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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 **1. Introduction**

2 Soy sauce, a widely consumed seasoning used in Asian food for thousands of years, 3 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 4 processes of soy sauce increases the incidence of several toxicants, and one of which, 5 ethyl carbamate (EC), is a group 2A carcinogen classified by the World Health 6 Organization's International Agency for Research on Cancer (IARC, 2010). EC is 7 8 commonly presented in high levels in fermented foods and alcoholic beverages since it 9 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 10 high levels of EC (Mo, He, Xu, Huang, & Ren, 2014; Wu, Pan, Wang, Shen, & Yang, 11 12 2012). Koh et at (2007) found that the average EC content in Japanese-style soy sauce was determined to be 53.8 μ g/L with a maximum of 128.9 μ g/L, that is much higher 13 than the EC in other fermented foods. In Korea, soy sauce has been found to be a major 14 15 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 16 become a key monitored substance by Food and Agriculture Organization (FAO) since 17 2002, and the upper limit was 20 μ g/L in food (Wu et al., 2012), it is valuable to develop 18 19 and implement practical approaches on an industrial scale to control the level of EC in soy sauce. 20

21 Previous studies have proven that EC is mainly formed under nonenzymatic
22 conditions via the reaction between ethanol and carbamyl compounds (urea, carbamyl

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

The available studies on EC reduction are heavily focuses on the control of 36 precursors, which has primarily been evaluated by screening low precursor-secreting 37 bacteria or by adding bacteria (or enzymes) with high precursor consumption (or 38 decomposition) ability (Kim, Lyu, Kim, & Lee, 2015; Liu et al., 2018; Zhang, Du, Chen, 39 & Fang, 2016). In addition, fermentation optimization and charcoal filtration have also 40 been adopted for EC reduction (Nout, Ruikes, Bouwmeester, & Beljaars, 1993; Park et 41 al., 2009). Although EC can be effectively reduced by some of these approaches, their 42 applications are still limited in practical ways because of the influence on soy sauce 43 flavor, complicated procedures, and relative low EC reduction efficiency (Jiao et al., 44

45	2014). Therefore, new approaches should be developed to reduce EC more easily and
46	safely. To date, no strategy has been developed to control EC formation during heat
47	treatment, when the majority of EC accumulation occurs during soy sauce production.
48	In recent years, phenols have been commonly added to food as antioxidants and to
49	reduce the levels of hazardous chemicals (such as furan and acrylamide) produced
50	during thermal treatment of food via competitive reactions with intermediates (Shen et
51	al., 2017; Y. Zhang & Zhang, 2007). Polyphenols have been used to decrease ethyl
52	carbamate-induced cytotoxicity and EC precursor accumulation during brewing (Chen,
53	Xu, Zhang, Su, & Zheng, 2016; Zhou, Fang, & Chen, 2017). In addition, phenolic
54	compounds, such as gallic and protocatechuic acid, may inhibit the conversion of
55	urea/citrulline to EC (Zhou et al., 2017). Therefore, the aim of this study was to
56	investigate the characteristics of EC formation during heat treatment of soy sauce under
57	different conditions and the impact of phenols on EC formation and to develop an easy
58	and efficient method to reduce EC levels via the addition of inhibitors. In addition, the
59	impacts on the quality and characteristics of soy sauce were assessed.

60 2. Materials and methods

61 **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-4methylphenol (BHT, 98%), butylated hydroxyanisole (BHA, 98%), tertbutylhydroquinone (TBHQ, 98%), carvacrol (CA, 99%), thymol (TM, 99%), protocatechuic acid (PA, 97%) and gallic acid (GA, 99%) were purchased from Aladdin

