Analysis and Identification of Astaxanthin and its Carotenoid Precursors from *Xanthophyllomyces dendrorhous* by High-Performance Liquid Chromatography

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This study presents an HPLC method for simultaneous analysis of astaxanthin and its carotenoid precursors from *Xanthophyllomyces dendrorhous*. The HPLC method is accomplished by employing a C_{18} column and the mobile phase methanol/water/acetonitrile/dichloromethane (70:4:13:13, v/v/v/v). Astaxanthin is quantified by detection at 480 nm. The carotenoid precursors are identified by LC-APCI-MS and UV-vis absorption spectra. Peaks showed in the HPLC chromatogram are identified as carotenoids in the monocyclic biosynthetic pathway or their derivatives. In the monocyclic carotenoid pathway, 3,3'-dihydroxy-β,ψ-carotene-4,4'-dione (DCD) is produced through γ-carotene and torulene.

Key words: Carotenoids, HPLC, *Xanthophyllomyces dendrorhous*

Introduction

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione), a particular carotenoid, is widely used as food colourant in aquaculture industries. Since the animals involved are unable to synthesize astaxanthin, this compound has to be supplemented to the feed to ensure proper pigmentation like their wild brethren (Tejera *et al.*, 2007). Moreover, there has recently been interest in the medical properties of astaxanthin. Many studies have demonstrated that astaxanthin may possess powerful antioxidant activity, which may be ten times stronger than that of other carotenoids such as β-carotene, zeaxanthin, and canthaxanthin (Nakano *et al.*, 1999). Therefore, astaxanthin exerts a protective effect against chronic diseases such as cancer and other degenerative diseases (Hussein *et al.*, 2006).

The red yeast *Xanthophyllomyces dendrorhous* (previously named *Phaffia rhodozyma*, a moderately psychrophilic yeast) has been regarded as one of the promising microbiological production systems for natural astaxanthin (Domínguez-Bocanegra *et al.*, 2007). In *X. dendrorhous*, astaxanthin represents about 85% of the total carotenoids content. Considerable research efforts have been devoted to improve the astaxanthin production. The production of astaxanthin in *X. dendrorhous* can be increased by classical muta-genesis (An *et al.*, 1989; Bon *et al.*, 1997). In our laboratory, *X. dendrorhous* strains were successively treated with UV radiation and mutagenic chemicals, and then screened for high-yield mutants. During that process, we isolated more than twenty mutants that produced more astaxanthin, but their colony's colour was obviously different, indicating different accumulation of carotenoids intermediates. In order to understand the changes in the carotenoids' synthesis pathway, it would be valuable to develop a reliable and applicable method to characterize the carotenoid profile of *X. dendrorhous*. Preferably, the method should be able to identify and quantify astaxanthin and other carotenoids simultaneously.

Over the years, high-performance liquid chromatography (HPLC) has been widely applied in carotenoids analysis using isocratic or gradient mobile phases in either normal-phase or reversed-phase mode (Su *et al.*, 2002). Rao *et al.* (2005) isolated and characterized some minor impurities of astaxanthin using a normal-phase silica gel column with *n*-hexane/acetone/tetrahydrofuran as mobile phase and identified related impurities by EMS and NMR spectroscopy. Lin *et al.* (2005) developed a reversed-phase HPLC method to analyse carotenoids in spear shrimp shells. Reversed-phase liquid chromatography with typical eluents, including methanol, acetonitrile, and water, was

used for separation of carotenoids in a variety of biological materials (Su *et al.*, 2002; Verdoes *et al.*, 2003). Weber *et al.* (2007) developed a reversed-phase HPLC method with acetone/water as solvent system to analyse and identify some carotenoids by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). However, it was often found that astaxanthin failed to yield a symmetrical peak, and the carotenoids peaks' resolution was poor. Abu-Lafi and Turujman (1999) have used a Pirkle (Regis, USA) covalent L-leucine column for rapid and direct resolution of the stereoisomers of all-*trans*-astaxanthin, but the reproducibility of this method was found to be poor when repeated with an identical L-leucine column. Subsequently, a reversed-phase HPLC method based on a C_{30} column was developed for separation of *cis/trans* isomers of different carotenoids (Su *et al.*, 2002; Dugo *et al.*, 2008). However, a C_{30} column is not suitable for routine analyses because of its high cost. So far, there are no reports on a method for simultaneous isolation and characterization of astaxanthin and carotenoids from *X. dendrorhous*.

