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An Effective Extract Method of Phospholipids from Antarctic Krill *Euphausea superba*

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Abstract

Phospholipids are one of the major bioactive ingredients of Antarctic krill *Euphausea superba*. A feasible and effective extraction method of Antarctic krill oil was investigated and modified by orthogonal test which the ratio of solid to liquid was 1:2.5, extraction time was 5 min, ratio of ethyl acetate (EA) and n-butanol (BuOH) was 1:1. With this method, the extract of krill oil has a higher phospholipids content of 27.7% - 42.3%, together with total oil yields of 4.15% - 6.18%.

Keywords

Phospholipids, Antarctic Krill, *Euphausea superba*, Extraction

1. Introduction

Phospholipids are not only a kind of important bioactive ingredients, but also essential nutrients in the metabolism of organisms. They were used in food, medicine, and industry widely [1] [2] [3]. At present, there were many preparation and purification methods of phospholipids, such as solvent extraction, supercritical fluid extraction, and column chromatography, as well as analytic methods including thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) [4]. Although the advanced phospholipid production technology has been applied in Europe, America, Japan and some other countries, it is still not popular in China.

According to the abundance phospholipids, *Euphausea superba* (**Figure 1**), one major species of Antarctic krill, is a biological enrichment library of phospholipids [3]. The main components of the phospholipids in Antarctic krill are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl

Figure 1. The picture of *E. superba.*

inositol (PI) and so on [5].

In our continuous investigation on feasible and effective extraction method of Antarctic krill oil, an improved solvent extraction and HPLC were used for extraction and detection of the phospholipid from *E. superba*. The high content phospholipid was therefore obtained.

2. Experimental Section

2.1. General Procedures

Homogenizer (DJ126-DEG1) was offered by Guangdong Midea boutique electrical appliance manufacturing co., LTD. Centrifugal machine(KUBOTA 7780) was obtained from Beijing dongxuntiandi medical instrument co. LTD. Agilent 1260 HPLC was used with the column of Atlantis HILIC Silica (4.6 × 150 mm, 5 μm). The ultraviolet spectrophotometer (752N) was purchased from Shanghai Precision and Scientific Instrument Corporation. L-*α*-phosphatidylcholine (P3556, ≥99%) and L-*α*-phosphatidylethanolamine (P7943, ≥97%) were obtained from Sigma. Astaxanthin was obtained from Dr. Ehrenstorfer GmbH. Methanol, acetonitrile and formic acid were chromatographic grade and obtained from J. C. Baker. Other reagents were analytical grade and obtained from Sinopharm chemical reagent Shanghai co., LTD.

2.2. Biological Source

E. superb were collected from the 30th Chinese Antarctic scientific expedition, and was identified by Prof. Hong-Liang Huang. The samples were flushed with plenty of water and then kept frozen at −18˚C before use.

2.3. Extraction

For better extraction of fat-soluble constituents, a solvent mixture of ethyl acetate (EA) and n-butanol (BuOH) was found to give a higher phospholipids content [6]. Then an orthogonal table of 3 factors and 3 levels is adopted in the design referred to Jian-Tong Zhang [7]. The ratio of solid to liquid, extraction time and ratio of solvent (EA/BuOH) were selected as the influencing factors, using the screening test to optimize the extraction process (**Table 1**).

Table 1. Factors and levels of orthogonal test.

Factor	level		
	1	2	3
Ratio of solid to liquid (A)	1:1.5	1:2	1:2.5
Extraction time (B)/min	3	5	8
Ratio of solvent (C)	1:1	2:1	4:1

The samples of *E. superba* were thus divided into 9 groups, and each group was 400 g. Every group was repeatedly extracted in homogenizer with ethyl acetate/n-butanol mixed solvent for 3 times at room temperature. Then the supernatants were combined and the solvent was removed in a rotary evaporator at 50˚C, then the fat-soluble extracts, namely krill oils, were obtained and weighed. The yields were calculated as in

$$\text{Yields} = \text{weight of extract}\,(\text{g})/\text{wet weight of}\ E.\ superba\,(\text{g}) \quad (1)$$

2.4. HPLC Analysis of Phospholipids

The standard solutions of L-α-phosphatidylethanolamine and L-α-phosphatidylcho-line in methanol were made up in two series of concentrations (0.2, 0.4, 0.6, 1.0, 1.4, 1.8 mg/mL and 0.06, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0 mg/mL), respectively. Krill oil samples (2.0 mg) were dissolved in methanol to 1.0 mg/mL concentration, respectively. Each reference and sample solutions was filtrated with 0.45 μm filter before injection to HPLC. The mobile phase was acetonitrile/ethanol/water containing 1% acetic acid (V:V:V = 30:69:1), with flow rate of 1.0 mL/min. The detection wavelength was 206 nm. The column temperature was room temperature and the injection volume was 10 uL.

