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

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Abstract: Toxic compounds such as heterocyclic amines (HAs) are formed during the processing of protein-rich foods, especially meat products. This study aims to investigate the formation of HAs during the canning of chicken and pork by using an ultra-performance liquid chromatograph coupled with tandem mass spectrometer (UPLC-MS/MS). Minced samples of both chicken and pork were separately subjected to marinating, stir-frying and degassing for subsequent canning and sterilization for 60 min at 115 °C (low temperature–long time, LL–ST) or 25 min at 125 °C (high temperature–short time, HS–ST) and analyzed for HAs. The results showed that both marinating and sterilization could significantly affect the HA formation in canned minced chicken and pork, with the LL–ST treatment being more liable to total HA formation than the HS–ST treatment, and the total HAs (especially, Harman and Norharman) was produced at a higher level in canned minced pork than in canned minced chicken under the same sterilization treatment. A reduction in reducing sugar, creatine, and amino acid contents resulted in HA formation in canned minced chicken and pork during processing. The results were confirmed by principal component analysis and showed that HAs were formed at significant levels in canned minced chicken and pork, with the level of major HA content following the order of Harman > Trp-P-1 > Norharman > DMIP. Although the presence of non-mutagenic HAs (Harman, Norharman and DMIP) and possibly carcinogenic HA (Trp-P-1) contributed to 95.8% of total HAs formed in both canned pork and chicken in this study, it is imperative to reduce the HA exposure to humans for improved public health by decreasing the consumption of processed meat and increasing the intake of fruits and vegetables, as well as incorporating natural antioxidant-rich ingredients into foods during processing to minimize the formation of HAs.

Keywords: HAs; canned minced chicken; canned minced pork; UPLC-MS/MS; HA precursors

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1. Introduction

Heterocyclic amines (HAs) are usually formed during the processing of protein-rich foods (meat products) at high temperatures. Structurally, HAs are polycyclic aromatic compounds with an amine group attached to the ring structure. They are mainly generated in food through the reaction of creatinine/creatine, reducing sugar and amino acid during heating [1–4]. The Ames test shows that the HA mutagenicity is higher than that of many common carcinogens such as aflatoxin B1 and benzo[a]pyrene, indicating that HAs may possess strong mutagenicity [5]. In addition, the most common HAs such as Norharman and Harman found in meat products are non-mutagenic, probably because of lack of the exocyclic amine groups. However, when Harman and Norharman co-existed with the

other HAs, the mutagenicity of the other HAs can be enhanced. Thus, both Norharman and Harman are generally considered to be co-mutagens [6].

The International Agency for Research on Cancer (IARC) has reported that some HAs may cause cancer in humans owing to their susceptibility to binding with cellular deoxyribonucleic acid (DNA) [7]. Of the various HAs, IQ belongs to the Group 2A category of "probably carcinogenic to humans", while MeIQ, 8-MeIQx, AαC, MeAαC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2 and PhIP are in the Group 2B category, meaning "possibly carcinogenic to humans" (all abbreviations are provided in their full form in Table 1) [7]. Although more than 25 HAs have been identified in processed foods, no maximum permissible limit has been recommended by any international regulatory agency as a safe level for HAs in processed foods [8]. Moreover, Lin et al. [9] reported that HAs may promote carcinogenesis caused by genetic changes and instability through the formation of HA-DNA adducts. Thus, the reduction in exposure to HAs is a vital issue in maintaining human health. Many factors, such as the oil type, food commodity, flavoring material, cooking method and condition, can affect the formation and reduction of HAs in food/food products [4]. In addition, the meat quality, postmortem aging and pH can significantly affect the formation of HAs in meat products [2,8,10]. For instance, Polak et al. [10] demonstrated an increase in the total HA content (1.35–3.49 ng/g) with aging (0–10 days) of pork muscle with no marked difference due to muscle quality (pale, soft and exudative), while Oz et al. [11] reported that HAs are not directly affected by variations in pH. Instead, HAs precursors are susceptible to pH changes, with a high pH being able to modify the formation pathways of HA precursors, leading to a decline in HA production [11].

Table 1. Retention time and SRM detection parameters of 20 HAs and internal standard (4,7,8-TriMeIQx) by UPLC-MS/MS.

HA Compound	Retention Time (min)	Precursor ion (*m*/*z*)	Quantitation		Confirmation	
			Product ion (*m*/*z*)	Collision Energy (V)	Product ion (*m*/*z*)	Collision Energy (V)
2-amino-1,6-dimethylimidazo [4,5-b]pyridine (DMIP)	0.50	163.10	148.10	24	105.09	37
2-aminodipyrido-[1,2-a:3′,2′-d]imidazole (Glu-P-2)	0.87	185.10	158.10	25	78.05	37
2-amino-1-methyl-imidazo[4,5-f]quinoline (iso-IQ)	0.70	199.09	184.12	25	156.10	21
2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ)	0.81	199.10	184.12	27	157.00	29
2-amino-3-methyl-imidazo[4,5-f]quinoxaline (IQx)	0.67	200.08	185.12	28	132.14	29
2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline (MeIQ)	1.45	213.11	198.09	27	145.15	29
2-amino-6-methyldipyrido-[1,2-a:3′,2′-d]imidazole (Glu-P-1)	2.15	199.10	92.10	36	172.14	26
2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (8-MeIQx)	1.23	214.10	131.07	41	173.18	24
2-amino-1-methyl-imidazo[4,5-b]quinoline (IQ[4,5-b])	2.10	199.11	183.92	27	115.19	46
2-amino-1,6-dimethyl-furo[3,2-e]imidazo[4,5-b]pyridine (IFP)	2.62	203.08	188.17	25	175.14	22
2-amino-3,7,8-trimethyl-imidazo[4,5-f]quinoxaline (7,8-DiMeIQx)	2.51	228.10	131.13	40	187.15	25
2-amino-3,4,8-trimethyl-imidazo[4,5-f]quinoxaline (4,8-DiMeIQx)	2.68	228.10	213.09	26	187.09	23
9H-pyrido[3,4-b]indole (Norharman)	2.82	169.06	115.09	33	89.05	48
2-amino-3,4,7,8-tetramethyl-imidazo[4,5-f]quinoxaline (4,7,8TriMeIQx) (IS)	2.82	242.13	145.09	42	201.21	26
1-methyl-9H-pyrido[3,4-b]indole (Harman)	2.83	183.09	115.15	34	89.09	49
2-amino-5-phenylpyridine (Phe-P-1)	3.02	171.09	127.13	30	154.07	21
3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2)	2.89	198.11	154.14	30	181.08	24
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)	3.02	225.10	210.05	30	140.08	54
3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1)	2.95	212.12	195.14	24	168.09	30
2-amino-9H-pyrido[2,3-b]indole (AαC)	3.13	184.07	140.13	33	167.07	24
2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC)	3.25	198.10	181.14	23	127.13	38

HAs, heterocyclic amines; UPLC-MS/MS, ultra-performance liquid chromatograph coupled with tandem mass spectrometer; SRM, selected reaction monitoring; *m*/*z*, mass-to-charge ratio; IS, internal standard.

The main purpose of canning is to destroy microorganisms and enzymes for the preservation of food products during storage with a long shelf life to be ≥2 years. However, the canning process involving high pressure and time–temperature conditions may generate HA formation, especially in meat products. Commercially, the sterilization temperature is often controlled at 120 °C and 100 °C, respectively, for low-acid and acid foods [12]. Consequently, in this study, we intend to explore the formation of HAs as affected by two sterilization temperatures and time length (115 °C for 60 min and 125 °C for 25 min) during canning process. Some recent studies have investigated the formation of toxic compounds

such as furan and polycyclic aromatic hydrocarbons in canned food products, with the former being reported to be significantly generated during processing in different canned foods and the latter being unaffected by sterilization conditions [12,13]. However, there is still a paucity of information on HAs formation during the canning of meat products. Therefore, the objectives of this study were to quantify the formation of HAs in canned minced pork and chicken, both of which are popular meat commodities in Taiwan, during the canning process, including marinating (MN), stir-frying (SF), degassing (DG) and sterilization (ST) at two time–temperature conditions (60 min at 115 °C and 25 min at 125 °C), for the elucidation of the probable mechanism by analyzing the level of HAs precursors.

2. Materials and Methods

2.1. Meat Raw Material

Raw minced pork and raw chicken leg muscle in lumpy form with a size of about 15 cm long, 4 cm wide and 3 cm high were obtained from a local supermarket in New Taipei City (Taiwan), with the raw chicken leg muscle being further minced in a blender to obtain raw minced chicken.

