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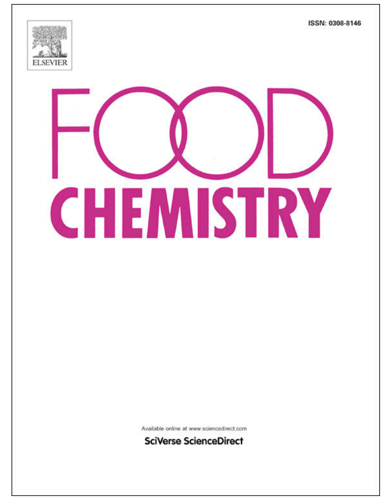

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Inhibition of Ethyl Carbamate Accumulation in Soy Sauce by Adding Quercetin and Ornithine during Thermal Process

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Abstract

Ethyl carbamate (EC), a genotoxic and carcinogenic compound in soy sauce accumulated during thermal processes, has raised public health concern for its multipoint potential carcinogenic risk to human. In this work, based on the analysis of EC accumulation during thermal processes of soy sauce, ornithine and quercetin were added before thermal processes to reduce EC accumulation. A reduction rate of 23.7-63.8% in simulated solution was founded. Kinetic studies indicated that ornithine was a byproduct of alcoholysis reaction when EC formed, while quercetin could compete with the precursor ethanol and react with carbamyl compounds, which therefore prevented EC accumulation. A maximum of 47.2% decrease of EC in soy sauce was achieved, and no remarkable changes in volatile compounds profile and color of soy sauce were found. In conclusion, the addition of quercetin and ornithine before thermal processes may be preferable for the controlling of EC content in soy sauce.

Keywords: ethyl carbamate; soy sauce; phenolic compound; inhibition mechanism

1. Introduction

Soy sauce, a widely consumed seasoning used in Asian food for thousands of years, is becoming increasingly popular worldwide, and the global consumption of soy sauce is estimated at 10 billion liters per year (Lee & Khor, 2015). However, the long brewing processes of soy sauce increases the incidence of several toxicants, and one of which, ethyl carbamate (EC), is a group 2A carcinogen classified by the World Health Organization's International Agency for Research on Cancer (IARC, 2010). EC is commonly presented in high levels in fermented foods and alcoholic beverages since it can be formed spontaneously during fermentation, distillation and storage (Gowd, Su, Karlovsky, & Chen, 2018; Liu et al., 2017). Soy sauce can be easily contaminated with high levels of EC (Mo, He, Xu, Huang, & Ren, 2014; Wu, Pan, Wang, Shen, & Yang, 2012). Koh et at (2007) found that the average EC content in Japanese-style soy sauce was determined to be 53.8 μg/L with a maximum of 128.9 μg/L, that is much higher than the EC in other fermented foods. In Korea, soy sauce has been found to be a major food contribution of EC exposure because of the high soy sauce consumption in the country (Choi, Ryu, Kim, Lee, Choi, & Koh, 2017; Koh et al., 2007). As EC have become a key monitored substance by Food and Agriculture Organization (FAO) since 2002, and the upper limit was 20 μg/L in food (Wu et al., 2012), it is valuable to develop and implement practical approaches on an industrial scale to control the level of EC in soy sauce.

Previous studies have proven that EC is mainly formed under nonenzymatic conditions via the reaction between ethanol and carbamyl compounds (urea, carbamyl phosphate and citrulline) or cyanide (Gowd et al., 2018; Jiao, Dong, & Chen, 2014; Zhao, Du, Zou, Fu, Zhou, & Chen, 2013). The major pathway of EC formation varies depending on the matrix. Many factors, such as the pH, temperature, light level, and copper ion and arginine concentrations which affect the formation of EC (Xia, Yang, Wu, Zhou, & Li, 2018). High temperature significantly increases EC formation, especially in the distillation and storage stages (Matsudo et al., 1993; Wu et al., 2014). Sterilization process has been confirmed to be a major contribution for the EC accumulation in soy sauce production (Matsudo et al., 1993; Zhou, Siroli, Patrignani, Sun, Lanciotti, & Xu, 2019), in fact, approximate 83.5% of the EC forms during heat treatment, including the extraction (refining) of raw soy sauce and pasteurization. These treatments are normally maintained above 60°C for tens of minutes to hours. However, the effect of these factors on the nonenzymatic reaction between the primary EC precursors has not been elucidated.

The available studies on EC reduction are heavily focuses on the control of precursors, which has primarily been evaluated by screening low precursor-secreting bacteria or by adding bacteria (or enzymes) with high precursor consumption (or decomposition) ability (Kim, Lyu, Kim, & Lee, 2015; Liu et al., 2018; Zhang, Du, Chen, & Fang, 2016). In addition, fermentation optimization and charcoal filtration have also been adopted for EC reduction (Nout, Ruikes, Bouwmeester, & Beljaars, 1993; Park et al., 2009). Although EC can be effectively reduced by some of these approaches, their applications are still limited in practical ways because of the influence on soy sauce flavor, complicated procedures, and relative low EC reduction efficiency (Jiao et al., 2014). Therefore, new approaches should be developed to reduce EC more easily and safely. To date, no strategy has been developed to control EC formation during heat treatment, when the majority of EC accumulation occurs during soy sauce production.

In recent years, phenols have been commonly added to food as antioxidants and to reduce the levels of hazardous chemicals (such as furan and acrylamide) produced during thermal treatment of food via competitive reactions with intermediates (Shen et al., 2017; Y. Zhang & Zhang, 2007). Polyphenols have been used to decrease ethyl carbamate-induced cytotoxicity and EC precursor accumulation during brewing (Chen, Xu, Zhang, Su, & Zheng, 2016; Zhou, Fang, & Chen, 2017). In addition, phenolic compounds, such as gallic and protocatechuic acid, may inhibit the conversion of urea/citrulline to EC (Zhou et al., 2017). Therefore, the aim of this study was to investigate the characteristics of EC formation during heat treatment of soy sauce under different conditions and the impact of phenols on EC formation and to develop an easy and efficient method to reduce EC levels via the addition of inhibitors. In addition, the impacts on the quality and characteristics of soy sauce were assessed.

