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NOTE

Tissue Distribution of Astaxanthin Geometrical Isomers in Male Sprague-Dawley Rats after Oral Administration of Astaxanthin Esters Derived from *Haematococcus lacustris*

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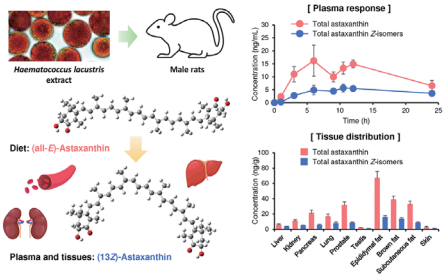
Abstract: Recent studies have revealed that the biological activity of astaxanthin differs among *E/Z*-isomers. Therefore, it is essential to investigate the distribution of astaxanthin isomers in the body to comprehensively elucidate their role. However, owing to the technical complexity of astaxanthin isomer analysis, detailed information regarding the precise distribution of isomers in the body remains limited. In this study, food-grade astaxanthin esters derived from *Haematococcus lacustris* (total *Z*-isomer ratio of astaxanthin = 34.5%) were administered to male Sprague-Dawley rats, and their plasma response and tissue distribution were investigated. Astaxanthin isomers were analyzed using normal-phase high-performance liquid chromatography, which can accurately measure the isomers. The maximum plasma concentration (C_{max}) and area under the curve from time 0 to 24 h ($AUC_{0–24\ h}$) values of total astaxanthin isomers were determined to be 20.4 ± 4.6 ng/mL (T_{max}: 8.3 ± 2.3 h) and 261.8 ± 16.4 ng·h/mL, respectively. The total *Z*-isomer ratio of astaxanthin in the plasma increased over time and reached approximately 55% at 24 h post-administration. Astaxanthin accumulated in the liver, kidneys, lungs, and testes exhibited a high total *Z*-isomer ratio (> 44.0%). In plasma and tissues, the predominant astaxanthin *Z*-isomer was the 13*Z*-isomer, and minor quantities of 9*Z*-, 15*Z*-, and two unidentified astaxanthin *Z*-isomers (potentially multi-*Z*-isomers) were observed. The composition of astaxanthin isomers in the plasma and tissues differed substantially from that of the diet. These findings suggest that astaxanthin *Z*-isomers, particularly the 13*Z*-isomer, possess higher absorbability than the all-*E*-isomer, or that a mechanism regulates astaxanthin isomer composition in the rat body.



Key words: carotenoid, isomerization, plasma response, disposition, bioavailability

1 Introduction

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione; $C_{40}H_{52}O_4$) is a keto-carotenoid pigment found predominantly in marine microorganisms and animals[1, 2]. This carotenoid exhibits potent antioxidant activity and has a wide range of health benefits, such as the reduction of cardiovascular disease risk and improvement in muscle function during body and eye strain[3, 4]. Although astaxanthin contains multiple conjugated double bonds, natural and synthetic astaxanthin occur predominantly in the all-*E*-configuration. For example, >85% of astaxanthin derived from *Haematococcus lacustris* and *Paracoccus carotinifa-*

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ciens exists in the all-*E*-configuration[5, 6]. In contrast, astaxanthin *Z*-isomers are abundant in the mammalian body, even when (all-*E*)-astaxanthin is ingested orally[7, 8]. It is believed that there is a mechanism in the mammalian body responsible for isomerizing astaxanthin into the *Z*-isomers, but the details remain unknown. Furthermore, there is limited information on the tissue distribution of the astaxanthin *E/Z*-isomers.

In recent years, astaxanthin *Z*-isomers have attracted attention because many studies have demonstrated that they exhibit greater biological activity than (all-*E*)-astaxanthin[9–11]. For example, astaxanthin *Z*-isomers show higher anti-inflammatory and anti-elastase activities than the all-*E*-isomer[10, 11]. The high *Z*-isomer ratio of astaxanthin in mammals may represent a biological regulatory function that allows astaxanthin to effectively exert its health benefits. Investigating the distribution of astaxanthin *E/Z*-isomers in the body is important for understanding the health benefits of the *Z*-isomers and the mechanism of *Z*-isomerization in the body. We have recently investigated the tissue distribution of astaxanthin isomers in male rats after oral administration of synthetic astaxanthin[8]. However, most astaxanthin currently on the market for use in the food industry is derived from *H. lacustris*[12]. Synthetic astaxanthin is principally present in the free form, whereas astaxanthin from *H. lacustris* predominantly occurs in the ester form, that is, the fatty acids bond to the hydroxyl group of astaxanthin, with the main component being a monoester[12].