2.5. Analysis of Astaxanthin

Astaxanthin standard (0.12 mg) was dissolved with chloroform and made up as 10.0 mL parent solution. Then it was gradually diluted to give a series of solutions of 0.12, 0.6, 1.2, 6, 12 μg/ml. Krill oil samples (20 mg) which was got from orthogonal test were dissolved in chloroform with concentration of 2 mg/mL. Each solution was tested in UV spectrophotometer under 478 nm with chloroform as blank control.

All statistical analyses were executed by using the Origin 9.1 software.

3. Results and Discussion

3.1. The Yields of Krill Oils

Orthogonal test design is a scientific method of arranging and analyzing multifactor experiments. The main advantage of the orthogonal test design is that it can significantly reduce the number of trials, but it still covers all the possible tests [8]. The result of extraction yields was shown in **Table 2**. Firstly, fat soluble ingredients from *E. superba* accounted for 4.15% - 6.18%. They were in accord

Table 2. The extraction yield of fat soluble ingredients from *E. superba*.

No.	A	B (min)	C (EA/BuOH)	Ratio (%)
1	1:1.5	3	1:1	4.68
2	1:1.5	5	2:1	4.55
3	1:1.5	8	4:1	4.15
4	1:2	3	2:1	4.60
5	1:2	5	4:1	5.45
6	1:2	8	1:1	5.25
7	1:2.5	3	4:1	5.15
8	1:2.5	5	1:1	6.18
9	1:2.5	8	2:1	5.25
K1	13.38	14.43	16.10	
K2	15.30	16.18	14.40	
K3	16.58	14.65	14.75	
κ1	4.46	4.81	5.37	
κ2	5.10	5.39	4.80	
κ3	5.53	4.88	4.92	
R	1.07	0.58	0.57	
The optimal level	A_3	B_2	C_1	

with Sun Lei's result [9]. Secondly, among all the three factors, ratio of solid to liquid was the most important factor which affected extraction yields because its value, the maximum range, was 1.07. The higher the ratio was, the larger the surface area of the solvent contact was. The influences of extraction time and solvent ratio were approximate, and were lower than that of solvent amount. However, they could not be neglected. At last, according to uniform design method, the best combination of experimental condition was A3B2C1, which meant the ratio of solid to liquid 1:2.5, extraction time 5 min, ratio of EA/BuOH 1:1, which was amazing the same to group 8th.

3.2. The Contents of Phospholipids and Astaxanthin in Krill Oil Samples

Standard curves (**Figure 2**) were constructed for peak areas and concentrations of standards L-*α*-phosphatidylethanolamine (t_R 3.9) and L-*α*-phosphatidylcholine (t_R 6.7), respectively. A good linear correlation was observed in the concentration range of 0.2 - 1.8 mg/mL of L-*α*-phosphatidylcholine, and its equation of calibration curve was $Y = 2462.7X - 95.488$ ($R^2 = 0.9987$). While for L-*α*-phosphatidylethanolamine, linear correlation was observed between concentrations of 0.06 and 2.0 mg/mL, with the equation of calibration curve $Y = 4554.5X - 30.893$ ($R^2 = 0.9989$).

The phospholipid contents of the 9 samples obtained from the orthogonal experiment were detected by HPLC with standards of L-*α*-phosphatidylcholine

