWB), Dealcoholized Wine+Herbs Beef (DWHB). One group remained unmarinated (control), codified as CB. Four hours of marinated time was according to results obtained in previous studies.[7]

Cooking conditions.

The meat samples, from *Longissimus dorsi* muscle of middle-aged bovine carcasses, were obtained from a major butchery in Porto, Portugal. Meat was chilled overnight in a cooling room (5 ± 1˚C). Following the chilling process, all trimmable fat and connective tissue were removed from the muscle. Steaks were cut manually with similar dimensions (1.2-1.5 cm thick) weighing about 100 g each. The relation amount of meat and volume of marinade was 1:1 (g/ml). Meat samples were marinated during 4 hours and pan-fried in a Teflon-coated pan 3 min on each side. The heat source was a gas cooker, and the temperature on the surface of the meat was monitored continuously during cooking with a meat thermometer; it was around 180°C. Meat was weighed before and after cooking to calculate the percent loss of weigh with cooking. Average cooking losses of around 40% were observed. The steaks were cut up using a knife, grinded with a food blender, and stored at -20 °C until analysis. Each sample was mixed in a kitchen blander (Moulinex, France) to produce a uniform sample. At the end, the homogenized samples were properly identified and frozen at -20 ºC until the analysis.

Determination of marinades radical-scavenging in DPPH reaction.

Extraction of phenolic compounds.

Extraction of phenolic compounds of the marinades was performed according to Zhao et al[24] and Xanthopoulou et al, [25] with some modifications. Briefly, marinades were degassed at room temperature and reduced pressure by intense stirring for 30 min. 15 ml of each batch was extracted during 30 min with 15 ml of ethyl acetate and centrifuged at 1500 rpm for 2 min (Eppendorf 5810 R centrifuge, Eppendorf, Hamburg, Germany), and supernatant was collected. Then, 6 g of NaCl were added and pH was set to 1 using HCl 0.05M and two extractions were performed, each with 15 ml of ethyl acetate. The supernatants were pooled and evaporated at 40 ºC and reduced pressure in a rotary evaporator and redissolved in 10 ml of ethanol 70% (v/v).

Antiradical activity using DPPH. Extracts of B, BH, W, WH, DW and DWH collected at different times were diluted in 96-well microplates (1:4; 1:8). 100µL of DPPH 150µM were added to the

extracts and absorbances at 517nm were recorded during 2 hours until reaction reached a plateau (Biotek microplate reader ELX 808, Biotek Corporation, USA). For each extract, two readings with DPPH, $A_{extract}$, and one without the radical, A_{blank1}, were performed. Wells with DPPH solution were used as control, $A_{control}$, and ethanol 70% was used as blank, A_{blank2}. Radical scavenging activity was expressed as percentage and calculated with the formula:

$$\%\ DPPH\ scavenging = 100 - \left(\frac{A_{extract} - A_{blank1}}{A_{control} - A_{blank2}} \times 100 \right)$$

To evaluate the contribution of sulfur dioxide to white wine antioxidant activity, excess of acetaldehyde was added to the wine, to scavenge sulfur dioxide before DPPH assay.[26]

Analysis of HAs.

Extraction and purification.

Extraction and purification of HAs were performed according to the method used in interlaboratorial exercises.[27] Sample preparation was as follows, a 5 g sample of pan-fried beef 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 VV3 (VWR International, West Chester, PA, USA). The alkaline solution was mixed with 16 g of diatomaceous earth, and was used to fill an empty 20 ml Extrelut column (Extrelut®, Merck, Darmstadt, Germany. After being preconditioned with 7 ml of dichloromethane, a PRS SPE column (Bond Elut PRS, 500 mg, 3 ml from Agilent Technologies, USA) 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, MeαAC, Trp-P-1, and Trp-P-2). After their organic solvent content was 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 500 mg C_{18} cartridge (Bond Elut C_{18}, from Agilent Technologies, USA), previously conditioned with 5 ml of MeOH and 5 ml of water, and "less polar" amines were concentrated. Finally, the C_{18} 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). To collect the "most polar" amines (IQ, Glu-P-1, MeIQx, 4,8-DiMeIQx), a 100 mg C_{18} cartridge (Bond Elut C_{18}, from Agilent Technologies, USA), was conditioned with 5 ml of MeOH and 5 ml of water and was then coupled online with the PRS cartridge. The "most polar" amines were eluted from the cationic exchanger with 20 ml of 0.5 M ammonium acetate at pH 8.5. Finally the C_{18} 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). Both final extracts containing each group of HAs

were gently evaporated under a stream of nitrogen and the analytes were redissolved in 80 µl of methanol.

Chromatographic conditions.

Separation and quantification of HAs were performed by liquid chromatography with diode array and fluorescence detection (HPLC-DAD/FLD).[5] The chromatographic analysis was carried out in an analytical HPLC equipped with all unities from Jasco (Japan): one PU-1580 HPLC pump, an autosampler AS-950 with a 20 µl loop and a MD 910 Multiwavelength detector (set at 263nm) coupled to a FP-920 fluorescence detector (excitation 307 nm; emission 370 nm). The software used was the Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France).

The separation through the TSK gel ODS80 column (Toyo Soda, Japan) (5 µm; 250 mm length; 4.6 mm internal diameter), at ambient temperature, was performed with the follow mobile phase: solvent A, 0.01M 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: 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.

Peak identification in food samples was carried out by comparing retention times and spectra of unknown peaks with reference standards, as well as co-chromatography with added standards and peak purity. Quantification of PhIP, Trp-P-1, MeAαC, and AαC was based on fluorescence peak area. Standard addition method was used for quantification of HAs using the non-spiked sample and two fortified levels (25 and 50 ng of "less polar" HAs; 50 and 100 ng of "most polar" HAs and PhIP) before extraction procedure.

(meat odor, and aroma intensity)

Sensory analysis.

Descriptive analysis was conducted by a trained panel (11-members) to evaluate the sensory characteristics of the meat samples. After cooking, the samples were served hot to the sensory panels. Analysis included the evaluation of color, meat odor, beer odor, wine odor, spicy odor, aroma intensity, juiciness, and overall quality. The sensory evaluation was conducted using a 1 to 7 scale, with 1 representing the lowest intensity and 7 the highest intensity, for all attributes. The sensory panel was composed by master students from University of Porto that had sensory analysis in their curriculum and expressed an interest and disposition to undertake the work. Panelists were trained using marinated and unmarinated beef samples, in four 1-hour sessions for term optimization and calibration for accuracy in interpretation and repeatability. Collected data were analyzed by analysis of variance (ANOVA), and panelist deviations were assessed to determine where additional training was needed. In evaluation sessions, samples, including, control and

marinated samples, were labeled with random three-digit codes. In each session, panelists received a maximum of five samples to evaluate.

Statistics.

The averages of triplicate analysis were calculated for each HAs. The results were statistically analyzed by analysis of variance. Comparison of mean values was made using the Duncan test. Statistical analyses were all performed with SPSS for Windows version 18 (SPSS Inc, Chicago, IL).

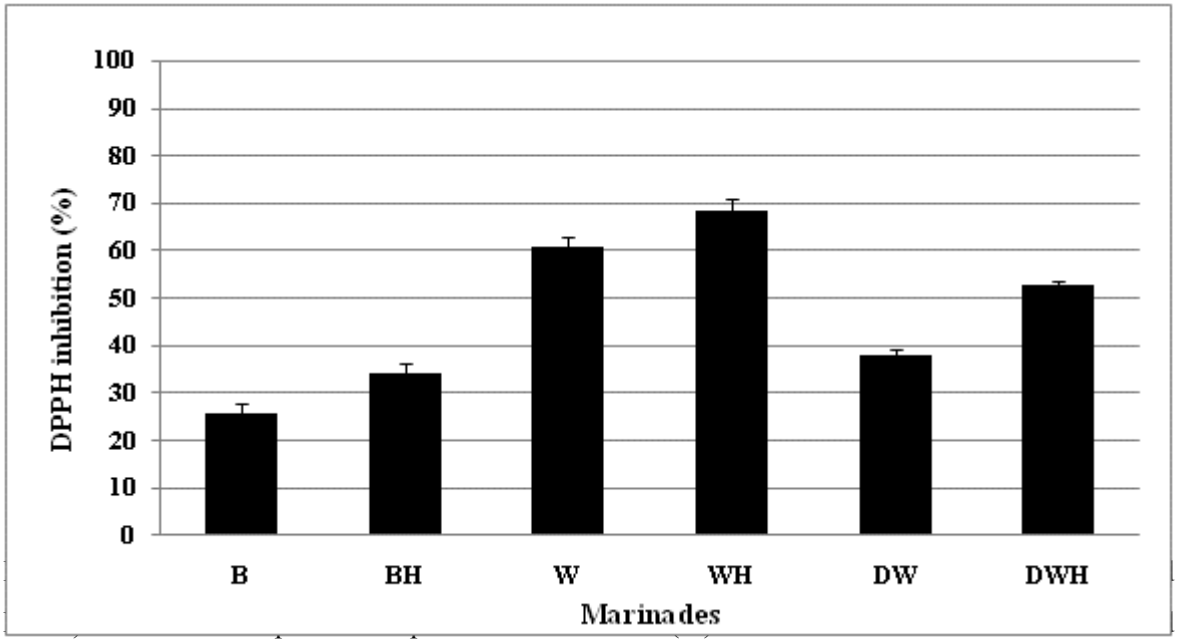
RESULTS AND DISCUSSION

Antiradical activity of marinades.

Antiradical activity expressed as % of inhibition of DPPH was determined in all marinades under study (B, BH, W, WH, DW, DWH) after 4 hours of marinating time, results are shown in **Figure 1**. As can be observed wine with herbs possessed the highest DPPH scavenging activity (68.3%), followed by wine (60,7%), dealcoholized wine with herbs (52.9%), dealcoholized wine (37.8%), beer and herbs (34.4%), and beer (25.9%). The addition of herbs increased the antioxidant activity of marinade medium.

The antiradical activity of the selected mixture of herbs in alcohol/water solution (13.5% v/V) and in 100% water (after 4 hours of exposure) was also evaluated. Using the same amount of herbs higher antiradical activity was observed in ethanol solution (27.4%) than in water (16.9%) antiradical activity. Wine added of acetaldehyde to bind free sulfur dioxide showed a quite similar value of antiradical activity when compared with dealcoholized wine (32.6%) (results not shown). During the alcohol evaporation free sulfur dioxide was evaporated also, which may be explaining the lower antioxidant activity in dealcoholized wine.

The antiradical activity of dealcoholized wine was similar (slightly higher) than beer. Antioxidant activity and phenolic content of beers are strictly correlated.[24, 28, 29] Lugasi and Hóvári[29] reported that beers antioxidant activity and total phenol content were similar to the white wines using the same test systems.



deviation obtained from triplicate analyses.

Effect of marinades in HAs formation.

Total content of HAs in control (unmarinated beef) and 4h marinated samples (expressed as nmol/g pan-fried meat) are presented in **Figure 2**. Total content of HAs is in agreement with literature related with real meat samples.[30] All marinade treatments inhibited the amount of total HAs to less than a half of the levels present in control beef. Of the six treatments evaluated, the less effective on reducing the total HAs content was white wine with herbs. Beer with herbs had the strongest inhibitory effect against total. No correlation was found between higher radical scavenging activity of marinades and decrease of total HAs formation. Cheng et al[12] highlighted that the role of phenolic compounds in Maillard reaction that occurred in HAs formation should be more complex than just being free radical scavenging. Many other factors might contribute to the inhibitory activities of phenolic compounds.

Control beef (unmarinated) exhibit clearly four thermic HAs: PhIP, IQ, MeIQx, and 4,8-DiMeIQx. **Table 1** displays the concentration, expressed as nanograms per gram. The HAs found comprise the three different thermic HAs with the same 2-aminoimidazole moiety: aminoimidazoquinoline (IQ), aminoimidazoquinoxaline (MeIQx and 4,8-DiMeIQx) and aminoimidazopyridine (PhIP).

Pyrolytic HAs, Trp-P-1, Trp-P-2, Glu-P-1 were identified only in concentrations near the detection limit, and the α-carbolines (AαC and MeAαC) were detected in control beef and all marinated samples at negligible levels (not quantified or around 1 ng/g). Although these HAs were included in total HAs estimation their contents were not used to evaluate the performance of marinades.

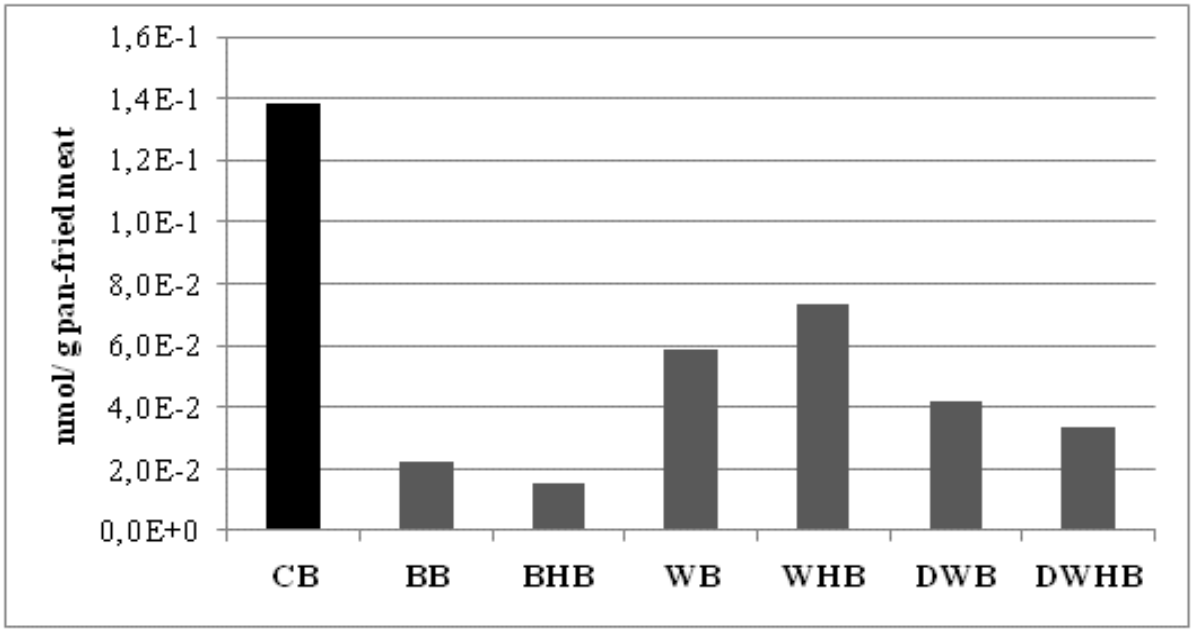


Figure 2. Inhibitory effect of six antioxidant rich marinades in Beer Beef (BB), Beer+Herbs Beef (BHB), Wine Beef (WB), Wine+Herbs Beef (WHB), Dealcoholized Wine Beef (DWB), Dealcoholized Wine+Herbs Beef (DWHB). Control Beef - unmarinated meat (CB). Results are expressed as nmol/ g of pan-fried meat.

