Food Chemistry 221 (2017) 404–411

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Inhibitory profiles of chilli pepper and capsaicin on heterocyclic amine formation in roast beef patties

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ARTICLE INFO

Article history:
Received 9 June 2016
Received in revised form 22 September 2016
Accepted 13 October 2016
Available online 14 October 2016

Keywords:
UPLC-MS/MS
Inhibitory profile
Chilli pepper
Capsaicin
Heterocyclic amine
Principal component analysis

ABSTRACT

The inhibitory profiles of chilli pepper and capsaicin, as well as their relationship to the formation of heterocyclic amines (HAs) in roast beef patties were investigated using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) combined with principal component analysis (PCA). HAs including 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine (1,5,6-TMIP), 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 1-methyl-9H-pyrido[3,4-*b*]indole (harman) and 9H-pyrido[3,4-*b*]indole (norharman) were detected and quantified in beef patties. Different levels of chilli pepper and capsaicin had different inhibitory profiles on HA formation, but had no significant ($P > 0.05$) effect on the texture of the patties. Furthermore, all levels of chilli pepper and capsaicin reduced total HA and PhIP concentrations dose-dependently, with the highest inhibitions of 80% and 98% at 2 mg of capsaicin. Moreover, capsaicin inhibited all HAs more than chilli pepper, implying that ingredients other than capsaicin in chilli pepper may promote the formation of HAs. These results could be useful for the reduction of HA, during food processing procedures, by spices

1. Introduction

Heterocyclic amines (HAs) are a group of mutagenic and carcinogenic compounds that are produced during high-temperature processing of protein-rich foods, such as meat (Shabbir, Raza, Anjum, Khan, & Suleria, 2015). More than 25 kinds of HA have been detected in various food systems (Alaejos & Afonso, 2011; Oz, 2011; Oz, Kizil, Zaman, & Turhan, 2016). The International Agency for Research on Cancer (IARC) has classified several HAs as possible, or probable, carcinogens (IARC, 1993). Exposure to HAs has been associated with several kinds of cancers in humans (Alaejos, Gonzalez, & Afonso, 2008). Thus, the inhibition of HA formation during food processing procedures has attracted much attention.

As free radicals have been proven to be involved in the formation of HAs, antioxidants are considered to be the most promising and effective inhibitors of HAs during food processing (Kikugawa, 1999). Spices are good sources of antioxidants (Madsen & Bertelsen, 1995) and are widely used in food, especially in meat processing. Several spices have been shown to reduce HA formation in cooked meat (Gibis & Weiss, 2012). In one of our former works (Zeng et al., 2014), we explored the effects of some spices on the imidazopyridine, imidazoquinoxaline, β-carboline and total HA profiles, so as to facilitate the selection of spices in meat processing to minimize HA formation. The results showed that some spices can significantly inhibit the formation of HAs, while other spices have no significant effects on HAs, and can even enhance the formation of HAs. In order to elucidate this phenomenon, it must be determined which ingredients of the spices could inhibit or enhance the formation of HA. Other studies have also demonstrated that some spices can significantly enhance the formation of HAs despite being rich in phenolic compounds (Puangsombat, Jirapakkul, & Smith, 2011). Antioxidants may also have pro-oxidative effects at certain concentrations (Yen, Duh, & Tsai, 2002). anti-oxidative actions, such as free radical scavenging activity, have been shown to have no correlation with total or individual HA formation (Viegas, Amaro, Ferreira, & Pinho, 2012; Zhu, Zhang, Wang, Chen, & Zheng, 2016). Thus, we wonder whether there are ingredients other than antioxidants, such as polyphenoic com-

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http://dx.doi.org/10.1016/j.foodchem.2016.10.061
0308-8146/

pounds, that in spices have significant effects on the formation of HAs and if it is necessary to explore the inhibitory profiles of spice constituents other than antioxidants on HA formation.

Chilli pepper is a widely used spice and has been screened as an inhibitor for 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethyl-3H-imidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in fried beef chops (Oz & Kaya, 2011). Capsaicin is a major pungent ingredient of chilli pepper, and may have clinical value in cancer prevention (Venier et al., 2014). However, there is a lack of information about the inhibitory profiles of chilli pepper and capsaicin, and about their relationship. As such, in this study we have investigated the inhibitory profiles of chilli pepper and capsaicin as well as their relationship during the formation of HAs in roast beef patties, using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and principal component analysis (PCA).

2. Material and methods

2.1. Reagents and materials

HA standards including 2-amino-9H-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 1-methyl-9H-pyrido[3,4-*b*]indole (harman), 9H-pyrido[3,4-*b*]indole (norharman), IQ, MeIQ, 2-amino-1-methylimidazo[4,5-*b*]quinoline (IQ[4,5-*b*]), PhIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine (1,5,6-TMIP), 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 4,8-DiMeIQx, 2-amino-3,7,8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), 2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-*f*]quinoxaline (4,7,8-TriMeIQx), 2-amino-5-phenylpyridine (Phe-P-1), 2-amino-6-methyldipyrido[1,2-*a*:3′, 2′-*d*]imidazole (Glu-P-1) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The capsaicin standard was bought from Sigma-Aldrich (St Louis, MO, USA). All standards had above 99.9% purity. The HA standards were used to prepare a mixed stock standard solution with a final concentration of 125 μg/ml in methanol.

LC-MS grade methanol, acetonitrile and HPLC grade formic acids were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Analytical grade diatomaceous earth were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The derivatization reagents O-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC-Cl) were purchased from Sigma-Aldrich (Shanghai, China). Oasis MCX cartridges (60 mg, 3 ml) were obtained from Waters (Shanghai, China). Chilli peppers were obtained from local markets, ground to a fine powder, and stored at −20 °C until use.

2.2. Meat preparation and cooking

Beef was obtained from the local market (Wuxi, China). Chilli pepper powder was added to ground beef at levels of 0.5, 1.0 and 1.5% according to previous research (Oz & Kaya, 2011), and 2, 4 and 6 mg of capsaicin were added to match the levels of capsaicin in chilli pepper (Barbero et al., 2014). Each patty was formed using a petri dish (6 cm × 1.5 cm) to ensure uniformity. The patties were roasted in a RATIONAL SCC 61 E Self-cooking Center (Landsberg, Munich, Germany). The hot plate was preheated for 5 min to 225 °C. The patties were then put into the preheated oven, and roasted for 10 min on each side. After the cooking procedure, the patties were cooled at room temperature and the cooked meat samples were cut into small pieces, freeze-dried and stored at −20 °C.

