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Liquid Chromatography–Tandem Mass Spectrometry Analysis of 2-Amino-1-methyl-6-(4-hydroxyphenyl)-imidazo[4,5-*b*]pyridine in Cooked Meats

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Several cooked meats such as beef (fried, coated-fried), pork (fried, coated-fried), and chicken (fried, griddled, coated-fried, roasted) were analyzed for the heterocyclic amine 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4′-OH-PhIP) not commonly determined in food and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). The highest content of 4′-OH-PhIP was found in fried and griddled chicken breast, the concentration being 43.7 and 13.4 ng/g, respectively, whereas the corresponding PhIP concentrations were 19.2 and 5.8 ng/g. The estimated concentration of both pyridines in fried pork loin, in fried pork sausages, and in coated-fried chicken was below 2.5 ng/g. In the rest of the samples, 4′-OH-PhIP was not detected. The analyses were performed by solid-phase extraction and LC-MS/MS. The fragmentation of 4′-OH-PhIP in an ion trap mass analyzer was studied in order to provide information for the identification of 4′-OH-PhIP. Additionally, the effect of red wine marinades on the formation of 4′-OH-PhIP in fried chicken was examined, finding a notable reduction (69%) in the amine's occurrence.

KEYWORDS: 4′-OH-PhIP; PhIP; heterocyclic amines; chicken; fried meat; wine marinades

INTRODUCTION

The Maillard reaction comprises a complex set of chemical reactions that take place between reducing sugars and amino acids. This amino–carbonyl reaction occurs during the heating process of foodstuffs. Among the wide range of compounds formed, there are some, such as heterocyclic amines (HCAs) (*1*), whose metabolically activated derivatives can form adducts with DNA (*2*). At present, it is difficult to establish a relationship between eating/cooking habits and cancer; however, better knowledge of the occurrence of food toxicants and their metabolism should lead to better understanding of their implication in cancer etiology. HCAs occur in protein-rich products cooked with methods involving efficient heat transfer (*3*). The formation of these cooking toxicants is affected by food composition, cooking procedures, and in some cases the ingredients used for the cooking. Today, over 20 HCA congeners have been detected in cooked food and in cooking residues (*4*–*7*) and also in cooking fumes (*8*, *9*). One of the most abundant mutagenic HCAs in cooked meat is 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (*5*, *10*, *11*). This compound has been demonstrated to be mutagenic (1800 revertants/μg) in *Salmonella typhimurium* TA98 with S9 mix (*12*), carcinogenic in animals (*13*, *14*), and to bind to DNA in humans (*15*–*18*). The International Agency for Research on Cancer classified PhIP as a possible human carcinogen (class 2B) (*19*), and recently PhIP together with IQ, MeIQ, and MeIQx were listed as "reasonably anticipated to be human carcinogens" by the U.S. Department of Health and Human Services (*20*). In contrast, the hydroxyl derivative of PhIP, 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4′-OH-PhIP, **Figure 1**), is one of the HCAs less frequently determined in food products probably because of the lack of a commercial standard. In fact, 4′-OH-PhIP has only been reported from two laboratories (*21*, *22*), where it was extracted from beef. With regard to 4′-OH-PhIP toxicity, it is known that it induces low mutagenicity in *S. typhimurium* TA98 with S9 mix (*21*), although it has not been tested in other toxicological assays. Nevertheless, Hatch et al. found dependence of HCA mutagenicity on structural factors, indicating that the number of fused rings, the number of heteroatoms in the nonimidazole ring, and also a methyl substitution on imidazo ring nitrogen atoms or on ring carbon atoms were of great relevance for mutagenicity (*23*). Moreover, a recent work has shown that the position of the methyl group in the imidazo ring seems to be crucial for the mutagenic activity

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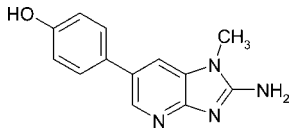

Figure 1. 2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4′-OH-PhIP).

since PhIP (methyl group at the 1-position) showed 100-fold higher mutagenic potency than its isomer 3-Me-PhIP (*24*). As the structure of 4′-OH-PhIP is very close to PhIP, its occurrence in some processed foods could constitute a human health risk. To our knowledge, this compound has only been reported in beef samples. Kurosaka et al. (*21*) described the occurrence of 4′-OH-PhIP in broiled beef using liquid chromatography with ultraviolet and fluorescent detection, and Reistad et al. (*22*) determined it in fried beef by GC-MS after a derivatization step. Furthermore, an additional reason for the analysis of 4′-OH-PhIP in food is that 4′-OH-PhIP (*22*, *25*) and also its sulfated derivative (*26*) have been identified as PhIP metabolites in human urine.

