Supplementary Material

for

A Study on Production of Canned Minced Chicken and Pork and Formation of Heterocyclic Amines during Processing

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Extraction and Purification of HAs in Raw, Marinated, Stir-Fried, Degassed and Canned Minced Chicken and Pork

HAs were extracted and purified simultaneously in raw, marinated, stir-fried, degassed and canned minced chicken and pork based on a QuEChERS method described by Lai et al. [1]. QuEChERS extraction and purification kits supplied by Yu-Ho Co. (New Taipei City, Taiwan) were used. Initially, 2 g of minced chicken or pork sample was mixed with deionized water (10 mL) in a centrifuge tube containing a ceramic homogenizer, followed by shaking (10 min) with 1% acetic acid in acetonitrile (10 mL), shaking (1 min) with 4-g magnesium sulfate and 1-g sodium acetate and collecting the supernatant after centrifuging at 4 °C for 10 min (4000xg). For purification, the supernatant was mixed with PSA (300 mg), magnesium sulfate (900 mg) and C18-EC (300 mg), after which this mixture was shaken (1 min), centrifuged, supernatant collected, dried under nitrogen, dissolved in methanol (0.2 mL) containing internal standard 4,7,8-TriMeIQx (1 ppb) and filtered through 0.22-μm membrane filter for HA analysis by UPLC-MS/MS.

Analysis of HAs in Raw, Marinated, Stir-Fried, Degassed and Canned Minced Chicken and Pork by UPLC-MS/MS

A total of 21 HA standards, including DMIP, IFP, PhIP, Phe-P-1, MeIQ, 4,8-DiMeIQx, 8-MeIQx, 7,8-DiMeIQx, IQx, IQ, IQ[4,5-b], Iso-IQ, MeAαC, AαC, Trp-P-2, Trp-P-1, Glu-P-2, Glu-P-1, Norharman, Harman and internal standard 4,7,8-TriMeIQx procured from Toronto Research Chemicals (Downsview, Ontario, Canada) were used for separation, identification, method validation and quantitation of HAs by an ultra-performance liquid chromatography (UPLC) system consisting of a degasser (G1379B), binary pump (G1312B), automatic sampler

(G1329B), column thermostat oven (G1316B) and diode array detector (G1215C) was from Agilent Technologies Co. (Palo Alto, CA, USA). An Acquity BEH C18 column (100 x 2.1 mm ID, particle size 1.7 μm) from Waters Corp. (Milford, MA, USA) was used for separation of 20 HAs and internal standard 4,7,8-TriMeIQx in both standard mixture and raw, marinated, stir-fried, degassed and canned minced chicken and pork within 4 min by using the same gradient mobile phase of 20 mM of ammonium acetate (pH 4.5) and acetonitrile as reported in a previous study [1]. The HA identification and quantitation were done in selected reaction monitoring (SRM) mode using a Dionex Ultimate 3000 model open sampler XSUPLC system coupled with TSQ Quantiva triple quadrupole tandem mass spectrometer with electrospray ionization from Thermo Fisher Scientific Co. (San Jose, CA, USA) with ion transfer tube temperature at 329°C, vaporizer temperature at 279°C, spray voltage at 2700 V, collision gas flow rate at 1.5 arbitrary units, sweep gas flow rate at 2 arbitrary units, sheath gas flow rate at 4 arbitrary units and auxiliary gas flow rate at 9 arbitrary units.

Amino Acid Determination

The individual amino acid contents in raw, marinated, stir-fried, degassed and canned minced chicken and pork were determined using a method as described by Taiwan Food and Drug Administration (TFDA) [1,2]. After derivatization of amino acid in standards or in chicken and pork with 1 N hydrochloric acid (10 mL), 0.4 M boric acid buffer (100 μL), phthaldehyde (20 μL), 9-fluorenylmethyl chloroformate (20 μL) and deionized water (1280 μL), various amino acids were separated using a Poroshell HPH-C18 column (10 cm x 3.0 mm internal diameter (ID), particle size 2.7 μm) with a gradient binary mobile phase of (A) 40 mM sodium dihydrogen phosphate (pH 7.8) and (B) acetonitrile/methanol/water (45:45:10, v/v/v) for subsequent identification and quantitation, with the former being done by comparing absorption spectra and retention time of amino acid standards with peaks on the high-performance liquid chromatogram, while the latter calculated using the formula: amino acid content (mg/100 g) = C × (V/M) x 10, where C is the concentration of amino acid (μg/mL) obtained based on standard curves, V is the final volume of sample extract (mL) and M is the sample weight (g).

