**Effects of phenolic compounds from blueberry leaves on the thermal decomposition of trimethylamine oxide in squid extract**

**Yingchang Li1**, **Fengxia Du1**, **Suzhen Song1**, **Shuangyan Li1**, **Xianqing Yang2**, **Shumin Yi1**

**Abstract**

The effects of chlorogenic acid and quercetin-3-D-galactoside on the decomposition of trimethylamine oxide (TMAO) in squid extract and the main mechanism of inhibition of thermal decomposition were studied. The results indicated that chlorogenic acid and quercetin-3-D-galactoside could inhibit decomposition of TMAO in squid extract. The amount of TMAO was increased by 11.79 % and 15.76 % in squid extract treated with chlorogenic acid and quercetin-3-D-galactoside from 0 g/L and 2.5 g/L, respectively. The contents of trimethylamine (TMA), dimethylamine (DMA), and formaldehyde (FA) were significantly decreased with increasing contents of chlorogenic acid and quercetin-3-D-galactoside. There were many free radicals in squid extract at high temperatures; however, the free radical signals were weakened after the addition of chlorogenic acid and quercetin-3-D-galactoside therein. This implied that chlorogenic acid and quercetin-3-D-galactoside could inhibit the thermal decomposition of TMAO in squid extract, which was associated with the scavenging of their free radicals. This result provides a theoretical basis for the development and utilization of blueberry leaf extract as an efficient FA inhibitor for aquatic products.

**Keywords** squid extract, chlorogenic acid, quercetin-3-D-galactoside, thermal decomposition, trimethylamine oxide, formaldehyde.

**1 Introduction**

In recent years, security issues relating to seafood have attracted widespread attention. Formaldehyde (FA) is both an industrial product and an essential metabolite of aquatic products and vegetables. The level of FA is especially high in squid, and squid products. FA is a reactive substance that can react with many functional groups of proteins, especially free amino groups, which contributes to proteins being denatured and aquatic products being damaged. Yeh *et al*. found that FA leads to changes in the secondary structure of squid collagen [1]. At the same time, Chanarat and Benjakul reported that FA present in surimi had a negative effect on gel improvement and cross-linking ability induced by transglutaminase [2]. FA could also cause DNA damage and loss of memory [3]. Furthermore, FA is found to be carcinogen and long-term exposure or inhalation of FA in humans increases the risk of lung cancer and brain cancer [4]. FA has been identified as carcinogenic by the World Health Organization, and can cause a variety of cancers, especially leukemia [5]. Some studies show that FA is able to injure arterial endothelial cells and cause arteriosclerosis [6].

Both dimethylamine (DMA) and FA are products of the decomposition reaction of trimethylamine oxide (TMAO). TMAO exists widely in marine biological tissues and is especially abundant in squid and crustaceans. TMAO plays an important role in cell osmoregulation of salt-water ﬁsh and stabilizing the folded state of the protein [7]. It is also the major source of fresh seafood flavor. As TMAO can be degraded to harmful DMA and FA during thermal processing, high TMAO concentrations in squid and crustaceans are both a technological and toxicological problem. Some studies have shown that TMAO can be transferred into DMA, TMA, and FA, by high temperatures, with the decomposition temperature being affected by many factors [8]. Large amounts of FA, DMA, and TMA were formed in squid muscle when heated [9]. The endogenous FA produced in aquatic products is closely related to TMAO decomposition in the processing of aquatic products at high temperatures, so it is unlikely to completely avoid the production of harmful FA during the processing of squid, which draws increasing attention as a crucial problem affecting food quality. It is important to control the pyrolysis of TMAO and improve the safety of marine aquatic food. Currently, the control of TMAO thermal decomposition in aquatic products focuses on processing technology and exogenous substances. Zhang *et al*. changed the TMAO degradation pathways to prevent FA production [10]. In addition, chitosan, calcium chloride, citric acid, and trisodium citrate were found to inhibit the formation of DMA and FA [11~~-~~12]. Trimethylamine oxide demethylase activity was significantly inhibited by low concentrations of polyphenols, phytic acid, and acetic acid in squid muscle [13]. As an FA scavenger, tea catechins had significant reactivity with FA at room temperature [14].