2.3. Analysis of free amino acids

1.0 g samples of the powdered cooked beef patties were ultrasonically extracted with 25 ml of 5% trichloroacetic acid for 2 h, then filtered through a 0.22 μm membrane. Then, 400 μl filtrate was used for HPLC analysis. Samples were analyzed using pre-column derivatization. 1 μl of sample, OPA and FMOC-Cl were added to 5 μl sodium borate buffer (pH 10.4) in reaction vessels from the autosampler and mixed for 20 s before injection. Analyses were performed with an Agilent 1200 HPLC system equipped with a quaternary pump (G1311A), autosampler (G1313A), online vacuum degasser (G1322A) and variable wavelength detector (G1314A), using a ODS HYPERSIL (5 μm particle size, 4.6 mm × 250 mm) column operated at 40 °C. The solvents were 27.6 mmol/l sodium acetate solution-trimethylamine-tetrahydro furan (500:0.11:2.5, pH = 7.2, Solvent A) and sodium acetate solution-acetonitrile-methanol (1:2:2, pH = 7.2, Solvent B) with a flow rate of 1.0 ml/min. The gradient was programmed as follows: 0 min, 8% B, 17 min, 50% B, 20.1 min, 100% B, 24.0 min, 0% B. Analysis was performed with a column temperature of 40 °C, an injection volume of 10 μl, and a monitoring wavelength of 338 nm, except for proline at 262 nm.

2.4. Proximate analysis and cooking loss

Crude protein contents, pH values, moisture, minerals (ash) and fat of each sample were determined according to literature (Bilek & Turhan, 2009). Protein concentrations were measured using the Kjeldahl method. The pH value was determined by blending and homogenizing 5 g of sample in 50 ml of KCl solution, then the mixture was filtered and the pH was measured using a digital pH meter. Moisture and ash contents were determined by muffle furnace. Total fat was determined by Soxhlet extraction method. After the cooking processes, samples were cooled at room temperature, surface-dried with filter paper and re-weighed. Cooking losses of the samples were calculated from the differences between patties before and after cooking.

2.5. Texture profile analysis

Texture profile analyses (TPA) of the beef patties were performed using a texture analyzer (TA-XT plus, Stable Micro System, Surrey, UK) fitted with a cylindrical probe (P/50, 50 mm stainless cylinder). The room temperature beef patties were cut into small cubes (1 × 1 × 1 cm) and subjected to a two-cycle compression test to determine the texture characteristic parameters: hardness, cohesiveness, gumminess, chewiness and resilience. Hardness (kg) is the maximum force required to compress the sample. Cohesiveness is the extent to which the sample could be deformed prior to rupture (A2/A1), A1 being the total energy required for the first compression and A2 the total energy required for the second compression. Chewiness (kg × cm) is the work needed to masticate the sample before swallowing (hardness × cohesiveness × springiness). The conditions were as follows: pre-test speed 3.0 mm/s; test speed 2.0 mm/s; post-test speed 3.0 mm/s; strain 50%; time 5.0 s; trigger type, auto; and trigger force 1.0 g for TPA measurement. Measurements were carried out in triplicate.

2.6. Extraction of HAs

Profiles of 17 polar and nonpolar HAs from 7 categories were screened as previously described (Zeng et al., 2014). Minced, freeze-dried meat (3 g) was homogenized with sodium hydroxide

(3 ml, 1 mol/l) for 1 min. The homogenate solution was mixed with 13 g of diatomaceous earth. Ethyl acetate (50 ml) was added and the sample was ultrasonically extracted twice for 30 min. The extracts were centrifuged at 12,000*g* for 10 min at 4 °C and the supernatant was collected. The ethyl acetate layer of the supernatant (10 ml) was loaded into Waters Oasis MCX cartridges, which were preconditioned with methanol (6 ml), distilled water (6 ml) and HCl (6 ml, 0.1 mol/l). The cartridges were then sequentially rinsed with HCl (6 ml, 0.1 mol/l) and methanol (6 ml). The retained HAs were eluted with 6 ml of a methanol-ammonia (19:1, v/v) mixture. The eluted mixtures were evaporated to dryness under nitrogen, filtrated through a 0.22 μm syringe filter and dissolved in 250 μl of methanol before UPLC-MS/MS analysis.

2.7. Identification and quantification of HAs

The analysis of HAs was performed on a Waters Acquity UPLC system equipped with a triple quadripole mass spectrometer (Waters, Milford, MA, USA) using positive electrospray ionization. Separation of the analytes was carried out on an ACQUITY UPLC BEH C_{18} reversed-phase column (1.7 μm particle size, 2.1 mm × 50 mm) at 35 °C. The gradient elution was achieved with a binary mobile phase of 10 mM ammonium acetate (pH 6.8) (A) and acetonitrile (B). The solvent protocol was 0–0.1 min, 90% A; 0.1–18 min, 10–30% B; 18–20 min, 30–100% B; 20–20.1 min, 100–10% B. The flow rate was 0.3 μl/min, and the injection volume was 1 μl. The mass spectrometric detection was conducted in multi-reaction monitoring (MRM) scan mode. The mass spectrometry operating conditions were: capillary voltage 3.5 kV, ion source temperature 120 °C, desolvation temperature 350 °C, cone gas (nitrogen) flow 60 L/h and desolvation gas (nitrogen) flow 650 L/h. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 4×10^{-3} mbar in the collision cell. Data acquisition was performed using Waters MassLynx 4.1 software (Milford, MA, USA). The HAs were quantified with calibration curves of each kind of HA at eight calibrant levels, ranging from 0.2–30.0 ng/ml.

