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# Effects of heating time and antioxidants on the formation of heterocyclic amines in marinated foods

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**Abstract**

The effect of heating time and antioxidants on the heterocyclic amine (HAs) formation in marinated foods were studied. Food samples were cooked at 98 ± 2 °C for 1, 2, 4, 8, 16 and 32 h in a closed pan in the presence of water, soy sauce and rock candy with or without antioxidants. The various HAs in marinated food samples and juice were analyzed by HPLC with photodiode-array detection. Results showed that the amount of HAs formed during heating followed an increased order for each increasing heating time. A larger variety and higher amount of HAs were generated in marinated pork when compared to marinated eggs and bean cake. In marinated juice, the levels of HAs were present in greater amount than in marinated foods. The incorporation of antioxidants Vitamin C, Vitamin E and BHT were found to be effective towards HAs inhibition, however, the effect was minor.


*Keywords:* Heating time; Food analysis; Antioxidants; Heterocyclic aromatic amines

## 1. Introduction

Heterocyclic amines (HAs) are important toxicological and carcinogenic compounds that could be formed during cooking of meat products. Many HAs have been characterized, however, several HAs such as 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) were the most commonly reported [1,2]. It has been well established that the precursors for HAs formation were creatine or creatinine, sugars and amino acids, all common components of muscle foods [3,4].

The amount and variety of HAs formed in cooked meat products depend primarily on processing conditions, of which time and temperature are the most important [5,6]. Knize et al. [7] reported that the major HAs formed were IQ, MeIQx and DiMeIQx, when beef patties were cooked at 200, 250 and 300 °C for 6 min. Skog et al. [8] further demonstrated that with cooking temperatures 150, 175, 200 and 225 °C and heating time 2–11 min, the levels of HAs formed in beef and pork increased with increasing time and temperature. A high amount and large variety of HAs often occur in cooked meat products under drastic conditions such as frying at 200 or 300 °C for 10 min [5,9]. Due to the complex nature of foods, many researchers have used model systems to study the HAs formation mechanism [3,4]. Although model studies may provide the basic knowledge about the precursors and reaction products formed, the results may be different from food systems. The effect of antioxidants on HAs formation has been controversial. Johansson and Jagerstad [10] reported that the addition of TBHQ, Vitamin C and Vitamin E did not show antioxidative effect towards MeIQx formation. In another study, Chen [11] found that both BHA and TBHQ could reduce MeIQx formation. Thus, the possible inhibitory effect of HAs formation by incorporating an appropriate level of antioxidants to foods prior to cooking needs to be further investigated.

Marinating is a traditional Chinese cooking method, which is often conducted at about boiling temperature for an extensive period of time to enhance color, flavor and texture attributes of foods in the presence of water and various ingredients such as soy sauce and sugar [12]. "Chinese marinating" means immersing the food items at about 98 ± 2 °C for 1 h or longer in a closed pan. This definition is different from what most people understand

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about marinating, which implies preincubation with a fluid of some sort to impart flavor prior to cooking. Several HAs, including IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-l) and 2-amino-9H-pyrido[2,3-*b*]indole (AαC) have been detected in Chinese marinated foods such as ground pork, eggs and bean cake [12]. The authors also reported that the amounts of HAs produced in marinated food samples followed an increased order for each increasing level of soy sauce or sugar [12]. However, in another study Salmon et al. [2] showed that marinating prior to grilling could reduce PhIP concentration in grilled chicken. Obviously, this outcome could be accounted for by the difference in preparation of food samples for marinating. Little research has focused on how the Chinese marinating method affects the HAs formation in meat products [12]. The objectives of this study were to determine the HAs formation in Chinese marinated foods as affected by heating time and antioxidants.

## 2. Materials and methods

### 2.1. Materials

Sixteen HAs standards: 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQx; CAS No. 108354-47-8); IQ (CAS No. 76180-98-6); MeIQx (CAS No. 77500-04-0); 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (CAS No. 77094-11-2); 4,8-DiMeIQx (CAS No. 95896-78-9); 2-amino-3,4,8-trimethylimidazo[4,5-*f*] quinoxaline (CAS No. 92180-79-9); 2-amino-9H-pyrido[2,3-*b*]indole (AαC; CAS No. 26148-68-5); 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC; CAS No. 68806-83-7); 2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole (Glu-P-1; CAS No. 67730-11-4); 2-aminodipyrido[1,2-a:3′,2′-*d*]imidazole (Glu-P-2; CAS No. 67730-10-3); 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1; CAS No. 62450-06-0); 3-amino-1-methyl-5H-pyrido-[4,3-*b*]indole (Trp-P-2; CAS No. 62450-07-1); 1-methyl-9H-pyriido[4,3-*b*]indole (Harman; CAS No. 486-84-0); 9H-pyrido[4,3-*b*]indole (Norharman; CAS No. 244-63-3); 2-amino-1-methyl-6- phenylimidazo[4,5-*f*]pyridine (PhIP; CAS No. 105650-23-5) and internal standard 2-amino-3,4,7,8-tetramethylimidazo[4,7,8-*f*]quinoxaline (4,7,8-TriMeIQx) were used. Chemicals including sodium hydroxide, trichloroacetic acid, ammonium acetate, lead acetate, potassium oxalate and hydrochloric acid were from Sigma (St. Louis, MO). Solvents such as acetonitrile, methanol and methylene chloride were from Merck (Darmstadt, Germany). The HPLC-grade solvent acetonitrile was degassed by sonication prior to use. Deionized water was made using a water purification system by Millipore (Bedford, MA). The propylsulfonic acid silica gel catridge (500 mg) was from Varian Co. (Harbor, CA) and C18 catridge (100 and 500 mg) were from J.C. Baker Co. (Philipsburg, NJ). A TSK-CEL ODS C18 column (250 mm × 4.6 mm i.d., 5 μm) from Tosoh Co. (Tokyo, Japan) was used for separation of HAs by HPLC. Raw materials such as ground pork, eggs and bean cakes as well as ingredients soy sauce and rock candy were purchased from a local supermarket in Taipei. Rock candy was used instead of sugar because it is composed of 95% sucrose and imparts a desirable color to marinated foods.

