Isomerization of *trans*-Astaxanthin to *cis*-Isomers in Organic Solvents

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The isomerization of *trans*-astaxanthin to *cis*-isomers in organic solvents was investigated. *trans*-Astaxanthin was dissolved in dimethyl sulfoxide, dichloromethane, chloroform, acetone, methanol, acetonitrile, and a mixture of dichloromethane and methanol (25:75) respectively, and heated at 35 °C followed by analyzing *cis*- and *trans*-astaxanthins in the solutions using HPLC. The isomerization rates of *trans*-astaxanthin were dependent on the solvent, and the following order was found: dichloromethane > chloroform > the mixture of dichloromethane and methanol (25:75) > methanol > acetonitrile > acetone > dimethyl sulfoxide. In different solvents, the relative contents of 9-*cis*- and 13-*cis*-astaxanthins formed during isomerization were different. In all solvents, 13-*cis*-isomer was the main *cis*-isomer from *trans*-astaxanthin. *trans*-Astaxanthin dissolved in dichloromethane or chloroform was very readily isomerized to *cis*-isomers, especially for dichloromethane, in which a maximum isomerization percentage was found and an equilibrium practically was reached after an appropriate time interval. Results also indicated that a higher temperature could promote markedly the isomerization of *trans*-astaxanthin.

Keywords: *Astaxanthin; cis−trans isomerization; organic solvent*

INTRODUCTION

Astaxanthin (3,3′-dihydroxy-β,β'-carotene-4,4′-dione) is a ketocarotenoid oxidized from β-carotene through echinenone, canthaxanthin, and adonirubin (Grung et al., 1992; Fan et al., 1995; Yokoyama and Miki, 1995; Fraser et al., 1997; Harker and Hirschberg, 1997; Yuan et al., 1997). Astaxanthin has an attractive pink color, antioxidative activity (Savore et al., 1995), and biological functions as a vitamin A precursor (Gobantes et al., 1998) and is therefore of interest both as a naturally food colorant and in medicine (Johnson and An, 1991). Recent studies have demonstrated that astaxanthin has possible immunomodulating activities (Okai and Higashi-Okai, 1996), and preventive effects against aflatoxin B_1 carcinogenicity (Gradelet et al., 1997). Astaxanthin has been permitted for use in the aquacultural industry by the Food and Drug Administration of the United States (Turujman et al., 1997).

All-trans natural astaxanthin is readily isomerized to *cis−trans* mixtures, especially the 9-*cis* and 13-*cis* unhindered isomers (Figure 1) for steric reasons (Quackenbush, 1987; Johnson and An, 1991). Increased temperature, exposure to light, or the presence of acids could cause the formation of *cis*-isomers. *cis*-Isomers of carotenoids might be naturally formed in certain organisms (Johnson and An, 1991). In the alga *Haematococcus pluvialis*, although astaxanthin exists mainly as *trans*-astaxanthin esters of various fatty acids, *cis*-astaxanthin esters were also detected in the algal pigment extracts (Yuan and Chen, 1997, 1998). The saponified extract solution from *H. pluvialis* contained *cis*-isomers rather than *trans*-isomers (Yuan and Chen, 1999). The free *cis*-astaxanthin might come from the hydrolysis of *cis*-astaxanthin esters or the isomerization of *trans*-astaxanthin. *cis*-Isomers of provitamin A carotenoids might have lower provitamin A activity (Chandler and Schwartz, 1987; Nyambaka and Ryley, 1996), which represented ∼50% or less of that of the corresponding *all-trans* carotenoids (Lessin et al., 1997).

To minimize the isomerization of *trans*-astaxanthin to form *cis*-isomers during the extraction of pigments, the saponification of *trans*-astaxanthin esters, the purification of *trans*-astaxanthin, and the preparation of *trans*-astaxanthin standard solution, it is imperative to investigate the isomerization of *trans*-astaxanthin in organic solvents. A reversed phase high-performance liquid chromatography (HPLC) method for the separation of *cis*/*trans*-isomers of astaxanthin has been achieved (Yuan and Chen, 1997, 1998) and may be used to study the isomerization of *trans*-astaxanthin. The major objective of the present work is to monitor the process of isomerization of *trans*-astaxanthin in various kinds of organic solvents by HPLC analysis and to study the reaction kinetics.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. HPLC grade methanol, acetonitrile, dichloromethane, and acetone were obtained from BDH Laboratory Supplies (Poole, U.K.). *trans*-Astaxanthin and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, MO). Chloroform was obtained from E. Merck KGaA (Darmstadt, Germany).