This study aimed to investigate the effect of oral administration of astaxanthin esters derived from *H. lacustris* in male rats on the plasma response and tissue distribution of astaxanthin isomers. In this study, astaxanthin isomers were analyzed using a recently developed normal-phase analysis method[6, 13].This analytical method can clearly separate astaxanthin *E/Z*-isomers such as the 13*Z*- and 15*Z*-isomers, which are difficult to separate using conventional reversed-phase analyses[5, 6, 10, 13]. Detailed analysis of astaxanthin isomers in the body should help elucidate the isomerization mechanism and biological roles of the *Z*-isomers in the body.

2 Materials and Methods

2.1 Materials

A *Haematococcus lacustris* extract containing astaxanthin esters (AstaREAL L10) was provided by Fuji Chemical Industries Co., Ltd. (Toyama, Japan)[14]. This astaxanthin source is commercially available and has an astaxanthin concentration of approximately 10%. The main component of this extract, besides astaxanthin, is triacylglycerol[12, 15]. High-performance liquid chromatography (HPLC)-grade organic solvents (acetone, ethyl acetate, and hexane), soybean oil, and cholesterol esterase were obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Analytical-grade butylated hydroxytoluene (BHT) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Anesthetics, ketamine hydrochloride (Ketalar) and xylazine hydrochloride (Seractal), were obtained from Daiichi Sankyo Propharma Co., Ltd., (Tokyo, Japan) and Bayer AG (Leverkusen, Germany), respectively.

2.2 Animal treatment

Ten-week-old male Slc/Sprague-Dawley rats (Japan SLC, Inc., Shizuoka, Japan) were used in this study. All animal experiments were performed in the I Tech Lab. Co., Ltd. (Gifu, Japan), and the protocols were approved by the Institutional Animal Care and Use Committee of this company (Permission Nos. ITL-23-RO-364-3 and ITL-23-RO-364-3). The rats were housed at 23 ± 5℃ with a 12-h light/dark cycle (light on at 7:00) with water and a carotenoid-free standard laboratory diet (AIN93M; Oriental Yeast Co., Ltd., Tokyo, Japan) ad libitum during the acclimation and testing periods. After a 7-day acclimation, single- and 14-day repeated-dose oral evaluations were performed. The astaxanthin esters (the *H. lacustris* extract; AstaREAL L10) used in the test were diluted to a prescribed astaxanthin concentration with soybean oil and were stored at −20℃ or below until just before use in the experiment. In the single-dose study, the rats were randomly divided into control (placebo, soybean oil, $n = 3$) and test (100 mg astaxanthin/kg body weight, $n = 4$) groups. Blood samples were taken from the abdominal aorta of rats and collected in heparinized tubes before administration and at 1, 3, 6, 9, 10.5, 12, and 24 h after the oral administration of soybean oil or astaxanthin esters, and plasma samples were obtained. In the 14-day repeated-dose study, the rats were randomly divided into control (placebo, soybean oil, $n = 3$) and test (10 mg of astaxanthin/kg body weight/day, $n = 5$) groups. Twenty-four hours after the rats were fed their last diet, they were sacrificed by exsanguination under anesthesia (ketamine hydrochloride and xylazine hydrochloride), and tissue (liver, kidney, adrenal, pancreas, lung, prostate, testis, eyes, hippocampus, cerebral cortex, epididymal fat, brown fat, subcutaneous fat, and skin) samples were collected. The brown fat was obtained from the back of the neck and the skin samples contained subcutaneous fat. These collected plasma and tissue samples were immediately stored at −80℃ until just before astaxanthin analysis.