PhIP and MeIQx, the most abundant HAs formed under normal cooking conditions,[4] were present in almost equal amounts, which is characteristic due the beef precursors. [31] PhIP, MeIQx and 4,8-DiMeIQx results were similar to pan-fried beef reported by other groups.[12, 32-34] The other HA present in **Table 1**, IQ, was found in control samples in relatively high amount, similar result was observed by Melo et al[5] and Balogh et al.[35]

Discriminative contribution of individual HAs in each treatment was shown in **Table 1**, additionally, results are expressed as % of inhibition towards control beef. When HAs content of marinated meat was below detection or quantification limits, the percentage of inhibition was the estimates using the respective limit of quantification (LOQ) or limit of detection (LOD) and is presented as higher than (>). LOQ and LOD were previously assayed.[7]

Only WHB exhibited the four HAs present in control, in other treatments at least IQ, the more carcinogenic HA[3] was reduced at levels below the detection limit, although IQ formation in WHB, was significantly lower than control (72%). 4,8-DiMeIQx, was the lowest HA presented in control samples (**Table 1**), all marinades reduced that HA significantly to values around 1 ng/g or no detected. IQ and 4,8-DiMeIQx were reduced efficiently by all marinades. Concerning these HAs, Ahn and Grün[36] observed similar behavior in pan-fried beef prior treated with grape seed and rosemary extracts.

Table 1. Effect of marinades on the formation of HAs in pan-fried beef at 180 ºC after 4h of treatment. (results are presented as mean ± standard deviation, *n*=3) followed by inhibition (%).

Meat samples	HAs (ng/g pan-fried beef) and inhibition (%)			
	PhIP	IQ	MeIQx	4,8-DiMeIQx
Control Beef (CB)	9.69 ± 2.27[a]	6.45 ± 0.35[a]	9.07 ± 0.6[a]	3.60 ± 1.64[a]
Beer beef (BB)	4.84 ± 0.93[b,c] (50)	nv.d.[c] (>97)	n.d.[c] (>99)	n.d.[b] (>98)
Beer + Herbs Beef (BHB)	0.83 ± 0.04[c] (91)	n.d.[c] (>97)	n.q.[c] (>76)	1.30 ± 0.62[b] (64)
Wine Beef (WB)	9.82 ± 2.19[a] (-1)	n.d.[c] (>97)	n.q.[c] (>76)	1.08 ± 1.52[b] (70)
Wine + Herbs Beef (WHB)	6.61 ± 2.14[a,b] (32)	1.82 ± 0.11[b](72)	6.09 ± 3.27[a,b] (33)	1.29 ± 0.48[b] (64)
Dealcoh. Wine Beef (DWB)	3.81 ± 1.03[b,c] (61)	n.d.[c] (>97)	4.38 ± 0.79[b] (52)	n.d.[b] (>98)
Dealcoh. Wine + Herbs (DWHB)	2.25 ± 1.32[c] (77)	n.d.[c] (>97)	4.44 ± 0.23[b] (51)	n.d.[b] (>98)

Means with the same letters in the same column are not significantly different ($p > 0.05$). Abbreviations n.d. and n.q. means not detected and not quantified, respectively. Limits of quantification (LOQ) and limits of detection (LOQ) were was previously determined.[7]

Concerning PhIP, it was formed in all treatments, all marinades reduced significantly its formation (more than 50%), except beef samples treated with white wine marinades (WB and WHB). The addition of herbs (BHB, WHB, DWHB) seems to be efficient in its reduction compared with the use of the respective marinade medium alone (BB, WB, DWB). Effect of dealcoholized wine and beer were statistically similar on inhibition of PhIP. Herbs explain around 30% of inhibition of PhIP formation. Murkovic[13] studied the individual application of some of these spices (rosemary, thyme and garlic) on the surface of meat, keep 24 h prior cooking that resulted in significantly lower amounts of PhIP. Smith et al[14] evaluated the effect of three different commercially available marinades and observed a reduction of MeIQx and PhIP due to the spice/herb effect.

MeIQx, the other most abundant HA formed, was reduced in all treatments, with no detection in beer beefs (BB and BHB), however herbs did not exhibit advantage in MeIQx inhibition, especially when added to the wine beefs (WB and WHB). Murkovic[13] reported a decrease in MeIQx levels owing to individual herb effect.

MeIQx and PhIP presented similar inhibition in WHB (around 30% of inhibition) and in BHB (inhibition higher than 76%). Dealcoholized wine marinades with and without herbs reduced both compounds but the effect was slightly stronger in PhIP, especially in the presence of herbs. Opposite behavior was observed in wine alone, with strong effect in MeIQx (higher than 70%) and no effect in PhIP. Oguri[11] suggested that the reaction processes responsible for formation of MeIQx and PhIP may be somewhat different.

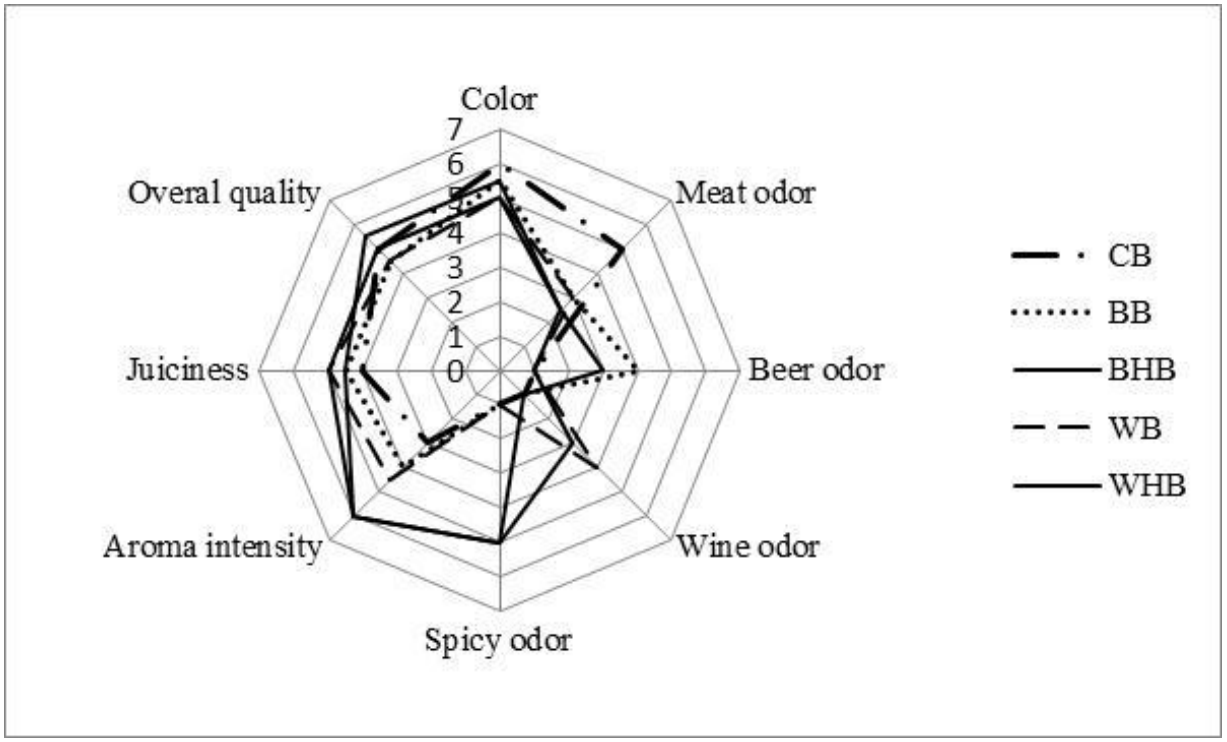
Concerning white wine, a strong decrease was observed in MeIQx formation, and no effect was observed on PhIP formation. Results obtained for white wine marinade are not in agreement with those obtained previously with red wine,[7,22] this work describes fairly inhibition on MeIQx and strong inhibition on PhIP formation. According to Busquets et al[22] the reducing effect on PhIP formation in red wine marinated may be related to the meat absorption of proline, which is an inhibitor of PhIP formation and was the most abundant amino acid in red wine.

Dealcoholized wine marinades promoted significant reduction of PhIP. Apparently, alcohol seems to perform a strong influence on PhIP formation. According to Busquets et al[22] marinating chicken with alcohol/water 1:7 increased of PhIP formation when compared with unmarinated samples. Recently, Wu et al[37] showed accelerating capability of ethanol on the formation of IQ and IQx in a dose-dependent manner in model systems, and advised that cooking with high ethanol content may not be safe. No studies were performed for other HAs in model systems.

Sensory analysis.

Sensory analysis was performed on CB, BB, BHB, WB, WHB samples. In general, data within each attribute were symmetric and mesocurtic. ANOVA performed using the sensory attribute scores was indicative of significant differences in some of the attributes considered. The aforementioned analysis of variance indicated that no significant differences were observed for color, juiciness; and overall quality, however, significant differences were noted for all other attributes (meat odor, beer odor, wine odor, spicy odor, and aroma intensity). The mean results obtained by the trained panel for the 8 sensory attributes assessed in CB, BB, BHB, WB, WHB samples are presented in **Figure 3**. CB samples presented higher meat odor ($p < 0.05$). BB and BHB presented a significantly different beer odor ($p < 0.05$), whereas WB and WHB presented a significantly different wine odor ($p < 0.05$), BHB and WHB presented a significantly different spicy odor ($p < 0.05$). However, no significant differences were noted for scores of overall quality ($p > 0.05$), although BHB samples presented the highest score for this attribute.

In conclusion, our data clearly show that all selected marinades exhibited a reduction in total HAs formation in pan-fried meat. In addition, all beef samples presented good overall quality. Beer marinades can be more efficient than white wine marinades and the addition of herbs provide a superior effect. No correlation was observed between antiradical activity of marinades and total or individual HAs formation. In the present study it was demonstrated that alcohol exerts an important effect on PhIP formation even when applied together with inhibitory ingredients, namely antioxidant polyphenols.



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2.3. Section C

To evaluate the chemopreventive potential of xanthohumol on HAs

2.3.1. Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells

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ABSTRACT

Previous studies showed that xanthohumol (XN), a hop derived prenylflavonoid, very efficiently protects against genotoxicity and potential carcinogenicity of the food borne carcinogenic heterocyclic aromatic amine (HAA) 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). In this study, we showed that XN was not mutagenic in *Salmonella typhimurium* TA98 and did not induce genomic instability in human hepatoma HepG2 cells. In the bacteria XN suppressed the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8 dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) induced mutations in a dose dependent manner and in HepG2 cells it completely prevented PhIP and MeIQx induced DNA strand breaks at nanomolar concentrations. With the QRT-PCR gene expression analysis of the main enzymes involved in the biotransformation of HAAs in HepG2 cells we found that XN upregulates the expression of phase I (*CYP1A1* and *CYP1A2*) and phase II (*UGT1A1*) enzymes. Further gene expression analysis in cells exposed to MeIQx and PhIP in combination with XN revealed that XN mediated up-regulation of *UGT1A1* expression may be important mechanism of XN mediated protection against HAAs induced genotoxicity. Our findings confirm the evidence that XN displays strong chemopreventive effects against genotoxicity of HAAs, and provides additional mechanistic information to assess its potential chemopreventive efficiency in humans.

1. Introduction

Epidemiological studies suggest that diet plays an important role in ethiology of human cancer (Doll and Peto, 1981). On one hand human diet often contains ingredients that cause DNA damage and are potentially carcinogenic, and on the other hand it contains numerous natural constituents with protective effects against cancer and other mutation-related diseases (Ferguson et al., 2004; Knasmuller et al., 2002; Zegura et al., 2011). An important class of compounds in the diet that are considered a dietary risk factor for human cancer are heterocyclic aromatic amines (HAAs) that are regularly formed in cooked meat products (Skog et al., 1998). Since their discovery 30 years ago, more than 20 HAAs have been identified in cooked meat, fish and poultry (Turesky, 2010), with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8 dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) being the most abundant (Costa et al., 2009; Polak et al., 2009; Salmon et al., 2006). HAAs are genotoxic carcinogens, and animal studies showed that they induce cancer in multiple species at multiple tissues (Sugimura et al., 2004). Several human epidemiological studies also indicated association between intake of HAAs and risk for important types of human cancer such as breast, colon or pancreas in meat-eating populations (Zheng and Lee, 2009). The International Agency for Research on Cancer (IARC) classified several HAAs as *probable* or *possible* human carcinogens (IARC, 1993).