### 2.2. Instrumentation

The HPLC instrument is made up of Jasco MD-915 photodiode-array detector (Jasco Co., Tokyo, Japan), a Rheodyne 7161 injector (Rheodyne Co., Rohert Park, CA), a Phenomenex DG-440 degassing system (Phenomenex Co., Torrance, CA), two Jasco PU-980 pumps and a Borwin computer software for processing data. The Beckman 6300 amino acid analyzer was from Beckman Co. (Fullerton, CA, USA). The homogenizer (Model HG-2800) was from Hsiang-Tai Co. (Taipei, Taiwan). The Sorvall RC5C high speed centrifuge was from Du Pont Co. (Wilmington, Delware).

### 2.3. Processing of marinated foods

Ground pork, eggs and bean cake were used as raw materials for marinating separately. Initially the 2 l juice was prepared for each saucepan by mixing 20 g (1%) sugar 200 ml (10%) soy sauce and 1780 ml deionized water. Each juice was then poured into a 4 l stainless-steel saucepan with a lid on the top and heated by gas. After boiling, 500 g raw ground pork was poured into the saucepan and cooking was continued for 1, 2, 4, 8, 16 or 32 h with a small fire. The temperature was controlled at $98 \pm 2\,^{\circ}$C and monitored with a thermometer all the time during marinating. Six saucepans were used for ground pork, and the cooked juice was referred to as “marinated juice”. Raw eggs and bean cakes were also prepared and heated separately in the same way as ground pork, except that the raw eggs were boiled at $100\,^{\circ}$C for 5 min and then shelled prior to marinating. After marinating, 50 g ground pork and 20 ml marinated juice were collected separately for HAs analysis. Prior to analysis, the ground pork was freeze-dried and ground into fine powder, and 10 g sample was obtained for HAs determination. Both eggs and bean cakes were cut into small pieces and freeze-dried before grinding into fine powder for HAs analysis. The marinated juice was analyzed directly for the HAs content. For each heating treatment, two eggs with an average weight of about 15 g each and four bean cakes with an average weight of about 25 g each were used. A total of 14 eggs and 28 bean cakes, which include two unheated samples of each were used.

To study the effect of antioxidant on the formation of HAs in marinated foods, the 2 l juice was prepared by mixing 200 ml (10%) soy sauce, 20 g (1%) sugar, 4 g antioxidant (0.2%) and 1776 ml deionized water. Antioxidants including ascorbic acid, α-tocopherol and BHT were added separately to the juice for comparison of antioxidative activity. Food samples were marinated and collected in the same way for

