# An improving method for extracting total carotenoids in an aquatic animal *Chlamys nobilis*

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ABSTRACT

In the present study, an improving method for extracting carotenoids in aquatic animals using ultrasound-assisted extraction (UAE) was developed. The theoretical optimum extraction condition (ultrasonic power, temperature and time of 246.77 W, 37.64 °C and 29.03 min, respectively) based on the maximum extracted total carotenoids content (TCC) (141.13 μg/g) was predicted by single-factor experiments and response surface methodology. Additional experiments were then carried out under practical extraction conditions to verify the predicted value for TCC. The optimum practical extraction condition was at ultrasonic power, extraction temperature and extraction time of 240 W, 38 °C and 29 min, respectively, where the TCC (137.88 ± 1.22 μg/g) similar to the predicted value. On the other hand, a much lower total carotenoid of 107.75 ± 2.60 μg/g was obtained by the 2 h conventional extraction of carotenoids (CEC). The UAE in carotenoids extraction has higher efficiency, shorter processing time, and less acetone than the CEC.

## 1. Introduction

Carotenoids are a group of fat-soluble pigments extensively existed in nature, which are synthesized by plants, algae, some bacteria and fungi. In general, animals are unable to synthesize carotenoids *de novo*, and must be obtained from diet (Torrissen, Hardy, Shearer, Scott, & Stone, 1990). A variety of biological functions have been found in carotenoids, including resisting oxidative damage (Astley, Hughes, Wright, Elliott, & Southon, 2004), regulating gene expression (Burri, 2000), and enhancing humoral immune response (Bendich, 1989), etc. Moreover, dietary intake of carotenoids also plays an important role in human health. Many studies have reported that carotenoids can be associated with reduced risks of cancers (Chatterjee, Roy, Janarthan, Das, & Chatterjee, 2012), cardiovascular diseases, metabolic syndrome, and obesity (Riccioni et al., 2010; Chaves et al., 2010), as well as cataract formation (Wegner & Khoramnia, 2011) and macular degeneration (Zhou et al., 2011).

Mollusk is the second phylum in the animal kingdom, and some species are important food sources for people in many countries. Carotenoids are enriched (ranges from 10 μg/100 g to 140 μg/100 g) in some mollusks including Polyplacophora, Gastropoda, Bivalvia, and Cephalopoda (Matsuno, 2001) (Kantha, 1989). Therefore, mollusks are important carotenoids source for human.

The noble scallop (*Chlamys nobilis* Reeve) is economically important bivalve species and has been commonly cultured in the Southern sea of China since 1980s. The rare individuals (3–4%) from the scallop' cultured stock not only possess orange shells and orange muscle, but also contains significantly higher total carotenoids content than others (Zheng et al., 2010). By a selection breeding project, a new variety named "Nan'ao Golden Scallop" was bred by our laboratory in 2015 (Zheng et al., 2015). The Golden scallops from the new variety not only possess golden shells and golden muscle, but also enrich carotenoids in their muscle. Moreover, the Golden scallops have stronger toleration to the low temperature (Han et al., 2016) and higher stress resistance to *Vibrio parahaemolyticus* (Lu, Zheng, Zhang, Yang, & Wang, 2016; Zhang, Cheng, Liu, & Zheng, 2018) than the other individuals with less carotenoids content. The market demand for Golden scallops is very high due to their attractive colour and rich in carotenoids. Since 2015, the Golden scallops have been widely cultivated in Sanya of Hainan Province, Zhanjiang, Shenzhen, Shanwei and Nan'ao of Guangdong Province, Dongshan, Zhangpu and Longhai of Fujian Province, China. Considering demand to determine carotenoids in the Golden scallop for researchers and industries, it is necessary to explore a rapid and efficient method to extract carotenoids.