Blueberry, which belongs to the plant group Ericaceae, is an evergreen or deciduous shrub. Blueberry leaves are used in medicine and food, moreover, recent research has indicated that polyphenols from blueberry leaves had functional activities such as antioxidant, antihypertensive, anti-hypolipidemic effects, and so on [15-17]. Tea polyphenols were found to be able to decrease the FA content in aquatic products because it could react with FA [18]. Polyphenol compounds from blueberry leaves differed from those in tea. Matsuo *et al*. proposed that polyphenols of rabbit-eye blueberry leaves were mainly composed of proanthocyanidins, caffeoylquinic acids, and flavonol glycosides. Oligomeric proanthocyanidins were degraded with mercaptoethanol, and the polymer had (+)-catechin and (-)-epicatechin as its terminal units [19]. It is worthy of discussion as to whether, or not, blueberry leaf polyphenols can inhibit the thermal decomposition of TMAO and react with FA.

Electron spin resonance (ESR) can detect unpaired electrons in atoms and free radicals. It was deemed promising, as a method, to detect those free radicals produced by the application of ESR spin-trapping techniques, which is also a valuable tool when seeking to increase the understanding of the underlying reaction mechanism. Formation of free radicals in whole wheat flour and white flour was determined during the heating by ESR spectroscopy [20]. Kreitman *et al*. found that TMAO produces FA, DMA, and TMA by free radical reaction under ferrous iron catalysis [21]. Zhu *et al*. also reported that the formation of DMA and FA was associated with free radicals by non-enzymatic reactions in the squid during thermal processing [12], but few reports have demonstrated the effects of blueberry leaf polyphenols on the thermal decomposition of TMAO and the formation mechanism of DMA and FA by non-enzymatic reactions in squid extract at high temperature.

Two compounds were extracted from blueberry leaves by semi-preparative high-performance liquid chromatography (SP-HPLC), which were identified as chlorogenic acid and quercetin-3-D-galctoside by UV, high-performance liquid chromatography-electrospray ionization/diode array detection tandem quadrupole-quadrupole-quadrupole mass spectrometry (HPLC-ESI/DAD-QQQMS), IR, and NMR. In the study, the effects of chlorogenic acid and quercetin-3-D-galactoside on the thermal decomposition of TMAO were analyzed. Meanwhile, the mechanism of chlorogenic acid and quercetin-3-D-galactoside inhibition of thermal decomposition of TMAO was also elucidated (Fig. 1).

**2 Materials and methods**

**2.1 Materials**

Jumbo squid (*Dosidicus gigas*), purchased from the Linxi aquatic product market, Jinzhou, China, was frozen and stored at -20 °C. The blueberry leaves were obtained from the blueberry planting base of Shenyang Academy for Development of Agricultural Science and Technology (Liaoning Province, China). TMAO was purchased by the Institute for Environmental Reference Materials of Ministry of Environmental Protection (China). N-tert-butyl-a-phenyl-nitrone (PBN) was provided from Sigma-Aldrich (St Louis, MO, USA). Methanol (chromatography grade) was also provided from Sigma-Aldrich (St Louis, MO, USA). Other chemicals used were of analytical grade and were sourced from Chinese Medicines Group Chemical Reagent Co., Ltd (Shanghai, China). HPLC (Agilent 1260, USA), Eclipse Plus C18 column (4.6 mm × 250 mm, 5 µm), a gas chromatograph (Agilent GC7890, Agilent Technologies, USA), and a UV-vis spectrophotometer (UV-2550, Shimadzu Co., Ltd, Japan) were used.