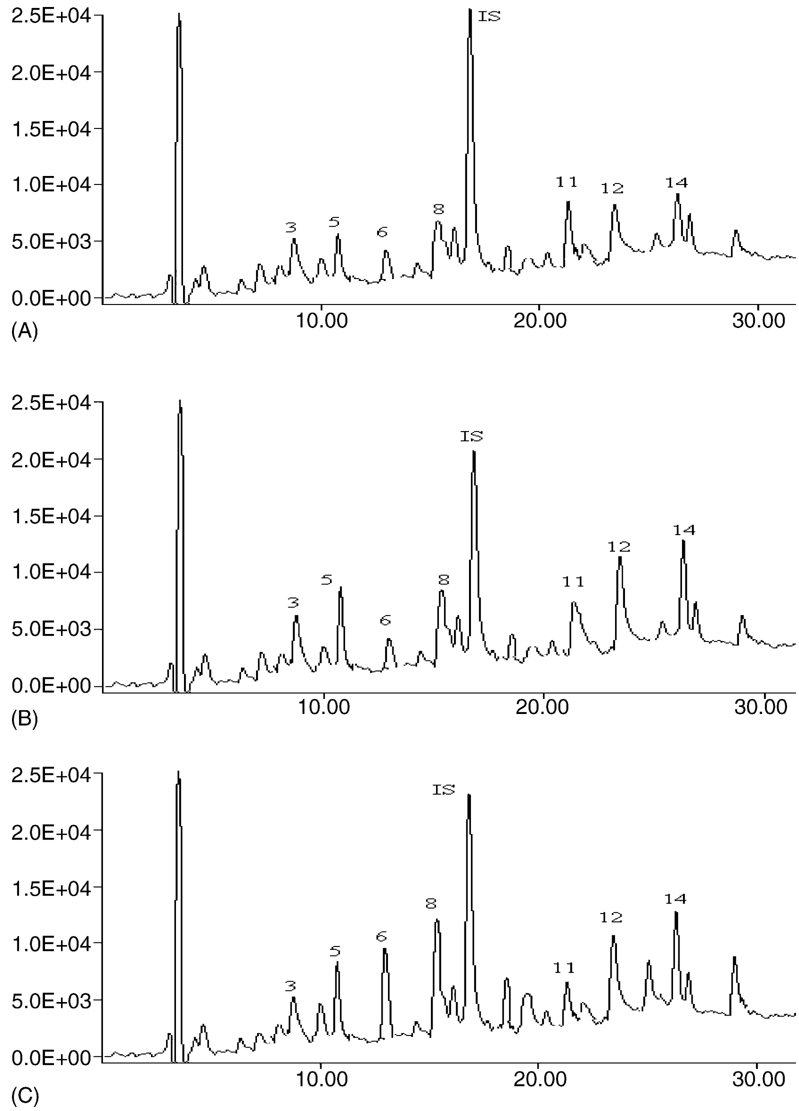


Fig. 1. HPLC chromatograms of HAs extracts from marinated pork after cooking for varied length of time. (A) 1 h, (B) 8 h, and (C) 32 h. Peaks: 3, IQ; 5, MeIQx; 6, MeIQ; 8, 4,8-DiMeIQx; 11, Trp-P-1; 12, PhIP; 14, AαC; IS, internal standard, 4,7,8-TriMeIQx.

HAs analysis as described above with the exception that the cooking time was 1 h.

### *2.4. Analysis of reducing sugars (glucose) in marinated juice and food samples*

The levels of glucose in marinated juice and food samples were measured based on a method in a previous report [6].

### *2.5. Analysis of creatinine in marinated juice and food samples*

The contents of creatinine in marinated juice and food samples were determined using a method described by Henry [13].

### *2.6. Analysis of amino acids in soy sauce and marinated food samples*

The levels of amino acids in soy sauce and marinated food samples were analyzed using a method described in a previous study [6].

### *2.7. Analysis of HAs in marinated food samples*

A method developed by Gross and Gruter [14] and modified by Chen and Yang [15] was used to analyze the HAs content in marinated food samples and juice. Briefly, 10 g of ground food sample or 20 ml of marinated juice was mixed with 10 ml of 1 M NaOH and homogenized for 3 min. Methylene chloride (50 ml) was added and the solution was centrifuged. The residue was extracted and centrifuged again. The two extracts were combined, concentrated to 10 ml and poured into a silica gel cartridge for purification. The apolar HAs were eluted with 15 ml methanol–0.1 M hydrochloric acid (40:60 (v/v)) and 2 ml water, while the polar HAs were eluted with 20 ml 0.5 M ammonium acetate solution (pH 8.0). The apolar extract was neutralized with ammonia solution and poured into a 500 mg C18 cartridge to remove the impurities. The cartridge was then dried with nitrogen gas and the apolar HAs eluted with 1.4 ml methanol–ammonia solution (9:1 (v/v)). Likewise, the polar extract was poured into a 100 mg C18 cartridge and 2 ml water was added to remove the impurities. The cartridge was dried with nitrogen gas and then the polar HAs were eluted with 0.8 ml methanol–ammonia solution (9:1 (v/v)). Both apolar and polar extracts were pooled and filtered through a 0.2 μm membrane filter. The combined extract was evaporated under nitrogen gas and dissolved in 100 μl methanol containing internal standard 4,7,8-TriMeIQx 1 ng/μl for HPLC analysis. For recovery determination, a mixture of 50 ng each of 15 HAs standards were added to samples for extraction. The recovery of each HA was obtained based on a ratio of the calculated concentration to the added concentration. The results were corrected for incomplete recovery. The identification of HAs in food samples was carried out by comparing retention times and spectra of unknown peaks with reference standards, as well as co-chromatography with added standards. Duplicate analyses were performed and the data were subjected to analysis of variance and Duncan's multiple range test using SAS [16].

## 3. Results and discussion

### 3.1. Quality control of the analysis

The coefficient of variation of the intra-day variability for retention times of 16 HAs standards were 0.05–0.18%, respectively, while the inter-day variability was 0.09–1.94%. The detection limits of 15 HAs standards based on a signal-to-noise ratio of three were: IQ (0.1 ng), IQx (0.2 ng), MeIQ (0.1 ng), MeIQx (0.2 ng), 4,8-DiMeIQx (0.05 ng), 7,8-DiMeIQx (0.1 ng), Glu-P-1 (0.1 ng), Glu-P-2 (0.2 ng), Harman (0.1 ng), Norharman (0.1 ng), Trp-P-1 (0.05 ng), Trp-P-2 (0.1 ng), AαC (0.1 ng), MeAαC (0.3 ng) and PhIP (0.02 ng). In food samples, the quantitation limits for IQ, MeIQx, MeIQ, 4,8-DiMeIQx, Trp-P-1, PhIP and AαC were 0.13, 0.30, 0.15, 0.06, 0.12, 0.03 and 0.21 ng, respectively [12]. The recoveries of 15 HAs based on duplicate analyses were found to be: IQ (88.6 ± 2.7), IQx (91.6 ± 3.1%), MeIQ (88.7 ± 6.2%), MeIQx (89.7 ± 6.2%), 4,8-DiMeIQx (86.4 ± 5.3%), 7,8-DiMeIQx (84.6 ± 5.1%), Glu-P-1 (85.7 ± 3.0%), Glu-P-2 (92.1 ± 5.2%), Harman (87.4 ± 1.8%), Norharman (90.3 ± 3.2%), Trp-P-1 (70.9 ± 3.6%), Trp-P-2 (84.0 ± 3.1%), AαC (74.8 ± 6.1%), MeAαC (73.2 ± 2.5%) and PhIP (62.1 ± 4.3%) [12].

