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Cooking with elaborate recipes can reduce the formation of mutagenic heterocyclic amines and promote comutagenic amines

Mohammad Rizwan Khan [†,‡], Rosa Busquets [§,‡] *, Mu Naushad [†] Lluís Puignou [‡]

[†] Department of Chemistry, College of Science, Bld. 5, King Saud University, Riyadh 11451, Saudi Arabia.

1. [§] School of Life Sciences, Pharmacy and Chemistry, Kingston University London, Penrhyn road, KT1 2EE, Kingston upon Thames, United Kingdom.

[‡] Department of Analytical Chemistry, University of Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain.

* Author to whom correspondence should be addressed:

Tel: + 44 (0) 2084177792; E-mail: r.busquets@kingston.ac.uk (R. Busquets)

Abstract

Heterocyclic amines (HCAs) are foodborne carcinogens which formation is highly dependent on cooking conditions. HCAs have been commonly quantified in food items prepared with simple procedures. This approach is suitable for elucidating HCAs' formation, but it reflects partially the contamination in consumed food. In the current investigation, the generation of HCAs has been investigated in fried beef items prepared with elaborated cooking recipes, and their occurrence has been compared with control beef fried without the addition of other ingredients than oil. The food recipes that included a variety of food ingredients had lower yields of mutagenic HCAs (≥ 47% reduction, with individual HCA levels ranging between 0.01 and 2.22 ng/g) with respect to the control beef. In contrast, the co-mutagens norharman and harman were formed generally at greater levels (up to 3 times the contamination in the control fried beef) in the items prepared including greater variety of ingredients.

Keywords: foodborne carcinogens, Maillard, PhIP, MeIQx, norharman; harman

Highlights

- Recipes including a variety of ingredients can minimize the yield of mutagenic HCAs
- Harman and norharman can increase their yield from the use of ingredients
- Epidemiologic studies to account for effect of ingredients on cancer risk from HCAs

Introduction

Heterocyclic amines (HCAs) are mutagenic compounds which are most commonly found in thermally processed protein-rich foods such are meat of fish (Alaejos et al. 2008; Khan 2015; Shabnam et al. 2018). HCAs form from the reaction of amino acids, sugar, creatine or creatinine via the Maillard reaction, which also produces compounds that give desirable taste and brown color to the food. Greater amounts of HCAs are produced when items are cooked for longer times at elevated temperatures. In addition, the type of meat and applied cooking process also affect HCAs' formation (Gibis 2016; Skog et al. 1998).

Epidemiologic studies assessing causal factors in the onset of different types of cancer are not conclusive about the contribution of the consumption of processed meat, or more specifically, the effect of individual HCAs from the cooked meat, despite that the activation of HCAs towards genotoxic metabolites and neurotoxicity have been reported (Bellamri et al. 2018; Turner and Lloyd 2017; Sadrieh and Davis 1998; Cruz-Hernandez et al. 2018). This is in part because the published levels of HCAs in food cannot represent entirely the consumed items (i.e have been prepared in absence of protective compounds (Turner and Lloyd 2017), and slight changes in cooking procedures can affect the yield, hence the intake, of HCAs). The use of biomarkers to assess the effective exposure to HCAs is expected to find a more robust relation between exposure and development of the disease. Adduct of some HCAs with protein (AαC) and DNA have been identified (Pathak et al. 2016; Ho et al. 2015). The HCA-DNA adducts indicate that the intake of the pro-mutagens, at least, enhances the risk of having mutations. Over 20 years ago, and based on experimental animal studies, the International Agency for Research on Cancer listed various HCAs as *possible and probable human carcinogens* (IARC 1993). The National

Toxicology Program classified four HCAs (MeIQ, MeIQx, IQ and PhIP) as *reasonably anticipated to be human carcinogens* (NTP 2016).

To clarify the extent of the threat to human health posed by HCAs and assess the gap between the level of HCAs in food cooked following simple and complex processes, it is important to quantify HCAs in diverse dishes prepared following widely used recipes. This comparison needs to consider that every cooking process involves a rate of heat transfer which can affect the yield of HCAs. Moreover, recipes involving the addition of a variety of ingredients to the raw meat/fish can also affect the transport of HCA's precursors within the item being processed and chemical reactions taking place within the food .This has resulted in reduced yields of some HCAs in a number of studies following traditional cooking styles (Zeng et al. 2017; Oz and Yuzer 2017; Busquets et al. 2006; Vitaglione and Fogliano 2004).

This work quantifies relevant HCAs in beef fried following traditional cooking recipes commonly used in Spain (Mendel 1997) and investigates how their levels were affected with respect to the preparation of the dish with oil as only ingredient. This research intends to support epidemiologic studies by highlighting the differences in HCAs' contamination in an item commonly consumed (Busquets et al., 2004) when fried with just oil or, in contrast, prepared following elaborated recipes. Identifying cooking practices that can minimize the exposure to HCAs, and new food safety risks, is important for defining healthy cooking guidelines.

