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Identification of Seafood as an Important Dietary Source of Heterocyclic Amines by Chemometry and Chromatography–Mass Spectrometry

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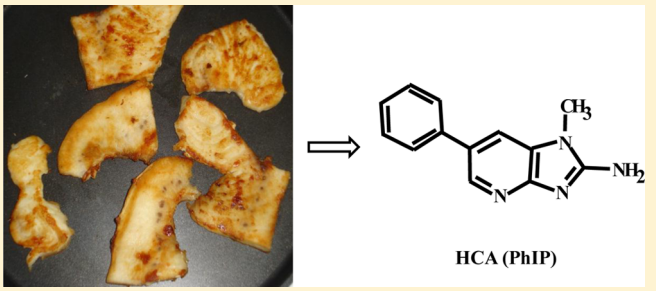
ABSTRACT: Heterocyclic amines (HCAs), which are formed during the cooking of protein-rich foods, are potent mutagens and a risk factor for human cancers. Levels of HCAs have been extensively investigated in meat products but not in fish products. Here, we report levels of HCAs in fried salmon, tuna, hake, sardine, angler fish, cod, sole, swordfish, squid, and cuttlefish. The HCA levels of some of these foods have not been previously analyzed. We employed multivariate factor-analysis tools, including principal components analysis (PCA) and partial least-squares (PLS) regression, to study the effects of cooking weight loss and levels of creatine, glucose, and free amino acids on HCA levels. The highest concentrations of mutagenic HCAs, 159.3 ng·g^{-1} total, where 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) accounted for 121 ng·g^{-1}, were found in fried swordfish (cooking loss of 51.8%). These levels are higher than those generally found in fried chicken, which is typically cited as the most contaminated food item. Thus, swordfish is among the richest known sources of HCAs. The other cooked seafood items contained from 0.4 to 35.4 ng·g^{-1} HCAs, comparable to concentrations typically reported for meat. Chemometric analysis showed that the fish species is the most influential parameter on the formation of HCAs such as DMIP, PhIP, and norharman. Concentrations of histidine, lysine, creatine, and glucose, as well as weight loss, also influence the yield of HCAs. These results suggest that seafood is an important dietary source of HCAs. The formation of HCAs in fish is influenced by multiple factors, some of which remain unknown.



INTRODUCTION

Proteinaceous foods cooked under domestic conditions contain HCAs.[1−4] Amino acids, creatine, and sugars are involved in the formation of these dietary toxins via the Maillard reaction.[5] The formation of HCAs depends greatly on the cooking method, temperature, and time as well as the composition of the raw protein-rich food.[6,7]

HCAs exhibit mutagenic activity in the Ames/*Salmonella* assay, and some are carcinogenic in rodents and non-human primates.[8,9] Although humans are exposed to HCAs at low levels, the exposure is chronic because cooked meat is in many cases consumed daily over a lifetime. Chronic exposure to mutagenic HCAs may be linked to the onset of certain cancers. For example, epidemiological studies have found a positive correlation between the consumption of cooked meat and the incidence of cancer.[10,11] The U.S. National Toxicology Program has classified IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline), MeIQ (2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) as reasonably anticipated human carcinogens.[12] The International Agency for Research on Cancer (IARC) has also classified IQ as a probable human carcinogen and MeIQ, MeIQx, PhIP, AαC (2-amino-9*H*-pyrido[2,3-*b*]indole), MeAαC (2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole), Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole), Trp-P-2 (3-amino-1-methyl-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole), and Glu-P-2 (2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole) as possible human carcinogens.[13] To investigate the association between HCAs and cancer, biomarkers of exposure in biological fluids must be identified, and the sources of these mutagens need to be determined.[14,15] Few studies of HCAs in cooked fish have been published, and few fish species have been analyzed for the presence of HCAs despite the fact that the cooking of fish leads to HCA formation and seafood is widely consumed.[16−19]

In this study, 10 fish and mollusk species (salmon, tuna, hake, sardine, angler fish, cod, sole, swordfish, squid, and cuttlefish) were fried and analyzed for HCAs by solid-phase extraction (SPE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS). The raw matrices were characterized in terms of glucose, creatine, and amino acid composition. The

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Table 1. Concentrations of HCAs, Weights of Raw Samples, and Weight Losses during the Cooking of Fried Seafood: Recoveries (R) of HCAs Are Given in Parentheses

HCAs	salmon	tuna	sardine	swordfish	hake	cod	angler fish	sole	squid	cuttlefish
weight, raw[a] (g)	200	183	205	197	176	212	185	212	91	112
weight loss (%)	46.1	29.5	44.4	51.8	44.9	52.4	55.1	39.6	39.6	34.8
					$ng \cdot g^{-1} \pm s^b$ (R, %)					
DMIP	8.3 ± 0.2 (32)	0.6 ± 0.1 (43)	0.5 ± 0.05 (37)	37.8 ± 2.8 (37)	11.9 ± 1.9 (40)	8.5 ± 1.2 (27)	15.9 ± 1.7 (42)	6.5 ± 0.7 (48)	1.1 ± 0.1 (36)	0.3 ± 0.01 (44)
IQ	0.04 ± 0.01 (110)	nq	nd	nq	nq	0.04 ± 0.01 (56)	0.03 ± 0.1 (25)	nq	nd	nd
MeIQx	0.6 ± 0.2 (51)	0.8 ± 0.1 (29)	0.5 ± 0.1 (24)	0.3 ± 0.01 (23)	nq	0.3 ± 0.1 (21)	0.6 ± 0.1 (32)	nq	nd	nd
MeIQ	nq[c]	nq	nd	nq	nq	0.2 ± 0.02 (26)	nq	nq	nd	nd
4,8-DiMeIQx	0.2 ± 0.02 (102)	0.9 ± 0.01 (73)	0.3 ± 0.1 (65)	0.2 ± 0.04 (64)	nq	nq	0.6 ± 0.1 (68)	nq	nd	nq
norharman	7.3 ± 1.2 (81)	2.1 ± 0.4 (96)	4.2 ± 0.4 (87)	51.3 ± 3.9 (70)	12.6 ± 2.1 (74)	12.2 ± 1.5 (40)	19.0 ± 1.5 (75)	5.3 ± 0.4 (84)	25.5 ± 1.5 (37)	17.6 ± 1.5 (67)
harman	1.2 ± 0.1 (92)	0.5 ± 0.1 (112)	2.4 ± 0.3 (44)	1.2 ± 0.7 (85)	0.6 ± 0.05 (81)	0.3 ± 0.04 (88)	nq	0.5 ± 0.1 (96)	1.0 ± 0.2 (48)	1.1 ± 0.3 (81)
PhIP	26.2 ± 1.6 (69)	0.7 ± 0.01 (99)	0.6 ± 0.05 (83)	121.0 ± 0.7 (73)	14.4 ± 2.0 (69)	19.1 ± 2.7 (44)	6.2 ± 0.6 (87)	4.2 ± 0.8 (94)	0.6 ± 0.1 (80)	0.2 ± 0.01 (91)
Trp-P-1	nq	nd	nd	nd	nd	nd	nq	nd	nd	nd
Trp-P-2	nd	nq	nq	nd	nd	nd	nq	nd	nd	nd
AαC	nq	nd	nd	nq	0.02 ± 0.01 (23)	nd	nq	nd	nd	nd
MeAαC	nd[d]	nq	nd	nq	0.04 ± 0.01 (21)	nd	0.04 ± 0.01 (23)	nq	nd	nd