2.2. Experiment Design for Processing of Canned Minced Pork and Chicken

A method as reported by Tsao et al. [12] and Inbaraj et al. [13] was used for processing of canned minced pork and chicken. Briefly, minced pork (2 kg) or minced chicken (2 kg) was mixed with sugar (60 g), allspice (10 g), soy sauce (400 mL), soybean oil (20 mL) and minced garlic (30 g) as marinade, followed by stirring for 10 min for marinating (MN) and pouring into a pan for stir-frying for 10 min at 95 °C (SF). This pretreatment is a standard method used for preparation of canned minced pork and chicken in Taiwan. Pretreated pork was then filled into 10 cans (No. 2, 307 × 201 mm) with each can containing 150 g of meat. Likewise, pretreated chicken was filled into 10 cans, followed by degassing 20 cans with hot steam for 15 min at 85 °C (DG). Of the 10 cans containing minced pork or chicken, 5 cans were subjected to ST treatment for 60 min at 115 °C (low temperature–long time sterilization, LL–ST) while the other 5 cans were sterilized for 25 min at 125 °C (high temperature–short time sterilization, HS–ST). After cooling to room temperature and homogenizing the meat sample, 2 g of pork or chicken sample in triplicate was collected for analysis of HAs by QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) coupled with UPLC-MS/MS (ultra-performance liquid chromatograph-tandem mass spectrometer). Figure 1 shows the flow chart of different processing steps including MN, SF, DG and ST (LL–ST and HS–ST) involved during canning of minced pork and chicken.

2.3. Extraction and Purification of HAs in Minced Chicken and Pork During Canning

For simultaneous extraction and purification of HAs in raw, MN, SF, DG and ST samples of minced chicken and pork, a QuEChERS method reported by Lai et al. [4] was used with the QuEChERS kits supplied by Yu-Ho Co. (New Taipei City, Taiwan). A detailed procedure is provided in the Supplementary Materials.

2.4. Analysis of HAs in Raw, MN, SF, DG and ST Samples of Minced Chicken and Pork by UPLC-MS/MS

A total of 20 HA standards and an internal standard 4,7,8-TriMeIQx, as listed in Table 1, were procured from Toronto Research Chemicals (Downsview, ON, Canada) and used for separation, identification, validation and quantitation of HAs by an UPLC system from Agilent Technologies Co. (Palo Alto, CA, USA) equipped with a diode-array detector and Acquity BEH C18 column (100 × 2.1 mm internal diameter, particle size 1.7 μm) from Waters Corp. (Milford, MA, USA). The same mobile phase of 20 mM of ammonium acetate

(pH 4.5) and acetonitrile with a gradient mode as reported by Lai et al. [4] was used for separation of all 21 HA standards as well as in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork, with the detailed experimental conditions being provided in the Supplementary Materials. For identification and quantitation, 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) was used in the selected reaction monitoring (SRM) mode, with the detailed experimental conditions being provided in the Supplementary Materials.

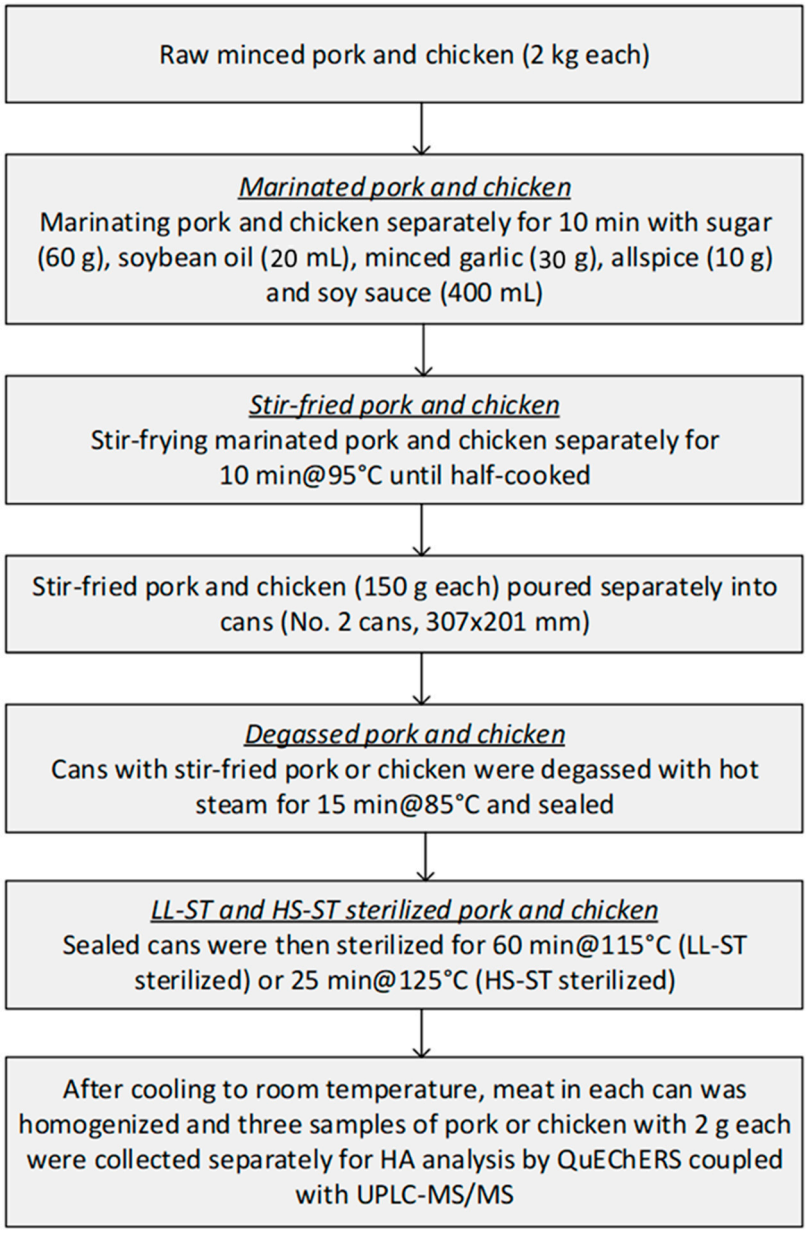


Figure 1. Processing steps for preparation of canned minced pork and chicken. LL–ST, low-temperature–long-time sterilization for 60 min at 115 °C; HS–ST, high-temperature–short-time sterilization for 25 min at 125 °C.

2.5. Matrix Effect, Method Validation and Quantitation of HAs in Raw Chicken and Pork

The determination of matrix effect, method validation parameters and quantitation of HAs was based on the methods reported by Lai et al. [4]. For determination of matrix effect, HA standard solutions at 5 concentrations (0.5, 1, 2, 5, and 10 ng/mL) were prepared in

methanol. Additionally, freeze-dried pork extract spiked with the same HA concentrations was prepared, followed by analyzing both solutions (HA standard and HA matrix solutions) by UPLC-MS/MS to prepare the standard calibration curve (SCC) and the matrix-matched calibration curve (MCC), respectively. The matrix effect was then calculated as the ratio of the MCC slope to SCC slope.

The limit of detection (LOD) was determined by preparing 7 concentrations (0.005, 0.01, 0.02, 0.03, 0.04, 0.05 and 0.1 ng/mL) of each HA standard and injecting into UPLC-MS/MS for analysis. The LOD was then obtained based on the signal-to-noise ratio (S/N) $\geq$ 3. For the limit of quantitation (LOQ), the same concentrations of HAs, excluding Harman and Norharman, were spiked into freeze-dried pork samples and subjected to extraction and analysis by UPLC-MS/MS, with the LOQ being determined based on S/N $\geq$ 10. Since Harman and Norharman were already present in the pork matrix, their LOQ was not determined in the spiked sample matrix but instead calculated from the standard solutions based on S/N $\geq$ 10.

The recovery study was done by spiking freeze-dried pork with two concentrations of each HA standard (1 and 10 ng/g), followed by extraction and analysis by UPLC-MS/MS. Recovery of each HA was calculated from the ratio of the detected amount to the spiked amount of the standard. The precision study involved determination of intra-day variability by spiking freeze-dried pork with HA standards at a concentration of 10 ng/g each, followed by extraction and analysis by UPLC-MS/MS in triplicate during the morning, afternoon and evening for a total of 9 analyses within the same day. Likewise, the inter-day variability was evaluated using the same procedure, with 9 analyses being performed 3 times a day for 3 successive days. Similarly, the LOD, LOQ, recovery and precision in freeze-dried chicken extract were determined by following the same procedure as described above.

Quantitation is performed by preparing standard calibration curves through injection of 10 concentrations (0.05, 0.1, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL) of HA standards in methanol, containing 1 ng/mL of internal standard (IS) 4,7,8-TriMeIQx, into UPLC-MS/MS for analysis. The concentration ratio (standard versus IS) was plotted against the peak area ratio (standard versus IS) to obtain the straight-line equations with their coefficients of determination (R^2). Next, matrix-matched calibration curves were prepared by extraction of freeze-dried pork samples by QuEChERS, followed by collecting supernatant (1 mL), evaporating to dryness, adding 5 concentrations of HA standards containing 1 ng/mL of 4,7,8-TriMeIQx and injecting into UPLC-MS/MS for preparation of straight-line equations with their R^2 values. However, the quantitation of Norharman and Harman was based on their respective standard calibration curves because both compounds were detected in the freeze-dried pork samples.

