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Possibility of Using Astaxanthin-Rich Dried Cell Powder from *Paracoccus carotinifaciens* to Improve Egg Yolk Pigmentation of Laying Hens

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Received: 29 April 2020; Accepted: 29 May 2020; Published: 2 June 2020

Abstract: The study investigated egg quality aspects such as astaxanthin concentration, *E*/*Z*-isomer ratio, and yolk color in laying hens fed with astaxanthin-containing diets. Dried *Paracoccus carotinifaciens* cell powder (Panaferd-AX) and fine cell powder (Panaferd-P) were used as sources of astaxanthin, with average particle diameters of approximately 100 μm and 10 μm, respectively. *Paracoccus carotinifaciens* contains valuable rare carotenoids such as adonirubin and adonixanthin, and thus the concentrations of these carotenoids in egg yolk were also evaluated. The *E*/*Z*-isomer ratios of the egg yolk carotenoids were determined by normal-phase high-performance liquid chromatography (HPLC) with an improved solvent system. Feeding diets containing *P. carotinifaciens* resulted in increased concentrations of astaxanthin, adonirubin, and adonixanthin in egg yolk, as well as a marked increase in the yolk color fan score; values associated with the Panaferd-P-containing diet were higher than those associated with the Panaferd-AX-containing diet. For example, the astaxanthin concentration in egg yolks of hens fed with the Panaferd-AX- and Panaferd-P-containing diets for 21 days were 1.21 μg/g and 1.85 μg/g, respectively. This indicates that the pulverization treatment of the *P. carotinifaciens* powder increased the efficiency of carotenoid accumulation in the egg yolk. Moreover, more than 95% of astaxanthin in *P. carotinifaciens* was present as the all-*E*-isomer. However, approximately 25% of astaxanthin in egg yolk was present as the *Z*-isomers. In recent years, astaxanthin *Z*-isomers have attracted substantial attention as they exhibit a greater bioavailability and bioactivity than the all-*E*-isomer. These data are important not only for understanding egg yolk pigmentation but also for improving the nutritional value of hens' egg yolk through the addition of *P. carotinifaciens* to their diet.

Keywords: *Paracoccus carotinifaciens*; astaxanthin; adonirubin; adonixanthin; geometric isomer; egg yolk

1. Introduction

The color of egg yolk is an important factor in determining whether the product will be acceptable to the consumer. Yolk with a high pigment content is in demand for egg-containing processed foods such as noodles, baked goods, and mayonnaise. Carotenoids are principally responsible for the yolk color, but because chickens cannot biosynthesize carotenoids, they obtain carotenoids from their diet [1–3]. Dietary sources of carotenoids have traditionally been corn,

alfalfa, and marigold flower, which contain lutein and zeaxanthin [1,4,5]. In recent years, the use of astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione; Figure 1a) as a carotenoid for improving egg yolk pigmentation has attracted substantial attention [6–8]. Astaxanthin efficiently pigments egg yolk and shows a strong antioxidant activity that is 10 times higher than β-carotene and 100 times higher than α-tocopherol [9,10]. Furthermore, carotenoid consumption offers various health benefits such as decreased risks of cardiovascular disease, certain cancers, and eye disease [9–11]. The yeast *Phaffia rhodozyma* has been used as a source of astaxanthin to allow pigmentation of egg yolk for commercial purposes [12,13]. In this study, we evaluated the use of an aerobic Gram-negative microorganism, *Paracoccus carotinifaciens* ASB-57 strain [14], as a source of astaxanthin (3*S*,3′*S* form). *Paracoccus carotinifaciens* contains not only high concentrations of astaxanthin but also two valuable rare carotenoids, adonirubin (3-hydroxy-β,β-carotene-4,4′-dione, 3*S* form; Figure 1b) and adonixanthin (3,3′-dihydroxy-β,β-carotene-4-one, 3*S*,3′*R* form; Figure 1c) [15,16]. These rare carotenoids have over 2.5 times more antioxidant activity than astaxanthin [17]. Moreover, microorganisms belonging to the genes *Paracoccus* are advantageous in their proliferation rates, and their carotenoid productivity is high compared to that of yeasts. Thus, the use of *P. carotinifaciens* should provide advantages in terms of the nutritional value and production cost of eggs compared to *P. rhodozyma* [18]. Dried *P. carotinifaciens* cell powder was recently commercialized by JXTG Nippon Oil & Energy Corporation (Tokyo, Japan) for use in the pigmentation of hens' egg yolk and seafoods such as salmon, trout, and shrimp. These cell powders are sold under the names Panaferd-AX and Panaferd-P. Panaferd-P is obtained by finely pulverizing Panaferd-AX; the average particle diameters of Panaferd-AX and Panaferd-P are approximately 100 μm and 10 μm, respectively [8,15]. Several studies indicated that the pulverization treatment of ingredients containing carotenoids, such as red pepper (*Capsicum frutescens*) and *P. rhodozyma*, enhanced the yolk pigmentation efficiency of laying hens [19,20]. Therefore, there is a possibility that Panaferd-P has a higher pigmentation efficiency than Panaferd-AX.