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Conventionally, the most common carotenoids extraction method in aquatic animals is using organic solvent. For instance, in salmonid, carotenoids were extracted in acetone for 2 days at 4–5 °C (Torrissen & Naevdal, 1984); in shrimp, carotenoids were extracted in acetone for 3 days at 4 °C (Yanar, Celik, & Yanar, 2004); in scallop, carotenoids were extracted in acetone for at least 2–12 h at 25 °C (Zheng et al., 2010; Lu et al., 2016). However, these conventional extraction methods are time-consuming (2–3 days at 4–5 °C, or 2–12 h at 25 °C) and requires large amount of solvent (at least 8 ml). Recently, novel extraction techniques such as microwave extraction (Hiranvarachat & Devahastin, 2014), supercritical fluid extraction (Zaghdoudi et al., 2016), and ultrasound-assisted extraction (UAE) (Ma et al., 2010) have been introduced in plants carotenoids extraction. Among them, the UAE technique is the most efficient due to the ability in causing some structural changes, such as cell deformations or cell membrane damages that allow greater penetration of solvent into the tissue, increasing the contact surface area between the solid and liquid phases (Corrales, Toepfl, Butz, Knorr, & Tauscher, 2008). However, the use of UAE in carotenoids extraction has not been reported in the aquatic animals.

The purpose of the present work was to improve a method in carotenoids extraction in the Golden scallops of the noble scallop *Chlamys nobilis*. Three experiments were carried out: 1) effects of ultrasonic power, extraction temperature and extraction time for total carotenoids content (TCC) were investigated by single-factor experiments, 2) optimal extraction conditions of UAE were determined by response surface methodology, and 3) efficiency of UAE extracting carotenoids was assessed as compared with conventional extraction of carotenoids (CEC).

## 2. Materials and methods

### 2.1. Sample collection and preparation

Adult Golden scallops used in the present study were obtained from a cultured stock at Nan'ao Marine Biology Experimental Station of Shantou University (Shantou, China). They possess golden shells and golden adductor and mantle (Fig. 1). Adductors of 40 scallops were sampled and stored at −80 °C for further used. The samples were dried in a vacuum freeze-dryer and then grinded to homogenized powder in mortars. Three biological replicates were employed for each testing, each sample was run in triplicate in the following experiments.

### 2.2. Conventional extraction of carotenoids (CEC)

Total carotenoids were conventionally extracted following the method of Zheng et al. (2010). Four milliliter of acetone was added in a glass tube containing 0.4 g tissue powder. Then the mixture was shaken at 200 rpm/min for 1 h in the dark at room temperature of 25 °C. The sample was centrifuged at 5000 rpm for 5 min, and the supernatant was collected. The carotenoid in the pallet was further extracted under the same conditions. The extracts from the two extractions were mixed.