Materials and methods

Chemicals and materials

Solvents used in this study were of analytical or HPLC grade and obtained from Merck (Darmstadt, Germany). Twelve HCAs (Figure 1), 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (4,7,8-TriMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-9H-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 9H-pyrido[3,4-*b*]indole (norharman) and 1-methyl-9H-pyrido[3,4-*b*]indole (harman) were investigated. The HCAs 4,7,8-TriMeIQx was used as internal standard in all analyzed samples. The co-mutagens norharman and harman were obtained from Sigma (St. Louis, MO, USA) with purity 98%. These rest of HCAs were purchased from Toronto Research Chemicals (Toronto, Canada) and were >99% pure. Stock standard solutions of each amine (100 µg/g) were prepared in methanol and diluted further forth the preparation of calibration standards and spiking solutions. The HCAs ranged between 0.01 µg/g, and l µg/g in the external calibration curve. Every standard and sample contained 4,7,8-TriMeIQx, 0.5 µg/g, as internal standard. Standard solutions were stored at 4 °C.

Nylon filters (Scharlab, Barcelona, Spain) of 0.22 µm pore size were used to filter the standard solutions and samples before their injection into the chromatographic system. Extraction cartridges Extrelut NT20 were supplied from Merck (Darmstad, Germany).

Octadecylsilane (C_{18}, 100 mg) and bond elut propylsulfonic acid (PRS, 500 mg) solid phase extraction cartridges, stopcocks and coupling pieces were supplied from Varian (Harbor City, USA). The refill material diatomaceous earth (Isolute HM-NTM) was purchased from International Sorbent Technology (Hengoed, United Kingdom).

Sample preparation

Fresh raw beef and food ingredients were obtained from a superstore in Barcelona. Prior to the food preparation, the visible fat was separated from the meat, which was then sliced in fillets of about 1cm in thickness. For the cooking procedure of the beef, frying was selected due to its frequent use in Spain (Busquets et al., 2004). The beef dishes were prepared following recipes from a popular cooking recipe book (Mendel 1997) and have been summarized in Table 1 and described in Supplemental material S1.

For cooking the samples, a teflon-coated frying pan with 270 mm × 270 mm dimensions and electric stove were used. The cooking temperature was monitored in the center of the frying pan using K type insulated-wire probes and the software Normadics TC6, all from Cole-Parmer (Vernon Hills, USA). The cooking started when the temperature in the center of the frying pan attained and was stabilized at 210 °C for a period of 12 min. Then the cooking began and it was kept between 210 and 225 °C during the cooking processes. The beef was processed for 4 min/side, and it was frequently rotated to ensure the mixture with the ingredients used. The weight loss was established as the weight difference of the beef between before and after cooking. The whole cooked beef samples (not only the crust), cooked in 3 batches, were then mixed and ground, bottled, labelled and stored at -18 °C until analysis.

HCAs extraction

149 The frozen cooked composite samples were allowed to attain room temperature. NaOH (1
150 M, 30-50 g) was added to the beef samples (15 g) followed by homogenization using a
151 blender (Ultra-Turrax® T25). Subsequently an amount corresponding to 1 g of cooked beef
152 sample were separated in 50 ml polypropylene centrifuge tubes (3 independent samples
153 were left unspiked and 3 were spiked). After 24h of spiking the samples, each sample was
154 carefully mixed with 13 g of diatomaceous earth and purified following validated
155 procedures (Toribio et al. 2007). Briefly, the diatomaceous earth impregnated with the
156 homogenized meat sample in NaOH was packed in empty Isolute columns (Hengoed, Mid-
157 Glamorgan, UK). The uncharged HCAs were eluted with 75 ml of ethyl acetate and
158 retained in a preconditioned Bond Elute PRS ion exchange cartridge (Varian, Harbor City,
159 USA). The cartridge was then washed with 7ml of ethyl acetate to remove fat and other
160 hydrophobic compounds, followed by methanol:water (15ml, 6:4), and a final wash with
161 water (2ml). The washing steps were carried out at 3ml/min and the eluate was discarded.
162 The HCAs were eluted from the PRS column by ammonium acetate solution (20ml, 0.5M
163 at pH 8.5) and the eluates were collected in a a C_{18} column (200mg) previously conditioned
164 with methanol (2 ml) and water (2 ml). Finally the HCAs were eluted with methanol:
165 ammonia (0.8 ml, 9:1); evaporated to dryness under a gentle stream of nitrogen; and
166 reconstituted with a solution containing internal standard (TriMeIQx) (0.3 ml, methanol:
167 30mM formic acid/ ammonium formate buffer at pH 3.7, 1:1).
168 In parallel with the samples of this study, laboratory reference materials were analysed for
169 the accuracy of the results. The laboratory reference materials were prepared in our group
170 and were based on freeze dried meat extract (Bovril) (Bermudo et al., 2004), and freeze-
171 dried chicken (Khan et al., 2009a). The former reference material had been part of an

interlaboratory study including main European teams working on the analysis of HCAs in meat (Santos et al., 2004). All reference materials and kept at -80°C

The concentrations of HCAs present in the cooked beef and reference materials were quantified by spiking the samples at 3 concentration levels (50%, 100% and 200%, where 100% implies an increase of the signal obtained for every HCA of 100%).