In the present work, a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed for the identification and quantitation of 4′-OH-PhIP in cooked meat. The first part of the present work is devoted to the study of the fragmentation pathway of 4′-OH-PhIP by means of multistep ion trap mass spectrometry. In the second part of the paper, 4′-OH-PhIP and PhIP were determined in several home-cooked meats using different cooking practices. Special attention was paid to the effect of marinating chicken breast with wine on the occurrence of 4′-OH-PhIP after frying.

MATERIALS AND METHODS

Chemicals. 2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4′-OH-PhIP) was synthesized at the National Cancer Center Research Institute (Tokyo). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-1-trideuteriomethyl-6-phenylimidazo[4,5-*b*]pyridine (D_3-PhIP), 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 9*H*-pyrido[3,4-*b*]indole (norharman), and 1-methyl-9*H*-pyrido[3,4-*b*]indole (harman) were purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). The chemical purity of HCAs was >99%, according to the manufacturers. The chemical purity of 4′-OH-PhIP was >97%. Individual stock standard solutions of 150 μg/g in methanol were prepared and used for further dilution. All chemicals and solvents were HPLC or analytical grade and were provided by Merck (Darmstadt, Germany). Materials for solid-phase extraction were diatomaceous earth, obtained from Isolute (Hengoed, Mid-Glamorgan, U.K.), and C_{18} (100 mg) and PRS (500 mg) cartridges, obtained from Varian Associates (Harbor City, CA).

Food Samples. Meat samples (see **Table 1**), olive oil, salt, golden bread crumbs, and eggs were bought in a local store. Cooking was performed using a gas cooker. A surface thermometer (Testo Instruments, Cabrils, Spain) was used for monitoring the temperature of the frying pan, griddle, or clay casserole. Initially, the surface temperature of the pan, griddle, or clay casserole was 200 °C. This temperature dropped to 170–175 °C when olive oil was added. During the cooking processes the temperature at the sample crust was 180–200 °C. For roasting, the temperature in the casserole was 160 °C when the cooking started and ranged from 140 to 160 °C at the meat crust during cooking. For griddling and roasting, olive oil was used to grease the surface of the griddle or the clay casserole. For frying, olive oil was used up to a level to almost cover the meat fillets. Steaks and burgers were seasoned with 1 g of salt per steak before cooking on the surface. The average weight of cooked fillets was 60 g for beef steaks, 44 g for beef burgers, 34 g for pork loin steaks, 135 g for pork sausages, 40 g for chicken fillets (griddled, fried, coated-fried), and 73 g for roasted chicken breast. When coating was performed, the surface of the steaks was covered with a layer of golden bread crumbs with egg prior to frying. **Table 1** shows the cooking conditions for the different meat products studied. Meat fillets were turned once during cooking in the half-cooking process. The frying pan and the griddle were washed after each cooking to avoid cross-contamination. Oil was not reused. The selected cooking conditions provided edible and appetizing products. The crust (3–4 mm) from the cooked samples was separated, frozen, and stored at −18 °C until analysis.

Table 1. Duration, Temperature, Cooking Method, and Estimated Concentrations of 4′-OH-PhIP and PhIP

meat sample	cooking method	total cooking time (min)	cooking temp (°C)	ng of 4′-OH-PhIP/g of cooked meat[a]	ng of PhIP/g of cooked meat[b]
beef burger	pan frying	11	180–200	<LOD	0.6
beef steak	pan frying	12	180–200	<LOD	1.1
beef steak	coating, frying	14	180–200	<LOD	0.2
pork loin	pan frying	10	180–200	0.7	2.5
pork loin	coating, frying	7	180–200	<LOD	1.3
pork sausage	pan frying	9	180–200	0.5	<0.1
chicken breast	pan frying	11	180–200	43.7	19.2
chicken breast	griddling	13	180–200	13.4	5.8
chicken breast	coating, frying	9	180–200	0.3	0.6
chicken breast	roasting	138	140–160	<LOD	0.9

[a] LOD of 4′-OH-PhIP = 0.08 ng/g. [b] LOD of PhIP = 0.02 ng/g.