Natural phytochemicals derived from dietary sources or medicinal plants have gained significant recognition in the potential management of several human clinical conditions including cancer. Xanthohumol (XN, 3′-[3,3-dimethyl allyl]-2,4,4′-trihydroxy-6′-methoxychalcone), the principal prenylated flavonoid present in the hop plant, *Humulus lupulus* L. (Yilmazer et al., 2001a; Stevens and Page, 2004), has been characterized as a potential "broad-spectrum" cancer chemo-preventive agent acting by multiple mechanisms in the initiation, promotion and progression stage of cancer development (Gerhauser et al., 2002). The antimutagenic effect of XN has been first shown in a bacterial test system with *Salmonella typhimurium* against the HAA 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Miranda et al., 2000a). In the test systems with metabolically active human hepatoma HepG2 cells and precision-cut rat liver slices, XN completely prevented formation of IQ and benzo(a)pyrene (BaP) induced DNA damage at concentrations as

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low as 10 nM (Plazar et al., 2007, 2008). XN showed protective effects also against oxidative DNA damage induced by *tert*-butyl hydroperoxide (*t*-BOOH) (Plazar et al., 2007, 2008), and menadione (Dietz et al., 2005). In addition, XN exhibited anti-inflammatory and antiproliferative effects at the promotional stage of carcinogenesis and prevented formation of carcinogen induced preneoplastic lesions in liver colon and mammary gland (Ferk et al., 2010; Gerhauser et al., 2002).

The protective effects of XN against IQ induced genotoxicity has been extensively studied, however there is no data on potential protective effect of XN against genotoxicity of other HAAs. Therefore, in this study we evaluated the potential protective effect of XN against the most abundant HAAs formed in fried and grilled meat, fish and poultry: PhIP and MeIQx. The protective potential of XN against PhIP and MeIQx induced genotoxicity was assessed in the bacterial test system with *S. typhimurium* TA98 and in the test system with human hepatoma HepG2 cells with the comet and cytokinesis block micronucleus (CBMN) cytome assay. To shed light whether the mechanism that account for the antigenotoxic effect of XN involves modulation of metabolism of HAAs, we evaluated the influence of XN on gene expression of the main enzymes involved in the biotransformation of MeIQx and PhIP in HepG2 cells. The test system with HepG2 cells has been shown to be particularly convenient for investigations of dietary anti-mutagens. Because these cells retain the activities of many metabolic enzymes in inducible form they enable detection of protective mechanisms that are not present in most of the conventional *in vitro* models (Knasmuller et al., 1998, 2002; Mersch-Sundermann et al., 2004).

2. Materials and methods

2.1. Chemicals

Williams' medium E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT [CAS 298-93-1]), cytochalasin B (Cyt-B), acridine orange (AO), dimethyl sulfoxide (DMSO [CAS 67-68-5]), EDTA [CAS 6381-92-6], ethidium-bromide solution (EtBr [CAS 1239-45-8]), Triton X-100 and benzo(a)pyrene [CAS 50-32-8], were obtained from Sigma–Aldrich (St. Louis, USA). Penicillin/streptomycin, fetal bovine serum (FBS), L-glutamine, phosphate-buffered saline (PBS) were from Euro Clone (Siziano, Italy) and trypsin from BD (Franklin Lakes, USA). PhIP and MeIQx were from Toronto Research Chemicals (Ontario, Canada); Xanthohumol was from the Nookandeh-Institut für Naturstoffchemie GmbH (Homburg/Saar, Germany). Normal melting-point (NMP) and low melting point (LMP) agarose [CAS 9012-36-6] and TRIzol® reagent were from Gibco BRL (Paisley, Scotland); High Capacity cDNA Archive Kit and Taqman Gene Expression Assays were from Applied Biosystems, Forest City, CA, USA, TaqMan Universal PCR Master Mix from Applied Biosystems, Branchburg, NJ, USA, and Human GAPDH from Applied Biosystems, Warrington, UK. Lyophilized Aroclor 1254 induced male rat liver post-mitochondrial fraction (S9) was obtained from Moltox, Boone, USA. All chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

XN, PhIP, MeIQx and BaP were dissolved in DMSO; the final concentration of DMSO in incubation mixtures was not higher than 1%.

2.2. Mutagenicity and antimutagenicity testing with the Salmonella/microsomal (Ames) assay

The mutagenicity/antimutagenicity studies were performed with the Salmonella/microsomal reverse mutation assay (Maron and Ames, 1983). For the mutagenicity testing 100 μl XN (final concentrations 2.5, 5, 10 and 20 μg/plate), 100 μl overnight culture of *S. typhimurium* strain TA98 and 500 μl of 4% S9 mix were added to 2 ml of molten top agar, mixed and poured onto minimal agar plates. For the antimutagenicity assay, 100 μl XN (final concentrations 2.5, 5, 10 and 20 μg/plate), 100 μl overnight culture of *S. typhimurium* strain TA98 and 500 μl of 10% S9 mix were added to 2 ml of molten top agar containing MeIQx (final concentration 0.4 μM/plate) or PhIP (final concentration 4 μM/plate), mixed and poured onto minimal agar plates. The number of His^+ revertants was scored after incubation for 72 h at 37 °C. Three plates were used per experimental point.

2.3. Human hepatoma HepG2 cells

HepG2 cells were provided by Prof. Firouz Darroudi, Department of Radiation Genetics and Chemical Mutagenesis, University of Leiden, The Netherlands. The cells were grown in Williams' medium E containing 15% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin at 37 °C in 5% CO_2. Cells were used at passages between 6 and 12.

2.4. Cytotoxicity assay

Cytotoxicity of XN was determined with the MTT assay, according to Mossmann (1983) with minor modifications. This assay measures the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan by dehydrogenase enzymes of intact mitochondria of living cells. HepG2 cells were seeded onto a 96-well microplate at a density of 7500 cells/well in 200 μl. The next day, the growth medium was replaced by complete growth medium, containing 0.1, 1, 10, 50 and 100 μM XN and incubated for 24 h. At the end of the exposure, the cells were washed with PBS and the medium was replaced by fresh growth medium. MTT was then added to a final concentration of 0.5 mg/ml. After 3 h, the medium was removed and the formazan crystals were dissolved in DMSO. The amount of formazan crystals directly correlates to the number of viable cells. The optical density (OD) was measured at 570 nm (reference filter 690 nm) using a spectrofluorimeter (Tecan, Genios). Relative cell survival (viability) was calculated by dividing the OD of the treated cells with the OD of the control cells. The cytotoxicity was measured in two independent experiments each time with five replicates per treatment point.

2.5. HepG2 cells treatment for the comet assay, CBMN cytome assay and QRT-PCR gene expression analysis

Genotoxicity, antigenotoxicity and the effects of mRNA expression of selected genes in HepG2 cells were determined using the same treatment conditions. The cells were exposed to XN alone (0, 0.01, 0.1, 1 or 10 μM) or in the combination with either 200 μM PhIP or 250 μM MeIQx for 24 h at 37 °C in 5% CO_2. 1% DMSO was used as the negative solvent control and BaP (30 μM) was as the positive control. At the end of the exposure the genotoxicity and antigenotoxicity were determined by the comet assay and by cytokinesis block micronucleus assay (CBMA), and mRNA expression of metabolic enzymes was determined with the QRT-PCR. The experiments were performed in three independent repetitions.

2.6. Comet assay

At the end of the exposure, the cells were washed with PBS, trypsinized, centrifuged at 115*g* for 5 min and resuspended in fresh medium. The comet assay was performed according to Singh et al. (1988). Briefly, 30 μL of cell suspension was mixed with 70 μL 1% LMP agarose and added to fully frosted slides coated with 80 μL of 1% NMP agarose. Subsequently, the cells were incubated in a lyses solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for at least 1 h at 4 °C, then the slides were placed into an alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min at 4 °C to allow DNA unwinding and electrophoresed for 20 min at 25 V (300 mA). Finally, the slides were neutralized in 400 mM Tris buffer (pH 7.5) for 15 min, stained with EtBr (5 μg/ml) and analyzed using a fluorescence microscope under 400x magnification (Nikon, Eclipse 800). Images of fifty randomly selected nuclei per experimental point were analyzed with the image analysis software (Comet Assay IV, Perceptive Instruments, UK). For each sample, three independent experiments were performed. The results from three independent experiments are expressed as % of tail DNA and are shown as box plots. One-way analysis of variance (ANOVA, Kruskal–Wallis) was used to analyze the differences between the treatments within each experiment. Dunnett's test was used for multiple comparison of treated versus the control cells; $p < 0.05$ was considered as statistically significant (∗).

2.7. Cytokinesis block micronucleus (CBMN) cytome assay

The CBMN cytome assay was performed according to Fenech (2000, 2006) with minor modifications (Straser et al., 2011). At the end of the exposure the treatment medium was removed and the cells were washed twice with PBS. Then the medium containing Cyt-B (final concentration 2 μg/ml) was added and the cells were incubated at 37 °C/5% CO_2 for additional 26 h. The cells were then trypsinised, washed with PBS, incubated in cold hypotonic solution (75 mM KCl) for 5 min and fixed with methanol/acetic acid (3/1) (v/v) and formaldehyde. Subsequently, the cells were put on microscope slides and air dried. All slides were randomised and coded prior to analysis. The slides were stained with acridine orange (20 μg/ml), and examined under the fluorescence microscope (Eclipse 800, Nikon, Japan) at 400× magnification. For each experimental point the total number of micronuclei (MNi), cells containing micronucleus (MNed cells), nucleoplasmic bridges (NPB) and nuclear buds (NB) were counted in 1000 binucleated cells (BNC) according to the criteria described by Fenech (2000). The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula [M1 + 2M2 + 3(M3 + M4)]/1000, where M1–M4 represent the number of cells with one to four nuclei, respectively. Statistically significant differences between the number of MN, NPB or NB in treated and control groups was determined by Student's *t*-test ; $p < 0.05$ was considered as statistically significant.

2.8. Real-time quantitative PCR (QRT-PCR) analysis

After the incubation, the cells were washed with 1x PBS and total RNA was isolated using TRIzol® reagent, according to the manufacturer's protocol with minor modifications. Glycogen (20 μg/ml) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at −20 °C to precipitate. All solutions needed for RNA isolation were prepared in RNase-free water.

The RNA was transcribed to cDNA using 1 μg of total RNA and cDNA High Capacity Kit, according to the manufacturer's protocol. Gene expression was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays were used (all from Applied Biosystems): *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1), Hs00153120_m1; *CYP1A2* (cytochrome P450, family 1, subfamily A, polypeptide 2), Hs01070374_m1; *UGT1A1* (UDP glucuronosyltransferase 1 family, polypeptide A1), *GSTA1* (glutathione S-transferase alpha 1) Hs00275575_m1; Hs02511055_s1; *NAT2* (N-acetyltransferase 2), Hs00605099_m1; *SULT1A1* (sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1), Hs00419411_m1. The preamplification of *SULT1A1* gene was performed with PreAmp Master Mix (Applied Biosystems) according to manufacturer's protocol before real-time quantitative PCR (QPCR). Amplification of *GAPDH* gene (Human Endogenous Controls, cat. No.: 4310884E, Applied Biosystems, USA) was used as an internal control. The conditions for PCR were 50 °C for 2 min, 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using the ΔΔCt algorithm. The expression levels of target mRNAs were normalized to the *GAPDH* mRNA level. Three independent experiments were performed each time on two duplicate samples. Statistical significance between treated groups and controls was determined by the Student's *t*-test and $p < 0.05$ was considered significant.

3. Results

3.1. Mutagenicity and antimutagenicity of XN in bacterial test system

In the bacterial mutagenicity assay with *Salmonella typhimurium* TA98 in the presence of standard S9-mix metabolic activation system, XN alone did not induce any increase in the number of revertants (Table 1). In the combined exposure experiments, XN strongly and in a dose dependent manner reduced the number of MeIQx and PhIP induced revertants (Table 1). The inhibition of PhIP induced mutagenesis was more efficient than inhibition of MeIQx induced mutagenesis.

3.2. Genototoxicity and antigenotoxicity studies in HepG2 cell

XN at concentrations up to 10 μM did not affect cell viability, whereas exposure to 100 μM reduced the viability of HepG2 cells by almost 50%, compared to untreated control (Fig. 1). The result is in agreement with those reported by Plazar et al. (2007, 2008) in which it has been also shown that XN at concentrations 0.01 to 10 μM did not induce DNA strand breaks. The viability of HepG2 cells exposed to MeIQx (250 μM) and PhIP (200 μM) was not reduced by more than 25% compared to untreated cells (Fig. 1) and was considered suitable for further antigenotoxicity and mRNA expression studies in HepG2 cells.

The effects of combined exposure to XN and MeIQx or PhIP on DNA strand break formation are shown in Figs. 2a and b. Exposure of HepG2 cells to MeIQx and PhIP for 24 h caused significant increase in DNA strand breaks. The formation of MeIQx induced DNA strand breaks was in the presence of XN nearly completely prevented at all tested XN concentrations (Fig. 2a). Similarly, XN prevented PhIP induced DNA strand breaks, however only at lower concentrations of XN (0.01–1 μM), while at the highest concentration (10 μM XN) no significant protection was observed.

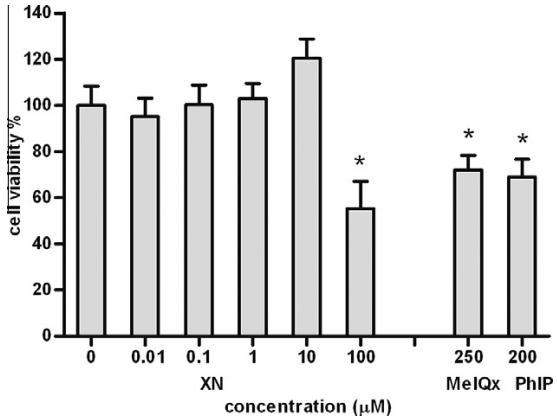

Fig. 1. The effect of XN, MeIQx and PhIP on the viability of HepG2 cells after 24 h exposure. Viability was determined using MTT assay as described in Section 2. Each column is the mean ± SD of three replicates. *Significant difference between the XN treated groups and control (Student's *t*-test; $p < 0.05$).

The results of the CBMN cytome assay showed that exposure to non-cytotoxic concentrations of XN did not induce any increase in the frequency of MNi, NPBs or NBs compared to nontreated control and also did not affect NDI (Table 2). Also MeIQx at the applied exposure conditions did not induce any increase in MNi, NPBs and NBs formation, and did not reduced NDI. PhIP induced slight increase in the frequency of MNi, NPBs and NB, however it was not significantly different from their frequencies in the control, non-treated, cells. The positive control (30 μM BaP) induced significant 3.5-fold increase in MN formation compared to nontreated control cells and reduced NDI by 40%, confirming the metabolic activity of HepG2 cells. Although MeIQx and PhIP gave negative response in CBMN cytome assay we performed also combined exposure to XN and MeIQx or PhIP and the result showed that the presence of XN had no effect on the frequencies of MNi, NPBs and NBs and on NDI (data not shown).

