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Occurrence of Heterocyclic Amines in Cooked Meat Products

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1 **ABSTRACT**

2
3 Heterocyclic amines (HCAs), potent mutagens and a risk factor for human cancers, are
4 produced in meats cooked at high temperature. The aim of this study was to determine the HCA
5 content in cooked meat products (beef, chicken, pork, fish) prepared by various cooking methods
6 (pan frying, oven broiling, and oven baking at 170 to 230 °C) that are preferred by U.S. meat
7 consumers. The primary HCAs in these samples were PhIP (2-amino-1-methyl-6-phenylimidazo
8 [4,5-*b*]pyridine) (1.49-10.89 ng/g), MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline)
9 (not detected-4.0 ng/g), and DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-*f*]quinoxaline) (not
10 detected-3.57 ng/g). Type and content of HCAs in cooked meat samples were highly dependent
11 on cooking conditions. The total HCA content in well-done meat was 3.5 times higher than that
12 of medium-rare meat. Fried pork (13.91 ng/g) had higher levels of total HCAs than fried beef
13 (8.92 ng/g) and fried chicken (7.00 ng/g). Among the samples, fried bacon contained the highest
14 total HCA content (17.59 ng/g).

15 **Keywords:** heterocyclic amines, cooking, beef, pork, chicken, fish
16
17

18 **1. Introduction**

19 Heterocyclic amines (HCAs) are mutagenic and carcinogenic compounds that are present
20 at parts per billion levels in cooked muscle foods, mainly meat and fish, via the Maillard reaction
21 with create(ni)ne, amino acids, and sugars as the precursors (Janoszka, Blaszczyk, Damasiewicz-
22 Bodzek, & Sajewicz, 2009; Pais, Salmon, Knize, & Felton 1999; Sugimura 2002). More than 25
23 HCAs have been isolated from different cooked muscle foods; however, the most common
24 HCAs found in foods are the thermic HCAs, which include 2-amino-3-methyl-imidazo [4,5-
25 f]quinoline (IQ), 2-amino-3-methylimidazo [4,5-f]quinoxaline (IQx), 2-amino-3,4-
26 dimethylimidazo [4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline
27 (MeIQx), DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-f]quinoxaline), and 2-amino-1-
28 methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) (Knize, Dolbeare, Carroll, Moore, & Felton,
29 1994). These HCAs are listed in the U.S. Department of Health and Human Services's 11th
30 Report of Carcinogens (2005) as compounds *reasonably anticipated to be a human carcinogen*.
31 The International Agency for Research on Cancer (1993) categorized MeIQ, MeIQx, and PhIP as
32 *reasonably anticipated to be a human carcinogen* and IQ as a *probable human carcinogen*. The
33 epidemiological studies over the past 10 years have shown that high intake of well-done meat
34 and high exposure to meat carcinogens, particularly HCAs, may increase the risk of stomach,
35 colon, and breast cancers in humans (Kampman, Slattery, Bigler, Leppert, & Samowitz, 1999).

36 The concentration and type of HCAs formed in thermally treated meat and fish depend on
37 many factors including cooking method, cooking time and temperature, the concentration of
38 precursors, and presence of water and fat in the raw product (Janoszka et al., 2009). The content
39 of HCAs formed increases with increasing temperature and time (Knize et al., 1994). High

40 cooking loss is related to the formation of large contents of HCAs (Knize et al., 1994; Skog,
41 Steineck, Augustsson, & Jägerstad, 1995), and the content of cooking loss during cooking
42 depends on several factors including the muscle tension and direction of muscle fibers (Pais et
43 al., 1999). Many cooking methods, including frying, roasting, smoking, broiling, and baking
44 have been reported to induce HCA formation, and the type HCAs formed can be different for
45 various cooking methods (Chen & Chiu, 1998). For example, IQ, MeIQx, PhIP were detected in
46 broiled beef, whereas MeIQx and DiMeIQx were detected in fried ground beef (Starvic, 1994).

47 The studies on HCA levels in cooked meat products have yielded inconsistent results, and
48 there are gaps in the available HCA data. It is difficult to directly compare results between
49 studies because of the differences in food items, cooking procedures (cooking methods, cooking
50 levels, fat or oil usage, frequency of turning), and food preparation. In some previous studies,
51 samples were cooked at high temperature or for a long time; these cooking conditions exceed
52 those needed to produce acceptable cooked meat products (Murkovic, Friedrich, & Pfannhauser,
53 1997; Pais et al., 1999). Reports from some previous studies did not include the information on
54 internal temperature of the cooked samples (Janoszka et al., 2009; Jo, Sim, Lee, Ryeom, &
55 Myung, 2008; Murkovic et al., 1997; Oz, Kaban, & Kaya, 2007). Internal temperature is usually
56 used to evaluate the safety of cooked meat products. Collecting this type of data would allow
57 researchers to better monitor HCA levels in meat products cooked under normal household
58 conditions and develop more accurate estimates of human HCA exposure. The main objective of
59 the study was to determine HCA contents of the major categories of cooked meat products
60 prepared with various cooking methods that are preferred by U.S. meat consumers. These data
61 can be combined with food consumption survey data to estimate exposure to HCAs due to meat

62 consumption.

63

64 **2. Materials and Methods**

65 2.1. Chemicals

66 The HCA standards IQ (2-amino-3-methyl-imidazo [4,5-*f*]quinoline), IQx (2-amino-3-
67 methyl-imidazo [4,5-*f*]quinoxaline), MeIQ (2-amino-3,4-dimethyl-imidazo [4,5-*f*]quinoline),
68 MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-
69 trimethyl-imidazo [4,5-*f*]quinoxaline, TriMeIQx (2-amino-3,4,7,8-tetramethyl-imidazo [4,5-
70 *f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine were obtained
71 from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate and triethylamine
72 were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Phosphoric acid was obtained
73 from Sigma Chemicals (St. Louis, MO, USA). Deionized water was processed by a
74 Sybron/Branstead PCS unit (Barnstead/Thermolyne, Dubuque, IA, USA). The solid-phase
75 extraction Extrelut NT 20 columns and diatomaceous earth refill material were purchased from
76 VWR International (Bristol, CT, USA). Bond Elut propyl-sulfonic acid (PRS) cartridges, C-18
77 cartridges, and coupling adaptors were purchased from Varian Sample Preparation (Harbor City,
78 CA, USA). Trichloroacetic acid, diacetyl, 1-naphthol, and NaOH were obtained from Sigma
79 Aldrich (St. Louis, MO, USA). Solvents and chemicals such as acetonitrile (HPLC grade),
80 methanol (HPLC grade), and sodium hydroxide (ACS-grade) were purchased from Fisher
81 Scientific (Fairlawn, NJ, USA).

