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Original Article

Extraction and purification of astaxanthin from shrimp shells and the effects of different treatments on its content

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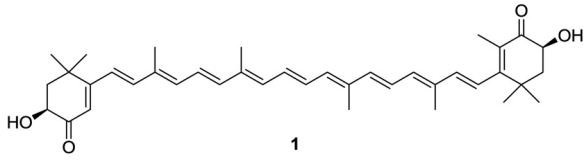
ABSTRACT

In this study, the extraction process and purification technology of astaxanthin from shrimp shells were established, and the effects of different treatments on the content of astaxanthin were studied. The determination results of astaxanthin in various shrimp/crab shells showed that the astaxanthin content in the *Procambarus clarkia* shell reached 239.96 μg/g. The effects of cool-ventilated, sun-dried and cooking conditions on the content of astaxanthin during the treatment of shrimp shells were investigated respectively and fresh shrimp shells as best extraction source was determined. The nine groups orthogonal test design with four factors and three levels ($L_9(3)^4$) was used to analyze the optimization of extraction process of astaxanthin from shrimp shells with ethanol as an environmentally friendly extraction solvent. The optimum experimental condition including the solid–liquid ratio (1:7), extraction time (20 min) and temperature (50 °C) was proposed with the maximum extraction yield of astaxanthin. Next, silica gel column chromatography was used to purify the crude extraction of astaxanthin, and the purity of astaxanthin increased from 0.34% to 85.1% (about 250 times), which has great applications in the high value utilization of shrimp shells resources and the development of astaxanthin-related products.

Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) (**1**) is a red carotenoid with scavenging ability of free radicals and more than 500 times the antioxidant activity of vitamin E (Dong et al., 2014; Papa et al., 2015; Elagamy et al., 2018; Zuluaga et al., 2018). Astaxanthin has proven to be excellent biological activities including anticancer and anti-aging, repairing central nervous system, improvement cardiovascular function and protecting eyesight, which become more and more popular in pharmaceutical, food industries and cosmetics (Wu et al., 2015; Visioli and Artaria, 2017; Zuluaga et al., 2018). At present, commercially available astaxanthin is mainly extracted from *Haematococcus pluvialis*, but its large-scale cultivation is difficult. However, seafood processing companies produce a large number of discarded shrimp/crab shells which are rich in astaxanthin, and the extraction of astaxanthin from them has great economic and social benefits.



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Different extraction methods of astaxanthin such as oil-soluble method, solvent extraction method, supercritical carbon dioxide extraction method and other kinds of extraction methods have been reported (Zhang et al., 2014; Dong et al., 2014; Radzali et al., 2014; Li et al., 2015). Supercritical fluid extraction method is safe and environmentally friendly with high requirements for equipment. Solvent extraction is the most commonly used method, and most studies focused on the screening of conventional solvents, but the extraction yield is low. Ethyl acetate was used to extract astaxanthin in *Prawn* shells and the yield was 30.258 μg/g (He et al., 2017). The present research results showed that the key factors of the astaxanthin extraction with high yield, content and stability by different type of treatment methods need to be further optimized, and there is also a lack of systematic research in this area. It was difficult to separate and purify due to its high antioxidant activity and similar structure to other carotenoids. Therefore, the

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establishment of high extraction rate method and purification technology of astaxanthin in shrimp shells is of great significance for the high value utilization of shrimp shells resources and the development of astaxanthin-related products.

Astaxanthin was usually determined by ultraviolet visible spectrophotometry and high-performance liquid chromatography (HPLC) with simple and rapid features. The spectrophotometric determination of astaxanthin was total carotenoids, but the content of astaxanthin cannot be determined accurately. The determination method of astaxanthin in shrimp shells was established by HPLC using C18 column with short retention time at 3.7 min which was easily interfered by other extractions and difficult to achieve baseline separation (Sun et al., 2010, Food Sci.). Although C30 chromatographic column obtained the good retention time at 9.2 min with favorable separation, the C30 column used was expensive (Sun et al., 2017, Food Safe. Qual. Detec. Technol.). Therefore, the determination of astaxanthin by HPLC with rapid, and accurate popular method need to be further established for the quality control of astaxanthin.