For marinating experiments chicken breast was marinated in three different red wines for 0–24 h before cooking with the same three wines (A, B, and C) as previously described (*27*). The samples were fried in a thermostat-controlled aluminum frying pan set at 220 °C for a total cooking time of 10 min. For each studied condition three independent replicates were marinated and cooked. Meat was turned once at half-cooking. Neither salt nor oil was used.

Extraction of HCAs. The crust of the cooked meat fillets was pooled, freeze-dried, and ground with a mortar. Two grams of ground crust was used for each extraction. Freeze-dried crust was mixed with 1 M NaOH (6–10 mL) and homogenized using an Ultra-Turrax T 25 basic (IKA, Staufen). HCAs were extracted, purified, and preconcentrated with the solid-phase extraction method developed by Toribio et al. (*28*) using ethyl acetate as extraction solvent. The purified extracts obtained after the cleanup of the freeze-dried ground crust were evaporated under a gentle stream of nitrogen and were reconstituted with a solution of internal standard d_3-PhIP in methanol (0.15 μg/g).

Quantitation. A screening to detect the presence of 4′-OH-PhIP was performed on several home-cooked beef, pork, and chicken samples. Both 4′-OH-PhIP and PhIP were quantified by standard addition in fried chicken breast and griddled chicken breast, the two meat products where the highest amount of this compound was found. Four spiking levels of both pyridines ranging between 16 and 128 ng/g of cooked chicken were used. To set the standard addition calibration curves within the linearity range of the instrument, up to 1 μg/g, the purified extracts were reconstituted using 1 mL of internal standard solution. Recoveries were calculated from the slope of the linear regression obtained between the added analyte concentration and the measured analyte concentration.

In those meat dishes with low concentration of both amines and also in the wine marinade samples, 4′-OH-PhIP concentration was estimated by comparison with the response of PhIP, which was quantified by standard addition as described before. The purified extracts obtained from the samples were reconstituted in 0.1 mL of internal standard solution in order to improve sensitivity. Some fried chicken marinated samples where high yield of the studied compounds was found required a dilution (1/6) to set the response in the linearity range of the method (0.005–1 μg/g). Blanks of the analytical process were analyzed to prevent false identification of 4′-OH-PhIP in the food samples.

LC-MS Conditions. A HPLC system Alliance 2695 from Waters (Milford, MA) and a Spectra Physics P2000 from Thermo Fischer

Table 2. Optimized CID Conditions in MS^2 and MS^3 and Main Product Ions Obtained for 4′-OH-PhIP

	MS spectra		MS^2 CID[a] conditions		MS^2 spectra		MS^3 CID[a] conditions		MS^3 spectra	
compound	*m/z* (rel ab, %)	tentative assignation	NCE[b] (%)	AQ[c]	*m/z* (rel ab, %)	tentative assignation	NCE[b] (%)	AQ[c]	*m/z* (rel ab, %)	tentative assignation
4′-OH-PhIP	241.2 (100)	$[M + H]^+$	49.0	0.45	241.2 (15)	$[M + H]^+$				
					226.2 (100)	$[M + H - CH_3]^{+\bullet}$	51.0	0.45	226.2 (25)	$[M + H - CH_3]^{+\bullet}$
									225.1 (12)	$[M + H - CH_3 - H]^+$
									224.1 (11)	$[M + H - CH_3 - H_2]^{+\bullet}$
									209.1 (16)	$[M + H - CH_3 - OH]^{+\bullet}$
									208.0 (16)	$[M + H - CH_3 - H_2O]^{+\bullet}$
									201.1 (31)	$[M + H - CH_3 - CN_2H_2 + OH]^+$
									200.1 (22)	$[M + H - CH_3 - CN_2H_3 + OH]^{+\bullet}$
									199.1 (100)	$[M + H - CH_3 - HCN]^{+\bullet}$
					224.2 (7)	$[M + H - NH_3]^+$				
					213.2 (24)	$[M + H - CNH_2]^{+\bullet}$				

[a] CID: collision-induced dissociation. [b] NCE: normalized collision energy. [c] AQ: trapping ratio frequency voltage.