2.6. Determination of HA Precursors in Raw, MN, SF, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork

2.6.1. Creatine and Creatinine

Each sample (20 g) of chicken or pork was mixed with distilled water (100 mL) for homogenization (24,000 rpm for 2 min) and, after standing for 20 min (18 °C), the mixture was filtered through a filter paper and the filtrate was mixed with perchloric acid and potassium hydroxide (pH 6.5) for subsequent quantitation of creatine and creatinine in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork using the assay kits [14].

2.6.2. Amino Acid

The variety and content of amino acids in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork were determined using a standard method by the

Taiwan Food and Drug Administration (TFDA) [15]. After derivatization of amino acid in standard chicken or 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 determined using a HPLC method described in a previous report [4]. A detailed procedure is provided in the Supplementary Materials.

2.6.3. Reducing Sugar

The contents of reducing sugar in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork was determined using a method described by Chen et al. [16] and Lai et al. [4], with the detailed procedure being provided in the Supplementary Materials.

2.7. Statistical Analysis

Three homogenized samples with 2 g each from raw, MN, SF, DG, LL–ST sterilized and HS–ST sterilized minced chicken/pork were separately extracted and analyzed by UPLC-MS/MS, with each sample being analyzed for a total of 36 samples. The data were subjected to statistical analysis by analysis of variance and Duncan's multiple range test for comparison of the statistical significance of the mean values at $p < 0.05$ using the statistical analysis software system (SAS) (version 6, SAS Institute Inc., Gary, NC, USA) [17].

The mean HA content data obtained during canning of minced chicken and pork samples were analyzed by multivariate principal component analysis (PCA) using Origin® 2019b (Version 9.65, OriginLab Corporation, Northampton, MA, USA). This involved grouping HA contents from various treatments and converting original correlated variables into a new smaller set of linearly uncorrelated variables called principal components by considering eigenvalues > 1. PCA was run to explore the similarities and differences in HA contents among raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork, with a KMO (Kaiser–Meyer–Olkin) test value of 0.80 and $p < 0.05$.

3. Results and Discussion

3.1. Proximate Analysis of Raw, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork During Canning

The proximate analysis data of raw, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork during the canning process was reported in a previous study [13], with the moisture, ash, crude fat and crude protein contents in minced chicken ranging, respectively, from 70.88 to 74.04%, 1.15 to 3.34%, 2.17 to 2.27% and 22.63 to 23.51%, while in minced pork, it ranged from 68.12 to 71.37%, 0.98 to 2.94%, 12.05 to 12.45% and 15.53 to 16.80%. The comprehensive proximate analysis data reported by Inbaraj et al. [13] are provided in the Supplementary Materials (Table S1). The results indicated that the moisture, ash and crude protein contents were higher in raw, DG and ST (LL–ST and HS–ST) samples of minced chicken, while the crude fat content was present in higher levels in raw, degassed and canned minced pork, which should facilitate the formation of more HAs in minced pork than in minced chicken during processing.

3.2. Analysis of HAs by UPLC-MS/MS in Raw, MN, SF, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork During Canning

After QuEChERS extraction/purification, the HAs formed in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork were analyzed by UPLC-MS/MS. A total of 21 HA standards, including internal standard 4,7,8-TriMeIQx, were separated within 4 min with the retention times ranging from DMIP (0.50 min) to MeAαC (3.25 min) (Table 1). The precursor ions (m/z 163.1–242.13) and product ions used for confirmation (m/z 78.05–201.21) and quantitation (m/z 92.1–213.09) of 20 HAs are also

shown in Table 1. The matrix effect ranged from 0.63 (DMIP) to 0.97 (IFP), which was evaluated for improvement in accuracy and precision of HA analysis (Table S2). The LOD ranged from 0.005 ng/g (DMIP and IFP) to 0.05 ng/g (IQ) and the LOQ from 0.01 ng/g (DMIP) to 0.10 ng/g (IQ, IQx and IQ[4,5-b]) in freeze-dried pork, while, in freeze-dried chicken, the LOD ranged from 0.003 ng/g (DMIP) to 0.04 ng/g (IQ[4,5-b] and 7,8-DiMeIQx) and the LOQ from 0.005 ng/g (DMIP) to 0.05 (IQ[4,5-b] and 7,8-DiMeIQx) (Table S2). The recovery of 20 HAs ranged from 74.4 to 95.4% for standards, 51.9 to 119.1% for freeze-dried pork and 65.1 to 116.6% for freeze-dried chicken, all of which met the requirement set by TFDA [18], stating that the acceptable recovery should be in a range of 50–125% for the analyte concentration at 0.001 ppm (Table S3). In addition, the coefficient of variation (CV) ranged from 5.2 to 10.5%, 5.9 to 28.4% and 7.3 to 18.3% for intra-day variability in HA standards, HAs in freeze-dried raw pork and HAs in freeze-dried chicken, respectively, as well as from 6.9 to 14.7%, 9.2 to 33.7% and 6.1 to 15.5% for inter-day variability (Table S4–S6), all of which met the requirement of CV at <35% for the former and <36% for the latter set by TFDA for the analyte concentration at $\leq$0.001 ppm [18].

3.3. HA Content Changes in in Raw, MN, SF, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork During Canning

Table 2 shows the changes in the contents of various HAs formed in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork during canning. Three HAs including DMIP (1.94 ng/g), Norharman (0.05 ng/g) and Harman (0.12 ng/g) were detected in raw chicken, which may arise from a polluted environment such as in water, air and soil. Following marinating, six more HAs including 7,8-DiMeIQx (0.82 ng/g), Phe-P-1 (0.09 ng/g), Trp-P-2 (2.31 ng/g), PhIP (3.49 ng/g), Trp-P-1 (14.92 ng/g) and MeAαC (trace) were generated in chicken. Meanwhile, the DMIP, Norharman, and Harman contents rose substantially to 22.15, 14.31 and 181.04 ng/g, respectively. The addition of soy sauce in a marinade may contribute to a large increase in Harman and Norharman [19]. However, the participation of sugar in the Maillard browning reaction for HA formation during marinating cannot be ignored. Additionally, the moisture absorption by marinade from chicken may result in a rise in total HAs in marinated chicken. In a previous study, Herraiz [20] also reported the presence of a high level of Norharman (44 ng/g) and Harman (187.6 ng/g) in soy sauce. After stir-frying in a pan for 10 min at 95 °C, most HAs showed an increased trend, with the exception of Harman and PhIP, both of which declined to 46.31 and 1.47 ng/g, respectively. Compared to the other HAs, a lower stability was shown for Harman and PhIP during heating, which can be attributed to a low activation energy of 8.2 and 12.7 kJ/mol, respectively [21]. Furthermore, the total HAs decreased from 239.13 to 145.69 ng/g during stir frying. Interestingly, during degassing for 15 min at 85 °C, most HAs showed a minor variation, except for DMIP and Harman, as evident by a drop of 9.22 ng/g for the former and an increase of 28.40 ng/g for the latter. At the same time, the total HAs rose to 170.94 ng/g. Following sterilization for 60 min at 115 °C, most HAs showed a rise phenomenon, especially DMIP, Norharman and Harman, with an increase by 10.23, 25.07 and 96.79 ng/g, respectively, being observed. Additionally, the total HAs also climbed to a maximum (300.66 ng/g). However, for sterilization for 25 min at 125 °C, a minor variation in most HAs was shown, except for DMIP (decreased by 9.27 ng/g), accompanied by a decline in total HAs to 290.74 ng/g. This outcome revealed that the LL–ST condition (60 min at 115 °C) can be more susceptible to HA formation than the HS–ST condition (25 min at 125 °C).

Table 2. Contents of HAs (ng/g) in raw minced chicken and pork as well as their marinated, stir-fried, degassed, LL–ST sterilized and HS–ST sterilized samples collected during the canning process [1,2].