2.8. Statistical analysis

In this work, three repeat samples were prepared for each treatment. For investigating the effects of chilli pepper on HA formation, PCA analysis of HA contents in beef patties of blank and that added with different amounts of chilli pepper were employed. The PCA score plot of blank and chilli pepper samples showed the differences between blank and chilli pepper samples, and the related loadings plot gave the HAs corresponding to this difference, thus revealing the effects of chilli pepper on HA formation. Similarly, we used PCA analysis of blank and capsaicin samples to illustrate the effects of capsaicin on HA formation, while chilli pepper and capsaicin samples revealed the difference in the inhibitory profiles between beef patties with added chilli pepper and capsaicin. The HA levels in beef patties were used to generate a data matrix, in which the rows and columns represent the observations (samples of beef patties) and the variables (HA levels of a single beef patty). The resulting data matrix was imported into SIMCA+13.0 (UMETRICS, Umea, Sweden) for PCA analysis. Two principal components (PCs) were used for calculating the PCA models, and the normal distribution of the variables was checked first to ensure that the data were suitable for conducting PCA. The scores plot was used to discern the trends in samples from different groups, and the loadings and contribution plots were used to explore the roles of different HAs in different groups.

3. Results and discussion

3.1. Proximate analysis

Crude protein contents, pH values, moisture, fat and minerals (ash) of the raw beef muscles were determined to be 22.09 ± 0.31%, 5.58 ± 0.01, 75.15 ± 0.09%, 0.13 ± 0.00% and 1.15 ± 0.02%, respectively. These values (listed in Table 1) coincide well with those in the literature (Oz et al., 2016), in which the water, crude protein, crude fat and pH values of raw beef M. L. dorsi muscle were measured as 75.72 ± 0.21, 17.80 ± 0.58, 4.65 ± 0.57 and 5.68 ± 0.13%. The content of fat (0.13%) was lower than usual, because the fat was removed before preparation to reduce its effects on HA formation.

Table 1 shows the chemical composition of beef patties with different levels of chilli pepper and capsaicin. The pH values of all groups were slightly acidic and increased significantly ($P < 0.05$) after roasting, but changed a little when chilli pepper and capsaicin were added. There was a negative relationship between cooking loss and moisture content. Higher levels of added chilli pepper and capsaicin reduced cooking loss and moisture content, although not always significantly ($P > 0.05$). Mineral (ash) and fat content did not differ significantly ($P > 0.05$) between roast control patties and those with added chilli pepper and capsaicin. The highest concentration of protein was found in beef patties with 6 mg capsaicin, at 46.23 ± 0.74 g/100 g dry matter.

3.2. Texture profile analysis

There was no statistically significant difference ($P > 0.05$) between roast control patties and those with added chilli pepper and capsaicin in terms of cohesiveness, gumminess, chewiness or resilience. Hardness increased significantly ($P < 0.05$) when chilli pepper and capsaicin were added, as shown in Table 2. Increasing chilli pepper and capsaicin level enhanced hardness, and the hardest patties were those with 1.5% chilli pepper (9997.01 ± 26.68 N) and 6 mg capsaicin (9993.45 ± 4.70 N). These values are comparable with moisture and cooking loss, as decreasing moisture content

Table 1
Chemical composition and pH values of raw beef, roast control patties and patties added with different levels of chilli pepper and capsaicin.

	pH	Cooking loss (g/100 g)	Protein (g/100 g)	Moisture (g/100 g)	Fat (g/100 g)	Minerals (g/100 g)
Raw beef	5.58 ± 0.01^{b}	–	22.09 ± 0.31^{a}	75.15 ± 0.09^{a}	0.13 ± 0.00^{c}	1.15 ± 0.02^{b}
Roast control patties	5.79 ± 0.01^{a}	51.15 ± 0.75^{a}	43.85 ± 1.38^{b}	49.61 ± 0.17^{b}	2.37 ± 0.02^{a}	2.81 ± 0.05^{a}
Chilli pepper-0.5%	5.85 ± 0.01^{a}	51.13 ± 1.16^{a}	43.88 ± 1.59^{b}	49.02 ± 0.42^{b}	2.32 ± 0.06^{ab}	2.73 ± 0.04^{a}
Chilli pepper-1.0%	5.82 ± 0.02^{a}	50.54 ± 0.53^{b}	46.00 ± 0.78^{c}	48.73 ± 0.27^{b}	2.28 ± 0.03^{b}	2.71 ± 0.00^{a}
Chilli pepper-1.5%	5.84 ± 0.01^{a}	50.29 ± 0.53^{c}	45.46 ± 0.42^{c}	48.96 ± 0.03^{b}	2.34 ± 0.06^{ab}	2.76 ± 0.06^{a}
Capsaicin -2 mg	5.86 ± 0.01^{a}	52.89 ± 2.27^{a}	46.20 ± 0.71^{d}	48.36 ± 0.37^{b}	2.33 ± 0.02^{ab}	2.73 ± 0.00^{a}
Capsaicin-4 mg	5.80 ± 0.01^{a}	52.26 ± 1.38^{a}	43.11 ± 1.00^{b}	48.41 ± 0.04^{b}	2.34 ± 0.05^{ab}	2.72 ± 0.00^{a}
Capsaicin-6 mg	5.82 ± 0.01^{a}	52.18 ± 1.31^{a}	46.23 ± 0.74^{c}	48.18 ± 0.07^{c}	2.31 ± 0.04^{ab}	2.72 ± 0.04^{a}

Comparisons are made to roast control patties within the same column;
Provides statistical significance at p value = 0.05. Data were presented as means ± standard deviations;
Means with different letters in each column are significantly different ($P < 0.05$).

Table 2
Texture characteristics of raw beef, roast control patties and patties added with different levels of chilli pepper and capsaicin.

	Hardness(N)	Cohesiveness(N)	Gumminess(N)	Chewiness(mJ)	Resilience(mm)
Raw beef	1036.54 ± 38.14[b]	0.45 ± 0.01[b]	492.07 ± 5.05[b]	210.42 ± 3.87[b]	0.16 ± 0.01[b]
Roast control patties	8518.23 ± 62.54[a]	0.61 ± 0.01[a]	5910.73 ± 62.38[a]	4859.57 ± 37.79[a]	0.25 ± 0.01[a]
Chilli pepper-0.5%	9850.83 ± 39.04[c]	0.62 ± 0.01[a]	5974.83 ± 69.35[a]	4763.32 ± 55.48[a]	0.24 ± 0.00[ab]
Chilli pepper-1.0%	9947.42 ± 76.13[c]	0.61 ± 0.00[a]	5818.66 ± 21.99[a]	4743.09 ± 90.54[a]	0.25 ± 0.01[b]
Chilli pepper-1.5%	9997.01 ± 26.68[c]	0.61 ± 0.00[a]	5829.67 ± 13.97[a]	4609.08 ± 9.91[a]	0.25 ± 0.00[b]
Capsaicin -2 mg	9836.54 ± 10.74[c]	0.61 ± 0.01[a]	5938.05 ± 4.08[a]	4831.99 ± 11.23[a]	0.24 ± 0.01[ab]
Capsaicin-4 mg	9910.30 ± 11.58[c]	0.61 ± 0.00[a]	5927.44 ± 3.23[a]	4730.02 ± 8.41[a]	0.24 ± 0.00[ab]
Capsaicin-6 mg	9993.45 ± 4.70[c]	0.61 ± 0.01[a]	5922.92 ± 2.52[a]	4873.94 ± 2.64[a]	0.24 ± 0.00[ab]

Comparisons are made to roast control patties within the same column;
Provides statistical significance at p value = 0.05. Data were presented as means ± standard deviations;
Means with different letters in each column are significantly different (P < 0.05).