[a]The thickness of raw fillets ranged between 0.8 and 1 cm. [b]Standard deviation as obtained from addition standard calibration. [c]nq: below the limit of quantification (0.02 ng of HCA/g of cooked seafood). [d]nd: not detected. Limit of detection: 0.01 ng of HCA/g of cooked seafood.

dependence of HCA levels on the type of fish, cooking weight loss, and concentrations of amino acids, creatine, and glucose in the raw material was chemometrically investigated for the first time to our knowledge by principal component analysis (PCA) and partial least-squares (PLS) regression methods.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. HCAs [IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx (2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline), PhIP, DMIP (2-amino-1,6-dimethylimidazo[4,5-*b*]-pyridine), AαC, MeAαC, Trp-P-1, and Trp-P-2] were purchased from Toronto Research Chemicals (Toronto, Canada). The stated purity of these HCAs was >99%. Harman (1-amino-9*H*-pyrido[3,4-*b*]indole) and norharman (9*H*-pyrido[3,4-*b*]indole) were obtained from Sigma-Aldrich (Steinheim, Germany); the purities were >98%. Stock solutions of 150 $\mu g \cdot g^{-1}$ were prepared in methanol and diluted as required. Solutions were stored at +4 °C. Standard mixtures of all amines with 4,7,8-TriMeIQx (0.5 $\mu g \cdot g^{-1}$) as an internal standard were prepared to establish the range of linearity and to prepare calibration curves. All solutions and purified fractions were passed through 0.22 μm nylon filters (Scharlab, Barcelona, Spain) prior to injection into the LC−MS system.

A glucose kit was supplied by Química Clínica Aplicada (Tarragona, Spain). Water was purified using a Milli-Q Simplicity 185 system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of HPLC or analytical grade and were supplied by Merck (Darmstadt, Germany).

Bond Elut propylsulfonyl silica PRS (500 mg) and octadecylsilane C_{18} cartridges (100 mg), coupling pieces, and stopcocks were purchased from Varian (Harbor City, USA). Extrelut NT20 extraction cartridges were provided by Merck (Darmstadt, Germany), and Isolute HM-N refill material was obtained from International Sorbent Technology (Hengoed, UK).

The cooking temperature was measured using type K insulated wire probes and monitored with Normadics TC6 software from Cole-Parmer (Vernon Hills, IL, USA). A Microtron MB 550 blender (Kinematica AG, Littau, Switzerland) was used to mince the cooked fish samples. An Ultra-Turrax T25 Basic instrument (IKA, Staufen, Germany) was used to homogenize the ground material mixed with 1 M NaOH solution. Supelco Visiprep and Visidry vacuum manifolds (Supelco, Gland, Switzerland) were used for sample purification and preconcentration. Glucose, creatine, and amino acids were analyzed as described elsewhere.[20]

Sample Preparation. Fresh seafood was purchased from a local retail market (Barcelona, Spain). Before cooking, scales were removed along with innards (salmon, tuna, sardine, hake, cod, and swordfish), skin (sole and angler fish), and ink sac and cartilage (mollusks). The fish were filleted (0.8 to 1.0 cm thickness), and the mollusks were sliced (0.8 cm). A total of 380 g of each type of raw item was cooked, and four cooking batches were carried out. Frying used only enough olive oil to cover the area of the pan in contact with the food (0.5 mL of olive oil/40 g of cooked fish). The heat source was an electric vitroceramic stove holding a Teflon-coated frying pan (270 mm × 270 mm). The surface temperature was 210−225 °C, as measured with a probe at the center of the pan. Cooking was started after the center of the pan had remained at 210 °C for 15 min. In every cooking batch, the temperature inside one of the fillets was monitored with four probes: two thermocouples were inserted in the center of the fillet, and the other two were fixed at 1 mm below the upper and lower surface. The temperature was recorded every 10 s. During the cooking process, the temperature measured inside the seafood fillets was constant at 100 °C, and the temperature measured at each side of the fillet increased from 25 to 130 °C, the temperature at which the item was flipped. All samples were cooked for 4 min·side^{-1}, except squid, which was cooked for 3 min·side^{-1}. The items were cooked to the preferred degree of doneness and browning selected by volunteers in previous studies by the authors: "brown" and "done" selected among (rare, medium, done, and well done) and browning (not brown, somewhat brown, brown, and dark brown).[14] Weight loss was determined by weighing samples before and after cooking. The cooked seafood (without bones and heads) was ground, bottled, and stored at −18 °C until analysis. Squid were peeled before being ground.

Sample Extraction. For each extraction, 1 g of ground sample was mixed with 12 mL of 1 M NaOH and homogenized using an Ultra-Turrax. HCAs were extracted in ethyl acetate and purified following a