Scientific (San Jose, CA) were used in the screening of 4′-OH-PhIP in cooked meats and in the marinating study, respectively. Separation was performed on a Symmetry C_8 5 μm (2.1 mm × 150 mm) column from Waters. The sample volume was 5 μL. The mobile phase was a gradient with acetonitrile (solvent A) and 30 mM acetic acid–ammonium acetate buffer adjusted at pH = 4.5 with ammonia (solvent B) at a flow rate of 0.3 mL/min. The gradient program was as follows: 0–0.5 min, 5% A; 0.5–15 min, 5–20% A; 15–18 min, 20–60% A; 18–24 min, 60% A; 24–27 min, return to initial conditions; 5 min postrun delay. Postcolumn addition of 0.1% formic acid in acetonitrile at a flow rate of 0.1 mL/min was applied. HCA detection and quantitation were done by ESI-MS/MS with an ion trap LCQ classic in the screening study and LCQ Deca in the marinating study, both from Thermo Fischer Scientific. Quantitative analyses were carried out in product ion scan mode. Ion source working parameters were as follows: spray voltage, 3 kV; sheath gas, 90 au; auxiliary gas, 60 au; heated capillary temperature, 280 °C. For 4′-OH-PhIP and PhIP, optimal values of collision-induced dissociation (CID) conditions, capillary voltage, and tube lens offset voltage were studied by infusing a standard solution of each analyte in methanol (5 μg/g) at 5 μL/min into the mobile phase at its corresponding eluting conditions: 17% acetonitrile in buffer (solvent B) for 4′-OH-PhIP and 60% acetonitrile in buffer for PhIP at 0.3 mL/min, with postcolumn addition of 0.1% formic acid in acetonitrile at 0.1 mL/min. An isolation width of *m/z* 1.5 was applied to isolate the precursor ion. The activation Q (AQ) was set at 0.45. The acquisition was performed in centroid mode, with a maximum injection time of 50 ms and one microscan for PhIP and maximum injection time of 100 ms and three microscans for 4′-OH-PhIP. The normalized collision energy values in MS/MS and MS^3, the main product ions of 4′-OH-PhIP, and their tentative assignment are given **Table 2**. For PhIP and d_3-PhIP, tandem mass spectrometry was performed at a normalized collision energy of 51% in both ion trap mass analyzers.

The masses scanned varied from *m/z* 110 to *m/z* 250, and quantitation was carried out using the most intense product ions for each compound. Quantitative analysis was performed in positive mode using the MS/MS transitions $[M + H]^+ \rightarrow [M + H - CH_3]^{+\bullet}$, $[M + H - CNH_2]^{+\bullet}$ for 4′-OH-PhIP and $[M + H]^+ \rightarrow [M + H - CH_3]^{+\bullet}$ for PhIP and d_3-PhIP. The identification of the analytes was carried out by means of the product ion scan spectra.

RESULTS AND DISCUSSION

During 2003–2007, more than 200 food samples were analyzed, searching for HCAs, but 4′-OH-PhIP has not been investigated in any of them (*29*) probably due to the lack of availability of 4′-OH-PhIP standard. In our case, during the analysis by LC-MS of several samples of fried chicken unmarinated and marinated with red wine (*27*), the presence of a relatively intense chromatographic peak eluting at 12.6 min and with a *m/z* of 241 was observed. In order to obtain additional information about this compound, MS^2 experiments were performed in that peak, and characteristic fragmentations previously described for HCAs such as the losses of $\cdot CH_3$, NH_3, HCN, or CN_2H_2 were observed (*30*). A review of the HCAs found in cooked food until the present time let us think that 4′-OH-PhIP could be the possible unknown HCA in fried chicken because its predicted mass spectra in positive ion mode would match the observed *m/z* of 241.

To confirm univocally the occurrence of 4′-OH-PhIP, its fragmentation was studied using multistep mass spectrometry, and some characteristic ions were proposed to be used for identification. The main product ions obtained in MS^2 and MS^3 experiments and their tentative assignments are given in **Table 2**. See also **Figure 2**. The ion at *m/z* 241, $[M + H]^+$, was exclusively obtained in MS mode, and it was used as the primary precursor ion. To perform MS^n experiments, the normalized collision energies were adjusted to provide a maximum intensity of the product ion, keeping a signal of the precursor ion around 15% of relative abundance. The MS^2 spectrum of the protonated molecule showed an intense fragment at *m/z* 226 that can correspond to the loss of a methyl group in agreement with fragmentation proposed for PhIP (*30*). Other ions were obtained in the MS^2 spectrum, and the one at *m/z* 224 with a relative abundance of less than 10% may be assigned to the loss of ammonia, as happens for other HCAs (*30*). An additional fragmentation not reported for PhIP was found at *m/z* 213 with a relative abundance of 24%. This ion can be originated by the loss of CNH_2 from the protonated molecule. This loss can be favored by the presence of the hydroxyl group in the phenyl moiety of the molecule that stabilizes the resulting ion, leading to conjugated and planar quinone-type structures. MS^3 experiments were performed using the MS^2 product ion (*m/z* 226) $[M + H - CH_3]^{+\bullet}$, which was conveniently isolated and fragmented. The most abundant MS^3 ion was found at *m/z* 199 and can be explained by the loss of HCN as happens for PhIP (*30*). Moreover, in the MS^3 spectrum several product ions appeared that seem to be characteristic of the hydorxy derivative such as those at *m/z* 209 and at *m/z* 208 that can be derived from the loss of $^{\bullet}OH$ and H_2O, respectively. The presence of ions showing these losses in the MS^3 spectra of an unknown compound would indicate that it contains a hydroxyl group in its structure. In addition, the hydroxyl group may lead to obtaining several product ions involving the loss of $^{\bullet}H$ such as *m/z* 225 and *m/z* 224, although this loss may also arise from the exocyclic amino group. In contrast to what happens with PhIP (*30*), the product ion produced by the loss of CN_2H_2 was not observed for 4′-OH-PhIP. However, the ion at *m/z* 201 can