Table 3
Levels of free amino acids in raw beef, roast control patties and patties that added with different levels of chilli pepper and capsaicin.

mg/g	Raw beef	Roast control patties	Chilli pepper-0.5%	Chilli pepper-1.0%	Chilli pepper-1.5%	Capsaicin-2 mg	Capsaicin-4 mg	Capsaicin-6 mg
asp	0.205 ± 0.001[b]	0.138 ± 0.001[a]	0.175 ± 0.001[a]	0.132 ± 0.002[a]	0.144 ± 0.001[a]	0.141 ± 0.001[a]	0.132 ± 0.001[a]	0.135 ± 0.001[a]
glu	0.788 ± 0.001[b]	0.218 ± 0.001[a]	0.316 ± 0.001[a]	0.215 ± 0.002[a]	0.212 ± 0.001[a]	0.216 ± 0.001[a]	0.204 ± 0.001[a]	0.182 ± 0.001[a]
ser	0.080 ± 0.000[b]	0.067 ± 0.000[a]	0.061 ± 0.001[a]	0.049 ± 0.001[a]	0.059 ± 0.001[a]	0.045 ± 0.001[a]	0.043 ± 0.001[a]	0.040 ± 0.001[a]
his	0.131 ± 0.001[b]	0.107 ± 0.001[a]	0.110 ± 0.001[a]	0.094 ± 0.001[a]	0.099 ± 0.000[a]	0.082 ± 0.000[a]	0.068 ± 0.001[a]	0.059 ± 0.001[a]
gly	0.238 ± 0.003[b]	0.138 ± 0.003[a]	0.163 ± 0.002[a]	0.135 ± 0.005[a]	0.129 ± 0.001[a]	0.145 ± 0.004[a]	0.138 ± 0.005[a]	0.127 ± 0.003[a]
thr	0.343 ± 0.003[b]	0.129 ± 0.002[a]	0.146 ± 0.003[a]	0.135 ± 0.003[a]	0.127 ± 0.002[a]	0.130 ± 0.002[a]	0.128 ± 0.002[a]	0.117 ± 0.004[a]
arg	0.286 ± 0.002[b]	0.153 ± 0.002[a]	0.186 ± 0.003[a]	0.141 ± 0.003[a]	0.152 ± 0.002[a]	0.134 ± 0.003[a]	0.130 ± 0.003[a]	0.138 ± 0.002[a]
ala	0.805 ± 0.003[b]	0.588 ± 0.005[a]	0.618 ± 0.002[a]	0.551 ± 0.002[a]	0.561 ± 0.003[a]	0.576 ± 0.004[a]	0.587 ± 0.006[a]	0.561 ± 0.003[a]
tyr	0.074 ± 0.002[b]	0.072 ± 0.001[a]	0.074 ± 0.001[a]	0.062 ± 0.002[a]	0.071 ± 0.004[a]	0.058 ± 0.001[a]	0.060 ± 0.002[a]	0.051 ± 0.003[a]
cys-s	0.028 ± 0.002[b]	0.020 ± 0.001[a]	0.017 ± 0.000[a]	0.015 ± 0.002[a]	0.013 ± 0.001[a]	0.015 ± 0.003[a]	0.023 ± 0.007[a]	0.016 ± 0.003[a]
val	0.201 ± 0.001[b]	0.134 ± 0.002[a]	0.139 ± 0.002[a]	0.109 ± 0.001[a]	0.115 ± 0.000[a]	0.103 ± 0.004[a]	0.100 ± 0.002[a]	0.091 ± 0.002[a]
met	0.131 ± 0.002[b]	0.115 ± 0.003[a]	0.116 ± 0.005[a]	0.106 ± 0.004[a]	0.106 ± 0.002[a]	0.073 ± 0.002[a]	0.079 ± 0.002[a]	0.072 ± 0.003[a]
phe	0.132 ± 0.002[b]	0.086 ± 0.003[a]	0.110 ± 0.002[a]	0.082 ± 0.003[a]	0.082 ± 0.001[a]	0.066 ± 0.002[a]	0.070 ± 0.004[a]	0.059 ± 0.003[a]
ile	0.101 ± 0.001[b]	0.071 ± 0.001[a]	0.087 ± 0.001[a]	0.065 ± 0.002[b]	0.068 ± 0.001[a]	0.060 ± 0.002[a]	0.060 ± 0.002[a]	0.052 ± 0.002[a]
leu	0.216 ± 0.003[b]	0.142 ± 0.003[a]	0.160 ± 0.000[a]	0.118 ± 0.003[a]	0.128 ± 0.005[a]	0.106 ± 0.003[a]	0.109 ± 0.003[a]	0.097 ± 0.004[a]
lys	0.179 ± 0.002[b]	0.091 ± 0.001[a]	0.101 ± 0.002[a]	0.072 ± 0.003[a]	0.073 ± 0.001[a]	0.076 ± 0.004[a]	0.073 ± 0.001[a]	0.065 ± 0.002[a]
pro	0.352 ± 0.003[b]	0.204 ± 0.002[a]	0.239 ± 0.003[a]	0.243 ± 0.002[a]	0.241 ± 0.003[a]	0.210 ± 0.003[a]	0.232 ± 0.002[a]	0.213 ± 0.004[a]

Comparisons are made to roast control patties within the same row;
Means with different letters in each row are significantly (P < 0.05) different;
Provides statistical significance at p value = 0.05. Data were presented as means ± standard deviations.

increases hardness, and there was a negative relationship between moisture content and hardness. Additives (chilli pepper and capsaicin) may improve the osmotic pressure of muscle tissue. Increased osmotic pressure increases water loss and hardness in the cooking process (Yildiz Terp et al., 2016).