### 3.2. Effect of heating time on the HAs formation in marinated pork and juice

Table 1 shows the change of HAs contents in marinated pork and juice during heating (see also Fig. 1). An increased order was found for the HAs contents in marinated pork and juice along with increasing heating time. Seven HAs, including IQ, MeIQx, MeIQ, 4,8-DiMeIQx, Trp-P-1, PhIP and AαC were present in marinated pork, and the levels increased by 1.39, 7.41, 0.39, 1.69, 7.40, 5.81 and 1.84 ng/g, respectively, after 32 h cooking. The total amounts of HAs showed the same trend with an increase of 25.93 ng/g. The marinated juice also showed an increase of 24.59 ng/g. In most pork and juice samples, MeIQx was produced in largest amount, followed by PhIP, Trp-P-1, 4,8-DiMeIQx, IQ, AαC and MeIQ during the initial heating period. However, Trp-P-l was more readily formed than PhIP after prolonged heating. The glucose contents in marinated juice of pork after cooking for 1, 2, 4, 8, 16 and 32 h were 0.94, 0.52, 0.41, 0.63, 0.12 and 0.08 mg/ml, respectively. The sharp decrease of glucose level after prolonged heating may indicate that a portion of glucose can react with amino acids and creatinine in pork for HAs formation. However, the other reaction routes cannot be excluded. The creatinine contents in marinated juice of pork were 0.16, 0.10, 0.04 and 0.06 mg/ml, respectively, after cooking for 1, 2, 4 and 8 h. No creatinine was detected after 16 h cooking, revealing that a part of creatinine was used for formation of HAs because the yield of HAs was much smaller than the amount of creatinine added.

### 3.3. Effect of heating time on the HAs formation in marinated eggs and juice

Table 2 shows the change of HAs contents in marinated eggs during marinating for varied length of time. Four HAs, MeIQx, 4,8-DiMeIQx, Trp-P-1 and PhIP were detected. An increased trend was observed for all the levels and total amounts of HAs in eggs during heating. For a cooking period of 1–32 h, the amounts of MeIQx, 4,8-DiMeIQx, Trp-P-1 and PhIP increased by 3.45, 2.34, 2.92 and 2.34 ng/g, respectively. Similarly, a large increase of 15.50 ng/g was shown for the total amount of HAs in marinated juice after 32 h heating. Also, the levels of most HAs in marinated juice were higher than in marinated eggs. In most egg samples PhIP was present in highest amount, followed by MeIQx, 4,8-DiMeIQx and Trp-P-1, while in most juice samples, a lowest amount was found for 4,8-DiMeIQx, followed by Trp-P-1, PhIP and MeIQx.

The glucose contents in marinated juice of eggs after cooking for 1, 2, 4, 8, 16 and 32 h were 0.86, 0.74, 0.36, 0.32, 0.48 and 0.14 mg/ml, respectively. As stated before, the drastic reduction of glucose after cooking is mainly due to reaction with amino acids in soy sauce and creatinine (0.03 mg/g) in eggs for HAs formation. Compared to the glucose content in pork juice, a higher amount was found in egg juice for heating time 2, 16 and 32 h, probably

Table 1
HAs (ng/g) in marinated pork after cooking for varied length of time (h) in the presence of 10% soy sauce and 1% rock candy

| Compound | Time (h)[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 4 | | 8 | | 16 | | 32 | |
| | Pork | Juice | Pork | Juice | Pork | Juice | Pork | Juice | Pork | Juice | Pork | Juice |
| IQ | 0.74 (0.57)[b] | 1.52 (1.48) | 0.87 (0.65) | 1.63 (1.85) | 1.28 (2.35) | 1.74 (3.45) | 1.85 (1.98) | 1.88 (2.75) | 2.05 (5.72) | 2.04 (3.62) | 2.13 (3.42) | 2.23 (5.87) |
| MeIQx | 1.44 (1.28) | 1.82 (1.37) | 2.64 (3.12) | 3.34 (4.56) | 3.43 (5.12) | 3.47 (5.62) | 6.26 (5.37) | 5.44 (7.23) | 7.35 (9.23) | 8.55 (10.76) | 8.85 (10.75) | 9.15 (13.67) |
| MeIQ | 0.44 (0.37) | 0.84 (1.12) | 0.55 (0.76) | 0.77 (0.98) | 0.64 (0.78) | 0.83 (1.25) | 0.72 (1.24) | 0.83 (1.86) | 0.76 (0.98) | 0.85 (1.23) | 0.83 (0.78) | 0.93 (2.87) |
| 4,8-DiMeIQx | 1.85 (2.04) | 1.48 (1.69) | 1.92 (2.35) | 3.47 (3.86) | 2.32 (1.85) | 3.53 (3.98) | 2.44 (4.56) | 3.62 (4.78) | 2.48 (5.24) | 3.83 (6.76) | 3.54 (6.58) | 3.92 (6.35) |
| Trp-P-1 | 0.63 (0.49) | 1.03 (0.85) | 1.23 (1.47) | 2.63 (1.78) | 2.45 (3.76) | 3.47 (4.59) | 4.34 (5.98) | 5.62 (6.35) | 5.66 (6.17) | 7.25 (11.23) | 8.03 (11.42) | 8.44 (11.86) |
| PhIP | 1.33 (1.58) | 2.47 (3.12) | 1.85 (2.08) | 3.03 (5.62) | 2.53 (2.98) | 3.66 (5.87) | 3.26 (2.87) | 5.35 (7.42) | 5.44 (7.36) | 6.49 (9.78) | 7.14 (12.53) | 7.36 (9.43) |
| AαC | 0.62 (0.57) | 0.93 (0.78) | 0.97 (1.23) | 1.33 (2.48) | 1.27 (2.56) | 1.85 (2.36) | 1.37 (2.26) | 2.15 (5.68) | 1.97 (3.18) | 2.45 (2.96) | 2.46 (5.87) | 2.65 (2.87) |
| Total | 7.05 | 10.09 | 10.03 | 16.20 | 13.92 | 18.55 | 20.24 | 24.89 | 25.71 | 31.46 | 32.98 | 34.68 |