2. Materials and methods

2.1 Reagents and sample

EC (99%), butyl carbamate (98%), urea (99%), citrulline (98%), arginine (98%), ornithine monohydrochloride (98%), quercetin (QC, 95%), 2,6-di-tert-butyl-4-methylphenol (BHT, 98%), butylated hydroxyanisole (BHA, 98%), tert-butylhydroquinone (TBHQ, 98%), carvacrol (CA, 99%), thymol (TM, 99%), protocatechuic acid (PA, 97%) and gallic acid (GA, 99%) were purchased from Aladdin

(Shanghai, China). Tea polyphenol (TP, 70% catechin hydrate) was extracted in our laboratory. Sodium hydroxide, anhydrous sodium sulfate and acetic acid were analytical grade and purchased from Heowns (Tianjin, China). The water used in this work was purified using an Elix ProGard TS2 system (Millipore, France).

Soy sauce *moromi*, which had been fermented for 165 days, was provided by a soy sauce manufacturer in Guangdong Province, China. The *moromi* was filtered through four layers of gauze to separate the fermented liquor from the soy sauce residue. After centrifugation at 2647 g for 10 min at 4°C, the supernatant was collected as raw soy sauce. selected constituents in the raw soy sauce were evaluated in terms of their initial concentrations in the matrix (Table S1).

2.2 Thermal processing of raw soy sauce and of a model solution

For simplicity, all reactions consisted of 20 mL of solution in a 30 mL sealed cylindrical glass vial with a narrow neck. The vials were heated in a water bath (or oil bath) at a set temperature to simulate the heat treatment of soy sauce. Afterwards, these vials were immediately cooled in an ice bath. Unless otherwise indicated, the pH was adjusted to 4.5 by using acetic acid/sodium acetate.

Preparation of the model solution: 2.36 g of citrulline and 36 mg of urea were transferred into a 1 L volumetric flask and diluted with 16.8% brine to volume. Except for ethanol (volatile component), the contents of the two main EC precursors (citrulline and urea), pH and NaCl content in the model solution were the same as those in the prepared raw soy sauce (Table S1). The prepared model solution was stored at 4°C while shielded from light. The raw soy sauce samples and model solution were spiked with absolute ethanol at a final concentration of 2% (V/V), and all samples were heated at 90°C and 80°C separately for 30 min.

2.3 Influence of temperature, pH, arginine, ornithine and metal ions on EC formation

The model solution was spiked with ethanol (2%, V/V) before use and then treated as follows: a) The model solution was heated at different temperatures (60-110°C) for 30 min. b) The pH of the model solution was adjusted to 4-6, and the solution was then heated at 90°C for 30 min. c) Aliquots of the model solution (20 mL each) were spiked with the following constituents: arginine (2-8 mg/L), ornithine (1-4 mg/L) and metallic ions ($CuCl_2$, $FeCl_3$, $ZnCl_2$, $CaCl_2$, $MgSO_4$, and $FeSO_4$; 50 mg/L each), and then the solutions were heated at 90°C for 30 min.

2.4 Influence of phenolic compounds on EC formation

Stock solutions of phenols were prepared in absolute ethanol at a concentration of 5 mg/mL. Then, 400 μL of each stock solution was added to the model solution for a final phenol concentration of 100 mg/L. In addition, quercetin solutions with concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.05 and 0.005 mg/mL were prepared by diluting the stock solution with absolute ethanol, and then 400 μL of each of these solutions was added to the model solutions as test groups. Model solutions with 400 μL of ethanol added were used as the control group (no phenols). These solutions were heated at 90°C for 30 min.

2.5 Kinetic experiments

The principal reactants (primary precursors) for EC formation during heat treatment were determined before the kinetic experiment. Ethanol (final concentration: 2% or 4%) and 1-4 mg/mL citrulline (or 10-80 mg/L urea) were added to 16.8% brine (pH 4.5), and the mixture was heated at 90°C for 30 min or 60 min. The constituents in different trials of the kinetic studies are shown in Table S2, and the heating duration in the trials was 30, 60, 90, 150, 210, 300, or 450 min. A pseudo-first-order-kinetic equation was selected to describe EC formation over the course of the heat treatment experiment. The maximum EC content ($[EC]^{\infty}$) was read from the plot of the EC concentration versus time. The rate constant K (observed rate constant) was calculated from plots of $\ln([EC]^{\infty}-[EC]_t)$ versus time (Galinaro, Ohe, da Silva, da Silva, & Franco, 2015).

2.6 Central composite design for the optimization of EC reduction

A central composite design (CCD) was applied to determine the optimal addition of quercetin and ornithine for EC reduction during soy sauce production, with two major factors (ethanol content and heat temperature). According to preliminary results and the soy sauce matrix, the process variables and their ranges were chosen, as shown in Table S3. To ensure alignment of the model with real samples, raw soy sauce was diluted 5-fold with distilled water and then fortified with all the ingredients in Table S1 except for ethanol and ornithine. Then, a total of 30 different combinations with a random order and 6 center points were evaluated according to a full CCD configuration for 4 factors, and the EC content determined after heat treatment was set as the response. The experimental design and data analysis were performed using Design Expert software, version 8.0.6.