67	(Shanghai, China). Tea polyphenol (TP, 70% catechin hydrate) was extracted in our
68	laboratory. Sodium hydroxide, anhydrous sodium sulfate and acetic acid were analytical
69	grade and purchased from Heowns (Tianjin, China). The water used in this work was
70	purified using an Elix ProGard TS2 system (Millipore, France).
71	Soy sauce moromi, which had been fermented for 165 days, was provided by a soy
72	sauce manufacturer in Guangdong Province, China. The moromi was filtered through
73	four layers of gauze to separate the fermented liquor from the soy sauce residue. After
74	centrifugation at 2647 g for 10 min at 4°C, the supernatant was collected as raw soy
75	sauce. selected constituents in the raw soy sauce were evaluated in terms of their initial
76	concentrations in the matrix (Table S1).
77	2.2 Thermal processing of raw soy sauce and of a model solution
78	For simplicity, all reactions consisted of 20 mL of solution in a 30 mL sealed
79	cylindrical glass vial with a narrow neck. The vials were heated in a water bath (or oil
80	bath) at a set temperature to simulate the heat treatment of soy sauce. Afterwards, these
81	vials were immediately cooled in an ice bath. Unless otherwise indicated, the pH was
82	adjusted to 4.5 by using acetic acid/sodium acetate.
83	Preparation of the model solution: 2.36 g of citrulline and 36 mg of urea were
84	transferred into a 1 L volumetric flask and diluted with 16.8% brine to volume. Except
85	for ethanol (volatile component), the contents of the two main EC precursors (citrulline
86	and urea), pH and NaCl content in the model solution were the same as those in the
87	prepared raw soy sauce (Table S1). The prepared model solution was stored at 4°C

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 (CuCl₂, FeCl₃, ZnCl₂, CaCl₂, MgSO₄, and FeSO₄; 50 mg/L each), and then the solutions were heated at 90°C for 30 min.

100 **2.4 Influence of phenolic compounds on EC formation**

Stock solutions of phenols were prepared in absolute ethanol at a concentration of 101 5 mg/mL. Then, 400 μ L of each stock solution was added to the model solution for a 102 final phenol concentration of 100 mg/L. In addition, quercetin solutions with 103 concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.05 and 0.005 mg/mL were prepared by 104 diluting the stock solution with absolute ethanol, and then 400 µL of each of these 105 solutions was added to the model solutions as test groups. Model solutions with 400 µL 106 of ethanol added were used as the control group (no phenols). These solutions were 107 heated at 90°C for 30 min. 108

109 **2.5 Kinetic experiments**

110 The principal reactants (primary precursors) for EC formation during heat

7

111	treatment were determined before the kinetic experiment. Ethanol (final concentration:
112	2% or 4%) and 1-4 mg/mL citrulline (or 10-80 mg/L urea) were added to 16.8% brine
113	(pH 4.5), and the mixture was heated at 90°C for 30 min or 60 min. The constituents in
114	different trials of the kinetic studies are shown in Table S2, and the heating duration in
115	the trials was 30, 60, 90, 150, 210, 300, or 450 min. A pseudo-first-order-kinetic
116	equation was selected to describe EC formation over the course of the heat treatment
117	experiment. The maximum EC content $([EC]^{\infty})$ was read from the plot of the EC
118	concentration versus time. The rate constant K (observed rate constant) was calculated
119	from plots of $ln([EC]^{\infty}-[EC]_t)$ versus time (Galinaro, Ohe, da Silva, da Silva, & Franco,
120	2015).

121 **2.6 Central composite design for the optimization of EC reduction**

A central composite design (CCD) was applied to determine the optimal addition 122 of quercetin and ornithine for EC reduction during soy sauce production, with two 123 major factors (ethanol content and heat temperature). According to preliminary results 124 and the soy sauce matrix, the process variables and their ranges were chosen, as shown 125 in Table S3. To ensure alignment of the model with real samples, raw soy sauce was 126 diluted 5-fold with distilled water and then fortified with all the ingredients in Table S1 127 except for ethanol and ornithine. Then, a total of 30 different combinations with a 128 random order and 6 center points were evaluated according to a full CCD configuration 129 for 4 factors, and the EC content determined after heat treatment was set as the response. 130 The experimental design and data analysis were performed using Design Expert 131 software, version 8.0.6. 132