In this study, astaxanthin of different shrimp shells was extracted by using ultrasound-assisted with grain alcohol, the extraction parameters were optimized by using single-factor experiment and orthogonal test to further improve the extraction rate of astaxanthin. The silica gel column chromatography would be selected for the purification of the astaxanthin extractive by using petroleum ether and ethanol as eluant to obtain high purity astaxanthin. The HPLC method for determination of astaxanthin was also established by optimizing the ratio of mobile phase. The effects of different kinds of shrimp/crab shells and different processing methods on astaxanthin were systematically investigated, and the optimized detection method, extraction and purification processes of astaxanthin were determined. This work will lay a foundation for development of astaxanthin health food, which not only reduced the environmental pollution by the dispose of discarded shrimp shells, but also improved the resource utilization rate and created enormous economic value.

Materials and methods

Samples and chemicals reagents

Astaxanthin standard (batch number: K23M7S15084, HPLC purity ≥98%) was obtained from Yuanye Biotechnology Company Ltd (Shanghai, China). Argentine Red Shrimp shells and Arctic Sweet Shrimp shells were obtained from Rongsense Company Ltd (Shandong, China). *Procambarus clarkia* and *Portunus* crab were bought from RT-Mart. Methanol and acetonitrile (HPLC grade) were obtained from Yuwang Industrial Company Ltd (Shandong, China). Dichloromethane (HPLC grade), 95% ethanol and ethyl acetate (analytical grade) were obtained from Kemiou Chemical Reagent Company Ltd (Tianjin, China). Petroleum ether and acetone (analytical grade) were obtained from Sinopharm Chemical Reagent Company Ltd (Shanghai, China). Silica gel (200–300 mesh) and silica gel plate were obtained from Qingdao Jiyida silica reagent Company Ltd (Qingdao, China). Potassium hydroxide was obtained from Kaitong Chemical Reagent Company Ltd (Tianjin, China). Hydrochloric acid was obtained from Laiyang Fine Chemical Factory.

Sample preparation

The effects of pretreatment on the extraction of astaxanthin were investigated in detail. Five raw materials: *Pandalus borealis* shells, *P. borealis* heads, Argentine red shrimp shells, *Procambarus clarkii* shells, *Portunus* crab back shells. Three treatment methods: drying under a ventilated and sunless place, drying under the sun and drying under the sun after boiling them. All the samples were crushed through the grinder and then passed through the 30 mesh screen, and marked respectively. Freshly smashed *P. borealis* shell was named sample 1. The *P. borealis* shell that was dried under the ventilated and sunless place was named sample 2. The *P. borealis* shell that was dried under the sun was named sample 3. The *P. borealis* heads that was dried under the ventilated and sunless place was named sample 4. The shrimp shells were boiled in water for 20 min and dried under the sun, *P. borealis* shells named sample 5, Argentine red shrimp shells named sample 6, *P. clarkii* shells named sample 7 and *Portunus* crab back shells named sample 8.

Astaxanthin extraction protocol

According to the optimized extraction scheme of astaxanthin, took 1 g of samples 1–8 to conical flask (sample 1 was converted into dry weight according to the water content), each added 7 ml ethanol, mixed evenly and extracted 20 min in 50 °C water by ultrasound equipment (40 kHz, Ningbo Scientz Biotechnology Company Ltd). After centrifugating at 1308.1 × *g* force for 5 min, the extraction was repeated three times and the supernatant was combined. Then 10% KOH–ethanol solution was added to each sample to adjust the pH to 10, shook evenly and saponified for 2.5 h at 4 °C in the refrigerator. Then the sample solution was adjusted to pH 7 with dilute hydrochloric acid, then filtered, and filtrate was evaporated under vacuum at 40 °C to get astaxanthin crude extract, dissolved with ethyl acetate and constant volume, overed 0.45 μm filter membrane and determined by HPLC.

Orthogonal test design

On the basis of single factor, orthogonal test $L9(3)^4$ was designed by four factors (solid-liquid ratio, temperature, extraction time and null column) and three levels as shown in Table 1.