2.7 Quantification of EC

Solid-phase extraction (SPE) (Chinese National Standards, Method for the Determination of Ethyl Carbamate in Food, GB 5009.223-2014) and liquid-liquid extraction (LLE) (Zhou et al., 2017) were used to extract EC from the raw soy sauce and model solution samples, respectively. The final extracts were diluted to 1 mL each with dichloromethane (Leca, Pereira, Pereira, & Marques, 2014) for GC/MS (Agilent 7890A-5975C, America) analysis with electron ionization (70 eV). A CP-WAX column (50 m, 0.32 mm, 1.2 μm, Agilent Technologies, the Netherlands) was used for separation. The oven temperature program was as follows: 80°C (5 min), followed by an increase to 180°C at a rate of 10°C/min (0 min) and then an increase to 220°C at a rate of 4.5°C/min (12 min). The inlet and detector interface temperatures were 250°C and 230°C, respectively. Sample aliquots of 1.0 μL were injected in splitless mode. Selected ion monitoring was used for the analysis of EC (SIM: m/z 44, 62, 74, and 89) and d_5-EC (SIM: m/z 44, 64, 76, and 89, internal standard). The recovery and coefficient of variation in matrix of GC-MS were tested, and the results were shown in Table S4. The rate of change in EC was calculated by Formula 1:

$$\text{Rate of change (\%)} = \frac{\text{EC content in treatment-EC content in control}}{\text{EC content in control}} \times 100 \qquad (\text{Formula 1})$$

2.8 Evaluation of volatile compounds and color

The volatile compounds and color after heat treatment were evaluated to investigate the impacts of quercetin and ornithine addition on the fundamental qualities of soy sauce. The volatile compounds were extracted by an SPME Trisplus automated sampler and then analyzed by GC/MS with a CP-WAX (50 m, 0.32 mm, 1.2 μm, Agilent

Technologies, the Netherlands) (Feng et al., 2015). Aliquots of sample (3 mL), 300 mg/L NaCl and internal standard (4-methyl-2-pentanol, 5 μg/L) were transferred into headspace vials, which were then sealed. Samples were equilibrated at 45°C for 20 min and extracted with 85 μm carboxen/polydimethylsiloxane fibers for 30 min at the same temperature. The separation program was as follows: 40°C for 2 min, ramped to 120°C at a rate of 5°C/min, held for 2 min, increased to 220°C at a rate of 7°C/min and held for 5 min. The injection temperature and ion source temperature were set at 250°C and 230°C, respectively. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. The scan range and scan rate applied to the analysis were 35-350 m/z and 3.00 scans/s, respectively. The electronic nose measured using a pen3 e-nose (Airsense Int, Schwerin, Germany) after 10-fold dilution with distilled water. The sample was placed into a 10 mL sealed glass bottle, and then balanced in the water bath (40°C) for 5 minutes. The response points at 110 s of electronic nose, which were the maximum response signal for each of the 10 sensors (Table S5), were used for analysis, each analysis was repeated 10 times. The color was detected using a colorimeter (ColorFlex EZ, HunterLab, America) after 10-fold dilution with distilled water. Values for L* (lightness), a* (redness) and b* (yellowness) were recorded, and ΔE was calculated as the primary descriptor (Gutiérrez, Tapia, Pérez, & Famá, 2015), each analysis was repeated 10 times.

2.9 Statistical analysis

All experiments were performed in triplicate unless otherwise noted, with the mean value and standard error reported. The data were analyzed by ANOVA using SPSS (version 22, IBM, USA), and significant differences among the mean values were determined by adopting Tukey's multiple range test, with significance defined at $P<0.05$.

3. Results and discussion

3.1 Thermal treatment of the raw soy sauce and the model solution

The contents of selected components in the raw soy sauce are shown in Table S1. The EC content in the final broth was 7.4 μg/L, which was only 16.5% of that in the same batch of soy sauce. The result strongly indicated that EC was primarily accumulated during heat treatment after fermentation, rather than during fermentation itself. The ethanol content in the raw soy sauce was 1.06%, which was lower than that in many other raw soy sauces and soy sauce products (Matsudo et al., 1993; Zhou et al., 2017). As reported in our previous research, ethanol was highly correlated with EC during thermal treatment (Zhou et al., 2019). In this study, the EC contents in the model solution and raw soy sauce heated at 90°C were increased by 68.5% and 61.8%, respectively, when compared to those in the two corresponding samples heated at 80°C (Table S6), indicating that the increase in temperature greatly increased EC formation. Notably, although there were other EC precursors (such as carbamyl phosphate and cyanate) in the raw soy sauce, the EC contents were lower in the raw soy sauce than in the model solution at both 80°C and 90°C, suggesting that components of raw soy sauce inhibit the formation of EC during heat treatment. According to previous studies, these components could be metal ions and amino acids related to arginine metabolism.

3.2 Factors influencing EC formation during heating

Metal catalysts are commonly added to the synthesis of carbamate, and previous research has revealed that some metal ions can catalyze the conversion of precursor into

EC (Aresta, Boscolo, & Franco, 2001). The effects of metal ions on EC formation are shown in Fig. 1A. In the model solution, all involved metallic ions promoted the formation of EC during heat treatment. But only Fe(III) and Cu(II) at 50 mg/L induced an increase in EC by more than 15%. However, the Cu(II) content in the soy sauce and fermented liquor was below 1 mg/L, and the total iron content in soy sauce, including Fe(III) and Fe(II), was approximately 50 mg/L (Table S1). Moreover, although the reaction between cyanic acid and ethanol could be catalyzed by Cu(II) or Fe(III) (Weber & Sharypov, 2009), cyanide was not the main precursor of EC in the soy sauce. Therefore, it was speculated that the metal ions had little effect on EC formation during the heat treatment in soy sauce production.

Citrulline and urea are the major precursors of EC in many fermented foods and are mainly derived from the catabolism of arginine via the arginine degradation pathway and urea cycle, respectively (Zhao et al., 2013). In addition, the degradation of arginine also generated ornithine (Araque, Gil, Carreté, Bordons, & Reguant, 2009). A high arginine content was maintained during soy sauce fermentation (Zhang, Fang, Chen, & Du, 2014), and considerable amounts of arginine were present in the raw soy sauce (3.48 mg/mL). Our previous study found a positive correlation between the total arginine and EC in soy sauce products, while free ornithine was negatively correlated with EC (Zhou et al., 2017). The phenomenon was confirmed again in the present study,, the addition of ornithine significantly decreased the EC content (23.7%-37.4%), but a high level of arginine (above 4 mg/mL) resulted in a high EC content (Fig. 1B). This suggested that free ornithine might be an inhibitor of EC formation. Therefore, the application of a screened strain with a high capability to convert arginine into ornithine might be an efficient method for EC reduction.