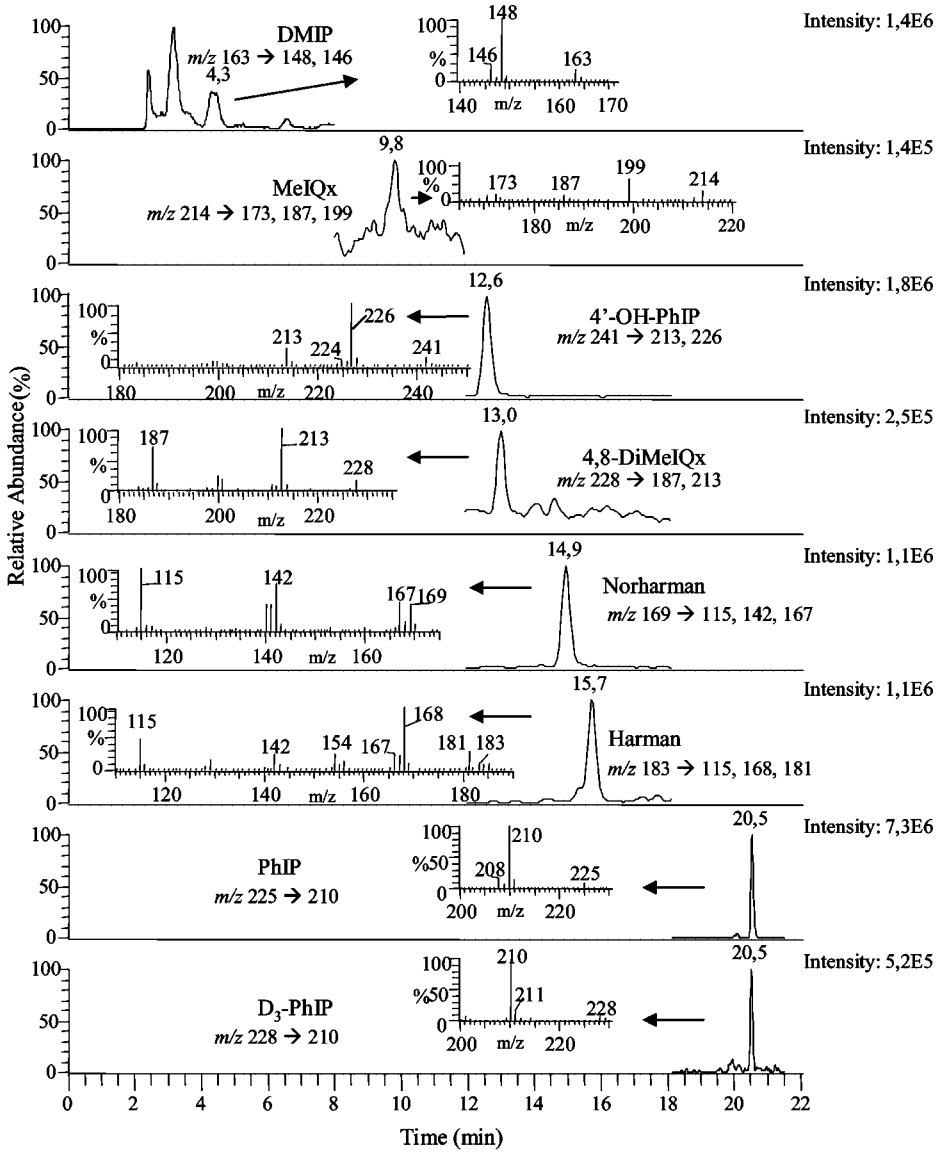

Figure 2. LC-MS/MS chromatograms of HCAs in fried chicken breast. Product ion scan spectra of the identified compounds are included. Chromatographic conditions as described in the Materials and Methods section.