### 2.3. Ultrasound-assisted extraction (UAE)

An amount of Four milliliter of acetone was added in a glass tube containing 0.4 g tissue powder. Next, ultrasound was applied using a probe (13 mm diameter) system (FN-1200, Shanghai Shengxi Ultrasonic Instrument Co. Ltd., Shanghai, China), which had a maximum power of 1200 W, and operated at 20 kHz. Duty cycle of ultrasound pulse was set at 50% (5 s on: 5 s off). 1 cm length of the ultrasonic probe tip was dipped into the solvent from its surface. The glass tube was covered using the aluminium foil to prevent loss of solvent by evaporation and then immersed in a thermostated water bath for a specified time varying from 5 to 40 min at predetermined ultrasonic power varying from 120 to 480 W. After ultrasonic extraction, the sample was centrifuged at 5000 rpm for 5 min, and the supernatant was collected for
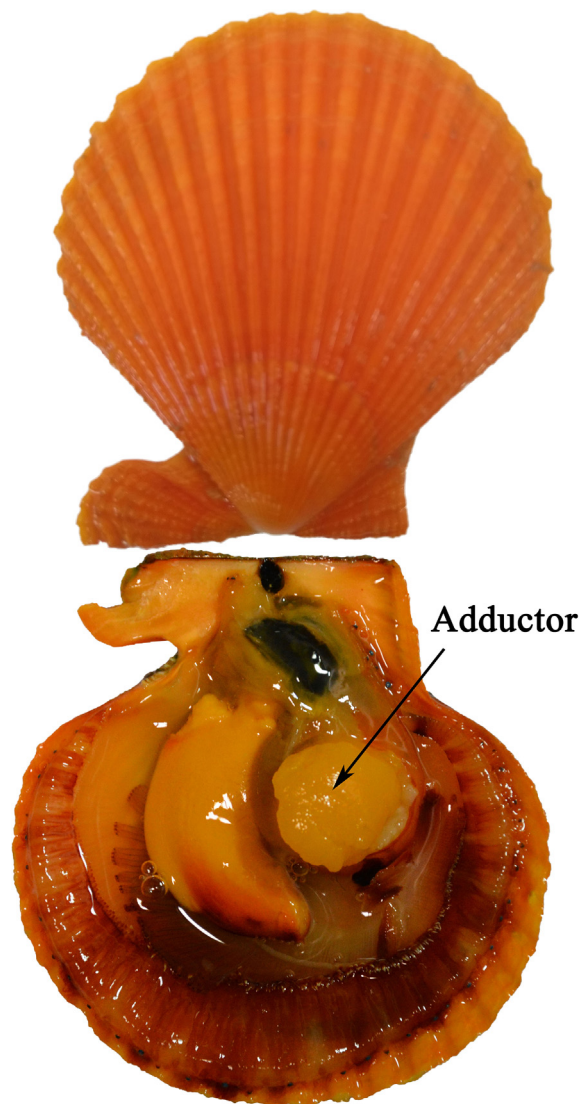


**Fig. 1.** Golden scallop of noble scallop *Chlamys nobilis*.

further analysis.

### 2.4. Determination of total carotenoids content (TCC)

The supernatants containing carotenoids from CEC and UAE were measured by using an Ultraviolet–visible (UV–vis) spectrophotometer (UV2501PC, Japan), based on the method reported by Dauqan, Sani, Abdullah, Muhamad, and Gapor (2011) with some modifications. The total carotenoids content was calculated according to the following equation described by Yanar et al. (2004):

$$\text{Total carotenoid content}\,(\mu gg^{-1}) = \frac{A(\text{total}) \times \text{volume}(mL) \times 10^4}{E_{1\,cm}^{1\%} \times \text{sample weight (g)}}$$

where A is the absorbance value of extract at 452 nm; and the extinction coefficient $E_{1\,cm}^{1\%} = 1900$ (Foss et al., 1984).

### 2.5. Experimental design and statistical analysis

Optimization of UAE parameters in total carotenoids extraction from dried scallop adductors was done using response surface methodology (RSM). Box Behnken design (BBD) is an efficient option for fitting response surfaces using three evenly spaced levels based on the construction of a balanced incomplete block design (Box & Behnken,

**Table 1**

Independent variables and their levels used for Box–Behnken rotatable design.

| Independent variables | Levels | | |
|---|---|---|---|
| | −1 | 0 | 1 |
| Ultrasonic power (X$_1$, W) | 120 | 240 | 360 |
| Extraction temperature (X$_2$, ℃) | 25 | 35 | 45 |
| Extraction time (X$_3$, min) | 10 | 20 | 30 |

**Table 2**

Box–Behnken design matrix and response values for total carotenoids content (TCC).