Reducing Sugar Determination

A method as described by Chen et al. [3] was used to determine reducing sugar content in raw, marinated, stir-fried, degassed and canned minced chicken and pork. Initially, the

glucose standard curve was prepared by mixing five concentrations (1.25, 2.5, 5, 8, 10 mg/mL) separately with a 100-mL solution mixture containing dinitrosalicylic acid (0.315 g), sodium hydroxide (0.2 g/mL) and sodium potassium tartrate (9.1 g), followed by heating the homogenized sample of chicken and pork (2 g) in deionized water with dinitrosalicylic acid (1 mL) at 100°C for 10 min, cooling and measuring absorbance at 570 nm for obtaining glucose content based on the glucose standard curve.

Table S1. Proximate analysis of raw minced chicken and pork as well as their degassed, LL-ST sterilized and HS-ST sterilized samples collected during the canning process (duplicate analyses).

	Moisture (%)	**Ash (%)**	**Crude fat (%)**	**Crude Protein (%)**
Chicken				
Raw	74.04±0.13	1.15±0.08	2.25±0.00	22.56±0.08
Degassed (15 min@85°C)	70.88±0.91	3.34±0.01	2.27±0.02	23.51±0.06
LL-ST (60 min@115°C)	72.13±0.43	2.99±0.09	2.21±0.02	22.67±0.06
HS-ST (25 min@125°C)	72.26±0.39	2.94±0.00	2.17±0.00	22.63±0.16
Pork				
Raw	71.37±0.24	0.98±0.00	12.05±0.01	15.60±0.21
Degassed (15 min@85°C)	68.12±0.35	2.93±0.12	12.15±0.04	16.80±0.12
LL-ST (60 min@115°C)	69.08±1.06	2.94±0.00	12.45±0.18	15.53±0.17
HS-ST (25 min@125°C)	69.16±1.12	2.75±0.01	12.14±0.02	15.95±0.16

LL-ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 60 min@115°C; HS-ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 25 min@125°C.

Table S2. Matrix effect as well as limit of detection (LOD) and limit of quantitation (LOQ) of 20 HAs in freeze-dried pork and chicken detected by UPLC-MS/MS.

HAs	Matrix effect[a]	Freeze-dried pork		Freeze-dried chicken	
		LOD (ng/g)[b]	LOQ (ng/g)[c]	LOD (ng/g)[b]	LOQ (ng/g)[c]
DMIP	0.63	0.005	0.01	0.003	0.005
Glu-P-2	0.86	0.02	0.05	0.005	0.01
iso-IQ	0.96	0.02	0.05	0.02	0.03
IQ	0.93	0.05	0.10	0.03	0.04
IQx	0.87	0.02	0.10	0.02	0.03
MeIQ	0.82	0.03	0.05	0.03	0.04
Glu-P-1	0.86	0.02	0.05	0.02	0.03
8-MeIQx	0.91	0.03	0.05	0.03	0.04
IQ[4,5-b]	0.95	0.04	0.10	0.04	0.05
IFP	0.97	0.005	0.02	0.005	0.01
7,8-DiMeIQx	0.84	0.04	0.10	0.04	0.05
4,8-DiMeIQx	0.95	0.02	0.05	0.02	0.03
Norharman	-	-	-	-	-
Harman	-	-	-	-	-
Phe-P-1	0.77	0.01	0.03	0.01	0.02
Trp-P-2	0.95	0.01	0.05	0.01	0.02
PhIP	0.92	0.01	0.03	0.01	0.02
Trp-P-1	-	-	-	-	-
AαC	0.81	0.01	0.03	0.005	0.01
MeAαC	0.90	0.01	0.03	0.01	0.02

[a]Matrix effect=$S_{matrix}/S_{standard}$, where S is the slope. [b]Limit of detection based on S/N≥3. [c]Limit of quantitation based on S/N≥10.