The purpose of the present study was to develop and validate a method for simultaneous analysis of astaxanthin and its carotenoid precursors from *X. dendrorhous* fermentation using C_{18} reversed-phase HPLC.

Material and Methods

Strains and chemicals

X. dendrorhous strain AS 2.1557 was obtained from China General Microbiological Culture Collection Center (CGMCCC, Beijing, China). It was subjected to successive mutagenesis according to the method described by An *et al.* (1989). Mutants were maintained at –70 °C in yeast malt (YM) broth and 30% glycerol. In this work, an astaxanthin-overproducing strain was used.

The shake flask medium was prepared in our laboratory; it was composed of 50 g glucose, 4 g yeast extract, 3 g KH_2PO_4, 3 g $MgSO_4 \cdot 7H_2O$ in 1 L distilled water, pH was adjusted to 5.5 using 1.0 M HCl. For solid medium, 2% agar was added. The yeast was incubated in 500-mL flasks containing 60 mL medium. Incubation was on an orbital shaker (200 rpm) for 5 d at 20 °C.

All reagents were of analytical reagent grade unless stated otherwise. HPLC grade solvents, in-

cluding methanol, acetonitrile, and dichloromethane, were purchased from Merck, Germany. Silica gel G60 for thin-layer chromatography was obtained from Qingdao Haiyang chemical company, Qingdao City, China. Astaxanthin and *β*-carotene standard were purchased from Sigma-Aldrich, Shanghai, China. Ultra-pure water was prepared using a Milli-Q Academic A10 system (Millipore).

Sample preparation

Preparation of crude carotenoids from *X. dendrorhous* was carried out according to the literature (Weber *et al.*, 2007) with minor modifications. In brief, 40 mL of culture broth were used. Cells were harvested by centrifugation. The obtained pellet was frozen at –20 °C, suspended in 3–5 mL DMSO by vortexing, and incubated at room temperature for 5 h. Following centrifugation and collection of the DMSO phase, the obtained pellet was suspended in 10 mL acetone with a spatula and by vortexing, and centrifuged. The acetone phase was decanted, and the DMSO and acetone extraction procedure was repeated further twice. All coloured DMSO and acetone phases were pooled and transferred to an extraction funnel containing an equal volume of light petroleum (b.p. range 30–75 °C), 10 mL distilled water and 5 mL saturated NaCl. Carotenoids were extracted into the light petroleum phase by gentle rotation, avoiding excessive agitation. The coloured light petroleum phase was washed three times with an equal volume of distilled water. NaCl was added, if required, for phase separation. The light petroleum phase was dried over Na_2SO_4, and rotary-evaporated to dryness at 40 °C in dim light.

The crude extract was taken up in acetone to 4.0 mL, giving a 10-fold volumetric concentration relative to the culture broth. Freezing was done at –20 °C to precipitate the colourless lipids. The defatted crude extracts were filtered through a 0.45-*μ*m syringe filter, and 10-*μ*L aliquots were subjected to reversed-phase HPLC analysis.