HPLC identification and quantification

A LC-10A HPLC (Shimadzu, Japan) equipped with LC-10ATvp binary pump, SIL-10A autosampler and SPD-M10Avp detector was used. The system was computer controlled installed with Lab Solutions chromatography workstation for the analysis of data. The chromatographic column (Shimadzu® C18 column (4.6 mm × 250 mm, 5 μm), Shimadzu, Japan) was used for the separation of astaxanthin. The flow rate was 1 ml/min, the detection wavelength was at 474 nm, the column temperature was 25 °C and the injection volume was 20 μl. The mobile phase was consisting of acetonitrile/methanol/dichloromethane (80:15:5, v/v/v).

Table 1
Factors and levels of orthogonal test.

Levels	Solid–liquid ratio (g/ml)A	Temperature (°C)B	Extraction time (min)C	Null columns
1	1:5	40	20	1
2	1:7	50	30	2
3	1:9	60	40	3

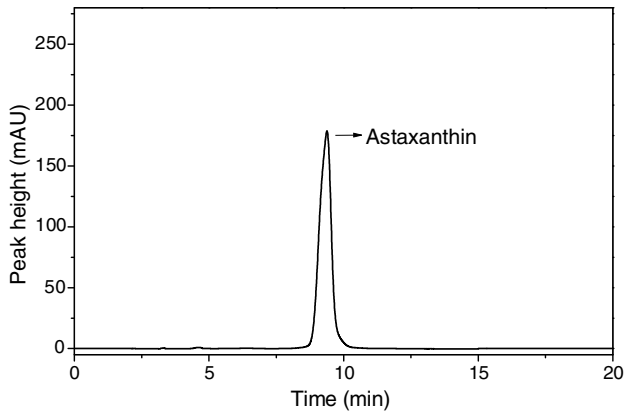

Fig. 1. The chromatogram of astaxanthin standard.

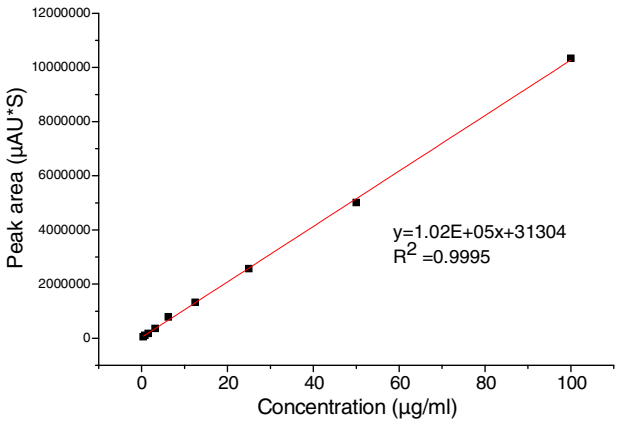

Fig. 2. Standard curve of astaxanthin.

Analysis of data

The experiments were performed three times unless otherwise mentioned. Analysis of data was performed by orthogonal test assistant.

Results and discussion

Validation of the extraction method

In order to optimize the extraction method, the level of linearity, precision, stability, reproducibility and recovery of experiments were performed. The standard chromatogram diagram is shown in Fig. 1, the astaxanthin peak appears at a retention time of 9.6 min, the theoretical plate number of the chromatographic peak is not less than 3000.

Linear range and standard curve determination

Astaxanthin standard substance was precisely weighed (10 mg) and dissolved in 100 ml ethyl acetate in a brown volumetric flask to obtain stock solution (contain astaxanthin 100 μg/ml) which sealed with nitrogen and stored in a −18 °C refrigerator without light. The stock solution was then diluted to the required concentrations (0.39, 0.78, 1.56, 3.15, 6.25, 12.50, 25, 50, 100 μg/ml) by acetonitrile/methanol/dichloromethane (80/15/5, v/v/v) to draw the standard curve under the chromatographic conditions as stated above. Linear regression was applied to calculate the standard curve. The coefficients of determination (R^2) value (0.9995) revealed a good linearity over the selected range for astaxanthin (0.39–100 μg/ml) was shown in Fig. 2.