3.3. Free amino acids analysis

The addition of chilli pepper and capsaicin had a small effect on the free amino acid content of the beef (Table 3). There was a significant difference in free amino acid content between raw beef and roast beef patties but no significant difference between roast control patties and those with added chilli pepper and capsaicin. Free amino acids are the precursors of HAs. HA levels increased gradually with roasting temperature and duration, whereas the precursors, such as free amino acids, decreased significantly, especially threonine (from 0.343 to 0.117 mg/g) and phenylalanine (from 0.132 to 0.059 mg/g).

3.4. Validation of the method

Linear range, limit of detection (LOD), limit of quantification (LOQ), recovery and matrix effects are listed in Table 4. These results are comparable with those in the literature (Oz et al., 2016). According to the literature, the recoveries of PhIP, IQx, MeIQx and 4,8-DiMeIQx were found to be 82.15%, 61.08%, 62.45% and 59.29%, while 68.52%, 53.67%, 58.33% and 80.63% were obtained in our work. As for LOD and LOQ of 4,8-DiMeIQx, 0.011 and 0.020 ng/g were obtained in this work, which are similar to 0.008 and 0.025 ng/g in the literature.

3.5. UPLC-MS/MS determination of HAs

Table 5 presents HA content in control beef patties and those with added chilli pepper and capsaicin. During the roasting of the beef patties, eight HAs including PhIP, DMIP, 1,5,6-TMIP, harman, norharman, IQx, MeIQx and 4,8-DiMeIQx were detected in the beef patties. However, the levels of DMIP and 1,5,6-TMIP in most of the samples were lower than LOQs. PhIP was the most prevalent (0.14 to 8.00 ng/g), followed by norharman (1.10 to 2.17 ng/g), MeIQx (0.23 to 1.10 ng/g), harman (0.24 to 0.44 ng/g), 4, 8-DiMeIQx (0.05 to 0.21 ng/g) and IQx (lower than LOD at 0.36 ng/g). The HAs quantified in this work coincide well with our former work (Zeng et al., 2014) and are similar to those found in other studies (Oz, 2011; Natale, Gibis, Rodriguez-Estrada, & Weiss, 2014; Szterk, 2013). The precision values for these 17 HAs upon three repeated samples were mostly within 13.04%. Recoveries were between 53.67% and 110.37%. In a previous study using Oasis MCX cartridges, the average recoveries for 4,8-DiMeIQx, MeIQx and PhIP were between 40% and 60%, which is comparable with the present work. The LOD values for the present study of PhIP, DMIP, 1,5,6-TMIP, harman, norharman, IQx, MeIQx and 4,8, DiMeIQx were 0.115, 0.090, 0.019, 0.036, 0.145, 0.106, 0.093 and 0.011 ng/g, and the LOQs were 0.209, 0.168, 0.096, 0.041, 0.208, 0.305, 0.129 and 0.020 ng/g, respectively. The relatively lower HA content measured in this study may be a result of different roasting

Table 4
Analytical characteristics of HA standard solutions and spiked samples.

HAs	Linear range (ng mL^{-1})	Coefficients (r^2)	LOD[a] (ng/g)	LOQ[b] (ng/g)	Matrix effects[c] ($R_n \pm SD$)	RSD (%)	Recovery (%)
DMIP	8.3–412.50	0.9992	0.090	0.168	0.95 ± 0.02	9.35	34.23
1,5,6-TMIP	0.99–508.00	0.9987	0.019	0.096	0.96 ± 0.16	9.17	65.92
IQ[4,5-*b*]	0.80–79.36	0.9991	0.021	0.044	1.16 ± 0.15	13.95	60.22
IQ	0.6–400.5	0.9955	0.012	0.021	0.91 ± 0.13	8.09	59.86
IQx	2.2–863.5	0.9988	0.106	0.305	0.90 ± 0.09	4.33	53.67
MeIQ	0.94–552.01	0.9987	0.049	0.137	0.98 ± 0.03	5.78	72.01
MeIQx	1.4–517.0	0.9995	0.093	0.129	0.96 ± 0.13	13.04	58.33
PhIP	1.1–440.5	0.9987	0.115	0.209	1.05 ± 0.12	19.35	68.52
7,8-DiMeIQx	3.3–679.0	0.9992	0.265	0.659	0.89 ± 0.02	20.67	52.71
4,8-DiMeIQx	0.53–139.55	0.9979	0.011	0.020	0.96 ± 0.03	7.30	80.63
4,7,8-TriMeIQx	0.67–163.05	0.9974	0.040	0.161	0.92 ± 0.25	12.98	65.50
Norharman	0.59–269.50	0.9996	0.145	0.208	0.92 ± 0.03	8.93	110.37
Phe-p-1	0.67–94.89	0.9997	0.011	0.037	0.91 ± 0.11	9.91	92.93
Harman	0.46–189.75	0.9993	0.036	0.041	0.92 ± 0.05	9.55	105.92
AαC	0.67–197.89	0.9968	0.016	0.039	0.89 ± 0.16	13.14	80.90
MeAαC	1.85–554.02	0.9996	0.020	0.045	0.94 ± 0.08	11.96	81.85
Glu-p-1	0.78–600.27	0.9996	0.087	0.102	0.96 ± 0.05	8.52	60.02

[a] Limit of detection.
[b] Limit of quantitation.
[c] Matrix effects are expressed as the slope ratios (Rn) of standard spiked calibration curve to pure standard calibration curves at the same analyte concentration. Values larger than 1.00 mean ionization enhancement, while lower than 1.00 indicate ionization suppression (n = 3).

Table 5
Heterocyclic amine levels (ng/g) of roast beef patties added with different levels of chilli pepper and capsaicin[a].

