JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isomerization of *trans*-Astaxanthin Induced by Copper(II) Ion in Ethanol

LIYAN ZHAO, FANG CHEN, GUANGHUA ZHAO,* ZHENGFU WANG, XIAOJUN LIAO, AND XIAOSONG HU

College of Food Science & Nutritional Engineering, China Agricultural University, Beijing 100083, China

Carotenoids are unstable and susceptible to disruption by environmental factors such as heat, light, and solvents. However, there is little information on the effect of metal ions on stability of carotenoids, especially those essential elements in human nutrition. Astaxanthin is one of the few carotenoids containing four oxygen donors. Usually, these oxygen donors can coordinate with heavy metal ions such as Cu(II) and Fe(III). In the present study, the interaction of *trans*-astaxanthin with Cu(II) was examined. It was found that Cu(II) markedly induces the conversion of *trans*-astaxanthin to its cis forms, which mainly consist of 9-*cis*-astaxanthin and 13-*cis*-astaxanthin as suggested by UV−visible spectra and HPLC measurements. Increasing either incubation time of Cu(II) and *trans*-astaxanthin in ethanol or the Cu(II)/astaxanthin ratio results in an increased percentage of cis isomers derived from *trans*-astaxanthin. All these results provide important information on the effects of dietary factors on the bioavailability and bioactivity of *trans*-astaxanthin.

KEYWORDS: Cu(II); astaxanthin; isomerization; carotenoids

INTRODUCTION

Carotenoids have received considerable attention because of their potential clinical use in treatment for cancer associated with reactive oxidative species (*1*). Anti-cancer activity of these carotenoids seems to be independent of pro-vitamin A activity (*1*). Of these, astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) occurs in certain marine animals and plants (salmon, trout, shrimp, lobster, and algae) (*2*−*5*). Although it lacks the pro-vitamin A activity, astaxanthin has been found to be more effective than β-carotene in preventing lipid peroxidation in solution and various biomembrane systems (*6*) and is more potent in the prevention of carcinogen-induced neoplastic transformation in 10T1/2 cells than any of the previously studied carotenoids (*7*). Recent studies suggest that astaxanthin exhibits about 100−500 times higher antioxidant activity than α-tocopherol (*8*−*10*). Due to its attractive pink color, it has been used as a colorant in aquaculture. Moreover, increasing studies on food, cosmetic, and medical application of astaxanthin have been made because of its high antioxidant activity (*10*, *11*) and other biological functions (*12*−*14*).

Cis isomers of carotenoids may be naturally formed in certain organisms (*15*, *16*); however, *trans*-astaxanthin is the biologically favored form, possibly because *cis*-astaxanthin is not utilized to the same extent as *trans*-astaxanthin (*17*). In contrast to *cis*-astaxanthin, *trans*-astaxanthin is unstable and readily isomerized to its cis analogues, especially the 9-cis and 13-cis unhindered isomers (**Figure 1**) by environmental factors such as heat, light, and oxygen (*15*, *16*). For example, high temperature markedly promotes the isomerization of *trans*-astaxanthin (*18*, *19*). Effects of different organic solvents on the isomerization of *trans*-astaxanthin in solutions have been investigated and the results showed that *trans*-astaxanthin dissolved in organic solvent also easily isomerized to its cis analogues, mainly 13-*cis*-astaxanthin (*19*, *20*). Moreover, iodine can be used as a catalyst to promote the isomerization of *trans*-astaxanthin (*17*) and its diacetate (*21*). However, to our knowledge, there is little information about the effect of metal ions on the isomerization of astaxanthin.

Trace amounts of certain metal elements such as copper, iron, and zinc are nutritionally required for organisms, including humans, in their diets. These metal ions serve a variety of functions in vitro, the most important of which are to enhance the structural stability of the protein in the conformation necessary for biological function and/or to take part in the catalytic processes of enzymes (*22*). Some of these metal ions such as Cu(II) and Fe(III) have the ability to coordinate with oxygen atom donors. Astaxanthin is one of few carotenoids that contains four oxygen atoms: two from hydroxyl groups and two from carbonyl groups. This raises the question of whether nutritionally essential metal ions can interact with astaxanthin and, if so, how this interaction influences on the isomerization of astaxanthin. To answer these questions, Cu(II) was chosen to determine its interaction with *trans*-astaxanthin in ethanol.