HPLC analysis

Analytical HPLC was performed with a Dikma (Tian Jin, China) Diamonsil TM-C_{18} column (5 *μ*m; 250 mm × 4.6 mm) on a Waters 2695 series instrument equipped with an online diode-array detector. The mobile phase was filtered through a 0.45-*μ*m membrane degassed before use. Under
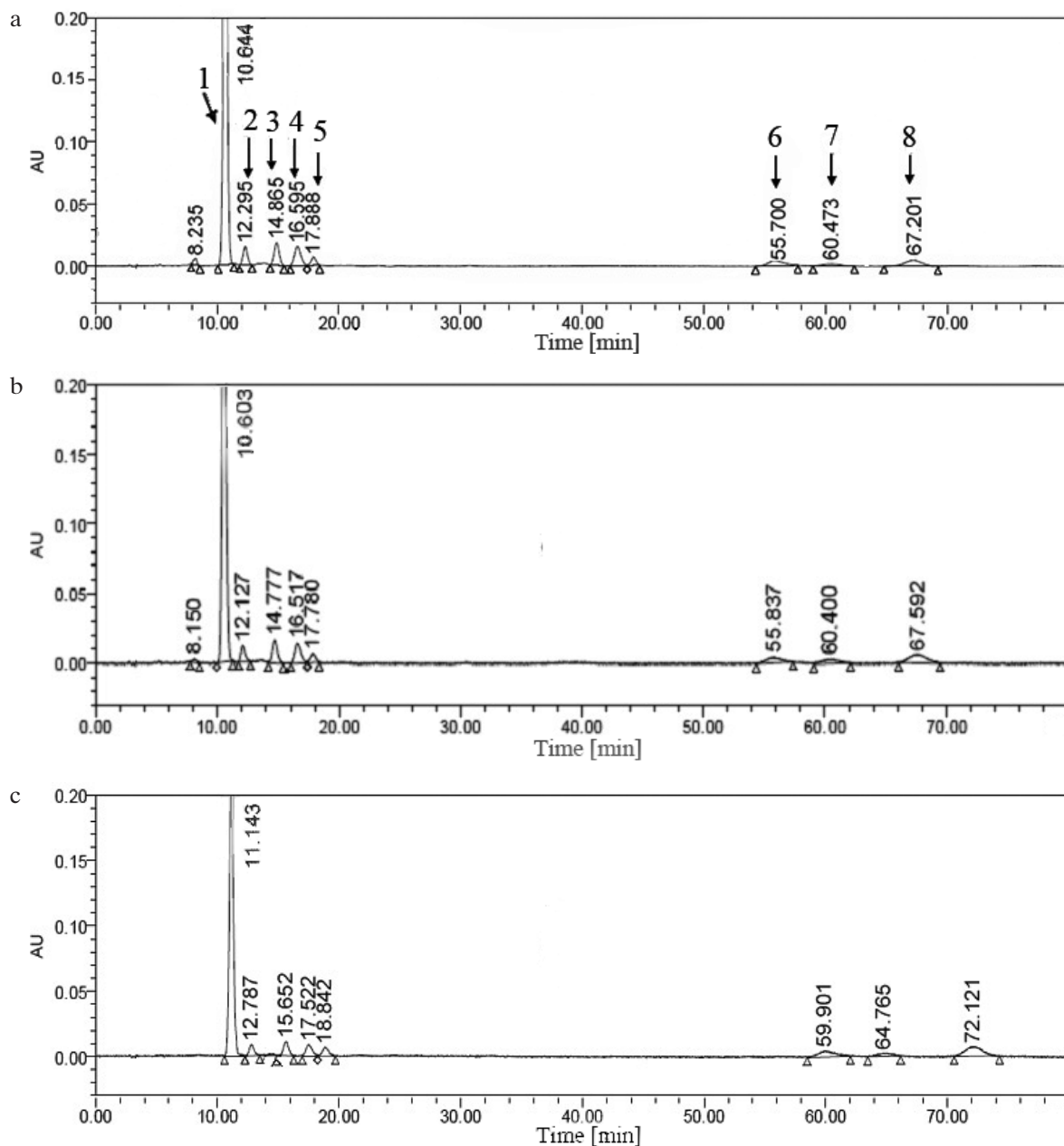
Fig. 1. HPLC chromatogram of carotenoid samples from *X. dendrorhous*. (a) Flow rate 1.0 mL/min, 20 °C, for peak identification see Table II; (b) flow rate 1.0 mL/min, 30 °C; (c) flow rate 0.8 mL/min.

isocratic conditions, the analysis was carried out at a flow rate of 1.0 mL/min at room temperature. Chromatograms were recorded at 480 nm, and UV-vis absorption spectra were recorded online with the photodiode-array detection system.

LC-APCI-MS analysis

In order to identify the main carotenoids appeared in the chromatogram, the mass spectra of carotenoids from *X. dendrorhous* yeast extracts

were recorded. After DMSO/acetone extraction, the concentrated oil product was firstly chromatographed on activated silica gel TLC plates (silica gel G60, 5 × 20 cm). The solvent system used for TLC was acetone/*n*-hexane (1:5, v/v). The bands were scraped off and eluted with acetone. This TLC purification procedure was not required for HPLC analysis, but greatly improved the signal-to-noise ratio of mass spectra. LC-APCI-MS analysis was preformed as described (Weber *et al.*, 2007). An Agilent LC-MSD-Trap-XCT instrument was operated at a fragmentor voltage of 140 V in the APCI-positive (PI) mode. The gas temperature was 350 °C, the vaporizer wasset to 400 °C, nitrogen was supplied as drying gas at 7 L/min, with a nebulizing pressure of 276 kPa. Spectra in the mass range between 100 and 800 were recorded. HPLC separation was carried out as described above.