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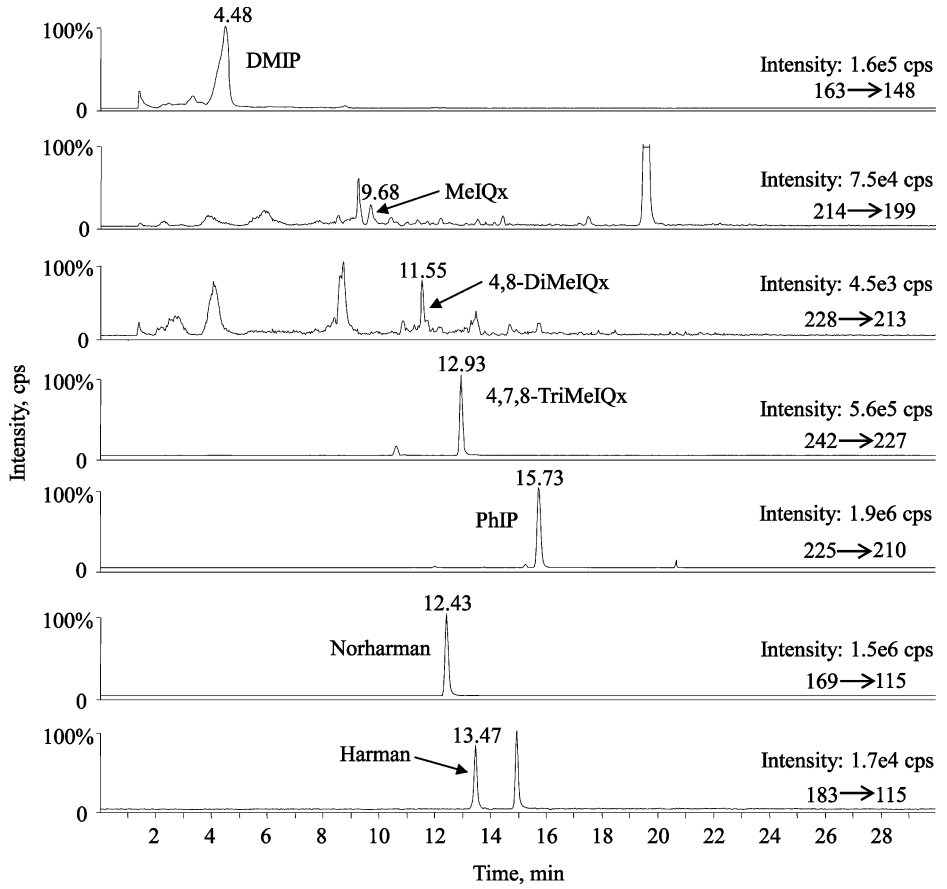

Figure 1. LC–MS/MS chromatogram of HAs in fried swordfish.

validated SPE method to yield HCAs in a single extract.[20−22] Purified extracts were evaporated to dryness under a stream of nitrogen and reconstituted with an internal standard (4,7,8-TriMeIQx, 0.5 $\mu g \cdot g^{-1}$) in methanol (0.1 mL).

Determination of HCAs. The separation, detection, and quantification were performed using a quaternary pump system from Agilent Technologies Series 1100 (Waldbronn, Germany) with a Symmetry C_8 column (5 μm, 150 mm × 2.1 mm, Waters Corporation, Milford, MA, USA) coupled to a PE Sciex API 3000 triple quadrupole mass spectrometer (Perkin-Elmer SCIEX, Concord, ON, Canada). The mobile phase, gradient elution, and MS operating conditions were as previously reported.[23]

HCA concentrations and recoveries were quantified by a standard addition method relying on two unspiked and two spiked samples. The spiking levels were 12.5 and 125.0 $ng \cdot g^{-1}$ for DMIP and PhIP and 1.5 and 15.0 $ng \cdot g^{-1}$ for each of IQ, MeIQ, MeIQx, 4,8-DiMeIQx, harman, norharman, Trp-P-1, Trp-P-2, AαC, and MeAαC.

Data Analysis. MATLAB (version 6.5) was used for the statistical calculations. PCA and PLS were performed using the PLS Toolbox.[24] A detailed description of these methods has been given elsewhere.[25] The map of the distribution of the seafood samples on the principal components (PCs) (plot of scores) was used to classify samples by the formation of the different HCAs during the cooking process. The PC plots revealed patterns that may be correlated to sample characteristics. The study on the distribution of the chemical variables in the raw fish and cephalopods such as concentrations of amines or amino acids (plot of loadings) provided information about their correlations' interdependence of these variables. Additionally, the simultaneous study of scores and loadings was used to explore relationships between samples and variables.

In the case of PLS, the algorithm was focused on determining relationships between the concentrations of particular HCAs and other fish characteristics, such as the amino acid composition, glucose, and creatine contents and weight loss. The calibration coefficient vector provided information on the variables that contribute most significantly, either positively or negatively, to the formation of HCAs. The validation of both the PCA and PLS models was performed using a leave-one-out cross-validation, in which each given fish or mollusk was predicted by using the remaining samples as standards for building the calibration model.

RESULTS AND DISCUSSION

HCAs in Seafood. The concentrations of HCAs in seafood, cooked as described in the Experimental Procedures section, are given in Table 1. The quantification of HCAs in seafood is toxicologically important; the levels of certain HCAs found here are comparable with those found in cooked meat.[16,17] HCAs have been analyzed in a wide range of cooked meats because meat is considered the main source of HCAs in contemporary diets. In contrast, analyses of HCAs in fish and mollusks (e.g., swordfish) remain scarce. Our results indicate that these foods are a rich source of mutagens. To illustrate the results reported in Table 1, Figure 1 shows the chromatogram of HCAs determined in fried swordfish.

The high level of HCAs found in swordfish stands out from the other values listed in Table 1 as well as earlier data on cooked salmon, sardine,[26] cod,[27,28] mackerel,[29] trout,[30] and herring.[28] The high level in swordfish is a consequence of the composition of the raw fish as well as the cooking process. Although similar cooking conditions were applied to the other species, the items underwent different weight losses due to their intrinsic characteristics (see Table 1). These differences suggest different mass transport mechanisms within the fillet, which

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Table 2. Levels of Free Amino Acids, Glucose and Creatine (mg·g^{-1} wet weight) in Raw Fish and Mollusk Samples[a]

amino acids	salmon	tuna	hake	squid	cuttlefish	cod	sardine	angler fish	sole	swordfish
taurine	0.23	0.08	0.27	0.90	1.27	0.07	0.68	0.06	0.80	0.24
alanine	0.31	0.09	0.10	1.00	0.75	0.05	0.27	0.05	0.13	0.21
cysteine	nd	nd	nd	nd	0.05	nd	nd	nd	nd	nd
aspartic acid	0.06	nd	0.01	0.15	0.03	0.01	0.01	0.01	nd	nd
glutamic acid	0.16	0.03	0.04	0.32	0.19	nd	0.07	0.05	0.03	0.11
phenylalanine	0.08	0.04	0.02	0.16	0.08	0.01	0.03	0.01	0.01	0.05
glycine	0.11	0.02	0.04	1.13	0.18	0.12	0.10	0.11	0.15	0.11
histidine	0.28	2.94	0.03	0.14	0.11	0.01	1.42	0.01	0.10	0.09
isoleucine	0.03	0.02	0.01	0.10	0.14	0.01	0.02	0.01	nd	0.05
lysine	0.13	0.05	0.12	0.18	0.18	0.01	0.21	0.02	0.05	0.23
leucine	0.10	0.05	0.03	0.28	0.25	0.02	0.07	0.02	0.02	0.11
methionine	0.03	0.03	0.01	0.22	0.15	0.01	0.03	0.01	0.01	0.05
asparagine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
proline	0.02	nd	0.02	4.25	2.10	0.01	0.02	0.01	0.03	0.28
glutamine	0.02	nd	nd	0.16	0.13	nd	nd	nd	0.02	0.01
arginine	0.05	0.01	0.02	0.99	1.00	nd	0.02	nd	0.01	0.04
serine	0.04	0.02	0.02	0.18	0.21	0.01	0.04	0.01	0.02	0.05
threonine	0.07	0.02	0.04	0.17	0.20	0.01	0.04	0.01	0.06	0.08
ornithine	nd	0.01	nd	0.04	0.47	0.01	0.04	0.01	0.02	nd
valine	0.10	0.05	0.03	0.13	0.22	0.01	0.05	0.01	0.01	0.07
tryptophan	nd	nd	nd	nd	0.02	nd	nd	nd	nd	nd
tyrosine	0.07	0.05	0.02	0.13	0.10	0.01	0.03	0.01	0.01	0.06
glucose	0.62	0.67	0.14	0.31	0.45	0.10	0.27	0.15	0.23	1.77
creatine	3.23	2.37	2.53	1.01	1.35	0.29	3.5	3.21	0.94	4.15