	Raw	Marinated	Stir-Fried (10 min at 95 °C)	Degassed (15 min at 85 °C)	LL–ST (60 min at 115 °C)	HS–ST (25 min at 125 °C)
			Chicken			
DMIP	1.94 ± 0.08 ^{g}	22.15 ± 1.19 ^{e}	34.01 ± 2.01 ^{a}	24.79 ± 1.2 ^{de}	35.02 ± 3.19 ^{a}	25.75 ± 2.24 ^{cde}
Glu-P-2	nd [3]	nd	nd	nd	trace	trace
8-MeIQx	nd	nd	nd	nd	0.59 ± 0.01 ^{a}	0.61 ± 0.02 ^{a}
IQ[4,5-b]	nd	nd	nd	nd	trace	trace
7,8-DiMeIQx	nd	0.82 ± 0.01 ^{d}	1.05 ± 0.05 ^{bc}	1.00 ± 0.03 ^{c}	1.09 ± 0.04 ^{bc}	1.11 ± 0.14 ^{abc}
Norharman	0.05 ± 0.01 ^{e}	14.31 ± 0.9 ^{d}	18.6 ± 1.81 ^{cd}	20.69 ± 1.16 ^{c}	45.76 ± 3.00 ^{b}	47.43 ± 5.62 ^{ab}
Harman	0.12 ± 0.01 ^{f}	181.04 ± 17.02 ^{b}	46.31 ± 2.91 ^{e}	74.71 ± 5.24 ^{d}	171.5 ± 16.77 ^{b}	167.26 ± 11.84 ^{b}
Phe-P-1	nd	0.09 ± 0.02 ^{e}	0.16 ± 0.01 ^{d}	0.20 ± 0.01 ^{cd}	0.20 ± 0.04 ^{cd}	0.24 ± 0.02 ^{ab}
Trp-P-2	nd	2.31 ± 0.05 ^{e}	4.86 ± 0.07 ^{cd}	5.73 ± 0.11 ^{b}	4.39 ± 0.17 ^{c}	4.69 ± 0.62 ^{bc}
PhIP	nd	3.49 ± 0.15 ^{d}	1.47 ± 0.05 ^{f}	2.16 ± 0.02 ^{e}	4.77 ± 0.32 ^{b}	4.75 ± 0.30 ^{b}
Trp-P-1	nd	14.92 ± 1.00 ^{c}	39.24 ± 1.60 ^{b}	41.67 ± 2.02 ^{b}	37.33 ± 1.90 ^{b}	38.90 ± 5.01 ^{b}
MeAaC	nd	trace [4]	trace	trace	trace	trace
Total	2.11 ± 0.20 ^{e}	239.13 ± 20.06 ^{c}	145.69 ± 7.30 ^{d}	170.94 ± 5.55 ^{d}	300.66 ± 21.50 ^{b}	290.74 ± 21.66 ^{b}
			Pork			
DMIP	0.30 ± 0.00 ^{g}	12.43 ± 1.44 ^{f}	30.04 ± 1.26 ^{b}	28.82 ± 3.49 ^{bc}	27.91 ± 3.65 ^{bcd}	23.14 ± 0.22 ^{e}
Glu-P-2	nd	nd	nd	nd	trace	trace
8-MeIQx	nd	nd	nd	nd	0.60 ± 0.02 ^{a}	0.58 ± 0.01 ^{a}
IQ[4,5-b]	nd	nd	nd	nd	trace	trace
7,8-DiMeIQx	nd	0.77 ± 0.01 ^{d}	1.25 ± 0.04 ^{a}	1.14 ± 0.07 ^{abc}	1.17 ± 0.14 ^{ab}	1.08 ± 0.07 ^{bc}
Norharman	0.07 ± 0.10 ^{e}	14.82 ± 1.10 ^{d}	20.98 ± 0.96 ^{c}	23.19 ± 1.58 ^{c}	51.98 ± 4.95 ^{a}	49.10 ± 2.82 ^{ab}
Harman	0.10 ± 0.00 ^{f}	162.31 ± 19.85 ^{b}	95.13 ± 4.31 ^{d}	120.83 ± 10.36 ^{c}	220.57 ± 31.72 ^{a}	167.25 ± 6.10 ^{b}
Phe-P-1	nd	0.09 ± 0.01 ^{e}	0.27 ± 0.02 ^{a}	0.23 ± 0.02 ^{bc}	0.19 ± 0.01 ^{cd}	0.23 ± 0.01 ^{bc}
Trp-P-2	nd	1.98 ± 0.09 ^{e}	6.53 ± 0.29 ^{a}	6.10 ± 0.15 ^{ab}	5.14 ± 0.75 ^{d}	4.82 ± 0.10 ^{cd}
PhIP	nd	3.72 ± 0.27 ^{d}	3.75 ± 0.10 ^{d}	4.28 ± 0.21 ^{c}	7.93 ± 0.62 ^{a}	5.40 ± 0.13 ^{a}
Trp-P-1	nd	14.02 ± 0.60 ^{c}	49.64 ± 1.46 ^{a}	48.77 ± 1.24 ^{a}	41.24 ± 6.6 ^{b}	39.46 ± 2.62 ^{b}
MeAaC	nd	trace	trace	trace	trace	trace
Total	0.48 ± 0.11 ^{e}	210.14 ± 21.79 ^{c}	207.60 ± 6.36 ^{c}	233.37 ± 16.37 ^{c}	356.74 ± 48.32 ^{a}	291.07 ± 8.54 ^{b}

[1] Data are presented as mean ± standard deviation of triplicate determinations and each HA content data with different small letters (a–g) in the same row are significantly different ($p < 0.05$). [2] The full name of each HA compound is shown in Table 1. [3] nd = not detected. [4] trace = limit of quantitation (LOQ) ≥ HAs levels ≥ limit of detection (LOD). LL–ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 60 min at 115 °C; HS–ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 25 min at 125 °C.

A similar trend was found for canned minced pork under various treatments (Table 2). Only three HAs including PHIP (0.30 ng/g), Norharman (0.07 ng/g) and Harman (0.10 ng/g) were detected in raw pork, and these rose greatly to 12.43, 14.82 and 162.317 ng/g, respectively. Following marinating, six more HAs including 7,8-DiMeIQx (0.77 ng/g), Phe-P-1 (0.09 ng/g), Trp-P-2 (1.98 ng/g), PhIP (3.72 ng/g), Trp-P-1 (14.02 ng/g) and MeAαC (trace) were generated, accompanied by a rise in total HAs to 210.14 ng/g. During stir-frying in a pan for 10 min at 95 °C, most HAs followed an increased trend except for Harmon, which dropped to 95.13 ng/g, but with a slight change in total HAs. Interestingly, degassing for 15 min at 85 °C showed only a minor change in most HAs except for Harman, which was raised to 120.83 ng/g, concomitant with a rise in total HAs to 233.37 ng/g. Following LL–ST sterilization treatment, the Norharman and Harman contents rose substantially to 51.98 and 220.57 ng/g, respectively, as well as to 49.10 and 167.25 ng/g for the HS–ST sterilization treatment.

By comparison, both the marinating and sterilization treatments showed a higher effect on total HA formation in canned minced chicken, with the former being more impactful, implying that the marinade composition and time length of marinating play a crucial role in HA formation. Nevertheless, among the various heating treatments, sterilization is the most important factor contributing to HA formation in canned minced chicken, especially for the LL–ST treatment. Furthermore, it appears that time length is more important than temperature in affecting HA formation in chicken during sterilization. Like with canned

minced chicken, the LL–ST sterilization treatment is more liable to total HA formation than the HS–ST sterilization treatment. However, under the same sterilization condition, the total HAs were produced at a higher level in canned minced pork than in canned minced chicken, probably caused by the difference in fat content.

As protein-bound iron exists in the form of soluble proteins, myoglobin, ferritin and some other iron-complexed proteins such as transferrin, ovotransferrin and lactotransferrin, cooking of meat causes a release of iron from heme proteins, while ferritin releases ferrous ion (Fe^{2+}) in the presence of reducing agents such as ascorbate and thiols [22]. Interestingly, ascorbate may act as a prooxidant at low concentration by reducing iron to a low valence state or as an antioxidant at high concentration by scavenging free radicals [22]. Additionally, cooking can disrupt cell membrane in meat and promote lipid oxidation through interaction of polyunsaturated fatty acids with ferrous ion for subsequent formation of HAs. Thus, heating and grinding raw meat accelerates lipid oxidation, while Maillard reaction products formed at >100 °C on meat surface was shown to inhibit lipid oxidation and development of warmed-over flavor [22]. The presence of spice in marinade may possess antioxidant activity as it contains polyphenols such as flavonoids and phenolic acids.

In a previous study Min et al. [23] reported that both the fat and unsaturated fatty acid content of raw pork tenderloin were higher than that of raw chicken breast, implying that the former was more liable to lipid oxidation than the latter. During heating of edible oil, Liu et al. [24] further reported that the degradation of Harman and Norharman during heating was dependent on oil type, temperature and time length. Thus, the reduction of Harman during stir-frying is probably due to reaction between β-carboline and lipid oxidation products during heating. Moreover, Harman should be more liable to react with lipid degradation products than Norharman because of higher activation energy of the latter [21]. But for some other HAs such as Glu-P-2, IQ[4,5-b], Trp-P-2 and MeAαC, they only showed insignificant changes during stir-frying, degassing and sterilization (LL–ST/HS–ST), which can be attributed to high activation energy of these HAs [21]. Thus, Harman is the dominant one responsible for HA formation in canned minced chicken and pork during sterilization. Yao et al. [25] analyzed HAs in commercially available braised beef sauce and found that the total amount of HAs in vacuum-packed braised beef samples (retort pouch) was higher than that in freshly cooked braised beef purchased from local restaurants, probably because that the former product requires a commercial sterilization step resulting in a higher level of HAs.

