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Research Article

Quantification of heterocyclic amines from thermally processed meats selected from a small-scale population-based study

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Heterocyclic amines (HAs) are potent mutagens that form at high temperatures in cooked, protein-rich food. Due to their frequent intake, these compounds are considered a risk factor for human cancer. Cooking conditions and eating habits strongly influence the level of HA exposure. Thus, it is difficult to assess the intake of HAs in a large population. Food-frequency questionnaires (FFQs), designed to provide data on parameters that affect HA formation, were used to survey a small population (459 persons) from Barcelona (NE Spain). Subsequently, the most-consumed food items named were cooked according to the preferences of the population surveyed and analyzed for HAs using SPE and LC-MS/MS. In the population studied, the estimated intake *via* consumption of 13 meat dishes was 285.6 ng of mutagenic HAs *per capita* and day. PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) was the HA to which the population was most exposed, mainly from fried chicken and griddled beef. When the co-mutagens norharman and harman are included, the mean daily intake of HAs rises to 475.6 ng *per capita* and day. A novel putative DMIP regioisomer was detected in the cooked meats, which was analyzed in the present study by multistage MS.

Keywords: Food mutagens / Heterocyclic amines / Mass spectrometry / Spanish diet

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1 Introduction

During the last few decades, different sorts of mutagens have been identified in popular and apparently innocuous food items [1]. Heterocyclic amines (HAs) are a highly mutagenic class of compounds that have been investigated since the detection of mutagenic activity to *Salmonella typhimurium* TA98 in the smoke produced by broiling fish [2]. HAs are formed from the Maillard reaction, involving creatine, creatinine, carbohydrates and free amino acids present in raw protein-rich food [3]. Other sources of exposure to HAs include diesel-exhaust particles [4, 5], tobacco fumes [6] and cooking fumes [7–9]. Based on evidences from animal experiments, the International Agency for Research on Cancer (IARC) classified eight HAs as possible human carcinogens (class 2B) and 1 as a probable human carcinogen (class 2A) [10]. At present, more than 20 HAs have been characterized in cooked food and in cooking residues [11]. Furthermore, current research on HAs is revealing the presence of novel HAs in cooked food [12–15].

Many parameters affect the formation of toxicants in heating processes, which is thus difficult to control. For

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Abbreviations: **4,8-DiMeIQx**, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoline, CAS no. 95896-78-9; **7,8-DiMeIQx**, 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline, CAS no. 92180-79-5; **AαC**, 2-amino-9*H*-pyrido[2,3-*b*]indole, CAS no. 26148-68-5; **D_3-PhIP**, 2-amino-1-trideuteromethyl-6-phenylimidazo[4,5-*b*]pyridine, CAS no. 210049-13-1; **DMIP**, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine, CAS no. 132898-04-5; **FFQ**, food-frequency questionnaire; **Glu-P-1**, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole, CAS no. 67730-11-4; **Glu-P-2**, 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole, CAS no. 67730-10-3; **Harman**, 1-amino-9*H*-pyrido[3,4-*b*]indole, CAS no. 485-84-0; **HAs**, heterocyclic amines; **IQ**, 2-amino-3-methylimidazo[4,5-*f*]quinoline, CAS no. 76180-98-6; **IARC**, International Agency for Research on Cancer; **MeAαC**, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole, CAS no. 68806-83-7; **MeIQ**, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, CAS no. 77094-11-2; **MeIQx**, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, CAS no. 77500-04-0; **NCE**, Normalized Collision Energy; **Norharman**, 9*H*-pyrido[3,4-*b*]indole, CAS no. 244-63-3; **PhIP**, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, CAS no. 105650-23-5; **Trp-P-1**, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, CAS no. 62450-06-0; **Trp-P-2**, 3-amino-1-methyl-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, CAS no. 62450-07-1

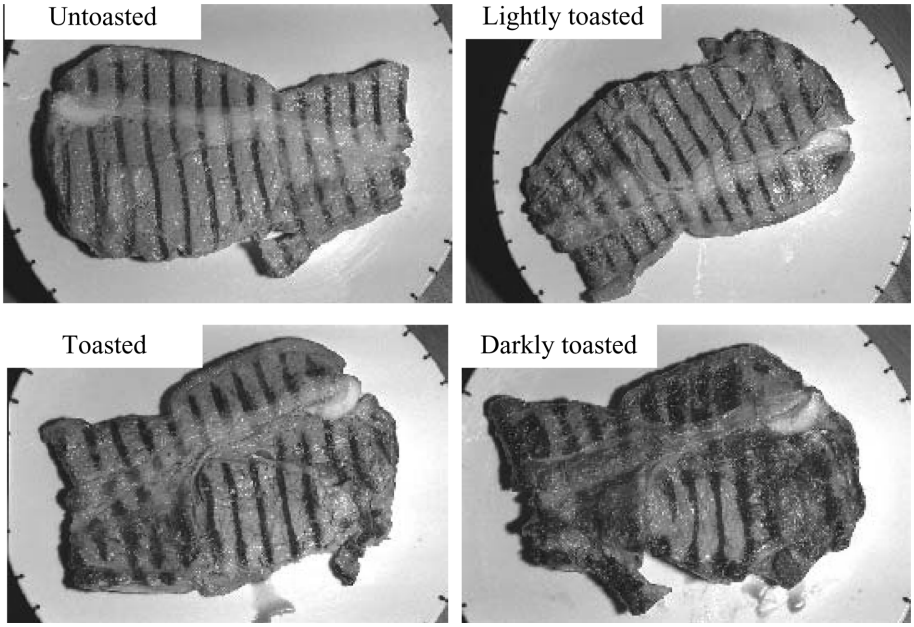

Figure 1. Beef fried to four levels of browning.

instance, temperature, heat transference and duration of the cooking process influence the levels of HAs [16 – 19]. Chemical factors such as meat composition or cooking ingredients also affect the formation of HAs [20 – 25]. The application of various cooking procedures to different kinds of meat and fish causes HAs to form in a range of concentrations; therefore, subsequent exposure differs from subject to subject. For example, the concentration of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine), which is one of the most ubiquitous HAs in cooked protein-rich food, usually ranges between 1 and 15 ng/g [11].