Table S3. Recovery (%) of 20 HA standards and HAs in freeze-dried pork and chicken detected by UPLC-MS/MS.[a,b]

HAs	Standards		Freeze-dried pork		Freeze-dried chicken	
	Low (1 ng/g)	High (10 ng/g)	Low (1 ng/g)	High (10 ng/g)	Low (1 ng/g)	High (10 ng/g)
DMIP	77.9 ± 0.4	81.9 ± 3.2	68.3 ± 6.1	79.1 ± 3.8	69.0 ± 7.5	82.2 ± 9.3
iso-IQ	74.4 ± 3.5	83.4 ± 2.6	70.9 ± 2.9	85.1 ± 8.5	69.7 ± 6.6	86.9 ± 7.9
Glu-P-2	86.0 ± 5.3	86.7 ± 2.2	86.3 ± 6.0	109.0 ± 5.0	92.0 ± 12.4	113.5 ± 11.7
IQ	74.9 ± 4.3	85.5 ± 4.6	73.2 ± 6.5	77.0 ± 3.4	65.4 ± 5.4	85.7 ± 7.8
IQx	67.5 ± 2.2	81.1 ± 1.8	75.7 ± 2.5	85.8 ± 8.5	65.1 ± 7.0	81.7 ± 6.7
MeIQ	76.9 ± 2.2	88.3 ± 0.9	61.0 ± 0.8	75.7 ± 2.5	71.2 ± 8.2	94.9 ± 10.6
8-MeIQx	86.2 ± 4.4	84.9 ± 5.0	78.8 ± 0.5	84.6 ± 3.0	97.2 ± 12.1	116.6 ± 11.4
Glu-P-1	87.2 ± 2.4	90.6 ± 2.5	61.5 ± 1.8	71.1 ± 1.4	98.1 ± 6.9	109.8 ± 10.6
IQ[4,5-b]	84.4 ± 4.1	88.8 ± 6.6	51.9 ± 1.3	75.8 ± 4.8	76.4 ± 7.1	99.6 ± 6.2
IFP	74.8 ± 5.3	82.1 ± 2.7	52.1 ± 1.6	66.7 ± 4.7	74.7 ± 4.1	88.8 ± 11.3
7,8-DiMeIQx	87.0 ± 6.8	89.6 ± 9.8	69.6 ± 4.3	70.2 ± 7.4	69.2 ± 13.8	93.1 ± 9.0
4,8-DiMeIQx	86.9 ± 5.7	95.4 ± 4.2	64.5 ± 2.2	82.3 ± 2.9	65.8 ± 10.3	89.0 ± 11.3
Norharman	86.7 ± 4.5	92.4 ± 4.0	80.2 ± 1.6	87.5 ± 3.2	95.7 ± 3.4	90.8 ± 9.7
Harman	85.6 ± 3.9	91.9 ± 2.0	88.8 ± 1.6	119.1 ± 7.3	85.7 ± 14.2	87.5 ± 11.8
Trp-P-1	78.8 ± 1.0	89.0 ± 1.8	58.0 ± 4.2	77.5 ± 2.8	66.6 ± 7.0	66.4 ± 13.4
Trp-P-2	76.6 ± 4.9	89.5 ± 3.1	85.5 ± 3.4	91.1 ± 3.0	90.3 ± 15.5	88.6 ± 9.2
PhIP	85.1 ± 4.7	92.7 ± 4.7	58.2 ± 6.7	81.5 ± 3.6	74.2 ± 5.8	80.8 ± 5.5
Phe-P-1	84.7 ± 6.4	91.8 ± 3.6	62.8 ± 1.9	78.1 ± 5.4	73.9 ± 11.5	88.0 ± 9.2
AαC	75.0 ± 4.7	80.6 ± 6.1	62.2 ± 2.6	72.3 ± 4.4	67.3 ± 5.2	73.6 ± 7.2
MeAαC	83.7 ± 2.9	90.2 ± 1.3	68.2 ± 5.0	65.3 ± 2.3	86.9 ± 7.7	73.7 ± 4.5

[a]Recovery (%) = [(amount found – original amount) / amount spiked] x 100.

[b]Average of triplicate analyses ± standard deviation.

Table S4. Intra-day variability and inter-day variability of 20 HA standards detected by UPLC-MS/MS.