Isomerization of *trans*-Astaxanthin in Organic Solvents. *trans*-Astaxanthin was dissolved in dimethyl sulfoxide, dichloromethane, chloroform, acetone, methanol, acetonitrile, and a mixture of dichloromethane and methanol (25:75), respectively. The isomerization of *trans*-astaxanthin was carried out in darkness at 25, 35, or 50 °C. The reaction mixtures were sampled and analyzed by HPLC for monitoring the progress of isomerization during storage.

HPLC. HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode

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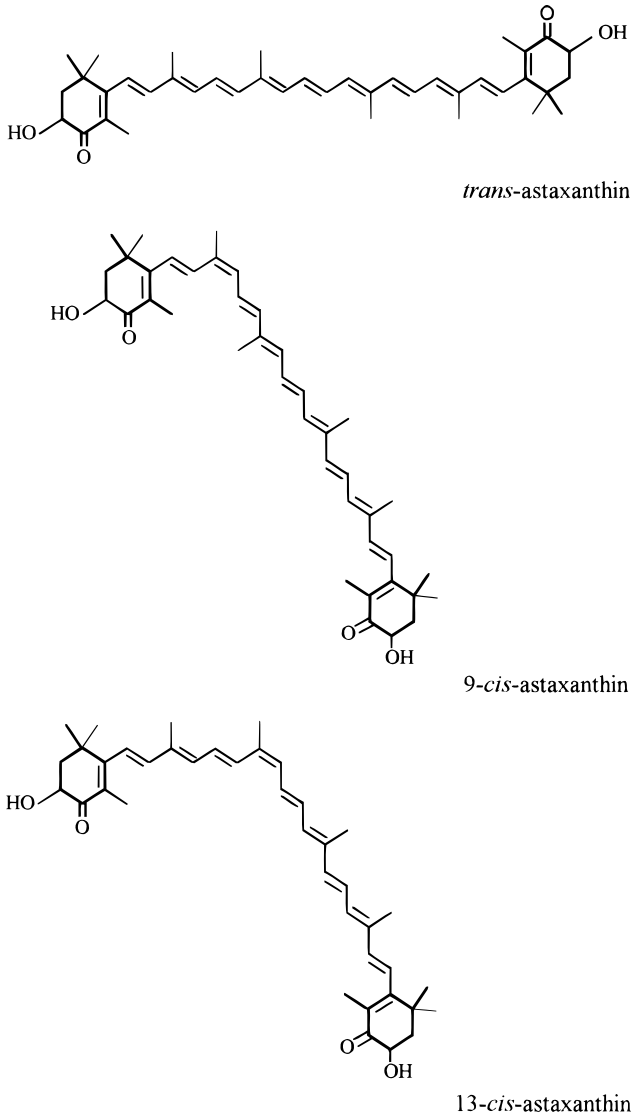

Figure 1. Structures of *trans*-astaxanthin, 9-*cis*-astaxanthin, and 13-*cis*-astaxanthin.

array detector. The isomers of astaxanthin were separated and analyzed (20 μL aliquots) by using a Beckman Ultrasphere C_{18} column (250 × 4.6 mm; 5 μm) at 25 °C. The mobile phase consisted of methanol (85%), dichloromethane (5%), acetonitrile (5.5%), and water (4.5%). The flow rate was set at 1.0 mL/min. The tridimensional chromatogram was recorded from 250 to 700 nm. Peaks were measured at a wavelength of 480 nm. The isomers of astaxanthin were identified according to their retention times and spectra by photodiode array detection (Yuan and Chen, 1997, 1998).