[a] Average of duplicate analyses.
[b] Values in parentheses represent ±S.D.

Table 2
HAs (ng/g) in marinated eggs after cooking for varied length of time in the presence of 10% soy sauce and 1% rock candy

| Compound | Time (h)[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 4 | | 8 | | 16 | | 32 | |
| | Egg | Juice | Egg | Juice | Egg | Juice | Egg | Juice | Egg | Juice | Egg | Juice |
| MeIQx | 0.63 (1.24)[b] | 0.94 (2.45) | 0.85 (1.86) | 1.44 (4.16) | 1.25 (4.56) | 2.18 (3.75) | 2.76 (5.16) | 3.26 (6.54) | 3.34 (6.24) | 4.84 (7.23) | 4.08 (6.72) | 5.62 (9.53) |
| 4,8-DiMeIQx | 0.32 (0.78) | 0.85 (1.16) | 0.85 (1.24) | 0.95 (2.78) | 1.23 (2.73) | 1.21 (2.76) | 1.85 (3.72) | 1.33 (4.28) | 2.33 (3.58) | 2.66 (5.68) | 2.85 (5.08) | 3.85 (4.89) |
| Trp-P-1 | 0.32 (0.96) | 0.63 (0.87) | 0.66 (0.78) | 1.33 (3.65) | 1.03 (1.89) | 1.42 (4.53) | 1.64 (4.18) | 1.68 (3.76) | 2.76 (4.92) | 2.85 (3.95) | 3.24 (6.73) | 3.47 (7.38) |
| PhIP | 0.53 (1.38) | 0.85 (1.23) | 0.96 (3.15) | 1.13 (1.98) | 1.66 (3.68) | 1.86 (3.89) | 2.13 (5.93) | 2.45 (5.78) | 3.47 (6.85) | 3.66 (4.87) | 5.42 (7.84) | 5.83 (6.75) |
| Total | 1.80 | 3.27 | 3.32 | 4.85 | 5.17 | 6.67 | 8.38 | 8.72 | 11.90 | 14.01 | 15.59 | 18.77 |

[a] Average of duplicate analyses.
[b] Values in parentheses represent ±S.D.

Table 3
Levels of HAs (ng/g) in marinated bean cake after cooking for varied length of time in the presence of 10% soy sauce and 1% rock candy

| Compound | Time (h)[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 4 | | 8 | | 16 | | 32 | |
| | Bean cake | Juice | Bean cake | Juice | Bean cake | Juice | Bean cake | Juice | Bean cake | Juice | Bean cake | Juice |
| IQ | 0.32 (1.39)[b] | 0.63 (2.75) | 0.44 (1.65) | 0.86 (2.34) | 0.66 (2.28) | 1.05 (3.62) | 0.85 (1.78) | 1.33 (3.28) | 1.75 (3.26) | 1.65 (2.78) | 1.96 (5.02) | 2.14 (5.28) |
| MeIQx | 0.68 (2.41) | 1.27 (2.18) | 1.66 (3.27) | 2.64 (5.28) | 3.53 (6.75) | 4.35 (7.28) | 4.33 (9.24) | 5.73 (9.26) | 5.72 (10.28) | 6.83 (10.37) | 6.74 (11.56) | 8.55 (11.26) |
| 4,8-DiMeIQx | 1.68 (3.56) | 1.13 (3.96) | 1.44 (5.89) | 1.41 (3.26) | 1.85 (3.24) | 1.86 (4.18) | 2.82 (5.86) | 2.03 (3.78) | 3.03 (5.23) | 2.75 (5.26) | 3.13 (5.89) | 3.18 (6.23) |
| PhIP | 1.07 (1.73) | 1.64 (4.24) | 1.93 (6.75) | 2.15 (4.93) | 2.35 (5.19) | 3.17 (6.98) | 3.47 (6.73) | 4.44 (9.28) | 4.65 (6.95) | 5.45 (9.38) | 6.45 (8.14) | 6.69 (10.57) |
| AαC | 0.45 (1.24) | 0.72 (1.38) | 0.52 (1.28) | 0.83 (2.54) | 0.83 (2.57) | 1.23 (3.47) | 1.33 (2.98) | 1.44 (2.78) | 1.72 (3.28) | 1.84 (4.17) | 1.85 (4.26) | 2.50 (4.67) |
| Total | 4.20 | 5.39 | 5.99 | 7.89 | 9.22 | 11.66 | 12.80 | 14.97 | 16.87 | 18.52 | 20.13 | 23.06 |