HAs	Intra-day variability		Inter-day variability	
	Content (ng/g)[a]	CV (%)[b]	Content (ng/g)[a]	CV (%)[b]
DMIP	0.881 ± 0.042	7.94	0.777 ± 0.083	9.18
iso-IQ	1.029 ± 0.083	9.18	0.818 ± 0.018	8.18
Glu-P-2	0.858 ± 0.088	5.99	0.810 ± 0.193	8.44
IQ	1.028 ± 0.097	8.24	1.153 ± 0.097	6.94
IQx	1.117 ± 0.017	8.11	1.518 ± 0.017	11.43
MeIQ	0.874 ± 0.174	10.42	0.850 ± 0.382	14.32
8-MeIQx	0.857 ± 0.036	9.11	0.821 ± 0.083	11.93
Glu-P-1	0.898 ± 0.187	5.18	0.766 ± 0.103	8.43
IQ[4,5-b]	0.911 ± 0.128	6.34	0.829 ± 0.037	9.27
IFP	0.749 ± 0.097	6.66	0.593 ± 0.066	10.84
7,8-DiMeIQx	0.925 ± 0.038	6.92	0.694 ± 0.074	14.28
4,8-DiMeIQx	0.930 ± 0.184	9.18	0.819 ± 0.184	10.82
Norharman	0.834 ± 0.074	10.48	0.628 ± 0.084	11.53
Harman	0.850 ± 0.082	7.17	0.820 ± 0.122	14.72
Trp-P-1	0.899 ± 0.083	6.94	0.874 ± 0.068	10.42
Trp-P-2	0.814 ± 0.072	8.17	0.875 ± 0.086	14.24
PhIP	0.839 ± 0.201	8.54	0.811 ± 0.184	9.42
Phe-P-1	0.929 ± 0.018	10.45	0.733 ± 0.177	9.18
AaC	0.717 ± 0.077	8.91	0.733 ± 0.097	8.17
MeAaC	0.917 ± 0.087	9.10	0.962 ± 0.184	10.47

[a]Average of 9 replicate analysis ± standard deviation.
[b]Coefficient of variation (%) = (standard deviation / average) x 100.

Table S5. Intra-day variability and inter-day variability of HAs in freeze-dried pork detected by UPLC-MS/MS.

HAs	Intra-day variability		Inter-day variability	
	Content (ng/g)[a]	CV (%)[b]	Content (ng/g)[a]	CV (%)[b]
DMIP	0.813 ± 0.432	28.44	0.618 ± 0.184	33.70
iso-IQ	0.829 ± 0.073	7.94	0.794 ± 0.183	16.48
Glu-P-2	0.729 ± 0.052	16.63	0.584 ± 0.097	19.53
IQ	0.722 ± 0.091	14.86	0.781 ± 0.088	17.38
IQx	0.721 ± 0.088	14.37	0.793 ± 0.154	11.42
MeIQ	0.868 ± 0.119	10.17	0.775 ± 0.105	15.38
8-MeIQx	0.833 ± 0.177	14.07	0.682 ± 0.186	9.17
Glu-P-1	0.794 ± 0.028	11.84	0.748 ± 0.088	14.83
IQ[4,5-b]	0.618 ± 0.036	17.04	0.684 ± 0.184	13.85
IFP	0.794 ± 0.179	16.62	0.737 ± 0.112	17.28
7,8-DiMeIQx	0.752 ± 0.099	11.56	0.684 ± 0.097	14.37
4,8-DiMeIQx	0.738 ± 0.174	19.03	0.668 ± 0.016	10.43
Norharman	0.993 ± 0.133	10.76	1.053 ± 0.226	19.45
Harman	0.916 ± 0.095	7.79	1.283 ± 0.188	16.93
Trp-P-1	0.668 ± 0.048	17.99	0.588 ± 0.069	13.55
Trp-P-2	0.682 ± 0.055	8.11	0.629 ± 0.018	17.93
PhIP	0.772 ± 0.198	17.94	0.637 ± 0.194	10.52
Phe-P-1	0.739 ± 0.065	5.88	0.793 ± 0.081	9.48
AaC	0.794 ± 0.095	10.74	0.714 ± 0.185	9.37
MeAaC	0.893 ± 0.085	9.04	0.799 ± 0.033	15.92

[a]Average of 9 replicate analysis ± standard deviation.
[b]Coefficient of variation (%) = (standard deviation / average) x 100.