RESULTS AND DISCUSSION

Isomerization of *trans*-Astaxanthin. The reduction in *trans*-astaxanthin dissolved in organic solvents was reflected by the increase in the amount of 9-*cis*-astaxanthin and 13-*cis*-astaxanthin, indicating that *trans*-astaxanthin could easily isomerize to its *cis*-isomers, 9-*cis*-astaxanthin and 13-*cis*-astaxanthin (Johnson and An, 1991; Yuan and Chen, 1997). Figure 2 shows typical chromatograms of *cis*−*trans* mixtures of astaxanthin after the *trans*-astaxanthin solutions were stored for 2 days. The isomers were identified by their absorbance spectra (Yuan and Chen, 1997). Figure 3 shows the absorption spectra of *trans*-astaxanthin, 9-*cis*-

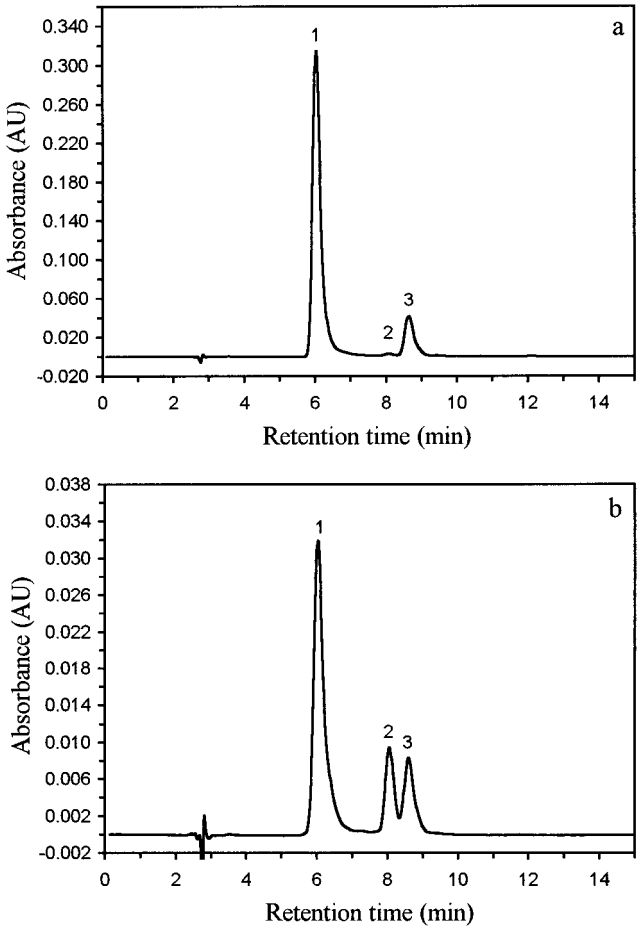

Figure 2. Typical chromatograms of *cis*−*trans* mixture of astaxanthins in dimethyl sulfoxide (a) and dichloromethane (b). Peaks: 1, *trans*-astaxanthin; 2, 9-*cis*-astaxanthin; 3, 13-*cis*-astaxanthin.

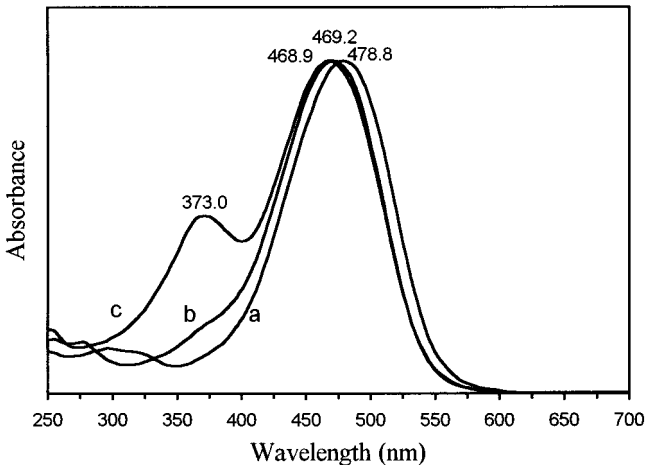

Figure 3. Absorption spectra of *trans*-astaxanthin (a), 9-*cis*-astaxanthin (b), and 13-*cis*-astaxanthin (c).

astaxanthin, and 13-*cis*-astaxanthin obtained by the photodiode array detector. The maximum absorption wavelength would shift and a new absorption maximum in the near-ultraviolet region of the spectrum (*cis*-peak) would occur when *trans*-carotenoids were isomerized to *cis*-carotenoids (Quackenbush, 1987; Nyambaka and Ryley, 1996; Yuan and Chen, 1997). Central *cis*-isomers have a higher *cis*-peak but with the least intense absorption in the visible region (Nyambaka and Ryley, 1996). The *Q* ratios, the absorbance at the maximum

wavelength/absorbance at the *cis*-peak, for the isomers of astaxanthin were obtained from the spectra.