Precision and stability experiments

The precision of the method was determined for the astaxanthin solution (12.5, 25 and 50 μg/ml) five times, respectively. The coefficient of variation (%RSD) value (2.2%, 1.8%, 1.6%) showed (Table 2) that the method is precise. The results showed that the precision of the instrument was good and it can meet the requirement of determination and analysis of astaxanthin content. The sample solution was placed at 25 °C for 12 h and analyzed every 2 h. The %RSD value (3.24%) displayed in Table 2 indicates that the samples were in good stability within 12 h. Due to the high antioxidant activity of astaxanthin, it was necessary to determine the sample solution in time to ensure the results accurate and reliable.

Table 2
Precision and stability experiments.

	Precision			Stability	Reproducibility
RSD (%)	2.2	1.8	1.6	3.24	1.45

Note: where $n = 5$.

Reproducibility and recovery of astaxanthin

The reproducibility of the method was determined for astaxanthin with 25 μg/ml. The process was repeated five times, and 1.45% RSD value showed that the repeatability of the method was good (Table 2).

The recovery of astaxanthin content was calculated for the same five samples (repeated six times) shown in Table 3. The recovery of astaxanthin was obtained from 0.101 mg of crude extract after adding astaxanthin (0.050 mg, 0.075 mg, 0.100 mg, 0.125 mg, 0.150 mg). The recovery rate was calculated by the following formula.

$$\text{Recovery rate } (\%) = \frac{(\text{detected value} - \text{added value})}{\text{original value}} \times 100$$

The recovery of the sample was 100.47% and the RSD was 2.92%, which indicated that the recovery rate of the method was good and the method was feasible.

Determination of sample content

Eight kinds of test solutions were prepared according to the section “Sample preparation”, and the astaxanthin content was determined according to the optimized chromatographic condition. The chromatograms of astaxanthin determination were shown in Fig. 3. According to the peak area and the standard curve, the content of astaxanthin in the sample was shown in Table 4.

The results showed that the content of astaxanthin in sample 1 (fresh *P. borealis* shells) reached 50.32 μg/g, and that in sample 2 (shrimp shells dried in ventilation) decreased by nearly 10 times, while in sample 3, the damage of astaxanthin was more serious under the condition of sun drying. The results of sample 4 showed that astaxanthin content in shrimp heads was slightly lower than that in shrimp shells. The astaxanthin content in shrimp shells was reduced to 2.69 μg/g after sample 5 was cooked at high temperature and dried in the sun. The above experimental results showed that fresh shrimp shells contain the highest amount of astaxanthin. Cool ventilated dried, sun-dried and cooked conditions can reduce obviously the content of astaxanthin in shrimp shells. The main reason may be that astaxanthin has strong antioxidant activity and the astaxanthin in shrimp shells will be destroyed by the oxygen

Table 3
Result of recovery of astaxanthin.

No.	Original (mg)	Added (mg)	Detected (mg)	Recovery (%)	Mean value (%)	RSD (%)
1	0.101	0.050	0.147	96.34	100.47	2.92%
2	0.101	0.075	0.178	101.74		
3	0.101	0.100	0.200	99.36		
4	0.101	0.125	0.227	100.64		
5	0.101	0.150	0.255	104.28		

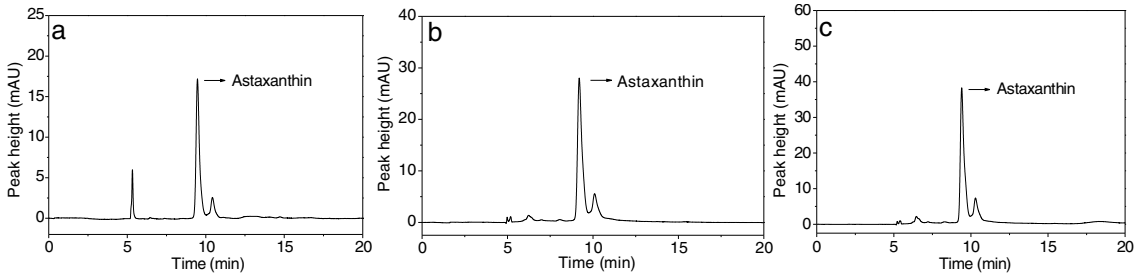

Fig. 3. The determination chromatograms of astaxanthin in shrimp shells: (A) *Pandalus borealis* shell; (B) *Procambarus clarkii* shell; (C) Argentine red shrimp shell.

Table 4
The determination results of astaxanthin in shrimp/crab shells by different methods.