[a]nd: not detected. Limit of detection: 0.003 mg ·g^{-1} wet weight.

may play a role in the formation of HCAs[31] together with the type of HCA precursors present during the cooking process.

The concentrations of the HCAs DMIP and PhIP varied by greater than 2 orders of magnitude among the tested samples. Similar findings have been reported for thermally treated meat items, showing that PhIP is formed at a wide range of concentrations.[16] Fried chicken can contain high concentrations of PhIP and DMIP[32] and is considered one of the main dietary sources of HCAs.[33] However, the levels of HCAs commonly detected in fried chicken are lower than the levels we detected in fried swordfish (e.g., PhIP in fried chicken is typically less than 30 ng·g^{-1}), indicating that fried swordfish may be among the richest dietary sources of HCAs. The levels of quinoxalines and α-carbolines in the studied samples were of the same order of magnitude as those reported for meat[34−37] and other fish samples.[26−28,30] The relatively high abundance of β-carbolines contrasts with the low levels of tryptophan detected in the raw matrices (given in Table 2). This amino acid has been identified as the main precursor of these amines.[38] Hence, these results suggest the formation of β-carbolines in the absence of free tryptophan, as previously reported in model systems.[39] Nonetheless, the cooking processes used in the present study are difficult to compare with those used in previous work.

Consistent with previous studies, IQ, MeIQ, Trp-P-1, Trp-P-2, AαC, and MeAαC have been identified in only a few of the studied samples. This finding may be due to the relatively low temperature and short cooking time used here, as both high temperatures and long cooking times appear to be necessary for the formation of these compounds.[40] However, these HCAs have been found at low levels in certain fish products after cooking at temperatures similar to those used in the present study.[29] Swordfish contained the highest total concentration of mutagenic HCAs (159.3 ng·g^{-1}), followed by salmon (35.4 ng·g^{-1}), cod (28.2 ng·g^{-1}), hake (26.4 ng·g^{-1}), and angler fish (23.6 ng·g^{-1}). Sole (10.7 ng·g^{-1}), tuna (3.0 ng·g^{-1}), sardine (1.9 ng·g^{-1}), squid (1.8 ng·g^{-1}), and cuttlefish (0.4 ng·g^{-1}) contained lower amounts. Levels of the mutagens harman and norharman were not included in this comparison.

The recoveries (21−112%), indicated in parentheses in Table 1, were used to assess the performance of the HCA analyses. Higher recoveries were obtained for PhIP, 4,8-DiMeIQx, harman, and norharman than for DMIP, AαC, and MeAαC. Extraction recovery depends on both the analytes and the matrix, as reported for meat samples. Thus, standard addition is the preferred quantification strategy. In this study, fatty species (salmon, tuna, sardine, and swordfish) did not yield lower recoveries of HCAs than low-fat species (hake, cod, angler fish, sole, squid, and cuttlefish).

Analyses of Precursors in Raw Seafood and Chemometric Interpretation of the Results. Free amino acids, glucose, and creatine were quantified in the raw fish and mollusks (Table 2) to study the influence of these precursors on HCA yield. The fish and mollusks have different ways of aquatic locomotion; among the invertebrates, cuttlefish and squid can use jet propulsion, and vertebrate fish swim by undulatory movements with their bodies and fins. These different propulsion methods have different energy demand, which require certain levels of creatine and glucose in the tissue for the synthesis of ATP. Besides, the concentration of glucose and creatine has a great influence on the formation of HCAs.[5] The levels of creatine and glucose have been found in the low-concentration range in the invertebrate samples, whereas in swordfish, which is a migratory fish that relies on its speed to catch its prey, these HCA precursors have been found at the highest level (Table 2). The high concentration of glucose and creatine may explain why swordfish has the highest yield of HCAs, PhIP in particular. However, the concentration of

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glucose and creatine in raw chicken, an item with generally the highest yield of PhIP, was determined to be 0.27 and 3.10 mg/g, respectively, in an earlier work by the authors.[41] These concentrations are slightly lower than the ones found in swordfish in this work but similar to that of sardine's. However, sardines are among the study items with a lower yield of HCAs (Table 1). These apparent contradictions illustrate that the formation of HCAs cannot be explained by single factors, and multivariate analysis is necessary.

Poor correlations were observed between each potential precursor and HCA levels using linear regression. As a representative example, the correlation coefficient (r^2) between weight loss and DMIP was 0.65. Reasonably high correlations were found between glucose and PhIP or DMIP concentrations, with r^2 values of 0.71 and 0.89, respectively. This finding illustrates the complexity and multivariate nature of the dependence of HCA levels on potential precursors. For example, concentrations of HCAs in the cooked samples are influenced by several factors, such as weight loss and concentrations of amino acids and glucose. Based on the results shown in Table 1, the nature of fish and mollusk species appears to be the most important factor in the formation of HCAs. For this reason, data in Tables 1 and 2 were studied using multivariate approaches (PCA and PLS) as a way of extracting global information on HCA formation. An initial PCA study focused on evaluating the distribution of fish and mollusk samples as a function of the HCA content. We found that 94 and 5% of the information, so-called data variance, was explained by PC1 and PC2, respectively.

As shown in Figure 2a, swordfish was the most different species, located on the right of PC1. This sample was characterized by higher levels of DMIP and PhIP than the other species, as shown by the loading coordinates of these amines in Figure 2b. Thus, PC1 was largely determined by the concentrations of DMIP and PhIP. PC2 was most strongly correlated with the concentration of norharman. Accordingly, samples with high PC2 values (i.e., mollusks and angler fish) contained relatively high levels of this amine. Further examination also revealed that levels of DMIP and PhIP were strongly correlated, whereas harman and norharman were not; they showed an independent behavior which could suggest that these amines might be formed from different reaction pathways. These amines could be formed through different reaction pathways.