		PhIP	DMIP	1,5,6-TMIP	IQx	MeIQx	4,8-DiMeIQx	Harman	Norharman	Total HAs
Control		8.00 ± 1.07	nq[b]	nq[b]	0.22 ± 0.01	0.24 ± 0.01	0.16 ± 0.01	0.24 ± 0.04	1.10 ± 0.02	10.08
Chilli pepper	0.5%	2.60 ± 0.20*(68%)	0.13 ± 0.01	0.24 ± 0.01*	nq[b]	0.68 ± 0.05*	0.10 ± 0.01*	0.44 ± 0.02*	1.21 ± 0.04*	5.49/(46%)
	1.0%	3.15 ± 0.09*(61%)	nd[c]	nq[b]	0.36 ± 0.02	0.99 ± 0.02*	0.21 ± 0.01*	0.28 ± 0.03*	1.49 ± 0.03*	6.53/(35%)
	1.5%	3.74 ± 0.32*(53%)	nd[c]	nq[b]	0.26 ± 0.01	0.83 ± 0.01*	0.16 ± 0.00	0.36 ± 0.05*	2.17 ± 0.19*	7.62/(24%)
Capsaicin	2 mg	0.14 ± 0.01*(98%)	nd[c]	nq[b]	nd[c]	0.23 ± 0.02	nq[b]	0.27 ± 0.05	1.36 ± 0.03*	2.03/(80%)
	4 mg	0.59 ± 0.06*(93%)	nd[c]	nq[b]	nq[b]	0.49 ± 0.06*	0.05 ± 0.00*	0.24 ± 0.01	1.80 ± 0.07*	3.26/(68%)
	6 mg	2.66 ± 0.25*(67%)	nd[c]	nq[b]	0.20 ± 0.03	1.10 ± 0.19*	0.09 ± 0.01*	0.28 ± 0.00	1.58 ± 0.04v	5.99/(41%)

[a] Comparisons are made to control within the same column. Data in parentheses are the inhibition rates compared to control;
[b] nq means the levels were lower than the limit of quantification;
[c] nd mean the levels were lower than the limit of detection;
* Provides statistical significance at p value = 0.05. Means ± standard deviations; n = 3 for all treatments.

conditions than those used in other studies (Alaejos & Afonso, 2011; Gibis, 2016). The method was reasonable for assessing and examining the inhibitory profiles of chilli pepper and capsaicin on HA profiles in roast beef patties and subsequent analysis.

3.6. Inhibitory profiles of chilli pepper on HA formation

To visualize and investigate the effects of different amounts of chilli pepper on HA formation from different categories, a multivariate method was necessary. Herein, PCA was used to screen the inhibitory patterns of chilli pepper on HA formation. The first two components accounted for 82.5% of the total variance. Fig. 1 shows the PCA scores and loadings plot (PC1 versus PC2) of the HAs formed in the roast beef patties with 0.5, 1 and 1.5% (g/g) chilli pepper added as well as blank patties, all of which were roasted for 10 min on each side. In the scores plot (Fig. 1A), samples of chilli pepper are mostly located at the top of the plot, and blank samples are located at the bottom. Furthermore, samples with different levels of chilli pepper formed three separate groups. These results indicate that chilli pepper could significantly affect the profiles of HAs in roast beef patties, and that the HA profiles of blank beef patties were greatly different from those with chilli pepper added. Furthermore, different chilli pepper concentrations had different HA profiles. The position of blank samples in the scores plot and PhIP in the loadings plot (Fig. 1B) were similar, indicating that PhIP levels in the blank samples were higher than in samples with added chilli pepper, showing that chilli pepper can significantly suppress the generation of PhIP in roast beef patties. The means of the PhIP levels on the addition of 0.5, 1 and 1.5% chilli pepper were significantly ($p < 0.05$) lower than those of the control samples, with inhibitions of 68, 61 and 53% as shown in Table 5. The results coincide well with a previous study, which reported that 1% red pepper added to the surface of beef chops and fried at 225 °C caused significant reduction of IQ, MeIQ, 4,8-DiMeIQx and PhIP (Oz & Kaya, 2011). DMIP and 1,5,6-TMIP, are also imidazopyridines, but their levels in the control beef patties were both lower than the LOQ, and significantly ($p < 0.05$) increased to 0.13 ± 0.01 and 0.24 ± 0.01 ng/g when 0.5% chilli pepper was added. When 1.0 and 1.5% of chilli pepper, with 2, 4, and 6 mg of capsaicin added, all levels were lower than the LODs or LOQs. As for other Has, such as IQx, MeIQx, 4,8-DiMeIQx, harman and norharman, most of them could be promoted by different amounts of chilli pepper (except for PhIP) but suppressed by capsaicin (except for MeIQx, harman and norharman) as shown in Table 5. These effects of chilli pepper or red pepper on the generation of 4, 8-DiMeIQx differ from a previous study (Oz & Kaya, 2011). Precursors could significantly affect the generation of HAs, by promoting or inhibiting their formation (Bordas, Moyano, Puignou, & Galceran, 2004). Thus, the difference between our research and the previous study may be caused by differences in HA precursors found in beef patties and beef chops. Furthermore, the spices used may also have different ingredients and interactions associated with HA generation, as different components may play significantly different roles, such as antioxidative or pro-oxidative effects (Yen et al., 2002).

