Factors affecting the formation of heterocyclic amines in sauced pork

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Abstract. To assess the factors affecting the formation of heterocyclic amines (HAs) in sauced pork, an orthogonal experiment about fat content, braising time and the number of braising times was designed, and the effects of common seasonings and seven Chinese spices on HAs formation were conducted. It was found that higher fat content, increasing braising time and the number of braising times would promote the formation of HAs. Sugar, salt and spices exhibited a reduction in HAs formation in sauced pork. 0.01% of clove could inhibit the formation of HAs by 36.21%. Notably, the inhibitory effect on HAs was significantly correlated with the concentration of spices.

1. Introduction

Sauced meat products are traditional food in China, which are deeply loved by Chinese consumers for their unique flavor. However, due to abundant amino acids and other substances in pork, it is easy to produce trace carcinogens during thermal processing, such as HAs.

HAs formation in meats depends on cooking conditions, precursors, seasonings and so on. Balogh et al\(^[1]\) found that under the same heating time, the HAs content increased as the temperature and HAs content would increase with time at the same temperature. HAs formation is the result of complex reactions between creatine, free amino acids, and sugars. Borgen et al\(^[2]\) effectively controlled the formation of HAs by reducing the contents of amino acids, creatine, creatinine and carbohydrates in raw meat. Recent years, special attention is given to the spices rich in antioxidant compounds that contribute to the inhibition of HAs formation. For example, the addition of red pepper before frying was found to reduce the formation of HAs in beef (the inhibition rate was 75%-100%) \(^[3]\).

To inhibit the formation of heterocyclic amines in sauced pork, the effects of cooking conditions and seasonings on the content of HAs were conducted, which could help improve the quality of traditional meat products and reduce the harm of meat products.

2. Materials and methods

2.1 Materials

Pork loin, pork belly, cinnamon (Cinnamomum japonicum), licorice (Glycyrrhiza uralensis), Red prickly ash (Zanthoxylum bungeanum), star anise (Illicium verum), clove (Eugenia caryophyllata),
galangal (Alpinia officinarum), fructus tsaoko (Amomum tsaoko) were purchased from Meilianmei Supermarket (Beijing, China). HAs standards (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhiP, AαC, Norharman, Harman, Trp-P-1 and 4,7,8-TriMeIQx) were purchased from Toronto Research Chemicals (Toronto North York, Canada).

Methanol, acetonitrile, ammonium formate and formic acid (chromatography grade), toluene, dichloromethane, ethyl acetate, hydrochloric acid, sodium hydroxide, ammonia (analytical grade) were obtained from Sinopharm Chemical Reagent Company (Beijing, China). Liquid chromatography-mass spectrometry (LC-MS/MS), Zorbax SB-C18 column (3.5 μm, 2.1×150 mm), a C18 SPE column (Bond Elut C18, 100 mg/mL), a PRS SPE column (Bond Elut PRS, 500 mg/3mL), nitrogen blowing apparatus were purchased from Agilent Technologies (United States).

2.2 Preparation of sauced pork
The frozen meat was defrosted at 4 °C for 24 h and cut into cubes weighting about 30 g each. Then rinsed the pork with warm water, boiled it for 15 min and took it out. Wrapped the following amounts with gauze: star anise (0.1%), licorice (0.1%), cinnamon (0.1%), fructus tsaoko (0.1%), galangal (0.05%), clove (0.05%), and red prickly ash (0.05%). 0.2% of salt, 1.8% of rock sugar, 2% of Soy sauce, 1% of cooking wine and the gauze were added in a pot filled with 1L of water boiling for 1h. Next kept water to 1L waiting to boil. Three pieces of pre-cooked pork were added, which were kept slightly boiling for 1h after boiling. When timeout, took out the pork and filtered the broth that was the new brine. The secondary old brine was obtained by adding the same amount of pork and half of the spices and seasonings in the new brine, adding water to 1L and boiling again. Repeated the above operation if the 10th pot of old brine was needed. Finally, the samples were cooled to room temperature, and then twisted and frozen at -18 °C.

2.3 Factors affecting the formation of HAs in sauced pork

2.3.1 Cooking conditions and fat content
Studies found that the braising time and the number of braising times were highly correlated with the content of HAs in sauced meat products. Fat content of pork also represented one of the properties. Therefore, an orthogonal experiment (L933) was designed to minimize HAs formation as Table 1.

| Levels | Fat content | Braising time (h) | Number of braising times (time) |
|--------|-------------|------------------|-------------------------------|
| 1      | Pork belly  | 1                | 1                             |
| 2      | Pork loin   | 2                | 5                             |
| 3      |             | 4                | 10                            |

2.3.2 Common seasonings
Five systems were tested in which water and pork was indispensable and salt, sugar, cooking wine, soy sauce and all spices were added respectively.

2.3.3 Seven different Chinese spices
Raw pork were cooked with Seven kinds of spices (cinnarmon, licorice, red prickly ash, star anise, clove, galangal, fructus tsaoko) respectively.