3.4. Amino Acid Content Changes in Raw, MN, SF, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork During Canning

Table 3 also shows the individual amino acid content (mg/g) changes in canned minced chicken and pork. Only a slight variation in individual and total amino acid contents was shown following marinating. However, after SF, DG and ST (LL–ST and HS–ST) treatments, a slight decline in total amino acid contents was found, implying that amino acids participated in HA formation through reaction with creatine/creatinine and reducing sugar. Compared with LL–ST sterilization treatment, a lower amount of total HAs in chicken and pork samples for the HS–ST sterilization treatment indicated that more amino acids involved in HA formation (Table 2). Under the same sterilization condition (LL–ST or HS–ST), the total amino acid content was lower in pork than in chicken, revealing the formation of a higher level of total HAs in canned minced pork as shown in Table 2.

Table 3. Contents of HA precursors including amino acids (mg/g), reducing sugar (mg/g), creatine (mg/100 g) and creatinine (mg/100 g) in raw minced chicken and pork as well as their marinated, stir-fried, degassed, LL–ST sterilized and HS–ST sterilized samples collected during the canning process [1].

	Raw	Marinated	Stir-Fried (10 min at 95 °C)	Degassed (15 min at 85 °C)	LL–ST (60 min at 115 °C)	HS–ST (25 min at 125 °C)
			Chicken			
Amino acid						
Aspartic acid	0.64 ± 0.08 ^{abc}	0.70 ± 0.03 ^{a}	0.63 ± 0.05 ^{bc}	0.59 ± 0.00 ^{cd}	0.55 ± 0.01 ^{d}	0.67 ± 0.00 ^{ab}
Glutamic acid	1.20 ± 0.06 ^{de}	1.48 ± 0.01 ^{a}	1.35 ± 0.05 ^{c}	1.41 ± 0.02 ^{b}	1.25 ± 0.03 ^{d}	1.18 ± 0.03 ^{e}
Serine	0.30 ± 0.02 ^{de}	0.33 ± 0.00 ^{ab}	0.27 ± 0.00 ^{fg}	0.30 ± 0.01 ^{de}	0.28 ± 0.00 ^{f}	0.26 ± 0.01 ^{g}
Glycine	0.66 ± 0.03 ^{a}	0.52 ± 0.00 ^{c}	0.50 ± 0.01 ^{c}	0.50 ± 0.04 ^{c}	0.50 ± 0.02 ^{c}	0.49 ± 0.01 ^{c}
Threonine	0.17 ± 0.01 ^{abc}	0.19 ± 0.02 ^{a}	0.17 ± 0.01 ^{ab}	0.16 ± 0.00 ^{bc}	0.13 ± 0.01 ^{d}	0.17 ± 0.00 ^{abc}
Arginine	0.60 ± 0.00 ^{bcd}	0.57 ± 0.00 ^{de}	0.57 ± 0.02 ^{de}	0.56 ± 0.02 ^{e}	0.62 ± 0.01 ^{b}	0.48 ± 0.01 ^{f}
Alanine	0.73 ± 0.03 ^{abc}	0.74 ± 0.01 ^{ab}	0.68 ± 0.03 ^{de}	0.69 ± 0.03 ^{cde}	0.65 ± 0.01 ^{ef}	0.62 ± 0.01 ^{f}
Tyrosine	0.33 ± 0.02 ^{a}	0.31 ± 0.00 ^{bc}	0.30 ± 0.02 ^{cd}	0.27 ± 0.00 ^{ef}	0.25 ± 0.00 ^{g}	0.28 ± 0.02 ^{de}
Cystine	0.24 ± 0.03 ^{b}	0.27 ± 0.01 ^{a}	0.23 ± 0.01 ^{b}	0.17 ± 0.00 ^{de}	0.16 ± 0.02 ^{ef}	0.20 ± 0.01 ^{cd}
Valine	0.41 ± 0.05 ^{d}	0.49 ± 0.01 ^{a}	0.46 ± 0.02 ^{b}	0.47 ± 0.00 ^{ab}	0.41 ± 0.01 ^{d}	0.42 ± 0.01 ^{cd}
Methionine	0.25 ± 0.01 ^{bc}	0.26 ± 0.00 ^{ab}	0.25 ± 0.01 ^{bc}	0.24 ± 0.01 ^{d}	0.20 ± 0.00 ^{f}	0.23 ± 0.01 ^{d}
Phenylalanine	0.35 ± 0.02 ^{e}	0.40 ± 0.01 ^{a}	0.37 ± 0.01 ^{bcd}	0.36 ± 0.00 ^{cde}	0.32 ± 0.01 ^{f}	0.33 ± 0.01 ^{f}
Isoleucine	0.38 ± 0.03 ^{fg}	0.47 ± 0.00 ^{a}	0.44 ± 0.02 ^{bc}	0.45 ± 0.00 ^{ab}	0.37 ± 0.01 ^{g}	0.40 ± 0.01 ^{def}
Leucine	0.93 ± 0.05 ^{ab}	0.95 ± 0.02 ^{a}	0.89 ± 0.03 ^{bc}	0.88 ± 0.04 ^{bc}	0.81 ± 0.01 ^{de}	0.81 ± 0.01 ^{de}
Lysine	0.90 ± 0.11 ^{a}	0.53 ± 0.06 ^{cd}	0.71 ± 0.13 ^{b}	0.59 ± 0.03 ^{bc}	0.46 ± 0.05 ^{de}	0.70 ± 0.05 ^{b}
Proline	0.39 ± 0.01 ^{a}	0.29 ± 0.00 ^{cd}	0.31 ± 0.05 ^{bc}	0.19 ± 0.00 ^{f}	0.19 ± 0.01 ^{f}	0.29 ± 0.01 ^{cd}
Total amino acid	**8.49 ± 0.01** ^{a}	**8.52 ± 0.07** ^{a}	**8.13 ± 0.12** ^{b}	**7.83 ± 0.19** ^{c}	**7.14 ± 0.07** ^{e}	**7.53 ± 0.16** ^{d}
Reducing sugar	**nd** [2]	**7.25 ± 0.07** ^{g}	**15.02 ± 0.18** ^{a}	**13.75 ± 0.37** ^{c}	**11.46 ± 0.12** ^{e}	**11.66 ± 0.32** ^{e}
Creatine	**407.08 ± 24.24** ^{a}	**358.24 ± 22.20** ^{b}	**320.17 ± 14.56** ^{c}	**301.39 ± 21.70** ^{cd}	**51.7 ± 1.85** ^{e}	**51.18 ± 5.61** ^{e}
Creatinine	**13.13 ± 0.95** ^{g}	**24.35 ± 0.44** ^{f}	**33.44 ± 0.56** ^{ef}	**56.34 ± 0.90** ^{d}	**204.07 ± 17.72** ^{b}	**214.62 ± 4.49** ^{b}
			Pork			
Amino acid						
Aspartic acid	0.60 ± 0.01 ^{cd}	0.70 ± 0.04 ^{a}	0.60 ± 0.02 ^{cd}	0.44 ± 0.01 ^{e}	0.62 ± 0.02 ^{bc}	0.55 ± 0.00 ^{d}
Glutamic acid	1.47 ± 0.02 ^{a}	1.41 ± 0.03 ^{b}	1.38 ± 0.03 ^{bc}	1.39 ± 0.00 ^{bc}	1.20 ± 0.02 ^{de}	1.21 ± 0.01 ^{de}
Serine	0.34 ± 0.00 ^{a}	0.34 ± 0.01 ^{a}	0.31 ± 0.00 ^{cd}	0.32 ± 0.00 ^{bc}	0.30 ± 0.01 ^{e}	0.26 ± 0.00 ^{g}
Glycine	0.59 ± 0.03 ^{b}	0.59 ± 0.07 ^{b}	0.52 ± 0.03 ^{c}	0.61 ± 0.03 ^{ab}	0.40 ± 0.01 ^{d}	0.53 ± 0.01 ^{c}
Threonine	0.16 ± 0.02 ^{bc}	0.17 ± 0.02 ^{abc}	0.17 ± 0.01 ^{abc}	0.15 ± 0.00 ^{bc}	0.16 ± 0.01 ^{bc}	0.15 ± 0.00 ^{c}
Arginine	0.60 ± 0.02 ^{bdc}	0.60 ± 0.03 ^{bcd}	0.58 ± 0.01 ^{cde}	0.66 ± 0.01 ^{a}	0.59 ± 0.01 ^{cd}	0.61 ± 0.00 ^{bc}
Alanine	0.76 ± 0.03 ^{a}	0.74 ± 0.05 ^{ab}	0.67 ± 0.02 ^{de}	0.70 ± 0.01 ^{bcd}	0.54 ± 0.01 ^{g}	0.65 ± 0.01 ^{ef}
Tyrosine	0.28 ± 0.01 ^{de}	0.32 ± 0.00 ^{ab}	0.28 ± 0.00 ^{de}	0.26 ± 0.00 ^{fg}	0.23 ± 0.02 ^{h}	0.26 ± 0.00 ^{efg}
Cystine	0.22 ± 0.02 ^{bc}	0.14 ± 0.00 ^{f}	0.17 ± 0.01 ^{ef}	0.18 ± 0.02 ^{de}	0.10 ± 0.01 ^{g}	0.16 ± 0.02 ^{ef}
Valine	0.46 ± 0.00 ^{b}	0.47 ± 0.01 ^{ab}	0.46 ± 0.00 ^{b}	0.46 ± 0.00 ^{b}	0.44 ± 0.01 ^{bc}	0.42 ± 0.01 ^{cd}
Methionine	0.25 ± 0.00 ^{bc}	0.27 ± 0.01 ^{a}	0.23 ± 0.00 ^{d}	0.21 ± 0.00 ^{e}	0.20 ± 0.00 ^{f}	0.21 ± 0.00 ^{e}
Phenylalanine	0.38 ± 0.01 ^{bc}	0.38 ± 0.00 ^{b}	0.36 ± 0.01 ^{e}	0.35 ± 0.00 ^{e}	0.33 ± 0.01 ^{f}	0.33 ± 0.01 ^{f}
Isoleucine	0.45 ± 0.00 ^{ab}	0.45 ± 0.00 ^{ab}	0.42 ± 0.01 ^{cd}	0.40 ± 0.00 ^{de}	0.39 ± 0.01 ^{efg}	0.38 ± 0.01 ^{fg}
Leucine	0.96 ± 0.04 ^{a}	0.94 ± 0.07 ^{ab}	0.86 ± 0.03 ^{cd}	0.83 ± 0.00 ^{de}	0.66 ± 0.02 ^{e}	0.80 ± 0.01 ^{e}
Lysine	0.66 ± 0.00 ^{bc}	0.66 ± 0.06 ^{b}	0.54 ± 0.00 ^{cd}	0.45 ± 0.10 ^{de}	0.37 ± 0.02 ^{e}	0.53 ± 0.05 ^{cd}
Proline	0.27 ± 0.02 ^{de}	0.33 ± 0.01 ^{b}	0.18 ± 0.00 ^{f}	0.17 ± 0.01 ^{f}	0.11 ± 0.01 ^{g}	0.25 ± 0.02 ^{e}
Total amino acid	**8.45 ± 0.06** ^{a}	**8.51 ± 0.14** ^{a}	**7.74 ± 0.14** ^{cd}	**7.59 ± 0.14** ^{d}	**6.64 ± 0.15** ^{f}	**7.31 ± 0.06** ^{e}
Reducing sugar	**nd**	**7.03 ± 0.14** ^{g}	**14.46 ± 0.22** ^{b}	**14.62 ± 0.13** ^{b}	**10.77 ± 0.12** ^{f}	**12.38 ± 0.24** ^{d}
Creatine	**429.14 ± 18.84** ^{a}	**327.17 ± 28.30** ^{c}	**319.86 ± 20.96** ^{c}	**287.1 ± 18.80** ^{d}	**48.72 ± 1.86** ^{e}	**60.72 ± 2.32** ^{e}
Creatinine	**17.54 ± 0.65** ^{g}	**28.16 ± 0.69** ^{ef}	**35.1 ± 0.75** ^{e}	**49.75 ± 0.44** ^{d}	**174.55 ± 4.10** ^{c}	**227.23 ± 4.77** ^{a}