133 **2.7 Quantification of EC**

Solid-phase extraction (SPE) (Chinese National Standards, Method for the 134 Determination of Ethyl Carbamate in Food, GB 5009.223-2014) and liquid-liquid 135 extraction (LLE) (Zhou et al., 2017) were used to extract EC from the raw soy sauce 136 and model solution samples, respectively. The final extracts were diluted to 1 mL each 137 with dichloromethane (Leca, Pereira, Pereira, & Marques, 2014) for GC/MS (Agilent 138 7890A-5975C, America) analysis with electron ionization (70 eV). A CP-WAX column 139 (50 m, 0.32 mm, 1.2 µm, Agilent Technologies, the Netherlands) was used for 140 141 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 142 rate of 4.5°C/min (12 min). The inlet and detector interface temperatures were 250°C 143 144 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) 145 and d₅-EC (SIM: m/z 44, 64, 76, and 89, internal standard). The recovery and coefficient 146 147 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: 148

149

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

150 2

2.8 Evaluation of volatile compounds and color

151 The volatile compounds and color after heat treatment were evaluated to 152 investigate the impacts of quercetin and ornithine addition on the fundamental qualities 153 of soy sauce. The volatile compounds were extracted by an SPME Trisplus automated 154 sampler and then analyzed by GC/MS with a CP-WAX (50 m, 0.32 mm, 1.2 μm, Agilent

155	Technologies, the Netherlands) (Feng et al., 2015). Aliquots of sample (3 mL), 300
156	mg/L NaCl and internal standard (4-methyl-2-pentanol, 5 μ g/L) were transferred into
157	headspace vials, which were then sealed. Samples were equilibrated at 45°C for 20 min
158	and extracted with 85 μm carboxen/polydimethylsiloxane fibers for 30 min at the same
159	temperature. The separation program was as follows: 40°C for 2 min, ramped to 120°C
160	at a rate of 5°C/min, held for 2 min, increased to 220°C at a rate of 7°C/min and held
161	for 5 min. The injection temperature and ion source temperature were set at 250°C and
162	230°C, respectively. Helium was used as the carrier gas with a flow rate of 1.0 mL/min.
163	The scan range and scan rate applied to the analysis were $35-350 \text{ m/z}$ and 3.00 scans/s ,
164	respectively. The electronic nose measured using a pen3 e-nose (Airsense Int, Schwerin,
165	Germany) after 10-fold dilution with distilled water. The sample was placed into a 10
166	mL sealed glass bottle, and then balanced in the water bath (40°C) for 5 minutes. The
167	response points at 110 s of electronic nose, which were the maximum response signal
168	for each of the 10 sensors (Table S5), were used for analysis, each analysis was repeated
169	10 times. The color was detected using a colorimeter (ColorFlex EZ, HunterLab,
170	America) after 10-fold dilution with distilled water. Values for L* (lightness), a*
171	(redness) and b* (yellowness) were recorded, and ΔE was calculated as the primary
172	descriptor (Gutiérrez, Tapia, Pérez, & Famá, 2015), each analysis was repeated 10 times.
173	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 177 determined by adopting Tukey's multiple range test, with significance defined at P<0.05.

178 **3. Results and discussion**

179 **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. 180 181 The EC content in the final broth was 7.4 μ g/L, which was only 16.5% of that in the same batch of soy sauce. The result strongly indicated that EC was primarily 182 accumulated during heat treatment after fermentation, rather than during fermentation 183 itself. The ethanol content in the raw soy sauce was 1.06%, which was lower than that 184 185 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 186 during thermal treatment (Zhou et al., 2019). In this study, the EC contents in the model 187 188 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 189 (Table S6), indicating that the increase in temperature greatly increased EC formation. 190 Notably, although there were other EC precursors (such as carbamyl phosphate and 191 cyanate) in the raw soy sauce, the EC contents were lower in the raw soy sauce than in 192 the model solution at both 80°C and 90°C, suggesting that components of raw soy sauce 193 inhibit the formation of EC during heat treatment. According to previous studies, these 194 components could be metal ions and amino acids related to arginine metabolism. 195