2.3 Astaxanthin analysis

Astaxanthin isomers in plasma and tissues were extracted using hexane and acetone containing 0.01% BHT, respectively, according to a previously described method[8]. To avoid isomerization of astaxanthin during this process, it was conducted under conditions that minimized expo-

sure to heat and light. The accuracy of this extraction process was evaluated by an addition-recovery test[16, 17] using standard (all-*E*)-astaxanthin for plasma and liver with recoveries between 95%–105% and 98%–104%, respectively. These results demonstrated that the sample matrix did not interfere with the astaxanthin extraction. Incidentally, astaxanthin *Z*-isomers exhibit higher solubility in acetone and hexane compared to (all-*E*)-astaxanthin[18], suggesting that the *Z*-isomers would also be recovered to nearly 100% using these extraction methods. Furthermore, it was confirmed that isomerization of astaxanthin did not occur during the extraction process. The resulting extracts were analyzed using normal-phase HPLC equipped with a photodiode array detector (SPD-M20A; Shimadzu, Kyoto, Japan) and a high-sensitivity detection cell (HS-Cell; Shimadzu)[6, 13], and the astaxanthin concentration and isomer ratio to total astaxanthin in the plasma and tissues were determined. To give a brief description of the HPLC conditions, a stationary phase and a mobile phase were Phenomenex silica gel column (Luna 5 μm Silica (2), 150 mm × 4.6 mm, 100 Å; two columns were connected in series) and a mixture of hexane/ethyl acetate/acetone (75:23:2, v/v/v), respectively[6, 13]. Astaxanthin isomers were identified based on HPLC retention times and spectral data (i.e., the absorption maximum wavelength, *Q*-ratio [relative intensity of the *Z*-peak to the absorption maximum peak], and mass spectrum)[6, 13, 19]. Mass spectral analysis of astaxanthin isomers was conducted using a Shimadzu LCMS-2050 mass spectrometer (Shimadzu Corp., Kyoto, Japan) in positive ion mode[19]. Incidentally, astaxanthin isomers in the diet (*H. lacustris* extract; ester form of astaxanthin) were analyzed using HPLC after de-esterification with cholesterol esterase according to a previously reported method[13, 19], i.e., the enzyme reaction was carried out in an isochoric mixture of acetone and 0.05 M Tris–HCl buffer (pH = 7.0) containing 4 U/mL cholesterol esterase at 30℃ for 15 min. It has been demonstrated that isomerization of astaxanthin does not occur under these enzyme treatment conditions[13]. Because astaxanthin isomers in plasma and tissues are present in the free form[7, 8], de-esterification was not performed.

2.4 Statistical analysis

All data were recorded as the means and standard errors of the means (mean ± SE). Statistical analyses were conducted using a one-way analysis of variance with Tukey's post-hoc test using EZR software (v. 1.54; Saitama Medical Center, Jichi Medical University, Saitama, Japan). A p-value < 0.05 was considered statistically significant.

3 Results and Discussion

3.1 Astaxanthin isomer composition in diet

In this study, a commercially available *H. lacustris* extract was used as the source of astaxanthin[14]. The HPLC chromatogram of the *H. lacustris* extract is shown in Fig. 1A. Six astaxanthin isomers were tentatively identified based on the HPLC retention time and spectral data[6, 13, 19]. The two peaks that eluted around 10 min were determined not to be astaxanthin isomers based on the spectral data. These components are thought to be other carotenoids such as astaxanthin precursors (e.g., adonirubin, canthaxanthin, and zeaxanthin)[5, 12]. The ratios of all-*E*-, 9*Z*-, 13*Z*-, and 15*Z*-isomers to total astaxanthin were 65.5%, 12.8%, 12.1%, and 1.4%, respectively. Two unknown astaxanthin *Z*-isomers (perhaps multi-*Z*-isomers) were observed, and the sum of these isomer ratios was 8.2%. Thus, the total *Z*-isomer ratio of the *H. lacustris* extract used in this study was 34.5%. It has been reported that the total *Z*-isomer ratio of astaxanthin in *H. lacustris* biomass is approximately 10%[5]. The higher *Z*-isomer ratio of astaxanthin in the *H. lacustris* extract used in this study was thought to be due to thermal *Z*-isomerization during the manufacturing process. (all-*E*)-Astaxanthin has been reported to be easily isomerized to the *Z*-isomers upon heating[6, 19], and the astaxanthin source used in this study was obtained using supercritical CO_2 extraction[14], which means it was exposed to high temperatures for long periods of time during the extraction process. Our recent study has demonstrated that the *Z*-isomer ratio of astaxanthin increased during the extraction process using supercritical CO_2[20]. Alternatively, the discrepancy in the total *Z*-isomer ratio of astaxanthin esters in *H. lacustris* between this study and previous reports might be explained, at least in part, by the fact that previous quantification methods, including the United States Pharmacopoeia monograph, only included the all-*E*-, 9*Z*-, and 13*Z*-isomers in the determination of astaxanthin[5, 12].