Sample number	1	2	3	4	5	6	7	8
Astaxanthin determination value (μg/g)	50.32	5.81	4.33	4.13	2.69	1.86	239.96	11.67

in the air and sunlight during the drying and high temperature process.

In addition, according to the edible methods of shrimp/crab, the *P. borealis* shells, Argentine red shrimp shells, *P. clarkii* shells and *Portunus* crab back shells were respectively treated after being cooked and dried in the sun. The results of astaxanthin determination showed that the astaxanthin content in the *P. clarkii* shells reached 239.96 μg/g, that in the back shells of *Portunus* crab is 11.67 μg/g, that in the Argentine red shrimp shells is 1.86 μg/g. These results showed that *P. clarkii* shells have great utilization value in astaxanthin extract.

Effects of solid–liquid ratio, extraction time and temperature on the yield of astaxanthin

According to the results in Table 4, it can be seen that fresh shrimp shells are the best raw material for extracting astaxanthin. As for the selection of solvents, ethanol has a better penetration effect on wet shrimp shells. Therefore, fresh *P. borealis* shells were used as raw materials and ethanol was used as extraction solvent to optimize the extraction of astaxanthin in the experiment, and the effects of solid–liquid ratio, temperature and extraction time on the yield of astaxanthin were further investigated.

Fig. 4A displays the effect of solid-liquid ratio on astaxanthin content. Solid–liquid ratio was fixed at 1:3, 1:5, 1:7, 1:9 and 1:11 with the temperature at 50 °C and extraction time at 10 min. When the solid–liquid ratio was 1:7, continued to decrease the solid–liquid ratio did not increase the yield significantly. Therefore, the experiment chose 1:7 as the optimum extraction solid–liquid ratio. Fig. 4B shows the effect of temperature on astaxanthin content. Temperature was fixed at 30 °C, 40 °C, 50 °C, 60 °C and 70 °C with the solid–liquid ratio at 1:7 and extraction time at 10 min. The yield of astaxanthin increased and reached a maximum value (36.4 μg/g) when temperature was 50 °C. The results showed that the appropriate temperature was between 40 °C and 60 °C. Therefore, the temperature at 40 °C, 50 °C and 60 °C was included in the orthogonal test. Fig. 4C displays the effect of extraction time on astaxanthin content. Extraction time was fixed at 10 min, 20 min, 30 min, 40 min and 50 min with solid–liquid ratio at 1:7 and temperature at 50 °C. The extraction yield of astaxanthin increased and reached a maximum value (45.3 μg/g) when extraction time was 30 min. The results showed that the appropriate extraction time was between 20 min and 40 min, and the extraction time (20 min, 30 min, 40 min) was included in the orthogonal test.

Optimization of the extraction parameters

The effects of solid–liquid ratio, temperature and extraction time on the yield of astaxanthin were optimized by orthogonal test. Orthogonal test has many advantages such as to reduce the testing times and obtain reliable data. The orthogonal test was designed to evaluate all the selected factors by nine groups of data, four factors and three levels, which named as $L9(3)^4$. Four factors are solid–liquid ratio, extraction temperature, extraction time and null column (Calculating variance), respectively. The *K* and *R* values and analysis of variance are displayed in Tables 5 and 6. It has been noted that the mean extraction yields of astaxanthin was good at A2 (solid–liquid ratio, 1:7), B2 (temperature, 50 °C) and C3 (extraction time, 40 min). In addition, analysis of variance showed that temperature was the most important factor in extraction process. It can be seen from Table 5 that the influence level of extracting conditions on astaxanthin yield varies in order: B > A > C. Considering the instability and loss of astaxanthin under high temperature for a long time, C1 can be selected. Therefore, the extraction scheme was A2B2C1.

Since this scheme was not in the orthogonal test, supplementary verification test was required. Three parallel experiments were conducted under this scheme to obtain an average astaxanthin yield of 43.7 μg/g, which was close to the 45.8 μg/g of the optimal combination A2B2C3. The crude extract was obtained at 0.632 g, and the purity was 3.4 mg/g (0.34%) by HPLC.