Quantifying the exposure to HAs of a large population requires access to reliable information on their eating and cooking habits, which may vary for geographical, economic, historical and cultural reasons. Therefore, a survey of a smaller population may be more informative as to the source of HAs, since the variables of eating and cooking habits are easier to monitor.

The aim of the present study was to carry out an exposure assessment of HAs in a small population from Barcelona (NE Spain). Extensive food-frequency questionnaire (FFQs) specifically designed to obtain information on the most-consumed dishes were given to a small population. On the basis of the survey results, foods were cooked and then analyzed using SPE and LC-MS/MS.

2 Materials and methods

2.1 Study population

The study is categorized into two parts: the first part based on a small survey in which the most frequently meat dishes were chosen and the second involving higher number of participants who have completed a semi-quantitative FFQ containing detailed information on their food intake frequency and cooking methods for the items that had previously been selected in the small survey.

The preliminary questionnaire was given to 220 randomly selected people living in Barcelona province. The semi-quantitative FFQ was completed by 800 randomly selected students and teachers from the Chemistry and Pharmacy faculties of the University of Barcelona who were living in Barcelona province. These populations were selected according to their work/study and resident places.

2.2 Dietary assessment

The preliminary questionnaires comprised ten blanks to list with: the most commonly eaten dishes, including meat, fish and mollusks; the way of cooking; the place where the food is eaten and personal information. An appendix was included with the definitions of different ways of cooking based on the classification recommended in the Eurocode description system (COST 99/Eurofoods). The most frequently consumed meat and fish items obtained from the small survey were contrasted with the most frequently eaten dishes in several canteens of the University of Barcelona according to the information provided by the executive chefs. The list of the most popular items according to both sources of information constituted the basis of the semi-quantitative FFQ.

The semi-quantitative FFQ included instructions for filling in the questionnaire and tables to describe the frequency of consumption and portion size of 14 different food items on the basis of photographs depicting four quantities of each item; to indicate the preferred way of cooking; to fill in with preferences on the degree of doneness and browning for each item; and questions about intrinsic characteristics

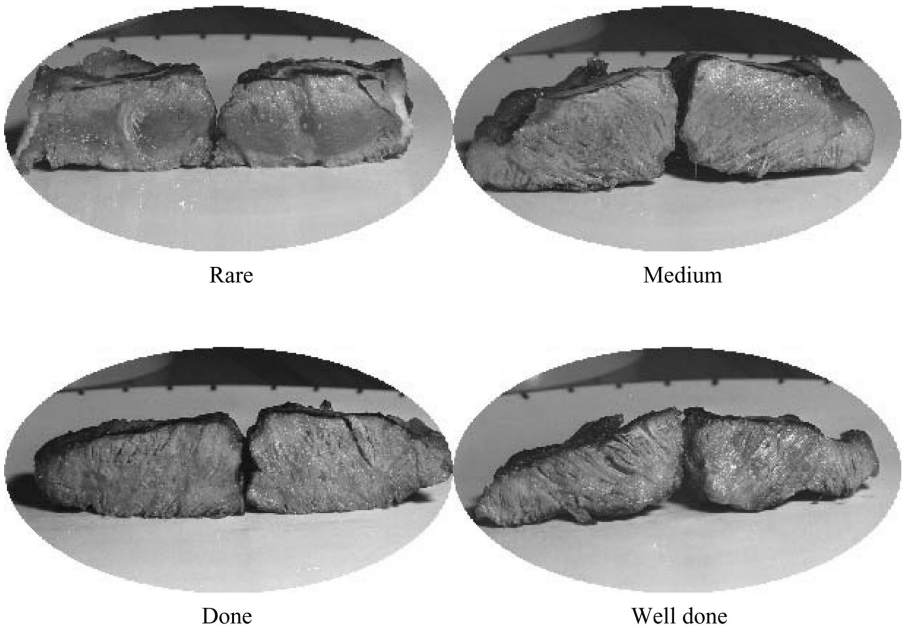

Figure 2. Beef fried to four levels of doneness.

of the food items and cooking procedures that could affect the yield of HAs, for instance, the type of frying fat used or the use of meat drippings for making sauces. To establish a scale in the preferred degree of browning and doneness, color photographs were provided; four depicting meat cooked to increasing degrees of browning (untoasted, lightly toasted, toasted and darkly toasted), shown in Fig. 1, and four color pictures showing a vertical section of steaks cooked to increasing degree of doneness (rare, medium, done and well done), shown in Fig. 2. The semi-quantitative FFQ was not validated for assessing exposure to HAs with other dietary methods (discussed in Section 3.4). The questionnaire was constructed on the basis of previous assessments of exposure to HAs [26, 27].

People participating in the survey were not informed about the objectives of the study before filling in the questionnaires. Both the preliminary and the semi-quantitative questionnaires were administered to the participants in June and considered the intake of meat dishes during the last 12 months.

2.3 Intake

Daily intake of HAs was estimated from particular foodstuffs. The intake of HAs from these foods was estimated by multiplying the daily amount consumed of each meat dish by the HA amount in each food item. The mean weight of cooked food corresponding to each portion size was measured after cooking the steaks following the cooking methods specified in the FFQ.

The analysis of HAs in 7 out of 13 food items used to estimate the intake in the present study was reported in previous works carried out by the authors [28, 29]. However, the consumed amounts of these quoted items were not used here to estimate the intake of HAs because the study populations were different in the previous (the Spanish population) and the present work.

The intake of HAs from fish dishes was not included in this study.