MATERIALS AND METHODS

Chemicals. HPLC-grade methanol, acetonitrile, ethanol, dichloromethane, and chloroform were purchased from Kangkede Chemical

* Corresponding author: tel +86-10-62737434; fax +86-10-62737434; e-mail gzhao318@yahoo.com.cn.

10.1021/jf0517750 CCC: $30.25
Published on Web 10/29/2005

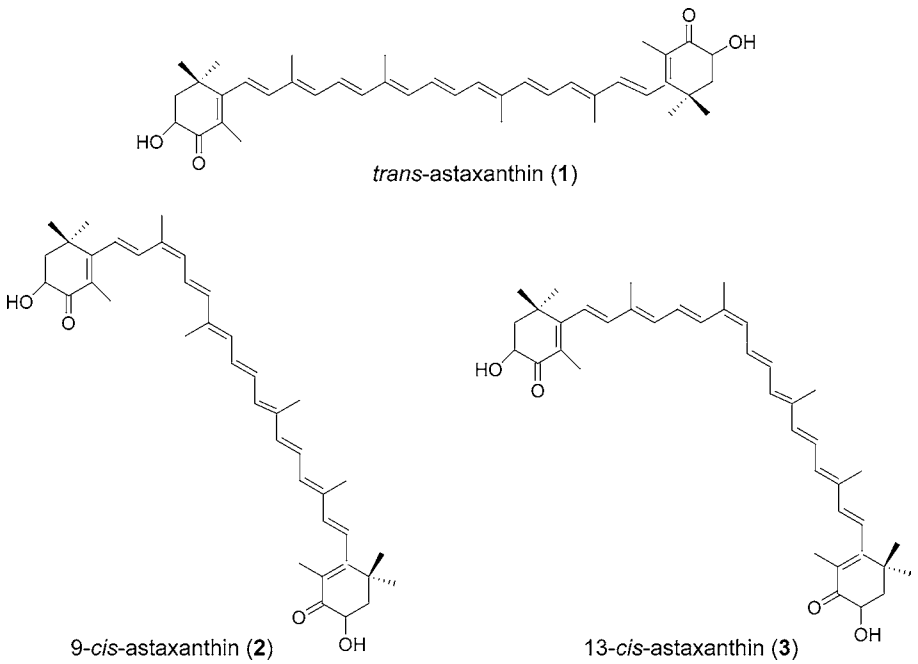

Figure 1. Structures of *trans*-astaxanthin (**1**), 9-*cis*-astaxanthin (**2**), and 13-*cis*-astaxanthin (**3**).

Co. (Tianjin, China). *trans*-Astaxanthin was purchased from Sigma Chemical Co. (St. Louis, MO).

Measurement of UV/Vis Spectra. The reaction of $CuCl_2$ with *trans*-astaxanthin (4.53 μM) was carried out in ethanol at 4 °C. To examine the effect of Cu(II) on the isomerization of *trans*-astaxanthin, a series of reaction mixtures with different molar ratio of Cu(II) to astaxanthin (4/1, 8/1, 16/1, 32/1, 64/1, and 128/1) were kept for 0.5, 1, 2, 4, 8, and 12 h. Then the mixtures were scanned by a model UV-190 UV/vis spectrophotometer (Beijing Puxi Universal Instrument Co.) from 300 to 700 nm. The concentration of astaxanthin used was 4.53 μM, based on its molar absorptivity, so that its absorbance at 480 nm changes between 0.2 and 0.8 upon interaction with Cu(II), the range that is the most sensitive for UV/vis spectrophotometry.