3.2 Plasma response of astaxanthin isomers

Although there are many reports on changes in plasma astaxanthin concentrations in mammals after astaxanthin administration[7, 8, 21–24], information on the *Z*-isomer ratios is limited. Østerlie et al.[7], Coral-Hinostroza et al.[23], and Zhou et al.[24] investigated the astaxanthin isomer ratio (i.e., the all-*E*-, 9*Z*-, and 13*Z*-isomer ratios) in human and mouse plasma, but the analysis was not accurate enough, that is, the HPLC analysis was performed under conditions where the 15*Z*-isomer and multi-*Z*-isomers could not be separated. Furthermore, many studies have used synthetic astaxanthin, which is not typically approved for food applications[7, 8, 23]. This research is valuable because astaxanthin esters derived from *H. lacustris*, which are commonly used in the food industry, were evaluated using a highly accurate analytical technique.

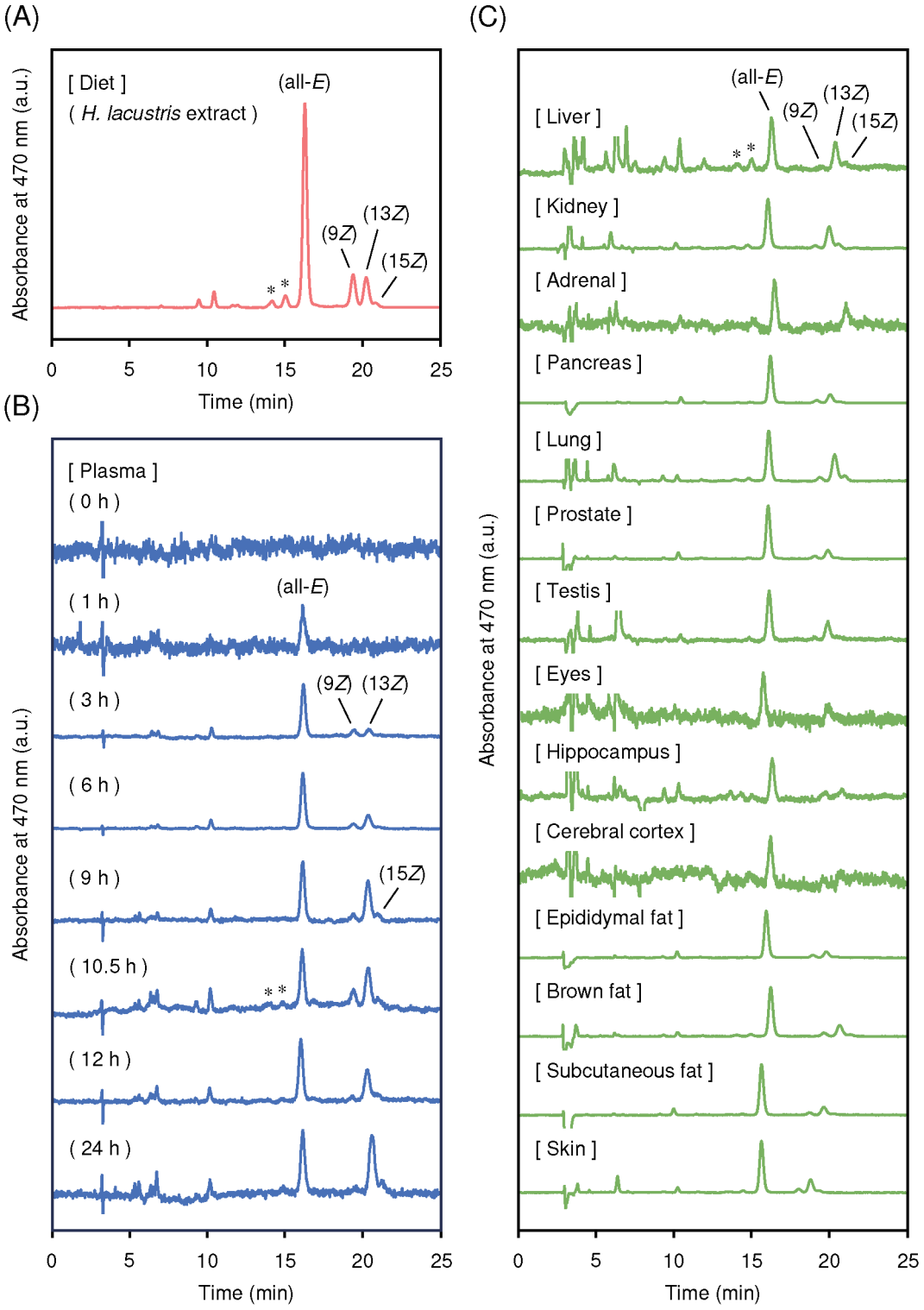

Fig. 1 Normal-phase HPLC chromatograms of (A) the diet (*H. lacustris* extract), (B) plasma samples before and after (1–24 h) oral administration of the diet, and (C) tissue samples after 14-day repeated oral administration of the test diet. Labels of (all-*E*), (9*Z*), (13*Z*), and (15*Z*) denote (all-*E*)-, (9*Z*)-, (13*Z*)-, and (15*Z*)-astaxanthin, respectively. The peaks of asterisk (*) were tentatively identified as astaxanthin *Z*-isomers according to the spectrum data[6, 13, 16].

Typical HPLC chromatograms of plasma samples before and after oral administration of the test diet are shown in Fig. 1B. Astaxanthin isomers were not detected in the control group fed soybean oil. In the test group fed *H. lacustris* extract (100 mg of astaxanthin/kg