However, the above conclusions on the influence of arginine on EC formation were contradicted to those of Matsudo et al (1993), who found that arginine had no effect on EC formation. We suspected that the addition of arginine (alkaline) increased the pH of the raw soy sauce and thus significantly hindered the formation of EC. Therefore, the effect of pH on EC formation was assessed, and presented in Fig. 1C. As expected, high pH significantly decreased the EC content, which probably offset the increase in EC by the addition of arginine. In other words, low pH was conducive to EC formation during heat treatment. The pH of the final soy sauce fermented broth was approximately 4.5, which was suitable for EC formation at 90°C. In the range of 70°C to 90°C (common temperatures of soy sauce heat treatment), a rise of 10°C could increase the EC content by more than 2-fold (Fig. 1D), which was consistent with the former results confirming that high temperature was critical for EC formation. Therefore, relatively low temperatures and high pH were favorable for EC control during soy sauce heat treatment.

3.3 Effect of phenolic compounds on EC formation during heat treatment

In the model solution, four polyphenols, four monophenols and one polyphenolic extract (Fig. S1) were added to test their effects on EC formation, and both positive and negative effects were observed (Fig. 2A). The polyphenols quercetin and TBHQ had an inhibitory effect on EC formation, whereas the monophenols CA, BHA and BHT improved EC formation, indicating that these three commonly used antioxidants should be avoided in soy sauce production. In a previous study, the transformation of urea/citrulline to EC was suppressed by GA and PA during wine brewing (Zhou et al., 2017). However, during thermal processing, GA, PA, TM and TP did not significantly reduce the EC content. In general, the tested monophenols promoted the formation of EC, some polyphenols had the opposite effect, and phenolic acids had no significant effects on EC formation. Among the tested phenolic compounds, quercetin significantly reduced the EC content by 48.38%, making it a potential inhibitor of EC formation. Quercetin is a flavonoid and often present as quercetin-3-O-rutinoside (rutin) in the human diet, many literatures have reported its strong antioxidant and anticancer activity (Jan et al., 2010; Dong et al. 2019), and the weight of the available evidence supports the safety of quercetin for addition to food (Harwood et al., 2007). The strong inhibitory ability of quercetin to against EC formation might be due to its preferential (over other EC precursors) reaction with carbamyl compounds under mild conditions. EC reduction had no dose-dependent relationship with the quercetin concentration (Fig. 2B). The addition of quercetin at 0.1 mg/L had no significant impact on the EC reduction compared to the control groups ($p<0.05$), and the addition of quercetin at 10-40 mg/L led to the optimal EC reduction ratio (approximately 62%).

3.4 Primary EC precursors during heat treatment

To further elucidate the crucial factors in the mechanism of EC reduction during heat treatment, the principal reactants contributing to EC formation in soy sauce were determined firstly. As shown in Fig. S2, the EC content increased with increasing citrulline, urea and ethanol contents, similar to the EC increase with prolonged heating time. The essence of EC formation is the alcoholysis reaction between ethanol and carbamyl compounds, and the EC level in citrulline and ethanol solutions was higher than that in urea and ethanol solutions. When the contents of citrulline (2000 mg/L) and urea (40 mg/L) in the model solution were similar to that in raw soy sauce, the majority of EC was formed in the reaction between citrulline and ethanol. These results were consistent with the findings of Matsudo et al. (1993), Zhang et al. (2014) and our former study (Zhou et al., 2019). The reasons for low EC formation in urea and ethanol solutions were speculated as follows: a) the molality of urea is much lower than that of citrulline in soy sauce, b) the formation of EC via catalytic alcoholysis of urea and ethanol requires high temperature and pressure, which leads to more by-products (Gu, 2013), and c) the generation of ammonia significantly increases the pH of the solution (Fig. 3A). Therefore, the model solution used in the kinetic experiments consisted of two principal precursors: 2% ethanol and 2000 mg/L citrulline.

3.5 Kinetic evaluation of temperature, ethanol, ornithine and quercetin in EC formation

The heating temperature and ethanol concentration in the raw soy sauce were the crucial factors affecting the EC formation during soy sauce production. As shown in Table 1, the $[EC]_{max}$ and reaction rate were significantly higher with 4% ethanol than that with 2% ethanol in the reaction between citrulline and ethanol at 90°C, which was different from the finding that the EC yield was not sensitive to the ethanol and citrulline concentrations in sugar cane spirit with an alcohol level of 13.3% (Galinaro et al., 2015). The same decrease in the $[EC]_{max}$ and reaction rate at low temperature was observed in our study and in a previous report (Galinaro et al., 2015). The higher the ethanol concentration and heat temperature, the more EC produced. However, the rates of EC formation were very low, with a yield of only 10^{-4}. Notably, the addition of quercetin led to a noticeable decrease in the EC yield and reaction rate. The rate of EC formation decreased upon addition of 1 mg/mL ornithine, but the $[EC]_{max}$ was similar to that of the control group, indicating that the EC reduction mechanisms related to the addition of quercetin were different from those related to the addition of ornithine. Based on the characteristics of the alcoholysis reaction and the reversible reaction between EC formation and hydrolysis during food production (Bogyoung Choi & Koh, 2016), we suspected that there were two steps in the reversible reaction during EC formation via alcoholysis between citrulline and ethanol (Fig. 3B). These observations suggested that ornithine was one of the intermediate products in the first step that can inhibit carbamic acid formation, but quercetin could compete with ethanol for reaction with carbamyl compounds.