PLS was subsequently used to determine the relationship between HCA levels (the Y data set) and potential precursors (amino acids, glucose, and creatine; the X data set). As an example, Figure 3 shows the results of the model relating the concentrations of PhIP to levels of precursors and cooking weight losses. Autoscaled data were used to construct the calibration model to prevent the magnitudes of the variables from influencing the results. Autoscaling is a data preprocessing treatment applied to a given chemical variable which consists of subtracting its average value and dividing by its standard deviation. Three latent variables captured more than 90% of the information contained in the data sets (latent variables 1−3 in Figure 3a). According to the representation of the captured variance (Figure 3a), two additional factors were needed to capture the relevant variance in the data sets. The map of samples on PC1 and PC2 (Figure 3b) showed one major group containing the majority of the fish species and two minor groups of species, swordfish and mollusks, at the right and left of the graph, respectively. In the loading plot (Figure 3c), most

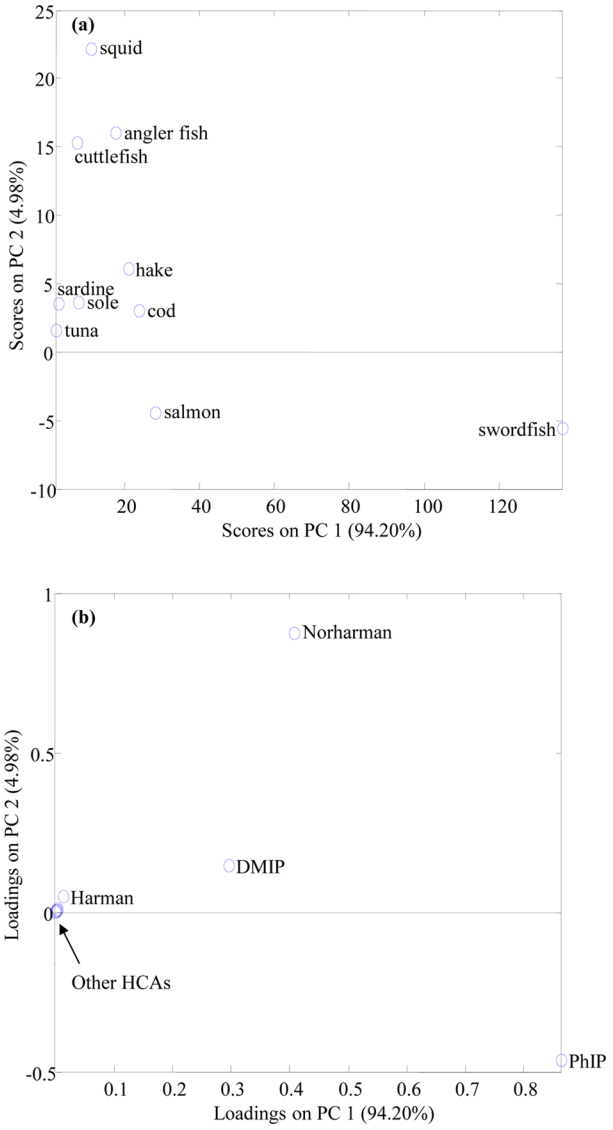

Figure 2. PCA of fish and mollusk samples as a function of HCA contents. (a) Scores plot. (b) Loadings plot.

amino acids (except histidine) appear in an area at the top left corner, indicating that this group of amino acids had a similar influence on PhIP formation. Histidine was located at the bottom of the plot (Figure 3c), indicating that its contribution to PhIP formation differed from those of the other amino acids. Glucose, creatine, and weight loss are plotted on the right side of the graph, indicating contributions to PhIP formation. It should be emphasized, however, that conclusions based on this scatter plot are tentative, as it was drawn from only 70% of the variance using the two most important latent variables to represent the results in a two-dimensional plot. The PLS regression vector (Figure 3d) provided important information on the influence of some chemical variables on PhIP formation. Based on the regression coefficients (with large positive or negative values), glucose appears to be the most important precursor to the amine, in agreement with earlier work.[42] PhIP levels may increase in glucose-rich samples. Lysine and cooking weight loss appear to have a similar degree of influence on the formation of PhIP. Histidine, with a negative coefficient, may protect against the formation of PhIP. To our knowledge, there

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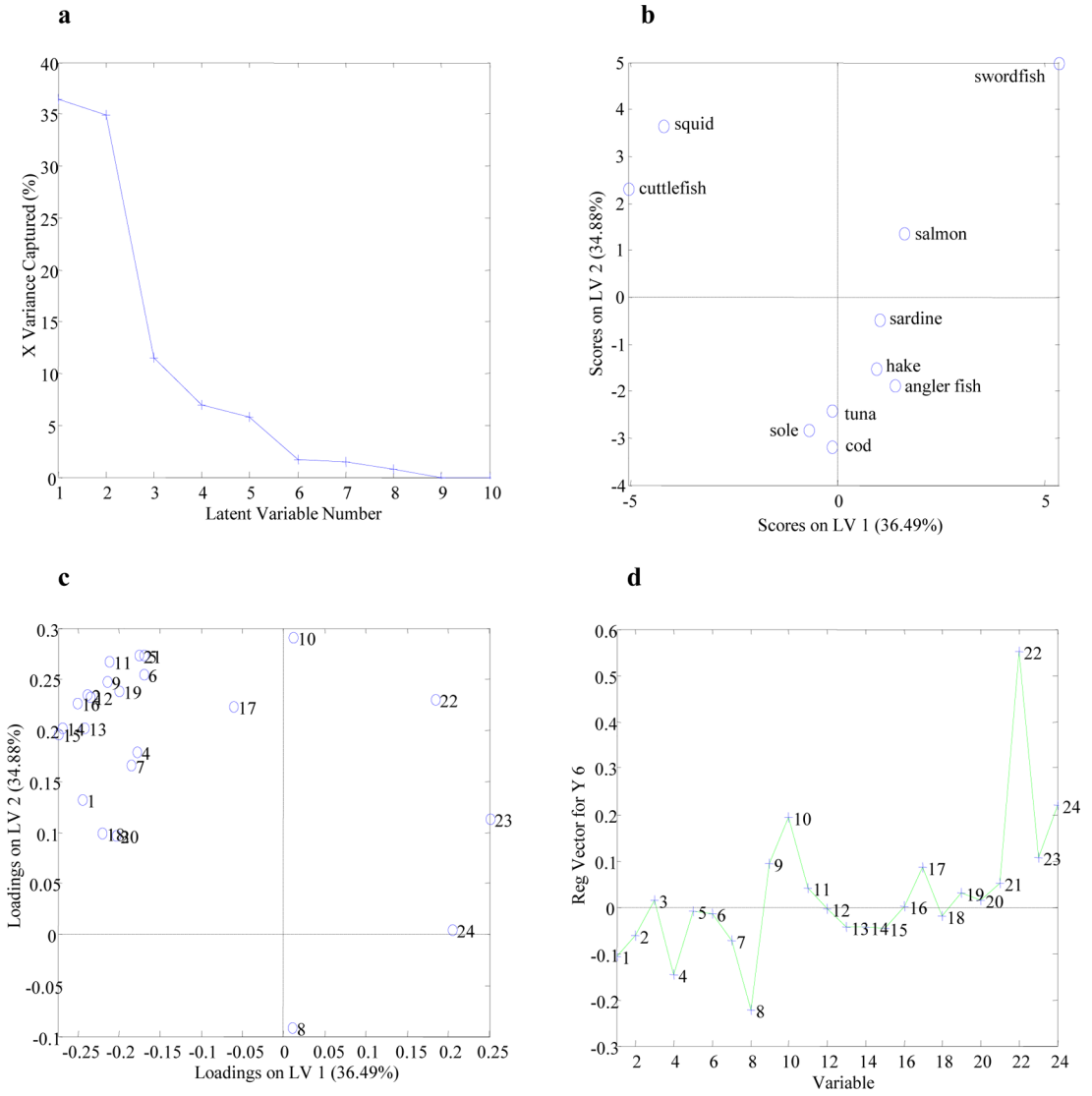