Results

Developing an HPLC method for determination of astaxanthin and carotenoids

In order to develop a fine analysis method for astaxanthin and carotenoids, we choose reversed-phase HPLC using a C_{18} column. Various mobile phases in isocratic or gradient mode were compared. Preliminary mobile phase studies were carried out with three solvents, *viz.*, methanol, acetonitrile, and water in different compositions. The results showed that the peak resolution between astaxanthin and the other carotenoids was not satisfactory, and the astaxanthin peak was tailing. Then dichloromethane was added to the mobile phase; the quaternary mobile phase system provided better resolution than the previously used ternary solvent mixture. The most appropriate mobile phase was found to be composed of methanol/water/dichloromethane/acetonitrile (70:4:13:13, v/v/v/v). The resolution of peaks was improved. Most peaks were adequately resolved within 70 min. On the basis of the retention behaviour and absorption spectrum compared with the standards, peak 1 and peak 8 were identified as *trans*-astaxanthin and *β*-carotene, respectively. Astaxanthin showed a symmetric peak, and the resolution of the other carotenoids was excellent (Fig. 1a). The tolerance of the method was tested by adjusting the temperature and flow rate slightly. The separation between carotenoids was still fine (Figs. 1b, c).

Method validation

The results obtained showed good performance for astaxanthin analysis. So further studies on linearity and precision were performed.

The linearity of the standard curve was expressed in terms of the determination coefficient (R^2) from plots of the integrated peak area *vs.* concentration of the standard (in mg/L). A concentration range in concordance with the level of astaxanthin found in *X. dendrorhous* yeast extracts was tested. Results showed that the linearity from 50 mg/L to 250 mg/L was good (Fig. 2). Calibration lines were constructed, and showed linear correlation of $R^2 = 0.9978$, with a regression equation as follows: $y = 59463.50 + 58936.51x$.

The method precision, as repeatability, was evaluated on the basis of the relative standard deviation (RSD) of astaxanthin, with determination in 5 replicates of the same sample prepared from *X. dendrorhous* yeast. The results are shown in Table I. The RSD is 0.19% which is very good for routine analyses of astaxanthin.

Related carotenoids identification by LC-APCI-MS

Peak 1 and peak 8 were identified to be *trans*-astaxanthin and *β*-carotene, respectively, by spiking the sample with individual standards and comparing the retention times. The result was also verified by the LC-APCI mass spectrum.

The other peaks were identified by LC-APCI-MS, UV-vis absorption and chemical characteristics. The molecular ions and the mass fragments
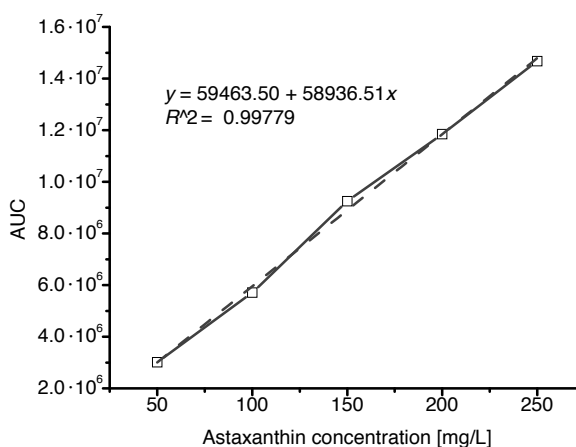
Fig. 2. Linear test of the HPLC method.

Table I. System precise test of the HPLC method.