HCAs determination

The chromatographic separation of HCAs was carried using a quaternary pump (model 1100 series from Agilent Technologies, Waldbronn, Germany), and a triple quadrupole mass spectrometer PE Sciex API3000 (SCIEX, Concord, Canada) with electrospray ionization source. The separation was carried out with a Symmetry® C_8 column with dimensions 150 × 2.1 mm and 5 μm particle size (Waters Corporation, Milford, USA). The separation of HCAs was achieved with a binary mobile phase of acetonitrile (solvent A) and formic acid-ammonium formate buffer (solvent B, 30 mM, pH 3.7). The elution program was: 5% A (0–1 min); 5–30% A (1–15 min); 30–60% A (15–18 min); 60% A (18–30 min) in B. The flow rate and column equilibration time was 0.3 mL/min and 10 min, respectively. The injection volume was 5 μL. The MS/MS system was applied in positive ionization mode and the detection was carried out in multiple reaction monitoring (MRM) mode. The working source parameters were: turbo ion-spray gas temperature, 450 °C; electrospray voltage, 2.5 kV; declustering potential, 30 V; curtain gas, 14 a.u.; nebulizer gas, 11 a.u.; turbo ion-spray gas flow rate, 7000 a.u. The protonated molecular ions $[M+H]^+$ were chosen as precursor ions. Two multiple reaction monitoring (MRM) transitions were monitored for every HCA. The most abundant product ions were used for quantification, and the second most abundant product ions were used to confirm the identity

of the detected HCAs. The MRM transitions are given in Table 2. The acquisition software was Analyst 1.4.2 (from SCIEX)

Results and Discussion

Cooking meat with differentiated approaches allow measuring the effect of elaborate cooking recipes on HCAs. In this research we have compared the effect on HCAs of pan frying beef using olive oil (control sample); with pan frying beef using a range of food ingredients following traditional Spanish recipes as described in a cookbook (Mendel 1997). The HCA levels in items cooked following elaborate recipes has been scarcely reported in research papers which are more focused on the identification of mechanisms of HCAs' formation which inherently requires moving away from complex cooking procedures. The weight loss of the cooked beef samples, reported in Table 2, shows that there was no major dissimilarity between the control beef (51%) and the beef cooked following more elaborate recipes (43-55%). The average weight loss of 48%, and the appearance of the dishes (photos shown in Figure 2), indicate that the fried beef dishes were not overcooked. High weight loss values entail greater transport of HCAs precursors to the meat surface during the cooking process and potentially greater formation of the foodborne mutagens (Persson and Sjöholm 2006). In terms of heat transfer, the temperature of the pan was kept within the same narrow range of temperatures in all the dishes. However, the presence of ingredient in dishes 2-8 led to greater amount of liquid in the pan, which could have reduced the temperature of localized parts of the beef when in contact with water.

217 The concentration of HCAs in beef samples cooked with different procedures
218 (described in Table 1) are given in Table 3. One of the most relevant differences observed
219 between the control sample and the rest, is the trend of greater concentration of the ß-
220 carbolines (harman and norharman) in the non-control dishes. Specifically, norharman
221 formed from similar level (dish 5) (P 0.05) to up to 2.6 times greater concentration (dish 8)
222 than in the control sample; and harman formed at greater levels in the most elaborated
223 dishes (up to 3.4 times in dish 8). The concentrations of both harman and norharman in
224 dish 8 were significantly greater (P 0.05) than those in the control sample (dish 1) (see
225 Supplemental information S2 for the overview of the concentration of HCAs in cooked
226 food with confidence intervals). Precisely the recipe applied in dish 8 was, among the
227 recipes tested, the one with the richest variety of ingredients, and some of these could be
228 responsible for the increase of harman. A precursor of norharman and harman is
229 tryptophan, which could have been released from the vegetables used (Rönner et al. 2000).
230 However, previous work from our group reported an increase in harman when using wine
231 marinades that did not contain that amino acid (Busquets et al. 2006). The presence of
232 precursors structurally close to β-carbolines, tetrahydro- β -carbolines (mainly 2,3,4-
233 tetrahydro- β -carboline-3-carboxylic acid), in raw meat and fish was found to be linked to
234 the content of β-carbolines in the cooked items (Herraiz, 2000), and the degree of meat
235 doneness correlated with the concentration of harman (Louis et al., 2007). Fe^{2+} and Cu^{2+},
236 potentially present at higher concentrations in the cooking mix than in absence of
237 ingredients, have been reported to enhance the formation of norhaman (Pfau et al. 2004).
238 The occurrence of harman and norharman is not exclusive from cooked meat and fish, they
239 are also present in a variety of processed food items with greatest levels detected so far in

240 brewed coffee, sauces; and toasted bread besides cooked meat and fish (Herraiz, 2002,
241 Herraiz 2004). Norharman was the most abundant β-carboline forming when roasting
242 coffee beans (Herraiz, 2002). The concentration of norharman was also greater than harman
243 in cooked fish (Khan et al., 2013). In contrast, there was not a clear prevalence of any β-
244 carboline in cooked meats (Busquets, 2012).