Figure 3. PLS study of the influence of potential precursors (amino acids, glucose, and creatine) and weight loss on the occurrence of PhIP. (a) Captured variance as a function of the number of latent variables. (b) Scores plot. (c) Loadings plot. (d) Regression vector. Variable assignment: 1 = taurine; 2 = alanine; 3 = cysteine; 4 = aspartic acid; 5 = glutamic acid; 6 = phenylalanine; 7 = glycine; 8 = histidine; 9 = isoleucine; 10 = lysine; 11 = leucine; 12 = methionine; 13 = proline; 14 = glutamine; 15 = arginine; 16 = serine; 17 = threonine; 18 = ornithine; 19 = valine; 20 = tryptophan; 21 = tyrosine; 22 = glucose; 23 = creatine; 24 = weight loss.

is little available evidence on the effects of these amino acids on PhIP yields.

Similar PLS studies were performed on potential precursors of other HCAs. For DMIP, the regression vector was similar to that determined for PhIP (Figure 4a), suggesting that similar processes lead to their formation. Again, glucose and weight loss contributed positively to the production of this amine. To a lesser extent, lysine and creatine were positively correlated with PhIP levels, whereas histidine may hinder PhIP formation. The precursors of norharman, deduced primarily from the loading plot in Figure 2, were similar to those of DMIP and PhIP (see Figure 4b). In contrast, the regression vector for harman was markedly different (Figure 4c). Creatine, lysine, and possibly taurine contributed to the formation of harman in fish. In contrast, glucose was important for reducing harman contents, an effect that to our knowledge has not been previously reported. The low concentrations of the remaining amines prevented extracting conclusions on their potential precursors.

Our results demonstrate that raw swordfish is a good matrix for the formation of HCAs. Cooked swordfish contained the highest levels of glucose, creatine, PhIP, DMIP, and norharman of the tested fish. These results are consistent with previous data showing a strong influence of sugars[43] and creatine[44] on the formation of PhIP. Multivariate analyses (PCA and PLS) showed that several variables (glucose, amino acids, and cooking weight loss) do not fully account for the formation of HCAs. However, glucose, creatine, and certain amino acids play a role in the formation of HCAs. To the best of our knowledge, this work presents the first evidence on the possible influence of certain amino acids on the formation of specific HCAs. Further model studies are required to confirm such an effect. In addition to the influence of these compounds, the type of fish or mollusk has been found to be an important factor that strongly corresponds to the formation of HCAs.

We demonstrated that cooked seafood is a rich source of HCAs, with concentrations of these mutagens that are comparable to those previously reported in meat and even

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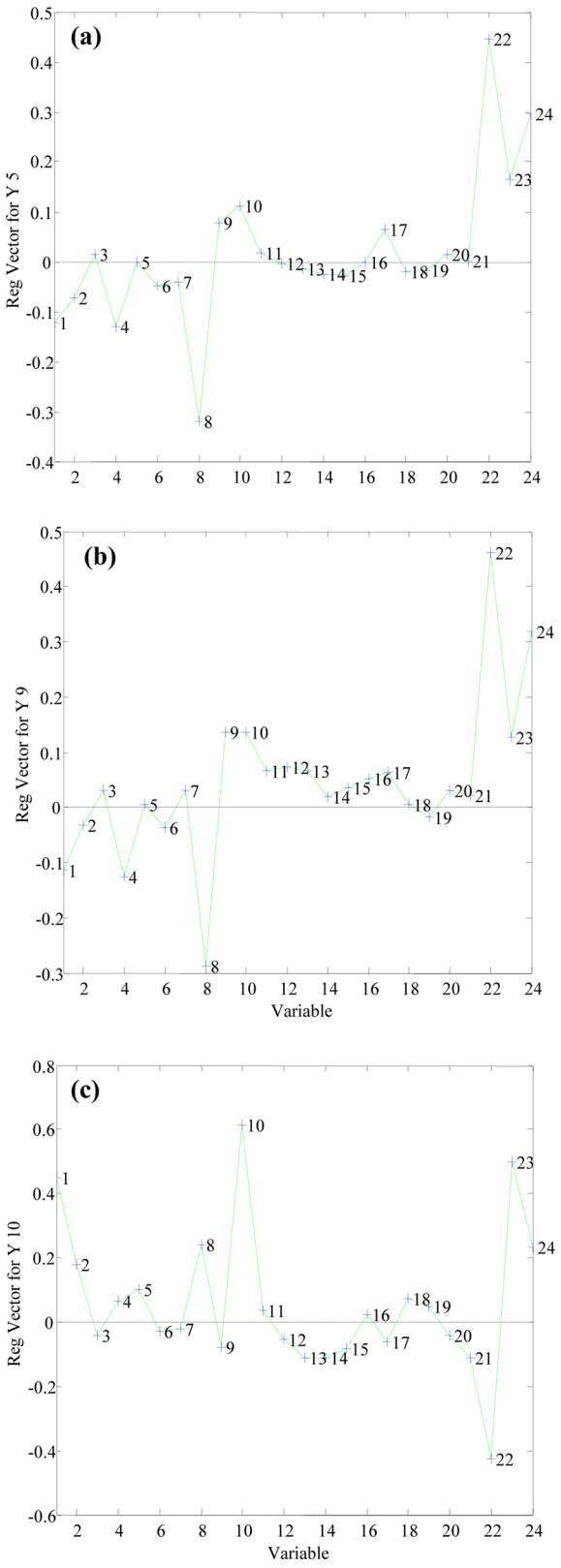