| Experimental number | X1 | X2 | X3 | Actual TCC (µg/g) | Predicted TCC (µg/g) |
|---|---|---|---|---|---|
| 1 | −1 | −1 | 0 | 111.78 ± 1.21 | 111.73 |
| 2 | 1 | −1 | 0 | 118.59 ± 5.51 | 118.91 |
| 3 | −1 | 1 | 0 | 125.86 ± 2.85 | 125.54 |
| 4 | 1 | 1 | 0 | 128.09 ± 1.77 | 128.14 |
| 5 | −1 | 0 | −1 | 116.01 ± 3.42 | 116.01 |
| 6 | 1 | 0 | −1 | 124.29 ± 4.42 | 123.93 |
| 7 | −1 | 0 | 1 | 132.27 ± 7.38 | 132.63 |
| 8 | 1 | 0 | 1 | 134.50 ± 4.44 | 134.50 |
| 9 | 0 | −1 | −1 | 110.89 ± 0.54 | 110.94 |
| 10 | 0 | 1 | −1 | 123.89 ± 0.98 | 124.21 |
| 11 | 0 | −1 | 1 | 126.60 ± 4.16 | 126.28 |
| 12 | 0 | 1 | 1 | 136.10 ± 3.99 | 136.05 |
| 13 | 0 | 0 | 0 | 138.27 ± 2.67 | 137.23 |
| 14 | 0 | 0 | 0 | 137.27 ± 0.79 | 137.23 |
| 15 | 0 | 0 | 0 | 137.22 ± 1.60 | 137.23 |
| 16 | 0 | 0 | 0 | 136.05 ± 0.42 | 137.23 |
| 17 | 0 | 0 | 0 | 137.33 ± 0.34 | 137.23 |

1960). According to the preliminary tests, ultrasonic power (W), extraction temperature (℃), and the extraction time (min) of 240 W, 38 ℃ and 29 min, respectively were chosen as design variables. A three-factors and a three levels BBD consisting of 17 experimental runs were applied to optimize carotenoids process. The actual and coded levels of the independent variables are given in Table 1. The experimental design and corresponding response data were listed in Table 2.

The modeling and statistical analyses were conducted using Design-Expert program (8.0.7.0 version). Experimental data were fitted to a quadratic polynomial model, as the following equation described by Zhang, Zhang, Yue, Fan, and Li (2009).

$$Y = \beta_0 + \sum_{i=3}^{3} \beta_i x_i + \sum_{i=3}^{3} \beta_{ii} X_i^2 \sum \sum_{i=3}^{3} \beta_{ij} X_i X_{ij}$$

where $\beta_0$, $\beta_i$, and $\beta_{ij}$ are the intercept, regression coefficients of the linear, quadratic, and interaction terms of the model, respectively, while $X_i$ and $X_j$ are the independent variables and Y is the dependent variable (Total carotenoids content). The quality of the fit of the polynomial model equation was expressed by the coefficient of determination $R^2$, and the significances of the regression coefficient were checked by F-test and P-value.