**2.2 Chlorogenic acid and quercetin-3-D-galactoside were isolated by SP-HPLC**

Polyphenols from blueberry leaves were prepared using the method of Li *et al*. [16]. Phenolic compounds were isolated by a SP-HPLC system (Waters 1525) with a photodiode array detector (Waters 2998). The condition of SP-HPLC was as follows: phenolic compounds from blueberry leaves were dissolved in methanol (50 %) with hydrochloric acid (0.5 %). A reversed-phase C18 column (xBridgeTM Prep C18 5 μm, OBDTM 19 × 100 mm column) was used to isolate individual compounds from blueberry leaf polyphenols at 28 °C. The mobile phases consisted of eluent A (aqueous solution, 0.1 % trifluoroacetic acid) and eluent B (methanol, 0.1 % trifluoroacetic acid). The A:B (v/v) linear gradient elution program was as follows. 5 % B to 40 % B over 4 min, 40-48 % over 16 min, 48-52 % over 7 min, 52-58 % over 6 min, and this was held for 10 min before returning to the initial conditions. The flow rate was 3.5 mL/min and detection was conducted at 360 nm. The concentration of polyphenols was 2.5 g/L and the injection volume was 5 mL. Peak fractions of blueberry leaf polyphenols were collected manually according to SP-HPLC chromatogram, concentrated at 50 °C under rotator evaporator, and then freeze-dried.

**2.3 Preparation of squid extract**

Jumbo squid (*Dosidicus gigas*), from the Linxi aquatic product market, Jinzhou, China was frozen and stored at -20 °C. The squid was thawed at room temperature, then skinned, de-boned, and gutted. The muscle tissues of squid were minced: 10 g minced squid was extracted with 20 mL of 20 mmol/L Tris-acetic acid buffer (pH 7.0), then homogenized by ultrasound for 30 min in an icy water bath, and finally centrifuged at 10,000 *g* for 15 min at 4 °C. The water-soluble fraction of squid was acquired as squid extract and stored at 4 °C for analysis of the thermal decomposition of TMAO.

**2.4 The effects of different concentrations of chlorogenic acid and quercetin-3-D-galactoside on the thermal degradation of TMAO in squid** **extract**

We mixed 2.0 mL of different concentrations of chlorogenic acid or quercetin-3-D-galactoside with 2.0 mL squid extract, respectively. The mixtures were heated at 100 °C for 15 min in a water bath. The sample tubes were cooled in running water to stop the reaction. 2.0 mL squid extract was mixed with 2.0 mL distilled water for use as a control. Then the contents of FA, DMA, TMA, and TMAO were determined, respectively.

**2.5 The effect of heating temperature on thermal degradation of TMAO in squid extract**

2.0 mL squid extract was mixed with 2.0 mL of 1 g/L chlorogenic acid or quercetin-3-D-galactoside. The mixtures were heated for 15 min at 20, 40, 60, 80, and 100 °C in a water bath, respectively. Then the sample tubes were cooled in running water. The contents of FA, DMA, TMA, and TMAO were identified using the following method.

**2.6 The effect of heating time on the thermal degradation of TMAO in squid extract**

2.0 mL squid extract was mixed with 2.0 mL of 1 g/L chlorogenic acid or quercetin-3-D-galactoside. The mixtures were heated for 15, 30, 45, 60, 75, and 120 min, respectively at 100 °C in a water bath. Then the sample tubes were cooled in flowing water. The contents of FA, DMA, TMA, and TMAO were obtained by the following detection methods, respectively.

**2.7 Determination of TMAO, FA, DMA, and TMA contents**

The content of TMAO was measured after reduction to TMA using the method described by Deng *et al*. [22]. The TMA content was measured using an ultraviolet-visible spectrophotometer according to the method described by Gou *et al*. [23]. The FA content was identified by the method described in Zhu *et al*. [12]. DMA was measured by gas chromatograph (GC) with hydrogen flame ionization detection (Agilent GC7890, Agilent Technologies, USA) using the method of Zhu *et al*. [24].