2.4 Analysis of HAs
Extraction and purification of HAs were performed by liquid chromatography-mass spectrometry (LC-MS/MS) according to Shen et al[4].
2.5 Statistical analysis
The averages of triplicate independent experiments were calculated. The results were statistically analyzed by ANOVA. Comparison of mean values was made using the Duncan test. Statistical analyses were all performed with SPSS Statistics 20.

3. Results and discussion

3.1 The relationship between HAs and cooking conditions and fat content
Fat content contributed more to the formation of polar HAs. As for non-polar HAs, braising time was the most important factor, followed by number of brining times and fat content. The effect on total HAs information was consistent with the results of non-polar HAs in that the content of non-polar HAs was much higher than that of polar HAs. Due to the oxidation of fat that can be decomposed into free radicals, which will participate in the Maillard reaction, certain pyrazines and pyridines will be produced. Generally non-polar HAs are generated by the pyrolysis of amino acids, which are not related to fat content. However, with the longer cooking time, the pyrolysis of amino acids may occur intensively leading the content of non-polar HAs increased.

The total amount of HAs in pork belly was higher than that of pork loin, indicating that higher fat content will promote the formation of HAs.

3.2 The relationship between HAs and common seasonings
The concentrations of HAs in different samples were presented in Table 2. Three non-polar HAs were detected that were Harman, Norharman and Trp-p-1 and others were polar HAs. 8-MeIQx, Harman and Norharman, the most abundant HAs formed in the system with cooking wine were significantly increased (p<0.05). Under the treatment of soy sauce, a promotion was observed in the formation of Harman and Norharman (p<0.05). No correlation was found between common seasonings and the formation of 4,8-DiMeIQx, PhIP, Trp-p-1 and AaC (p>0.05). Salt, sugar, and spices had an inhibitory effect on the formation of Harman (75.35%, 22.71% and 81.18%) and Norharman (83.28%, 64.72% and 72.91%). However, the difference was not significant compared with the control group (p>0.05).

To identify whether HAs come from exogenous sources or raw meat, the discriminative contribution of individual HAs in each spice treatment was showed in Figure 1, and results were expressed as % of inhibition toward control pork.

Salt and sugar had inhibitory effects on HAs formation, while cooking wine promoted increase of HAs and the similar results were observed by other group[5]. Reducing the amount of soy sauce, the content of HAs could be reduced. Previous literature[6] has shown that salt could inhibit the conversion of creatine into creatinine during frying to inhibit the formation of HAs.

3.3 The relationship between HAs and seven different Chinese spices
Control samples exhibited clearly two HAs: Norharman and Harman, as shown in Table 3. Although lacked of exocyclic amino groups, they were still carcinogenic as the auxiliary mutagens. Not all spices reduced the amount of different HAs.

| Table 2. Effects of different seasonings on the content of HAs in sauced pork. |
|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Control         | Salt            | Sugar           | Cooking wine    | Soy sauce       | Spices          |
| 8-MeIQx          | 0.05±0.02\(^b\) | 0.04±0.00\(^b\) | 0.05±0.01\(^b\) | 0.16±0.09\(^a\) | 0.05±0.04\(^b\) | 0.03±0.02\(^b\) |
| 4,8-DiMeIQx      | 0.08±0.01\(^a\) | 0.05±0.01\(^a\) | 0.06±0.01\(^a\) | 0.07±0.02\(^a\) | 0.08±0.04\(^a\) | 0.08±0.01\(^a\) |
| PhIP             | 0.06±0.02\(^a\) | 0.04±0.00\(^a\) | 0.04±0.01\(^a\) | 0.08±0.02\(^a\) | 0.05±0.03\(^a\) | 0.07±0.03\(^a\) |
| Harman           | 26.08±8.30\(^c\) | 6.43±1.59\(^b\) | 20.15±19.34\(^c\) | 239.33±64.75\(^a\) | 125.96±28.21\(^b\) | 4.91±2.14\(^c\) |
| Norharman        | 23.13±1.64\(^c\) | 3.87±1.69\(^c\) | 8.25±3.92\(^c\) | 74.78±22.50\(^b\) | 101.63±9.93\(^a\) | 6.27±2.28\(^c\) |
| Trp-p-1          | Nd              | Nd              | Nd              | 0.01±0.02\(^a\) | Nd              | Nd              |

\(^a\)p<0.05 compared to control; \(^b\)p<0.05 compared to control but not compared to other groups; \(^c\)p<0.05 compared to other groups.
|                | AaC     | Polar HAs | Non-polar HAs | TotalD |
|----------------|---------|-----------|---------------|--------|
|                | 0.01±0.00a | 0.19±0.05b | 49.21±10.85c       | 49.41±10.90c |
|                | 0.01±0.00a | 0.14±0.01b | 10.3±3.17c        | 10.44±3.17c  |
|                | 0.01±0.00a | 0.15±0.01b | 28.42±23.13c       | 28.56±23.12c  |
|                | 0.01±0.00a | 0.31±0.11a | 314.12±86.73a      | 314.43±86.84a |
|                | 0.02±0.01a | 0.17±0.10b | 227.60±36.33b      | 227.77±36.34b |
|                | 0.01±0.00a | 0.18±0.04b | 11.19±4.29c        | 11.37±4.24c   |

A value means mean ± standard deviation (n=3), different letters show significant differences (p < 0.05)
Nd means not detected

Figure 1. The rate of HAs formation under treatment of different seasonings in sauced pork.