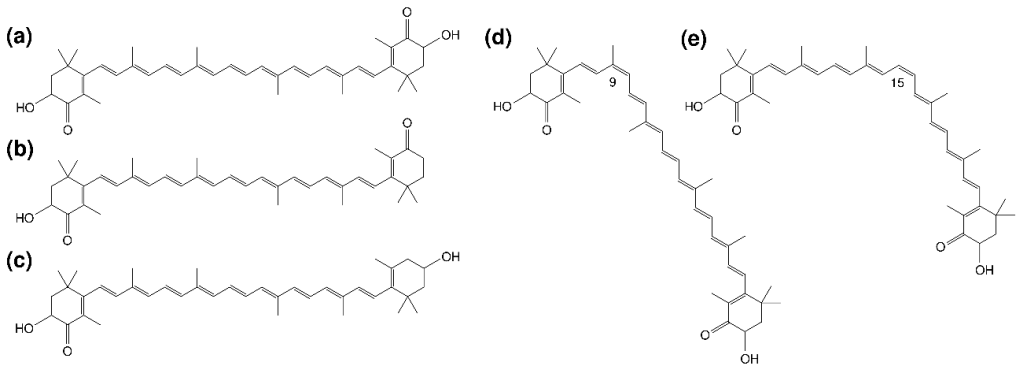

Figure 1. Chemical structures of astaxanthin isomers: (**a**) (all-*E*)-astaxanthin; (**b**) (all-*E*)-adonirubin; (**c**) (all-*E*)-adonixanthin; (**d**) (9*Z*)-astaxanthin; (**e**) (15*Z*)-astaxanthin.

The aim of this study was to investigate the effects of feeding laying hens with diets containing *P. carotinifaciens* powder of different particle sizes on the concentrations of astaxanthin, adonirubin, and adonixanthin in egg yolk and the yolk color. We also evaluated the impact of the feeding on some egg qualities such as egg weight, yolk weight, albumen height, and the Haugh unit. Moreover, we investigated the *E*/*Z*-isomer ratios of astaxanthin, adonirubin, and adonixanthin in egg yolk using an improved high-performance liquid chromatography (HPLC) system. Apart from some notable exceptions, the carotenoid all-*E*-isomer is the most predominant geometric isomer in plants and microorganisms such as *P. carotinifaciens* (Figure 1a–c) [16,21–23]. In contrast, *Z*-isomers of carotenoids exist in considerable quantities in humans and other animals (Figure 1d,e) [21,24–26]. Several studies have reported that *Z*-isomers of carotenoids are found in egg yolk [2,6,27,28]. For example, we have recently reported that even though most dietary lycopene represents the all-*E*-isomer, more than 65% of lycopene in egg yolk is present as the *Z*-isomers [27]. Walker et al. reported that when laying hens were

fed with astaxanthin-rich diets, several types of astaxanthin *Z*-isomers could be detected [6]. However, they did not provide the exact value of the *Z*-isomer ratio, likely because of the difficulty in accurately analyzing carotenoid isomers in egg yolk. Recently, several studies have reported that astaxanthin *Z*-isomers had a higher bioavailability and showed stronger bioactivities, such as antioxidant and anti-inflammatory activities, than those by the all-*E*-isomer [29–31]. Hence, the evaluation of the carotenoid *E*/*Z*-isomer ratio in foods, especially that of astaxanthin, is very important.