[a] Average of duplicate analyses.
[b] Values in parentheses represent ±S.D.

because less glucose is available for HAs formation. It has been well documented that HAs could be formed both in the absence and presence of glucose [17,18]. Interestingly, no creatinine was detected in egg juice after cooking time reached 2 h and above. A greater creatinine loss occurred for egg juice than pork juice after heating, which could be attributed to a much lower content of creatinine in raw eggs (0.03 mg/g). It may also be inferred that creatinine in eggs was leached into juice during marinating, which in turn reacted with glucose and amino acids for HAs formation. In addition, a large surface of ground pork was exposed during cooking, and the leaching of creatinine from pork into juice should be more proned to occur than eggs. Chen and Meng [4] reported that no HAs were formed when heating creatinine alone. Instead, by heating a mixture of glucose and creatinine, and of glucose, creatinine and phenylalanine, several HAs such as IQx, AαC and MeAαC were generated.

### *3.4. Effect of heating time on the HAs formation in marinated bean cake and juice*

Table 3 shows the change of HAs contents in marinated bean cake and juice during heating for 1, 2, 4, 8, 16 and 32 h. Five HAs, including IQ, MeIQx, 4,8-DiMeIQx, PhIP and AαC were present. During heating for 1–32 h, the amounts of IQ, MeIQx, 4,8-DiMeIQx, PhIP and AαC increased by 1.64, 6.06, 1.45, 5.38 and 1.40 ng/g, respectively, while the total amounts of HAs rose by 15.93 ng/g. Likewise, a larger increase of 17.67 ng/g occurred in marinated juice, which was much higher than marinated bean cake. In most bean cake and juice samples, MeIQx was the most abundant, followed by PhIP, 4,8-DiMeIQx, AαC and IQ. Surprisingly, the glucose content in marinated juice showed an inconsistent change. After cooking for 1, 2, 4, 8, 16 and 32 h, the glucose contents were 0.28, 0.60, 0.18, 0.54, 0.62 and 0.36 mg/ml, respectively. As explained before, the formation rate of glucose from rock candy or the reaction rate with amino acids or creatinine could be varied depending upon length of heating time. Yoshida et al. [19] reported that both AαC and MeAαC were formed when heated a model system containing soybean protein. As bean cake is a rich source of protein, the formation of AαC is probably due to reaction of amino acids because no creatinine was detected in marinated bean cake and juice. In another study, Arvidsson et al. [20] found that the level of HAs decreased with increasing heating time when heated a model system containing creatinine, glucose and amino acids at 150 °C. This result seemed to be contradictory to our observation, which may be postu1ated that in our experiment the heating temperature (98 ± 2 °C) was not high enough to cause HAs degradation. It is also possible that all the food samples were cooked in an environment full of amino acids and glucose, which should lead to a more HAs formation.

In most studies the HAs formed in cooked meat products belonged to the polar types such as MeIQx and PhIP [5,7–9], but there is an increase interest in the apolar HAs. In our study some polar HAs such as IQ, MeIQx, MeIQ, 4,8-DiMeIQx and PhIP were also present. This may be explained by the addition of various ingredients such as soy sauce and rock candy to the food samples during marinating, which could make the reaction for HAs formation more complex. In our experiment seven HAs were detected in marinated pork, while five HAs in eggs and four HAs in bean cake. The formation of IQ, PhIP, MeIQx or 4,8-DiMeIQx could be explained through heating a mixture containing creatinine and glucose or amino acids such as phenylalanine and serine [3], and the formation mechanism could involve several key steps such as dehydration, a retro-aldol reaction and the Strecker degradation. The effect of temperature on HAs formation in meat products has been controversial. For instance, no HAs were detected in ground beef when cooked at 100 °C for 20 min [22]. In contrast, Manabe et al. [23] found that PhIP was present when a liquid mixture of creatinine, phenylalanine and sugar were heated at 37 and 60 °C for 4 weeks. Arvidsson et al. [24] also reported that both Harman and Norharman were formed in a meat juice system after 100 °C heating for 150 min. Obviously the formation of HAs in a food commodity or in a model system as affected by various temperatures can be quite different. As the marinated food samples used in our study were found to contain an adequate amount of amino acids, reducing sugar and creatinine, the formation of a different variety of HAs is expected. Soy sauce was found to contain the following amino acids: aspartic acid 4.21 mg/100 g, threonine 1.39 mg/100 g, serine 2.05 mg/100 g, asparagine 0.04 mg/100 g, glutamic acid 11.0 mg/100 g, glutamine 0.06 mg/100 g, proline 2.21 mg/100 g, glycine 1.49 mg/100 g, alanine 2.35 mg/100 g, valine 1.53 mg/100 g, cystine 0.01 mg/100 g, methionine 0.49 mg/100 g, leucine 3.05 mg/100 g, tyrosine 0.85 mg/100 g, phenylalanine 1.74 mg/100 g, tryptophan 0.09 mg/100 g, lysine 2.18 mg/100 g, histidine 0.41 mg/100 g and arginine 1.57 mg/100 g. The levels of several amino acids in ground pork such as glycine, alanine and histidine were found to decline during marinating, mainly because of participation in the browning reaction for HAs formation. In addition, the basic amino acids such as histidine may undergo degradation more readily during heating because of presence of a basic nitrogen atom in the side chain. In contrast, an increase was shown for phenylalanine, serine and proline, which may be attributed to the degradation rate of protein being greater than the formation rate of HAs. The other reactions such as Maillard reaction may also be involved. The degradation of HAs would be difficult to occur in Chinese foods marinated at 100 °C unless a long heating time was used [12]. Moreover, a larger surface area was exposed in ground pork during marinating, and some more varieties of HAs should be produced. As time and temperature are the most important parameters affecting HAs formation, the cooking condition has to be carefully controlled. Some authors suggested that cooking at 85 °C for a short time could minimize HAs formation [21].