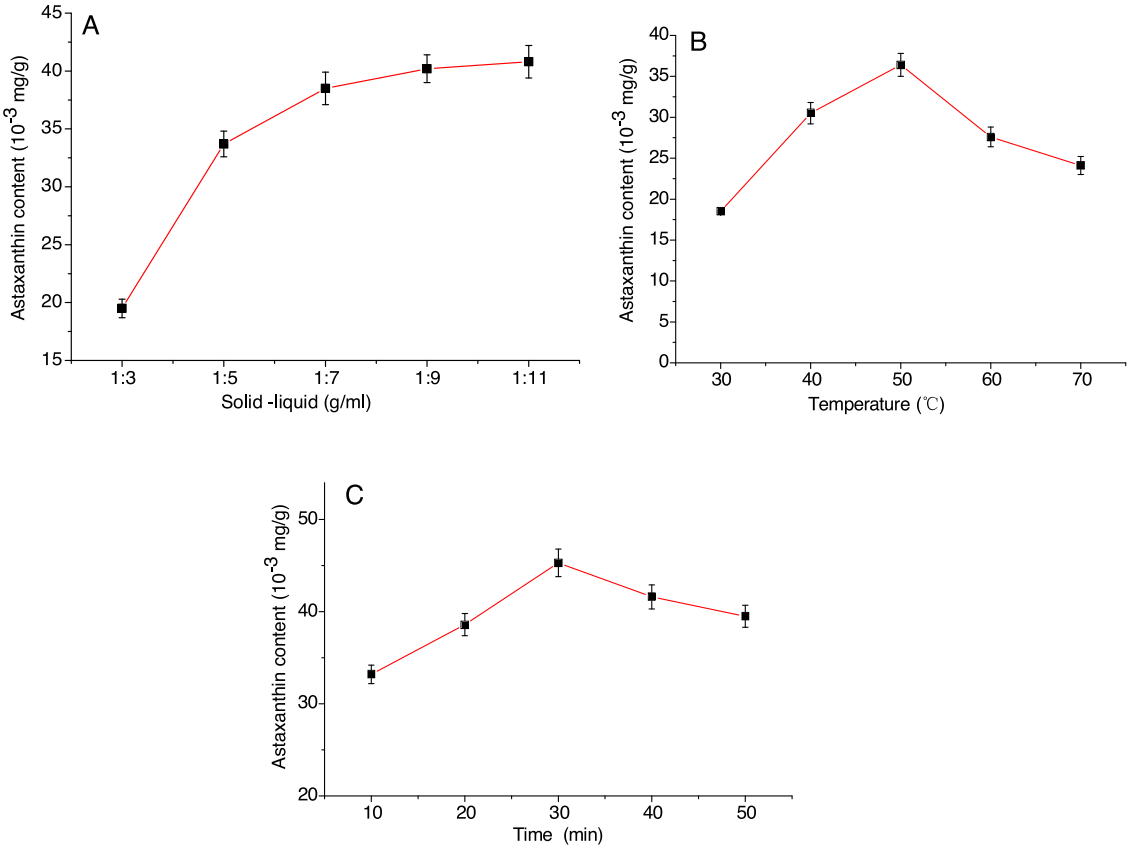

Fig. 4. The effects of (A) solid–liquid ratio, (B) temperature, and (C) extraction time on astaxanthin content (whereas $n = 3$).

Table 5
Orthogonal test of astaxanthin extraction.

Level	A	B	C	Null column	Astaxanthin content (μg/g)
1	1	1	1	1	31.4
2	1	2	2	2	41.7
3	1	3	3	3	28.6
4	2	1	2	3	35.5
5	2	2	3	1	45.8
6	2	3	1	2	31.7
7	3	1	3	2	35.8
8	3	2	1	3	43.5
$9K_1K_2K_3R$	333.90037.66737.6333. 767	334.23343.66731.30012.700	234.53336.93336.0672.400	136.93336.40035.8671.066	33.6

Table 6
Analysis of variance.

Factor	Sum of squares	Freedom	*F*	Conspicuousness
A	28.127	2	0.396	–
B	250.527	2	3.531	a
C	3.440	2	0.048	–
Error	283.80	8		

"a" means that the factor is significant, "–" means that the factor is not significant.

Therefore, this scheme was proved as the effective extraction condition.