## 3. Results and discussion

### 3.1. The optimized conditions for extracting total carotenoids

#### 3.1.1. Effect of ultrasonic power on TCC

In the present work, four levels of ultrasonic power of 120, 240, 360, and 480 W were set. The extraction temperature and extraction time were maintained at 25 ℃ and 10 min, respectively. The extraction temperature and time of 25 ℃ and 10 min were selected based on preliminary studies. The result is shown in Fig. 2A. The TCC increased significantly from 120 to 240 W, but then decreased significantly at higher powers. Therefore, the optimum ultrasonic power is at 240 W.
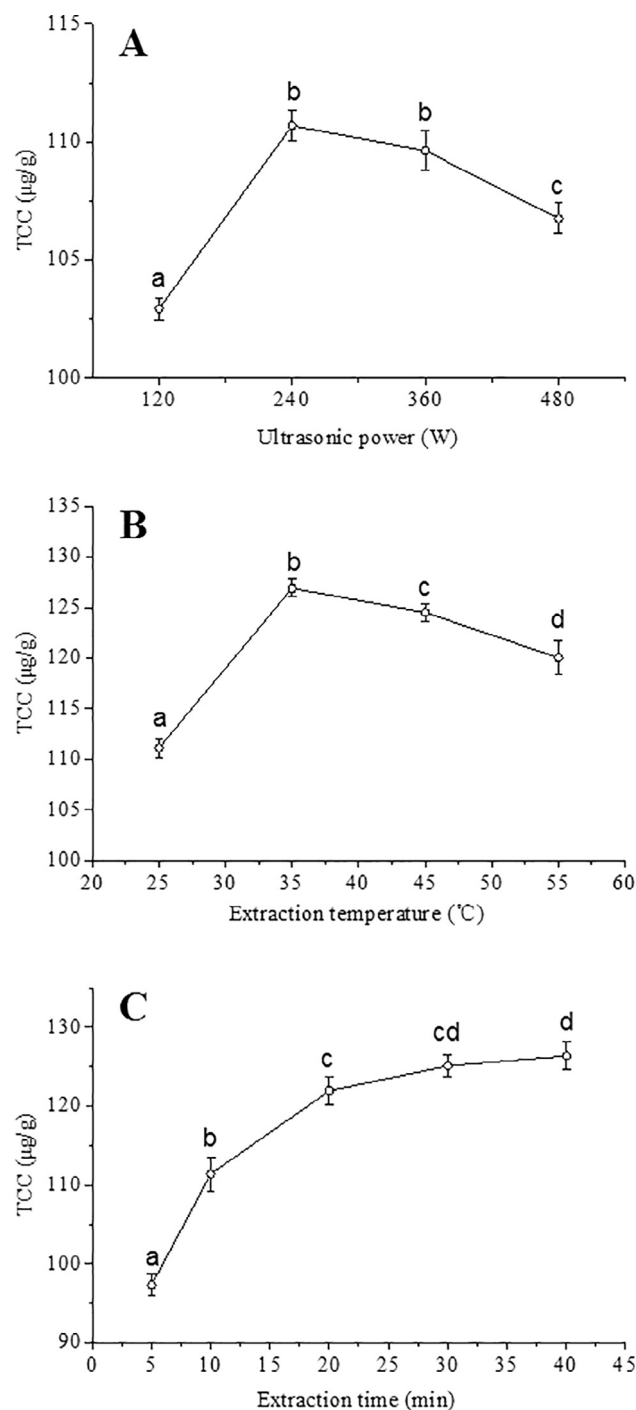


**Fig. 2.** Effects of different extraction conditions on the TCC. TCC was represented as mean ± SD (n = 3), and bars without shared letters were significantly different (P < 0.05). (A) effect of ultrasonic power on the TCC. (B) effect of extraction temperature on the TCC. (C) effect of extraction time on the TCC.

Similar observations have been reported in plants (Ye, Feng, Xiong, & Xiong, 2011; Yan, Fan, He, & Gao, 2016), where the efficiencies of total carotenoids extraction is dependent on the ultrasonic power used. The ultrasonic wave could disrupt and penetrate the cell, and thus accelerate the release of intracellular products into the solvent, as well as increase mass transfer rate (Xu et al., 2016). Therefore, in the present study, the extracted TCC increased with increasing in ultrasonic power from 120 to 240 W. However, TCC decreased when ultrasonic power increased further, probably due to formation of bubbles which

**Table 3**
Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters.

| Source | df | MS | F value | P |
|---|---|---|---|---|
| Model | 9 | 152.94 | 338.33 | < 0.0001 |
| Ultrasonic intensity, $X_1$ | 1 | 47.78 | 105.68 | < 0.0001 |
| Temperature, $X_2$ | 1 | 265.42 | 587.14 | < 0.0001 |
| Extraction time, $X_3$ | 1 | 369.78 | 818.00 | < 0.0001 |
| $X_1X_2$ | 1 | 5.24 | 11.60 | 0.0114 |
| $X_1X_3$ | 1 | 9.15 | 20.24 | 0.0028 |
| $X_2X_3$ | 1 | 3.06 | 6.77 | 0.0353 |
| $X_1^2$ | 1 | 199.03 | 440.27 | < 0.0001 |
| $X_2^2$ | 1 | 362.04 | 800.87 | < 0.0001 |
| $X_3^2$ | 1 | 54.12 | 119.72 | < 0.0001 |
| Residual | 7 | 0.45 | | |
| Lack of Fit | 3 | 0.23 | 0.36 | 0.7836 |
| Pure error | 4 | 0.62 | | |
| Cor total | 16 | | | |

$r^2 = 0.9977$, Adj-$r^2 = 0.9948$, Pred-$r^2 = 0.9893$, Adeq Precision = 50.986, CV = 0.53%.

hampered the propagation of ultrasound waves (Entezari & Kruus, 1996; Lou, Wang, Zhang, & Wang, 2010), or the heat produced by excessive ultrasonic power may not completely dissipate in a short period of time, leading to the degradation of carotenoids (Xu & Pan, 2013).