3.3. QRT-PCR gene expression analysis

The results of the changes in the gene expressions of the enzymes involved in the biotransformation of HAAs (*CYP1A1, CYP1A2, NAT2, SULT1A1, UGT1A1* and *GSTA1*) after exposure of HepG2 cells to XN alone and in combination with MeIQx or PhIP compared to corresponding control groups are shown in Table 3. XN alone exerted the most pronounced effect on mRNA expression of *CYP1A1* and *CYP1A2* that were at the highest concentration (10 μM XN) up-regulated by more than 40 and 8-fold, respectively compared to control cells. In a dose dependent manner XN up-regulated also the expression of *UGT1A1* that was at the highest concentration

Table 1
Antimutagenicity of XN against MeIQx and PhIP induced His^+ revertants in *Salmonella typhimurium* TA98 in the presence of metabolic activation.

XN (μg/plate)	– Revertants/plate[a]	MeIQx (0.4 μM/plate) Induced revertants/plate[b]	PhIP (4 μM/plate) Induced revertants/plate[b]
0	33 ± 5	258 ± 28	116 ± 22
2.5	40 ± 3	175 ± 8 (32)	46 ± 13 (60)
5	39 ± 1	167 ± 9 (35)	44 ± 6 (62)
10	33 ± 8	110 ± 10 (57)	27 ± 8 (77)
20	37 ± 4	72 ± 7 (72)	21 ± 10 (82)

[a] Values are mean number of revertants ± SD of triplicate plates.
[b] Values are mean number of MeIQx and PhIP induced revertants ± SD of triplicate plates: the total number of revertants per plate corrected for the number of spontaneous revertants (33 ± 5). The values in parentheses are percent inhibition of MeIQx or PhIP induced revertants in the presence of XN.

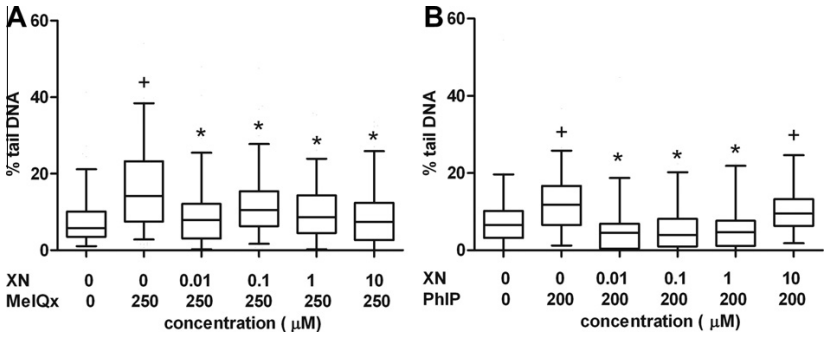

Fig. 2. Protective effect of XN on DNA damage induced by MeIQx and PhIP in HepG2 cells. Cells were exposed to different concentrations of XN (0.01, 0.1, 1 and 10 μM) in combination with (A) 250 μM MeIQx and (B) 200 μM PhIP. The level of DNA strand breaks is expressed as the percentage of DNA in the comet tails. Fifty cells were analyzed per experimental point in each of the three independent experimental cultures. The data are presented as quartile box plots. *Significant differences between treated groups and the negative control. *Significantly different from the group treated with MeIQx and PhIP alone (Kruskal–Wallis test, Dunn's Post-test, $p < 0.05$).

Table 2
The effect of XN, MeIQx and PhIP on chromosomal instability in HepG2 cells determined by CBMN cytome assay.

XN (μM)	MNed/10^3 BNC	MNi/10^3 BNC	NPBs/10^3 BNC	NB/10^3 BNC	NDI
0	27.0 ± 3.0	30.7 ± 4.0	0.3 ± 0.6	32.3 ± 4.7	1.9 ± 0.0
0.01	27.0 ± 3.6	29.7 ± 5.5	0.3 ± 0.6	35.3 ± 11.2	1.9 ± 0.1
0.1	29.0 ± 1.0	30.0 ± 1.0	0.7 ± 1.2	44.0 ± 14.8	2.0 ± 0.0
1	29.0 ± 2.6	32.0 ± 3.5	0.7 ± 1.2	41.3 ± 8.5	2.0 ± 0.0
10	30.0 ± 4.6	32.3 ± 6.0	0.3 ± 0.6	47.0 ± 14.5	1.9 ± 0.0
MeIQx (250 μMl)	29.5 ± 3.5	32.5 ± 3.5	3.0 ± 2.8	45.5 ± 14.8	1.6 ± 0.1*
PhIP (200 μM)	43.7 ± 13.8	48.7 ± 13.7	1.7 ± 1.2	56.7 ± 15.9	1.9 ± 0.0
BaP (30 μM)	90.0 ± 9.5*	105.7 ± 14.6*	1.3 ± 1.2	31.0 ± 6.2	1.1 ± 0.0*

BNC – binucleated cells; MNed – micronucleated cells; MNi – micronuclei; NPB – nucleoplasmatic bridges; NB – nuclear buds; NDI – nuclear division index.
* Significantly different from control cells (Student's *t*-test, $p < 0.05$).

about two fold higher than in control cells. The expression of *NAT2* was by XN not significantly changed, while the expressions of *GSTA1* was dose dependently down regulated. Also the expression of *SULT1A1* appear to be down-regulated at the highest two XN concentrations however, the background expression of *SULT1A1* was in HepG2 cells very low, so that pre-amplification was required for its detection, which is the reason for the very high variability of the results.

Furthermore, we were interested how XN affects the expression of these metabolic genes in MeIQx (250 μM) and PhIP (200 μM) exposed HepG2 cells. MeIQx strongly up regulated the expression of *CYP1A1*, *CYP1A2* and *UGT1A1* and nearly completely down-regulated expression of *GSTA1*, whereas the expressions of *NAT2* and *SULT1A1* were slightly but not significantly down- and up-regulated, respectively. XN did not change MeIQx induced expressions of *CYP1A1*, *SULT1A1* and *GSTA1*. Interestingly, even though XN alone up-regulated expression of *CYP1A2*, it significantly reduced MeIQx induced expression of this gene at all concentrations; at the highest XN concentration by about 50%. The expressions of *NAT2* and *UGT1A2* were in combined exposure up-regulated compared to the expression of these two genes in cells exposed to MeIQx alone. The differences in the expression profiles observed in cells exposed to MeIQx alone to that of the cells exposed to combination of MeIQx and XN may indicate that XN mediated preventive effect against MeIQx induced DNA damage was associated with down-regulation of *CYP1A2* and up-regulation of *UGT1A1*.

PhIP up-regulated gene expressions of *CYP1A1* and *1A2*, and *UGT1A1*, however to lesser extent than MeIQx, and down-regulated expressions of *NAT2, SULT1A1* and *GSTA1*. In the combined exposure XN did not significantly affect expressions of PhIP induced *CYP1A2*, *NAT2* and *SULT1A1*, while it up-regulated expressions of *CYP1A1, UGT1A1* and *GSTA1*. It is notable that the expression of *CYP1A1* was in cells exposed to PhIP in combination with XN significantly higher from that induced by PhIP alone at all concentrations of XN. At concentrations 0.01 to 1 μM XN *CYP1A1* expression was by about 1.4-fold higher from that induced by PhIP alone and the effect on the expression was synergistic at 0.01 and 0.1 μM XN, and additive at 1 μM XN. At the highest XN concentration (10 μM), the expression of *CYP1A1* was 2.6-fold higher from the expression of *CYP1A1* induced by PhIP alone and was at the same level as the expression induced by XN alone. The up-regulation of *GSTA1*, which was in PhIP exposed cells down regulated by about 30% compared to nonexposed cells, was observed only at lower concentrations of XN, while at the highest it was not different from the expression in cells exposed to PhIP alone. Based on the gene expression profiles observed in cells treated with PhIP alone to that observed in cells treated with the combination of PhIP and XN it can be speculated that at low concentrations of XN the protective effect against PhIP induced DNA damage was associated by increased expression of detoxifying enzymes *UGT1A1* and *GSTA1*, while at the high XN concentration the lack of the effect might be associated high expression of *CYP1A1* and concurrent lack of up-regulation of *GSTA1*.

4. Discussion

Previous studies showed that XN, a bioactive ingredient in hop and beer, very efficiently protects against genotoxicity and potential carcinogenicity of the HAA IQ (Ferk et al., 2010; Miranda et al., 2000a; Plazar et al., 2007, 2008). In this study, we showed that in the experimental model with metabolically active HepG2 cells XN exerts protective effect also against the genotoxicity of the most abundant HAAs in grilled meat: MeIQx and PhIP, and the analysis of the modulation of the expression of genes involved in metabolic activation and detoxification of HAAs by XN gave us new information on molecular mechanisms underlying XN mediated protective effect.

Table 3
Effect of XN alone and in combination with MeIQx and PhIP on expression of mRNAs of selected genes encoding HAA metabolizing enzyme in HepG2 cells.

	XN (μM)	HCA bioactivating enzymes				HCA detoxifying enzymes	
		CYP1A1	*CYP1A2*	*NAT2*	*SULT1A1*	*UGT1A1*	*GSTA1*
Control XN	0	1.00 ± 0.00	1.01 ± 0.01	1.00 ± 0.00	1.00 ± 0.01	1.00 ± 0.00	1.02 ± 0.04
	0.01	1.20 ± 0.03	1.33 ± 0.19	0.95 ± 0.06	0.70 ± 0.14*	1.24 ± 0.41	0.79 ± 0.17
	0.1	1.29 ± 0.33*	1.16 ± 0.17	0.79 ± 0.07	1.24 ± 0.17*	1.45 ± 0.45	0.69 ± 0.21
	1	5.23 ± 0.52*	2.79 ± 0.17*	0.82 ± 0.10	0.41 ± 0.03*	1.78 ± 0.47*	0.71 ± 0.04*
	10	42.32 ± 0.29*	8.48 ± 1.42*	0.80 ± 0.01	0.56 ± 0.22*	2.22 ± 0.16*	0.67 ± 0.10*
PhIP	0	15.35 ± 1.29 *	12.35 ± 3.20*	0.67 ± 0.10*	0.31 ± 0.16*	1.40 ± 0.17*	0.66 ± 0.11*
	0.01	21.73 ± 1.35*+	13.40 ± 0.93*	0.56 ± 0.14*	0.03 ± 0.01*	1.47 ± 0.18*	0.72 ± 0.03*
	0.1	20.66 ± 0.81*+	14.01 ± 0.48*	0.62 ± 0.07*	0.84 ± 0.34	1.68 ± 0.07*	1.25 ± 0.09*+
	1	21.22 ± 4.43*+	13.10 ± 1.20*	0.72 ± 0.12*	0.86 ± 0.28	1.90 ± 0.19*+	1.15 ± 0.17*+
	10	40.30 ± 0.89*+	13.46 ± 1.12*	0.77 ± 0.12*	0.45 ± 0.20*	2.19 ± 0.67*	0.65 ± 0.19*
MeIQx	0	156.76 ± 7.38*	41.41 ± 0.22*	0.81 ± 0.09	1.23 ± 0.07	4.77 ± 0.31*	0.07 ± 0.02*
	0.01	142.18 ± 6.26*	35.47 ± 9.52*	0.98 ± 0.07	1.00 ± nd	5.17 ± 0.59*	0.07 ± 0.02*
	0.1	148.21 ± 6.53*	51.18 ± 1.25*+	0.95 ± 0.09+	0.71 ± nd	4.62 ± 0.29*	0.07 ± 0.02*
	1	136.55 ± 5.23*	33.59 ± 6.56*+	1.05 ± 0.08+	0.73 ± 0.53	5.56 ± 0.56*+	0.07 ± 0.01*
	10	162.60 ± 4.84*	20.98 ± 1.75*+	1.20 ± 0.03*+	0.99 ± 0.19	7.49 ± 1.14*+	0.13 ± 0.01*+

Cells were treated with 1% DMSO (vehicle control), XN (0.01–10 μM), MeIQx (250 μM), PhIP (200 μM) and the combinations: MeIQx + XN, and PhIP+XN for 24 h. The data are expressed relative to mRNA levels expressed in control (1% DMSO) cells. Data are means ± SD of three independent experiments.

* Significant difference vs. control.

\+ Significant difference of combined exposure vs. exposure to MeIQx or PhIP alone (Student's *t*-test, $p < 0.05$).

While XN alone was not mutagenic in the bacterial/microsomal tests system with *Salmonella typhimurium* TA98, it strongly suppressed the formation of MeIQx and PhIP induced mutations. The suppression was comparable to that previously observed against IQ induced mutations (Kac et al., 2007; Miranda et al., 2000a). The antimutagenic activity of pure XN against MeIQx and PhIP induced mutagenicity has not been studied before however, Nozawa et al. (2004) showed, that beer strongly suppressed MeIQx and PhIP as well as IQ induced mutagenicity in *Salmonella typhimurium* TA98. They also showed that the suppressive effect was associated with the content of the hop in beer, confirming that hop constituents are responsible for the antimutagenic effect.