In Figure 2, peak 1, which had a maximum absorption wavelength of 478.8 nm, was inseparable from the *trans*-astaxanthin standard and thus was identified as *trans*-astaxanthin. Peak 2, which had a larger spectral shift (469.2 nm) and a much lower *cis*-peak (*Q* ratio = 5.0), was identified as 9-*cis*-astaxanthin. Peak 3, which had a larger spectral shift (468.9 nm) with a higher *cis*-peak (*Q* ratio = 1.9), was identified as 13-*cis*-astaxanthin. The utilization of *Q* ratios to identify *cis*-isomers of carotenoids is important (Quackenbush, 1987; Saleh and Tan, 1991). The *Q* ratio of *cis*-isomers is a function of the molecular shape, and the most pronounced *cis*-peak has the least *Q* ratio (Nyambaka and Ryley, 1996).

Iodine had been used as a catalyst to promote the isomerization of *trans*-astaxanthin (Bjerkeng et al., 1997) and its diacetate (Englert and Vecchi, 1980). Three mono-*cis*-isomers and six di-*cis*-isomers of astaxanthin diacetate were separated and identified by Englert and Vecchi (1980). The results of Bjerkeng et al. (1997) showed that the mixture of isomerization reaction consisted mainly of *all-trans*-, 13-*cis*-, and 9-*cis*-astaxanthin and the total content of di-*cis*-astaxanthin was only 7.2%. For di-*cis*- and poly-*cis*-carotenoids, a large hypsochromic shift in the main absorption band might be seen (Britton et al., 1995) and could be used to identify tentatively di-*cis*- or poly-*cis*-isomers. In our work, only two *cis*-isomers (13-*cis*- and 9-*cis*-isomers) were separated and identified and no di-*cis*-isomers were found (Figure 2). It was possible that a very small amount of di-*cis*-isomers (e.g., 9,13-; 9,9′-; 13,13′-; and 13,15-di-*cis*-astaxanthins) were not separated from *trans*-, 9-*cis*-, or 13-*cis*-astaxanthins and then were not detected.

Effect of Solvents on the Isomerization of *trans*-Astaxanthin. Different organic solvents were used for the investigation of the isomerization of *trans*-astaxanthin in solutions. Because astaxanthin is insoluble in aqueous solution and most organic solvents, some solvents that could easily dissolve astaxanthin at room temperature, such as dichloromethane (~30 g/L), chloroform (~10 g/L), dimethyl sulfoxide (~0.5 g/L), and acetone (~0.2 g/L) (Johnson and An, 1991), and some solvents that are often used in HPLC analysis, such as methanol and acetonitrile, were chosen as solvents. Because the solubility of astaxanthin in methanol and acetonitrile was low, only a low concentration of astaxanthin in methanol or acetonitrile solution could be obtained. In comparison with free astaxanthin, *trans*-astaxanthin esters in the pigment extract were stable and did not easily form their isomers according to our previous work (Yuan and Chen, 1998).

Figures 4−6 show the isomerization process of *trans*-astaxanthin in different solvents at 35 °C. The results indicated that the relative contents of 9-*cis*- and 13-*cis*-astaxanthin formed during the isomerization were different in different solvents. For all the solvents, the 13-*cis*-isomer was the main *cis*-isomer from *trans*-astaxanthin.

As shown in Figures 4 and 5, the plots of the isomerization percentage of *trans*-astaxanthin versus time gave a straight line, indicating that the isomerization of *trans*-astaxanthin is a zero-order reaction in all solvents but dichloromethane (Figure 6).