3.6 Optimization of ornithine and quercetin addition

The CCD results revealed the detailed impacts of the major factors affecting EC formation during heat treatment of raw soy sauce, and the optimal addition of quercetin and ornithine can be predicted using the "Point Optimization" tool of Design Expert software. As shown in Table S7, the ethanol content and temperature had significant effects on EC accumulation ($p<0.0001$). Notably, when the heating temperature was set between 70°C and 75°C or the ethanol content of the raw soy sauce was below 1.3%, the EC content was below 20 μg/L (Fig. 4A), which met the international standard set by the Food and Agriculture Organization (FAO) in 2002 (Wu, Chen, Pan, Tu, Zhou, & Mo, 2012). Ornithine had a significant negative effect on EC formation, especially at high temperatures (Fig. 4B). As the optimal quercetin content range was used in the CCD experiment, the addition of 10-40 mg/L quercetin led to no significant change in the EC yield (p=0.1214). When the ethanol content exceeded 2.4%, the addition of quercetin at a high concentration could effectively suppress the formation of EC, whereas when the heating temperature was higher, quercetin at a low concentration could better decrease the EC content (Fig. 4C and 4D). To achieve practical EC reduction with ornithine and quercetin, we choose 90°C and 80°C as the sterilization temperatures in this study, and the ethanol content was 2%. For 80°C sterilization, the optimal ornithine and quercetin contents obtained by inputting the above criteria in the Design Expert software were 0.67 mg/mL and 18.63 mg/L, respectively, with a predicted EC content of 18.96 μg/L. When the temperature was set at 90°C, the optimum ornithine and quercetin contents were 0.85 mg/mL and 10 mg/L, respectively, with a predicted EC content of 23.58 μg/L.

3.7 Practical EC reduction, volatile flavors and color related to soy sauce

The EC content was significantly lower in the soy sauce samples containing the optimal amounts of quercetin and ornithine than in the control groups, with EC reductions of 42.1% and 47.2% at 80°C and 90°C, respectively, as shown in Fig. 5. However, the increases in the EC content in the treatment groups (1.5-3.3 μg/L) were slightly lower than the predicted values, suggesting that other unidentified EC precursors were occurred in the soy sauce (the raw soy sauce was diluted 5-fold in the

CCD experiment). Approximately 76 volatile compounds were identified by GC/MS (Fig. 5). There were high levels of alcohols, volatile acids, esters, and aldehydes in the soy sauce after heat treatment, while some ketones, furan(ones), phenols, and sulfur-containing compounds were present at low percentages. The aldehydes (both 80°C and 90°C) and furan(ones) (90°C) in the treatment groups were higher than the aldehydes and furan(ones) in the control groups, and the other major volatile compounds did not significantly differ between the treatment and control groups. The 4-ethyl-2-methoxyphenol, 2-methylbutanal, 3-methylbutanal and benzene acetaldehyde contents were higher in the treatment groups than that in the control groups, and these compounds could contribute to the aroma of ‘‘smoky, bacon, soy sauce’’, ‘‘malty, almond’’, ‘‘malty, almond’’ and “honey-like,” respectively, in high-salt liquid fermented soy sauce (Feng, Cai, Su, Zhao, Wang, & Zhao, 2014). For data analysis of e-nose determination, the difference between ten sensors response value of four samples is not significant (Fig. 5), the R1, R3, R5. R7. R8. R9 sensor which sensitive to aromatic compounds, sulfur compounds and pyrazines were changed slightly. PCA was used for discriminating samples based on the difference of volatile compounds and signal intensity of the sensor array. The data showed that only a slight shift of different samples along the first principal component, PC1, which represented 91.29% of the total variance with value 97.37%. The differences were not evident between the samples of 80°C control, 80°C treatment and 80°C control. In the color evaluation (Fig. 5), the significant decrease in L* in the heated samples indicated that the raw soy sauce turned black after heat treatment (Gutiérrez et al., 2015). There were no differences in ΔE and

L* between the control group and the soy sauce with added quercetin and ornithine. In general, the addition of ornithine and quercetin had limited effects on the volatile flavor compounds and color of soy sauce but might induce the generation of key volatile compounds.

4. Conclusions

In this study, we found that ornithine and quercetin could significantly decrease EC formation during soy sauce heat treatment, and speculative EC inhibition mechanisms were proposed on the basis of the results of kinetic experiments. Then, a creative and practical method for EC reduction was developed via the simple addition of quercetin and ornithine before heat treatment during soy sauce production. With the optimized contents of quercetin and ornithine, the EC concentration in soy sauce was reduced by 42.1% and 47.2% during heat treatment at 80°C and 90°C, respectively. The proposed method had limited effects on the volatile flavor compounds and color of soy sauce. The discovery of more efficient inhibitors of EC formation is still underway.

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Conflict of interests

The authors declare that they have no conflicts of interest.

Supporting information

The supporting information includes Figs. S1-S2 and Tables S1-S5.