Further studies of the protective activity of XN against MeIQx and PhIP induced genotoxicity were performed with the test system with metabolically active human hepatoma HepG2 cells. With the comet assay we detected complete prevention of the DNA damage induced by MeIQx at all tested concentrations of XN that ranged from 10 nM to 10 μM. The result is in accordance with our previous study in which we showed that XN, in HepG2 cells and in rat liver slices, at the same concentration range, completely prevented DNA damage induced by IQ (Plazar et al., 2007, 2008). Also PhIP induced DNA damage was by XN completely prevented, however only at the lower three concentrations (10 nM–1 μM), while at the highest one (10 μM) no prevention was observed. Similar protective response at low doses of XN and the lack of protection at 10 μM XN was reported against *t*-BOOH, induced oxidative DNA damage in rat liver slices (Plazar et al., 2008). The U- or J-shaped response curves where protective effect is observed at low dose level and no protection or even increase in DNA damage at higher concentrations are not uncommon in antimutagenicity studies (Knasmuller et al., 2002). Possible explanation for this phenomenon might be, that antimutagens that may be DNA reactive by themselves induce at low dose a sort of adaptive response, similar to the one observed with ionizing irradiation, which is due to induction of DNA repair enzymes and detoxifying enzymes (Weisburger, 1999). XN is an antioxidant (Gerhauser et al., 2002), and another explanation may be the pro-oxidant activity of flavonoids at elevated doses in combination with agents that induce oxidative DNA damage (Galati and O'Brien, 2004). This may explain why at high XN concentration no protection against t-BOOH induced DNA damage was observed and may also explain the difference in XN protection against MeIQx and PhIP induced DNA damage. PhIP has been clearly shown to induce oxidative stress (Breinholt et al., 2000; Morel et al., 1999), whereas MeIQx was not (Hirose et al., 1999).

The CBMN cytome assay is relatively new method that enables simultaneous detection of multiple parameters associated with chromosomal instability: MN induction that reflects structural and numerical chromosomal aberration, NPB formation that are indicator of chromosomal rearrangements, DNA miss-repair, or telomere end-fusions, and NBs that are marker of gene amplification (Fenech, 2006). With this assay we showed that XN at the applied exposure conditions did not induce genomic instability. This result corroborates our previous studies in which we showed that XN at non cytotoxic concentrations does not induce DNA strand breaks in HepG2 cells (Plazar et al., 2007) and in rat liver slices (Plazar et al., 2008). Several researchers reported induction of MNi by MeIQx and PhIP in HepG2 cells (Majer et al., 2004; Sanyal et al., 1997), however in this study MeIQx and PhIP gave negative response in the CBMN cytome assay. Similarly, positive response in the comet assay and no induction of MNi by PhIP was also observed in recently developed metabolically active human liver derived cells HepaRG cells (Le Hegarat et al., 2010).

Genotoxicity of HAAs in a great deal depends on the balance between the metabolic activation and detoxification pathways (Airoldi et al., 2004). Metabolic activation of HAAs to genotoxic metabolites is a two-step process that in the first step involves *N*-oxidation of the exocyclic amine groups of HAAs to the *N*-hydroxy-derivatives. This reaction is in the liver catalyzed predominantly by the inducible cytochrome CYP1A2 although CYP1A1 and other CYPs are involved too (Boobis et al., 1994; Hammons et al., 1997). The *N*-hydroxy-derivatives can themselves react with DNA, however subsequent *O*-esterification catalyzed by phase II enzymes, mainly by *N*-acetyltrasferases (NATs) and sulfotransferases (SULTs), produce *O*-derivatives that after heterocyclic cleavage produce DNA reactive nitrenium ion (Turesky, 2010). The *N*-hydroxy HAAs derivatives are in addition to NATs and SULTs also substrates of enzymes belonging to UDP-glucuronosyltranspherases (UGTs) and glutathione S-transferase (GSTs) superfamilies that catalyze conjugation of reactive species subsequently enabling their elimination (Hammons et al., 1997).

The antimutagenicity of XN against HAAs in bacterial test system has been explained by inhibition of the activity of cytochrome

P450 enzymes that catalyze metabolic activation of these mutagens (Henderson et al., 2000; Miranda et al., 2000a). However, in our previous study in which we demonstrated protective effect of XN against IQ and BaP induced DNA damage in HepG2 cells and in rat liver slices we found that XN mediated inhibition of CYP1A activity was observed only in isolated liver microsomes, but not in intact cells or tissue (Plazar et al., 2007, 2008). This indicates that in mammalian cells the protective effect is mediated through mechanisms other than those operative in bacteria. Therefore, in this study we applied QRT-PCR based gene expression analysis to explore whether protective effects of XN against MeIQx and PhIP induced DNA damage can be explained by its interference with the expression of genes coding for the enzymes involved in HAAs activation and detoxification.

In HepG2 cells exposed to XN we observed clear dose dependent increase in gene expression of phase I enzymes *CYP1A1* and *CYP1A2* and phase II enzyme *UDP1A1*. The result is expected as XN has been shown to be the substrate of several CYPs enzymes (Yilmazer et al., 2001b), and that it undergoes glucuronidation by different UGTs including UGT1A1 (Ruefer et al., 2005; Yilmazer et al., 2001a). However, in previous studies XN failed to induce gene or protein expression of CYP1A1 and CYP1A2 in mouse Hepa 1c1c7 cells (Gerhauser et al., 2002; Miranda et al., 2000b) and in rat liver slices (Plazar et al., 2008). Currently we do not have explanation for this inconsistency. One of the reasons might be the test model with human derived cells versus rodent experimental models.

It is interesting that XN differently modulated expressions of *CYP1A1* and *CYP1A2* induced by MeIQx or by PhIP. XN down-regulated MeIQx induced expression of *CYPA1A2*, which could contribute to the observed protection against MeIQx induced DNA damage. On the other hand the expression of *CYP1A1* was in PhIP exposed cells in the presence of XN up-regulated, and despite it PhIP induced DNA damage was at lower three concentrations completely prevented. The possible explanation could be concurrent up-regulation of *GSTA1*. Although HAAs have been shown to be poor substrates of GSTs; *N*-acetoxy-PhIP is an exception (Coles et al., 2001; Coles and Kadlubar, 2003). More than 2-fold higher expression of *CYP1A1* in cells exposed to 10 μM XN together with PhIP compared to its expression in cells exposed to PhIP alone and no up-regulation of *GSTA1* may explain the lack of the protective effect of XN against PhIP induced DNA damage at this concentration. High up-regulation of the expression *CYP1A1* also indicates that XN at higher concentrations may not only lose protective effect but may even increase genotoxicity of certain indirect acting mutagens and carcinogens, particularly those that are activated by CYP1A1.

The effect of XN on phase II enzymes involved in bio-transformation of HAAs has so far not been studied. The expression of *UGT1A1* was up-regulated in cells exposed to XN alone and in cells exposed to MeIQx or PhIP alone. In the cells exposed to MeIQx or PhIP in the presence of XN, the expression of *UGT1A1* was elevated over the expressions induced by HAAs alone. This result strongly suggests that induction of *UGT1A1* may play an important role in XN mediated protection against HAAs induced genotoxicity. UGTs are, like many antioxidant and detoxifying enzymes, activated via nuclear factor-erythroid 2 (NF-E2)-related factor-2 (Nrf2)-Kelch-like ECH-associated protein (Keap1) signalling pathway (Copple et al., 2008). In this context, Dietz et al. (2005) showed that the XN mediated elevation of NAD(P)H:quinone oxidoreductase (NQO) activity, which was associated with the protection against menadione induced DNA damage, occurred in an ARE-dependent manner via activation of Nrf2 through alkylation of Keap1. Keap1 modification by XN has been confirmed also by liquid-chromatography–tandem mass spectroscopy (LC–MS/MS) (Luo et al., 2007).

In conclusion, in this study with metabolically active HepG2 cells we showed that XN at non toxic concentrations, at which it did not induce chromosomal instability and DNA damage, exerts strong protection against genotoxicity of MeIQx and PhIP, the two most abundant HAAs in grilled meat. The expression analysis of the main genes involved in bio-transformation of HAAs showed that XN up-regulates the gene expression of phase I (*CYP1A1* and *CYP1A2*) and phase II (*UGT1A1*) enzymes. This suggests that XN is a bifunctional rather than monofunctional phase II enzyme inducer as has been previously suggested (Miranda et al., 2000b). Further gene expression analysis in cells exposed to MeIQx and PhIP in combination with XN revealed that induction of *UGT1A1* may be important mechanism of XN mediated protection against HAAs induced genotoxicity, however this remains to be confirmed also at the level of protein expression and activity. The protective efficiency of XN against MeIQx and PhIP induced genotoxicity was the same as previously observed against IQ. Complete prevention of DNA damage occurred already at nanomolar concentrations suggesting that we may expect protective effects against HAAs induced carcinogenicity also in humans. This assumption is supported by the study showing that feeding rats with XN at concentrations relevant for humans reduced IQ induced liver and colon preneoplastic lesions and DNA damage (Ferk et al., 2010), and by the study in which feeding rats with freeze-dried beer reduced the formation of PhIP induced preneoplastic lesions in the colon (Nozawa et al., 2004). However, the protection against HAAs induced genotoxicity and preneoplastic lesions by XN may not be only due to its interference with activation and detoxification of HAAs as also other mechanisms such as interference with DNA repair, cell cycle regulation and apoptosis may be involved. Thus, further studies addressing the role of these mechanisms in antigenotoxicity and cancer preventive activities of XN are encouraged.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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3. Discussion

Since the late 1970s' a number of research studies have been performed aiming to alleviate human health risk associated with HAs. These studies contribute to the understanding of their formation, characterization, and quantification in foods; strategies to inhibit their formation, either modifying cooking conditions or incorporating different modulators; and their mutagenesis/carcinogenesis, and the search for mechanisms of antimutagenesis by chemical or phytogenic modulators. However HAs remain a challenge for scientists from all these areas (Knasmüller, et al., 2004; Cheng et al., 2006).

Furthermore several researchers have highlighted an urgent need of studying HAs and other concomitant mutagens at the same time, to obtain more realistic exposure levels (Sugimura, 2000; Jägerstad and Skog, 2005; Ferguson, 2010). These compounds occur in mixtures, information about combined effect of compounds from the same group or interactions with concomitant carcinogens from different groups, especially those who have similar metabolic pathways, as in the case of HAs and PAHs (Dumont et al., 2010; Tarantini et al., 2011), need to be taken into account when considering their risk.

For all the above referred HAs will remain one of the foremost areas of research in the field of food chemistry and safety (Cheng et al., 2006). In the present dissertation all areas were taking into account in order to clarify or to add some new information to HAs risk assessment.

3.1. Occurrence of HAs and PAHs in muscle foods cooked in various ways

The levels of HAs and PAHs in different cooked muscle foods and different cooking procedures, from sections A (grilled) and B (pan-fried) in a general way are discussed together, since they provide valuable information concerning the occurrence of those contaminants on Portuguese household cooking procedures. Furthermore, the HAs extraction and detection is the same in both sections and it is pertinent a brief comment of that methodology together with the PAHs methodology validated in the present dissertation (**B2**).

The accurate determination of HAs and PAHs levels is a difficult analytical task, since their occurrence at part per billion has to be determined in highly complex matrices. In the present PhD dissertation HAs extraction and purification was performed according to the Gross method (1990) further modified by Galcerán et al. (1996) (**A1**, **A3**, **B1**, **B2** and **B3**). This multi-step sample preparation method was the recommended method in the inter-laboratory studies due to the reliable results obtained by the experienced analysts (Santos et al., 2004). Separation was carried out by HPLC and detection by DAD and FLD (for some HAs) in order to obtain unequivocal identification and high sensitivity, respectively. Quantitative determination by standard addition

method was selected in order to correct analyte losses and matrix interferences (Gross and Grüter, 1992).

Concerning PAHs in cooked muscle foods, no reference method or inter-laboratory studies were performed, however, their presence in charcoal grilled meat/fish should not be neglected. The need for reliable data about the concentration of PAHs in food is increasing, namely the PAH8 from EFSA (2008). Taking this aspect into consideration it was ascertained a methodology for determination of PAHs levels in charcoal broiled muscle foods (extraction, chromatographic conditions and quantification method) (**A2**). The analytical strategy consisted in extraction using sonication followed by purification on SPE. It was based on an extraction procedure developed for smoked foods (Purcaro et al., 2009) and clean-up for oils (Moret and Conte, 2002), with appropriate modifications for the matrix under study. Standard addition method was selected because it compensates the PAHs losses at different stages of sample preparation, and decreases the contribution of systematic errors as described for HAs, and improves the accuracy of the results. After clean-up and dryness, the PAHs residue is able to be separated and detected by both outstanding techniques HPLC-FLD or GC-MS (/MS), which have been the basis of different official methods (Plaza-Bolãnos et al., 2010). In the present work the HPLC-FLD method was selected, because it provides a higher degree of selectivity between structural isomers than GC-MS, e.g. chrysene and triphenylene give unresolved peaks (Plaza-Bolãnos et al., 2010). EFSA (2008) highlighted that interferences coming from non-target PAHs such as triphenylene that may interfere with chrysene, the most frequent PAH8, should also be considered. The HPLC-FLD developed in the present work provides good chromatographic separation of PAHs, and offers an alternative elution of others that require acetonitrile as the eluent. Analytical performance of HPLC-FLD method indicates that the precision (repeatability and reproducibility) was good and the LODs and LOQs comply with the requirements of the criteria for the chromatographic separation found in the European guidelines (European Commission, 2011). The extraction procedure proposed is not very laborious and uses only one silica cartridge per extraction, although the standard addition method was required for quantification of low levels of analytes in complex matrices. Application on meat and fish samples permitted to prove its suitability and to collect data on PAHs profile in this type of foods (**A2**).

The results from section A and B provide HAs and PAHs levels in different cooked meat and fish dishes using different cooking procedures. The occurrence of HAs and PAHs in cooked muscle foods were not evaluated by other Portuguese research group. Our previous work concerning HAs in Portuguese meat dishes (Melo et al., 2008) was a first effort concerning this matter. Since then the methodology was improved and new information was obtained, which comprises the present PhD dissertation. In addition, the evaluation of HAs and PAHs formation in the same samples are scarce worldwide.

To the present knowledge only two works evaluated the formation of both classes of carcinogens in the same paper, the analytical procedure developed by Rivera et al. (1996) to determine some HAs and some PAHs, and the Knize et al. (1999) study, evaluating the formation of both classes of carcinogens on laboratory cooked hamburgers. Knize et al. (1999) concluded that open flames are required to make PAHs but high variety of heat sources can form HAs. They observed that in pan-fried 30% fat hamburger no PAHs were formed, but MeIQx and PhIP were quantified (3.8 and 16 ng/g, respectively), whereas in charcoal grilled hamburger three of the PAHs were formed but no HAs were detected.