Determination of *trans*-Astaxanthin Concentration. A stock solution (635.3 μM) was made by dissolving 37.92 mg of *trans*-astaxanthin in 100 mL of chloroform. A series of dilutions was prepared: 1.588, 3.176, 6.353, 12.706, 19.06, 25.413, and 31.766 μM with ethanol. These solutions were quantitatively analyzed by a LC-10AT HPLC (Shimadzu, Kyoto, Japan) equipped with a RID-10A photodiode array detector (Shimadzu, Kyoto, Japan) at 480 nm. On the basis of a standard curve of *trans*-astaxanthin concentration versus HPLC peak area, the concentrations of the *trans*-astaxanthin in the mixtures were determined by HPLC at the same condition as standard samples after the *trans*-astaxanthin was mixed with $CuCl_2$ at different incubation times (0−12 h) or molar ratios of Cu(II)/pigment (4/1−128/1). The concentration of *trans*-astaxanthin between 1.588 and 31.766 μM exhibits good linear dependence on the peak area (R^2 = 0.9999). The detection limit is 0.0168 μM. The chromatography conditions were 250 × 4.6 mm i.d. Kromasil C18 column (Eka Chemicals, Bohus, Sweden), a mixture of dichloromethane, methanol, acetonitrile, and water (5:85:5:5) as mobile phase; flow rate (1 mL/min), detection wavelength (480 nm); and column temperature 25 °C.

Separation and Identification of Isomers of Astaxanthin. The isomers of astaxanthin were separated under the same conditions as above. The UV/vis spectra of all peaks were recorded by the photodiode array detector from 190 to 800 nm. Isomers of astaxanthin were assigned according to their retention times and spectra (*17*, *19*, *20*, *23*).

RESULTS AND DISCUSSION

Interaction of Cu(II) with *trans*-Astaxanthin. Since some organic solvents have been reported to induce the conversion of *trans*-astaxanthin to its cis forms and ethanol is used as solvent during the interaction of Cu(II) with *trans*-astaxanthin (*19*, *20*), the effect of ethanol on isomerization of *trans*-astaxanthin was examined. It was found that the UV/vis spectrum of *trans*-astaxanthin (4.53 μM) was unchanged during its 12 h incubation with ethanol (data not shown), suggesting that *trans*-astaxanthin is stable in ethanol within 12 h. In contrast, marked changes were observed in the UV spectrum of *trans*-astaxanthin (**Figure 2A**) after *trans*-astaxanthin (4.53 μM) was treated with a 16/1 molar ratio of Cu(II)/astaxanthin for different times. As the time of the interaction of Cu(II) with *trans*-astaxanthin increased from 0.5 to 12 h, the maximum absorbance

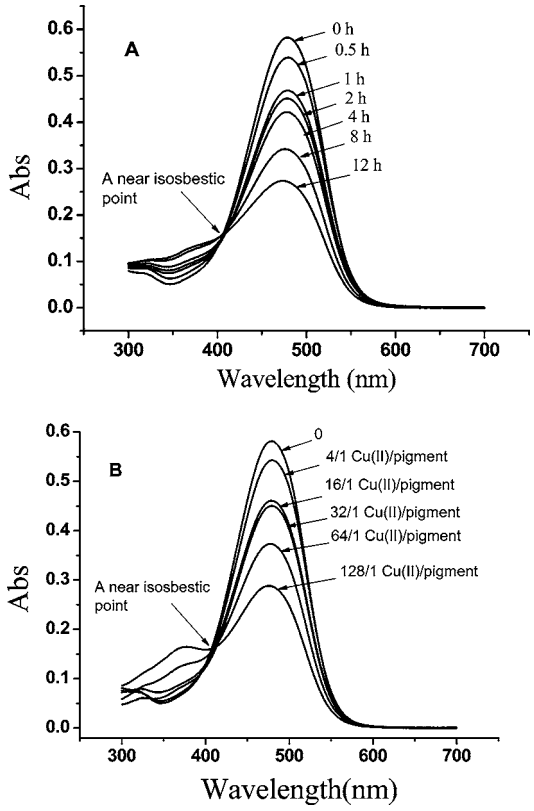

Figure 2. UV spectra from 300 to 700 nm of the reaction mixture of $CuCl_2$ and *trans*-astaxanthin as a function of reaction time (**A**) or of the molar ratio of Cu(II)/*trans*-astaxanthin (**B**).