Table 4
Contents of HAs (ng/g) in marinated ground pork, egg and bean cake after cooking for 1 h in the presence of 10% soy sauce and 1% rock candy with or without ascorbic acid

| Compound | Ascorbic acid (0.2%)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Ground pork | | Egg | | Bean cake | |
| | Without ascorbic acid | With ascorbic acid | Without ascorbic acid | With ascorbic acid | Without ascorbic acid | With ascorbic acid |
| IQ | 0.74 (1.28)[b] | 0.72 (1.76) | N.D. | N.D. | 0.26 (0.78) | 0.24 (0.96) |
| MeIQx | 1.26 (1.43) | 1.31 (0.98) | 0.62 (1.08) | 0.68 (0.89) | 0.68 (1.47) | 0.53 (2.18) |
| MeIQ | 0.30 (0.65) | 0.28 (1.14) | N.D. | N.D. | N.D. | N.D. |
| 4,8-DiMeIQx | 1.28 (1.18) | 1.26 (0.59) | 0.26 (0.89) | 0.24 (0.43) | 0.70 (2.56) | 0.76 (1.85) |
| Trp-P-1 | 0.58 (0.24) | 0.31 (0.75) | 0.25 (0.64) | 0.22 (0.38) | N.D. | N.D. |
| PhIP | 1.26 (0.98) | 1.28 (1.53) | 0.46 (1.23) | 0.32 (0.59) | 1.12 (2.34) | 0.86 (1.75) |
| AαC | 0.56 (0.78) | 1.26 (1.75) | N.D. | N.D. | 0.40 (0.98) | 0.36 (1.04) |
| Total | 5.98 | 6.42 | 1.59 | 1.46 | 3.16 | 2.75 |

N.D.: not detected.
[a] Average of duplicate analyses.
[b] Values in parentheses represent ±S.D.

However, for the Chinese marinated foods, both the levels of soy sauce and rock candy should be reduced in order to prevent HAs formation [12]. In a previous study, Lan and Chen [12] further demonstrated that soy sauce played a more important role for HAs formation than rock candy.

### *3.5. Effect of antioxidants on HAs formation in marinated foods*

The retardation of HAs formation by incorporation of antioxidants is probably due to inhibition of free radicals. Johansson and Jagerstad [10] studied the effect of various antioxidants TBHQ, ascorbic acid, α- and γ-tocopherol on HAs inhibition and found that with the exception of ascorbic acid, the other antioxidants could promote HAs formation. The authors also suggested that the prooxidant or antioxidant effect was dependent on concentration. Table 4 shows the effect of ascorbic acid on HAs formation in marinated food samples. Without ascorbic acid, the total amounts of HAs in marinated pork, eggs and bean cake were 5.89, 1.59 and 3.16 ng/g, respectively. However, with ascorbic acid (4 g), an increase of 0.44 ng/g was found for marinated pork, while a decrease of 0.13 and 0.41 ng/g was observed for marinated eggs and bean cake, respective1y. The enhanced effect of HAs formation in marinated pork is probably because during marinating, the high fat content (24.6%) in pork may retard the penetration and thus reduce the antioxidative activity of water-soluble ascorbic acid. It is also possible that the formation of IQ-type HAs may be facilitated through Maillard reaction products such as pyridine from lipid degradation products [12]. For the individual HAs, an antioxidant effect was found for Trp-P-1 in marinated pork, Trp-P-1 and PhIP in eggs, as well as MeIQx, PhIP and AαC in bean cake.

The effect of α-tocopherol on the HAs formation in marinated food samples is shown in Table 5. In the presence of 4 g α-tocopherol, the total amounts of HAs declined by

