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Liquid Chromatographic Determination of Astaxanthin in *Phaffia rhodozyma*

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HPLC에 의한 *Phaffia rhodozyma* 중의 Astaxanthin의 측정법

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Abstract

A sensitive and accurate method for the detection of astaxanthin was developed. The optimum HPLC system for the extraction and quantification of astaxanthin was based on a reversed phase column, and a quaternate mobile phase contained dichloromethane. The amount of astaxanthin can be accurately quantified in *Phaffia rhodozyma* cultures. The results of this study suggest that the mixture solution of dimethylsulphoxide and acetone is the optimal extraction solvent for astaxanthin and samples treatment for astaxanthin analysis must control low temperature and absent light operation strictly.

Key words : astaxanthin, *Phaffia rhodozyma*, HPLC, stability.

INTRODUCTION

Astaxanthin is a useful carotenoid for inducing pigment formation in fish during aquaculture. The use of astaxanthin as colourant in aquaculture (especially as feed supplement in farmed trout, salmon and prawns) is necessary to obtain the natural red-pink colours since they are not capable of *de novo* synthesis of carotenoids [1]. In addition, astaxanthin was also found to have antioxidation[2~4] and antitumor activity[5]. Astaxanthin possesses a higher antioxidative activity than β-carotene and α-tocopherol, so that astaxanthin is attractive as a powerful antioxidative reagent that may delay aging and degenerative diseases in human and animals[6]. The biotechnological production of astaxanthin can be based on the utilization of microalgae, bacteria or yeast. The

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Phaffia rhodozyma is one of the most promising micro-organisms for the industrial production of astaxanthin[7]. The biotechnological process for astaxanthin production is an alternative to the chemical synthesis, owing to their high market price and growing demand. However, the yield of astaxanthin in *P. rhodozyma* was quite a few, add to insensitive and inaccurate analysis method using, make many researcher and producer get negative or inaccurate result. It was the goal of this work to study the liquid chromatographic properties and stability of astaxanthin in detail in order to develop an accurate and sensitive method to determine astaxanthin in yeast cultures.

MATERIALS AND METHODS

1. Chemicals and Materials

Commercial pure astaxanthin was obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All solvents used in chromatography were of HPLC grade; solvents used for spectroscopy were of spectroscopic grade. Other reagents used were of analytical grade. Water was of Milli-Q (Milli-Q® Gradient, Millipore Corporation, Billerica, MA, USA) quality water. All the samples produced in flask cultures were obtained from our laboratory.

2. Preparation of Astaxanthin Standard Solution

Stock solution of astaxanthin was prepared by dissolving 1.0 mg astaxanthin in 1 ml dichloromethane solution. It was stored at - 20°C in the dark place before use. Stock solution of astaxanthin standard was gradient diluted as below: 500 μg/ml, 200 μg/ml, 100 μg/ml, 50 μg/ml, 10 μg/ml, and 5 μg/ml, these were used as working solutions.

3. Preparation of Samples from *P. rhodozyma*

After 30 days flask cultivation of *P. rhodozyma*, the cultures were harvested, then were centrifuged at 5,000×g for 10 min at 4℃, the supernatant was discarded, the remaining pellets were washed twice with sterilized water and were used in measurement of both biomass (as dry weight) and carotenoids. The washed pellets were frozen to dryness by freezing dryer (Ecospin 3180C, Hanil Science Industrial, Inchon, Korea). Then the lyophilized powder were accurately weighed 50±0.02 mg, then were added into 1.5 ml eppendorf tube, extracted with 1 ml dimethylsulphoxide (DMSO) /acetone=1/1(v/v) by ultrasonic power at 20℃ for 20 min, then centrifuged and transferred the supernatant to a 5 ml-vial, and repeated extracting the residue with 1 ml extracting solvent for another 4 times. Finally, 5 times extraction were combined and made it up to 5 ml with the responding extracting solvent. The extraction was centrifuged at 5,000× g for 5 min at 4℃. 1 ml the clear supernatants solution was subjected to 4 mm syringe filter (0.2 μm PTFE media, Whatman International Ltd, Maidstone, England). 20 μl of the filtrated solutions were directly injected into the HPLC system. Each test was indented three times. Above processes were conducted in darkness.

4. Yeast and Culture Conditions

The *P. rhodozyma* ATCC 24228 was grown on a YM agar media with the following composition: yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; glucose, 10 g/l; agar, 15 g/l. Plate culture was performed in incubator at 21℃. The seed culture was prepared by inoculating the yeast from a fresh plate into a 250 ml Erlenmeyer flask containing 100 ml YM broth, and incubating for 72 hr in a rotary shaker (21.5℃, 150 rpm). The yeasts were centrifuged, washed twice with distilled water, and re-suspended in 100 ml YM broth.

Flask cultures were carried out on 100 ml YM media with the following composition: yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; glucose, 10 g/l. They were shaking at 150 rpm in an orbital shaker incubator (K.M.C-8480SFN, Virsion Scientific Ltd, Bucheon, Korea) at 21±0.5℃ in 250 ml Erlenmeyer flasks.