2. Materials and Methods

2.1. Reagents

Two forms of carotenoid-rich dried cell powders from the *P. carotinifaciens* ASB-57 strain [14], Panaferd-AX (average particle diameter, approximately 100 μm; astaxanthin content, 2.0%; adonirubin content, 0.61%; adonixanthin content, 0.22%) and Panaferd-P (average particle diameter, approximately 10 μm; astaxanthin content, 1.9%; adonirubin content, 0.63%; adonixanthin content, 0.20%) were obtained from JXTG Nippon Oil & Energy Corporation (Tokyo, Japan) [8,14,15]. Panaferd-P was obtained by finely pulverizing Panaferd-AX [8]. High-purity (all-*E*)-astaxanthin was purchased from Sigma-Aldrich Co. Ltd. (Dorset, United Kingdom), and (all-*E*)-adonirubin and (all-*E*)-adonixanthin were obtained from JXTG Nippon Oil & Energy Corporation. HPLC-grade acetone, ethyl acetate, hexane, and dichloromethane (CH_2Cl_2), and analytical-grade acetone were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The basal diet used in this study was purchased from Kameya Syoji Co., Ltd. (Ginan, Japan), and its ingredients and chemical composition are shown in Table 1. The chemical composition of the feed was analyzed as a mixed condition. Although the amino acid composition of the feed was not analyzed in this study, the feed was mixed by the feed company so as to meet the amino acid requirements for layers producing 56 g of egg mass a day. The amino acid requirements were based on the Japanese Feeding Standard for Poultry (2011), edited by the National Agriculture and Food Research Organization.

Table 1. Ingredients and nutrient composition of the basal diet.

Ingredient	g/kg
Corn	566.0
Soybean meal	174.0
Limestone	93.4
Corn gluten meal	50.0
Rice bran	30.0
Vegetable oil	27.9
Fish meal	25.0
Corn gluten feed	20.7
Calcium phosphate	8.60
Sodium chloride	4.40
Chemical Composition (Dry Matter Basis)	**g/kg**
Metabolizable energy (kcal/kg)	2720
Dry matter	898.4
Organic matter	850.3
Crude protein	183.4
Ether extract	69.2
Crude fiber	39.3
Neutral detergent fiber	797.2
Acid detergent fiber	297.6
Nitrogen free extract	558.4
Crude ash	149.7
Calcium	15.5
Phosphorus	9.6

2.2. Feeding Experiments

Lohmann Julia Lite hens (24 weeks old) housed in individual cages (19.5 × 39.0 × 39.5–43.0 cm) were used in this study. The laying hens were randomly assigned into three groups: basal diet alone, basal diet with Panaferd-AX, or basal diet with Panaferd-P (astaxanthin content: 8 mg/kg diet). The dietary astaxanthin content used in this study was determined to provide sufficient egg yolk pigmentation in accordance with previous studies [12,13]. After moving the hens to their individual cages, they were fed with the basal diet for a week, followed by the experimental diets for 21 days. During the experiment, the laying hens were allowed free access to water and each diet. The evaluation of the egg quality was conducted at 4, 7, 14, and 21 days from the start of experimental feeding. The concentrations of astaxanthin, adonirubin, and adonixanthin, and their *Z*-isomer ratios in yolk were determined using normal-phase HPLC as described below (Section 2.3). The egg yolk color, assessed by the yolk color fan score and lightness (L*), redness (a*), and yellowness (b*) values, as well as the other egg qualities such as egg weight, yolk weight, albumen height, and the Haugh unit, were evaluated using an Egg Multi Tester (EMT-7300; Robotmation Co., Ltd., Tokyo, Japan) and a color spectrophotometer (CM-700d; Konica Minolta, Inc., Tokyo, Japan). These experiments were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Meijo University.