at 480 nm, characteristic of *trans*-astaxanthin, significantly decreased while the absorbance at 373 nm, which is characteristic of cis isomers of astaxanthin (*17*, *19*, *20*, *23*), gradually increased, resulting in a near-isosbestic point at ~410 nm as shown in **Figure 2A**. The lack of a pure isosbestic point suggests that *trans*-astaxanthin is replaced by more than one product. When the interaction time was kept constant at 0.5 h, the UV/vis spectra were also recorded as a function of the molar ratio of Cu(II) to *trans*-astaxanthin (**Figure 2B**). It was observed that as the ratio of Cu(II) to *trans*-astaxanthin increased, the absorbance at 480 nm decreased whereas the absorbance at 373 nm increased, again producing a near-isosbestic point at ~410 nm. Thus, both results strongly indicate that Cu(II) can induce the conversion of *trans*-astaxanthin to its cis forms. Although the exact mechanism remains to be determined, the coordination of Cu(II) with the *trans*-astaxanthin may trigger the isomerization. In support of this proposal comes from the structures of *trans*-astaxanthin and its 9-cis and 13-cis forms. As shown in **Figure 1**, four oxygen donors are at the same side of the two cis isomers while they are symmetrically distributed at two sides of *trans*-astaxanthin. Since the four oxygen donors of the two cis isomers are in a more compact array with respect to those of *trans*-astaxanthin, complexes from the coordination of the cis forms with Cu(II), respectively, are more stable than that from *trans*-astaxanthin with Cu(II). Such a different stability possibly results in the isomerization of *trans*-astaxanthin to its cis analogues in the presence of Cu(II).

Change of *trans*-Astaxanthin Concentration upon Interaction with Cu(II). The absorbance at 480 nm of *trans*-astaxanthin decreased as both the interaction time and the Cu(II)/pigment molar ratio increase (**Figure 2**), implying that the concentration of *trans*-astaxanthin decreases with increasing interaction time or molar ratio. To confirm this conclusion, the concentration of *trans*-astaxanthin as a function of the reaction time or the Cu(II)/pigment molar ratio was determined by HPLC, and the results are shown in **Figure 3**. It was observed that the *trans*-astaxanthin concentration decreased in two phases when the reaction time of Cu(II) with *trans*-astaxanthin increased while the Cu(II)/ pigment molar ratio is fixed at 16/1. The original concentration of *trans*-astaxanthin was 11.309 μM. As the time of its interaction with Cu^{2+} increased, the *trans*-astaxanthin concentration rapidly decreased by ~30%, corresponding to the first fast phase; then it continued to decrease by ~15%, representing the second slow phase (**Figure 3A**). Thus after 12 h of incubation with Cu(II), the concentration of *trans*-astaxanthin decreased to 6.26 μM, a result in accord with the above UV measurements (**Figure 2A**). In contrast to Cu(II), organic solvents exhibited a slower isomerization induction of *trans*-astaxanthin and only one phase was observed after 12 h of incubation of the *trans*-astaxanthin with different organic solvents at 20 °C or higher (*19*, *20*), suggesting that Cu(II) is more effective in inducing the isomerization of *trans*-astaxanthin.

Likewise, a similar profile of the decrease of *trans*-astaxanthin concentration was obtained with increasing ratio of Cu(II) to *trans*-astaxanthin (**Figure 3B**). The concentration of the *trans*-astaxanthin decreased from 11.31 to 4.189 μM upon addition of Cu(II) to *trans*-astaxanthin solution with a ratio of 128/1, in agreement with the UV measurements (**Figure 2B**).