2.4 Chemicals

Solvents and chemicals were HPLC or analytical grade and were purchased from Merck (Darmstadt, Germany). The HAs 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 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), 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), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-1,4-dimethyl-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) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were provided by Toronto Research Inc. (North York, ON, Canada). 1-Amino-9*H*-pyrido[3,4-*b*]indole (Harman) and 9*H*-pyrido[3,4-*b*]indole (Norharman) were from Sigma (Steinheim, Germany). 2-Amino-1-trideuteromethyl-6-phenylimidazo[4,5-*b*]pyridine (D_3-PhIP), used as internal standard, was purchased from Toronto Research Inc. The chemical purity of the reference

Table 1. MS/MS parameters used with ion trap mass spectrometer

Segment	Time (min)	HA	Precursor ions $[M+H]^+$ (*m/z*)	NCE (%)	Product ion scan range (*m/z*)	Product ions used for quantification	
						m/z	Tentative assignation (*m/z*) [40]
1	0 – 9	DMIP	163	46	[140 – 170]	148	$[M+H-CH_3]^{+\bullet}$
		Glu-P-2	185	50	[150 – 190]	158	$[M+H-HCN]^+$
		IQ	199	47	[150 – 205]	184	$[M+H-CH_3]^{+\bullet}$
2	9 – 14	MeIQx	214	49	[165 – 220]	199	$[M+H-CH_3]^{+\bullet}$
						173	$[M+H-C_2NH_3]^+$
		MeIQ	213	47	[165 – 220]	198	$[M+H-CH_3]^{+\bullet}$
		Glu-P-1	199	50	[165 – 210]	184	$[M+H-CH_3]^{+\bullet}$
						172	$[M+H-HCN]^+$
		7,8-DiMeIQx	228	48.5	[180 – 235]	213	$[M+H-CH_3]^{+\bullet}$
						187	$[M+H-C_2NH_3]^+$
		4,8-DiMeIQx	228	48.5	[180 – 235]	213	$[M+H-CH_3]^{+\bullet}$
						187	$[M+H-C_2NH_3]^+$
3	14 – 17.6	Norharman	169	52	[110 – 175]	167	$[M+H-2H]^+$
						142	$[M+H-HCN]^+$
						115	$[M+H-2HCN]^+$
		Harman	183	53	[110 – 190]	181	$[M+H-2H]^+$
						168	$[M+H-CH_3]^{+\bullet}$
						115	$[M+H-C_3H_4N_2]^+$
4	17.6 – 21.2	Trp-P-2	198	40	[175 – 225]	222	$[M+H-NH_3+CH_3CN]^+$
						199	$[M+H-NH_3+H_2O]^+$
						181	$[M+H-NH_3]^+$
		Trp-P-1	212	47	[190 – 240]	236	$[M+H-NH_3+CH_3CN]^+$
						213	$[M+H-NH_3+H_2O]^+$
						195	$[M+H-NH_3]^+$
		PhIP	225	51	[200 – 230]	210	$[M+H-CH_3]^{+\bullet}$
		D_3-PhIP	228	51	[200 – 230]	210	$[M+H-CD_3]^{+\bullet}$
5	21.2 – 25	AaC	184	46	[165 – 215]	208	$[M+H-NH_3+CH_3CN]^+$
						185	$[M+H-NH_3+H_2O]^+$
						167	$[M+H-NH_3]^+$
		MeAaC	198	46	[175 – 225]	222	$[M+H-NH_3+CH_3CN]^+$
						199	$[M+H-NH_3+H_2O]^+$
						183	$[M+H-CH_3]^{+\bullet}$
						181	$[M+H-NH_3]^+$

compounds was higher than 99%. Stock standard solutions of 130 μg/g were prepared in methanol and used for further dilution.

Isolute diatomaceous earth refill material was obtained from IST (Hengoed, Mid-Glamorgan, UK). Propylsulfonate silica PRS (500 mg) cartridges and endcapped Bond Elut C_{18} (100 and 500 mg) cartridges were from Varian (Harbor City, CA, USA).

2.5 Instrumentation

An HPLC system from Waters (Milford, MA, USA) model Alliance 2695 coupled to an IT mass spectrometer LCQ (ThermoFisher, San Jose, CA, USA) equipped with electrospray (ESI) interface operating in positive ionization mode was used for the determination of HAs. In addition, a single pump (Pharmacia, Uppsala, Sweden) was used to perform post-column addition.

A surface thermometer (Testo Instruments, Cabrils, Spain) was used to measure the pan, griddle and clay casserole temperatures. A common blender was used for grinding sample crusts. An Ultra-Turrax® T 25 basic (IKA, Staufen, Germany) was used to homogenize ground sample crust mixed with 1 M sodium hydroxide solution. Supelco Visiprep™ and Visidry™ vacuum manifolds (Supelco, Gland, Switzerland) were used for SPE and solvent evaporation.

2.6 Analytical conditions

HAs were separated using a reversed-phase column Symmetry® C_8 (5 μm, 150 mm × 2.1 mm; Waters Corporation, Milford, MA, USA) equipped with a C_8 (4 mm × 2.0 mm) precolumn (Phenomenex, Cheshire, UK). The gradient elution program and the MS conditions were as previously described [30]. Normalized collision energies (NCE), time schedule and MS/MS parameters are given in Table 1.

Table 2. Cooking conditions and mean values of thickness cooking loss, weight of cooked food, portion size, frequency of consumption and daily intake are given. Samples were cooked at "well done" and "toasted" degree of cooking. Information obtained from a survey of 459 persons

Meat sample	Cooking method	Thickness (raw meat) (cm)	Total cooking time (min)	Cooking temperature (°C)	Cooking loss (%)	Weight of cooked steaks (g)	Portion size (number of steaks consumed)	Frequency of consumption (times/month)	Daily intake of cooked meat (g)
Beefsteak	pan-frying	0.5	12	180 – 200	45	56	1.1	0.8	1.7
Beefsteak	griddling	0.5	4	180 – 210	40	56	1.1	3.5	7.2
Beefsteak	coating-frying	0.5	14	180 – 200		67	1.1	0.3	0.6
Beef hamburger	pan-frying	0.8	11	175 – 200	34	44	1.3	0.9	1.8
Pork loin	pan-frying	0.5	10	175 – 200	36	27	2.4	0.6	1.2
Pork loin	griddling	0.5	10	180 – 200	30	27	2.4	1.4	3.1
Pork loin	coating-frying	0.5	7	180 – 200		47	2.4	1.0	3.8
Pork sausages	pan-frying	2	9	175 – 200	16	135	1.1	1.9	9.6
Chicken breast	pan-frying	0.8	12	175 – 200	38	34	1.3	1.4	2.0
Chicken breast	griddling	0.8	13	175 – 200	27	33	1.3	2.3	3.1
Chicken breast	coating-frying	0.8	9	180 – 200		48	1.3	1.4	2.8
Chicken breast	roasting	0.8	138	140 – 160	43	74	1.3	1.5	4.7
Lamb steak	griddling	0.8	11	175 – 200	12	55	1.1	0.8	1.6