82

83

84 2.2. Fresh meat samples

85 The following fresh meat samples were purchased from local grocery stores: consisting
86 of beef (top loin, round tip, and ground beef), pork (top loin, ground pork, and bacon), chicken
87 (breast without skin, breast with skin, thigh without skin, and thigh with skin), and fish (catfish,
88 salmon, and tilapia).

89

90 2.3. Chemical analyses

91 The pH of uncooked samples was measured according to the method of Jang et al. (2008)
92 with slight modifications. Five grams of fine ground sample was added to 45 mL of distilled
93 water and blended for 30 s at medium speed in a Waring blender (Waring Laboratory,
94 Torrington, CT, USA). The pH of each sample was measured with an Accumet AP115 portable
95 pH meter (Fisher, Pittsburgh, PA, USA).

96 Fat and moisture for each sample were determined by rapid microwave drying and
97 nuclear magnetic resonance using the CEM Smart Trac system (CEM Corporation, Matthews,
98 NC, USA). Crude protein was determined with a LECO FP-2000 protein analyzer (Leco Corp, St
99 Joseph, MI, USA).

100 Creatine content was determined according to the method described by Polak, Došler,
101 Žlender, and Gašperlin (2009) with slight modifications. A 0.25-g finely ground sample was
102 homogenized for 5 min at 9500 rpm (IKA, Ultra-Turrax T18, Wilmington, NC, USA) in 100 mL
103 trichoroacetic acid (30 g/L in distilled water), and then the samples were filtered through
104 Whatman #4 filter paper. Twenty milliliters of the filtrate was defatted with 10 mL diethylether,
105 and then samples were shaken vigorously and allowed to stand for 10 min to separate the phases.

106 After the phases were separated, 4 mL of defatted extract (bottom layer) was mixed with 2 mL of
107 diacetyl (0.2 g/L in distilled water) and 2 mL of 1-naphthol (25 g/L in 20 g/L of sodium hydroxide
108 solution). The mixture heated for 5 min at 40 °C. Each sample's absorbance was measured at 520
109 nm against a reagent blank. The creatine content was expressed as milligrams per gram of meat
110 sample. The chemical analyses of raw meat samples are summarized in Table 1.

112 2.5. Cooking procedure and cooking loss

113 Fresh meat products were removed from refrigerator and allowed to approach room
114 temperature before they were cooked (Infrared thermometer model ST20XE, Raytek, Santa
115 Cruz, CA, USA). Spear-point thermocouple temperature probes were inserted horizontally to the
116 midpoint of samples, and temperature was monitored with a data logger (USB-TC model,
117 Measurement Computing, Norton, MA, USA). Each meat sample was cooked as described in
118 Table 2. All meats were cooked one sample at a time, except for fried bacon, which was cooked
119 three slices at a time. No salt, spice, food additive, or oil was used in the cooking procedures.
120 Cooked sample were allowed to cool at room temperature for approximately 30 min, and then
121 cooking loss was determined using the following equation:

$$122 \text{ \% cooking loss} = [(\text{before cook weight} - \text{after cook weight}) / \text{before cook weight}] \times 100$$

123
124 The cooking information for each type of meat consists of the data for uncooked meat
125 (weight of raw meat, thickness of raw meat), and cooking data (desired cooking internal
126 temperature, cooking temperature, cooking time, and cooking loss); this information is
127 summarized in Table 3. After samples were cooled at room temperature, they were refrigerated

128 overnight. For the samples of chicken with skin, the skin was removed from the muscle.

129 Approximately 2 mm of the surface was removed from chilled meats with a commercial-grade
130 meat slicer (Cebela's commercial grade slicer, 1/3 hp, Sidney, NE, USA). The meat surface was
131 ground and homogenized with a food processor (KitchenAid, model KFP 750), and refrigerated
132 at 4 °C, and extraction of HCAs in meat samples was performed on the next day.

133 134 2.6. Extraction and analysis of HCAs

135 The HCAs were extracted from meat samples and purified using the method described by
136 Gross and Grüter (1992) except that ethyl acetate was used as the extraction solvent
137 (Puangsombat & Smith, 2010; Santos et al., 2004; Smith, Ameri, & Gadgil, 2008). Each sample
138 (3 g) was homogenized with 12 mL of 1 M NaOH in a commercial Waring blender (Fisher,
139 Pittsburgh, PA, USA). The homogenate was then mixed with 24 g of Extrelut refill material
140 (Merck, Darmstadt, Germany) and poured into an empty Extrelut 20 column. For determination
141 of recovery, selected homogenate samples were spiked with 50 ng of each of the HCA standards.
142 The HCAs were eluted from the Extrelut columns with 60 mL ethyl acetate into a PRS cartridge
143 conditioned with 7 mL of ethyl acetate. The PRS cartridge was then rinsed with 6 mL of 0.1 M
144 HCl, 15 mL of methanol/0.1 M HCl (45:55 v/v), and 2 mL of distilled water to wash out the
145 nonpolar HCAs and other impurities. The HCAs were eluted from the PRS cartridge with 20 mL
146 of 0.5 M ammonium acetate pH 8.5 into 100-mg C-18 cartridges preconditioned with 5 mL of
147 methanol followed by 5 mL of distilled water. The HCAs were then eluted from the C-18
148 cartridge with 1 mL of methanol/ammonium hydroxide (9:1, v/v) into the vial. The HCA extract
149 was concentrated until dry under a stream of nitrogen and dissolved in 25 µL of methanol before

150 it was injected into the HPLC. The HCAs were analyzed on an HP1090A Series II HPLC
151 (Agilent Technologies, Santa Clara, CA, USA) coupled with a photodiode array UV-visible
152 detector (HP 1040) and an HP 1046A programmable fluorescence detector. The HCA separation
153 was performed on a reversed-phase TSK gel ODS-80 TM column (25 cm × 4.6 mm, 5 μm, 80 Å,
154 Tosohass, Montgomeryville, PA, USA) with a mobile phase of 0.01 M triethylamine pH 3.6 (A)
155 and acetonitrile (B). The HCA separation was achieved using a linear gradient that started with
156 95% A and 5% B and changed to 75% A and 25% B in 30 min at a flow rate of 1 mL/min and a
157 column temperature of 40 °C. After 30 min, the mobile phase returned to its original ratio (95%
158 A, 5 % B) for 10 min to allow the column to equilibrate before the next injection. The UV
159 detector was set at 252 nm for IQ, IQx, MeIQ, MeIQx, and DiMeIQx, and the fluorescence
160 detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm
161 for PhIP. Data were analyzed with an HP 9000 series 300 ChemStation. The identities of HCA
162 peaks were confirmed by comparing the retention times and the UV absorbance spectrum of each
163 peak with library spectra acquired from standard solutions.