body weight), similar to the diet, all-*E*, 9*Z*-, 13*Z*-, 15*Z*-, and two unknown astaxanthin *Z*-isomers (perhaps multi-*Z*-isomers) were observed. The maximum plasma concentration (C_{max}: 20.4 ± 4.6 ng/mL) of total astaxanthin isomers, including the all-*E*-isomer was reached at 8.3 ± 2.3 h (T_{max}) after administration of the test sample, while the maximum concentration (C_{max}: 6.6 ± 1.3 ng/mL) of total astaxanthin *Z*-isomers was reached at 10.5 ± 1.5 h (T_{max}) after the administration (Fig. 2A). The calculated area under the curve from time 0 to 24 h ($AUC_{0-24\,h}$) values of total astaxanthin isomers including the all-*E*-isomer and total astaxanthin *Z*-isomers was 262.7 ± 33.3 and 99.3 ± 16.4 ng·h/mL, respectively. The total *Z*-isomer ratio in plasma tends to increase over time. For example, 3, 9, and 24 h after administration, the total *Z*-isomer ratios of astaxanthin in plasma were approximately 25%, 45%, and 55%, respectively. Considering that the total *Z*-isomer ratio of astaxanthin in the diet is 34.5%, it is possible that the *Z*-isomers are more absorbable than the all-*E*-isomer or that some of the all-*E*-isomer is converted to the *Z*-isomers after absorption. Numerous studies have shown that carotenoid *Z*-isomers are more absorbable than the all-*E*-isomers. Furthermore, (all-*E*)-carotenoids tend to isomerize into the *Z*-isomers as they circulate throughout the body[8, 9, 24, 25]. On the other hand, it is also possible that the ratio of *Z*-isomers in plasma has elevated over time because the all-*E*-isomer is more readily absorbed by certain tissues, broken down by certain enzymes, and excreted from the body than the *Z*-isomers.