be formed by an ion–molecule reaction between the ion resulting from the aforementioned loss and the hydroxyl radical generated in the formation of the ion at *m*/*z* 209. The ion at *m*/*z* 200 can be also explained by an ion–molecule reaction with the ion formed by the loss of CN_2H_3. The loss of an additional $^{\bullet}H$ can also be related to the presence of the hydroxyl group in the molecule.

Several quality parameters such as limit of detection, run-to-run precision and medium term or day-to-day precision, and linearity range were studied to check the performance of the LC-MS/MS method for 4′-OH-PhIP. Limits of detection (LODs) were established as the amount of analyte that produces a signal to noise ratio of 3:1. In standards, the LOD for 4′-OH-PhIP was 24 pg injected, and for PhIP, 5 pg injected. In meat samples, the corresponding LODs for 4′-OH-PhIP and PhIP were estimated to be 0.08 and 0.02 ng/g of cooked meat, respectively. The lower LOD obtained for PhIP is due to the narrow chromatographic peak obtained at the chromatographic conditions used for this compound (60% ACN, **Figure 2**). Postcolumn addition of 0.1% formic acid in acetonitrile improved the sensitivity by increasing the ionic evaporation efficiency and the ionization of HAs in the liquid phase. Run-to-run precision was determined by consecutive replicate injections ($n = 6$) of a standard (50 ng/g) in the same day. Day-to day precision was estimated by analyzing two replicates of the standard throughout three nonconsecutive days ($n = 6$). Relative standard deviations obtained for run-to-run and day-to-day precisions studied were 8% and 10%, respectively. Good linearity, $r^2 = 0.9988$, was obtained for both compounds from the limit of detection up to 1 μg/g.

The comparison of the retention time of the unknown compound and that of the 4′-OH-PhIP standard and also the agreement of the MS, MS^2, and MS^3 spectra allowed identifying 4′-OH-PhIP in fried chicken breast. Therefore, the presence of 4′-OH-PhIP in different types of cooked meat (pork, beef, and chicken) was evaluated. The highest amount of 4′-OH-PhIP occurred in fried chicken and in griddled chicken breast. As an example, **Figure 2** shows the LC-MS/MS chromatograms of HCAs detected from a fried chicken breast sample where the product ion spectrum of each HCA is included. 4′-OH-PhIP eluted between MeIQx and 4,8-DiMeIQx, which provides clues for its identification in other samples. **Figure 3** displays chromatograms and the corresponding MS, MS^2, and MS^3 spectra for the unknown compound eluted at 12.5 min from a