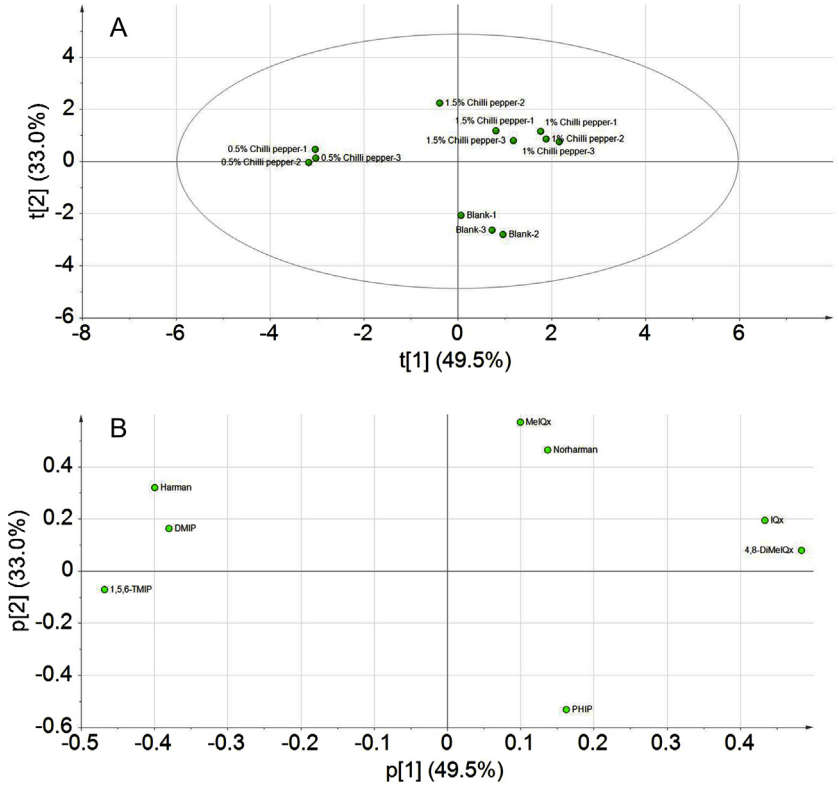

Fig. 1. Scores plot (A) and loadings plot (B) of PCA analysis (PC1 versus PC2) of control beef patties vs those with 0.5, 1.0 and 1.5% added chilli pepper.

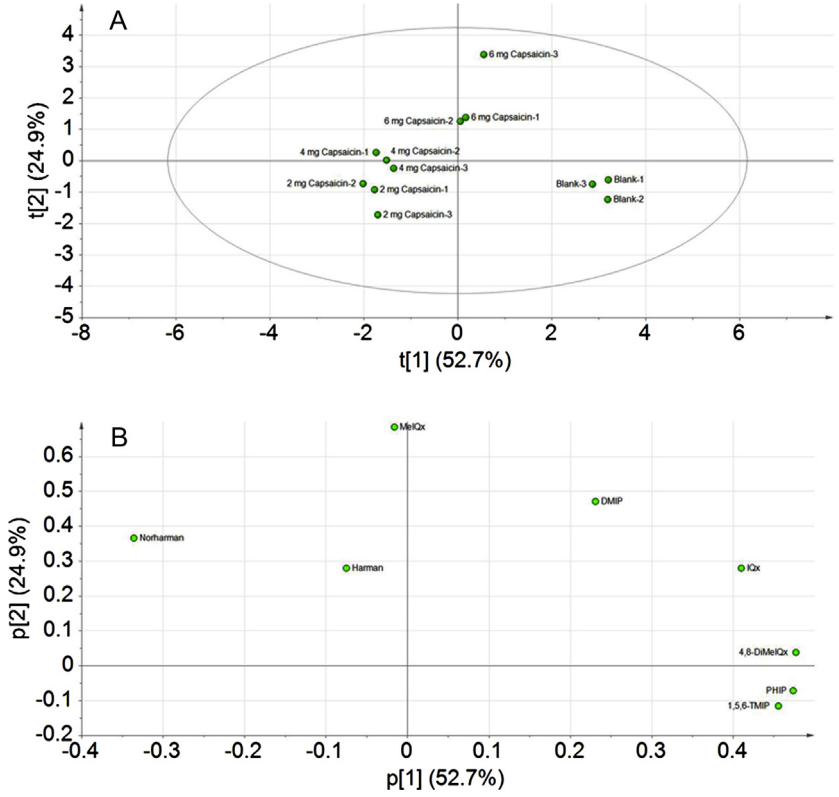

Fig. 2. Scores plot (A) and loadings plot (B) of PCA analysis (PC1 versus PC2) of control beef patties vs those with 2, 4 and 6 mg of capsaicin added.

The total HA level in the control was 10.08 ng/g, and 5.49, 6.53 and 7.62 ng/g when 0.5, 1.0 and 1.5% chilli pepper was added, showing inhibitions of 46, 35 and 24%, respectively. PhIP inhibitions were 68, 61 and 53% for the same concentrations. These results indicate that the total HAs and the most prevalent HA, PhIP, could be inhibited by chilli pepper dose-dependently, and that

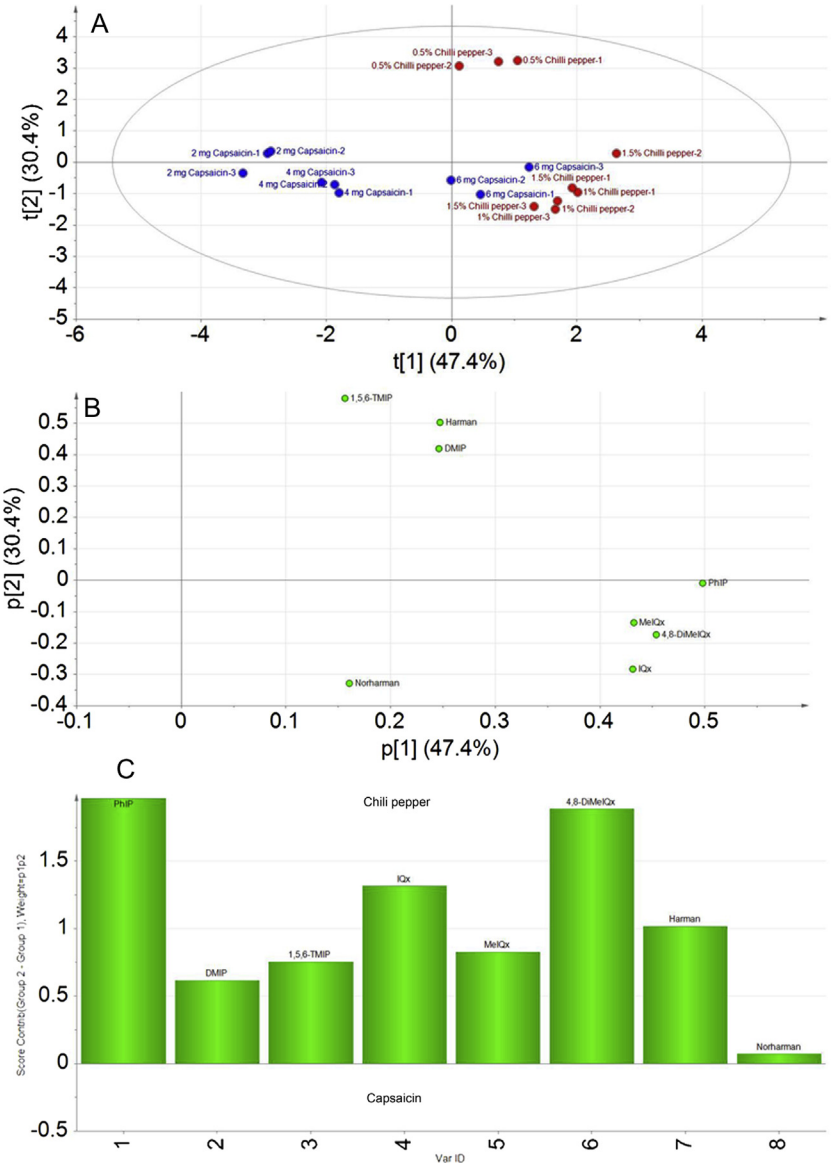

Fig. 3. Scores plot (A), loadings plot (B) and contribution plot (C) of PCA analysis (PC1 versus PC2) of beef patties with 0.5, 1.0 and 1.5% added chilli pepper as well as 2, 4 and 6 mg of capsaicin.