When focusing on the individual astaxanthin *Z*-isomers (Fig. 2B), the 13*Z*-isomer was more abundant than the other *Z*-isomers. In the astaxanthin diet, the amounts of (9*Z*)- and (13*Z*)-astaxanthin was almost the same (Fig. 1A). Thus, it is possible that the 13*Z*-isomer has higher absorbability than the 9*Z*-isomer, that some astaxanthin isomers are converted to the 13*Z*-isomer in the body, or that the 9*Z*-isomer is converted into other isomers or metabolites. A higher ratio of the 13*Z*-isomer to the 9*Z*-isomer in plasma has also been observed in previous studies using mammals other than rats, such as humans and mice[7, 23, 24]. These trends are observed regardless of the astaxanthin form (free or ester form)[7, 8, 23, 24]. The amount of the 15*Z*- and unknown astaxanthin *Z*-isomers (perhaps multi-*Z*-isomers) in the plasma was lower than that of the 9*Z*- and 13*Z*-isomers and a nearly constant concentration was maintained for 24 h after the administration of astaxanthin. This is the first study to observe the plasma response of astaxanthin *Z*-isomers other than the 9*Z*- and 13*Z*-isomers. The mechanism by which the astaxanthin isomer ratio is controlled in mammalian bodies has not yet been elucidated; thus, further research is needed to understand this mechanism.

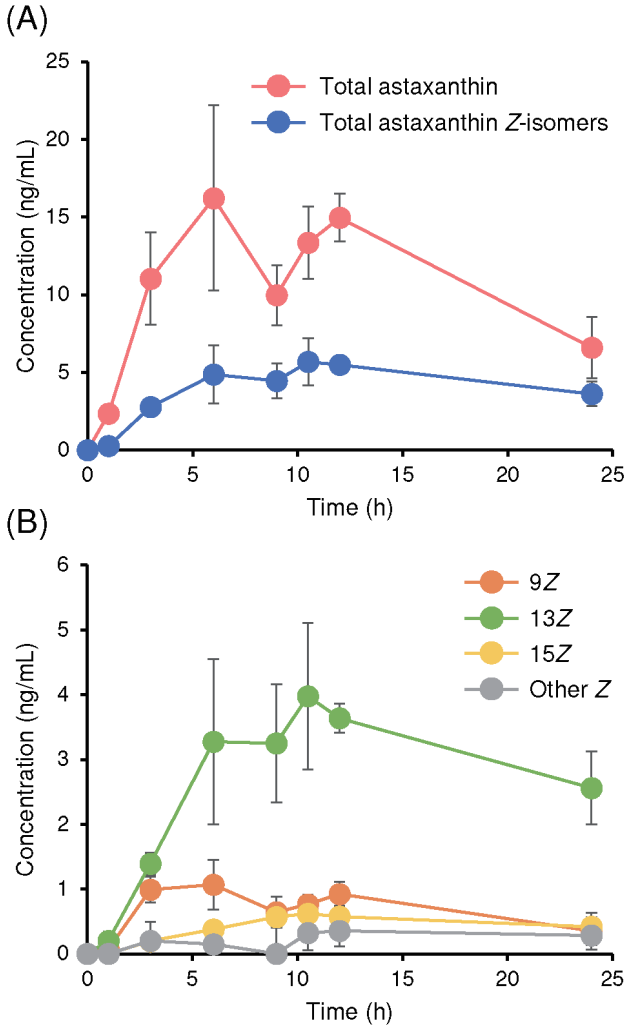

Fig. 2 Plasma concentration (ng/mL) of (A) total astaxanthin including the all-*E*-isomer and total astaxanthin *Z*-isomers and (B) individual astaxanthin *Z*-isomers. Labels of (all-*E*), (9*Z*), (13*Z*), and (15*Z*) denote (all-*E*)-, (9*Z*)-, (13*Z*)-, and (15*Z*)-astaxanthin, respectively, and (other *Z*) denotes the sum of unknown astaxanthin *Z*-isomers (perhaps multi-*Z*-isomers).

3.3 Tissue distribution of astaxanthin isomers

Several studies have reported that the concentration of accumulated astaxanthin varies depending on the tissue type[8, 24, 26–28]. Astaxanthin tends to accumulate in the lungs, liver, and kidneys, but the degree of accumulation varies greatly depending on the animal species and the astaxanthin form (free or ester form)[8, 24, 26, 27]. To the best of our knowledge, there are no reports on the distribution of astaxanthin isomers in tissues after the administration of

astaxanthin esters to mammals. In this study, the distribution of astaxanthin isomers in peripheral tissues such as the liver, kidney, adrenal gland, pancreas, lung, prostate, testis, epididymal fat, brown fat, subcutaneous fat, and skin, as well as in central nervous system tissues such as the eyes, hippocampus, and cerebral cortex, was investigated after a 14-day repeated dose of astaxanthin esters derived from *H. lacustris* (10 mg of astaxanthin/kg body weight/day).