Optimization of process amplification

In order to verify the feasibility of the test parameters in the production process, the optimum conditions according to the above results were chosen (solid–liquid ratio, 1:7; temperature, 50 °C and extraction time, 20 min) and the yield of astaxanthin and crude extract were calculated. The results of 100 times more of the sample tested were shown in Table 7 under the above optimum extracting conditions, which confirmed that the optimized extraction process is reliable and precise.

Table 7
Optimum scale-up test.

	1	2	3
Pandalus borealis shells (dry weight, g)	500	500	500
Astaxanthin content (μg/g)	45.65	42.83	43.18
Total crude product yield (%)	7.29	7.87	8.05

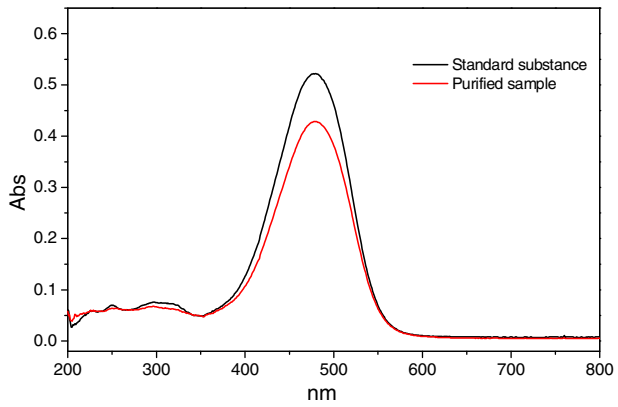

Fig. 5. Ultraviolet visible scanning spectrum.

Purification of astaxanthin

Although silica gel has excellent separation and purification ability, irreversible adsorption is unavoidable. In order to determine the irreversible adsorption of astaxanthin on silica gel, astaxanthin standard solution was loaded, and petroleum ether and ethanol as mixed solvent to desorb astaxanthin from silica gel. The eluate was determined by ultraviolet visible spectrophotometer (Shimadzu UV2450), and irreversible adsorption rate was calculated to be 14.05% by the standard curve method. In the actual purification process, a large amount of fatty acid and other polar substances in the crude extract of astaxanthin compete with astaxanthin for the adsorption center of silica gel. In fact, the irreversible absorption of astaxanthin on silica gel during the purified experiment was probably less than the dead adsorption 14.05%. In view of the cheap price of silica gel and its strong ability to purify astaxanthin, it was feasible to choose silica gel as the separation medium.

The saponified astaxanthin was purified with silica gel as the separation medium, petroleum ether (boiling range: 60–90 °C): ethanol = 95:5 (v/v) as the eluting solvent, received the red color band, concentrated, evaporated solvent and freeze-dried to obtain 10.08 mg of deep fuchsia astaxanthin powder with a purity of 85.1% (HPLC). The characteristic absorption spectrum of purified sample and standard astaxanthin were scanned at 200–800 nm by ultraviolet visible spectrophotometer. The comparative results showed that the all bands of purified astaxanthin were basically consistent with that of the standard astaxanthin simple (Fig. 5), and obvious absorption band of the impurity was not found in the ultraviolet region. Therefore, the purity of astaxanthin with 85.1% was reliable.

Conclusion

HPLC method for rapid and accurate determination of astaxanthin was established by using C18 chromatographic column. By comparing the effect of different treatment methods of shrimp/crab shells on astaxanthin content, it is determined that fresh shrimp shell is the best raw material for astaxanthin extraction. The extraction scheme of astaxanthin in shrimp shells was determined through the single factor experiment and orthogonal test: ethanol as extraction solvent, solid–liquid ratio is 1:7, temperature is 50 °C, extraction time is 20 min, astaxanthin yield is 43.7 μg/g. The astaxanthin extract with 0.34% content was further purified by silica gel column chromatography, and the purity of astaxanthin was 85.1%. This work lays a foundation for the comprehensive utilization of shrimp/crab shells resources and the development of astaxanthin related products.

Ethical statement

All the experiments protocols were performed in accordance with guidelines and approved protocols of Jining Medical University.

Authors contribution

LW designed and supervised experiments; WL, LM, RD and YW performed experiments; JH and LM contributed to the writing of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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