Identification of Products Isomerized from *trans*-Astaxanthin. To determine how many species were generated from isomerization of *trans*-astaxanthin by Cu^{2+}, 16/1 and 128/1 molar ratio Cu(II)/pigment samples with standard *trans*-astaxanthin as a control were analyzed by HPLC after a 30 min incubation, and their corresponding chromatograms are displayed in **Figure 4**. As expected, only one peak appeared at 6.2 min in the HPLC chromatogram of the control sample; this corresponds to *trans*-astaxanthin (**Figure 4A**). This assignment was further confirmed by its UV spectrum (**Figure 5A**) exhibiting a maximum absorbance at 480 nm (*18*−*20*, *24*). In contrast, there were three peaks appearing at 6.2, 9.5, and 10.4 min for the 16/1 molar ratio Cu(II)/pigment sample (**Figure 4B**). Besides the three peaks, the fourth peak appeared next to peak 1 for the 128/1 molar ratio Cu(II)/pigment sample (**Figure 4C**). With increasing ratio of Cu(II) to *trans*-astaxanthin from

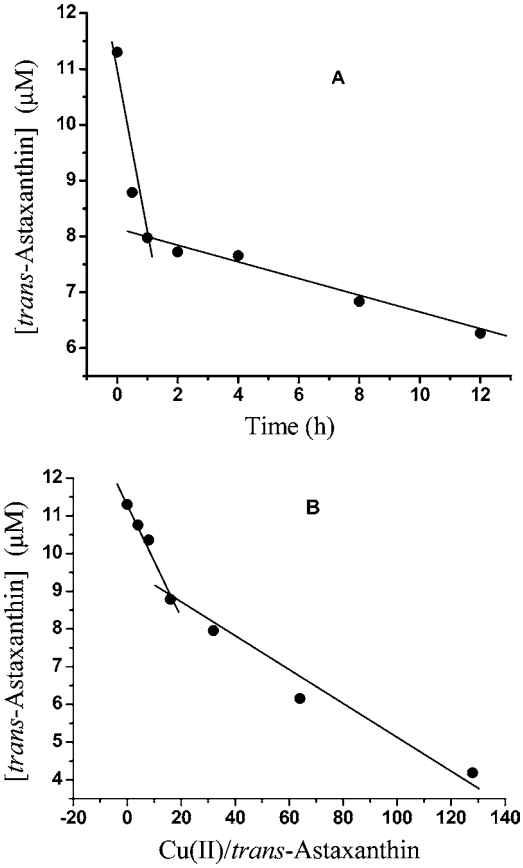

Figure 3. *trans*-Astaxanthin concentration versus either reaction time (**A**) or molar ratio of Cu(II)/*trans*-astaxanthin (**B**).

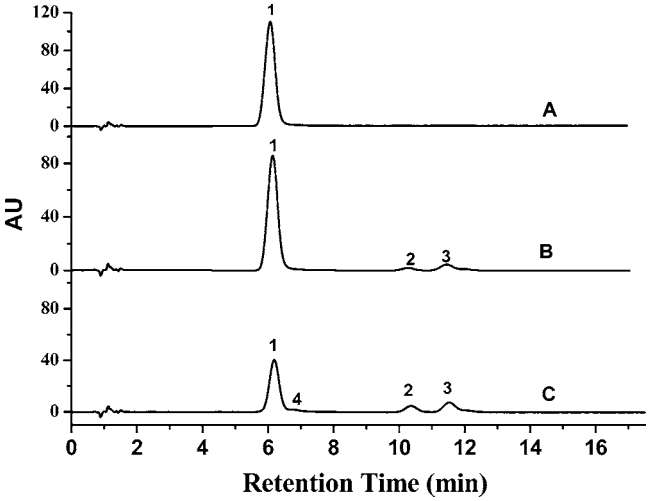

Figure 4. Chromatogram of *trans*-astaxanthin and the mixture of $CuCl_2$ with astaxanthin: (**A**) *trans*-astaxanthin; (**B**) 16/1 Cu(II)/*trans*-astaxanthin; (**C**) 128/1 Cu(II)/*trans*-astaxanthin.