245 PhIP is known to be the main contributor to our daily intake of mutagenic HCAs,
246 and of its concentration in cooked meat samples usually varies from 1 to 10 ng/g (Oz and
247 Yuzer 2017; Khan et al. 2017). However, unlike other mutagenic HCAs, peak
248 concentrations of PhIP have been reported, mainly in poultry meat products 27 ng/g (Khan
249 et al. 2009a), 47 ng/g (Busquets et al., 2004), 70 ng/g (Sinha et al. 1995), and recently, high
250 concentration of PhIP were found in cooked swordfish (121 ng/g) (Khan et al. 2013). In
251 contrast, the lowest levels of PhIP were found in cooked sausages, offal, kebabs and
252 hamburgers (Khan et al. 2017; Iwasaki et al. 2010; Khan et al. 2009b; Borgen and Skog
253 2004). Murkovic et al. demonstrated that PhIP could originate from the condensation of
254 phenylacetaldehyde (degradation product of phenylalanine) with creatine (Murkovic et al.
255 1999). In the present study, a general significant reduction of PhIP levels was observed for
256 the elaborate dishes (P 0.05).This is overall a positive outcome and indicates that the
257 formation of this amine can be inhibited with common cooking ingredients. Hence, the
258 levels reported in papers using simple cooking methods which do not include a variety of
259 ingredients may be indicative of top concentration values at which that mutagen can be
260 found in the study conditions of temperature and cooking time, and this needs to be
261 considered when using these values in epidemiology studies.

Besides PhIP, the other mutagenic HCAs produced under the effect of cooking recipes including a range of ingredients were generally at lower concentrations in agreement with the amounts found in previous works (Zeng et al. 2017; Jinap et al. 2018; Gibis and Weiss 2012), but the difference with the control sample (dish 1) was not significant in all cases (see Supplemental Information S2). The antioxidants from the ingredients used might have inhibited radical reactions leading to the formation of the quinoxalines (Murkovic et al. 1998), although these reaction mechanisms are complex and there is no yet clear understanding or capacity to predict the effect of ingredients on the yield of quinoxalines. Indeed, there is research reporting no correlation between the radical scavenging activity of the ingredients used in marinades and the formation of quinoxalines (Viegas et al. 2012), and also recipes that led to significant positive correlation between increasing antioxidant properties and enhancement of the formation of quinoxalines when using red wine marinades for short marinating time (Busquets et al. 2006).

The HCAs IQ, MeIQ, Trp-P-1, Trp-P-2, AαC and MeAαC have not been frequently identified in meat products, this could be because the precursors for these amines present in the meat may be limited, or react towards the formation of other products, and the high cooking temperatures required for the formation of the α- and γ-carbolines (Sinha et al. 1998; Jägerstad et al. 1998). These carbolines have been found generally at <1 ng/g in cooked meat and fish (Busquets 2012). The formation of HCAs could also be affected by rotating the meat samples repeatedly when cooking (Salmon et al. 2000). The LC-MS/MS chromatograms of HCAs detected in dish 5 are shown in Figure 3 as an example that illustrates the quality of the analysis. The recovery values were calculated for studied HCAs

and ranged from 30 to 60%. These recoveries were similar to those in previous studies (Toribio et al. 2007).

The control of the Maillard reaction is of high importance to achieve quality in the cooked food and minimize the formation of harmful compounds (Rannou et al 2016). The inhibitory character of some ingredients onto the formation particular quinoxalines and PhIP has been demonstrated by ingredients used in different cultures (Busquets et al. 2006; Viegas et al. 2012; Rannou et al 2016; Tengilimoglu-Metin et al. 2017). This work has shown that among different traditional Spanish cooking recipes, the ones with greater variety of ingredients (see dishes 3 and 8 in Table 2), presented the greatest reduction rates of mutagenic HCAs: 88 and 99%, respectively. Hence, using a broad range of food ingredients can be beneficial and hinder the formation of mutagenic HCAs. However, the promotion of the co-mutagens norharman and harman, which may have neurotoxicity in humans and play a role in Parkinsons' disease (Pfau et al. 2004), can be enhanced with recipes involving a range of ingredients, according to this research and previous work with other cooking procedures and meats (Busquets et al. 2006; Gibis and Weiss 2012; Tengilimoglu-Metin et al. 2017; Zeng et al. 2016). A recent review has explained the current knowledge on the toxicology of β-carbolines and related compounds (Herraiz, 2016).

The reduction of the formation of mutagenic HCAs caused by using recipes including a broad range of ingredients was between 47 and 96% in this study. Lower HCA contamination than levels reported in papers trying to investigate HCAs' formation (which usually requires using simpler cooking procedures) should be considered when trying to link the intake of cooked meat or fish/ HCAs and types of cancer. These results show that

a healthier diet, in terms of lower intake of mutagenic HCAs, can be achieved by including a range of ingredients of vegetable origin in the cooking process. Further research will help to gain greater understanding on the generation of the co-mutagens harman and norharman .

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Figure captions

Figure 1: Investigated heterocyclic amines (structures and acronyms).

Figure 2: Images from the beef dishes in the present study.

Figure 3: Liquid chromatography–tandem mass spectrometry chromatograms of HCAs detected in dish 5 (beef cooked with eggplant, bread crumbs, olive oil and salt), acquired in MRM mode.