5. Instrumental and Chromatographic Conditions

The content (μg/ml) of astaxanthin was determined by reverse-phase high-performance liquid chromatography (HPLC). HPLC was conducted with a Prostar HPLC equipped with two Prostar 210/215 Solvent Delivery Modules and Prostar 320 UV/VIS detector (Prostar, Varian Inc., Greek, CA, USA). The pigment extract was analyzed using a 250×4.6 mm C_{18} (5 μm) column (Ultrasphere®, Beckman Instruments Inc., Fullerton, CA, USA).

The identification and quantification of astaxanthin was carried out by using reverse phase column. The quaternate mobile phase was consisted of methanol (82%), dichloromethane (6.5%), acetonitrile (7.5%) and water (4.0%) by volume. The flow-rate was 0.8 ml/min. The detection was monitored at 482 nm. The injection volume was 20 μl, and the run time was 30 to 45 min. All the mobile phases were filtrated through a 0.45 μm filter membrane (Sigma-Aldrich Inc., St. Louis, MO, USA), and were degassed by air pump while sonicated for 15 min.

6. Screening of Optimum Extracted Solvent

Among dimethylsulphoxide, methanol, acetone, dichloromethane and their mixed solutions, *P. rhodozyma* culture on YM medium as sample was chosen for screening the optimum extracting solvent for astaxanthin analysis. For each treatment, the extracting steps just followed the procedures described above. The filtered extracted solution was subjected to the screened optimum HPLC condition. The extracting efficiency of dimethylsulphoxide was relatively considered as 100%, and the relative percentages of other solvents were calculated and used to estimate their extracting efficiency. Each treatment was repeated up to three times.

7. Recovery of the Screened HPLC System

The accuracy and the precision of the screened optimum HPLC system were estimated by recovery rate and relative standard deviation (R.S.D.) respectively, calculated from the running of 100 μg/ml standard astaxanthin six times. The concentration of 100 μg /ml was considered as true value to calculate the recovery rate.

RESULTS AND DISCUSSION

1. The Optimum Extracted Solvent

The relative extracting efficiency (R.E.F.) of dimethylsulphoxide for astaxanthin was considered as 100%. And mixture solution of dimethylsulphoxide and acetone was screened as the optimum extraction solvent for astaxanthin with the relative extracting efficiency of 135.53%. The result was shown in Table 1.

Though astaxanthin owns highest solubility in dichloromethane (30 g/l) and relatively low solubility in acetone (0.2 g/l) and dimethylsulphoxide (0.5 g/l) were referred in previous study[8], dimethylsulphoxide own higher cell penetrability than other solvents, this make dimethylsulphoxide and acetone quickly penetrate cell wall into yeast cell, therefore break cell wall, extract astaxanthin into extracellular solution effectively, so mixture solvents contained dimethylsulphoxide own higher extracting efficiency than that of single solvents.

2. Stability Analysis of Standard Solution and Samples

Our research suggested that temperature was an important sensitive factor for stability of astaxanthin. Under room temperature, astaxanthin can be oxidated into other compounds; under low temperature (4 or - 20°C), stability of astaxanthin can be preserved. In addition, astaxanthin exists mainly as free form in *P. rhodozyma*,

Table 1. The screening for the optimum extracting solvent

Extract solvents (1ml)	Avr. conc.* (μg /ml)	R.S.D.** (%)	R.E.E. ***(%)
DMSO-acetone (1:1)	25.3085±2.458	1.22	135.53
DMSO	18.6728±4.007	1.60	100.00
Acetone	3.0983±0.235	1.39	16.59
DMSO-dichloromethane (1:1)	16.2406±3.645	1.33	86.97
Dichloromethane	4.1064±2.476	1.29	21.99
Methanol-dichloromethane (3:1)	13.1798±1.728	1.50	70.58

* Avr. conc.: Average concentration, mean ± S.D. (n=3).

** R.S.D.: Relative standard deviation.

*** R.E.E.: Relative extracting efficiency, comparing to the extracting efficiency of DMSO.

it had been proved by us and previous study[9], it is different with microalgae *Haematococcus pluvialis*, which astaxanthin exists in the form of astaxanthin esters[1], astaxanthin esters own fine stability, so free form of astaxanthin existed in *P. rhodozyma* is instable, the HPLC determination of astaxanthin of *P. rhodozyma* must control low temperature operation strictly, decrease the time of samples and standards existing under room temperature as less as possible, and avoid thermal operation procedure in case of getting negative or wrong results. Our trials and results were shown in Table 2.

The results suggest that all manipulations should be performed in the condition of absent light and low temperature, as carotenoids are very sensitive to light, oxygen and heat. So the manipulations should be performed in a dark room while keeping the temperatures at 4 °C, and standard solutions should be hermetically stored in refrigeratory at - 20℃.

3. Assay Evaluation of HPLC System

With a 100 μg/ml of astaxanthin, the accuracy and precision of this screened optimum HPLC system was estimated by recovery rate and R.S.D., the recovery rate and R.S.D. were calculated from the data obtained from six parallel tests. The average recovery rate of astaxanthin was 98.29%, and the R.S.D. was 1.11% (n=6), which indicated that this HPLC system had a very high accuracy and precision. The results were shown in Table 3.