164 2.6. Quantitation, recovery, and spectral matching

166 The HCA concentrations were quantitated by the internal standard method to compensate
167 for variations in injection volume and also for small changes in detector sensitivity that might
168 occur (Lindsay, 1992). A known content of TriMeIQx (used as internal standard) was added to
169 samples before they were injected into the HPLC. The limit of detection for the HCAs was 0.5
170 ng/mL for IQ, IQx, MeIQ, MeIQx, and PhIP. The HCA identities were verified in the cooked
171 meat extracts by online UV spectral matching to a spectral library made from pure standards.

172 Match factors typically were observed at 95% or greater (Tsen, Ameri, & Smith, 2006). Average
173 recoveries for the HCAs were 72% for IQ_x, 61% for IQ, 63% for MeIQ, 68% for MeIQ_x, 60%
174 for DiMeIQ_x, and 65% for PhIP. The HCA identities were verified in the cooked meat extracts
175 by online UV spectral matching to a spectral library made from pure standards. Match factors
176 typically were observed at 95% or greater (Puangsombat & Smith, 2010). Average recoveries for
177 the HCAs were 72% for IQ_x, 61% for IQ, 63% for MeIQ, 68% for MeIQ_x, 60% for DiMeIQ_x,
178 and 65% for PhIP. The recoveries of MeIQ_x and PhIP are in agreement with previous reports
179 from this laboratory (Puangsombat & Smith, 2010; Smith et al., 2008; Tsen et al., 2006) and
180 from Cheng et al. (2007).

181 182 2.7. Statistical analyses

183 The experimental design was a randomized complete block with repeated measurements,
184 and each experiment was replicated four times. Duplicate measurements taken on the same
185 experimental unit were averaged for statistical analysis. All statistical significance tests were
186 analyzed using SAS version 9.1. Data were examined by analysis of variance (ANOVA)
187 followed by Tukey's multiple comparison test (Tukey, 1993), and means were considered
188 significant at $p < 0.05$.

189 190 **3. Results and discussion**

191 The choice of meat samples in our study was based on a previous internet-based survey
192 of U.S. consumers' preference for method of cooking and degree of doneness of meat and fish.
193 The survey was conducted by Exponent, Inc. developed to assess customer's HCA intake

194 (unpublished data). Meat samples selected for the present study included beef (fried beef and
195 broiled beef cooked to medium-rare and well-done, baked beef, and fried beef patty), pork (fried
196 pork, baked pork, fried pork patty, and fried bacon), chicken (fried-chicken breast and fried-
197 chicken thigh with skin and without skin), and fish (fried and baked catfish, salmon, and tilapia).

198 199 3.1. Chemical analyses

200 Table 2 summarizes the results of chemical analyses in the selected fresh meat products.
201 The pH of beef samples (5.47 to 5.89) was lower than that of pork samples (6.01 to 6.71) and
202 chicken samples (6.19 to 6.70); fish samples had the highest pH (6.94 to 7.91). The moisture
203 level of fresh meat products ranged between 69 to 82%, except in the high fat parts (bacon,
204 breast skin, and thigh skin), which contained low moisture levels (approximately 37%). The fat
205 levels of raw meat samples ranged from 1.07 to 53.67%; tilapia contained the lowest content of
206 fat and skin of chicken breast contained the highest contents of fat. The protein levels of raw
207 meat samples ranged from 9.04 to 23.37%; chicken thigh skin contained the lowest content of
208 protein, and skin of chicken breast contained the highest content of protein. Creatine in the
209 uncooked meat samples ranged from 1.02 to 2.95 mg/g. There was not much difference in
210 creatine level among these samples.

211 212 3.2. Identification and quantification of HCAs

213 Because consumption of undercooked meat and fish has been linked epidemiologically to
214 foodborne outbreaks, the U.S. Department of Agriculture Food Safety and Inspection Service
215 (USDA-FSIS, 1998) has established guidelines for both consumers and the food service industry

216 for safe handling and preparation of cooked meat and fish. USDA-FSIS recommends a minimum
217 instantaneous internal cooking temperature of 63 °C (145 °F) for beef steak and fish, 71 °C (160
218 °F) for pork and ground beef, and 74 °C (165 °F) for chicken. According to the survey by
219 Exponent Inc. (unpublished data), people mostly consume meat and fish that are cooked until
220 their internal temperatures reach the minimum temperature recommended by USDA-FSIS,
221 except for steak and bacon. Two doneness levels of steak, medium-rare and well-done, are most
222 often consumed. In our study, all samples except beef steak and bacon were cooked until their
223 internal temperature reached the temperatures recommended by USDA-FSIS. Fried and broiled
224 beef steak samples were cooked until their internal temperature reached 57 °C (135 °F) for
225 medium-rare and 71 °C (160 °F) for well-done. Fried bacon was cooked at 172 °C for 3 min on
226 each side (three slices at a time) as recommended to minimize carcinogenic nitrosamine level
227 (Ikins et al., 1986). We investigated the presence of five HCAs (IQ, IQx, MeIQ, MeIQx, and
228 PhIP) that can be found in cooked meat products and have been commonly studied and reported
229 in many papers. Level of HCAs in each sample was analyzed the outer layer (2 mm) of meat
230 samples to increase analysis sensitivity because HCAs are mainly present on the outer surface
231 (Busquets, Bordas, Toribio, Puignou, & Galceran, 2004). The content of HCAs on the surface
232 was then used to calculate the content of HCAs in the whole cooked meat samples (Busquets et
233 al., 2004). The values were corrected for incomplete recovery. To our knowledge, this is the first
234 report of HCA contents in food samples commonly consumed in the U.S. and prepared by
235 domestic cooking procedures to internal temperatures recommended by USDA to eliminate
236 foodborne illness.

237 HCAs were extracted and quantified by HPLC. The UV and FLD chromatograms of

238 HCA standards, sample, and spiked sample are shown in Figure 1. The quantitative analyses of
239 HCAs in cooked meat products are summarized in Tables 4 to 8. The total content of HCAs
240 ranged from 1.72 ng/g (medium-rare broiled-beef) to 17.59 ng/g (fried-bacon). In all meat
241 samples, PhIP was found at the highest level (1.49 to 10.89 ng/g), followed by MeIQx (not
242 detected to 4.00 ng/g), DiMeIQx (not detected to 3.57 ng/g), and IQx (not detected to 3.11 ng/g);
243 neither IQ nor MeIQ was found in any sample. The highest level of PhIP was found in fried
244 tilapia (10.89 ng/g), followed by fried pork (9.20 ng/g). IQx was not found except in fried bacon
245 (3.11 ng/g) and baked fish (0.38 to 0.85 ng/g).