Table 1. Details of the food processing methods used following traditional Spanish recipes (Mendel 1997). In all cases, fillet thickness was ~1 cm; the temperature of the pan was 210−225 °C. The cooking processes were carried out 3 times and the cooked beef (from each dish) was combined and blended. The average volume or weight of ingredients and cooked meat is provided.

	Ingredients	Raw beef meat (g)	Total cooking time (min)	Cooked meat (g)	Meat crust (g)	Weight loss (%)
Dish 1[a]	Olive oil (5 mL)	200	6	98	56	51
Dish 2	Green pepper (400 g), butter (40 g), olive oil (30 g), Flour (¼ broth cube, 3g), chopped parsley (10 g), salt (5 g).	315	20	179	57	43
Dish 3	Sweet potato (3 units, 450g), onion (2 units, 410g, tomato (1unit, 80 g), leek (1 unit, 110g), carrot (2 units, 120g), green pepper (1 unit, 100g), garlic (2 cloves, 10g), corn flour (1 spoonful, 8g), olive oil (30 g). Salt (6 g).	393	35	211	130	46
Dish 4	Black pepper (2 g), salt (4 g), olive oil (4 g).	225	14	101	63	55
Dish 5	Eggplant (1 unit, 200g), bread crumbs (10 g), olive oil (15 g), salt (5 g).	296	8	139	94	53
Dish 6	Onion (1 unit, 205g), garlic (2 cloves), chopped parsley (10 g), olive oil (5), salt (5 g).	292	20	148	96	49
Dish 7	Mushroom (150 g), garlic (1 clove, 5g), olive oil (60 g), chopped parsley (6 g), salt (4 g), black pepper (2 g).	199	6	108	44	46
Dish 8	Onion (1 unit, 205), green pepper (200 g), garlic (1 clove, 5g), salt (4 g), black pepper grain (10 unit), bay leaves (2 units), cloves (5 units, 2 g).	226	20	128	49	43

[a]Control sample, thermally processed without food ingredients

Table 2: Acquisition parameters in the analysis of HCAs with mass spectrometry in multiple reaction monitoring mode.

HCAs	Precursor ion $[M+H]^+$, *m/z*	Quantitation precursor→ product ion, *m/z*	Confirmation precursor→ product ion, *m/z*	Collision voltage, V
DMIP	163	163→148	163→147	37
MeIQ	213	213→198	–	38
IQ	199	199→184	199→157	39
MeIQx	214	214→199	214→173	38
4,8-DiMeIQx	228	228→213	228→187	40
4,7,8-TriMeIQx	242	242→227	242→201	38
Norharman	169	169→115	–	49
Harman	183	183→115	183→168	49
PhIP	225	225→210	225→183	43
AαC	184	184→167	184→140	38
MeAαC	198	198→181	198→154	35
Trp-P-1	212	212→195	212→168	36
Trp-P-2	198	198→181	198→154	35

[a]Dwell time:150 ms;
[b]Interchannel delay time:5 ms

Table 3: Levels of HCAs in fried beef dishes prepared with cooking processes including broad range of ingredients (dishes 2-8) and with a simpler process without ingredients. Cooking recipes are detailed in Table 1 and in Supplemental material S1.