### 3.1.2. Effect of extraction temperature on TCC

Literatures documented that temperature affects the carotenoids extraction period in aquatic animal specimens. For instance, it requires a longer extraction time of 2–3 days at 4–5 °C (Torrissen and Naevdal (1984); Yanar et al., 2004), but only requires 2–12 h at 25 °C (Zheng et al., 2010; Lu et al., 2016). After taking degradation of carotenoids under higher temperature and lower boiling point of acetone into consideration, four levels of temperature (25, 35, 45 and 55 °C) were set in the present study, while ultrasonic power and extraction time were maintained at 240 W and 10 min, respectively. The result showed TCC significantly increased from 25 to 35 °C and then significantly decreased at higher temperature of 35 and 55 °C (Fig. 2B). Therefore, the optimum extraction temperature is at 35 °C. The higher solvent diffusion coefficients with lower viscosity at high temperature resulting in higher solubility of carotenoids (Hemwimol, Pavasant, & Shotipruk, 2006). This could partially explain the higher TCC extraction when the temperature increased from 25 to 35 °C. Carotenoids degradation begins at temperature close or higher than 40 °C (Oliveira, Carvalho, Nutti, Carvalho, & Fududa, 2010). In addition, high temperature can cause extensive isomerization and some *trans*-formers are converted into various *cis*-isomers, which is considered as a negative effect of processing since *cis*-isomers have less provitamin A activity than *trans*-isomers (Norshazila et al., 2017). Therefore, these could explain the decreasing TCC when increasing extraction temperature from 35 to 55 °C.