246 We investigated the occurrence of HCAs for three types of fried meat (beef, pork,
247 chicken) and the results of the HCA quantitative determinations are summarized in Table 4. All
248 of these samples contained MeIQx, DiMeIQx, and PhIP. Although the target internal
249 temperatures of the meat samples fried at 204 °C were slightly different, total HCAs in fried pork
250 (13.91 ng/g, PhIP accounting for 9.20 ng/g) were significantly higher than those in fried beef
251 (8.92 ng/g, PhIP accounting for 6.60 ng/g) and fried chicken (7.06 ng/g, PhIP accounting for
252 6.06 ng/g). There were no significant differences in total HCAs between fried beef and fried pork
253 ($p > 0.05$). This is in agreement with results of Skog, Augustsson, Steineck, Stenberg, and
254 Jägerstad (1997) who reported higher contents of total HCAs in fried pork (21.3 ng/g) than fried
255 chicken breast (10.7 ng/g) when cooked at 225 °C. In contrast, our result is not in agreement
256 with data from Pais et al. (1999), who reported that total HCAs were higher for chicken (38.2
257 ng/g) than for pork (8.6 ng/g) and beef (2.83 ng/g) when cooked at 275 °C for 30 min. Also,
258 Iwasaki et al. (2010) reported a lower content of total HCAs in fried chicken (1.01 ng/g), fried
259 pork (0.5 ng/g), and fried beef (0.1 ng/g) when cooked to well-done (internal temperature 75 °C

260 for chicken, 88 °C for pork, and 78 °C for beef). The inconsistent results could be due to
261 different cooking methods, different weight/thickness of meat samples, and different ways of
262 preparing meat before cooking, as well as the efficiencies of heat transfer. The high level of
263 HCAs in the fried pork in present study is an important finding because of the three meats
264 studied, the consumption of pork is growing the fastest (1.6 % annually) (FAPRI 2010 U.S. and
265 world agricultural outlook, 2010).

266 Table 5 shows the HCA levels of cooked chicken samples. MeIQx, DiMeIQx, and PhIP
267 were detected in all chicken samples and the values were in agreement with the HCA found in
268 fried chicken reported by Solyakov and Skog (2002) and Liao, Wang, Xu, and Zhou (2010). All
269 chicken samples had more PhIP than MeIQx and DiMeIQx. For the chicken samples without
270 skin, total HCA levels in the chicken breasts (7.06 ng/g) were higher than those in the chicken
271 thighs (5.58 ng/g); this may be because the weight of raw chicken breast (250 to 280 g) was
272 higher than that of raw chicken thigh (140 to 180 g), therefore, a longer cooking time was needed
273 for chicken breast to reach the same internal temperature of 74 °C, leading to higher cooking loss
274 (Table 2) and increasing the level of HCAs. This result is in agreement with data by Pais et al.
275 (1999), who reported that chicken breast had more total HCAs (38.2 ng/g) than chicken thigh
276 (8.07 ng/g). For the chicken samples with skin (both breasts and thighs), the meat and skin were
277 analyzed separately. MeIQx, DiMeIQx, and PhIP levels in the skin were much higher than the
278 levels detected in the meat ($p < 0.05$). Total HCAs were 7.06 ng/g for skin and 2.89 ng/g for
279 meat in chicken breast and 4.87 ng/g for skin and 2.07 ng/g for meat in chicken thigh. The
280 cooking loss of chicken breast with skin (24.39%) was lower than that of chicken breast without
281 skin (27.88%); the cooking loss of chicken thigh with skin (22.74%) was lower than that of

282 chicken thigh without skin (24.96%) (Table 2). This suggests that the skin present at the surface
283 acts as an insulating layer for the meat and can help retain moisture during frying, thus
284 decreasing HCA formation. This is in agreement with results of Chiu, Yang, and Chen (1998) and
285 Solyakov and Skog (2002). The high level of HCAs in the skin can be explained by the direct
286 exposure to the cooking surface. In addition, the higher fat content in skin might affect HCA
287 formation. Lipids are known to conduct heat more efficiently into the product, which favors the
288 formation of HCAs (Johansson & Jägerstad, 1994). It is possible that lipids, and perhaps lipid
289 oxidation, form products that may enhance the formation of certain Maillard reaction products and
290 lead to an increased content of HCA formation (Barnes, Maher, & Weisburger, 1983; Johansson
291 & Jägerstad, 1994). Removing the skin portion before consumption could significantly reduce
292 total HCA levels from 7.06 to 2.89 ng/g in chicken breast ($p < 0.05$), and from 5.58 to 2.06 ng/g
293 in chicken thigh ($p < 0.05$). Although chicken skin contains a high level of HCAs, the weight of
294 skin portion is much less than meat portion. The total HCA levels in chicken cooked with skin
295 (3.13 ng/g in breast and 2.33 ng/g in thigh) were still significantly lower than the levels in
296 chicken cooked without skin (7.06 ng/g in breast and 5.58 ng/g in thigh) ($p < 0.05$). These results
297 agree with studies by Gašperlin, Lukan, Žlender, & Polak (2009), who reported a lower content
298 of total HCAs in chicken with skin (3.49 ng/g) than in chicken without skin (4.75 ng/g) grilled at
299 a temperature of 220 °C to an internal temperature of 82 °C, and Chiu et al. (1998), who reported
300 a lower content of total HCAs in chicken with skin (6.67 ng/g) than in chicken without skin
301 (12.71 ng/g) fried at 200 °C for 15 min. Taken together, these results indicated that presence of
302 skin reduces HCA formation. However, it is still best to remove skin before consuming chicken
303 to minimize HCA intake.