2.3. HPLC Analysis

The determination of the carotenoid (astaxanthin, adonirubin, and adonixanthin) concentrations and their *E*/*Z*-isomer ratios in egg yolk were performed using normal-phase HPLC with a Phenomenex silica gel column (Luna 5 µm Silica (2), 150 mm × 4.6 mm, 100 Å) [16,32,33]. Several studies successfully separated astaxanthin, adonirubin, and adonixanthin isomers by normal-phase HPLC with the Phenomenex silica gel column using hexane/acetone (83:17, v/v) [32] and hexane/ethyl acetate/acetone (70:20:10, v/v/v) solvent systems [16,33]. However, these solvent systems cannot differentiate the peaks of the carotenoid isomer and the peaks derived from components originally contained in the egg yolk. Hence, to clearly separate them, a new hexane/ethyl acetate/acetone (75:23:2, v/v/v) solvent system was used in this study. The flow rate and column temperature were set at 1.2 mL/min and 40 °C, respectively. The detection and quantification of the carotenoid isomers were performed by peak area integration at 470 nm using a photodiode array detector (SPD-M20A; Shimadzu, Kyoto, Japan). The peaks of astaxanthin, adonirubin, and adonixanthin isomers were identified according to HPLC retention times, visible spectral data (absorption maxima and shape of spectrum), and relative intensities of the *Z*-peak (350–370 nm) compared to the main absorption peak of the isomer (Figure S1; *Q*-ratio) [16,32–34]. The carotenoid *Z*-isomer ratio (%) was estimated as the amount of total *Z*-isomers to the amount of total carotenoid isomers including the all-*E*-isomer.

2.4. Carotenoid Extraction from Egg Yolk

The extraction of astaxanthin, adonirubin, and adonixanthin isomers from egg yolk was performed using acetone, following previously established protocols [22,27]. All procedures were performed at room temperature unless otherwise indicated, and light exposure was kept to a minimum throughout the extraction. Approximately 1 g of egg yolk was weighed into a 100-mL screw-capped glass bottle, and 50 mL of acetone was added to the bottle. Carotenoid extraction was performed using ultrasonic treatment (CPX1800H-J; Yamato Scientific Co., Ltd.) at 80 W and 38 kHz for 15 min on ice (approximately 5 °C). The residue was removed with a 0.22-µm polytetrafluoroethylene (PTFE) membrane filter (Osaka Chemical Co., Ltd., Osaka, Japan), and the filtrate containing carotenoid isomers was dried under reduced pressure at 35 °C for 5 min. The obtained extract was dissolved in a defined amount of ethyl acetate and filtered through a 0.22-µm PTFE membrane filter for HPLC analysis.

2.5. Statistical Analysis

Data were statistically analyzed by analysis of variance (ANOVA) and the Tukey's multiple comparison tests using a commercially available computer program (Excel Statistics; SSRI Co., Ltd.). Differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

3.1. Profile of Carotenoid Isomers in Egg Yolk

The use of reversed-phase HPLC with C_{18} or C_{30} columns and normal-phase HPLC with a silica gel column to separate astaxanthin isomers has been well documented [16,32–38]. However, ample studies have shown that normal-phase HPLC exhibits a greater separation ability than that by reversed-phase HPLC when separating carotenoid isomers [16,32–38]. In addition, a sufficient separation of adonirubin and adonixanthin isomers can be obtained in normal-phase HPLC [16,32–34]. Thus, this study used normal-phase HPLC with a Phenomenex silica gel column to separate astaxanthin, adonirubin, and adonixanthin isomers. However, previously reported solvent systems using hexane/acetone (83:17, v/v) [32] and hexane/ethyl acetate/acetone (70:20:10, v/v/v) [16,33] do not clearly separate peaks corresponding to the carotenoid isomer and components originally contained in the egg yolk. Hence, we used an improved solvent system using hexane/ethyl acetate/acetone (75:23:2, v/v/v). On the other hand, we also evaluated other solvent systems, such as hexane/ethyl acetate (82:12, v/v), hexane/acetone (82:12. v/v), and hexane/ethyl acetate/acetone (76:16:8, 75:15:10, and 75:20:5, v/v/v), but their good separation could not be obtained. The typical chromatograms of a mixture of astaxanthin, adonirubin, and adonixanthin isomers, obtained by the thermally *Z*-isomerized treatment of their all-*E*-isomer standards at 80 °C for 3 h in CH_2Cl_2 [38–40], as well as extracts from Panaferd-AX, Panaferd-P, and egg yolks before and after the 21-day feeding of the experimental diets, are shown in Figure 2. This improved HPLC method could not only clearly separate astaxanthin, adonirubin, and adonixanthin isomers (peaks 1–10; peaks 5 and 6 were tentatively identified as (9*Z*)-astaxanthin and a mixture of (13*Z*)- and (15*Z*)-astaxanthin according to previous studies [16,32–34]; Table S1), but also their isomers and egg yolk-derived peaks (denoted by asterisks).