**2.8 The effects of different concentrations of chlorogenic acid and quercetin-3-D-galactoside on free radicals in squid extract**

0.25 mL of different concentrations of chlorogenic acid (0.5 g/L, 1 g/L) or quercetin-3-D-galactoside (0.5 g/L, 1 g/L) was mixed with 0.25 mL squid extract and 15 mg of N-tert-butyl-a-phenyl-nitrone (PBN), respectively. The mixtures were incubated at 100 °C for 15 min in a water bath, respectively. The change in free radical content was measured by ESR.

**2.9 The effect of temperature on free radicals in squid extract**

0.25 mL squid extract was mixed with 0.25 mL of chlorogenic acid (1 g/L) or quercetin-3-D-galactoside (0.5 g/L) and 15 mg of PBN. The mixtures were incubated at 80 and 100 °C, respectively for 15 min in a water bath. Taking deionized water as a control, the change of free radical content was determined by ESR.

**2.10 The effect of reaction time on free radicals in squid** **extract**

0.25 mL squid extract was mixed with 0.25 mL of chlorogenic acid (1 g/L) or quercetin-3-D-galactoside (0.5 g/L) and 15 mg of PBN. The mixtures were incubated for 15 and 75 min, respectively at 100 °C in a water bath. Taking deionized water as a control, the change in free radical content was determined by ESR.

**2.11 The method of determination of free radical content**

Free radicals were assayed using the method of Zhu *et al*. [12]. The ESR spectra of the aforementioned reaction samples were recorded at room temperature (298 K) on a Bruker ESP A300 spectrometer. The determination conditions were as follows: center field 3512 G, sweep width 100 G, modulation frequency 100 kHz, modulation amplitude 2.00 G, microwave power 12.87 mW, and a spectrum scan time of 40.96 s.

**2.12 Statistical analysis**

All experiments were repeated in triplicate with data reported as mean ± standard deviation. Statistical analysis (*t*-testing) was performed using SPSS 19. The significance level was *p* < 0.05.

**3 Results and discussion**

**3.1 SP-HPLC chromatography**

There were two major peaks and other diminutive peaks that indicated traces of compounds according to SP-HPLC, as shown in Fig. 2(A). The two major peaks were collected by an automatic collector every 30 s. The collected fluid was isolated by SP-HPLC until purified for analysis by HPLC with DAD. Thereafter, the mobile phase in pure substances was moved by rotary evaporation followed by drying to powder using vacuum freeze-drying. The structures of the resulting compounds were confirmed by UV, HPLC-DAD/ESI-QQQ MS, FT-IR, and NMR spectra. Two compounds identified were chlorogenic acid and quercetin-3-D-galactoside, as shown in Fig. 2(B).

### 3.2 Effects of different concentrations of chlorogenic acid and quercetin-3-D-galactoside on thermal degradation of TMAO in squid extract