196 **3.2 Factors influencing EC formation during heating**

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

11

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

Citrulline and urea are the major precursors of EC in many fermented foods and 209 210 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 211 of arginine also generated ornithine (Araque, Gil, Carreté, Bordons, & Reguant, 2009). 212 A high arginine content was maintained during soy sauce fermentation (Zhang, Fang, 213 Chen, & Du, 2014), and considerable amounts of arginine were present in the raw soy 214 sauce (3.48 mg/mL). Our previous study found a positive correlation between the total 215 arginine and EC in soy sauce products, while free ornithine was negatively correlated 216 with EC (Zhou et al., 2017). The phenomenon was confirmed again in the present study,, 217 the addition of ornithine significantly decreased the EC content (23.7%-37.4%), but a 218 high level of arginine (above 4 mg/mL) resulted in a high EC content (Fig. 1B). This 219 suggested that free ornithine might be an inhibitor of EC formation. Therefore, the 220

application of a screened strain with a high capability to convert arginine into ornithinemight be an efficient method for EC reduction.

223 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 224 EC formation. We suspected that the addition of arginine (alkaline) increased the pH of 225 the raw soy sauce and thus significantly hindered the formation of EC. Therefore, the 226 effect of pH on EC formation was assessed, and presented in Fig. 1C. As expected, high 227 pH significantly decreased the EC content, which probably offset the increase in EC by 228 229 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, 230 which was suitable for EC formation at 90°C. In the range of 70°C to 90°C (common 231 232 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 233 that high temperature was critical for EC formation. Therefore, relatively low 234 235 temperatures and high pH were favorable for EC control during soy sauce heat treatment. 236

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

243	be avoided in soy sauce production. In a previous study, the transformation of
244	urea/citrulline to EC was suppressed by GA and PA during wine brewing (Zhou et al.,
245	2017). However, during thermal processing, GA, PA, TM and TP did not significantly
246	reduce the EC content. In general, the tested monophenols promoted the formation of
247	EC, some polyphenols had the opposite effect, and phenolic acids had no significant
248	effects on EC formation. Among the tested phenolic compounds, quercetin significantly
249	reduced the EC content by 48.38%, making it a potential inhibitor of EC formation.
250	Quercetin is a flavonoid and often present as quercetin-3-O-rutinoside (rutin) in the
251	human diet, many literatures have reported its strong antioxidant and anticancer activity
252	(Jan et al., 2010; Dong et al. 2019), and the weight of the available evidence supports
253	the safety of quercetin for addition to food (Harwood et al., 2007). The strong inhibitory
254	ability of quercetin to against EC formation might be due to its preferential (over other
255	EC precursors) reaction with carbamyl compounds under mild conditions. EC reduction
256	had no dose-dependent relationship with the quercetin concentration (Fig. 2B). The
257	addition of quercetin at 0.1 mg/L had no significant impact on the EC reduction
258	compared to the control groups (p< 0.05), and the addition of quercetin at 10-40 mg/L
259	led to the optimal EC reduction ratio (approximately 62%).

260

3.4 Primary EC precursors during heat treatment

To further elucidate the crucial factors in the mechanism of EC reduction during 261 heat treatment, the principal reactants contributing to EC formation in soy sauce were 262 determined firstly. As shown in Fig. S2, the EC content increased with increasing 263 citrulline, urea and ethanol contents, similar to the EC increase with prolonged heating 264

