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Determination of Zeaxanthin in New Food Raw Materials

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Abstract: The new food raw material zeaxanthin has been used in many food industries. However, the state has not yet formulated relevant detection methods for the detection of zeaxanthin raw material content. There are many reports on the extraction and purification of zeaxanthin in the existing literature, but there is no report on the method for the determination of zeaxanthin in the raw material of zeaxanthin. As a new food raw material, the content of zeaxanthin itself directly affects the content of the product. Therefore, developing a determination method of zeaxanthin in zeaxanthin raw material is urgently needed for the field of added zeaxanthin functional food research and product quality assurance. This study describes a method for the determination of zeaxanthin in new food raw materials by high performance liquid chromatography. Samples were dissolved with water and extracted with ultrasonication at a temperature range of 58–62°C, and then precipitated with mixed solvent of ethanol and trichloromethane. The supernatant was diluted by ethanoland and then filtered through a 0.45μm pore nylon filter, prior to injection into the HPLC system. Calibration of the chromatographic system was carried out by the external standard method. Analyses was carried out using an HPLC-UV Agilent 1260 system equipped with DP-4 gradient pump, and C_{30} column (250mm×4.6, 5μm) was used. The quantitative limit of zeaxanthin was 0.1g/100g and this method was accurate, repeatable, sensitivity.

Keywords: Zeaxanthin, Liquid Chromatography, New Food Raw Materials, C_{30} Column

1. Introduction

Zeaxanthin is an oxygen-containing carotenoid [1], Zeaxanthin is a beta-carotene derivative, soluble in organic solvents such as acetone, petroleum ether, ether and esters, insoluble in water, with poor thermal and photostability [2]. Many studies have shown that Zeaxanthin has many physiological functions, such as antioxidant [1], visual protection [3-4], free radical scavenging, anti-cancer [5], cardiovascular disease reduction, prevention of Senile cataract and macular [1, 6], etc., Zeaxanthin has also been shown to have unique physiological functions in enhancing immunity [1, 7]. It can be seen that zeaxanthin has a high nutritional value, and the human body cannot synthesize zeaxanthin by itself, which must be obtained with food or supplements. According to the Measures for the Safety Examination of New Food Raw Materials and the Food Safety Law, The National Health and Family Planning Commission, in accordance with legal procedures, organized experts to review the safety assessment materials of Zeaxanthin, it is considered that Zeaxanthin can be used as a new food raw material, and can be produced and used according to the contents of the announcement, which meets the requirements of food safety [8]. At present, the new food raw material zeaxanthin has been used in many food industries. However, the state has not yet formulated relevant detection methods for the detection of zeaxanthin raw material content. There are many reports on the preparation [9-11] and purification [12-14] of zeaxanthin in the existing literature, some determination of zeaxanthin in wolfberry [15], sea thorn [16], eye care products [17], lutein microcapsule powder [18] and marigold [19] have also been reported. but there is no report on the method for the

determination of zeaxanthin in the raw material of zeaxanthin, As a new food raw material, the content of zeaxanthin itself directly affects the content of the product. Therefore, it is urgent to develop a method for the determination of zeaxanthin in the zeaxanthin raw material for the research in the field of adding zeaxanthin to functional foods. In this paper, a method for the determination of zeaxanthin content in raw materials by liquid chromatography is established, which provides a solution for the determination of zeaxanthin in raw materials, and provides a certain reference for enterprises that need to monitor zeaxanthin raw materials.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals were of analytical grade or higher purity unless otherwise stated, Ultra-pure water (Milli-Q Plus System, Millipore, 18.2 MΩ) was prepared in laboratory. Standards of zeaxanthin (≥74.5%) was obtained from Chromadex (USA), Anhydrous ethanol and Trichloromethane were from Da Mao (Tianjin, China), HPLC-grade methanol and Methyl tert-butyl ether were purchased from CNW (Shanghai, China).

2.2. Instruments and Equipment

1260 high performance liquid chromatograph (with fluorescence detector) was purchased from Agilent Technologies (USA), AB265-S analytical balance was purchased from Mettler-Toledo (Switzerland), Ultrapure water machine was purchased from Thermo Fisher (USA).

2.3. Methods

2.3.1. Sample Preparation

0.2g of raw materials sample was weighed and dissolved with 5 mL of water in 100 mL brown volumetric flask, vortex-mixed and then extracted with ultrasonication at a temperature range of 58–62°C for 5 min. Then added 40 mL of ethanol, vortex-mixed to dissolve fully and added 50 mL of chloroform, maked up to volume with ethanol and let stand for 30 min. The supernatant was taked 0.5 mL to 50 mL of brown volumetric flask, dissolved with ethanol and maked up to volume. The Diluent was collected and filtered through a 0.45μm pore nylon filter, prior to injection into the HPLC system.