[1] Data are presented as mean ± standard deviation of triplicate determinations and each HA precursor content data with different small letters (a–g) in the same row are significantly different ($p < 0.05$). [2] nd, not detected. LL–ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 60 min at 115 °C; HS–ST, raw minced chicken or pork after marinating, stir-frying, degassing and sterilizing for 25 min at 125 °C.

3.5. Reducing Sugar Content Changes in Raw, MN, SF, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork During Canning

The reducing sugar content (mg/g) changes in canned minced chicken and pork during processing are shown in Table 3. No reducing sugar was detected in raw chicken and pork, but the reducing sugar contents rose to 7.25 and 7.03 mg/g, respectively, following marinating. After stir-frying for 10 min at 95 °C, the reducing sugar content also increased significantly to 15.02 and 14.46 mg/g in chicken and pork, respectively, which should be due to decomposition of sucrose into glucose and fructose. Then the reducing sugar contents slightly decreased to 13.75, 11.46 and 11.6 mg/g in chicken as well as to 14.62,

10.77 and 12.38 mg/g in pork, respectively, following the DG, LL–ST and HS–ST treatments. Compared with the HS–ST treatment, a lower amount of reducing sugar in both chicken and pork samples was found for the LL–ST treatment, implying more reducing sugar participated in HA formation through reaction with creatine/creatinine and amino acid. However, for the LL–ST treatment (60 min at 115 °C), a lower reducing sugar content in pork indicated that a higher level of total HAs was produced in canned minced pork as shown in Table 2. But this phenomenon was not shown for the HS–ST treatment for 25 min at 125 °C, revealing that the LL–ST treatment possessed a higher impact on formation of total HAs in chicken. Like reducing sugar, some other reducing compounds such as vitamin C, vitamin E and natural antioxidants can also inhibit the formation of HAs [2,26,27]. However, the tendency of vitamin C and vitamin E to act as an antioxidant or prooxidant may depend on their concentration as mentioned above.

3.6. Creatine and Creatinine Content Changes in Raw, MN, SF, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork During Canning

Table 3 shows creatine and creatinine content changes in canned minced chicken and pork during processing. Theoretically creatine can be hydrolyzed to creatinine, a precursor for HA formation, when muscle tissue is heated. Consequently, the formation and degradation reactions of creatinine can occur simultaneously during processing of meat products [4]. In raw chicken, a high amount of creatine (407.08 mg/100 g) and a low level of creatinine (13.13 mg/100 g) was shown. Following marinating, the creatine content dropped to 358.24 mg/100 g, accompanied by a rise of creatinine to 24.35 mg/100 g, apparently caused by hydrolysis of creatine. The creatine content further declined to 320.17, 301.39, 51.70 and 51.18 mg/100 g in minced chicken after SF, DG, LL–ST and HS–ST treatments, respectively, accompanied by an increment of creatinine to 33.44, 56.34, 204.07 and 214.62 mg/100 g. Among the various heating treatments, sterilization is the most important one contributing to creatinine formation. Compared to the LL–ST treatment, a higher level of creatinine was produced for the HS–ST treatment, indicating a higher content of total HAs in minced chicken generated for the former treatment (Table 2). Similar outcome was found for the creatine and creatinine contents in canned minced pork during processing. Following MN, SF, DG, LL–ST and HS–ST treatments, the creatine contents dropped to 327.17, 319.86, 287.10, 48.72 and 60.72 mg/100 g, respectively, concomitant with formation of creatinine at 28.16, 35.10, 49.75, 174.55 and 227.23 mg/100 g, respectively.

Like canned minced chicken, a higher level of creatinine was generated in canned minced pork for the HS–ST sterilization treatment when compared with the LL–ST sterilization treatment. Additionally, a lower creatinine content was shown in pork than in chicken for the LL–ST sterilization treatment, leading to a higher content of total HAs in canned minced pork as shown in Table 2. However, this phenomenon was not observed for the HS–ST sterilization treatment. In other words, the LL–ST sterilization showed a higher impact on formation of total HAs in pork.

3.7. Composition of Fatty Acid in Raw, MN, SF, DG and ST (LL–ST and HS–ST) Samples of Minced Chicken and Pork During Canning

The composition of fatty acids in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork during canning was determined in a previous study [13] and the results are provided in the Supplementary Materials (Table S7). The fat content was shown to be higher in raw and sterilized pork than in raw and sterilized chicken. More specifically, the polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) in raw, LL–ST and HS–ST chicken accounted for 0.30% and 0.48%, 0.66% and 0.58%, and 0.59% and 0.49%, respectively, while the saturated fatty acids accounted for 0.35%, 0.48% and 0.37%. An analogous trend was shown in raw, LL–ST and HS–ST sterilized pork,

with PUFA accounting for 1.71%, 2.52% and 2.09%, respectively, as well as 4.73%, 5.53% and 4.79% for MUFA, and 3.84%, 4.64% and 4.03% for saturated fatty acid. Comparatively, MUFA and PUFA contributed to the largest portion of fat in LL–ST sterilized chicken and pork, followed by HS–ST sterilized chicken and pork as well as raw chicken and pork. As linoleic acid (PUFA) is more prone to oxidation than oleic acid (MUFA), the former should play a vital role in formation of HAs in canned minced chicken and pork, as evident by the data shown in Table 2. Compared to LL–ST and HS–ST sterilized chicken, a much higher level of MUFA and PUFA was found in pork with the same treatments, which should result in a higher level of total HAs in pork under the same treatment (Table 2).