The thermal degradation of TMAO in squid extract could generate FA and DMA at high temperatures [12]. Zhang *et al*. also reported that the non-enzymatic decomposition of TMAO was crucial during the thermal processing of squid product [10]. As shown in Fig. 3(A), the TMAO content of squid extract treated with chlorogenic acid and quercetin-3-D-galactoside was significantly higher than that of squid extract without chlorogenic acid and quercetin-3-D-galactoside treatment (*p* < 0.05). The amount of TMAO in squid extract treated with chlorogenic acid and quercetin-3-D-galactoside increased with increasing concentrations of chlorogenic acid and quercetin-3-D-galactoside. The TMAO content increased (*p* < 0.05) from 3531.82 ± 50.03 mg/L and 3556.32 ± 53.33 mg/L to 3996.55 ± 28.16 mg/L and 4116.62 ± 55.56 mg/L, respectively. The TMAO content was increased by 13.16 % and 15.76 % when the concentrations of chlorogenic acid and quercetin-3-D-galactoside were increased from 0 g/L and 2.5 g/L, respectively. The amount of TMAO in squid extract treated with quercetin-3-D-galactoside was significantly (*p* < 0.05) higher than that in squid extract treated with chlorogenic acid at the same concentration (increasing from 0.5 g/L to 2.5 g/L). The results showed that chlorogenic acid and quercetin-3-D-galactoside could inhibit the thermal decomposition of TMAO in squid extract. As shown in Figs. 3(B) to (D), the amounts of TMA, DMA, and FA in squid extract decreased with increasing concentrations of chlorogenic acid and quercetin-3-D-galactoside. The FA content decreased in squid extract from 8.66 ± 0.46 mg/L and 8.49 ± 0.48 mg/L to 1.36 ± 0.08 mg/L and 0.96 ± 0.11 mg/L, respectively. Lee *et al*. reported that sodium tripolyphosphate (STPP) and tetrasodium pyrophosphate (TSPP) have an inhibitory effect on TMAO and reduce the formation of FA [25], which was consistent with our results. The amounts of TMA, DMA, and FA in squid extract by quercetin-3-D-galactoside treated were lower than those in chlorogenic acid treated at the same concentration. Chlorogenic acid and quercetin-3-D-galactoside could decrease the FA content and inhibit thermal degradation of TMAO in squid extract. This was in agreement with the findings of Dong *et al*. [26], who found that tea polyphenols could restrain the increase of DMA and FA. The FA content was reduced because chlorogenic acid and quercetin-3-D-galactoside could inhibit the thermal degradation of TMAO.

**3.3 Effects of chlorogenic acid and quercetin-3-D-galactoside on the thermal decomposition of TMAO in squid extract at different heating temperatures**

The effects of chlorogenic acid and quercetin-3-D-galactoside on the thermal degradation of TMAO in squid extract at different heating temperatures (for 15 min) are shown in Fig. 4(A). As the heating temperature increased, the TMAO content decreased both in control, and treated groups. The higher the temperature, the lower the TMAO content. TMAO-N in squid was converted to TMA-N and DMA-N, and that the thermal conversions were accelerated by heat [8]. Our study also showed that the thermal decomposition of TMAO was accelerated as the temperature rose, while the content of TMAO in chlorogenic acid and quercetin-3-D-galactoside groups was significantly (*p*< 0.05) higher than that in the control group. The TMAO content decreased from 4333.68 ± 28.50 mg/L to 3451.97 ± 10.63 mg/L in the control group, however, the heating temperature increased, and decreased by about 20.35 % as the TMAO content decreased from 4451.22 ± 5.99 mg/L and 4490.27 ± 24.69 mg/L to 3947.64 ± 4.43 mg/L and 4051.68 ± 14.47 mg/L in the chlorogenic acid and quercetin-3-D-galactoside groups respectively (decreases of only 11.31 % and 9.77 %). Compared with the control group, the amount of trimethylamine oxide in the chlorogenic acid and quercetin-3-D-galactoside groups was increased by 14.36 % and 17.37 %, respectively. This indicated that thermal degradation of TMAO in squid extract could be inhibited by chlorogenic acid and quercetin-3-D-galactoside at high temperatures.