4. Sample Analysis

With the screened optimum HPLC conditions and the screened extracting solvent system, seven samples were analyzed. The results were presented in Table 4. The minimum and maximum detected amounts of astaxanthin were 0.014 mg/g and 1.69 mg/g, respectively.

In conclusion, we have developed an HPLC system for the separation and quantification of astaxanthin in *P. rhodozyma*. The system was based on a reversed-phase column, and a quaterate mobile phase contained dichloromethane, this method was shown here to provide producible, accurate and sensitive results, allowing astaxanthin to be determined using a simple extraction procure. In addition, temperature, light and oxygen are important influencing factors on stability of astaxanthin standard and sample astaxanthin from *Prhodozyma*. HPLC manipulations should be performed in the conditions of absent light and low temperature.

요 약

Astaxanthin은 수산양식에 필수적인 carotenoid 첨가물일 뿐 아니라 우수한 항암 및 항산화 물질로서 알려

Table 2. The stability analysis of astaxanthin

Trials No.	Original conc. (μg /ml)	HPLC quantify conc.* (μg /ml)	Treatments memo
1[a]	50	49.7024±1.346	sealed, dark condition
2[a]	50	48.6728±1.027	sealed, bright condition
3[a]	50	30.0315±0.222	unsealed, dark condition
4[a]	50	22.7965±1.087	unsealed, bright condition
5[b]	50	49.6520±2.345	sealed
6[c]	50	45.3450±2.774	sealed
7[d]	50	49.8898±1.728	sealed
8[e]	16.9	16.7756±1.348	sealed

* Average concentration, mean ± S.D. (n=3).

[a] standard of 50 μg /ml was stored at room temperature for one day.

[b] standard of 50 μg /ml was stored at 4℃ in refrigeratory for one day.

[c] standard of 50 μg /ml was stored at 4℃ in refrigeratory for one week.

[d] standard of 50 μg /ml was stored at - 20℃ in refrigeratory for one day.

[e] sample of 16.9 μg /ml was stored at - 20℃ in refrigeratory for one day.

Table 3. Recovery rate and R.S.D. of standard astaxanthin

Operation times	100 μg/ml standard astaxanthin[a]			
	Detected conc. (μg/ml)	Recovery rate[b] (%)	Average recovery rate[c] (%)	R.S.D[d] (%)
1	97.479	97.479		
2	95.080	95.080		
3	101.965	101.965	98.29±2.624	1.11
4	97.408	97.408		
5	105.072	105.072		
6	100.377	100.377		

[a] true value concentration.

[b] Recovery rate=(detected concentration/true value concentration)×100%.

[c] Average recovery rate is average value of recovery rate of six operation, value is mean ± S.D. (n=6).

[d] Relative standard deviation.

Table 4. Astaxanthin content in *P. rhodozyma* cultures

Sample ID	Sample amount (μg)	Astaxanthin concentration (μg/ml)	Astaxanthin content* (mg/g dry cells)
323-tomato[a]	50×10^3	8.8204±0.933	0.8890±0.057
323-malt[b]	50×10^3	16.900±1.503	1.6900±0.073
NA[c]	50×10^3	0.145±0.081	0.0145±0.006
YEPD[d]	50×10^3	0.700±0.573	0.0700±0.004
YM[e]	50×10^3	10.430±3.774	1.0430±0.055
323-carrot [f]	50×10^3	10.088±5.727	1.0080±0.096
PDBY[g]	50×10^3	2.845±0.003	0.2840±0.002

* astaxanthin content = astaxanthin concentration×5ml/sample amount (mg/g dry cell).

[a] Sample come from *P. rhodozyma* cultures on 323 medium contained tomato extract.

[b] Sample come from *P. rhodozyma* cultures on 323 medium contained malt extract.

[c] Sample come from *P. rhodozyma* cultures on NA medium.

[d] Sample come from *P. rhodozyma* cultures on YEPD medium.

[e] Sample come from *P. rhodozyma* cultures on YM medium.

[f] Sample come from *P. rhodozyma* cultures on 323 medium contained carrot extract.

[g] Sample come from *P. rhodozyma* cultures on PDB medium contained yeast extract.

져 있다. 현재 astaxanthin은 *P. rhodozyma*에 의해 생산할 수 있으나 *P. rhodozyma*에 함유된 유효성분인 astaxanthin의 정량적인 분석 방법이 제시되지 못하였다. 본 연구에서는 astaxanthin에 대한 정확한 정성법과 정량분석법을 제안하였다. 이 방법은 HPLC의 역상 컬럼을 사용하여 dichloromethane 및 4개의 혼합 용매를 이동상으로 사용하는 것이다. 이 제시된 방법을 활용했을 때 *P. rhodozyma* 배양액 중의 astaxanthin의 함유량 을 정확하게 검출할 수 있다. DMSO 와 acetone의 혼합용매가 최적의 astaxanthin 추출용매이었고 또한 낮은 온도와 어두운 환경이 최적의 시료처리조건이었다.

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