In general very low amounts of HAs are formed in preparation methods which involve gentle cooking conditions such as boiling, steaming, oven roasting, and deep-frying. In contrast the cooking methods associated with an increased cancer risk, which involve high temperature: pan-frying, grilling/barbecuing, and oven-broiling seem to generate the highest HAs concentrations (Solyakov and Skog, 2002; Alaejos and Afonso, 2011). Considering these aspects the work presented in this dissertation was carried out in muscle foods prepared by the last referred cooking method.

Meat (Melo et al., 2008) and fish (**A1**) cooked at rare browning surface (low doneness degree) presented traces or not detected amounts of HAs. However, undercooked meat and fish have been also linked epidemiologically to food born occurrence, and minimum internal cooking temperatures recommended to each type of muscle foods needs to be reached to decrease the microbial content (Puangsombat et al., 2012). The safety internal temperatures were achieved in the doneness degrees applied in our experiments. Fourteen HAs were evaluated, including all with human toxicological relevance (IARC, 1993) which comprises thermic and pyrolytic ones.

Concerning the occurrence of HAs in cooked muscle foods, without addition of ingredients, PhIP and AαC were formed in all samples (**A1, A3, B1, B2** and **B3**). In a general view the most abundant HA was PhIP (1.45-33.8 ng/g), followed by AαC (1-19 ng/g) and MeIQx (not detected-9.07ng/g). The HAs, IQ, 4,8-DiMeIQx, MeAαC, Trp-P-1, Trp-P-2 and Glu-P-1 were also formed in some muscle foods and cooking procedures with relative significance. In contrast IQx, MeIQ, 7.8-DiMeIQx, 4,7,8-TriMeIQx, and Glu-P-2 were not detected at all.

In literature PhIP and MeIQx are the most frequent and abundant HAs detected in cooked beef, fish and poultry (Turesky, 2007; Alaejos and Afonso, 2011). However, data from bibliographic are usually incomplete because HAs comprises numerous compounds and the quantification of each of them in a large number and variety of food samples is not an easy task. Furthermore, in some cases cooking methods are poorly defined or severe conditions have been used in order to maximize HAs production without attention to the palatability (Keating et al., 1999; Alaejos and Afonso, 2011). Additionally, a comparison between results obtained by different analytical methods must be made

with caution. It should be highlighted that all the presented experiments had the acceptance of sensorial panels (**B1**, **B2** and **B3**) or the careful judgment of the authors favoring the edibility (**A1**, **A2** and **A3**) instead of maximizing the hazard compounds formation.

Pyrolytic HAs described as formed under severe conditions (>300ºC) were overlooked in many studies. Exception are the non-mutagenic ß-carbolines, frequently reported (Busquets et al., 2004; Alaejos and Afonso, 2011) and included together with PhIP, MeIQx, and 4,8-DiMeIQx. These HAs are sometimes reported as the only HAs formed under domestic cooking conditions (Solyakov and Skog, 2002; Jautz and Morlock, 2007). In the last years pyrolyic HAs have been receiving more attention (Alaejos and Afonso, 2011) probably due to the improvement of analytical methods (Skog, 2002). However, recent works aiming either occurrence (Iwasaki et al., 2010; Jahurul et al., 2011; Puangsombat et al., 2012) or improvement of analytical methodology (Zhang et al., 2012) of HAs still neglecting this type of HAs. Puangsombat et al. (2012) screened the occurrence of HAs in different type of muscle foods, in several cooking conditions but only thermic HAs were evaluated. Iwasaki et al. (2010) measured HAs concentrations in commonly consumed meats and fish cooked at vary degrees of doneness by the methods typically used in Brazil, which include pan-fried, grilled and barbecued, still pyrolytic HAs were neglected. Dietary exposure to HAs by Malaysian population was achieved after the analysis of six thermic HAs in high temperature cooked foods (Jahurul et al., 2011). In the present dissertation it is clearly shown that mutagenic pyrolytic HAs should be always considered, since they can be formed at temperatures below 200 ºC as observed in article **A1** (for samples of salmon B and C) and articles **B1**, **B2** and **B3**. In a recent study Liao et al. (2010) analyzed the formation of both types of HAs in chicken and duck breast in several cooking procedures at temperatures around 180-200 ºC and in all cases mutagenic pyrolytic HAs were detected, especially the amino- α- carbolines.

Since 1990 several studies have been performed regarding the estimation of dietary HAs. In the beginning it was performed with one to three HAs (Skog, 1998). Later more HAs have been included in estimation of HAs intake. Two studies estimated the average intake of HAs in the US (Layton et al., 1995) and New Zealand (Thomson et al., 1996) diets based on HAs data from literature. According to these studies the three principal HAs in descending order were: PhIP > AαC > MeIQx, and together with 4,8-DiMeIQx and IQ comprised the five most abundant HAs. However, pyrolytic HAs are still being neglected in the intake estimations probably as consequence of scarce information concerning their occurrence. Only thermic HAs accounted to evaluate HAs exposures in different countries further 2000, namely, Switzerland (Zimmerli et al., 2001), Sweden (Olsson et al., 2005), Singapore (Wong et al., 2005; Salmon et al., 2006), Malaysia (Jahurul et al., 2011) and some US (Sinha et al, 2000; Nowell et al., 2002) studies. In the case of the Japanese study performed by Kobayashi et al. (2002) six thermic HAs and Trp-P-1 were analyzed and considered to the total HA intake. Keating and Bogen (2004) estimated the HA intake in the US

population considering the five compounds referred by Layton et al. (1995), however the three dominating HAs were all thermic (PhIP > MeIQx > 4,8-DiMeIQx). The most complete in the wide range of HAs analyzed was achieved by Busquets et al. (2004), the authors screened in their laboratory fifteen HAs from both HAs types in the seven most popular home-cooked meat dishes of the Spanish diet.

In spite of the diversity of HAs in each study, analytical methodologies or cultural differences, PhIP was in all the works described above the most abundant mutagenic HA as in the present thesis. Even without the preferences or intake of muscle foods in Portuguese population, the contribution of "pyrolytic HAs", especially the AαC should not be neglected in HAs exposition.

In relation to PAHs the highest concentration is usually found in charcoal grilled foods (Philips, 1999; EFSA, 2008). Considering cooking methods other than grilled only trace amounts of PAHs are produced. In the present thesis PAHs analyzes was carried out only in charcoal grilled foods, namely, beef (**A2** and **A3**), salmon (**A2** and **A3**) and grilled chicken (**A3**). All samples presented all the PAHs analyzed (15 fluorescent US-EPA PAHs) including the carcinogenic ones that correspond to PAH8.

Troughout Europe the estimated exposure of 279 ng/day of PAH8 by meat and meat products was based on the average consumption of these foods (132 g/day) and the occurrence data on PAHs concentrations in this food group (EFSA, 2008). Considering these consumption, the intake *per* day of PAH8, from barbecued beef, salmon and chicken samples analyzed in the present PhD thesis was extremely higher (**A3**). Prevalence of charcoal grilling is quite variable in different populations (Keating and Bogen, 2004). However, if barbecued chicken (3296 ng/ 132g) or salmon (5718 ng/ 132 g) are consumed in one meal taking into account the average consumption, theoretically the PAH8 intake would exceed the overall dietary exposure of high consumers all over Europe (range: 1415-2136 ng/ day) estimated by EFSA (2008). However, this regulatory body highlighted that the consumption of certain barbecued foods, fatty meat, may lead to an exposure to PAHs that considerably exceeds the above estimated.

3.1.1. Concentration of HAs in meat

Meat samples analyzed, pan-fried beef (**B1**, **B2** and **B3**), charcoal grilled beef (**A3**), and barbecued chicken (**A3**) the values obtained are quite variable even in samples prepared in similar conditions. Concerning pan-fried beef from **B1**, **B2** and **B3**, the total HAs content and the amounts of each HA were within literature levels (see Table 3 in Introduction), however some differences between the results described in the three articles should be highlighted. Pan-fried beefs from **B1** (PhIP, 33.8 ng/g; AαC, 19 ng/g; MeIQx, 3.6 ng/g; 4,8-DiMeIQx, 1.3 ng/g) and **B2** (PhIP, 33.8 ng/g; AαC, 14.7 ng/g; MeIQx, 4.1 ng/g; 4,8-DiMeIQx, 1.3 ng/g) presented similar quantitative and qualitative

profile whereas differences were observed in **B3** (PhIP, 9.69 ng/g; MeIQx, 9.07 ng/g; IQ, 6.45 ng/g; 4,8-DiMeIQx, 3.60 ng/g**)**.

Experiments from **B1** and **B2** were performed chronologically and meat used were cut in a way to achieve the same shape (0.8-1.0 cm) and weight (90-100 g), and pan-fried during 4 minutes *per* side, although higher weight losses were reported in **B1**. Experiment **B3** was performed with beefs weighing 100 g and the thickness was larger (1.2 and 1.5 cm), and to reach the same degree of doneness meat were pan-fried 3 minutes *per* side. As referred in article **B2** and observed in experiments from **A1**, similar surface browning degree do not mean similar content and profile of HAs. The cut is one parameter which can affect the HAs formation (Alaejos and Afonso, 2011). Since HAs are produced mainly in the crust (Skog et al., 1998), higher levels of HAs were obtained owing to larger surface area-to-mass ratio of the beef. In experiments **B1** and **B2** PhIP and AαC were highly produced in contrast with MeIQx and 4,8-DiMeIQx.

Meat from experiment **B3**, showed inversely behavior, for example the pyrolytic AαC was around 1ng/g, and more IQ was formed. These differences can be justified by Skog et al. (2000) that observed, in experiments performed under dry conditions, AαC formation at normal cooking temperatures and a dramatic increase of the yield of PhIP, in contrast to the IQ-compounds whose formation is favored by wet conditions. As described above the dimensions of the beef samples, especially the thickness is of the major importance for HAs formation. Thus, beef samples from **B1** and **B2** exhibited higher larger surface area in contact with the dry heated surface of the pan than **B3** beef samples.

Concerning the charcoal grilled beef (**A3)**, beef samples with 2.5 cm thick and weighing around 400 g were selected, in order to obtain a succulent grilled meat. Temperatures in all our beef experiments were around 200 ºC, the fewer HAs amounts reported in charcoal grilled beef (**A3**) than pan-fried (**B1**, **B2** and **B3**) can be explained by the low surface area-to-mass ratio of the beefs, furthermore the type of heat may also explain the HAs differences. Keating et al. (1999) reported that, usually, grilling results in higher amounts of total HAs formation than pan-frying, however this is true if the same size portion was under study using the same temperature (Liao et al., 2010) or grilling involving high temperatures (Sinha et al., 1998; Ni et al., 2008). Skog et al. (1997) observed in experiments performed at the same temperature (200 ºC), that the heat transferred to the food by air, produces fewer HAs in meat than when the food is in direct contact with the heated pan.

Other type of meat studied was the chicken meat. Chicken is one of the most important protein-rich muscle food sources available today and its consumption and consequent production have been rising rapidly (Solyakov and Skog, 2002; Gašperlin et al., 2009).

Concerning chicken meat, in the present dissertation only barbecued chicken was evaluated (**A3**). Barbecued whole chicken (*frango de churrasco*) is a popular meat dish in Portugal and can be prepared at home or acquired in ready-to-eat commercial houses (restaurants and supermarkets). Chicken samples (**A3**) were collected in a traditional restaurant for this type of meat and all grilling conditions were monitored. The first charcoal grilled chicken samples cooked were considered equivalent to household prepared and in this way could be compared with other muscle foods. The predominant HA formed was PhIP, followed by Trp-P-1.

Literature data show that in a general way PhIP seems to be formed more easily in chicken than in other muscle foods during cooking, while the amount of other HAs, namely MeIQx, is generally lower in cooked chicken than in cooked red meats (Skog and Solyakov 2002; Alaejos et al., 2011; Puangsombat et al., 2012). Differences in composition of the natural precursors could explain these HAs profiles, not only the amino acid pattern and the content of glucose, but also creatine and fat (Pais et al., 1999; Borgen et al., 2001). Several works showed that chicken cooked with skin exhibit lower content of HAs than cooked without skin, the explanation was that skin acts as an insulating layer for the meat, and due to the low levels of creatine in the skin (Solyakov and Skog, 2002; Gašperlin et al., 2009; Puangsombat et al., 2012).

It is interesting to observe the profile of HAs in chicken samples, PhIP was the most abundant followed by Trp-P-1 and α-carbolines and no MeIQx was detected (only in the 2nd period samples). The temperatures reached in these samples were higher, which can explain the Trp-P-1 formation and the absence of MeIQx, which can be degraded or not formed due to the dry conditions (Skog et al., 2000). Pyrolytic amines can also be formed in the lack of creatine, which may explain their presence even with skin. Liao et al. (2010) observed similar profile in charcoal grilled chicken breast. Ni et al. (2008) screened in chicken samples several thermic HAs and α-carbolines. PhIP followed by pyrolytic HAs (α-carbolines) were mainly formed, however no information concerning γ-carbolines is presented.

3.1.2. Concentration of HAs in fish

The fish samples selected salmon (**A1** and **A3**) and sardines (**A1**) are fatty fish species widely consumed. Grilling and barbecuing are the most common methods for preparation of fatty fish, which usually require high temperatures. Experiments from **A1** provided valuable information in relation to the grilling conditions and also a wide spectrum of HAs comprising both HAs types which are very scarce in literature (Alaejos and Afonso, 2011).

Different qualitative and quantitative profiles of HAs were observed in sardine and salmon samples cooked under similar conditions of temperature and doneness. 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 (**A1**). Puangsombat et al. (2012) observed no significant difference in content of HA among three fish species pan-fried or baked, however only thermic HAs were analyzed and no information about the weight/thickness or skin were referred.