265	time. The essence of EC formation is the alcoholysis reaction between ethanol and
266	carbamyl compounds, and the EC level in citrulline and ethanol solutions was higher
267	than that in urea and ethanol solutions. When the contents of citrulline (2000 mg/L) and
268	urea (40 mg/L) in the model solution were similar to that in raw soy sauce, the majority
269	of EC was formed in the reaction between citrulline and ethanol. These results were
270	consistent with the findings of Matsudo et al. (1993), Zhang et al. (2014) and our former
271	study (Zhou et al., 2019). The reasons for low EC formation in urea and ethanol
272	solutions were speculated as follows: a) the molality of urea is much lower than that of
273	citrulline in soy sauce, b) the formation of EC via catalytic alcoholysis of urea and
274	ethanol requires high temperature and pressure, which leads to more by-products (Gu,
275	2013), and c) the generation of ammonia significantly increases the pH of the solution
276	(Fig. 3A). Therefore, the model solution used in the kinetic experiments consisted of
277	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 287 concentration and heat temperature, the more EC produced. However, the rates of EC 288 formation were very low, with a yield of only 10⁻⁴. Notably, the addition of quercetin 289 led to a noticeable decrease in the EC yield and reaction rate. The rate of EC formation 290 291 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 292 of quercetin were different from those related to the addition of ornithine. Based on the 293 characteristics of the alcoholysis reaction and the reversible reaction between EC 294 295 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 296 alcoholysis between citrulline and ethanol (Fig. 3B). These observations suggested that 297 298 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 299 compounds. 300

301 **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 μ g/L (Fig. 4A), which met the international standard set

309 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 310 311 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 312 313 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, 314 whereas when the heating temperature was higher, quercetin at a low concentration 315 could better decrease the EC content (Fig. 4C and 4D). To achieve practical EC 316 reduction with ornithine and quercetin, we choose 90°C and 80°C as the sterilization 317 temperatures in this study, and the ethanol content was 2%. For 80°C sterilization, the 318 optimal ornithine and quercetin contents obtained by inputting the above criteria in the 319 320 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 321 optimum ornithine and quercetin contents were 0.85 mg/mL and 10 mg/L, respectively, 322 with a predicted EC content of 23.58 μ g/L. 323

324 **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 μ g/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

331	CCD experiment). Approximately 76 volatile compounds were identified by GC/MS
332	(Fig. 5). There were high levels of alcohols, volatile acids, esters, and aldehydes in the
333	soy sauce after heat treatment, while some ketones, furan(ones), phenols, and sulfur-
334	containing compounds were present at low percentages. The aldehydes (both 80°C and
335	90°C) and furan(ones) (90°C) in the treatment groups were higher than the aldehydes
336	and furan(ones) in the control groups, and the other major volatile compounds did not
337	significantly differ between the treatment and control groups. The 4-ethyl-2-
338	methoxyphenol, 2-methylbutanal, 3-methylbutanal and benzene acetaldehyde contents
339	were higher in the treatment groups than that in the control groups, and these
340	compounds could contribute to the aroma of "smoky, bacon, soy sauce", "malty,
341	almond", "malty, almond" and "honey-like," respectively, in high-salt liquid
342	fermented soy sauce (Feng, Cai, Su, Zhao, Wang, & Zhao, 2014). For data analysis of
343	e-nose determination, the difference between ten sensors response value of four samples
344	is not significant (Fig. 5), the R1, R3, R5. R7. R8. R9 sensor which sensitive to aromatic
345	compounds, sulfur compounds and pyrazines were changed slightly. PCA was used for
346	discriminating samples based on the difference of volatile compounds and signal
347	intensity of the sensor array. The data showed that only a slight shift of different samples
348	along the first principal component, PC1, which represented 91.29% of the total
349	variance with value 97.37%. The differences were not evident between the samples of
350	80°C control, 80°C treatment and 80°C control. In the color evaluation (Fig. 5), the
351	significant decrease in L* in the heated samples indicated that the raw soy sauce turned
352	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.

357 **4. Conclusions**

In this study, we found that ornithine and quercetin could significantly decrease 358 EC formation during soy sauce heat treatment, and speculative EC inhibition 359 mechanisms were proposed on the basis of the results of kinetic experiments. Then, a 360 creative and practical method for EC reduction was developed via the simple addition 361 of quercetin and ornithine before heat treatment during soy sauce production. With the 362 optimized contents of quercetin and ornithine, the EC concentration in soy sauce was 363 364 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 365 sauce. The discovery of more efficient inhibitors of EC formation is still underway. 366

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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 \pm 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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 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:

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

2% ethanol, 90°C, 0 mg/L quercetin, 0.5 mg/mL ornithine and pH 4.5).

Highlight

- $\hfill \bullet$ 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.

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