In a study dealing with the effect of canning condition (100 min at 115 °C) on tuna in olive oil and sardine in tomato juice, the degree of lipid oxidation of sardine meat was less due to presence of low lipid content (1.16%) [28]. Similarly, Naseri et al. [29] used silver carp as raw material to prepare canned product and studied the effect of different filling media including brine, soybean oil, sunflower oil and olive oil on lipid oxidation, no significant difference ($p > 0.05$) was shown in the level of thiobarbituric acid reactive substances (TBARs), a secondary product produced during lipid oxidation, between raw fish and precooked fish (48 min at 102 °C), but the TBARs value rose substantially after sterilization at 65 min at 121 °C. A similar result was observed in canned eel when sterilized at 30 min at 118 °C [30]. Obviously, the sterilization treatment can facilitate lipid oxidation for hydroperoxide formation and degradation, especially for oils rich in PUFA, leading to formation of more free radicals and secondary products such as malondialdehyde for subsequent generation of HAs such as Harman in canned meat products through Maillard reaction. In a previous study Jing et al. [31] also demonstrated that acrolein, the simplest unsaturated aldehyde formed through oxidation of PUFA during heating, can contribute to PhIP formation through formation of some key intermediate such as aldol condensation product. Furthermore, the oxidation of tilapia fish rich in PUFA was shown to result in a higher content of PhIP during roasting of fish patties [31]. This should explain why a higher level of PhIP was observed in sterilized chicken and pork under LL–ST/HS–ST condition in our study when compared with the other treatments including marinating, stir-frying and degassing. Most importantly, the prevention of formation of lipid oxidation products may lead to a reduction of HAs in meat products such as beef patties during cooking [32].

3.8. Principal Component Analysis (PCA)

Figure 2 shows the PCA for formation of HAs in raw, MN, SF, DG and ST (LL–ST and HS–ST) samples of minced chicken and pork during canning as affected by different processing treatments. A total of four principal components including PC 1 (64.95%), PC 2 (19.03%) and PC 3 (10.39%) were respectively obtained for an eigenvalue >1 at 10.39, 3.05 and 1.66. Among the three PCs, PC 1 and PC 2 contributed to a total variation of 83.99% in HAs formation at the studied conditions. The score plot in Figure 2A reveals that the principal component data can be grouped into four groups based on the extent of separation of one group from the others in four quadrants. Groups 1 and 2 represent various HAs formed at 12 different treatments including raw chicken (rc) or raw pork (rp), marinated chicken (mc) or pork (mp), stir-fried chicken (fc) or pork (fp), degassed chicken (dc) or pork (dp), LL–ST sterilized chicken (sc1) or pork (sp1) for 60 min at 115 °C and HS–ST sterilized chicken (sc2) or pork (sp2) for 25 min at 125 °C. Furthermore, both ‘rc’ and ‘rp’ in group 1 distinctly separated from the remaining 10 treatments in group 2, revealing a significantly higher level of HA formation in the latter group (145.69–300.66 ng/g in chicken and 207.60–356.74 ng/g in pork) than in raw meat (2.11 ng/g in chicken and 0.48 ng/g in pork), as evident from Table 2. Likewise, the total amount of HAs formed in chicken and pork (c and p) regardless of processing condition were clustered in group 3, confirming that

significant levels of HAs were produced at all studied conditions, with a distinct separation of data points between 'c' and 'p' indicating that HAs were formed at significantly different levels in chicken and pork (Table 2). In the final group 4, the total amount of HAs formed at different treatments including marinating, stir-frying and degassing regardless of meat type represented as 'o' was largely separated from the amount of HAs formed at LL–ST and HS–ST sterilization conditions represented as 's', implying that HAs formed under sterilization conditions (290.74–300.66 ng/g in chicken and 291.07–356.74 ng/g in pork) are much higher than that formed under the other processing conditions (145.69–239.13 ng/g in chicken and 207.60–233.37 ng/g in pork) (Table 2).

Figure 2B illustrates the biplot consisting of both loading plot and score plot with a larger deviation in angle between groups 1 and 2 confirming that significant levels of HAs were formed under different processing conditions compared to their levels in raw chicken and raw pork. Also, the loading plots corresponding to total HA contents formed in chicken and pork in group 3 as well as that formed in raw chicken and raw pork in group 1 were separated by an angle of deviation, revealing a significant difference in HAs formation between the two types of meat. A larger degree of angle between the loading plots represents a higher variation or smaller correlation in HAs formed under a specific processing condition [13]. Most importantly, a larger degree of angle between the loading plot of HAs formed under sterilization conditions ('s') and the other processing conditions ('o') in group 4 corroborates again that HAs were formed at higher levels under sterilization conditions compared to the other processing conditions.

Figure 2B also shows the score plots of individual HA formation with four asterisks in quadrants I and II denoting that the formation of four individual HA compounds, regardless of meat type and processing condition, highly impacted the PC 1 and the asterisk approaching the vertical zero line at the center of Figure 2B represents their decreasing levels of formation: Harman > Trp-P-1 > Norharman > DMIP. The remaining five HA compounds denoted as five asterisks in quadrants III and IV highly impacted the PC 2 with the asterisk positions approaching the vertical zero line at the center of Figure 2B representing their decreasing levels of formation: Trp-P-2 > PhIP > 7,8-DiMeIQx > 8-MeIQx > Phe-P-1. The above trend is in line with the respective total content of each HA along with the row in Table 2 regardless of meat type and processing condition, with the four HA compounds in quadrants I and II contributing to 95.8% of total HAs formation while the remaining five HA compounds in quadrants III and IV contributing to only 4.2% of total HAs formation (Table 2). All in all, the PCA confirmed the results that a significant amount of HAs was generated in minced chicken and pork during the canning process with HAs being more susceptible to formation under LL–ST and HS–ST sterilization conditions.

Taken together, HAs are usually formed during thermal processing of protein-rich foods especially meat products, posing a serious health risk in people exposed to HAs through dietary intake. They act as mutagenic and carcinogenic compounds causing detrimental effects in the human body with several HAs being reported to be 10-fold more carcinogenic than the other food toxicants including benzo[α]pyrene, aflatoxins B1 and nitrosamines [33]. In this study we mainly aim to investigate the processing of canned minced pork and chicken based on the standard pretreatment used for preparation of canned minced pork and chicken in Taiwan, which involved marinating (10 min) minced pork or chicken (2 kg) with sugar (60 g), allspice (10 g), soy sauce (400 mL), soybean oil (20 mL) and minced garlic (30 g), followed by stirring (10 min) and pouring into a pan for stir-frying (10 min at 95 °C, SF), degassing with hot steam (15 min at 85 °C) and sterilizing separately at low temperature-long time treatment (60 min at 115 °C, LL–ST) and high temperature-short time sterilization (25 min at 125 °C, HS–ST). The results showed that both marinating and sterilization treatments generated a higher HA formation in canned minced

chicken and pork with the LL–ST treatment being more liable to total HA formation than the HS–ST treatment, implying that time length is a more important factor than temperature affecting HA formation. Under the same sterilization condition, total HAs was produced at a higher level in canned minced pork than in canned minced chicken, probably due to the difference in fat content (2.17–2.27% crude fat in canned chicken and 12.05–12.45% in canned pork), with the level of Harman being generated the most, followed by Trp-P-1, Norharman and DMIP. Specifically, the formation of non-mutagenic DMIP and comutagens Harman and Norharman, as well as the possible carcinogen Trp-P-1, contributed to 95.8% of total HAs in both canned minced pork and chicken; additionally, low levels of some other possible carcinogens such as 8-MeIQx, Trp-P-2, PhIP and MeAαC were detected.