As in meat, the amines IQx, MeIQ, 7,8-DiMeIQx, 4,7,8-TriMeIQx, and Glu-P-2 were not detected, however in all fish samples analyzed no 4,8-DiMeIQx were found (**A1** and **A3**), which is different from meat results (**A3**, **B1**, **B2** and **B3)** and in part surprising because in several reports this HA has been reported as the third most abundant (Gibis and Weiss, 2012). Some works reported the presence of 4.8-DiMeQx in pan-fried fish samples (Skog et al., 1997; Salmon et al., 2006; Puangsombat et al., 2012), however in grilled fish this HA in general was to low or not found (Tikkanen et al., 1993; Pais et al., 1999; Iwasaki et al., 2010).

Concerning charcoal grilled sardines (**A1**) by the same grilling conditions (280-300 ºC; 12-15 cm from charcoal) the cooking time was decisive in both amounts and profile of HAs. Slightly differences in grilling time caused large differences in amounts and variety of HAs and differences in weight losses (sardines A: 27%; sardines B: 33%; sardines C: 38%), probably due to the sardines shape: larger surface area-to-mass ratio. No HAs were formed in sardines cooked at "rare" level (sardines A). To reach the "medium" degree (sardines B) only one minute more in each side was needed, around 12 ng/g of HAs (MeIQx> PhIP> AαC = IQ) were formed. In relation to well-done level sardines, one and two minutes/ side more than A and B sardines respectively was needed and around 50 ng/g HAs were formed (AαC> MeAαC> Trp-P-2> PhIP> MeIQx> Trp-P-1> IQ). Thermic amines are the most abundant in sardines B, whereas pyrolytic amines are the most abundant in sardines C. Pyrolytic HAs increased from 2 ng/g (B) to 38.3 ng/g (C). In sardines C the α-carbolines account for more than a half of total HAs. Concerning the thermic HAs, they were found in similar amount (9.6 ng/g) but the profile was different in sardines B (MeIQx> PhIP> IQ) and C (PhIP> MeIQx> IQ).

Longer time and higher temperatures were reported to be needed to produce the initial amount of PhIP compared with MeIQx in fried beef patties (Knize et al., 1994; Balogh et al., 2000), and the same can be concluded for sardines (**A1**). The IQ-type compounds were higher in sardines B than C, especially for MeIQx and can be the result of degradation of thermic HAs with the increasing heating time and crust dryness. No HAs were detected in sardines D grilled at 180-200 ºC to similar surface browning than sardines B. Usually other cooked muscle foods present at least thermic HAs at this temperature, however the sardines skin may act as an insulating layer for the muscle, as described in chicken samples skin can act as a meat protection. The presence of HAs in sardines B and C can be explained by the closeness and rough conditions which damaged part of the skin exposing the precursors to the heat.

Concerning salmon grilled at different conditions to obtain the similar doneness (**A1**), levels of PhIP, and α-carbolines were significantly higher in salmon samples barbecued near the charcoal (at 280 to 300 ºC) (salmon C) than salmon samples grilled at barbecued at longer distance from the heat source (180 to 200 ºC) (salmon B). No significant differences were found between levels of HAs using either charcoal (salmon B) or an electrical griddle (salmon C) at the same temperature. The same degree of doneness can present larger differences in HAs content. HAs formation on salmon was basically dependent on the temperature, at 280 to 300 ºC salmon formed almost 20 ng/g and at 180 to 200 ºC, independently of the heat source, the values of total HAs were around 6 ng/g. Thermic amines were the mainly HAs formed in "medium done" salmon, and PhIP was always the most abundant HA. In electric grilled salmon C, AαC was the only pyrolytic HA formed, but in charcoal grilled salmon B Glu-P-1 was also detected.

Considering the charcoal grilled salmon from experiment **A3**, it can be situated somewhere between salmon A and B (**A1**) conditions, 200 to 220 ºC (18 cm from heat source), exhibiting a total HAs amount around 14 ng/g. The HAs profile was similar to previous reported, however instead Glu-P-1 another pyrolytic were formed, Trp-P-1, as in charcoal grilled beef and chicken, and in sardines. PhIP amounts (7.8 ng/g) formed in **A3** experiment are between the salmon A (13.0 ng/g) and B (4.3 ng/g), as expected. It was observed in the article **A1**, that salmon A presented significantly lower levels of MeIQx (0.5 ng/g) when compared with salmon B (1.3 ng/g), and it was justified either by degradation with the increasing temperature or lower formation due to the dry conditions of the crust. Results from **A3** showed the highest MeIQx formation in salmon grilled (200 to 220 ºC - 4.0 ng/g). This behavior is in agreement with literature since it is reported that in model systems, at temperatures higher than 250 ºC, the concentrations of IQ-type compounds decrease (Felton et al., 1999; Pais et al., 1999), and in beef MeIQx reached the highest formation at 220 ºC (Ahn and Grün, 2005a).

To obtain the same degree of doneness ("medium") at the same distance from charcoal (25 cm) no HAs were formed in sardines, whereas in salmon both types of HAs were measured. Salmon fillets due to the dimension need more time to attain the same doneness and together with the absence of skin justify this difference.

α-Carbolines were ubiquitous in muscle food samples, namely the AαC, other pyrolytic HAs can be formed significantly in high temperature charcoal grilled foods. Although the high temperature/ proximity to the heat source explain the larger formation of pyrolytic HAs through the direct pyrolysis on crust. The presence of other pyrolytic HAs in few amounts in charcoal grilled samples at low temperatures (around 200 ºC) may are a result of pyrolysis of dripping muscle juices in charcoal suffering the reaction at high temperatures and dragged on by smokes to the muscle food.

The present results suggested that the temperatures of formation of α-carbolines classified as pyrolytic HAs should be changed to "formed at normal household conditions" and should be always considered in HAs analyzes in food. The other pyrolytic HAs, γ-carbolines and the δ-carbolines should be accounted into charcoal grilled analysis, their significant formation agrees with the usually reported to pyrolytic HAs (formation at temperatures > 250 ºC). The ß-carbolines (harman and norharman) was already referred as formed at lower temperatures.

Evaluation of pyrolytic HAs in food samples has been neglected, probably due to their lower mutagenicity, however, recent studies describe similar carcinogenicity when compared with thermic ones (Skog et al., 1998; Frederiksen, 2005). Consequently, more studies are necessary to relate the intake of pyrolytic HAs.

The variability on amounts and profiles of polled samples from all papers in the present dissertation could be due to different cooking methods, different weight/thickness of muscle samples, as well as the type and efficiencies of heat transfer. If the selections of different muscle foods to apply the same conditions in laboratory are necessary to understand the HAs formation, it is also fundamental to prepare the muscle dishes in a way that reflects regular household and restaurant cooking conditions to obtain realistic exposures.

3.1.3. Concentration of PAHs in different muscle foods

Quantitative PAHs profiles were different in beef and salmon charcoal grilled (**A2** and **A3**). Between **A2** and **A3**, the same visually doneness was achieved, however some cooking conditions were different. **A2** samples were grilled near the charcoal (280 to 300 ºC) and **A3** samples were grilled distant from charcoal (200 to 220 ºC).

Concerning salmon samples in both experiments (**A2** and **A3**) the raw samples were similar. To obtain the desirable doneness 15 minutes were needed in **A2**, and 23 minutes were needed in **A3**. In relation to beef, beef samples used in **A2** were smaller than in **A3**, however the surface area-to-mass ratio were the same. To obtain the same doneness in **A2** the grilling time was 9 minutes, and 18 minutes in **A3** beef samples. Concerning PAHs formation, surprisingly salmon from **A3** experiments presented higher PAHs level than salmon from **A2**. Regarding beef samples, **A2** presented slightly higher amount of PAHs than samples from **A3**. Since temperature was lower, one possible explanation to higher formation on **A3** salmon samples is the longer time needed to obtain similar doneness.

As reported in **1.3.2**, three mechanisms are possible to PAHs presence in muscle foods: direct pyrolysis of organic matter, such as fat and protein, of the food surface; contamination from heat source; and pyrolysis of melted fat from food on the heat source that can carry back to the food

surface. The possible explanation to higher PAHs formation in salmon at lower temperature (**A3**) is the longer time needed to reach similar doneness as salmon grilled in higher temperature (**A2**), during this period the salmon surface was accumulating PAHs generated by pyrolysis of melted fat. In lean beef no relevant fat could drop and the longer time was not favorable to PAHs increase, however the higher direct pyrolysis of organic matter such as protein in the surface exposed to higher temperature may justify the higher PAHs measured in beef samples from **A2** compared with low cooking temperature selected to **A3** experiments.

In **A3**, salmon exhibited the highest amount of total PAH8 and, followed by chicken and much lower amount was quantified in beef. PAH8 content is significantly correlated (p<0.05) with fat content (INSA, 2006) of these muscle foods.

3.2. Mitigation strategies to reduce the HAs and PAHs

In general, human diets include muscle foods, thus it is impossible to avoid the risk of exposure to the carcinogenic compounds generated from the cooking of these foods. As HAs and PAHs 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 from the viewpoint of food safety.

3.2.1. Mitigation of HAs and PAHs in barbecued foods

The studies included in Section A (**A1, A2** and **A3**) showed several conditions to minimize the formation of the main heat generated hazard compounds in barbecued foods.

Fish consumption presents well-established health benefits, in particular due to the intake of omega-3 polyunsaturated fatty acids, however a number of recent studies have shown that fish might also be a potential source of human exposure to toxic contaminants, including the heat generated toxicants (Domingo et al., 2011).

Scarce information exists concerning HAs formation in fish when compared with cooked meat (Alaejos and Afonso, 2011). The experiments from work **A1**, showed the HAs formation in fatty fish at varying degrees of doneness and grilling conditions (temperature/ distance to the heat source and type of the heat source) in order to accurate the grilling conditions which produce less HAs, making such cooked foods safer for human consumption. In fish samples the varieties and amounts of HAs increase along with increasing temperature and/or time, from the rare to well doneness (sardines A: not detected < sardines B: 11.6ng/g < sardines C: 47.9 ng/g). Even avoiding the very- or well-done grilled fish, precautions must be taken into account when barbecuing fish such as keeping the muscle away from the charcoal heat (sardines D: not detected *vs* sardines B: 11.6 ng/g;

salmon B: 6.97 ng/g *vs* salmon A: 18.94 ng/g) or using an electrical griddle equipment (salmon C: 5.81 ng/g) to reduce the formation of HAs. Fish scales and skin can act as a protective layer in preventing the formation of HAs. Since no HAs were detected in the inner part of grilled salmon, only in crust, avoiding the crust also reduce the exposition to HAs.

In the study **A3** two types of charcoal were compared concerning the formation of HAs and PAHs on salmon and beef. To attain the same temperature (200 to 220 ºC) in the wood charcoal (the same type used in **A1** and **A2**) samples were grilled at18 cm from the heat source whereas in the coconut shell charcoal samples were grilled at 8 cm. No significant differences were observed for HAs and PAHs in beef samples grilled with both charcoal types, whereas salmon grilled with coconut shell charcoal presented significantly lower amounts of HAs and PAHs than salmon grilled with usual wood charcoal. Salmon grilled on coconut charcoal inhibit at least 70% of the PAH8 formation and 60% of thermic HAs, however no differences were observed concerning the pyrolytic HAs. Coconut charcoal is labeled as flameless and smokeless charcoal, this justifies lower amounts of HAs and PAHs in salmon samples grilled with this type of charcoal probably due to the way that charcoal coconut absorb fat that drips from the cooking food. The formation of HAs in salmon grilled on coconut charcoal was quite similar to that observed in salmon grilled on electrical griddle. For fatty fish samples, and may be to other fatty muscles, coconut shell charcoal can be a safety option to barbecue.

The continuous barbecuing with the same charcoal was also evaluated and it was observed that combustion of fat that dropped along the grilling period contributed to higher formation of HAs and PAHs. Concerning the total light PAHs no differences were observed between 1st and 2nd period chicken samples, whereas for the heavy PAHs an increase of 65% was observed. In relation to PAH8 60% increase was observed from the 1st to 2nd period samples. The replacement of charcoal should be frequent in order to avoid the contamination from fatty samples to further grilled samples.

3.2.2. Mitigation of HAs in pan-fried beef by antioxidant rich ingredients

Establishment of adequate databases of HAs content in foods from each population needs to take into account all the factors prior reported. Another important aspect that can affect the HAs formation is the treatment applied before cooking, such as the addition of ingredients which can affect the HAs formation.

As depicted in section **1.2.7.**, the addition of natural products containing antioxidants is considered the main promising strategy to reduce HAs exposure, especially in conditions as close to possible to practical applications.

Marinating meat with several ingredients before grilling or frying is a practice that gives better flavor and texture and differs in function of cooking habits and culture. Compared with the other antioxidant rich-food applications, marinades are an advantage of cooked food is not over spiced and the muscle food do not acquire negative sensory properties (Gibis and Weiss, 2010).

The use of antioxidant rich beverages, such as wine and beer, in marinades are a common practice in Portugal and Spain. In the present dissertation the effect of these beverages in HAs formation on pan-fried beef was evaluated (**B1**). Furthermore, with the knowledge that extracts or phenolic compounds from green tea plant (*Camellia sinensis*) (Oguri et al., 1998; Weisburger et al. 2002; Cheng et al., 2007a) are the most promising inhibitors on HAs formation, the effect of Portuguese (from Azorean island) green tea prepared in household conditions was tested in beef marinades (**B2**).

The last work in Section B (**B3**) was designed aiming to know the behavior of the common used spices on HAs formation when applied in meat marinades. Beer was selected as marinade liquid to suspend the spices taking into account the favorable results from prior works (**B1** and **B3**) on total HAs inhibition and overall acceptance by panel. Since the use of white wine in cooking meat is frequent it was also evaluated the effect of the white wine marinades and the effect of spices together with this alcoholic beverage. Four hours of marinating time was selected for **B3** experiments because it showed the best HAs inhibition time to beer marinade (**B1** and **B2**).