The zero-order reaction rate constants (the slope of the plot) of the isomerization of *trans*-astaxanthin in different solvents at 35 °C are shown in Table 1. The isomerization reaction in dichloromethane was fast and changeable and could not be represented by a zero-order reaction. The result showed that the isomerization reaction rate was highest in dichloromethane. The

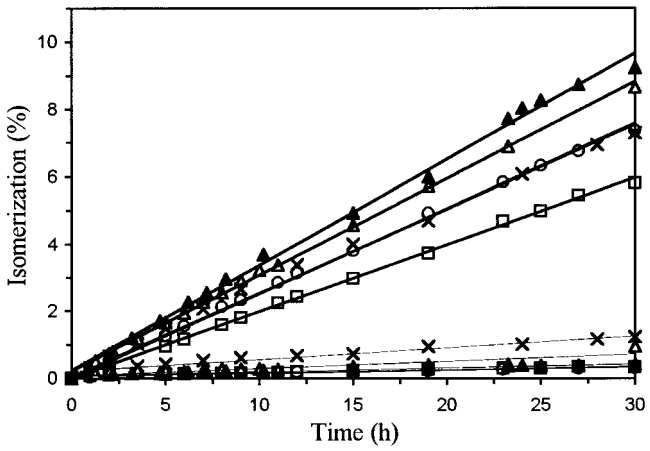

Figure 4. Changes in the content percentage of 13-*cis*-astaxanthin (dark line) and 9-*cis*-astaxanthin (light line) during the isomerization process of *trans*-astaxanthin in the mixture of 25% dichloromethane and 75% methanol (▲), methanol (△), acetonitrile (×), acetone (○), and dimethyl sulfoxide (□) at 35 °C.

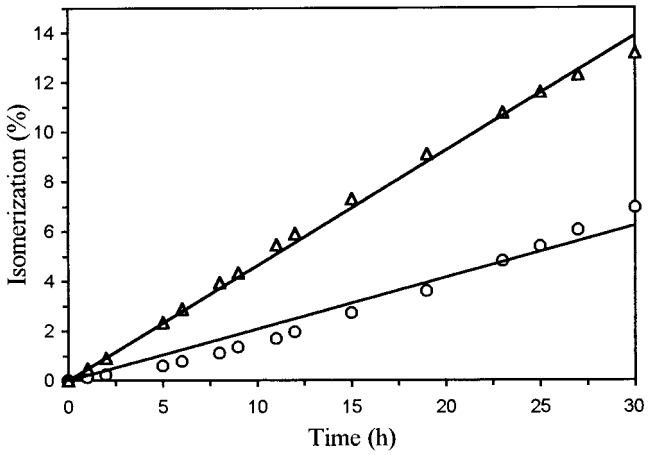

Figure 5. Changes in the content percentage of 9-*cis*-astaxanthin (○) and 13-*cis*-astaxanthin (△) during the isomerization process of *trans*-astaxanthin in chloroform at 35 °C.

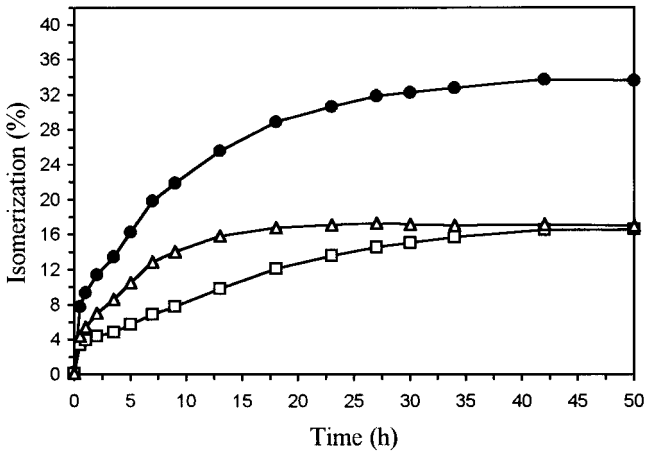

Figure 6. Changes in the content percentage of *cis*-astaxanthin isomers during the isomerization process of *trans*-astaxanthin in dichloromethane at 35 °C: (□) 9-*cis*-astaxanthin; (△) 13-*cis*-astaxanthin; (●) 9-*cis*- + 13-*cis*-astaxanthin.

Table 1. Zero-Order Reaction Rate Constants (h^{-1}) of the Isomerization Reaction of *trans*-Astaxanthin to *cis*-Astaxanthin in Organic Solvents at 35 °C

	13-*cis*-astaxanthin	9-*cis*-astaxanthin	*cis*-isomers
dimethyl sulfoxide	0.199	0.013	0.212
acetone	0.253	0.012	0.265
acetonitrile	0.241	0.035	0.276
methanol	0.287	0.022	0.309
dichloromethane + methanol (25:75)	0.313	0.012	0.325
chloroform	0.463	0.208	0.671
dichloromethane	—[a]	—[a]	—[a]

[a] No zero-order reaction.