The effects of chlorogenic acid and quercetin-3-D-galactoside on the contents of TMA, DMA, and FA in squid extract at different temperatures are illustrated in Figs. 4(B) to (D). The decomposition of TMAO to TMA, DMA, and FA was increased with increasing temperature in both control and treated groups. The FA content significantly (*p* < 0.05) increased from 2.48 ± 0.21 mg/L to 8.45 ± 0.61 mg/L in the control group as the temperature rose from 20 °C to 100 °C. The FA content in the chlorogenic acid and quercetin-3-D-galactoside groups increased slowly, only reaching 4.57 ± 0.12 mg/L and 3.07 ± 0.15 mg/L at 100 °C. Compared with the control group, the FA content decreased by 45.99 % and 63.87 % in the chlorogenic acid and quercetin-3-D-galactoside groups at 100 °C, respectively. As the temperature increased, the contents of DMA and TMA also increased in control and treated groups; however, the DMA and TMA contents in the treated group were smaller than those in the control. The highest DMA and TMA contents in the control group were 46.55 ± 1.06 mg/L and 60.66 ± 0.92 mg/L while DMA contents in the chlorogenic acid and quercetin-3-D-galactoside group were 32.13 ± 0.92 mg/L and 25.46 ± 0.46 mg/L, and TMA contents were 41.65 ± 1.13 mg/L and 33.96 ± 0.43 mg/L, respectively. The DMA and TMA contents were decreased by 30.98 % and 31.33 % in the chlorogenic acid group, however the DMA and TMA contents were decreased by 45.31 % and 44.01 % in quercetin-3-D-galactoside group.

The contents of FA, DMA, and TMA in the treated group were lower than those in the control group because chlorogenic acid and quercetin-3-D-galactoside could inhibit the thermal degradation of TMAO at high temperatures. Quercetin-3-D-galactoside could suppress the thermal decomposition of TMAO to a greater extent than that of chlorogenic acid. Zhu *et al*. proposed that the production of TMA and DMA from the degradation of TMAO by non-enzymatic pathways depended on the heating temperature in dried squid [27]. Our result showed that temperature exerted a significant effect on the decomposition of TMAO in squid extract.

**3.4 Effects of chlorogenic acid and quercetin-3-D-galactoside on thermal degradation of TMAO in squid extract for different times**

As shown in Fig. 5(A), the TMAO content decreased in squid extract, in the control group, chlorogenic acid group, and quercetin-3-D-galactoside group, as the heating time increased. Zhu *et al*. reported that the decomposition of TMAO-N in squid was accelerated to FA, TMA, and DMA with increasing heating time [12]. The reduction of TMAO in squid extract in chlorogenic acid and quercetin-3-D-galactoside groups was smaller than that in the control group. The TMAO content decreased slowly in squid extract by chlorogenic acid and quercetin-3-D-galactoside treated (decreases of 26.53 % and 17.52 %, respectively), which were significantly lower than that of the control group (41.64 %) when the heating time reached 60 min. The TMAO content decreased (*p*< 0.05) in squid extract when the heating time reached 120 min. The TMAO content in the quercetin-3-D-galactoside group was higher than that in the chlorogenic acid group. The relative decreases of TMAO contents were 27.32 % and 32.93 % in chlorogenic acid and quercetin-3-D-galactoside groups, respectively, while it was 51.34 % in the control group, compared with that at 60 min. TMAO content in the quercetin-3-D-galactoside group significantly decreased at the higher temperature for a longer time, which was attributed to the decreased quercetin-3-D-galactoside activity.

The effects of chlorogenic acid and quercetin-3-D-galactoside on the contents of TMA, DMA, and FA in squid extract at different heating times are demonstrated in Figs. 5(B) to (D): as the heating time increased, the FA content of squid extract significantly (*p* < 0.05) increased in the control group and reached 19.69 ± 0.35 mg/L after heating for 120 min. In comparison with the control group, the FA content in the chlorogenic acid and quercetin-3-D-galactoside groups slowly increased, reaching 9.24 ± 0.25 mg/L and 8.44 ± 0.14 mg/L, respectively after heating for 120 min. This indicated that the FA content in squid extract could be reduced by chlorogenic acid and quercetin-3-D-galactoside due to the reaction of FA, and the inhibition of the decomposition of TMAO. As the heating time increased, both the DMA and TMA contents of squid extract increased (*p* < 0.05) in the control, chlorogenic acid, and quercetin-3-D-galactoside groups. The increased DMA and TMA contents in the chlorogenic acid and quercetin-3-D-galactoside groups were smaller than that in the control group. The FA, DMA, and TMA contents showed a slow increase in the treated group (after 60 min) compared with that at 15, 30, 45, and 60 min due to the decrease in activities of chlorogenic acid and quercetin-3-D-galactoside after extending the heating time. The inhibitory effects of chlorogenic acid and quercetin-3-D-galactoside on TMAO were enhanced when the treatment time was less than 60 min at 100 °C.