2.7 Food samples

The raw meat products (meat steaks) and the cooking ingredients (olive oil, salt, golden bread crumbs and eggs) were bought in a local store. Cooking was carried out by lab staff. All meat dishes, including the quoted items [28, 29], were cooked according to the most frequent cooking habits, degree of doneness and degree of browning reported in the questionnaires. Meat was seasoned with 1 g salt/steak. For each studied food item 0.5 – 1 kg of meat was cooked. Cooking was carried out on a gas cooker using a frying pan, a griddle and a clay casserole for frying, griddling and roasting, respectively. Each food item was cooked at different sessions and four steaks were cooked at a time. The temperature of the pan, griddle and casserole was monitored with a surface thermometer. During the cooking, the surface temperature of the pan was 180 – 200°C. When roasting, the temperature ranged from 140° to 160°C on the surface of the casserole. Olive oil was used to grease the surface of the griddle and the clay casserole in griddling and roasting processes. For frying, olive oil was used up to a level so as almost to cover the meat steaks and it was never reused. Table 2 shows the sample characteristics and cooking conditions. After cooking, the food was drained on absorbent paper to remove residues. A scalpel was used to separate the crust (3 – 4 mm), which was blended using a domestic blender. The ground crust was kept at – 18°C until analysis.

2.8 Extraction of HAs

The extraction and purification of HAs formed in the meat crust during cooking was carried out with a SPE method developed by Gross and Grüter [31] and slightly modified by Galceran *et al*. [32]. Dichloromethane was used as extracting solvent. Through the clean-up applied, the less polar HAs (norharman, harman, Trp-P-2, Trp-P-1, PhIP, AαC, MeAαC) and the most polar HAs (DMIP, Glu-P-2, IQ, MeIQx, MeIQ, Glu-P-1, 7,8-DiMeIQx, 4,8-DiMeIQx) were obtained in two separate extracts. Although the crust was the only part analyzed, the ultimate concentrations were expressed as the concentrations of HAs in the cooked meat.

2.9 Quantification

HAs from each food item were quantified by standard addition method. As a consequence, all results given in the text or in the tables are corrected by recovery. The standard addition method comprised two unspiked and four spiked samples. The spiking levels were experimentally selected for the analysis of each cooked meat. For instance, the spiking levels used to determine PhIP in fried beefsteak were 0.0, 1.0, 2.2, 3.5 and 5.0 ng/g cooked meat. Recovery rates were calculated as the slope that was obtained when representing the amount of HAs added per gram of cooked sample and the amount of HAs found per gram of cooked sample. The external calibration curve ranged between the limit of quantification, 0.01 μg/g, and the limit of the linearity range 1 μg/g.

3 Results and discussion

3.1 Selection of the most-consumed meat dishes

The number of preliminary questionnaires returned was 143. Of those who answered the questionnaire, 74% were women and 26% men; 59% were ≤25 years old, 28% were 26 – 50 years old, 13% >50 years old. The items most frequently selected from the small survey were: pork (chop,

Table 3. Cooking methods of the most-consumed food items. Results of the semi-quantitative FFQ

Food item	Way of cooking (%)						
	Fried	Barbecued	Griddled	Coated-fried	Roasted	Stewed	Boiled
Beefsteak	15	8	66	5	3	3	0
Beef hamburger	40	6	53	1	0	0	0
Pork loin	17	7	43	29	3	1	0
Pork sausages	44	19	32	0	2	1	2
Chicken breast	9	9	31	19	21	9	2

loin and sausage); beef (steak, hamburger and meatball); chicken (leg and breast); rabbit (shoulder and leg); white fish; blue fish; and mollusks. The cooking methods mostly used were frying, griddling, coating-frying, barbecuing and roasting. The most frequently selected items in the university canteens were coincident with the results of the preliminary questionnaire; with the particularity that coated-fried beefsteak was the most-consumed dish in the canteens.

The list of the most popular items according to both sources of information constituted the basis of the semi-quantitative FFQ. A total of 459 FFQs were received. The population that completed the FFQ comprised 70% women and 30% men; 40% were $\leq$20 years old, 43% were 21–30 years old, 6% were 31–40 years old, 11% were $\geq$41 years old, which was representative of the studied population.

Above 99% of the 459 completed FFQ corresponded to people who eat meat or fish. Half of the fish/meat consumers eat meat or fish more than 6 times/week, while 36% of the meat/fish consumers eat meat or fish 4–6 times/week. The most-consumed dishes, ordered from more to less frequency of consumption are pork (11.1 times/month), veal (9.5 times/month), chicken (7.3 times/month), white fish (6.4 times/month), fatty fish (4.3 times/month), mollusks (2.9 times/month), lamb (2.5 times/month) and rabbit (1.8 times/month). The frequency of use of the reported cooking methods to prepare the most-consumed food items is shown in Table 3.

The consumption of different items from the same type of meat was also specified by the participants in our study. With regard to pork, sausages (4.4 times/month) and loin (3.5 times/month) were the favorite items. Regarding veal/beef, steak (5.4 times/month) and hamburger (2.4 times/month) were the most-consumed dishes. Under chicken products, breast meat (6.0 times/month) was more consumed than thigh meat (1.3 times/month).

Degree of doneness and browning in the consumed food items were reported for each specific food item. "Well done" (42–50%) was the most frequently preferred degree of doneness, and "toasted" (42–58%) was the favorite degree of browning, grades to which the meats were cooked for the estimation of the HA daily intake. Comparing the photographs used in this work to establish the degree of browning and doneness with previous works, the lowest level of toasting and doneness considered here was more toasted and cooked to higher degree of doneness than the samples cooked at the respective level in other works [26, 33, 34]. Moreover, the most toasted sample in our work, with no charred part, was less toasted than the corresponding samples in these works.