Typical HPLC chromatograms of the tissue samples from the test groups are shown in Fig. 1C. Astaxanthin isomers were detected in all the tissue samples. Using normal-phase HPLC, astaxanthin isomers (i.e., the all-*E*, 9*Z*-, 13*Z*-, 15*Z*-, and two unknown astaxanthin *Z*-isomers) in the tissues were accurately analyzed. The total astaxanthin concentration in the tissues is shown in Fig. 3A. Although astaxanthin isomers were detected in the adrenal gland, eyes, hippocampus, and cerebral cortex using HPLC analysis (Fig. 1C), these data are not included in Fig. 3 because their concentrations were too low for accurate quantification (note that in some tissues, the sample amount was too small to allow for accurate analysis). As with the previous reports, a large amount of astaxanthin was accumulated in the lung (16.7 ± 3.3 ng/g), liver (5.9 ± 1.1 ng/g), and kidney (10.9 ± 1.7 ng/g). Higher astaxanthin concentration was observed in the pancreas (21.1 ± 3.9 ng/g), prostate (31.4 ± 4.4 ng/g), and adipose tissues (epididymal fat, 67.0 ± 8.7 ng/g; brown fat, 38.7 ± 4.6 ng/g; subcutaneous fat, 32.7 ± 4.1 ng/g) than in other tissues. This may provide health benefits to the tissues containing high levels of astaxanthin. Indeed, ample studies have demonstrated that astaxanthin exhibits anti-tumor effects in the pancreas and prostate as well as inhibits inflammation and fibrosis in adipose tissues[12, 29–31]. On the other hand, it has been documented that the administration of free-form (all-*E*)-astaxanthin to mice and rats results in the accumulation of the highest concentrations of astaxanthin in the liver among the tissues examined[8, 24]. Thus, the astaxanthin form (free or ester form) may affect its accumulation in tissues.

The total (*Z*)-astaxanthin concentration and the total *Z*-isomer ratio of astaxanthin in the tissues are shown in Figs. 3A and 3B. Liver, kidney, lung, and testis showed higher *Z*-isomer ratios (44%–57.3%) than those of other tissues (23.2%–34.4%), that is, 57.3% ± 3.0%, 45.2% ± 1.3%, 46.5% ± 4.1%, and 44.0% ± 2.1%, respectively. The high astaxanthin *Z*-isomer ratio in these tissues is similar to the previous study in which free-form astaxanthin was administered to rats[8]. Their total *Z*-isomer ratios were higher than those in the astaxanthin diet (34.5%). The liver showed the highest *Z*-isomer ratio, possibly because of its metabolic activity, which promotes isomerization via oxidative and enzymatic processes[32, 33]. The total *Z*-isomer ratio of the pancreas (26.9% ± 0.8%), prostate (26.4% ± 0.8%), epididymal fat (23.2% ± 0.2%), and subcutaneous fat