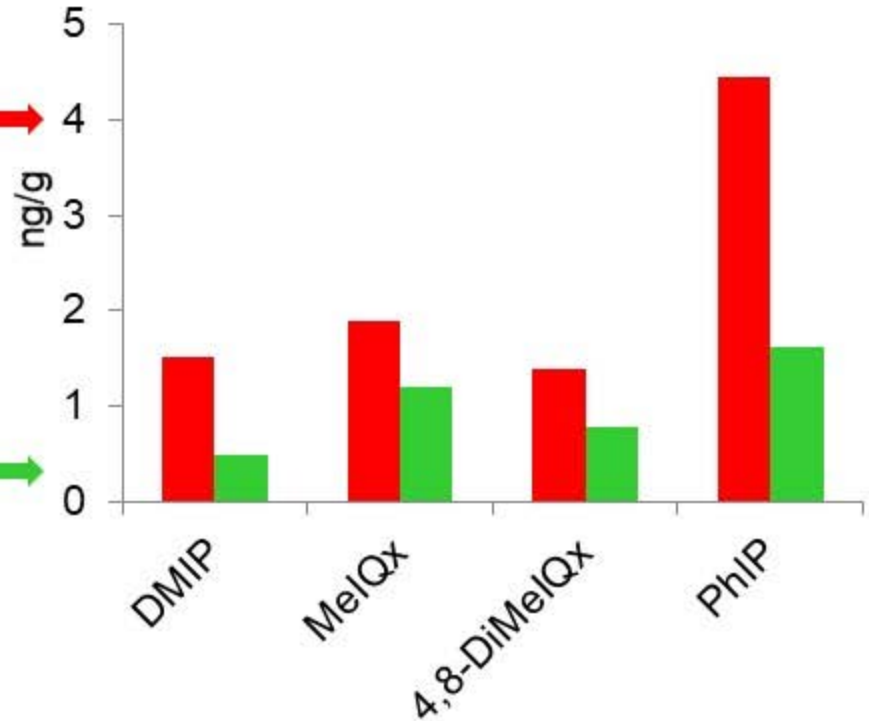
HCAs	Dish 1[a] ng/g ± SD[b], (R%)	Dish 2 ng/g ± SD, (R%)	Dish 3 ng/g ± SD, (R%)	Dish 4 ng/g ± SD, (R%)	Dish 5 ng/g ± SD, (R%)	Dish 6 ng/g ± SD, (R%)	Dish 7 ng/g ± SD, (R%)	Dish 8 ng/g ± SD, (R%)
DMIP	1.51 ± 0.25, (47)	0.19 ± 0.02, (43)	0.08 ± 0.02, (40)	0.28 ± 0.05, (40)	0.79 ± 0.09, (45)	0.09 ± 0.02, (41)	0.48 ± 0.03, (42)	0.06 ± 0.02, (45)
MeIQ	nq[c], (35)	–, (30)	–, (32)	–, (31)	–, (35)	–, (32)	–, (30)	–, (33)
IQ	nq, (38)	–, (32)	–, (33)	–, (30)	–, (36)	–, (33)	–, (33)	–, (35)
MeIQx	1.89 ± 0.87, (46)	0.45 ± 0.14, (42)	0.09 ± 0.10, (38)	0.89 ± 0.42, (41)	2.22 ± 0.55, (43)	0.96 ± 0.07, (40)	1.21 ± 0.45, (39)	0.14 ± 0.07, (43)
4,8-DiMeIQx	1.40 ± 0.56, (60)	0.25 ± 0.12, (55)	0.52 ± 0.38, (53)	1.22 ± 0.26, (53)	1.10 ± 0.18, (55)	1.04 ± 0.32, (54)	0.79 ± 0.42, (54)	0.01 ± 0.01, (57)
Norharman	5.87 ± 0.84, (55)	12.32 ± 1.54, (49)	9.37 ± 1.68, (46)	10.22 ± 1.86, (47)	5.65 ± 0.91, (49)	14.53 ± 1.98, (48)	6.20 ± 1.20, (51)	15.22 ± 2.20, (53)
Harman	2.31 ± 0.46, (63)	5.31 ± 0.68, (60)	3.66 ± 0.65, (54)	3.89 ± 0.82, (61)	3.68 ± 0.65, (56)	7.62 ± 1.10, (55)	3.34 ± 0.35, (53)	7.77 ± 1.14, (59)
PhIP	4.45 ± 0.43, (58)	0.87 ± 0.33, (54)	0.42 ± 0.16, (48)	1.09 ± 0.52, (52)	0.77 ± 0.22, (54)	0.65 ± 0.52, (47)	1.62 ± 0.40, (49)	0.18 ± 0.05, (55)
AαC	–[d], (61)	–, (55)	–, (50)	–, (56)	–, (58)	–, (52)	–, (53)	–, (56)
MeAαC	–, (64)	–, (58)	–, (53)	–, (59)	–, (60)	–, (56)	–, (55)	–, (58)
Trp-P-1	nq, (42)	–, (36)	–, (34)	–, (34)	–, (40)	–, (38)	–, (38)	–, (38)
Trp-P-2	nq, (47)	–, (43)	–, (39)	–, (41)	–, (44)	–, (42)	–, (40)	–, (42)

[a]Control sample, thermally processed without food ingredients;
[b]standard deviation from a standard addition quantification consisting of 3 unspiked and 3 spiked samples.
[c]nq: below limit of quantification (<signal-to-noise ratio of 10): <0.01 ng/g
[d]–: not detected (limit of detection 0.003 ng/g)
R=Recovery

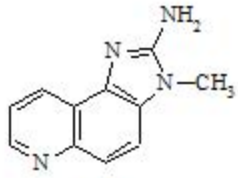
210-225 °C, 4 min/side

Cooked with oil only

Cooked with a range of ingredients



Quinolines



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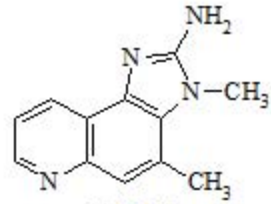
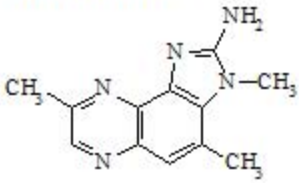


MeIQ

Quinoxalines



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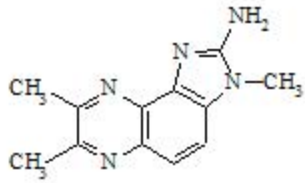

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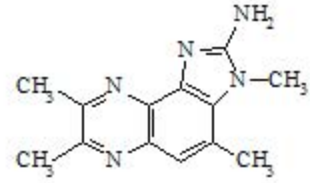
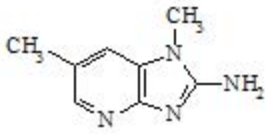


4,7,8-TriMeIQx (IS)

Pyridines



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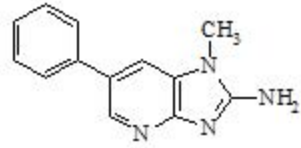
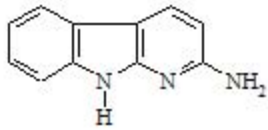