Only 11.3% of the respondents used drippings or pan residues to make gravies or sauces when frying, whereas 38.5% used them when stewing or roasting. The studied population ate lean meat, 76.1%, without visible fat. Skin was not frequently present in poultry when cooking (10% of the time). The use of fat to cook meat or fish was very common; only 4% did not use fat to cook meat or fish. Olive oil was used by 87.5% of subjects, sunflower oil was used by 8.3% and butter was only used by 0.2% of subjects. Regarding some other cooking practices that influence HA formation, obtained from the FFQ, 93% of subjects never used aluminum foil for cooking. The setting of the stove most often used was "medium" according to 65% of the subjects, 32.6% used "high" and only 2.4% used "low". Of the subjects, 20.1% did not change the setting of the stove during the whole cooking process, 36.2% turned it down immediately after starting to cook and 43.7% turned it down later.

The meat dishes more prone to cause high exposure to HAs due to the frequency and amount of consumption, in addition with the potential high yield of the mutagens due to the cooking method used (see Section 3.3), were selected for the exposure assessment and are described in Table 2.

3.2 Analysis of HAs

Analysis was performed on a number of the most-consumed meat dishes listed in Table 2, e.g. griddled pork loin, coated-fried pork loin, fried beef, coated-fried beef, roasted chicken and coated-fried chicken. Variable recoveries in HA extraction were obtained, depending on the matrix, which were very low in some cases. In particular PhIP and DMIP were the analytes most affected; 10–20% for DMIP and 16–77% for PhIP (see Table 4). This fact was also observed in previous works using the same clean-up or other SPE methods based on it and by using different extraction solvents such as dichloromethane, toluene or ethyl acetate [28, 29, 35–38]. The low recovery obtained for DMIP could be due to the low polarity of the extraction

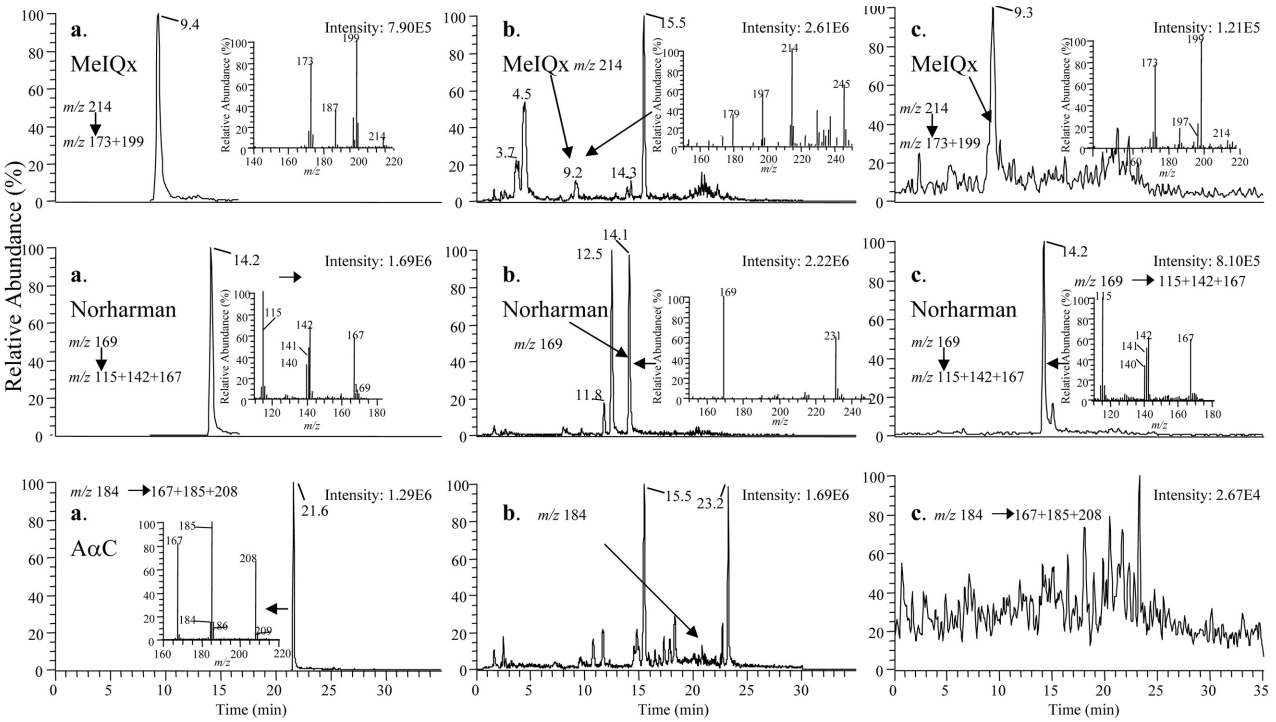

Figure 3. Comparison of the detection of some HAs using different acquisition modes. (a) Standard acquired in product ion scan; (b) purified sample from coated-fried pork loin acquired in full scan mode; (c) the same sample as in (b) acquired in product ion scan mode.

solvent, which makes difficult the purification of such a polar HA in the first clean-up step. HAs were separated in two extracts on the basis of their polarity according to the reference method (see section 2.8). However, we notice that in most meat matrices norharman, harman and PhIP were present in both types of extract. For example, in coated-fried beef, 50% of PhIP was found in the extracts containing the less polar HAs and 30% of PhIP was found in the other purified extract. This loss of analytes can be avoided by using SPE methods where the purified HAs are collected in a single extract.

Regarding the quantification strategy, the decrease of the slope of the calibration curve due to the presence of matrix indicates that standard addition is a suitable way to measure HAs in meat samples, as described in previous works [31, 32, 39].

Mass spectrometry is a powerful tool for the determination of these analytes at low concentrations in foodstuffs. Tandem mode is more selective and sensitive than full-scan mode. Figure 3 shows the improvement provided by tandem mode in the determination of MeIQx and norharman in coated-fried pork loin; where it can be compared the full scan acquisition (b) with the product ion scan acquisition (c), and the prevention of false identification of species that appear at a similar retention time to that of the target analyte in the standard (see AαC in Fig. 3).