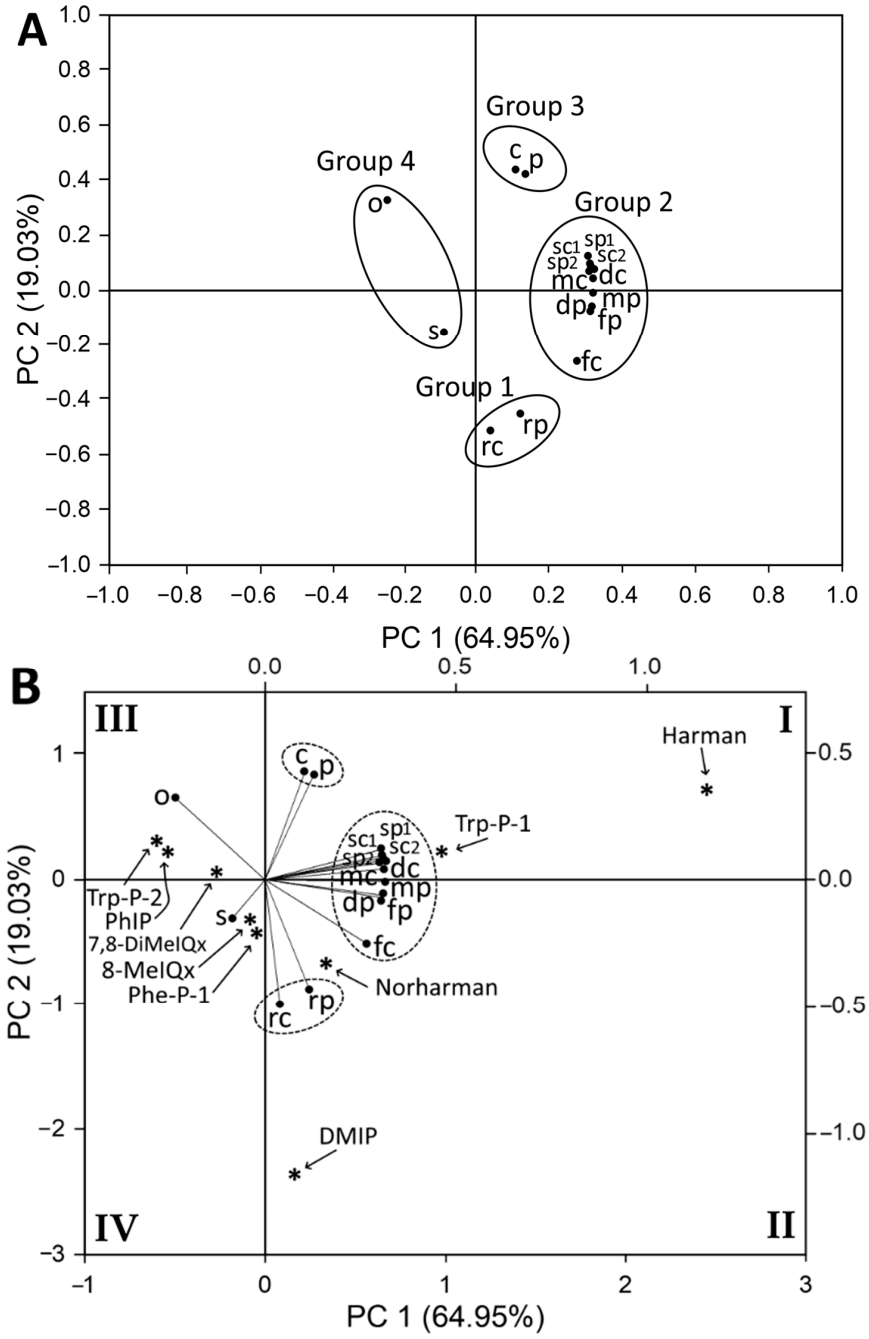


Figure 2. Principal component analysis for HAs formation in canned minced chicken and minced pork as affected by different processing conditions with the plots showing the score plot (**A**) and the biplot

consisting of loading plot and score plot (**B**). HAs, heterocyclic amines; rp, the amount of HAs formed in unprocessed raw chicken and raw pork; mc and mp, the amount of HAs formed in marinated chicken and pork; fc and fp, the amount of HAs formed in stir-fried chicken and pork; dc and dp, the amount of HAs formed in degassed chicken and pork; sc1 and sc2, the amount of HAs formed in LL–ST sterilized chicken for 60 min at 115 °C and HT-ST sterilized chicken for 25 min at 125 °C; sp1 and sp2, the amount of HAs formed in LL–ST sterilized pork for 60 min at 115 °C and HT-ST sterilized pork for 25 min at 125 °C; c and p, the amount of HAs formed in chicken and pork regardless of processing condition; o, the total amount of HAs formed in marinated meat, stir-fried meat and degassed meat regardless of meat type and processing condition; s, the total amount of HAs formed in LL–ST sterilized meat for 60 min at 115 °C and HS–ST sterilized meat for 25 min at 125 °C regardless of meat type and sterilization condition. The dark dot (●) symbol denotes principal component data for the formation of HAs in chicken or pork under the above processing conditions. The asterisk (∗) symbol represents the principal component data of individual HA formed under the above processing conditions.

Several epidemiological studies have shown that HAs are one of the leading causes of various types of cancers including colon, pancreatic, breast, prostate and other cancers [34–37]. For instance, a meta-analysis study by Gibis et al. [38] demonstrated a positive association of dietary intake of HAs including PhIP and DiMeIQx with the risk of colon cancer, while a high intake of MeIQ and total HAs was shown to increase the risk of colorectal cancer in women [39]. Both epidemiological investigations and surveys using questionnaires have linked a high dietary intake of red meat and processed meat with a high risk of several types of human cancers including colorectal, gastric and breast cancers [37,38,40,41]. The consumption of processed meat was shown to increase the probability of breast, pancreatic, prostate and colon cancers by 9%, 19%, 4% and 18%, respectively [42]. Moreover, HAs exposure was shown to exert mutagenic effects on *Salmonella typhimurium* and mammalian cells, with HAs such as IQ, MeIQ and MeIQx exhibiting much higher mutagenic activity than PhIP, AαC and MeAαC, implying that a marked difference in mutagenic activity may be exerted among various types of HAs [5,33]. As mentioned above, the negative Ames test results for Harman and Norharman revealed the non-mutagenic nature of these HAs. However, their presence was shown to enhance the mutagenicity of the other HA compounds by acting as comutagens [43]. Thus, dietary HA exposure to humans can cause both carcinogenic and mutagenic effects, as demonstrated in several animal models including mice, rats and monkeys [33,44].

In addition, the HAs may also increase the risk of obesity and cardiovascular disease through activating oxidative stress [45]. After absorption into the human body, HAs are metabolized by various enzymes to generate a reactive product N-acetoxyamine, resulting in DNA damage [36]. After further mutations, the generated metabolites are conjugated with DNA to form DNA adducts or excreted via urine and feces [33]. To reduce dietary HA exposure in humans, three major strategies can be employed, which include (1) controlling the level of precursors, (2) regulating the cooking conditions and (3) incorporating natural ingredients rich in antioxidants [46–49]. These strategies can reduce the formation of HAs as well as modulate both the bioaccessibility and metabolism of HAs [46]. Several comprehensive review articles have addressed these mitigation strategies [1–3,8,33,43,46,47] and their appropriate application may protect humans from HA exposure through various dietary sources.

4. Conclusions

A QuEChERS extraction method coupled with UPLC-MS/MS was successfully employed to determine the formation of HAs in canned minced chicken and pork as affected by different processing conditions including marinating, stir-frying, degassing, LL–ST sterilization for 60 min at 115 °C and HS–ST sterilization for 25 min at 125 °C. Compared to

the other processing conditions, both marinating and sterilization had a high impact on the formation of HAs in canned minced chicken and pork, with the LL–ST sterilization resulting in a higher level of total HAs in canned minced pork compared with canned minced chicken. The HA formation can be attributed to a decline in the contents of reducing sugar, creatine and amino acids during processing. PCA corroborated the results, showing that HAs were formed at significant levels in canned minced chicken and pork during processing, with the levels of major HAs following the order of Harman > Trp-P-1 > Norharman > DMIP. Although IQ, listed in the Group 2A category (probably carcinogenic to humans) by the IARC, remained undetected during the canning process of pork and chicken in this study, as well as low levels of 8-MeIQx, Trp-P-1, Trp-P-2, PhIP and MeAαC being classified in the Group 2B category (possibly carcinogenic to humans), and non-mutagenic DMIP, Harman and Norharman being detected in canned pork and chicken after sterilization, it is important to reduce the consumption of processed meat, increase the intake of fruits and vegetables and incorporate natural antioxidant-rich ingredients into foods during processing to minimize HA exposure in humans.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr13010153/s1. Experimental methods for QuEChERS extraction/purification of HAs, HAs analysis by UPLC-MS/MS and determination of HA precursors such as amino acid and reducing sugar in raw, marinated, stir-fried, degassed and canned minced chicken and pork. 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). 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. Table S3—Recovery (%) of 20 HA standards and HAs in freeze-dried pork and chicken detected by UPLC-MS/MS. Table S4—Intra-day variability and inter-day variability of 20 HA standards detected by UPLC-MS/MS. Table S5—Intra-day variability and inter-day variability of HAs in freeze-dried pork detected by UPLC-MS/MS. Table S6—Intra-day variability and inter-day variability of HAs in freeze-dried chicken detected by UPLC-MS/MS. 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.

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