**3.5 Effects of concentration of chlorogenic acid and quercetin-3-D-galactoside on the production of free radicals in squid extract**

As shown in Fig. 6, free radicals were produced from 3460.00 G to 3560.00 G at 100 °C. The primary signal was an ESR spectrum with six peaks. There were also other ESR spectra with negligible peaks. Zhu *et al*. found that free radicals were produced by the degradation of TMAO in the presence of ferrous iron, causing a series of free radical chain reactions to produce FA and DMA [24]. Free radical signals were also produced because there were trace iron ions in the squid extract. It was verified that the production of FA in squid extract was closely related to free radicals. The free radical signal intensity was weak in the treated squid extract with the increasing concentrations of chlorogenic acid and quercetin-3-D-galactoside. ESR signals in squid extract could be weakened by chlorogenic acid and quercetin-3-D-galactoside. The higher the concentration of chlorogenic acid and quercetin-3-D-galactoside, the weaker signals of the free radicals. ESR signals were decreased by approximately 23.84 % and 78.13 % when the chlorogenic acid content was 0.5 g/L chlorogenic acid and 1 g/L, respectively. At the same time, ESR signals were decreased by approximately 46.04 % and 88.77 % when the quercetin-3-D-galactoside content was 0.5 g/L chlorogenic acid and 1 g/L, respectively. The free signals were weaker in squid extract treated by quercetin-3-D-galactoside than that treated by chlorogenic acid at the same concentration, which was consistent with the change in FA content. Reber, *et al*. also reported that quercetin-3-glucoside was proved to be effective in the scavenging of free radicals [28]. Quercetin, a phenolic compound, and its semi-synthetic derivatives have exhibited high anti-oxidative ability [29, 30]. This result showed that free radical activity could be reduced by the chemical reaction with chlorogenic acid and quercetin-3-D-galactoside, reducing the production of FA and DMA. There were only base-line noise peaks in the signals when the concentration of quercetin-3-D-galactoside reached 1 g/L. The results also implied that the conversion of TMAO to TMA, DMA, and FA marked the initial formation of ammonium radicals and then the intermediate was reduced to TMA by ferrous iron or oxidized to DMA and FA by ferric iron [24]. Chlorogenic acid and quercetin-3-D-galactoside could inhibit the decomposition of TMAO and the production of FA in squid extract because they could react with the free radicals at high temperature.

**3.6 Effect of temperature on the production of free radicals in squid extract**

Temperature played an essential role in the formation of free radicals during the decomposition of TMAO in squid extract. As shown in Fig. 7, the intensity of the free radical signals was enhanced in squid extract in both the control, chlorogenic acid, and quercetin-3-D-galactoside groups as the temperature rose. Strong radical signals (3.33 × 105) were detected in squid extract when the temperature reached 100 °C. This was consistent with the TMAO content in the squid extract being decreased, and the FA content increasing with the increase of temperature. Similarly, the concentrations of DMA and FA increased, indicating that the formation of FA and DMA was correlated with free radical production in squid extract during thermal processing. This result also showed that the formation of free radicals was related to the temperature. The signals from the free radicals in squid extract of chlorogenic acid and quercetin-3-D-galactoside groups were weak compared with that of the control group at 80 and 100 °C. Chlorogenic acid and quercetin-3-D-galactoside inhibition of endogenous FA in squid extract was related to the scavenging of their free radicals. Zhu *et al*. found that the TMAO to FA ratio varied depending on both the anion used, and the ferrous iron concentration; an excess of iron (II) could depress the yield of FA [24]. It was inferred that a little ferrous iron in squid extract could promote ammonium radical formation, leading to the difference in the formation of FA and DMA at high temperatures.