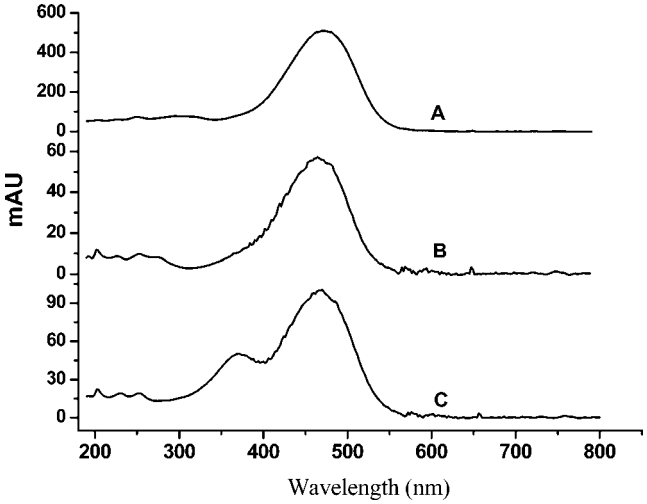

Figure 5. UV/Vis spectra of peaks of chromatogram of *trans*-astaxanthin and the mixture of $CuCl_2$ with astaxanthin: (**A**) peak 1 of **Figure 4A**; (**B**) peak 2 of **Figure 4C**; (**C**) peak 3 of **Figure 4C**.

0 to 16/1 to 128/1, the intensity of peak 1 significantly decreased, while the intensities of peaks 2 and 3 increased gradually, a result directly suggesting that two new species corresponding to peaks 2 and 3 are derived from *trans*-astaxanthin. This observation coincided with UV measurements showing the appearance of a near-isosbestic point at 410 nm upon addition of Cu(II) to *trans*-astaxanthin at different times or with different Cu(II)/pigment ratios (**Figure 2**). To identify the two species appearing at 9.5 (peak 2) and 10.4 min (peak 3), their UV−visible spectra are obtained by photodiode array detection and are displayed in **Figure 5B,C**. The assignments of the two species generated from *trans*-astaxanthin are based on previous work on astaxanthin (*18*−*20*, *24*). The UV/vis spectrum of peak 2 had a maximum absorbance at ∼470 nm and is assigned to 9-*cis*-astaxanthin. In contrast to peaks 1 and 2, there were two maximum absorbances appearing at ∼470 and ∼373 nm in the spectrum of peak 3, which is identified as 13-*cis*-astaxanthin. The overlap of peak 4 with peak 1 excludes assignment of peak 4. In addition, it is worth noting that peak 3 is larger than peak 2 in **Figure 4A,C**, a finding indicating that *trans*-astaxanthin is converted to 13-*cis*-astaxanthin more than 9-*cis*-astaxanthin, agreeing with recent observations showing that 13-*cis*-astaxanthin was the main cis isomer produced from *trans*-astaxanthin through isomerization induced by all tested organic solvents (*19*).

The present studies demonstrate that Cu(II) significantly induces the conversion of *trans*-astaxanthin to its analogues in which 9-*cis*-astaxanthin and 13-*cis*-astaxanthin are the major cis isomers. The isomerization increases proportionately with increasing Cu(II)/pigment ratio or interaction time.

ACKNOWLEDGMENT

We thank Chris Janus-Chandler for her corrections to the manuscript.

LITERATURE CITED

(1) Britton, G. Structure and properties of carotenoids in relation to function. *FASEB J.* **1995**, *9*, 1551−1558.

(2) Yokoyama, A.; Miki, W. Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. *FEMS Microbiol. Lett.* **1995**, *128*, 139−144.

(3) Fraser, P. D.; Miura, Y.; Misawa, N. *In vitro* characterization of astaxanthin biosynthetic enzymes. *J. Biol. Chem.* **1997**, *272*, 6128−6135.

(4) Osterlie, M.; Bjerkeng, B.; Liaaen-Jensen, S. Accumulation of astaxanthin all-*E*, 9*Z* and 13*Z* geometrical isomers and 3 and 3′ *RS* optical isomers in rainbow trout (*Oncorhynchus mykiss*) is selective. *J. Nutr.* **1999**, *129*, 391−398.

(5) Hagen, C.; Braune, W.; Birckner, E.; Nuske, J. Functional aspects of secondary carotenoids production of *Haematococcus pluvialis* (Girod) Rostafiniski (Volvocales). *New Phytol.* **1993**, *125*, 625−633.

(6) Goto, S.; Kogure, K.; Abe, K.; Kimata, Y.; Kitahama, K.; Yamashita, E.; Terada, H. Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochim. Biophys. Acta* **2001**, *1512*, 251−258.