Moreover, by means of LC-MS/MS, further information can be obtained about species other than analytes that have also been purified from food samples. We detected a chromatographic peak whose multistage spectrum is similar to that of DMIP when working in our specific chromatographic conditions and in low resolution mass spectrometry (see Fig. 4). The fragmentation pathway of this chromatographic peak was studied and compared with the peak at 4.3 minutes that corresponded to DMIP. Based on an early study by our group, where the fragmentation of DMIP was reported [40], similarities were observed between the unknown and DMIP, such as tentative loses of $CH_3^{\bullet}$ (m/z = 148), NH_3 (m/z = 146) and CN_2H_2 (m/z = 121) in the MS^2 as well as important coincidences in both MS^3 spectra. Thus, the unknown could be structurally related with DMIP. This unknown was detected in all the meat dishes included in the present study and in a previous study carried out by the authors [29]. Putative DMIP regioisomers have not been reported previously in cooked foodstuffs to our knowledge; however, additional studies are necessary to elucidate the structure of this unknown.

3.3 HA determination in meat samples

The amounts of HAs in the cooked meats analyzed in this work are given in Table 4. In previous studies [28, 29], HAs

Table 4. Concentration of HAs in cooked meat dishes and their corresponding intake

A	Griddled pork loin		Coated-fried pork loin	
Compounds	Content (R)[a] ng/g ± s[b]	Intake ng[c]	Content (R)[a] ng/g ± s[b]	Intake ng[c]
DMIP	1.0 ± 0.3 (20)	3.1	ND	–
MeIQx	0.5 ± 0.1 (64)	1.5	<0.3 (73)	<1.1
MeIQ	ND[d]	–	ND	–
7,8-DiMeIQx	ND	–	ND	–
4,8-DiMeIQx	0.1 ± 0.1 (72)	0.3	ND	–
Norharman	2.2 ± 0.3 (63)	6.8	6.7 ± 1.6 (99)	25.4
Harman	1.1 ± 0.3 (82)	3.4	0.7 ± 0.3 (101)	2.5
Trp-P-2	ND	–	ND	–
Trp-P-1	ND	–	ND	–
PhIP	2.1 ± 0.3 (30)	6.5	1.3 ± 0.3 (77)	5
AaC	<0.1	0.3	ND	–
MeAaC	ND	–	ND	–

B	Fried beefsteak		Coated-fried beefsteak	
Compounds	Content (R)[a] ng/g ± s[b]	Intake ng[c]	Content (R)[a] ng/g ± s[b]	Intake ng[c]
DMIP	1.1 ± 0.8 (12)	1.8	<0.3 (10)	< 0.2
MeIQx	0.8 ± 0.4 (47)	1.3	0.3 ± 0.1 (48)	0.2
MeIQ	ND	–	ND	–
7,8-DiMeIQx	ND	–	ND	–
4,8-DiMeIQx	0.7 ± 0.4 (50)	1.1	ND	–
Norharman	6.0 ± 0.8 (38)	9.8	15.4 ± 0.8 (40)	9.9
Harman	4.3 ± 0.3 (55)	7.1	6.7 ± 0.4 (50)	4.3
Trp-P-2	<0.3 (11)	<0.5	<0.1 (13)	<0.1
Trp-P-1	<0.5 (18)	<0.9	<0.1 (22)	<0.1
PhIP	1.1 ± 0.3 (46)	1.8	0.2 ± 0.1 (50)	0.1
AaC	<0.1 (29)	<0.2	ND	–
MeAaC	0.7 ± 0.1 (54)	1.2	ND	–

C	Roasted chicken breast		Coated-fried chicken breast	
Compounds	Content (R)[a] ng/g ± s[b]	Intake ng[c]	Content (R)[a] ng/g ± s[b]	Intake ng[c]
DMIP	<0.04 (13)	< 0.2	ND	–
MeIQx	<0.1 (63)	< 0.3	<0.2 (59)	<0.5
MeIQ	<0.1 (42)	< 0.3	<0.1 (51)	<0.3
7,8-DiMeIQx	<0.05 (37)	< 0.2	ND	–
4,8-DiMeIQx	<0.04 (13)	< 0.2	0.1± 0.4 (61)	0.4
Norharman	0.9 ± 0.3 (47)	4.3	9.7 ± 0.5 (74)	26.8
Harman	0.9 ± 0.4 (41)	4.3	1.3 ± 0.4 (81)	3.6
Trp-P-2	<0.3 (22)	< 1.3	ND	–
Trp-P-1	<0.2 (27)	< 0.7	<0.1 (56)	<0.4
PhIP	0.9 ± 0.3 (16)	4.3	0.6 ± 0.4 (65)	1.8
AaC	ND	–	ND	–
MeAaC	ND	–	ND	–

a) Recovery, expressed in %, is given in brackets.
b) Standard deviation of the standard addition calibration curve.
c) Intake per day and person.
d) ND: not detected (S/N <3).

were determined in other frequently consumed items that are presented in Table 2. The HAs most frequently found in the different cooked meats are PhIP, MeIQx, norharman and harman, as in dishes from other areas [41 – 43]. In general, the food items cooked under the selected cooking conditions generated mutagenic HAs at a total concentration lower than 5 ng/g. Since norharman and harman are not mutagenic on their own [22], PhIP was the most abundant mutagenic HA at 2.1 ng/g or below, although it has been reported at higher concentrations in some studies, especially in fried poultry meat (for a review see Skog and Solyakov, 2004 [44]). This fact shows that not only cooking methods, but probably also concentrations of precursors and cooking additives influence the occurrence of HAs.

In general, cooking methods where the heat is transferred by conduction, such as pan-frying, griddling and coating-frying, yielded higher amounts HAs than when the heat is transferred by air, for instance in roasting processes. However, the latter is a less efficient mechanism of heat transfer than conduction. In addition, the heat transfer can be decreased by coating, which insulates the meat from the heat source to some extent; therefore, lower levels of HAs may be produced by coating-frying than by frying [45]. In our study, even though the cooking conditions were not comparable (see Table 2), it was observed that coated-fried chicken, beefsteak, pork loin and also roasted chicken were among the meat dishes that generated the lowest amount of HAs, which is in agreement with previously reported data [16, 19, 34, 42, 46]; however, HAs have rarely been reported for coated-fried items. The levels of some HAs in the pork loin are in the same range than those reported in steaks cooked in restaurants [47], although the amount of PhIP determined in the present study was lower. The content of HAs found in fried beefsteak was similar than the levels in previous works [15, 48, 49] but at lower level than in samples purchased from restaurants [50].