### 3.1.3. Effect of extraction time on TCC

Generally, the extraction time of CEC in the noble scallop needs 2–12 h at 25 °C (Zheng et al., 2010; Lu et al., 2016). According to the previous results in the animals (Torrissen & Naevdal, 1984; Yanar et al., 2004), there is an adverse correlation between extracting time and temperature. Therefore, extraction time was set at 5, 10, 20, 30, and 40 min with ultrasonic power of 240 W and extraction temperature of 25 °C in the present study. From Fig. 2C, we found that TCC rapidly increased from 5 to 20 min and then tended to become steady up to 40 min. With elongating extraction time, ultrasound wave disrupts the cell effectively, leading to a better mass transfer of intracellular products into solvent (Wang, Sun, Cao, Tian, & Li, 2008). However, extraction time should be set between 10 and 30 min to save time and cost
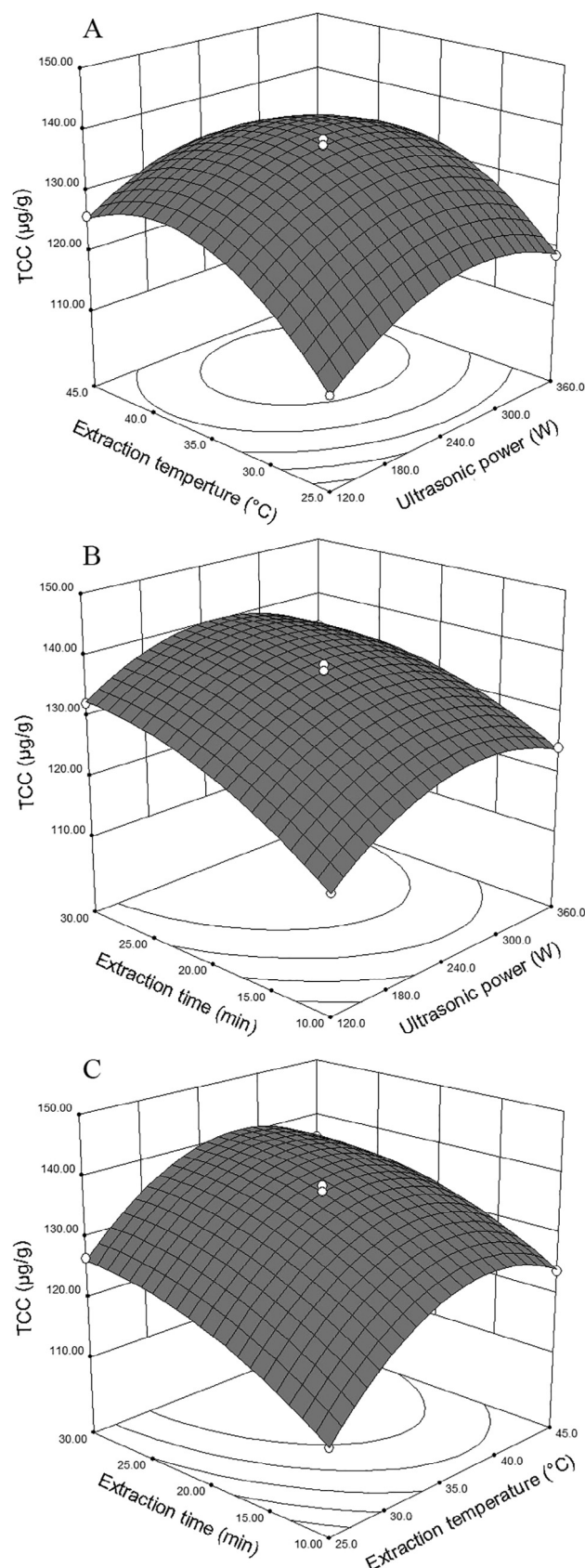
**Fig. 3.** Response surface plots of different extraction conditions on TCC. (A) ultrasonic power and extraction temperature. (B) ultrasonic power and extraction time. (C) extraction temperature and extraction time.

**Table 4**

Comparisons of extraction conditions and TCC between UAE and CEC.

| Extraction method | | Ultrasonic power (W) | Extraction temperature (°C) | Extraction time (min) | Acetone (mL) | TCC (µg/g) |
|---|---|---|---|---|---|---|
| UAE | Optimum conditions | 246.77 | 37.64 | 29.03 | 4 | 141.131 (predicted) |
| | Modified conditions | 240 | 38 | 29 | 4 | 137.88 ± 1.22 (actual) |
| CEC | / | / | 25 | 120 | 8 | 107.75 ± 2.60 |

of energy.

### 3.2. Response surface analysis

#### 3.2.1. Statistical analysis and the model fitting

Optimization of ultrasound-assisted carotenoids was conducted by a $3^3$ factorial BBD. The design matrix and experimental responses are shown in Table 1. 17 experiments were designated, from which 12 factorial experiments and 5 zero-point tests were performed to estimate the errors.

Table 2 shows the treatments with code levels and the experimental results of total carotenoids content in the Golden scallop. Predicted response Y for the TCC could be expressed by the following second-order polynomial equation in terms of coded values:

$$Y = 137.23 + 2.44X_1 + 5.76X_2 + 6.80X_3 - 1.15X_1X_2 - 1.51X_1X_3 - 0.88X_2X_3 - 6.88X_1^2 - 9.27X_2^2 - 3.59$$

where Y is the total carotenoids content (µg/g), $X_1$, $X_2$ and $X_3$ are the coded variables for ultrasonic power (W), extraction temperature (°C), extraction time (min), respectively.

Table 3 shows the analysis of variance (ANOVA) for the regression equation. The linear term and quadratic term were highly significant ($P < 0.01$). The lack of fit was used to verify the adequacy of the model and was not significant ($P > 0.05$), indicating that the model could adequately fit the experiment data. The adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this study, the ratio was found to be 50.986, which indicates that this model can be used to navigate the design space. The value of adjusted $R$-squared (0.9977) for the equation is reasonably close to 1, indicated a high degree of correlation between the observed and predicted values, therefore the model is suitable. A very low value of coefficient of the variance (C.V.%) (0.53) clearly indicated a very high degree of precision and reliability of the experimental values.