304 Table 6 shows the HCA levels of fried beef and broiled beef cooked to medium-rare
305 (internal temperature 57 °C) and well-done (internal temperature 71 °C). MeIQ_x, DiMeIQ_x, and
306 PhIP were detected in fried and broiled beef. There was a dramatic increase in total HCAs
307 (approximately 3.5-fold) for both fried beef and broiled beef with the increase in cooking time
308 (degree of doneness) from medium-rare to well-done (from 2.73 ng/g to 8.92 ng/g for fried beef
309 and from 1.72 ng/g to 6.04 ng/g for broiled beef) ($p < 0.05$). We observed approximate increases
310 of 2-fold for MeIQ_x level, 8-fold for DiMeIQ_x, and 6-fold for PhIP when comparing medium-
311 rare with well-done fried beef. This result is in agreement with studies by Skog et al. (1997) and
312 Janoszka et al. (2009). When cooking time increases, more proteins are denatured, pressing more
313 water, which contains water-soluble HCA precursors, out of the protein network to the meat
314 surface. Thus more of these precursors are transferred to the surface for HCA formation
315 (Persson, Oroszvári, Tornberg, Sjöholm, & Skog, 2008; Skog et al., 1997). Different cooking
316 methods affected total HCA formation. Total HCAs in fried beef (2.73 ng/g) were slightly higher
317 than those in broiled beef (1.72 ng/g) for medium-rare samples, but the difference was not
318 significant ($p > 0.05$). Total HCAs of fried beef (8.92 ng/g) were significantly higher than those
319 of broiled beef (6.04 ng/g) for well-done samples ($p < 0.05$). Cooking time may have more
320 influence on HCA formation than cooking temperature because the cooking temperature used for
321 broiling (232 °C) was higher than that used for frying (204 °C); however, the cooking time used
322 for broiling was less than that used for frying. Also, in oven broiling, the heat is transferred to the
323 meat by air, this produces fewer HCAs than frying, in which the meat is in direct contact with a
324 heated pan (Skog et al., 1997). This result clearly indicates that controlling cooking temperature
325 is a way to minimize HCA formation.

326 The HCA quantitative determination in fried beef and pork patties, baked beef and pork,
327 and fried bacon is summarized in Table 7. The level of total HCAs did not differ much between
328 fried beef patty and fried pork patty, and between baked beef and baked pork. Baked beef had a
329 lower content of HCAs than fried and broiled beef (Table 6) because baking is done at a lower
330 temperature and because a higher weight of raw beef was used for baking. The level of HCAs in
331 fried bacon was the highest of all meat samples in present study. The total content of HCAs in
332 fried bacon was 17.59 ng/g (6.91 ng/g PhIP, 4.00 ng/g MeIQx, 3.57 ng/g DiMeIQx, and 3.11
333 ng/g IQx). The content of HCAs in fried bacon in the present study was much higher than that of
334 fully cooked bacon, ready-to-eat meat product included in our previous studies. Fully cooked
335 bacon (heated in a microwave for 30 s according to package direction) had only 0.91 ng/g HCAs
336 (0.14 ng/g PhIP, 0.14 ng/g MeIQx, 0.60 ng/g IQ, and 0.04 ng/g IQx). We believed that the low
337 content of HCAs in fully cooked bacon is due to the precooking process. Industrial fully cooked
338 bacon is cooked at low temperature (162 °C) in the presence of steam induced high humidity
339 either by using a continuous microwave oven or a continuous linear circulating oven. Cooking
340 loss of fried bacon in the present study was high (71.94%). This may explain the high level of
341 HCAs, especially PhIP, in fried bacon compared with fully cooked bacon. PhIP formation
342 increases dramatically in the cooking conditions that generate high cooking loss (Messner &
343 Murkovic, 2004). The content of cooking loss and total HCAs of fried bacon in the present study
344 agree with results of a study by Johansson and Jägerstad (1994), who reported 50.3 to 71.4%
345 cooking loss and 16.7 ng/g total HCAs of bacon fried at 150 °C for 5 min per side.

346 Table 8 summarizes the results of HCA quantitative determination in fried and baked fish
347 (catfish, salmon, and tilapia). There was no significant difference in content of HCAs among the

348 three fish species ($p > 0.05$). Concentrations of MeIQx, DiMeIQx, and PhIP in fried fish (catfish,
349 salmon, and tilapia) were similar to those reported earlier for fried mackerel (Gu et al., 2002) and
350 fried salmon (Iwasaki et al., 2010). For all three fish species, total HCAs in fried fish (13.09 to
351 16.29 ng/g) were significantly higher than those in baked fish (7.85 to 8.70 ng/g) ($p < 0.05$);
352 however, the small contents of IQx (0.38 to 0.52 ng/g) were detected only in baked fish samples.

353 The total content of HCAs can be used to order these cooked meat products from low to
354 high. Low levels of total HCAs (less than 5 ng/g) were found in baked beef (2.34 ng/g), fried
355 chicken thigh with skin (2.33 ng/g), medium-rare fried beef (2.73 ng/g), fried chicken breast with
356 skin (3.13 ng/g), baked pork (3.29 ng/g), and fried pork patty (4.12 ng/g). Intermediate levels of
357 total HCAs (5 to 10 ng/g) were found in fried beef patty (5.46 ng/g), fried chicken thigh (5.58
358 ng/g), well-done broiled beef (6.04 ng/g), fried chicken breast without skin (7.06 ng/g), baked
359 fish (8.32 ng/g), and well-done fried beef (8.92 ng/g). High levels of total HCAs (higher than 10
360 ng/g) were found in fried pork (13.91 ng/g), fried fish (14.91 ng/g), and fried bacon (17.91 ng/g).
361 The high levels of HCAs in some cooked meat products in the present study raises several
362 interesting issues related to HCA intake and cancer aetiology. Data from the National Health and
363 Nutrition Examination Survey 2003-2006 (unpublished data), which estimated meat
364 consumption of U.S. populations, indicated that chicken breast without skin was the most
365 frequently consumed meat item in the U.S. (9.57 g/day), followed by beef steak (8.52 g/day),
366 pork chops (2.89 g/day), and bacon (1.39 g/day). Thus, according to our study, the high levels of
367 HCAs found in fried bacon and fried pork and the intermediate levels of HCAs found in fried
368 chicken breast without skin and well-done fried beef steak indicate that people consuming these
369 products frequently have a high exposure to HCAs that could lead to the possibility of an

370 increased risk of cancers.

372 **4. Conclusions**

373 The HCA content in cooked meat depends on type of meat, cooking methods, and
374 cooking time and temperature. The primary HCAs in these samples were PhIP, MeIQx, and
375 DiMeIQx. Our results indicated that type and content of HCAs in cooked meat samples were
376 highly dependent on cooking conditions. The total HCA contents in cooked meat were 3.5 times
377 lower if cooked to medium-rare rather than well-done degree of doneness. Fried pork showed
378 higher total HCAs than fried beef and chicken. The skin of fried chicken contained a significant
379 HCA contents, therefore removing the skin before consuming can reduce HCA exposure. Total
380 HCAs were briefly ranked in a decreasing order as follows: low HCA contents (< 5 ng/g) found
381 in baked beef, fried chicken without skin, medium-rare steak, and fried pork patty; intermediate
382 HCA contents (5-10 ng/g) found in fried beef patty, fried chicken with skin, baked fish, and
383 well-done steak; and high HCA contents (> 10 ng/g) found in fried pork, fried fish, and fried
384 bacon. Our data can help food safety professionals recommend cooking methods to be used at
385 home or in the food industry to reduce HCA formation in cooked meat products, will provide
386 important information for use in estimating HCA exposure, and will facilitate investigation of the
387 role of HCAs in the etiology of cancer of population in the United States.