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Figure captions

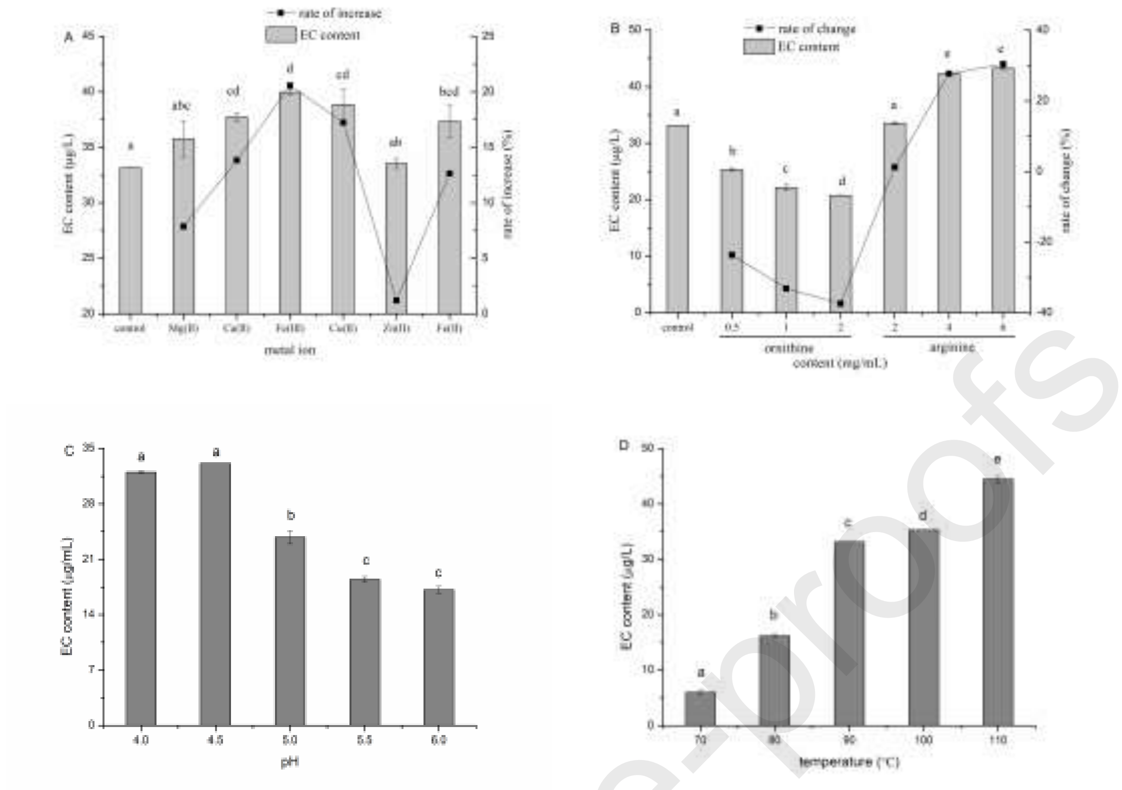
Fig. 1. The effect of metallic ions (A), arginine, ornithine (B), pH (C) and temperature (D) on EC formation (data represent the mean ±SD of three independent experiments, bars with different letters are significantly different from the control group ($p < 0.05$), similarly hereinafter).

Fig. 2. The effect of phenolic compounds on EC formation during heat treatment.

Fig. 3. The proposed formulation of citrulline (A) and urea (B) reacting with ethanol to form EC, and the speculative re-alcoholysis reaction mechanism of ornithine and quercetin.

Fig. 4. The contour of EC formation as a function of the ethanol content and temperature (A), the ornithine content and temperature (B), the ethanol and quercetin contents (C) and the temperature and quercetin content (D). Other parameters: 1.75% ethanol, 80°C, 25 mg/L quercetin and 0.5 mg/mL ornithine.

Fig. 5. The effect of ornithine and quercetin on the EC content, volatile flavor compounds (Volatile component categories and E-nose data), characteristic odorants and color of soy sauce.



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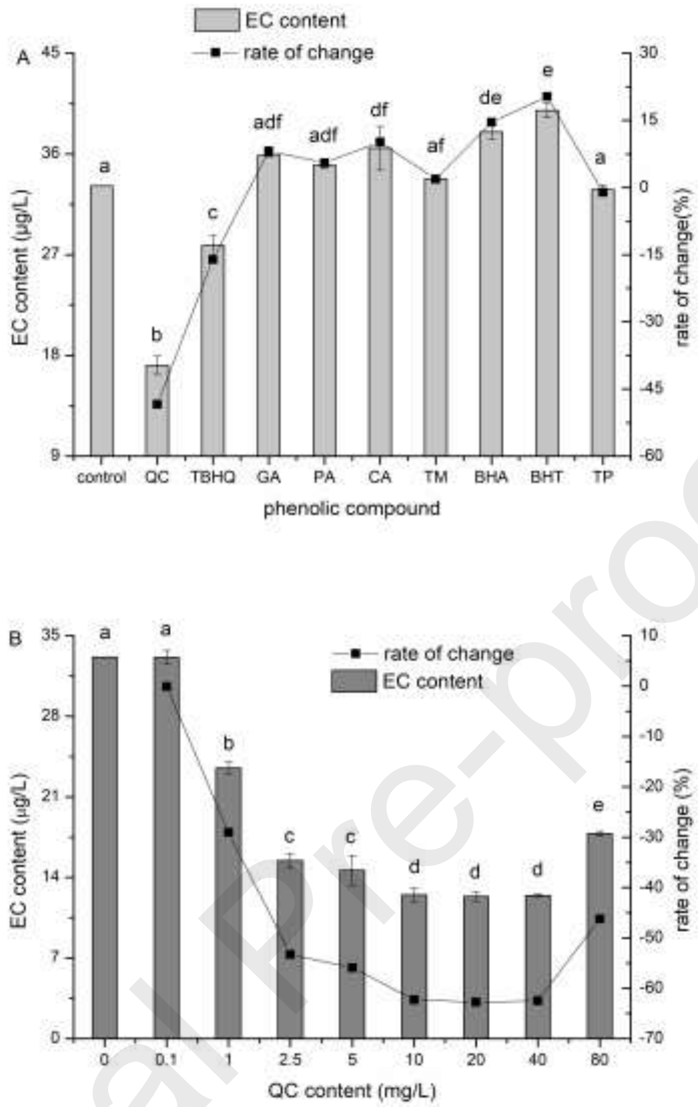
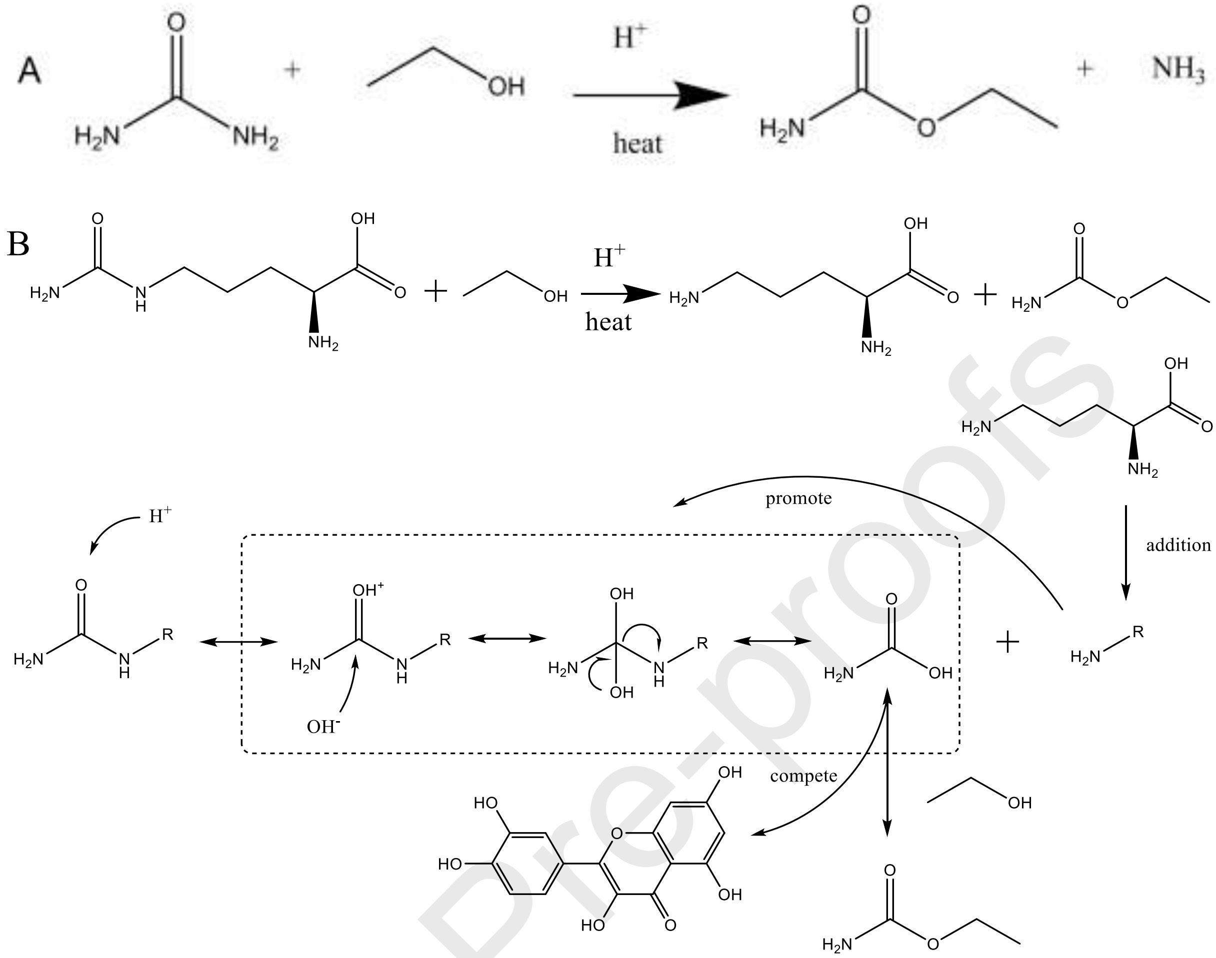