In Panaferd-AX and Panaferd-P dried cell powders, large peaks from (all-*E*)-astaxanthin, (all-*E*)-adonirubin, and (all-*E*)-adonixanthin were observed (Figure 2b,c), as in previous studies [16,41], and minor peaks from the Z-isomers of astaxanthin and adonirubin were detected. Before the feeding with the experimental diets, the astaxanthin, adonirubin, and adonixanthin isomer peaks were not detected in egg yolk, but large peaks from lutein and zeaxanthin were observed (Figure 2d). After 21 days of feeding diets containing Panaferd-AX and Panaferd-P, the peaks from astaxanthin, adonirubin, and adonixanthin isomers could be detected (Figure 2e,f). To the best of our knowledge, this is the first study to show that feeding laying hens with valuable rare carotenoids such as adonirubin and adonixanthin causes their accumulation in the hens' egg yolks. Furthermore, it is interesting that the egg yolk contained an abundance of astaxanthin *Z*-isomers despite the hens being fed with diets rich in (all-*E*)-astaxanthin. This data contributes to improving the nutritional value of egg yolk by using *P. carotinifaciens* for pigmentation.

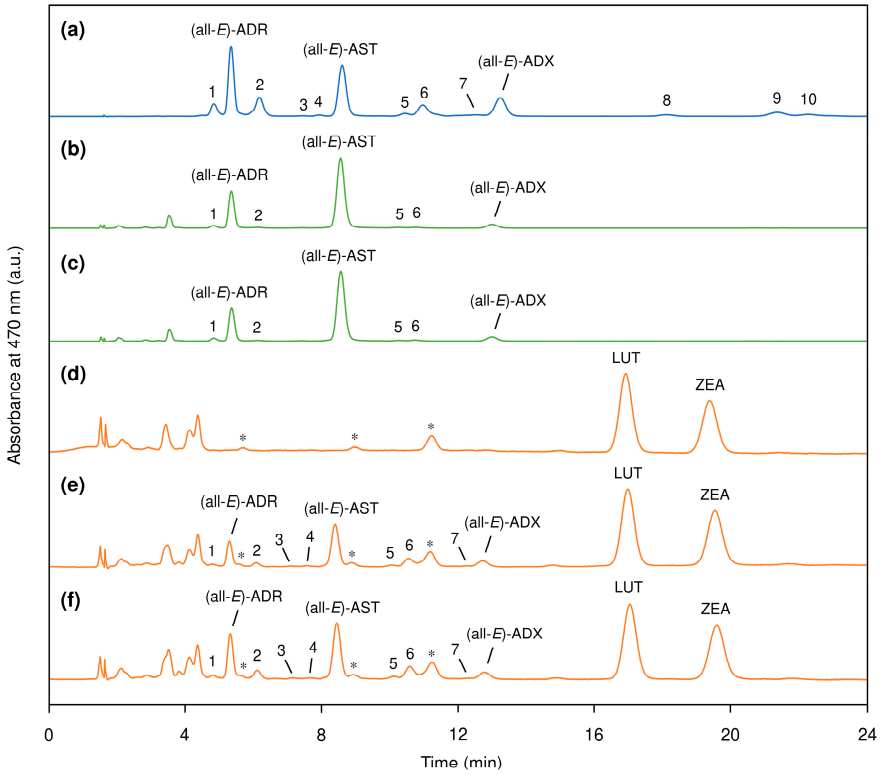

Figure 2. Normal-phase HPLC chromatograms of (**a**) a standard mixture of (all-*E*)-astaxanthin (AST), (all-*E*)-adonirubin (ADR), and (all-*E*)-adonixanthin (ADR), which were treated with CH_2Cl_2 at 80 °C for 3 h in order to increase their *Z*-isomers, extracts from (**b**) Panaferd-AX, (**c**) Panaferd-P, (**d**) egg yolk before feeding the experimental diets, and egg yolks after feeding diets containing (**e**) Panaferd-AX and (**f**) Panaferd-P. Peak identifications: 1, 2 = adonirubin *Z*-isomers, 3, 4 = astaxanthin *Z*-isomers, 5 = (9Z)-astaxanthin, 6 = (13Z + 15Z)-astaxanthin, 7–10 = adonixanthin *Z*-isomers, LUT = lutein, ZEA = zeaxanthin [16,32–34]. Asterisk symbols (*) show several egg yolk-derived compounds. Several peaks (1–10) were tentatively identified, as shown in Table S1.

3.2. Evaluation of Carotenoid Concentration and Z-isomer Ratio in Egg Yolk

The effects of feeding laying hens diets containing *P. carotinifaciens* of different particle sizes (Panaferd-AX and Panaferd-P) on the concentrations of astaxanthin, adonirubin, and adonixanthin and their total *Z*-isomer ratios in egg yolk are shown in Figures 3–5. Astaxanthin, adonirubin, and adonixanthin could be detected in egg yolk from the fourth day of feeding, and the carotenoid concentrations reached their maximum levels around 14–21 days in both Panaferd-AX- and Panaferd-P-containing diets. When hens were fed with a diet containing Panaferd-P, the egg yolk concentrations of astaxanthin, adonirubin, and adonixanthin were significantly higher than those in hens fed with a diet containing