IQ, Glu-P-1 and Glu-P-2 were not detected in any of the cooked meats analyzed in the present work, and MeIQ and 7,8-DiMeIQx were not frequently detected in the analyzed cooked meats, which is in accordance with literature data [36, 51 – 53]. The LC-MS/MS chromatograms of one of the food items where MeIQ has been detected, coated-fried chicken breast, are shown in Fig. 5.

On the basis of the present study, the quantification of PhIP and MeIQx would cover over 50% of the total mutagenic amines analyzed in this work, and by determining the four most abundant HAs, DMIP, MeIQx, 4,8-DiMeIQx and PhIP, over 90% would be covered.

3.4 Estimation of daily HA exposure

The mean daily intake of mutagenic HAs, considering the intake from the 13 food items selected from among the most-consumed items identified by the studied populations

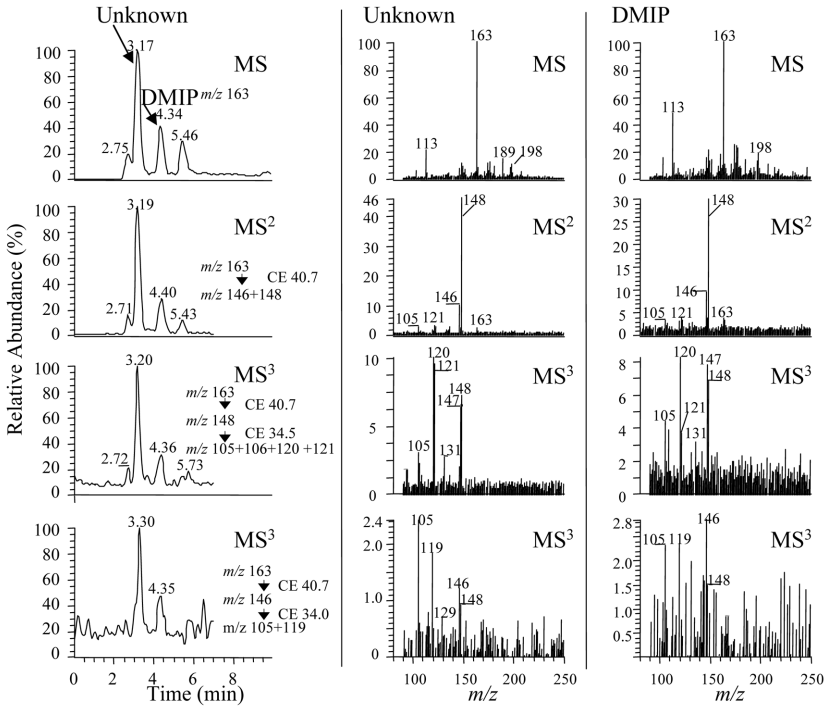

Figure 4. Chromatograms showing the MS fragmentation pathway of an unknown compared to DMIP in griddled pork loin. Full scan, MS^2 and two different MS^3 in product ion scan are presented.

and with the most probable presence of HAs, was 285.6 ng of HAs *per capita* and day. The mean daily intake of HAs goes up to 475.6 ng *per capita* and day when the co-mutagens norharman and harman are included. This estimated daily intake comprises the exposure to HAs from the consumption of 63% of the total cooked meat consumed according to our small-scale study. The average daily intake of cooked meat was 68.6 g, intake that was within the range of the consumption of meat reported for a population from NE of Spain [54]. However, the comparison must be done with caution because the compared populations have different characteristics and the dietary assessments have been performed using different methods. The contribution of each selected food item to the daily intake *per capita* of the studied mutagenic HAs has been indicated in Table 5. The cooked meat items with the highest contribution of mutagenic HAs were fried chicken and griddled beef, representing 54% and 20% of the daily intake of mutagenic HAs, respectively. Among the mutagenic HAs studied, PhIP, DMIP, MeIQx and 4,8-DiMeIQx were the most consumed, with estimated daily intakes *per capita* of 162.1 ng, 87.9 ng, 23.1 ng and 14.1 ng. We underscore the finding that the high intakes of DMIP and PhIP are mainly due to the high concentrations found in fried chicken breast, with 29.7 ng/g and 46.9 ng/g cooked food, respectively [29]. Of the daily intake *per capita* of PhIP, 57% comes from the consumption of fried chicken, with 92.8 ng PhIP, but griddled beef also appears as an important source of PhIP, with a contribution of 29.1 ng PhIP to the daily intake *per capita*, which represents 18% of the PhIP daily intake. Fortunately, the intake of the most mutagenic HAs, IQ and MeIQ, has been estimated as less than 1 ng/*per capita* and day. The Trp-P-2 and AαC contribution is about 6 ng each, and MeAαC accounts for 2 ng *per capita* and day, its main source being griddled beef. Harman and norharman are the most abundant HAs found in meat, but they are also found in other types of food [22]. In this study, the daily intake *per capita* of norharman and harman from cooked meat was estimated as 261.3 ng and 94.3 ng respectively. This is important to note, not only because their co-mutagenic activity but also because of their affinity with receptors in the central nervous system [22].

This study has several advantages. The questionnaires were specifically designed to obtain data related with the intake of HAs from cooked meat. Exposure to HAs depend not only on the type of meat, frequency or amount consumed but also on the cooking habits; therefore, the questionnaire also sought out such information. Data on the preferred portion size and the degree to which meat was cooked both externally and internally were obtained using photographs (Figs. 1 and 2), which helped to both express the habits of the respondents and to reproduce the dishes for their analysis.