To some extent, 0.1% of cinnamon, licorice, star anise, clove, galangal can inhibit the total content of HAs. And 0.1% of clove had the strongest inhibitory effect (36.21%). 0.05% of clove and ginger had the same effects (25.78% and 22.91%), while the other spices could promote the formation of HAs. Different investigations of model system and experiments[7-9] have shown that HAs formation was the result of complex reactions through the Maillard reaction. The molecular structure of the antioxidant components, such as phenolic acids and flavonoids, contained active hydrogen, which could bind with active peroxy radicals and scavenge free radicals.

As the change of concentrations, antioxidants may convert to prooxidant. That was why no significant inhibitory effect of spices on HAs could be observed. Controversial findings on the effect of antioxidant capacity of phenolic compounds and HAs formation have been conducted. Zeng et al[10] have shown that the total phenolic content was positively correlated with ABTS + · scavenging ability. However, in this study, the inhibition rate of clove on HAs was only 13.1%, which was lower than that of licorice. Additionally, information about the effect of mixtures containing antioxidant-rich ingredients is still a challenge for researchers.

4. Conclusions
The results clearly showed that sugar, salt and spices exhibited a reduction in HAs formation in sauced pork, while soy sauce and cooking wine represented the opposite effects. In addition, the inhibitory effect of spices with higher concentration (0.1%) was more significant than that with lower concentration (0.05%) on HAs formation. The current study would support the inhibition of HAs.
Table 3. HAs content under different addition levels of spices in sauced pork.

| Spice                  | 8-MeIQx (ng/g) | 4,8-DiMeIQx (ng/g) | PhIP (ng/g) | Norharman (ng/g) | Harman (ng/g) | Trp-p-1 (ng/g) | Total (ng/g) |
|------------------------|----------------|--------------------|-------------|------------------|---------------|---------------|--------------|
| Black                  | 0.02±0.01d     | 0.02±0.00b         | 0.01±0.01bc | 3.17±0.71e       | 5.5±1.2s      | Nd            | 8.77±1.88s   |
| Control                | 0.02±0.01d     | 0.03±0.01b         | 0.02±0.00e  | 84.99±5.41ef     | 140.21±12.41ef| 0.07±0.13a    | 225.29±17.26ef|
| Innarmon               | 0.02±0.01d     | 0.04±0.01b         | 0.01±0.01f  | 73.27±5.11ef     | 136.38±11.24ef| Nd            | 209.75±16.25ef|
| Licorice               | 0.04±0.03ef    | 0.06±0.01b         | 0.05±0.03e  | 64.03±5.11f      | 113.42±8.47ef | Nd            | 177.61±13.63f|
| Red prickly ash Star anise | 0.12±0.16abc  | 0.14±0.13a         | 0.13±0.16bc | 113.46±14.38ge   | 168.72±14.99e | Nd            | 282.66±28.77e|
| Galangal               | 0.05±0.04d     | 0.05±0.03b         | 0.05±0.04e  | 76.27±16.46ef    | 139.43±24.18ef| Nd            | 215.88±40.63f|
| Fructus tsaoko         | 0.01±0.01d     | 0.04±0.00b         | 0.02±0.01c  | 76.09±2.08ef     | 122.16±2.68ef | Nd            | 198.35±3.19f |
| Innarmon               | 0.04±0.01ef    | 0.05±0.01b         | 0.03±0.00b  | 91.93±10.32ef    | 135.04±11.93ef| Nd            | 227.11±22.24ef|
| Licorice               | 0.15±0.05a     | 0.16±0.05a         | 0.17±0.08a  | 136.25±9.3d      | 284.6±13.35d  | Nd            | 421.38±17.45d|
| Red prickly ash Star anise | 0.04±0.01ef    | 0.05±0.01b         | 0.05±0.02b  | 503.33±75.71a    | 934.31±115.87a| Nd            | 1437.83±191.10a|
| Clove                  | 0.07±0.03bcd   | 0.04±0.01b         | 0.04±0.01b  | 213.14±22.33c    | 414.53±44.48a | Nd            | 627.83±66.82c|
| Galangal               | 0.02±0.01d     | 0.05±0.01b         | 0.03±0.01b  | 60.37±5.33ef     | 106.72±5.53ef | Nd            | 173.7±10.89f |
| Fructus tsaoko         | 0.14±0.04ab    | 0.14±0.07a         | 0.16±0.01a  | 250.49±14.81b    | 553.31±70.13b | Nd            | 804.26±81.79b|

*value means mean ± standard deviation (n=3), different letters show significant differences (p < 0.05)
Nd means not detected

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