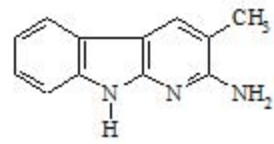
PhIP

Pyridoindoles

α-carbolines

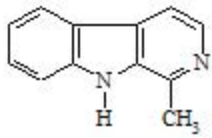


AαC

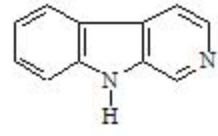


MeAαC

β-carbolines

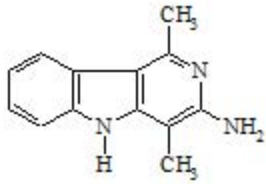


Harman

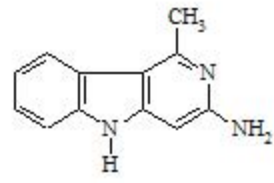


Norharman

γ-carbolines



Trp-P-1



Trp-P-2

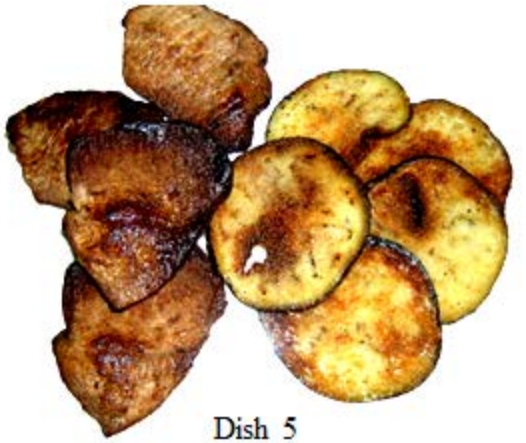
Raw beef

Dish 1

Dish 2

Dish 3

Dish 4

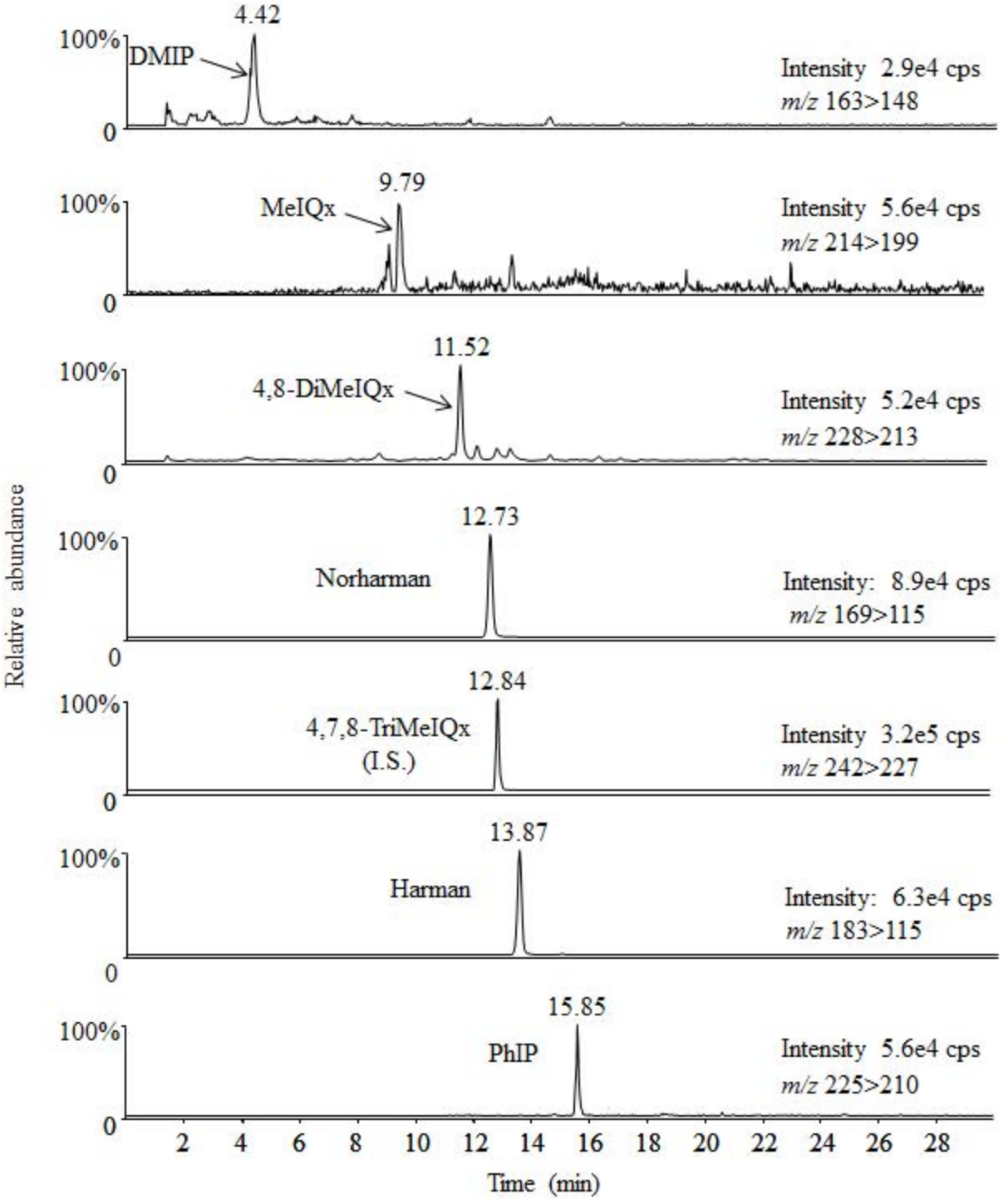


Dish 5

Dish 6

Dish 7

Dish 8


4.42
100%
DMIP
Intensity 2.9e4 cps
m/z 163>148
0
9.79
100%
MeIQx
Intensity 5.6e4 cps
m/z 214>199
0
11.52
100%
4,8-DiMeIQx
Intensity 5.2e4 cps
m/z 228>213
0
12.73
100%
Norharman
Intensity: 8.9e4 cps
m/z 169>115
0
12.84
100%
4,7,8-TriMeIQx
(I.S.)
Intensity 3.2e5 cps
m/z 242>227
0
13.87
100%
Harman
Intensity: 6.3e4 cps
m/z 183>115
0
15.85
100%
PhIP
Intensity 5.6e4 cps
m/z 225>210
0
Relative abundance
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (min)

Food Additives and Contaminants

Supplemental material

Cooking with elaborate recipes can reduce the formation of mutagenic heterocyclic amines and promote comutagenic amines

Mohammad Rizwan Khan [†,‡], Rosa Busquets [§,‡] *, Mu Naushad [†] Lluís Puignou [‡]

[†] Department of Chemistry, College of Science, Bld. 5, King Saud University, Riyadh 11451, Saudi Arabia.

1. [§] School of Life Sciences, Pharmacy and Chemistry, Kingston University London, Penrhyn road, KT1 2EE, Kingston upon Thames, United Kingdom.

[‡] Department of Analytical Chemistry, University of Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain.

* Author to whom correspondence should be addressed:

Tel: + 44 (0) 2084177792; E-mail: r.busquets@kingston.ac.uk (R. Busquets)

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S1 Description of the cooking procedures followed to prepare the samples. In every process, oil was added to the pan once the temperature of the pan had remained stable at 210 °C for 12 min.

	Brief description of the cooking procedure
Dish 1	Olive oil was added to the pan. The meat was placed in the oil when it started boiling. The meat was flipped after 3 min.
Dish 2	The meat fillets were brushed with olive oil and were cooked in the oven (12 min, 90 °C). Following, the fillets were coated with flour and were pan-fried (4 min/side, 8 min total) with melted butter, oil and ¼ broth cube. After the cooking time, the meat was placed in a dish together with green peppers (cut into broad strips), chopped parsley and salt.