The respondents reported frequencies of intake over a year, which may be a difficult cognitive task, and thus it was a source of error. Misclassification errors for the developed FFQ cannot be calculated because the questionnaire was not validated following traditional validation strategies such as weighed records, 24-h recalls or another FFQ, which is a limitation. For example, Sinha *et al.* [55] used multiple food diaries to validate HA intake estimated by an FFQ. However, validation methods based on additional

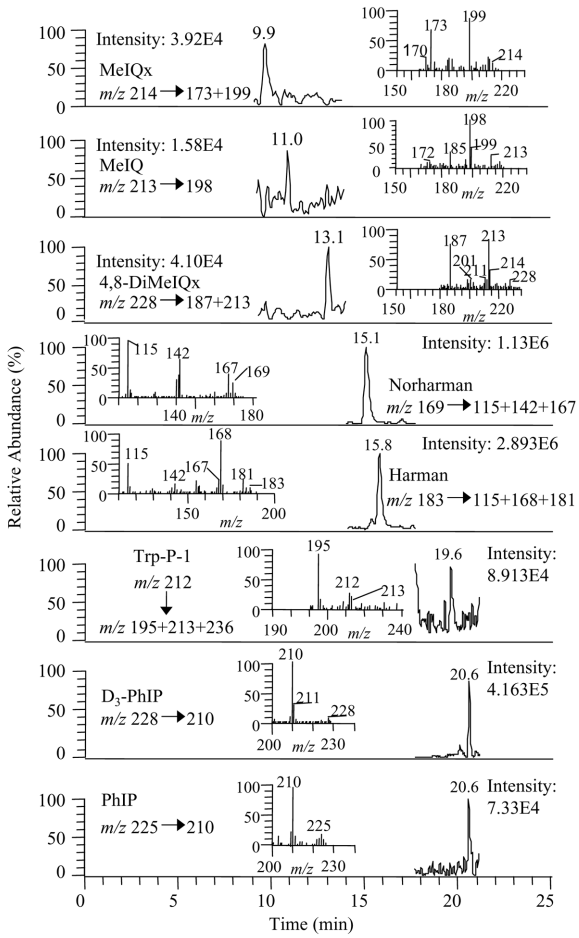

Figure 5. Chromatograms of HAs from a sample of coated-fried chicken breast obtained with LC-MS/MS. The amounts of HAs are given in Table 4. The purified samples were reconstituted in 0.1 mL internal standard D_3-PhIP (0.10 μg/g) in methanol.

dietary assessments may not be more accurate than the FFQ being evaluated [56] and the ideal way to validate the questionnaire would be by quantifying biomarkers [55]. At present, scientists are working hard to identify biomarkers of exposure to HAs [57 – 66].

In our earlier study in which the population studied was Spanish and where EPIC (European Prospective Investigation into Cancer and Nutrition) data on consumption patterns were used, the estimated mean value calculated without co-mutagens harman and norharman was 606 ng *per capita* and day, and this increased to 934 ng *per capita* and day if these co-mutagens were included [29]. One of the main differences between the previous study on large scale and the present study on a small-scale Spanish population is that the amount of fried chicken consumed was much higher in the first case (6.6 g *per capita* and day) rather than the amount consumed in the small-scale population (2.0 g *per capita* and day). These differences may be due to the

Table 5. Contribution of each food item to the daily intake *per capita* of mutagenic HAs

Meat sample	Cooking method	Intake per day and person of mutagenic HAs[a)] (ng)
Beefsteak	Pan-frying	6.0
Beefsteak	Griddling	57.7
Beefsteak	Coating-frying	0.3
Beef hamburger	Pan-frying	2.3
Pork loin	Pan-frying	11.4
Pork loin	Griddling	11.4
Pork loin	Coating-frying	5.0
Pork sausages	Pan-frying	0.0
Chicken breast	Pan-frying	153.2
Chicken breast	Griddling	16.2
Chicken breast	Coating-frying	1.8
Chicken breast	Roasting	4.3
Lamb steak	Griddling	15.5

a) Intake corresponding to the studied mutagenic HAs determined over the limit of quantification (S/N =10).

different origins of the participants and different characteristics of the studied population (age, sex). Thus, the amount of fried chicken consumed has important repercussions on the final estimation of the daily HA intake estimation.

The estimated daily intake of HA *per capita* from the analysis of the most-consumed meat dishes in a small population living in Spain, 285.6 ng, was higher than that estimated in Asian populations: 50 ng in China [43] and 72 ng in Japan [67], higher than the estimation carried out in Heidelberg (Germany), with 69 ng [68], but it was in the range of other European populations: 160 ng in Stockholm (Sweden) [26]; 690 ng in Malmö (Sweden) [69]; 330 ng in Switzerland [42]; 606 ng in Spain [29], and lower than in the United States: 1690 ng [70]; 455 ng [71]; 585 ng [72]; 715 – 1293 ng [73], intakes estimated considering an average body mass of 65 kg. In this work, meat was the only source of HAs considered to estimate the daily intake of HAs *per capita.* Fish consumption would increase the exposure to HAs in our population; which is currently being studied by our group.

4 Concluding remarks

An FFQ specifically designed to provide information related to the intake of HAs was completed by a small population from NE Spain (459 questionnaires answered). From the analysis of the most-consumed meat dishes among the dishes named in the completed questionnaires with most probability of contributing to mutagenic HA intake, a mean daily intake of 285.6 ng *per capita* was estimated. The meat dishes included in the mean daily intake estimation were fried chicken breast, griddled chicken breast, coated-fried chicken breast, roasted chicken breast, fried pork loin, griddled pork loin, coated-fried pork loin,

fried pork sausages, fried beefsteak, griddled beefsteak, coated-fried beefsteak, fried beef hamburger and griddled lamb. These dishes represented 63% of the total meat consumption in the studied population. Fried chicken breast was the main source of mutagenic HAs, with 153.2 ng *per capita*. PhIP was the most-consumed mutagenic HA, with a daily intake estimated as 162.1 ng, 75% of which came from the consumption of fried chicken and griddled beef. An unknown with similar MS multistage spectra as DMIP was detected in the analyzed cooked meats. Further work should be carried out to include the exposure to HAs from fish dishes in the studied population because their contribution may be far from negligible.

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