Injection	RT [min]	AUC	RSD (%)
1	10.114	11273460	
2	10.140	11214719	
3	10.185	11238643	0.19
4	10.266	11227006	
5	10.279	11214389	

are listed in Table II. In the positive ion mode, the most prominent ion corresponded to $[M+H]^+$ in the mass spectrum of carotenoids (Weber *et al.*, 2007). The peaks 1, 2, 3, 4 and 5 had the fragments $[M+H–18]^+$ and $[M+H–36]^+$, which indicated that these carotenoids had two hydroxy groups (Ren and Zhang, 2008). The relative molecular weights of peak 3 and peak 4 were 596 according to *m/z* 597 $[M+H]^+$, which were the same as that of *trans*-astaxanthin. These two peaks were identified by their online PDA UV-vis absorption maxima. Peak 3 was identified as 3,3'-dihydroxy-β,ψ-carotene-4,4'-dione (DCD), which was first discovered by An *et al.* (1999) in the monocyclic carotenoid biosynthetic pathway. Peak 4 was identified as one or a mixture of astaxanthin isomers. Peak 5 showed the molecular weight of 594 and UV-vis spectra with absorption maxima at 461 and 429 nm. This absorption character was not found in the literature, but considering the existence of DCD in the system, we tentatively identified it as dehydro-DCD. Peak 6 and peak 7 were identified by their

molecular weights and UV-vis spectra as torulene and γ-carotene, respectively.

Discussion

An earlier work using C_{18} reversed-phase HPLC to analyse carotenoids from yeasts has been reported in the literature. Weber *et al.* (2007) developed a C_{18} reversed-phase HPLC method by means of simple water/acetone gradient elution. The method has been applied successfully in the analysis of astaxanthin from *X. dendrorhous* within less than 20 min, however, the separation of other carotenoids peaks was not satisfactory. Our method described here, in contrast, permits an efficient chromatographic separation of typical carotenoids from *X. dendrorhous*.

For this purpose, an HPLC method based on a C_{30} column was developed; it was more effective in separation of structurally related carotenoids than the C_{18} column. However, several drawbacks occur with this method, such as the high cost of a C_{30} column and the longer running time. Therefore, this method was usually only used for the study of carotenoids from botanical materials, because the composition of carotenoids in these materials is generally complicated (Su *et al.*, 2002). But most microorganisms produce only a limited number of carotenoids. Our results showed that a C_{18} column with an optimized mobile phase would enable to achieve enough resolving power in the analysis of microbial samples.

Table II. Peak identification results.

Peak no.	Name	RRT	λ_{max} [nm]	λ_{max} [nm] (reported)	APCI-PI (*m/z*)
1	*trans*-Astaxanthin	1.0	478	478 (Weber *et al.*, 2007)	597.3 $[M+H]^+$, 579.3 $[M+H–H_2O]^+$, 561.4 $[M+H–2H_2O]^+$
2	Dehydro-astaxanthin	1.15	469	470 (Rao *et al.*, 2005)	595.4 $[M+H]^+$, 577.3 $[M+H–H_2O]^+$, 559.4 $[M+H–2H_2O]^+$
3	DCD	1.39	461, 489, 524	461, 490, 523 (An *et al.*, 1999)	597.5 $[M+H]^+$, 579.4 $[M+H–H_2O]^+$, 561.4 $[M+H–2H_2O]^+$
4	*cis*-Astaxanthin	1.56	367, 469	371, 470 (Yuan and Chen, 1997)	597.4 $[M+H]^+$, 579.3 $[M+H–H_2O]^+$, 561.4 $[M+H–2H_2O]^+$
5	Dehydro-DCD	1.68	461, 429	NA	595.4 $[M+H]^+$, 577.4 $[M+H–H_2O]^+$, 559.4 $[M+H–2H_2O]^+$
6	Torulene	5.23	464, 483, 515	460, 485, 518 (Weber *et al.*, 2007)	534.2 $[M+H]^+$
7	γ-Carotene	5.68	434, 463, 495	430, 460, 489 (Weber *et al.*, 2007)	537.4 $[M+H]^+$
8	β-Carotene	6.31	430, 452, 481	425, 452, 478 (Weber *et al.*, 2007)	537.4 $[M+H]^+$

NA, not available in the literature.

There are two carotenoids biosynthetic pathways postulated in *X. dendrorhous*, namely the monocyclic pathway and the dicyclic pathway (An *et al.*, 1999). According to our result, most identified carotenoids were compounds of the monocyclic biosynthetic pathway or their derivatives. It is presumed that the monocyclic biosynthetic pathway may be the dominating biosynthetic pathway in this astaxanthin-overproducing *X. dendrorhous* mutant.

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