Dish 3	The meat was salted and stir-fried in boiling olive oil together with chopped carrots, leek, green pepper, onion, garlic, tomato for 35 minutes. After this time, the meat together with ¾ of the vegetables were separated and placed on a dish. The remaining portion of vegetables and juices, including meat drippings, were cooked for 5 additional minutes; mixed with flour, blended and filtered using a strainer. The resulting sauce was poured onto the cooked meat. Separately, sweet potatoes were peeled, sliced and fried in boiling oil for 8 minutes and added to the dish.
Dish 4	The meat was sprinkled with black pepper and salt. Olive oil (a fine layer) was added to the pan. The meat was added to the oil once the oil started boiling. The meat was stirred continuously and flipped every 4 min.
Dish 5	The meat was salted and added to a pan with boiling olive oil. At the same time, sliced eggplant coated with bread crumbs was added to the same pan and fried together with the meat. The mixture was continuously stirred, and the meat was flipped every 4 minutes.
Dish 6	Garlic was crushed in a mortar together with chopped parsley. Following, olive oil and salt was added to the mortar and the mixture was stirred for 3 minutes. The fillets were introduced in the mortar and stirred in that mixture of garlic, parsley and oil. The fillets impregnated with garlic, parsley and oil were added to a pan with boiling oil. The meat was stirred continuously and flipped every 4 minutes. The final dished included fried onion rings which had been cooked separately from the meat.

Dish 7 The fillets were salted. Following, they were fried one at a time (2 min/side) and removed from the pan. Sliced mushrooms and chopped garlic were stir-fried together (5 minutes) in the same pan which contained the meat drippings and oil from frying the meat. Finally, the pre-fried fillets were added to the pan with the mixture of mushrooms and garlic and were stir-fried for 2 minutes.

Dish 8 The garlic was heated in a pan (with its peel) for 5 minutes. Onion, green pepper and the pan-fried garlic were chopped and added to the raw meat. The mixture was heated in a pan for 5 minutes. Water was then added to cover the meat. Pepper grains, clove and bay leaves were added to the mixture, which was covered with a lid and cooked for additional 15 minutes.

S2 Concentrations of HCAs, expressed in ng HCA/g cooked beef, quantified by standard addition. The error bar corresponds to the confidence interval (uncertainty in quantity of HCAs calculated from the regression line multiplied by the Student t for n-2 degrees of freedom) of the quantified value. The Student t value was 2.776 for 4 degrees of freedom (n-2) and P 0.05.