Fig. 2. The effect of phenolic compounds on EC formation during heat treatment.



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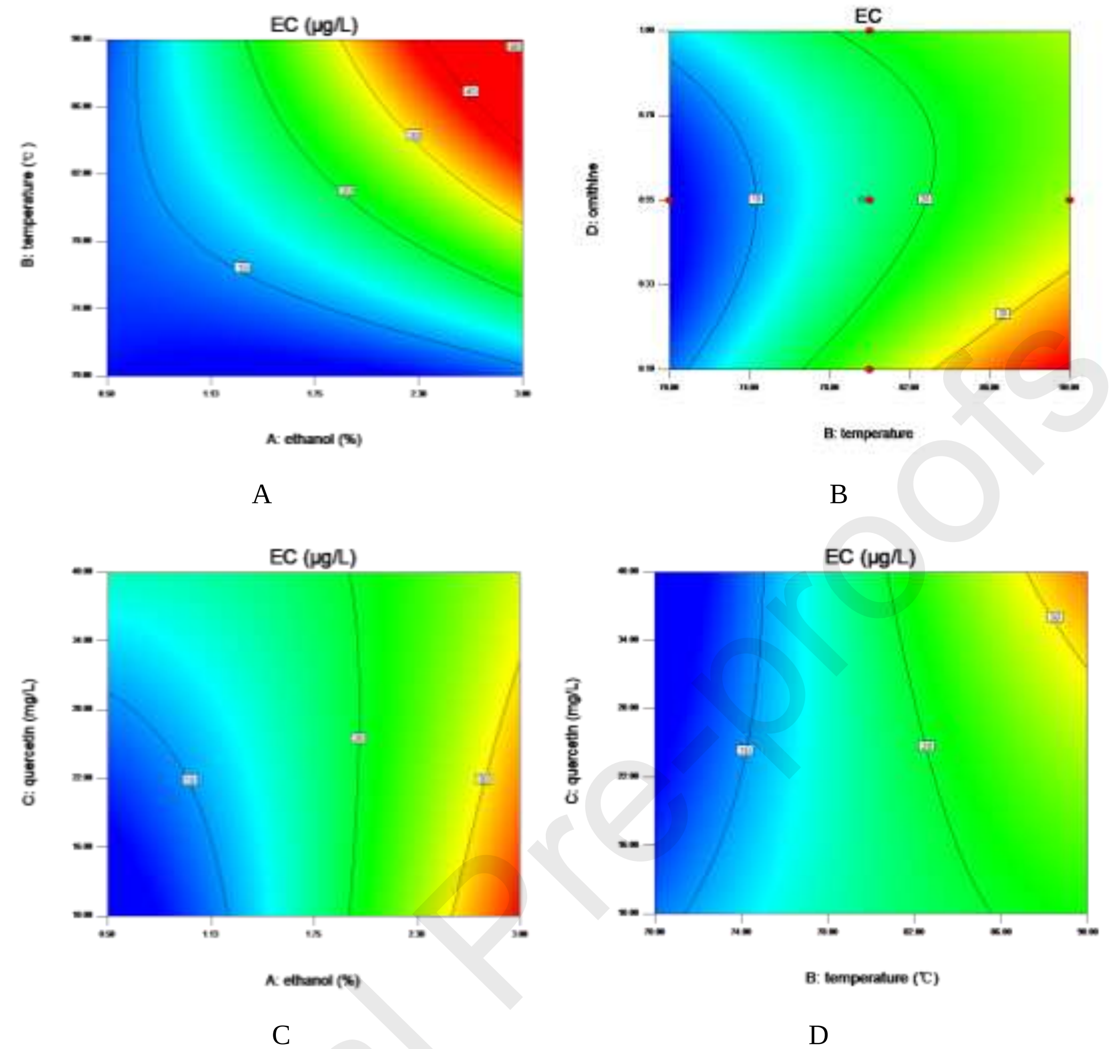