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Table 1: Chemical analyses (pH, moisture, fat, protein, and creatine) of raw meat samples prior to cooking

| Sample | | pH | moisture (%) | fat (%) | protein (%) | creatine (mg/g) |
|---------|-------------|-------------|--------------|--------------|--------------|-----------------|
| Beef | Top loin | 5.62 ± 0.05 | 69.32 ± 0.83 | 7.25 ± 0.01 | 21.29 ± 0.18 | 2.93 ± 0.06 |
| | Round tip | 5.47 ± 0.04 | 71.21 ± 1.22 | 4.61 ± 2.27 | 22.50 ± 0.64 | 2.95 ± 0.23 |
| | Ground beef | 5.89 ± 0.04 | 69.79 ± 0.79 | 9.22 ± 1.46 | 19.66 ± 0.54 | 2.53 ± 0.09 |
| Pork | Top loin | 6.01 ± 0.36 | 75.07 ± 0.45 | 7.73 ± 0.32 | 20.90 ± 1.01 | 1.88 ± 0.69 |
| | Ground pork | 6.23 ± 0.08 | 60.12 ± 1.15 | 21.42 ± 0.59 | 15.48 ± 0.10 | 1.79 ± 0.14 |
| | Bacon | 6.71 ± 0.05 | 37.74 ± 2.14 | 47.99 ± 0.16 | 11.83 ± 1.15 | 1.23 ± 0.33 |
| Chicken | Breast meat | 6.19 ± 0.16 | 74.63 ± 0.62 | 4.87 ± 0.71 | 23.37 ± 0.25 | 2.21 ± 0.17 |
| | Breast skin | 6.35 ± 0.07 | 37.06 ± 3.56 | 53.67 ± 4.36 | 11.04 ± 1.56 | 1.02 ± 0.16 |
| | Thigh meat | 6.70 ± 0.10 | 74.44 ± 1.47 | 4.58 ± 0.64 | 19.85 ± 0.54 | 2.51 ± 0.07 |
| | Thigh skin | 6.64 ± 0.04 | 37.36 ± 2.47 | 52.98 ± 3.80 | 9.04 ± 1.29 | 1.18 ± 0.22 |
| Fish | Catfish | 6.94 ± 0.11 | 77.98 ± 0.39 | 4.99 ± 0.13 | 15.49 ± 0.13 | 2.81 ± 0.15 |
| | Salmon | 6.80 ± 0.05 | 78.66 ± 2.91 | 1.12 ± 1.11 | 18.21 ± 2.71 | 2.66 ± 0.23 |
| | Tilapia | 7.91 ± 0.14 | 82.03 ± 2.64 | 1.07 ± 1.02 | 15.72 ± 1.01 | 1.80 ± 0.12 |

Each value is represented as mean ± standard deviation ($n = 3$).

Table 2 : Cooking description

| Cooking Method | Food item | Description |
|-----------------------|---------------------------|--|
| Frying | Beef, pork, chicken, fish | Meat was fried in a Teflon-coated frying pan without adding oil at a surface temperature of 204 °C. Meat was fried, turned once, and removed from the pan when the desired temperature was reached. |
| Broiling | Beef | Oven (convection, top and bottom heat) was preheated to 232 °C (monitored with oven thermometer). The meat was placed on a broiler pan to keep the broiled beef out of the drippings. The meat was removed when a final internal temperature was achieved. |
| Baking | Beef, Pork, Fish | Oven (convection, top and bottom heat) was preheated to 177 °C (monitored with oven thermometer). The meat was placed on a baking pan. The meat was removed when a final internal temperature was achieved. |

*Convection oven with top and bottom heat (top heat 1.0 W and bottom heat 1.2 W)

Table 3: Cooking conditions and cooking loss in cooked meat samples

| Meat type | Types/cuts of meat | Type of cooking | Raw meat (g) | Thickness, raw meat (cm) | Desired internal temperature (°C) | Cooking temperature (°C) | Cooking time (min per side) | Cooking loss (%) |
|------------------|---------------------------|------------------------|---------------------|---------------------------------|--|---------------------------------|------------------------------------|-------------------------|
| Beef | Top loin | Frying (medium rare) | 350-400 | 3.8 | 57 | 204 | 6 | 17.50 ± 1.84 |
| | Top loin | Frying (well done) | 350-400 | 3.8 | 71 | 204 | 12 | 31.86 ± 1.66 |
| | Top loin | Broiling (medium rare) | 350-400 | 3.8 | 57 | 232 | 5 | 23.57 ± 1.66 |
| | Top Loin | Broiling (well done) | 350-400 | 3.8 | 71 | 232 | 10 | 33.81 ± 2.19 |
| | Round tip | Baking (well done) | 650-680 | 9.0 | 71 | 177 | 80 (total) | 30.75 ± 3.75 |
| | Ground beef | Frying | 140-160 | 2.3 | 71 | 204 | 6 | 35.30 ± 2.35 |
| Pork | Top loin | Frying | 230-250 | 2.3 | 71 | 204 | 8 | 26.12 ± 1.70 |
| | Top loin | Baking | 650-680 | 9.0 | 71 | 177 | 70 (total) | 26.48 ± 2.24 |

| | | | | | | | | |
|---------|------------------------------|--------|---------|-----|----|-----|------------|--------------|
| | Ground pork | Frying | 130-135 | 2.3 | 71 | 204 | 6 | 22.20 ± 1.93 |
| | Bacon | Frying | 18-25 | 0.3 | - | 172 | 3 | 71.94 ± 1.26 |
| Chicken | Breast without skin | Frying | 250-280 | 2.5 | 74 | 204 | 10 | 27.88 ± 1.26 |
| | Breast with skin | Frying | 280-310 | 2.5 | 74 | 204 | 10 | 24.39 ± 4.71 |
| | Thigh without skin (bone-in) | Frying | 140-180 | 2.5 | 74 | 204 | 7 | 24.96 ± 3.09 |
| | Thigh with skin (bone-in) | Frying | 150-200 | 2.5 | 74 | 204 | 7 | 26.74 ± 2.99 |
| Fish | Catfish | Frying | 170-190 | 1.8 | 63 | 204 | 6 | 27.28 ± 2.46 |
| | Salmon | Frying | 180-200 | 1.8 | 63 | 204 | 6 | 21.60 ± 2.39 |
| | Tilapia | Frying | 140-160 | 1.5 | 63 | 204 | 6 | 23.65 ± 1.84 |
| | Catfish | Baking | 170-190 | 1.8 | 63 | 177 | 15 (total) | 20.68 ± 2.45 |
| | Salmon | Baking | 180-200 | 1.8 | 63 | 177 | 14 (total) | 18.41 ± 2.24 |
| | Tilapia | Baking | 140-160 | 1.5 | 63 | 177 | 12 (total) | 18.55 ± 3.73 |