Table 5
Contents of HAs (ng/g) in marinated ground pork, egg and bean cake after cooking for 1 h in the presence of 10% soy sauce and 1% rock candy with or without α-tocopherol

| Compound | α-Tocopherol (0.2%)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Ground pork | | Egg | | Bean cake | |
| | Without α-tocopherol | With α-tocopherol | Without α-tocopherol | With α-tocopherol | Without α-tocopherol | With α-tocopherol |
| IQ | 0.74 (1.28)[b] | 0.52 (0.83) | N.D. | N.D. | 0.26 (0.78) | 0.24 (0.58) |
| MeIQx | 1.26 (1.43) | 0.64 (1.16) | 0.62 (1.08) | 0.52 (1.08) | 0.68 (1.47) | 0.76 (2.18) |
| MeIQ | 0.30 (0.65) | 0.28 (0.57) | N.D. | N.D. | N.D. | N.D. |
| 4,8-DiMeIQx | 1.28 (1.18) | 0.64 (2.08) | 0.26 (0.89) | 0.38 (0.76) | 0.70 (2.56) | 0.56 (1.19) |
| Trp-P-1 | 0.58 (0.24) | 0.36 (0.34) | 0.25 (0.64) | 0.32 (0.58) | N.D. | N.D. |
| PhIP | 1.26 (0.98) | 1.36 (1.07) | 0.46 (1.23) | 0.38 (1.13) | 1.12 (2.34) | 0.98 (2.04) |
| AαC | 0.56 (0.78) | 0.42 (1.24) | N.D. | N.D. | 0.40 (0.98) | 0.41 (0.76) |
| Total | 5.98 | 4.22 | 1.59 | 1.60 | 3.16 | 2.95 |

N.D.: not detected.
[a] Average of duplicate analyses.
[b] Values in parentheses represent ±S.D.

Table 6
Contents of HAs (ng/g) in marinated ground pork, egg and bean cake after cooking for 1 h in the presence of 10% soy sauce and 1% rock candy with or without BHT

| Compound | BHT (0.2%)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Ground pork | | Egg | | Bean cake | |
| | Without BHT | With BHT | Without BHT | With BHT | Without BHT | With BHT |
| IQ | 0.74 (1.28)[b] | 0.72 (1.04) | N.D. | N.D. | 0.26 (0.78) | 0.24 (0.98) |
| MeIQx | 1.26 (1.43) | 1.23 (2.18) | 0.62 (1.08) | 0.52 (0.47) | 0.68 (1.47) | 0.52 (0.67) |
| MeIQ | 0.30 (0.65) | 0.26 (0.75) | N.D. | N.D. | N.D. | N.D. |
| 4,8-DiMeIQx | 1.28 (1.18) | 1.29 (3.17) | 0.26 (0.89) | 0.11 (0.34) | 0.70 (2.56) | 0.68 (1.25) |
| Trp-P-1 | 0.58 (0.24) | 0.42 (0.58) | 0.25 (0.64) | 0.35 (1.96) | N.D. | N.D. |
| PhIP | 1.26 (0.98) | 1.01 (2.46) | 0.46 (0.23) | 0.45 (0.83) | 1.12 (2.34) | 1.16 (2.85) |
| AαC | 0.56 (0.78) | 0.52 (1.28) | N.D. | N.D. | 0.40 (0.98) | 0.46 (0.76) |
| Total | 5.98 | 5.45 | 1.59 | 1.43 | 3.16 | 3.06 |

N.D.: not detected.
[a] Average of duplicate analyses.
[b] Values in parentheses represent ±S.D.

1.76 and 0.21 ng/g in marinated pork and bean cake, respectively, while no significant change was in marinated eggs. The greater inhibitory effect of α-tocopherol towards HAs formation in marinated pork could be attributed to a higher fat content of pork when compared to eggs or bean cake, and thus the solubility of α- tocopherol in pork was enhanced. In addition, a pronounced inhibition effect was found for IQ, MeIQx, 4,8-DiMeIQx, Trp-P-1 and AαC in pork, MeIQx and PhIP in eggs, as well as IQ, 4,8-DiMeIQx and PhIP in bean cake. This result seems to be contradictory to a report by Johansson and Jagerstad [10], who reported that both α- and γ-tocopherol could promote MeIQx formation when heated a model system containing creatinine, glycine, glucose and corn oil at 180 °C for 10 min. This difference could be due to the fact that only polar HAs were investigated in a model system by Johansson and Jagerstad [10]. In our experiment, a real food system was used and a different outcome may occur because of complex nature of food components. Similar to the result shown above, the total amounts of HAs decreased by 0.23, 0.16 and 3.06 ng/g in marinated pork, eggs and bean cake, respectively, in the presence of 4 g BHT (Table 6). The formation of MeIQ, Trp-P-1, PhIP and AαC was also inhibited in marinated pork, while in marinated eggs and bean cake, both MeIQx and 4,8-DiMeIQx, and MeIQx showed the same phenomenon, respectively.

By comparison of the results shown above, in most food samples the addition of antioxidants was effective against HAs formation. However, the activity was not pronounced because a dilution effect may occur for a small amount of antioxidant (4 g) in a large volume (2 l) of marinated juice. We also have to point out here that the Maillard reaction may proceed slowly after incorporation of ascorbic acid, however, the production of reducing sugar from sucrose under acidic condition can still participate in the Maillard reaction and resulted in HAs formation. Chen [11] reported that both BHA and TBHQ could reduce MeIQx formation while BHT could promote 4,8-DiMeIQx formation in ground beef during frying. Apparently the antioxidant activity could be affected by many parameters such as antioxidant concentration, method of cooking and variety of antioxidants and food systems.

## 4. Conclusion

In conclusion, a larger variety and higher amount HAs were produced in marinated pork than marinated eggs or bean cake. The HAs were present at a higher amount in marinated juice than in marinated foods, and the levels of HAs in food samples increased with increasing heating time. The incorporation of antioxidants was effective towards HAs inhibition during marinating, however, the effect was minor.

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