Comparison between marinades effect was done taking into account only 4 hours of marinating time that was common in all works (Section B). Despite some differences in unmarinated beef (control), beer was the stronger marinade liquid, inhibiting around 80% of the total HAs formation (**B1** and **B2**: 79%; **B3**: 84%). All other liquid marinades exhibited at least 50% of inhibitory behavior on total HAs formation. Since beer was the common marinade in all works (**B1**, **B2** and **B3**), results obtained using other marinades were normalized in relation to beer values. Relative efficiencies of liquid marinades were: 1 to beer, 0.9 to dealcoholized white wine, 0.7 to red wine, 0.7 to white wine and 0.6 to green tea marinade. In experiments **B3** also spices were included in marinades, relative efficiency were: 1.1 to beer with herbs, 0.95 to dealcoholized white wine with herbs, 0.6 to white wine with herbs.

Unmarinated samples were chosen as control, since this type of sample is usually used by consumers. Additionally, Busquets et al. (2006) studied the physical effect due to marinating media and analyzed meat samples marinated (30 min, 3 h, and 24 h) prior to cooking in an ethanol/water mixture, with similar alcoholic composition as wine, and no reduction of HAs content was observed as a result of liquid media.

In relation to green tea marinade, it was prepared according to the conventional tea brewing method as infusion to beverage, with brewing temperature and time selected in order to extract the

maximum of catechins. Compared with the unmarinated samples, marinating in green tea resulted in a significant decrease of levels of PhIP and AαC, the effect was stronger with the increase of marinating time. No reduction was observed in 4,8-DiMeIQx and MeIQx, however, the reduction of total HAs were around 50% and 70% at 4h and 6h of marinade. Analysis of organoleptic attributes revealed no significant differences between control samples and tea marinated samples. Since the catechins are natural products present in green tea consumed worldwide without any human disease risk, this procedure might well be introduced in the future in the cooking of meat practices.

Concerning beer and the different wines under study, beer was by far the more effective on HAs inhibition. In experiments from **B1** it could be evaluated the effect of beer and red wine in PhIP, AαC, MeIQx and 4,8-DiMeIQx formation along the time marinade in pan-fried meat. Beer and red wine marinades reduced the amount of PhIP at similar level. The reducing effect (88% after 4 h of the marinating time) of wine marinade on the formation of PhIP was according with Busquets et al. (2006) that also found a pronounced reduction of PhIP (83-88%) at 24h of marinating time by three different red wines. The reducing effect of red wine or beer marinating on the formation of MeIQx was not as remarkable as that observed for PhIP. 4,8-DiMeIQx was completely reduced by beer since the first marinating hour, but red wine needed the six hours to achieve similar performance. The pyrolytic AαC, were reduced by both marinades with oscillations in performance along marinating time, at 4h of marinade time beer was clearly more efficient in the reduction of that HA formation. Beer marinade was the most adequate for maintaining with better scores in usual overall appearance and quality of the pan-fried steaks.

Experiments from **B3** added information about the effect of beer against IQ formation, and showed the effect of white wine and dealcoholized white wine in PhIP, MeIQx, 4,8-DiMeIQx and IQ. Also the effect of herbs added to the liquids under study in **B3** was studied on the last HAs referred. IQ and 4,8-DiMeIQx were reduced efficiently by all marinades. MeIQx, was reduced by all treatments, beer and beer with herbs. Herbs did not exhibit advantage in MeIQx inhibition. Concerning PhIP, in a general way marinades reduced significantly its formation (>50%), except beef samples treated with white wine marinades, alone (no effect) or with herbs (~ 30%). The addition of herbs to the liquid marinades seems to improve PhIP reduction compared with the respective marinade medium alone. The herbs mixture explains around 30% of inhibition of PhIP formation. Beer marinades can be more efficient than white wine marinades and the addition of herbs provides a superior effect. No correlation was observed between antiradical activity of marinades and total or individual HAs formation. **B3** also demonstrated that alcohol exerts an important effect on PhIP formation even when applied together with inhibitory ingredients, namely antioxidant polyphenols. In a general view, all beef samples prepared in **B3** presented good overall quality.

Red and white wine marinades presented very similar reduction effect on total HAs formation, however the performance on each HA was somewhat different. Red wine marinade promoted pronounced PhIP inhibition and fairly inhibition of other HAs, namely in MeIQx. While white wine did not affect the PhIP formation but was very effective in MeIQx and other IQ-type HAs. Oguri et al. (1998) suggested that the reaction processes responsible for formation of MeIQx and PhIP may be somewhat different. However, the same white wine when dealcoholized promoted significant reduction of PhIP. Apparently, alcohol seems to perform a strong influence on PhIP formation. According to Busquets et al. (2006) marinating chicken with alcohol/water 1:7 increased PhIP formation. Wu et al. (2011) showed accelerating capability of ethanol on the formation of IQ and IQx in a dose-dependent manner in model systems.

Based on Skog et al. (2000) observation, that proline inhibits PhIP formation, Busquets et al. (2006) concluded that the reducing effect on PhIP formation by red wine marinade may be related to meat absorption of proline, which was the only amino acid found at high concentrations in the three red wines tested. However, red wines are richer in proline than white ones, as this amino acid is present in the pulp and peel of grapes, thus it is more efficiently extracted during vinification of red wines (Cataldi and Nardiello, 2003). Furthermore, red wine presents considerably higher polyphenols content and antioxidant activity when compared with white wine and beer. The different effects of wine marinades in PhIP formation can be explained considering: alcohol, proline and polyphenols content, which may promote or retard the formation of HAs. Beer also contain alcohol, but at lower level than white wine, but beer also contains higher sugar content, including maltotriose, and 24 g/L of dextrines (Jackson, 2000; Ferreira, 2008), that may inhibit the HAs formation (Jägerstad et al., 1998).

PhIP is the most abundant HA, thus it is very important to evaluate the effect of ordinary seasonings, including marinades in its formation. Clearly, red wine and beer are the best inhibitors of that HA, and the use of the selected herbs improves inhibition of PhIP.

Green tea marinade might well be introduced in the future in the cooking of meat practices, especially for children and consumers that do not use alcoholic marinades owing to medical requirements, food allergies or religious practices (Mateus et al., 2011).

3.3. Modulation of HAs metabolism by xanthohumol

The potential modulator effect of beer in HAs was also evaluated in its metabolism, which is presented in Section C. According to literature beer was shown to inhibit DNA-adduct formation by HAs in several target organs (see Table 4 in Introduction). The key for beer antimutagenicity appears to be the inhibition of the metabolic activation, as detailed in **1.2.7.2.2** from Introduction. It is not known which of the many components contained in beer account for the effect, but several

studies indicate that prenylflavonids from beer may be interesting candidates to the beer chemoprevention against HAs carcinogenicity in rodents.

Previous studies showed that XN, a bioactive ingredient in hop and beer, very efficiently protects against genotoxicity and potential carcinogenicity of the supermutagen IQ (Miranda et al., 2000; Plazar et al., 2007, 2008; Ferk et al., 2010). In Plazar et al. (2007; 2008) studies XN revealed strong inhibition of genotoxicity of IQ in human hepatoma HepG2 cells, in addition similar effect was also observed in BaP DNA damage induced.

As shown in results from Sections A and B, IQ was not so frequent in muscle food, thus, it is important to evaluate the XN effect on HAs more frequent in diet. The use of recent methods to evaluate geno- and antigenotoxic effects in human cell lines is needed to obtain more realistic approaches to human. If there is no doubt about PhIP to be the most abundant, it is important to evaluate the XN effect on its metabolism. MeIQx was also selected, due to the high frequency on foods and also due to the higher mutagenicity, furthermore there are some differences in biotransformation and target organ between both HAs (Turesky et al., 2007; Dumont et al., 2010).

In the present study (**C1**) using metabolically competent human cell line (HepG2), it was shown that XN prevents completely the PhIP induced DNA damage at lower concentrations (10 nM to 1 μM) may mediated by detoxifying enzymes (GSTA1 and UGT1A1), while at 10 μM no prevention was observed, maybe the lack of the effect might be associated with high expression of *CYP1A1* and concurrent lack of up-regulation of *GSTA1*. In relation to MeIQx, XN prevents completely the DNA damage at all concentrations in the study, mediated by down-regulation of *CYP1A2* and up regulation of *UGT1A1*.

In a general way, in human HepG2 liver cells XN up-regulates the expression of phase I (*CYP1A1* and *CYP1A2*) and phase II (*UGT1A1*) enzymes. However, gene expression analysis in cells exposed to MeIQx and PhIP in combination with XN revealed that XN mediated up-regulation of *UGT1A1* expression may be an important mechanism of XN mediated protection against HAs induced genotoxicity.

XN exerts protective effect also against the genotoxicity of the characterized most abundant HAs: MeIQx and PhIP. It was shed light for the first time the modulator effect of XN on the expression of genes involved in metabolic activation and detoxification of HAs by XN, which gave us new information on molecular mechanisms underlying XN mediated protective effect.

In a recent study (Ferk et al., 2010), developed by some co-authors from the present study (**C1**) (B. Žegura, and M. Philipič), it was concluded that XN prevents *in vivo* lesions induced by IQ at low doses. When extrapolated to humans, the XN dose required corresponds to 4.97 mg in a 70 kg person, through the ingestion of 200 ml of the recent developed beers with increased XN levels (up to 20 mg/L) this amount can be uptake.

4. Conclusions

4.1. Main Conclusions

Data from this thesis provide valuable information concerning the occurrence of HAs and PAHs on muscle foods cooked reflecting Portuguese household procedures.

In the present thesis it was established a methodology (extraction, chromatographic conditions and quantification method) suitable to collect data on PAHs, namely the PAH8 from EFSA, in grilled meat and fish. The technique is not expensive or laborious, and complies with the requirements of the criteria for the chromatographic separation found in the European guidelines.

In what concerns HAs occurrence, in general the most abundant HA was PhIP, as described in cooked foods worldwide. Other predominant HAs were AαC and MeIQx, the relevance of each of them in different samples seems to be somewhat opposite. Whereas AαC was rather formed with increasing temperature and/or crust dryness, for MeIQx these conditions were disadvantageous. The present work provides information concerning HAs formation in grilled fish using different conditions, this information is scarce in literature.

In the present dissertation it is clearly shown that mutagenic pyrolytic HAs, frequently neglected, should be always considered in HAs occurrence. Even without the preferences or intake of muscle foods in Portuguese population, the contribution of the AαC it is crucial in HAs exposition.

Concerning PAHs occurrence in grilled muscle foods, quantitative PAHs profiles were different among salmon> chicken> beef. PAH8 content is significantly correlated with fat content of these muscle foods.

As HAs and PAHs are candidates in the etiology of human cancer, the search for ways to minimize their intake was an important issue in the present dissertation.

The barbecuing conditions affect the formation of HAs and PAHs in muscle foods. Efficient mitigation strategies of HAs in fatty fish were to grill at longer distance of charcoal or substitute the wood charcoal by coconut shell charcoal or using an electrical grill device. Considering the high PAHs formation in this muscle food, the last two options seem to be also effective in prevention of PAHs formation. The fish scales and “skin” can also act as a protective layer in preventing the formation of HAs. Concerning lean beef samples no differences between grill on wood or coconut charcoal were observed concerning HAs and PAHs formation.

The continuous barbecuing with the same charcoal contributed to higher formation of HAs and PAHs in chicken samples along the grilling period. The replacement of charcoal should be frequent in order to avoid HAs and PAHs contamination promoted by dripping juices from previous grilled samples.

Meat marinades usually applied in Portuguese cuisine, such as wine and beer marinades, are a useful strategy for HAs reduction. Since the use of alcohol marinades should be avoided by certain population groups, green tea marinade might well be introduced, which gave inhibitory HAs formation and also good acceptance by a trained sensorial panel. Beer showed the best inhibitory results in the total HAs formation. The addition of selected herbs (garlic, ginger, thyme, rosemary, red chili pepper) to beer improved the inhibitory effect. Concerning wine marinades, either red wine or white wine were effective on HAs reduction, however some differences were observed on individual HAs formation depending on wine type. Advantages on PhIP reduction were observed by the application of herbs in marinades. Concerning this HA, it was shown that alcohol affected its formation; however other components present in these alcoholic beverages can explain the inhibitory effects.

In the present thesis it was shown that xanthohumol (XN), a compound found in beer, displays a strong chemopreventive effect against genotoxicity of PhIP and MeIQx. It was shed light for the first time the modulator effect of XN on the expression of genes involved in metabolic activation and detoxification of HAs by XN, which gave us new information on molecular mechanisms underlying XN mediated protective effect. Gene expression analysis in cells exposed to MeIQx and PhIP in combination with XN revealed that XN mediated up-regulation of *UGT1A1* expression. This may be an important pathway of XN mediated protection against HAs induced genotoxicity.

There is no doubt concerning the high variety of heat sources that can contribute to HAs exposition. PAHs seem to be a relevant contaminant in barbecued muscle foods. Taken together, results show that cooking conditions, type of muscle foods and ingredients influence HAs in muscle foods. Grilling conditions also affect PAHs levels in muscle food. Marinades usually applied in Portuguese cuisine, such as wine and beer marinades, is a useful strategy on HAs reduction. Green tea marinades is an option to some population groups that should avoid alcohol.

Beer is one of the most widely consumed alcoholic beverages worldwide and it contains bioactive compounds that can prevent HAs carcinogenesis, either by inhibiting their formation or also as chemopreventing agent.

4.1. Future trends

Data from this thesis highlight the importance of assessing the real risk associated to the intake of HAs and PAHs, in barbecued muscle foods.

In the future, for assessment of the eating habits of Portuguese population concerning muscle foods, it should be applied a food frequency questionnaire taking into account the type of muscle,

the different culinary treatments applied before cooking, the ingredients used in recipes, the cooking procedures, and also the preference doneness (including the inside and outside food appearance). These parameters will be useful to establish a database in HAs and also PAHs reflecting the real heating habits.

Assays concerning the behavior of HAs and PAHs after intake, not only bioaccessibility but also metabolism should be considered in the evaluation of the real risk effect. It is important to understand in which way the food matrix/compound influences the bioaccessibility, and also to consider the interaction from other food items.

XN completely prevented the DNA damage induced by HAs (PhIP and MeIQx) in HepG2. The potential of other prenylflvonoids should be evaluated, namely IX. Since IX is the most abundant prenylflavonoid in beer, resulting from the isomerisation of XN during the brewing, its effect on HAs toxicity should also be evaluated.

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