lower doses have higher inhibition. The results could be useful for helping to reduce HA formation during meat processing procedures using chilli pepper.

3.7. Inhibitory effects of capsaicin on HA profiles

Capsaicin is a pungent alkaloid that is present in large quantities in the placental tissue, internal membranes and other fleshy parts of *Capsicum* fruits (chilli peppers). Capsaicin has biological effects of pharmacological relevance (Venier et al., 2014), for example, inhibition of lipid peroxidation and scavenging of DPPH radicals (Venier et al., 2014). Radicals are thought to be involved in HA formation (Gibis & Weiss, 2012). As such, the effects of capsaicin on HA formation must be investigated. As far as we know, no research has been conducted on the inhibitory profiles of capsaicin on HA formation. Thus, we added different amounts of capsaicin to the beef patties to explore its inhibitory effects on HA formation. Fig. 2 shows the PCA scores (A) and loadings (B) of the HA profiles of blank and beef patties with 2, 4 and 6 mg capsaicin added. As shown in the figure, blank samples are located on the right of the scores plot, and the capsaicin samples are on the left. This means that the HA profiles of blank and capsaicin groups were significantly different. Furthermore, the samples of 2 mg capsaicin are located at the bottom of the plot, the 6 mg samples are at the top and the 4 mg samples are in the middle of the plot, indicating that the HA profiles differed with the level of capsaicin. In the loadings plot (Fig. 2B), samples of PhIP, IQx and 4,8-DiMeIQx are all located on the right, which coincides well with the scores plot (Fig. 2A) of the blank samples, indicating that the levels of these HAs were higher in the blank samples than in samples with added capsaicin, and that these three HAs could be inhibited by capsaicin. As shown in Table 5, the mean values of the levels of PhIP, IQx, MeIQx, 4,8-DiMeIQx, harman and norharman were 8.00 ± 1.07, 0.22 ± 0.01, 0.24 ± 0.01, 0.16 ± 0.01, 0.24 ± 0.04 and 1.10 ± 0.02 ng/g in control beef patties, respectively. When capsaicin was added, PhIP and total HAs were reduced. 2, 4 and 6 mg capsaicin had inhibitions of 98, 93 and 67% for PhIP, as well as 80, 68 and 41% for the total HAs. The inhibition effect of capsaicin on PhIP and total HAs was dose-dependent. Lower levels of capsaicin showed higher inhibition of HAs, which coincides well with the effects of chilli

pepper on total HAs and PhIP. Other Has, such as IQx and 4,8-DiMeIQx, were not significantly inhibited ($p < 0.05$), and MeIQx, harman and norharman were promoted. Although the effect of capsaicin on HA formation has not previously been investigated, capsaicin is considered to be a free radical scavenger (Embuscado, 2015). However, whether its HA inhibitory effects can be attributed to its free radical scavenging activity needs to be investigated further.

3.8. Differences of the inhibitory profiles between chilli pepper and capsaicin in HA formation

Chilli pepper contains many components other than capsaicin, which may interact with capsaicin and alter the HA inhibitory profile. Thus, differences in inhibitory profiles between chilli pepper and capsaicin must be investigated. PCA scores, loadings and contribution plots were used to explore the differences in HA inhibition between chilli pepper and capsaicin (Fig. 3). In the scores plot (Fig. 3A), the chilli pepper samples are almost all located to the right of the plot and the capsaicin samples are all to the left, indicating that the HA profiles of chilli pepper and capsaicin are different. In the loadings plot (Fig. 3B), the HAs investigated are all located on the right-hand side of the plot, just like the chilli pepper samples in the scores plot, meaning that the levels of these HAs in the chilli pepper samples were higher than in the capsaicin samples. These results are confirmed by the contribution plot (Fig. 3C), which shows that the contribution scores in the chilli pepper group were higher than in the capsaicin group, demonstrating that capsaicin had more inhibitory effects on HA formation compared to chilli pepper. The opposite effects of chilli pepper and capsaicin may be due to the promotion effects of other components in the chilli pepper or their products from roasting, which may have antagonistic effects on HA formation. Some phenolic compounds, such as p-coumaric acid and ferulic acid, have been detected at high levels in chilli pepper, and were proven to be HA inhibitors in roast beef patties (Zeng, Li, He, Qin, & Chen, 2016).

4. Conclusions

In general, chilli pepper and capsaicin affected the profiles of HAs in roasted beef patties, and different concentrations had different inhibitory profiles. Chilli pepper and capsaicin mainly inhibited the formation of total HAs, and had no significant ($P > 0.05$) effect on the texture of the patties. Chilli pepper and capsaicin suppressed PhIP and total HAs dose-dependently. Lower concentrations of chilli pepper or capsaicin inhibited HAs more strongly. Furthermore, PCA analysis showed that capsaicin inhibited HAs more than chilli pepper, which indicates that ingredients in chilli pepper other than capsaicin could promote the formation of IQx, MeIQx, 4,8-DiMeIQx, harman and norharman. Thus, other components in chilli pepper or their products must be screened for promotion of HA formation during roasting. These results may provide us with important information for safety control of the use of chilli pepper in high-temperature meat processing in the interests of HA reduction.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

This work was supported financially by the National Natural Science Foundation of China (Grant No. 31101287), the National Basic Research Program of China (Grant No. 2012CB720801), the National Key Scientific Instrument and Equipment Development Project (Grant No. 2011YQ170067), and the research project of the State Key Laboratory of Food Science and Technology (SKLF-ZZA-201604).

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