512 Table 4: Heterocyclic amine content (MeIQx, DiMeIQx, PhIP, and total) of fried meat samples

| Cooked items | Internal temperature (°C) | Heterocyclic amines (ng/g) | | | |
|-------------------------------------|---------------------------|----------------------------|-------------|-------------|--------------|
| | | MeIQx | DiMeIQx | PhIP | Total |
| Fried beef (well done) | 77 | 3.33 ± 0.38 | 0.33 ± 0.38 | 5.27 ± 0.81 | 8.92 ± 1.08 |
| Fried pork | 71 | 2.39 ± 0.50 | 2.33 ± 0.52 | 9.20 ± 1.20 | 13.91 ± 1.81 |
| Fried chicken (breast without skin) | 74 | 0.46 ± 0.34 | 0.54 ± 0.19 | 6.06 ± 0.10 | 7.06 ± 0.56 |

513 Each value is represented as mean ± standard deviation ($n = 4$). Means with different superscript letters within the same
 514 column are significantly different at $p < 0.05$.

515

Table 5: Heterocyclic amine content (MeIQx, DiMeIQx, PhIP, and total) of fried chicken samples

| Cooked items | | | Heterocyclic Amines (ng/g) | | | |
|--------------|--------------|---------------|----------------------------|--------------------------|--------------------------|--------------------------|
| | | | MeIQx | DiMeIQx | PhIP | Total |
| Breast | without skin | meat | 0.46 ± 0.34 ^b | 0.54 ± 0.19 ^a | 6.06 ± 0.10 ^a | 7.06 ± 0.56 ^a |
| | with skin | meat | 0.23 ± 0.15 ^b | 0.05 ± 0.01 ^c | 2.61 ± 0.63 ^c | 2.89 ± 0.72 ^b |
| | | skin | 1.61 ± 0.72 ^a | 0.93 ± 0.50 ^a | 4.52 ± 0.37 ^b | 7.07 ± 1.43 ^a |
| | | meat and skin | 0.31 ± 0.15 ^b | 0.10 ± 0.02 ^b | 2.72 ± 0.60 ^c | 3.13 ± 0.67 ^b |
| Thigh | without skin | meat | 0.09 ± 0.05 ^b | 0.06 ± 0.04 ^b | 5.43 ± 0.43 ^a | 5.58 ± 0.38 ^a |
| | with skin | meat | nd | nd | 2.06 ± 0.04 ^c | 2.07 ± 0.05 ^b |
| | | skin | 0.47 ± 0.18 ^a | 0.24 ± 0.14 ^a | 4.16 ± 0.42 ^b | 4.87 ± 0.65 ^a |
| | | meat and skin | 0.05 ± 0.03 ^b | 0.02 ± 0.02 ^b | 2.25 ± 0.10 ^c | 2.33 ± 0.14 ^b |

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Each value is represented as mean ± standard deviation ($n = 4$). Means with different superscript letters within the same column are significantly different at $p < 0.05$.

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Table 6: Heterocyclic amine content (MeIQx, DiMeIQx, PhIP, and total) of fried beef and broiled beef

| Cooked items | | Heterocyclic amines (ng/g) | | | |
|--------------|-------------|----------------------------|--------------------------|--------------------------|--------------------------|
| | | MeIQx | DiMeIQx | PhIP | Total |
| Fried beef | Medium rare | 1.75 ± 1.43 ^a | 0.04 ± 0.07 ^a | 0.94 ± 0.70 ^b | 2.73 ± 2.01 ^c |
| | Well done | 3.33 ± 0.38 ^a | 0.33 ± 0.38 ^a | 5.27 ± 0.81 ^a | 8.92 ± 1.08 ^a |
| Broiled beef | Medium rare | 0.08 ± 0.07 ^b | 0.06 ± 0.04 ^a | 1.58 ± 0.36 ^b | 1.72 ± 0.43 ^c |
| | Well done | 0.12 ± 0.07 ^b | 0.11 ± 0.02 ^a | 5.63 ± 0.95 ^a | 6.04 ± 0.97 ^b |

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Each value is represented as mean ± standard deviation ($n = 4$). Means with different superscript letters within the same column are significantly different at $p < 0.05$.

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Table 7: Heterocyclic amine content (IQ_x, MeIQ_x, DiMeIQ_x, PhIP, and total) of fried beef and pork patties, baked beef and pork, and fried bacon

| Cooked items | Heterocyclic amines (ng/g) | | | | |
|------------------|----------------------------|-------------------|---------------------|-------------|--------------|
| | IQ _x | MeIQ _x | DiMeIQ _x | PhIP | Total |
| Fried beef patty | nd | 3.11 ± 0.69 | ND | 2.35 ± 0.30 | 5.46 ± 0.78 |
| Fried pork patty | nd | 1.09 ± 0.16 | 1.24 ± 0.75 | 1.80 ± 0.10 | 4.12 ± 0.72 |
| Baked beef | nd | 0.33 ± 0.05 | 0.53 ± 0.12 | 1.49 ± 0.10 | 2.34 ± 0.11 |
| Baked pork | nd | 0.23 ± 0.06 | 0.86 ± 0.24 | 2.20 ± 0.12 | 3.29 ± 0.36 |
| Fried bacon | 3.11 ± 1.38 | 4.00 ± 1.46 | 3.57 ± 1.12 | 6.91 ± 2.06 | 17.59 ± 5.18 |

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Each value is represented as mean ± standard deviation ($n = 4$).
nd = not detected

Table 8: Heterocyclic amine content (IQx, MeIQx, DiMeIQx, PhIP, and total) of fried and baked fish

| Cooked items | | Heterocyclic amines (ng/g) | | | | |
|--------------|---------|----------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| | | IQx | MeIQx | DiMeIQx | PhIP | Total |
| Fried | Catfish | nd | 2.31 ± 0.10 ^b | 2.72 ± 0.08 ^a | 10.31 ± 0.83 ^a | 15.35 ± 0.78 ^a |
| | Salmon | nd | 2.05 ± 0.50 ^b | 1.93 ± 0.12 ^{ab} | 9.11 ± 1.25 ^a | 13.09 ± 0.90 ^a |
| | Tilapia | nd | 3.11 ± 0.42 ^a | 2.29 ± 0.35 ^a | 10.89 ± 1.35 ^a | 16.29 ± 1.98 ^a |
| Baked | Catfish | 0.85 ± 0.45 ^a | 2.95 ± 0.70 ^{ab} | 0.51 ± 0.03 ^c | 4.40 ± 0.64 ^b | 8.70 ± 1.61 ^b |
| | Salmon | 0.38 ± 0.19 ^a | 2.03 ± 0.85 ^b | 1.66 ± 0.77 ^b | 4.34 ± 0.48 ^b | 8.41 ± 1.09 ^b |
| | Tilapia | 0.52 ± 0.21 ^a | 1.27 ± 0.16 ^c | 0.29 ± 0.23 ^c | 5.67 ± 0.44 ^b | 7.85 ± 0.65 ^b |