2.3.2. Chromatographic Conditions

Analyses was carried out using an HPLC-UV Agilent 1260 system equipped with DP-4 gradient pump, and C_{30} column (250 mm×4.6, 5μm) was used. The mobile phase consists of 0.1% (v) methanol solution of BHT (A) and 0.1% (v) Methyl tert-butyl ether solution of BHT (B). Total analysis time for a single sample was 28 min at 450 nm wavelength with column temperature at 25°C. The flow rate was set to be 1.0 mL/min and the sample injection volume was 50 μL. The gradient elution conditions are shown in Table 1.

Table 1. *Mobile phase gradient elution program table.*

Time /min	Flow rate/mL/min	A/%	B/%
0	100	0	0
18	10	90	18
18.1	100	0	18.1
28	100	0	28

2.3.3. Preparation of Standard Solutions

Zeaxanthin standard stock solution (concentration about 0.1 mg/mL): Weigh a certain amount of zeaxanthin standard, add ethanol to dissolve and dilute to 10 mL, as a stock solution. Stored in a refrigerator at -16 to -20°C (or lower temperature), the validity period is 1 month (ethanol is not completely dissolved, and chloroform can be added to dissolve).

Preparation of zeaxanthin intermediate solution: Pipette 0.5 mL of stock solution and dilute to 10 mL with 0.1% BHT ethanol.

Zeaxanthin working solution: Pipette 950, 900, 800, 500, 200, 0 μL of 0.1% BHT ethanol into a brown injection bottle, respectively, add 50, 100, 200, 500, 800, 1000 μL of zeaxanthin liquid, shake well and test on the machine.

2.4. Result Calculation

The determination of zeaxanthin content in the sample is calculated according to formula (1):

$$X = \frac{C \times V \times D}{m \times 1000 \times 1000} \times 100 \quad (1)$$

where:

X—sample content, g/100g,
C—Sample concentration, μg/mL,
V—Constant volume of sample treatment solution, mL,
D—Dilution factor of sample treatment solution,
m—sample weight, g.

3. Results and Analysis

3.1. Selection of Preprocessing Methods

Zeaxanthin is lively and easy to oxidation. Therefore, it is embedded during the raw material production for the stability of zeaxanthin, the common carotenoid embedding agent is ocenyl succinate starch sodium. The synthesis [20], properties [21] and preparation [22] of sodium ocenyl succinate are studied to obtain suitable pretreatment methods. It is determined to break the embedding agent with warm water, zeaxanthin is then extracted with ethanol and impurities are precipitated using trichloromethane, finally, the purification and miscellaneous removal of zeaxanthin are realized.

3.2. Selection of Flow Phase Conditions and Detection Wavelengths

Zeaxanthin is a carotenoid compound, which has some interference by spectrophotometer. Therefore, the maximum absorption wavelength of 450nm and the C30 chromatcolumn with good carotenoid separation were selected for analysis and detection. To obtain suitable separation conditions, the 2 flow

phase conditions were compared: Mobile phase system A is methanol / methy ltert-butyl ether / water = 80 / 15 / 5 and methanol / methyl tert-butyl ether = 1 / 10. The results are shown in Figure 1, you can see from the figure: In the case of no obvious difference in separation, the mobile phase system B is simpler to prepare, shorter peak out time and avoid the water phase, thus avoiding potential damage to the chromatcolumn, so the mobile phase system B is used for the experiment.