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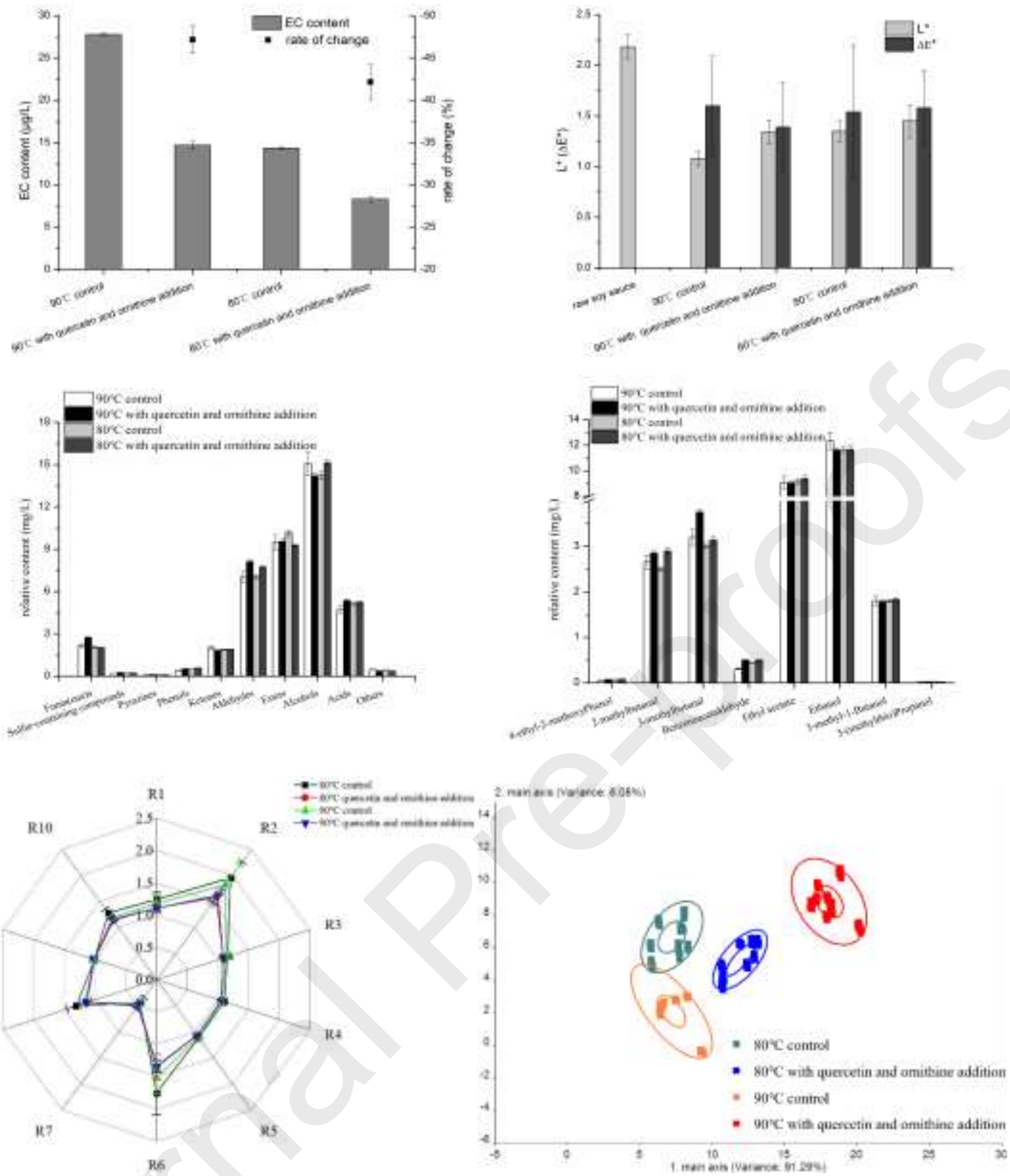

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Table 1 Maximum concentration of EC formed and the observed rate constant at different temperatures and ethanol, and quercetin and ornithine concentrations during heat treatment (control: 2% ethanol, 90°C, 0 mg/L quercetin, 0.5 mg/mL ornithine and pH 4.5).

	$[EC]_{max}$ (μg/L)	k (min^{-1})
Control	498.53±33.76	2.68×10^{-3}
Ethanol (4%)	611.44±30.42	3.22×10^{-3}
Temperature (80°C)	275.69±25.13	2.25×10^{-3}
Quercetin (40 mg/L)	417.31±35.23	2.53×10^{-3}
Ornithine (1 mg/mL)	492.00±57.90	2.50×10^{-3}

Highlight

- Ethyl carbamate formation during thermal process of soy sauce were studied.
- Ornithine and quercetin were found to be effective for reducing ethyl carbamate.
- The possible mechanisms were explained.
- No remarkable difference in volatile compounds profile and color of soy sauce were found.

Kai Zhou**:** Conceptualization, Methodology, Investigation, Writing **-** Original Draft

Francesca Patrignani**:** Methodology, Investigation, Validation, Writing **-** Review & Editing

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