Figure 4. PLS regression vectors of the models relating precursors and DMIP: (a,b) harman and (c) norharman. Variable assignment as in Figure 3.

higher in the case of swordfish. PhIP, DMIP, and norharman were detected at higher concentrations than the other HCAs studied for all analyzed samples. Multivariate analyses using PCA and PLS showed that the type of fish is the most differentiating parameter on the formation of HCAs such as DMIP, PhIP, and norharman. The concentrations of certain amino acids and creatine, along with weight loss, were also shown to be influential in the formation of HCAs. Histidine appears to have a chemoprotective effect against the formation of PhIP and DMIP, but this needs to be confirmed in further studies with chemical models.

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Notes

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ABBREVIATIONS

HCAs, heterocyclic amines; IARC, International Agency for Research on Cancer; LC−MS/MS, liquid chromatography coupled to tandem mass spectrometry; PLS, partial least-squares; PCA, principal component analyses; SPE, solid-phase extraction; DMIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]-pyridine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; 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-tetramethyl-imidazo[4,5-*f*]quinoxaline); Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole; Trp-P-2, 3-amino-1-methyl-1,4-dimethyl-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; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; Harman, 1-amino-9*H*-pyrido[3,4-*b*]indole; Norharman, 9*H*-pyrido-[3,4-*b*]indole

REFERENCES

(1) Puangsombat, K., Gadgil, P., Houser, T. A., Hunt, M. C., and Smith, J. S. (2012) Occurrence of heterocyclic amines in cooked meat products. *Meat Sci. 90*, 739−746.

(2) Iwasaki, M., Kataoka, H., Ishihara, J., Takachi, R., Hamada, G. S., Sharma, S., Marchand, L. L, and Tsugane, S. (2010) Heterocyclic amines content of meat and fish cooked by Brazilian methods. *J. Food Comp. Anal. 23*, 61−69.

(3) Sun, L., Zhang, F., Yong, W., Chen, S., Yang, M., Ling, Y., Chu, X., and Lin, J. (2010) Potential sources of carcinogenic heterocyclic amines in Chinese mutton shashlik. *Food Chem. 123*, 647−652.

(4) Khan, M. R., Bertus, L. M., Busquets, R., and Puignou, L. (2009) Mutagenic heterocyclic amine content in thermally processed offal products. *Food Chem. 112*, 838−843.

(5) Murkovic, M. (2004) Formation of heterocyclic aromatic amines in model systems. *J. Chromatogr., B 802*, 3−10.