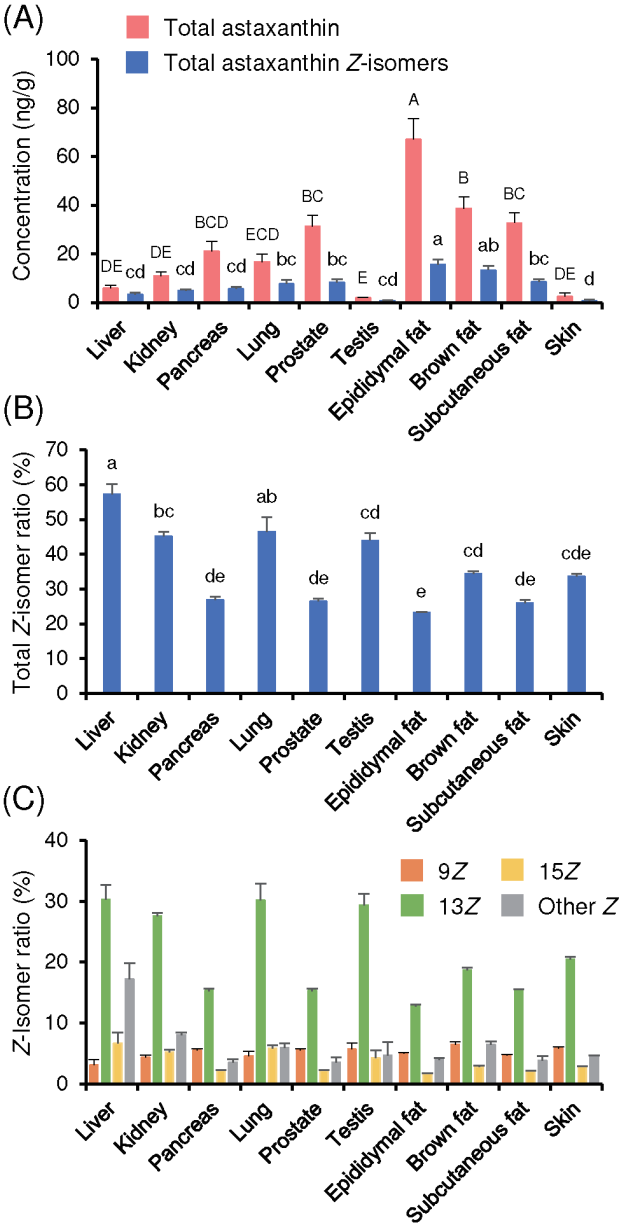

Fig. 3 Tissue concentration (ng/g) of (A) total astaxanthin and astaxanthin *Z*-isomers and (B) total and (C) individual *Z*-isomer ratios (%) of astaxanthin in the tissues. Labels of (all-*E*), (9*Z*), (13*Z*), and (15*Z*) denote (all-*E*)-, (9*Z*)-, (13*Z*)-, and (15*Z*)-astaxanthin, respectively, and (other *Z*) denotes the sum of unknown astaxanthin *Z*-isomers (perhaps multi-*Z*-isomers). The means with different letters for each tissue are significantly different ($p < 0.05$), whereas the means with similar letters are not different from each other.

(25.9% ± 0.9%) was lower than that of the diet astaxanthin (34.5%). In these tissues, the all-*E*-isomer may be preferentially accumulated, or the *Z*-isomers may be converted

to the all-*E*-isomer. Similar phenomena have been reported in rainbow trout (*Oncorhynchus mykiss*)[34]. When *Z*-isomer-rich astaxanthin (total *Z*-isomer ratio = 56.6%) was fed to rainbow trout, approximately 50% of astaxanthin that accumulates in the liver was the *Z*-isomers, whereas more than 95% of astaxanthin that accumulates in the flesh was the all-*E*-isomer. The possibility that (all-*E*)-carotenoids are converted to *Z*-isomers in the body has been discussed in many studies; however, research on the reverse phenomenon is limited. To elucidate the details of the body kinetics of astaxanthin isomers, oral administration tests using each astaxanthin isomer and labeled astaxanthin need to be performed.

When focusing on individual astaxanthin *Z*-isomers (Fig. 3C), as with plasma samples, the 13*Z*-isomer ratio was the highest in all tissues. The liver had a higher ratio of unknown astaxanthin *Z*-isomers (perhaps multi-*Z*-isomers) than the other tissues. The *Z*-isomer composition of the tissues differed from that of dietary astaxanthin. As mentioned in the previous section (*section 3.2*), there may be a mechanism in the rat body that controls the ratio of individual *Z*-isomer. The high ratio of (13*Z*)-astaxanthin in the body may be the mechanism by which the body has it in place because the 13*Z*-isomer may have useful biological activities. However, information on the differences in the biological activities of individual astaxanthin isomers is lacking and should be investigated in the future.

4 Conclusions

The body kinetics of astaxanthin isomers in male rats administered astaxanthin esters derived from *H. lacustris* were investigated. The plasma response test revealed that the total *Z*-isomer ratio of astaxanthin in the plasma increased over time. Furthermore, several tissues, such as the liver, kidneys, lungs, and testes, contained large amounts of astaxanthin *Z*-isomers, and the total *Z*-isomer ratio of these tissues (>44.0%) was higher than that of the diet (34.5%). This suggests that there is a mechanism in rats that regulates the astaxanthin isomer ratio and that the *Z*-isomers may play a beneficial role in the body. As there are many unknown aspects of the biological activity of carotenoid *Z*-isomers, future research is expected to shed light on these aspects.

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Conflict of Interest

The authors declare no conflicts of interest.

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