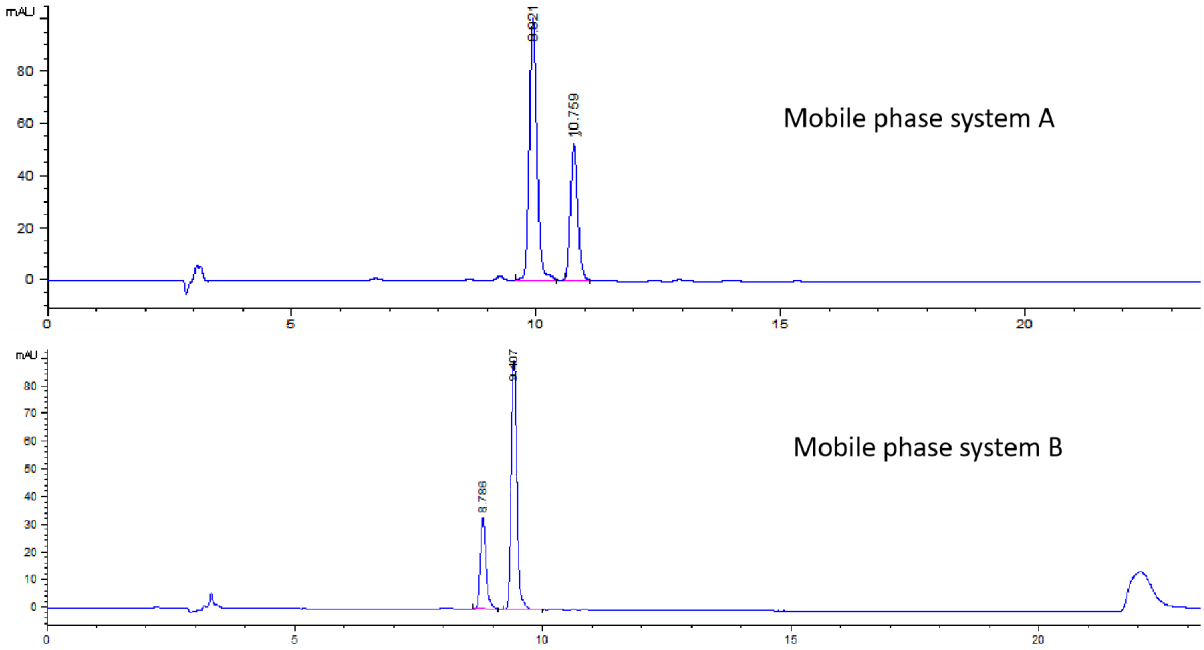


Figure 1. *Chromatogram of zeaxanthin in mobile phase system B and mobile phase system A.*

3.3. Linear Range

Table 2. *Standard curve concentration point table.*

Standard concentration ug/ml	Standard peak area	Standard curve Equation	Linear relationship R^2
0.1173	74.9		
0.2347	146.3		
0.4693	302.8	y=617.0x+2.3888	0.999
1.1733	706.3		
1.8772	1170.5		

Zeaxanthin has a good linear relationship with its peak area within the concentration range of 0.1μg/mL-2μg/mL, all of which meet the requirements of the national standard GB/T 27417-2017 "Guidelines for Confirmation and Verification of Chemical Analysis Methods for Conformity Assessment", The correlation coefficient of the linear regression equation is not lower than 0.99, indicating that the method has a good linear relationship.

3.4. Repeatability

A certain brand of zeaxanthin in the market was selected for the repetitive detection of zeaxanthin content. The specific results are shown in Table 3.

Table 3. *Zeaxanthin content and relative standard deviation (n=6).*

raw material name	Sample content (g/100 g)	average value (g/100 g)	RSD %
1-1	4.52		
1-2	4.56		
1-3	4.63		
1-4	4.44	4.55	1.77
1-5	4.51		
1-6	4.67		
1-7	4.49		

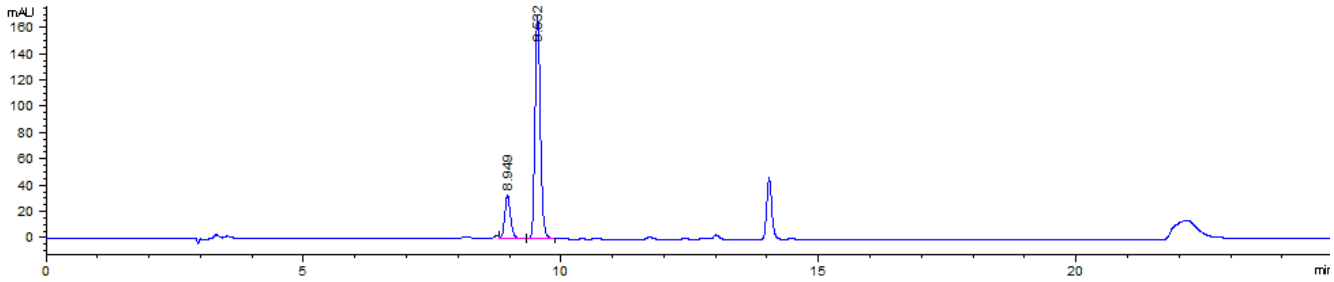
The national standard GB/T 27417-2017 "Guidelines for the Confirmation and Verification of Chemical Analysis Methods for Conformity Assessment" requires: when the content is 1%, RSD≦2.7%; when the content is 10%, RSD≦2.0%, when the content in this experiment is about 4.5%, The RSD was 1.77, all of which met the requirements of GB/T 27417-2017, indicating that the method has good precision for the determination of zeaxanthin content in raw materials.

3.5. The Limit of Quantitation

According to the method, the signal-to-noise ratio was greater than 10:1 when the raw material was re-diluted by 50 times, and the corresponding raw material content was about 0.1% at this time, so 0.1% could be the quantitative limit of this method.

3.6. Selectivity

According to the requirements of 0512 high-performance liquid chromatography in the 2015 edition of the Chinese Pharmacopoeia, the resolution was greater than 1.5, and the actual target and adjacent peaks were separated by 2.46, which met the selectivity requirements, indicating that the method had good selectivity, As shown in Figure 2.



Note: The retention time is 9.532 min for zeaxanthin, 8.949 min for lutein, and the rest are impurity peaks.

Figure 2. *Chromatogram of zeaxanthin in raw materials.*

4. Conclusion

This paper provides a liquid chromatography method for the determination of zeaxanthin content in raw materials, which is simple, rapid, precise and repeatable, and provides a solution and a determination scheme for the determination of zeaxanthin content in raw materials. In this paper, we optimize the method for pretreatment and flow phase conditions, the optimal detection step for zeaxanthin in raw material were determined. At the same time, it is hoped that people should pay attention to the beneficial functions of new food raw materials, but also pay attention to the detection method of the content of new food raw materials, which is more conducive to the quality assurance of products and human health.

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