(6) Liao, G. Z., Wang, G. Y., Xu, X. L., and Zhou, G. H. (2010) Effect of cooking methods on the formation of heterocyclic aromatic amines in chicken and duck breast. *Meat Sci. 85*, 149−54.
(7) Sugimura, T., Wakabayashi, K., Nakagama, H., and Nagao, M. (2004) Heterocyclic amines: mutagens/carcinogens produced during cooking of meat and fish. *Cancer Sci. 95*, 290−299.
(8) Takayama, S., Thorgeirsson, U. P., and Adamson, R. H. (2008) Chemical carcinogenesis studies in nonhuman primates. *Proc. Jpn. Acad. Ser., B: Phys. Biol. Sci. 84*, 176−188.
(9) De Stefani, E., Ronco, A., Mendilaharsu, M., Guidobono, M., and Deneo-Pellegrini, H. (1997) Meat intake, heterocyclic amines, and risk of breast cancer: a case-control study in Uruguay. *Cancer Epidemiol. Biomarkers Prev. 6*, 573−581.
(10) Sinha, R., Kulldorff, M., Chow, W. H., Denobile, J., and Rothman, N. (2001) Dietary intake of heterocyclic amines, meat-derived mutagenic activity, and risk of colorectal adenomas. *Cancer Epidemiol. Biomarkers Prev. 10*, 559−562.
(11) Martínez, M. E., Jacobs, E. T., Ashbeck, E. L., Sinha, R., Lance, P., Alberts, D. S., and Thompson, P. A. (2007) Meat intake, preparation methods, mutagens and colorectal adenoma recurrence. *Carcinogenesis 28*, 2019−2027.
(12) 11th Report on Carcinogens (2005). National Toxicology Program. US Department of Health and Human Services, Public Health Services, Washington DC.
(13) International Agency for Research on Cancer (1993) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Natural Occurring Substances: Food Items and Constituents. Heterocyclic Amines and Mycotoxins*; No. *56*, pp 163−242, Lyon, France.
(14) Busquets, R., Frandsen, H., Jönsson, J. Å., Puignou, L., Galceran, M. T., and Skog, K. (2013) Biomonitoring of dietary heterocyclic amines and metabolites in urine by liquid phase microextraction: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a possible biomarker of exposure to dietary PhIP. *Chem. Res. Toxicol. 26*, 233−240.
(15) Bessette, E. E., Yasa, I., Dunbar, D., Wilkens, L. R., Marchand, L. L., and Turesky, R. J. (2009) Biomonitoring of carcinogenic heterocyclic aromatic amines in hair: a validation study. *Chem. Res. Toxicol. 22*, 1454−1463.
(16) Skog, K. I., Johansson, M. A. E., and Jägerstad, M. I. (1998) Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurrence and intake. *Food Chem. Toxicol. 36*, 879−896.
(17) Busquets, R. (2012) Food borne carcinogens: a dead end? In *Carcinogen* (Pesheva, M., Ed.) pp 163−184, InTech, Croatia.
(18) Kasai, H., Yamaizumi, Z., Wakabayashi, K., Nagao, M., Sugimura, T., and Yokoyama, S. (1980) Structure and chemical synthesis of Me-IQ, a potent mutagen isolated from broiled fish. *Chem. Lett. 9*, 1391−1394.
(19) Welch, A. A., Lund, E., Amiano, P., Dorronsoro, M., Brustad, M., Kumle, M., Rodriguez, M., Lasheras, C., Janzon, L., Jansson, J., Luben, R., Spencer, E. A., Overvad, K., Tjønneland, A., Clavel-Chapelon, F., Linseisen, J., Klipstein-Grobusch, K., Benetou, V., Zavitsanos, X., Tumino, R., Galasso, R., Bueno-De-Mesquita, H. B., Ocké, M. C., Charrondière, U. R., and Slimani, N. (2002) Variability of fish consumption within the 10 European countries participating in the European Investigation into Cancer and Nutrition (EPIC) study. *Public Health Nutr. 5*, 1273−1285.
(20) Khan, M. R., Milà, A., Busquets, R., Santos, F. J., and Puignou, L. (2009) Preparation and characterisation of fried chicken as a laboratory reference material for the analysis of heterocyclic amines. *J. Chromatogr., B 877*, 1997−2002.
(21) Toribio, F., Moyano, E., Puignou, L., and Galceran, M. T. (2002) Ion-trap tandem mass spectrometry for the determination of heterocyclic amines in food. *J. Chromatogr., A 948*, 267−281.
(22) Toribio, F., Busquets, R., Puignou, L., and Galceran, M. T. (2007) Heterocyclic amines in griddled beef steak analysed using a single extract clean-up procedure. *Food Chem. Toxicol. 45*, 667−675.
(23) Khan, M. R., Busquets, R., Santos, F. J., and Puignou, L. (2008) New method for the analysis of heterocyclic amines in meat extracts using pressurised liquid extraction and liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A 1194*, 155−160.
(24) Wise, B., and Gallager, N. B. (1992), *PLS Toolbox for use with MATLAB* (version 2.0), Eigenvector Research Inc., Mason, WA.
(25) Massart, D. L., Vandeginste, B. G. M., Buydens, L. M. C., De Jong, S., Lewis, P., and Smeyers-Verbeke, J. (1997) *Handbook of Chemometrics and Qualimetrics*, Elsevier, Amsterdam.
(26) Costa, M., Viegas, O., Melo, A., Petisca, C., Pinho, O., and Ferreira, I. M. (2009) Heterocyclic aromatic amine formation in barbecued sardines (*Sardina pilchardus*) and Atlantic salmon (*Salmo salar*). *J. Agric. Food Chem. 57*, 3173−3179.
(27) Wakabayashi, K., Ushiyama, H., Takahashi, M., Nukaya, H., Kim, S. B., Hirose, M., Ochiai, M., Sugimura, T., and Nagao, M. (1993) Exposure to heterocyclic amines. *Environ. Health Perspect. 99*, 129−134.
(28) Skog, K., Augustsson, K., Steineck, G., Stenberg, M., and Jägerstad, M. (1997) Polar and non-polar heterocyclic amines in cooked fish and meat products and their corresponding pan residues. *Food Chem. Toxicol. 35*, 555−565.
(29) Ristic, A., Cichna, M., and Sontag, G. J. (2004) Determination of less polar heterocyclic aromatic amines in a standardised beef extract and cooked meat consumed in Austria by HPLC and fluorescence detection. *J. Chromatogr., B 802*, 87−94.
(30) Oz, F., Kaban, G., and Kaya, M. (2007) Effects of cooking methods on the formation of heterocyclic aromatic amines of two different species trout. *Food Chem. 104*, 67−72.
(31) Persson, E., Sjöholm, I., Nyman, M., and Skog, K. (2004) Addition of various carbohydrates to beef burgers affects the formation of heterocyclic amines during frying. *J. Agric. Food Chem. 52*, 7561−7566.
(32) Busquets, R., Bordas, M., Toribio, F., Puignou, L., and Galceran, M. T. (2004) Occurrence of heterocyclic amines in several home-cooked meat dishes of the Spanish diet. *J. Chromatogr., B 802*, 79−86.
(33) Knize, M. G. (2006) Assessing human exposure to heterocyclic aromatic amines. In *Acrylamide and Other Hazardous Compounds in Heat-Treated Foods* (Skog, K., and Alexander, J., Eds.) pp 231−246, Woodhead Publishing Ltd., Cambridge, UK.
(34) Pais, P., Salmon, C. P., Knize, M. G., and Felton, J. S. (1999) Formation of mutagenic/carcinogenic heterocyclic amines in dry-heated model systems, meats, and meat drippings. *J. Agric. Food Chem. 47*, 1098−1108.
(35) Solyakov, A., and Skog, K. (2002) Screening for heterocyclic amines in chicken cooked in various ways. *Food Chem. Toxicol. 40*, 1205−1211.
(36) Turesky, R. J., Taylor, J., Schnackenberg, L., Freeman, J. P., and Holland, R. D. (2005) Quantitation of carcinogenic heterocyclic aromatic amines and detection of novel heterocyclic aromatic amines in cooked meats and grill scrapings by HPLC/ESI-MS. *J. Agric. Food Chem. 53*, 3248−3258.
(37) Busquets, R., Mitjans, D., Puignou, L., and Galceran, M. T. (2008) Quantification of heterocyclic amines from thermally processed meats selected from a small-scale population-based study. *Mol. Nutr. Food Res. 52*, 1408−1420.
(38) Friedman, M., and Cuq, J. L. (1988) Chemistry, analysis, nutritional value, and toxicology of tryptophan in food. *J. Agric. Food Chem. 36*, 1079−1093.
(39) Chen, B. H., and Meng, C. N. (1999) Formation of heterocyclic amines in a model system during heating. *J. Food Prot. 62*, 1445−1450.
(40) Arvidsson, P., Vanboekel, M. A. J. S., Skog, K., and Jägerstad, M. I. (1997) Kinetics of formation of polar heterocyclic amines in a meat model system. *J. Food Sci. 62*, 911−916.
(41) Khan, M. R., Mila, A., Busquets, R., Santos, F. J., and Puignou, L. (2009) Preparation and characterisation of fried chicken as a laboratory reference material for the analysi of heterocyclic amines. *J. Chromatogr., B 877*, 1997−2002.
(42) Skog, K., and Jägerstad, M. (2006) Modifying cooking conditions and ingredients to reduce the formation of heterocyclic amines. In *Acrylamide and Other Hazardous Compounds in Heat-*

592 *Treated Foods* (Skog, K., and Alexander, J., Eds.) pp 407−424,
593 Woodhead Publishing Ltd., Cambridge, UK.
594 (43) Skog, K., and Jägerstad, M. (1991) Effects of glucose on the
595 formation of PhIP in a model system. *Carcinogenesis 12*, 2297−2300.
596 (44) Murkovic, M., Weber, H. J., Geiszler, S., Frohlich, K., and
597 Pfannhauser, W. (1999) Formation of the food associated carcinogen
598 2-amino-1-methyl-6- phenylimidazo(4,5-*b*)pyridine (PhIP) in model
599 systems. *Food Chem. 65*, 233−237.