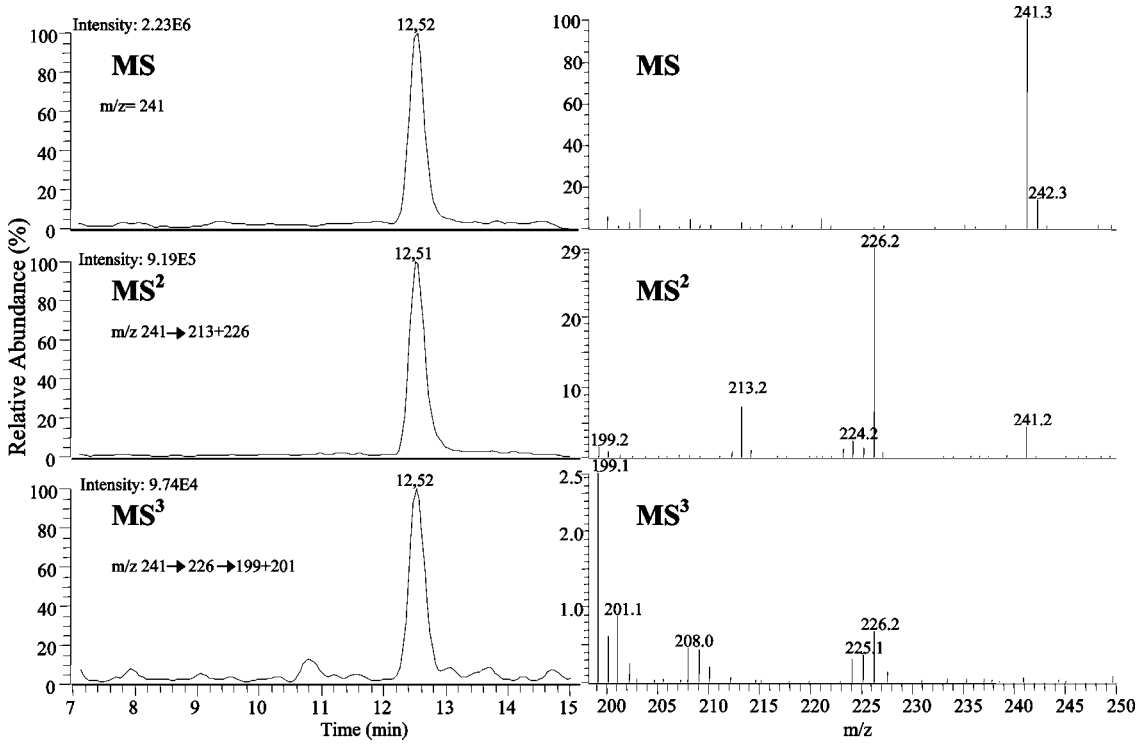

Figure 3. Identification of 4′-OH-PhIP from griddled chicken breast by full scan and product ion scan spectra MS^2 and MS^3.

Table 3. Determination of 4′-OH-PhIP and PhIP in Fried and Griddled Chicken Breast[a]

	4′-OH-PhIP			PhIP		
food item	ng/g cooked food ± ts	recovery ± ts	standard addition r^2	ng/g cooked food ± ts	recovery ± ts	standard addtition r^2
fried chicken	43.7 ± 13.8	65.7 ± 8.0	0.9901	19.2 ± 12.3	61.8 ± 8.5	0.9898
griddled chicken	13.4 ± 3.1	42.2 ± 2.3	0.9982	5.8 ± 2.7	42.6 ± 4.4	0.9973

[a] $p = 0.05$. Student's t-test = 2.78 (four degrees of freedom). s = standard deviation. Quantitation carried out by standard addition.

griddled chicken breast sample. These spectra agree with that of the 4′-OH-PhIP standard.

For fried chicken and griddled chicken, accurate quantitation of both pyridines, 4′-OH-PhIP and PhIP, was carried out by means of standard addition (**Table 3**). The concentration of 4′-OH-PhIP was found to be 43.7 ng/g in fried chicken and 13.4 ng/g in griddled chicken, respectively. The amount of this amine was about double that of PhIP, which was found to be 19.2 ng/g in fried chicken and 5.8 ng/g in griddled chicken. The higher content of pyridines in the fried meat could be due to the better heat transfer to the meat due to oil. Recoveries of both pyridines in the same food matrix were not significantly different (**Table 3**). As a consequence, the recovery obtained for PhIP can be used to estimate 4′-OH-PhIP concentration in food samples since both compounds are extracted and purified simultaneously. Amounts of 4′-OH-PhIP and PhIP found in different meat dishes are given in **Table 1**. Due to the diverse sample sizes and different cooking performances used, the amount of HAs cannot be strictly compared in the different food items. Estimated concentrations of 4′-OH-PhIP were 0.3 ng/g in coated-fried chicken breast, 0.7 ng/g in fried pork loin, and 0.5 ng/g in fried pork sausages while the corresponding amount of PhIP in these dishes was 0.6 ng/g, 2.5 ng/g, and <0.1 ng/g. In the other samples 4′-OH-PhIP was not detected at concentrations higher than 0.08 ng/g whereas the corresponding amount of PhIP in these cooked meats was in the range of 0.2–1.3 ng/g. 4′-OH-PhIP was not detected in any of the beef samples analyzed, although in the literature there are some data indicating that this compound was found at levels of 21.0 ng/g in broiled beef (*21*) and approximately at 2 ng/g in beef fried at 180–200 °C using soya oil as frying fat (*22*). In contrast, the content of 4′-OH-PhIP found in the cooked chicken samples (**Table 1**) analyzed in this work is within the same range as the values found in the literature for beef. In fact, several studies have shown that fried chicken breast is the food item in which PhIP is most frequently detected, at concentrations ranging from traces to 40 ng/g (*6, 11*). Our results show that 4′-OH-PhIP can also occur in cooked chicken at relatively high amounts, at the same level or even higher than PhIP. Since tyrosine and phenylalanine, precursors of 4′-OH-PhIP and PhIP, respectively, may be found at similar concentration, 0.6–0.8 μmol/g wet tissue, in chicken breast (*11*) they may potentially generate comparable concentrations of the respective pyridine-type HCAs, as was found in the present work. Moreover, the cooking method decisively influences the formation of the studied HCAs since a low yield of the studied pyridines (lower than 1 ng/g) was found in roasted and in coated-fried chicken breasts.