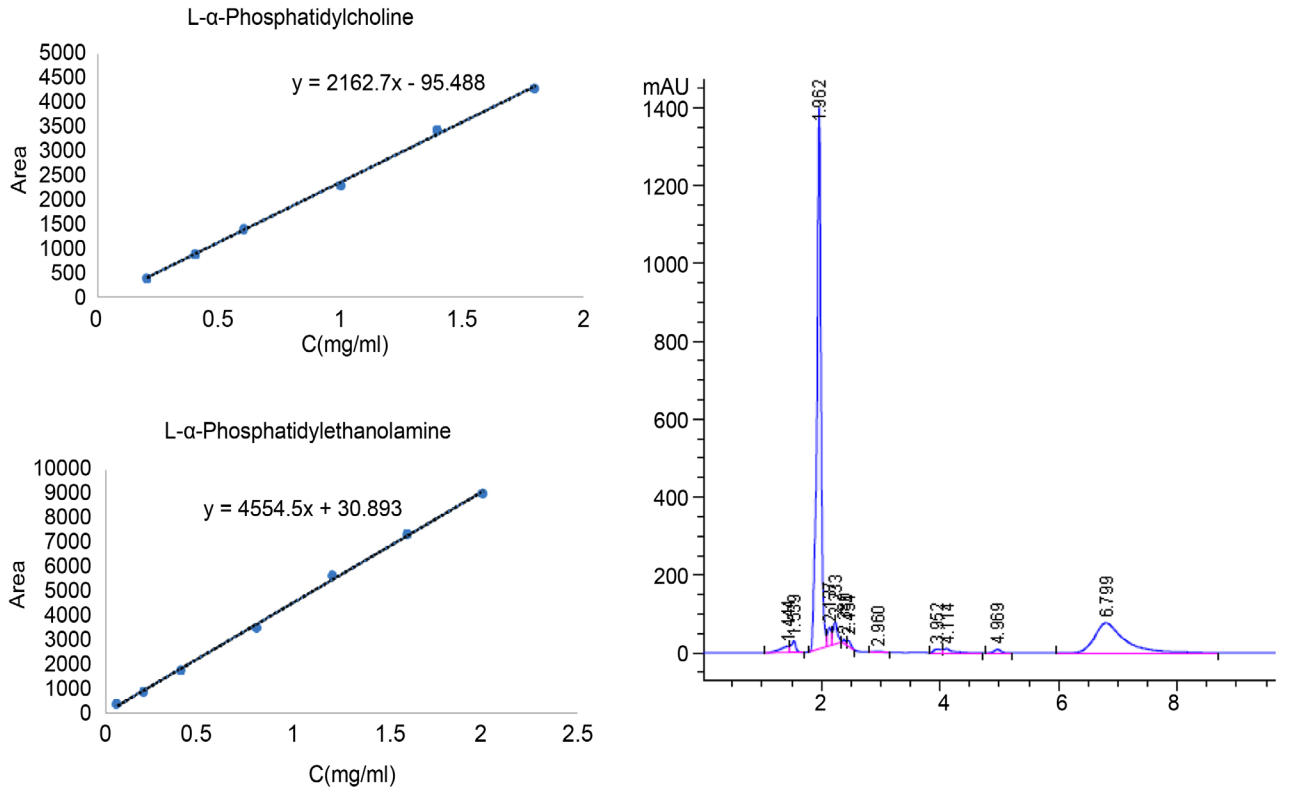

Figure 2. Standard curves of L-*α*-phosphatidylethanolamine and L-*α*-phosphatidylcholine, and HPLC of krill oils.

and L-*α*-phosphatidylethanolamine (**Table 3**), which can be separated from other impurities completely in orthogonal sample (**Figure 1**). The contents of L-*α*-phosphatidylcholine and L-*α*-phosphatidylethanolamine in the krill oils accounted for 25.79% - 38.79%, 0.92% - 3.54%, respectively. The results were close to the results of Bjørn Winther [10]. It should be mentioned that L-*α*-phosphatidylcholine was more than 90% of the total phospholipids.

The contents of astaxanthin in these samples were also determined by UV spectrophotometer. Standard curves were constructed for absorbance and concentrations of standard astaxanthin. A good linear correlation was observed in the concentration range of 0.12 - 12 μg/mL. The equation of calibration curve was A = 0.1710C + 0.0601 (R^2 = 0.9956). The contents of astaxanthin in these krill oil samples accounted for 37.9 ~ 49.3 ppm. Astaxanthin was a kind of natural pigment in the *E. superba*, which was easier to be esterified. The average content is about 180 ppm (132 - 250 ppm) [11]. These experiment results were less thanthe average range. Therefore, to raise the contents of astaxanthin in extraction of *E. superba* will be the following research emphasis.

On the basis of all above, a feasible and effective extraction method of Antarctic krill oil with a solvent mixture of ethyl acetate and n-butanol were established, and modified by orthogonal test. It was found to extract krill oil with a high phospholipids content of 27.7% - 42.3%, together with a total oil yields of 4.15% - 6.18%.

Table 3. The contents of astaxanthin and phospholipid of the krill oil samples.

No.	Astaxanthin (ppm)	L-*α*-phosphatidylcholine (%)	L-*α*-phosphatidylethanolamine (%)
1	49.3	32.72	2.94
2	40.2	30.46	2.00
3	40.9	38.79	3.54
4	41.3	34.90	2.93
5	37.9	26.77	0.92
6	45.3	25.79	2.82
7	45.1	32.51	2.18
8	42.2	28.59	2.60
9	41.1	28.61	1.65

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