The significance of each coefficient was checked using $P$-value in Table 3. The $P$ value is used as a tool to check the significance of each coefficient. The corresponding variable will be more significant if the $P$-value becomes smaller (Chen et al., 2010). In this case, the linear coefficients ($X_1$, $X_2$, and $X_3$), quadratic term coefficients ($X_1^2$, $X_2^2$, and $X_3^2$) and cross product coefficients ($X_1X_2$, $X_2X_3$, and $X_1X_3$), with very small $P$-value ($P < 0.05$), are significant model. The result indicated ultrasonic power, extraction temperature and extraction time were significant single parameters influencing the TCC.

#### 3.2.2. Response surface plots

Response surface plots are very useful to judge interaction effects of the factors on the responses (Fig. 3A–C). These plots show effects of two factors on the response at a time with the other factor kept at level zero. Fig. 3A shows that TCC was significantly affected by ultrasonic power and extraction temperature. Higher TCC is strongly favored when extraction temperature is kept between 30 °C and 40 °C for a given ultrasonic power. TCC increases with increasing temperature, reaching a maximum value at intermediate ultrasonic power level (levels 0 or 240 W), and then decreases at high ultrasonic power level (levels 1 or 480 W). This is a result of negative effects of extraction temperature and ultrasonic power quadratic coefficients. Fig. 3B shows ultrasonic power and extraction time effects on TCC. When extraction time was 10 min, TCC increased with ultrasonic power, the maximum was achieved at

intermediate ultrasonic power level. However, when extraction time was 30 min, TCC was higher. Thus, extraction time is one of the most important factors in improving TCC. From Fig. 3C, we found that the TCC was significantly affected by extraction time and extraction temperature. And the TCC was always higher at intermediate extraction temperature from 30 °C to 40 °C with elongating extraction time.

### 3.3. Comparisons of extraction conditions and TCC between UAE and CEC

The optimal values of the selected variables were obtained by solving the regression equation. After calculation by Design Expert software (8.0.7.0-verision), the optimal conditions for extracting carotenoids in the Golden scallop were the ultrasonic power of 246.77 W, extraction time of 29.03 min, and extraction temperature of 37.64 °C, with the corresponding $Y = 141.13$ µg/g as shown in Table 4. However, considering feasibility in practice, the optimal extraction conditions were slightly modified as follows: ultrasonic power of 240 W, extraction time of 29 min and extraction temperature of 38 °C. An actual value of $137.88 ± 1.22$ µg/g was gained, which was significantly in agreement with the predicted value 141.13 µg/g ($P > 0.05$), implying that the optimal conditions are reliable.

TCC extracted by CEC was also listed in Table 4, which is significantly lower than the actual value by UAE. And 2 h time-spend by CEC is far longer than 29 min time-spend by UAE. Moreover, acetone consumption by CEC (8 ml) is two-fold of that by UAE (4 ml). Therefore, the UAE is more efficient than the CEC in terms of TCC, time-spend and acetone-consumption.

## 4. Conclusion

In the present study, an improving method for extracting carotenoids using ultrasound-assisted extraction (UAE) was the first time reported in aquatic animals. The optimal conditions for extracting carotenoids by UAE were determined, and the maximum value of total carotenoids content (TCC) was predicted to be 141.13 µg/g, which was agreed closely with the actual value of $137.88 ± 1.22$ µg/g obtained by modified conditions. While TCC extracted 2 h by conventional extraction of carotenoids (CEC) was $107.75 ± 2.60$ µg/g. The UAE has three advantages including higher efficiency for extracting carotenoids, shorter processing time, and less acetone requirement than the CEC.

### Declaration of Interest Statement

None.

### Acknowledgements

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