**3.7 Effect of treatment time on the production of free radicals in squid extract**

As shown in Fig. 8, the longer the heating time, the stronger the signals representing the free radicals. The formation of free radicals in the squid extract was consistent with the decomposition of TMAO to DMA and FA during thermal processing with prolonged heating time. When the heating time was extended to 75 min, compared with the control group, the signal intensity was decreased by 57.18 % and 23.48 %, in squid extract treated by the chlorogenic acid and quercetin-3-D-galactoside, respectively. The signals representing the free radicals were reduced after adding chlorogenic acid or quercetin-3-D-galactoside to the squid extract. This was also in line with the decomposition of TMAO to DMA and FA being slow when chlorogenic acid or quercetin-3-D-galactoside was added to the squid extract. Additionally, the free radical signal intensity was stronger when the heating time was 75 min when adding chlorogenic acid or quercetin-3-D-galactoside to the squid extract compared with that at 15 min. This suggested that the stability of chlorogenic acid and quercetin-3-D-galactoside decreased when the heating time reached 75 min, and the reaction capacity of chlorogenic acid and quercetin-3-D-galactoside with free radicals decreased as the heating time increased. This showed that the production of free radicals was related to heating time and proved that chlorogenic acid and quercetin-3-D-galactoside could inhibit the production of free radicals in squid extract, and thus could inhibit the decomposition of TMAO to FA.

**4 Conclusion**

Chlorogenic acid and quercetin-3-D-galactoside could inhibit the decomposition of TMAO in squid extract, and decreased the contents of FA, DMA, and TMA. There was a large amount of FA, DMA, and TMA produced when the temperature reached 100 °C in both control and treated groups. Compared to the control, the TMAO content was increased by 14.36 % and 17.37 % in chlorogenic acid and quercetin-3-D-galactoside groups, respectively, however, the FA content was decreased by 45.99 %, and 63.69 % in chlorogenic acid and quercetin-3-D-galactoside groups at 100 °C. The TMAO content decreased, and the contents of FA, DMA, and TMA increased in the control, chlorogenic acid, and quercetin-3-D-galactoside groups as the heating time increased. The TMAO content was high, and the contents of FA, DMA, and TMA were low in chlorogenic acid and quercetin-3-D-galactoside groups compared with those in the control group. There were many free radicals generated in the squid extract with increased heating temperature and time, but signals representing the free radicals were weakened after the addition of chlorogenic acid and quercetin-3-D-galactoside. This showed that chlorogenic acid and quercetin-3-D-galactoside could inhibit the thermal decomposition of TMAO in squid extract, which was associated with the scavenging of their free radicals.

**Acknowledgement**

This work was supported by National Key R & D Programme of China (Grant No. 2017YFC1600706) and the National Natural Science Foundation of China (Grant No. 31201308).

**Figure captions**

Fig. 1 Technical map adopted for the present study

Fig. 2(A) The SP-HPLC spectra of blueberry leaf polyphenols; (B) The structures of compounds obtained from blueberry leaves

Fig. 3 Effects of different concentrations of chlorogenic acid and quercetin-3-D-galactoside on thermal degradation of TMAO in squid extract

Fig. 4 Effects of chlorogenic acid and quercetin-3-D-galactoside on the thermal decomposition of TMAO in squid extract at different heating temperatures

Fig. 5 Effects of chlorogenic acid and quercetin-3-D-galactoside on thermal degradation of TMAO in squid extract at different times

Fig. 6 Effects of different concentrations of chlorogenic acid and quercetin-3-D-galactoside on the generation of free radicals in the thermal decomposition of TMAO

Fig. 7 Effects of chlorogenic acid and quercetin-3-D-galactoside on the generation of free radicals in the thermal decomposition of TMAO at different heating temperatures

Fig. 8 Effects of chlorogenic acid and quercetin-3-D-galactoside on the generation of free radicals in the thermal decomposition of TMAO at different heating times