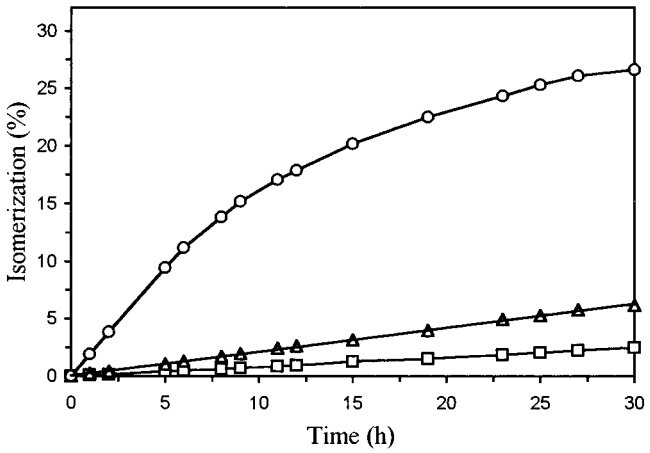

Figure 7. Changes in the content percentage of *cis*-astaxanthin (9-cis + 13-*cis*-astaxanthin) during the isomerization process of *trans*-astaxanthin in dimethyl sulfoxide at 25 (□), 35 (△), and 50 °C (○).

isomerization of *trans*-astaxanthin dissolved in a mixture of dichloromethane and methanol (25:75) was much slower than that in dichloromethane. Therefore, in the preparation of a standard solution, *trans*-astaxanthin was first dissolved with dichloromethane and then diluted with methanol. Although the solubility of astaxanthin in dimethyl sulfoxide is lower than that in dichloromethane, dimethyl sulfoxide may also be used as solvent for preparing astaxanthin standard solution.

In dichloromethane, *trans*-astaxanthin was very readily isomerized to its *cis*-isomers. After *trans*-astaxanthin in dichloromethane was kept for a longer time, the percentage of *trans*-astaxanthin would almost remain unchanged. The results showed that a maximum isomerization percentage was found to be ~33.5% and the contents of 9-cis and 13-*cis*-isomers were almost equal (Figure 6). Because of degradation resulting from traces of hydrochloric acid in dichloromethane (Khachik et al., 1988), the total concentration of astaxanthins slowly decreased, but this value (33.5%) was almost unchanged. This indicated that the isomerization reaction of *trans*-astaxanthin had almost reached an equilibrium state, with *trans*-astaxanthin, 9-*cis*-astaxanthin, and 13-*cis*-astaxanthin present in a balance condition.

Effect of Temperature on the Isomerization. To test the effect of temperature on the isomerization of *trans*-astaxanthin, the experiments for the isomerization of *trans*-astaxanthin were carried out at different temperatures. Figure 7 shows the progress of isomerization of *trans*-astaxanthin at 25, 35, and 50 °C in dimethyl sulfoxide. The results indicated that a higher temperature could promote markedly the isomerization

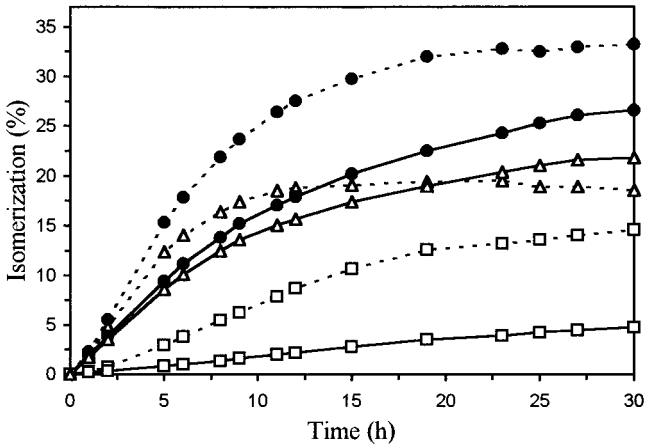

Figure 8. Changes in the content percentage of astaxanthin isomers in dimethyl sulfoxide without (—) and with (- - -) added NaCl at 50 °C: (□) 9-*cis*-astaxanthin; (△) 13-*cis*-astaxanthin; (●) 9-*cis* + 13-*cis*-astaxanthins.

of *trans*-carotenoids (Nyambaka and Ryley, 1996; Lessin et al., 1997).