In a previous investigation we studied the effect of wine marinades on the formation of HCAs and concluded that this treatment reduced PhIP formation (*27*). Because of the similar chemical structure between PhIP and 4′-OH-PhIP, it was expected that the formation of 4′-OH-PhIP would also be minimized. So, the same samples were reanalyzed. The selected wines, named A, B, and C, had similar pH, reducing sugars, and metal composition but different antioxidant capacities, 35, 42, and 47 mM Trolox equivalents, respectively (*27*). The percentage of reduction obtained for both pyridines using the three wines is given in **Table 4** where it can be observed that

Table 4. Reduction (%) of 4′-OH-PhIP and PhIP in Fried Chicken by Marinating with Wine

marinating time (h)	reduction of 4′-OH-PhIP (%)			reduction of PhIP (%)		
	wine A	wine B	wine C	wine A	wine B	wine C
0.5	52	22	2	63	36	22
3	51	32	28	81	47	74
24	69	45	50	83	88	87

wine marinades caused a reduction of both pyridines, up to 69% for 4′-OH-PhIP and up to 88% for PhIP at the longest marinating time studied (24 h). These results seem to indicate that the reaction mechanism causing a decrease on the formation of the pyridines was controlled by the diffusion of wine components from the wine matrix to the surface of the raw meat. The increasing antioxidant properties of the wines A, B, and C may be correlated with a decrease on the reduction of the pyridines at short marinating time;s however, this effect is not clear for longer marinades possibly because other mechanisms may have counteracted the enhancing effect. In the preceding work competitive reactions of amino acids from the wines with precursors of HAs were pointed out as possible causes of the decrease of some HAs (*27*).

Humans are exposed to HCAs principally by means of the intake of cooked meat and fish. However, only a fraction of the consumed toxicants are activated to electrophilic structures that can cause DNA damage because of their bioaccessibility from the meat matrix (*31*); enzyme polymorphisms influencing its balance between toxicant activation/detoxification and DNA repair efficiency are playing a role in the rate of activation process (*32*). The search for biomarkers of exposure to HCAs in biological matrices such as urine, hair, DNA, or blood proteins is a matter of current research. Particularly, PhIP and its metabolites are being studied for this purpose (*25*, *32*–*35*). The present paper shows that 4′-OH-PhIP can be present in different types of cooked meat. Hence, 4′-OH-PhIP coming from PhIP metabolism and also that coming from dietary sources could undergo phase II metabolism, be converted into a more polar compound by conjugation with sulfate or glucuronide (*36*, *37*), and be excreted in urine. As a consequence, the amount of PhIP-derived metabolites present in human urine cannot be directly related to PhIP because of the presence of 4′-OH-PhIP metabolites coming from dietary 4′-OH-PhIP. Therefore, the intake of 4′-OH-PhIP should be considered in risk assessment studies involving the determination of PhIP-related compounds in urine.

In summary, in this study, chromatographic retention time, multistep mass spectra, and tentative product ion mass assignations are reported as clues for 4′-OH-PhIP identification in future research. The LC-MS/MS method developed was used to identify 4′-OH-PhIP in several cooked meats, where it was reported for the first time. The highest levels of 4′-OH-PhIP, 43.7 and 13.4ng/g, were found in fried chicken and in griddled chicken, respectively. In the other studied samples 4′-OH-PhIP was not detected or present at concentrations lower than 1 ng/g. Wine marinades caused an important reduction in the formation of 4′-OH-PhIP, estimated up to 69%, at the longer marinating time (24 h). The effect of the different wines used in the marinades was similar for both 4′-OH-PhIP and PhIP.

ABBREVIATIONS USED

LC, liquid chromatography; MS, mass spectrometry; au, arbitrary units; HCAs, heterocyclic amines; 4′-OH-PhIP, 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; d_3-PhIP, 2-amino-1-trideuteriomethyl-6-phenylimidazo[4,5-*b*]pyridine; DMIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; harman, 1-methyl-9*H*-pyrido[4,3-*b*]indole; norharman, 9*H*-pyrido[3,4-*b*]indole.

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