(7) Hix, L. M.; Lockwood, S. F.; Bertram, J. S. Upregulation of connexin 43 protein expression and increased gap junctional communication by water soluble disodium disuccinate astaxanthin derivatives. *Cancer Lett.* **2004**, *211*, 25−37.

(8) Miki, W. Biological functions and activities of animal carotenoids. *Pure Appl. Chem.* **1991**, *63*, 141−146.

(9) Shinidzu, N.; Goto, W.; Miki, W. Carotenoids as singlet oxygen quenchers in marine organisms. *Fish. Sci.* **1996**, *61*, 134−137.

(10) Naguib, Y. M. A. Antioxidant activity of astaxanthin and related carotenoids. *J. Agric. Food Chem.* **2000**, *48*, 1150−1154.

(11) Terao, J. Antioxidant activity of β-carotene-related carotenoids in solution. *Lipids* **1989**, *24*, 659−661.

(12) Chew, B. P.; Park, J. S.; Wong, M. W.; Wong, T. S. A comparison of the anticancer activities of dietary β-carotene, canthaxanthin and astaxanthin in mice *in vivo*. *Anticancer Res.* **1999**, *19*, 1849−1853.

(13) Jyonouchi, H.; Sun, S.; Iijima, K.; Gross, M. D. Antitumor activity of astaxanthin and its mode of action. *Nutr. Cancer* **2000**, *36*, 59−65.

(14) Jacobsson, L. S.; Yuan, X. M.; Ziedén, B. Olsson, A. G. Effects of α-tocopherol and astaxanthin on LDL oxidation and atherosclerosis in WHHL rabbits. *Arteriosclerosis* **2004**, *173*, 231−237.

(15) Qackenbush, F. W. Reverse phase HPLC separation of *cis*-and *trans*-carotenoids and its application to β-carotenoids in food materials. *J. Liq. Chromatogr.* **1987**, *10*, 643−653.

(16) Johnson, E. A.; An, G. H. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.* **1991**, *11*, 297−326.

(17) Bjerkeng, B.; Folling, M.; Alsted, N., Lagocki, S.; Storebakken, T.; Olli, J. J.; Alsted, N. Bioavailability of all-*E*-astaxanthin and *Z*-isomers of astaxanthin in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **1997**, *157*, 63−82.

(18) Nyambaka, H.; Ryley, J. An isocratic reversed-phase HPLC separation of the stereoisomers of the provitamin A carotenoids (α- and β-carotene) in dark green vegetables. *Food Chem.* **1996**, *55*, 63−72.

(19) Yuan, J. P.; Chen, F. Isomerization of *trans*-astaxanthin to *cis*-isomers in organic solvents. *J. Agric. Food Chem.* **1999**, *47*, 3656−3660.

(20) Yuan, J. P.; Chen, F. Kinetics for the reversible isomerization reaction of *trans*-astaxanthin. *Food Chem.* **2001**, *73*, 131−137.

(21) Englert, G.; Vecchi, M. *trans*/*cis* Isomerization of astaxanthin diacetate. Isolation by high-performance liquid chromatography and identification by ^{1}H NMR spectroscopy of three mono-*cis*- and six di-*cis*-isomers. *Helv. Chim. Acta* **1980**, *63*, 1711−1718.

(22) Glusker, J. P. Structural aspects of metal liganding to functional groups in proteins. *Adv. Protein Chem.* **1991**, *42*, 1−76.

(23) Yuan, J. P.; Chen, F. Identification of astaxanthin isomers in *Haematococcus lacustris* by HPLC-photodiode array detection. *Biotechnol. Tech.* **1997**, *11*, 455−459.

(24) Saleh, M. H.; Tan, B. Separation and identification of *cis*/*trans* carotenoid isomers. *J. Agric. Food Chem.* **1991**, *39*, 1438−1443.

Received for review July 23, 2005. Revised manuscript received September 25, 2005. Accepted September 27, 2005. The project (2002AA248011) was supported by the National High-Tech Research and Development Plan of China.

JF0517750