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Each value is represented as mean ± standard deviation ($n = 4$). Means with different superscript letters within the same column are significantly different at $p < 0.05$.

nd = not detected

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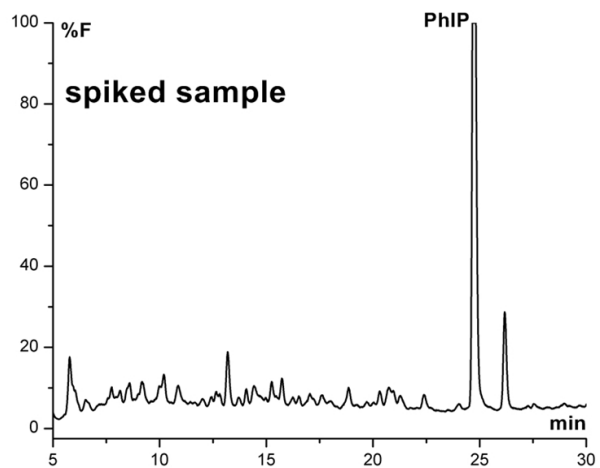
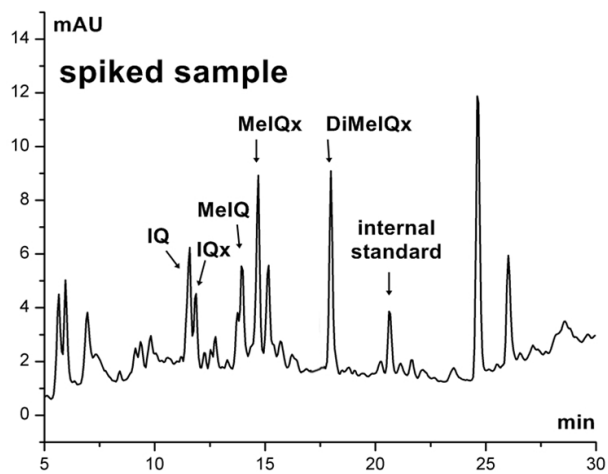
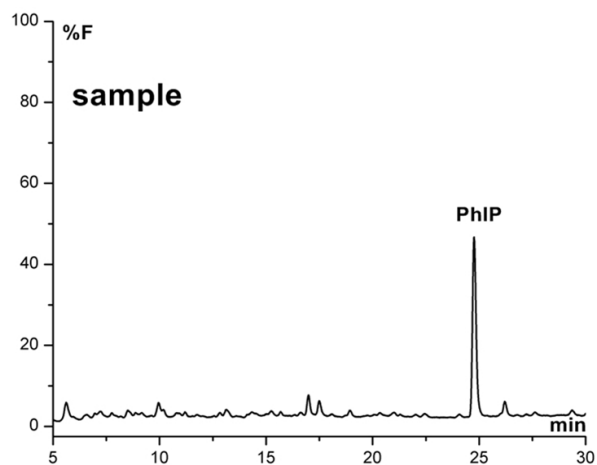
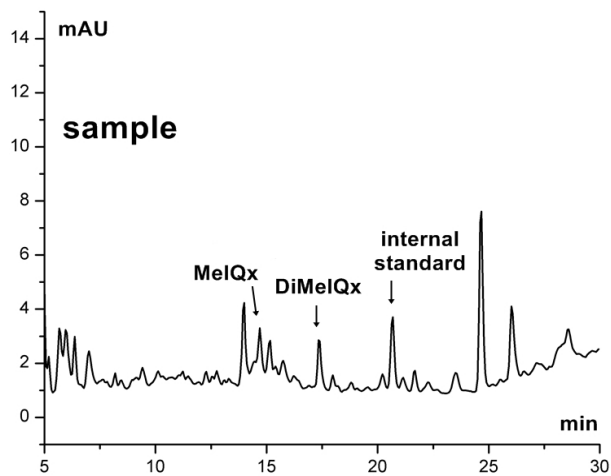
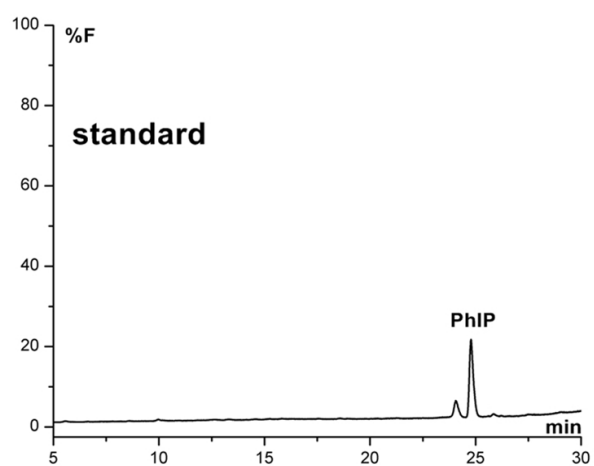
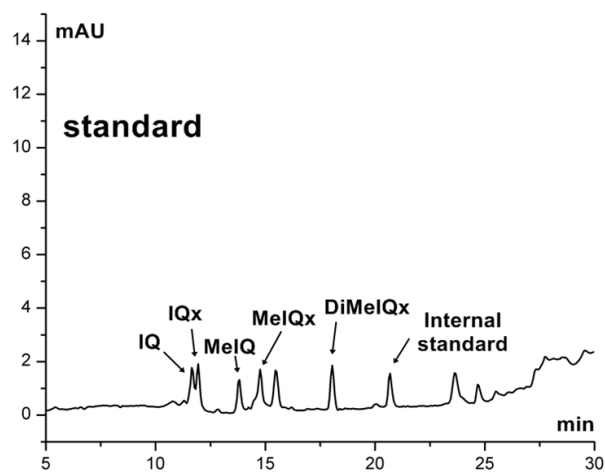
Figure Legend

531

532 Figure 1. UV chromatogram (a) and FLD chromatogram (b) of HCA standards (250 ppb each),
533 sample (fried pork), and spiked sample at concentration 25 ppb of each

534

HCA.



(a)

(b)