Effect of Inorganic Solute on the Isomerization. A small amount of NaCl was added to *trans*-astaxanthin in dimethyl sulfoxide to test the effect of inorganic solute on the isomerization of *trans*-astaxanthin. Figure 8 shows changes in the content percentage of astaxanthin isomers in dimethyl sulfoxide without and with added NaCl at 50 °C. As can be seen from the figure, the addition of NaCl resulted in significant isomerization of *trans*-astaxanthin and increased markedly the percentage of 9-*cis*-astaxanthin, indicating that NaCl could promote the isomerization of *trans*-astaxanthin, like iodine catalyzes the isomerization of carotenoids (Quackenbush, 1987; Bjerkeng et al., 1997). Like the addition of NaCl, the addition of a small amount of water also increased significantly the isomerization of *trans*-astaxanthin and the percentage of 9-*cis*-astaxanthin (data not shown), but unlike NaCl and water, the addition of HCl would result in the fast degradation of both *trans*- and *cis*-astaxanthin (data not shown), indicating that acids were particularly detrimental to carotenoids (Johnson and An, 1991).

Non-Zero-Order Reaction. When dissolved in dichloromethane at 35 °C (Figure 6) or in dimethyl sulfoxide without and with added NaCl at 50 °C (Figures 7 and 8), *trans*-astaxanthin was very readily isomerized to its *cis*-isomers. Under these conditions, zero-order kinetics were not applicable to the isomerization reactions. The results suggested that the isomerization of *trans*-astaxanthin to *cis*-astaxanthin was a reversible reaction and an equilibrium situation had almost been reached or would be reached. As can be seen in Figures 6−8, at the beginning of the reaction, the rate (dc/dt) of isomerization reaction was high, and then the isomerization reaction rate gradually decreased as the reaction proceeded. According to Drenth and Kwart (1980), the rate for first-order with a reverse reaction was proportional to the distance removed from equilibrium. Therefore, for the reversible isomerization reaction, first-order reaction kinetics are suggested and will be studied in detail in further work.

Under a mild condition for isomerization, the isomerization reaction was slow and the change in concentration of *trans*-astaxanthin was small (Figure 4), and then the reverse reaction from *cis*-astaxanthin to *trans*-astaxanthin was negligible. Therefore, the isomerization

reaction might be represented by a zero-order reaction kinetics (Table 1).

To minimize the isomerization of *trans*-astaxanthin to *cis*-isomers during the extraction of pigments, the saponification of *trans*-astaxanthin esters, the purification of *trans*-astaxanthin, and the preparation of *trans*-astaxanthin standard solution, chlorinated solvents should be avoided or only incorporated as a solvent modifier. However, because of the ability of dichloromethane to solubilize highly lipophilic carotenoids, the mixture of dichloromethane and methanol (25:75), in which the isomerization rate of *trans*-astaxanthin was much lower than that in dichloromethane and almost the same as that in dimethyl sulfoxide at 25 °C (data not shown), was used to extract pigments from the alga *H. pluvialis* at ambient temperature (Yuan and Chen, 1997, 1998, 1999). It was important to control air, light exposure, and temperature to reduce the isomerization and degradation of *trans*-astaxanthin during the storage of standard solution of *trans*-astaxanthin (Scita, 1992).

Conclusion. In organic solvents, *trans*-astaxanthin could be partially isomerized to its *cis*-isomers, mainly 13-*cis*-astaxanthin. The isomerization rates were different in different solvents. The following order was found: dichloromethane > chloroform > the mixture of dichloromethane and methanol (25:75) > methanol > acetonitrile > acetone > dimethyl sulfoxide. The chlorinated solvents, that is, dichloromethane and chloroform, should be avoided or only incorporated as a solvent modifier in pigment extract and HPLC analysis due to the ability to solubilize highly lipophilic carotenoids (i.e., dichloromethane). In addition, a high temperature should be avoided to minimize the isomerization of *trans*-astaxanthin to *cis*-astaxanthin.

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