Table S6. Intra-day variability and inter-day variability of HAs in freeze-dried chicken detected by UPLC-MS/MS.

HAs	Intra-day variability		Inter-day variability	
	Content (ng/g)[a]	CV (%)[b]	Content (ng/g)[a]	CV (%)[b]
DMIP	1.000 ± 0.153	15.35	1.029 ± 0.156	15.14
iso-IQ	0.907 ± 0.131	14.46	1.008 ± 0.062	6.13
Glu-P-2	1.000 ± 0.149	14.89	0.980 ± 0.125	12.78
IQ	0.990 ± 0.134	13.58	0.971 ± 0.128	13.19
IQx	0.894 ± 0.164	18.32	0.955 ± 0.108	11.31
MeIQ	0.843 ± 0.105	12.48	0.739 ± 0.062	8.37
8-MeIQx	0.966 ± 0.081	8.37	0.933 ± 0.095	10.21
Glu-P-1	0.855 ± 0.087	10.20	0.807 ± 0.125	15.54
IQ[4,5-b]	0.934 ± 0.164	17.60	0.890 ± 0.136	15.29
IFP	1.029 ± 0.076	7.34	1.135 ± 0.103	9.03
7,8-DiMeIQx	1.018 ± 0.149	14.60	1.110 ± 0.137	12.33
4,8-DiMeIQx	1.022 ± 0.137	13.40	1.093 ± 0.130	11.88
Norharman	0.905 ± 0.128	14.12	0.935 ± 0.084	9.03
Harman	0.849 ± 0.104	12.26	0.896 ± 0.081	9.00
Trp-P-1	0.864 ± 0.124	14.34	0.834 ± 0.095	11.37
Trp-P-2	0.902 ± 0.164	18.16	0.956 ± 0.117	12.21
PhIP	0.938 ± 0.127	13.52	0.980 ± 0.149	15.16
Phe-P-1	0.810 ± 0.098	12.16	0.783 ± 0.079	10.08
AaC	0.743 ± 0.085	11.40	0.760 ± 0.059	7.78
MeAaC	0.640 ± 0.112	17.50	0.639 ± 0.082	12.87

[a]Average of 9 replicate analysis ± standard deviation.
[b]Coefficient of variation (%) = (standard deviation / average) x 100.

Table S7. Composition and percentage of fatty acids in raw chicken/pork as well as their LL-ST sterilized and HS-ST sterilized samples collected during the canning process.

Fatty acids	Chicken			Pork		
	Raw	Sterilization		Raw	Sterilization	
		LL-ST	HS-ST		LL-ST	HS-ST
saturated fatty acid						
14:0	nd	nd	nd	0.12	0.15	0.13
16:0	0.27	0.34	0.27	2.44	2.97	2.56
18:0	0.08	0.14	0.1	1.28	1.52	1.34
Total	0.35	0.48	0.37	3.84	4.64	4.03
trans fatty acid	nd	nd	nd	nd	nd	nd
cis-MUFA[a]						
9c-16:1	nd	nd	nd	0.2	0.22	0.21
9c-18:1	0.48	0.58	0.49	4.2	4.92	4.25
11c-18:1	nd	nd	nd	0.25	0.29	0.25
11c-20:1	nd	nd	nd	0.08	0.1	0.08
Total	0.48	0.58	0.49	4.73	5.53	4.79
cis-PUFA[b]						
18:3 ω3 (ALA)	nd	0.06	0.05	0.08	0.14	0.11
18:2 ω6	0.30	0.60	0.54	1.51	2.25	1.87
20:2 ω6	nd	nd	nd	0.06	0.07	0.06
20:4 ω6	nd	nd	nd	0.06	0.06	0.05
Total	0.30	0.66	0.59	1.71	2.52	2.09
Total fatty acid	1.13	1.72	1.45	10.28	12.69	10.91

LL-ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 60 min@115°C; HS-ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 25 min@125°C; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; nd, not detected. Reproduced from the open-access article published by Inbaraj et al. [4].

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