Panaferd-AX. This is likely due to the smaller particle size of Panaferd-P. Several studies have shown that carotenoids' bioavailability depends on their particle size, such that the micronization of carotenoids effectively enhances their bioavailabilities [42,43]. In addition, since Panafed-P was obtained by pulverizing Panafed-AX, the release of carotenoids from the microorganism cell by pulverization treatment would also improve their bioaccessibilities [44]. This theory is supported by Li et al. [19]; that is, the breakup of the cell wall by pulverization treatment would increase the ability of hens to access the carotenoids in the feeds, facilitating their uptake and incorporation into the egg yolk. These improvements to carotenoid bioaccessibility and/or bioavailability would result in increased concentrations of astaxanthin, adonirubin, and adonixanthin in the egg yolk of hens fed with Panafed-P. When fed with diets containing Panaferd-AX and Panaferd-P (astaxanthin content: 8 mg/kg diet) for 21 days, the astaxanthin concentration in egg yolk reached 1.21 μg/g and 1.85 μg/g, respectively. Akiba et al. [12] reported similar concentrations of astaxanthin in egg yolk upon feeding

a diet containing *P. rhodozyma*, a yeast that has been commercially used for pigmenting hens' egg yolk. They found that laying hens fed with this diet (astaxanthin content: 8 mg/kg diet) for 28 days produced eggs with yolk astaxanthin concentrations of 1.39 μg/g. These results indicate that, similar to *P. rhodozyma*, *P. carotinifaciens* is commercially suitable for pigmenting a hen's egg yolk.

The total *Z*-isomer ratios of astaxanthin, adonirubin, and adonixanthin in Panaferd-AX were 3.9%, 11.3%, and 5.0%, respectively, and those in Panaferd-P were 4.2%, 11.6%, and 5.3%, respectively. In contrast, in both diets, the *Z*-isomer ratios of astaxanthin, adonirubin, and adonixanthin in egg yolk were approximately 25%, 20%, and 7%, respectively (Figure 3b, Figure 4b, and Figure 5b). These results suggest that the carotenoid *Z*-isomer ratio in egg yolk differs depending on the types of carotenoids. For example, astaxanthin and lycopene (reported in our previous study [26]) had higher *Z*-isomer contents. This may be related to the fact that the *Z*-isomers of astaxanthin and lycopene are more bioavailable than the all-*E*-isomers [24,28]. Since the mechanisms underlying the differences in carotenoid *Z*-isomer ratios in egg yolk remain unknown, future studies should be conducted on this topic. In any case, the fact that high amounts of astaxanthin are present as *Z*-isomers (over 25%) in egg yolks of hens fed with astaxanthin-rich sources (Panaferd-AX and Panaferd-P) is a very important finding in relation to their nutritional value.

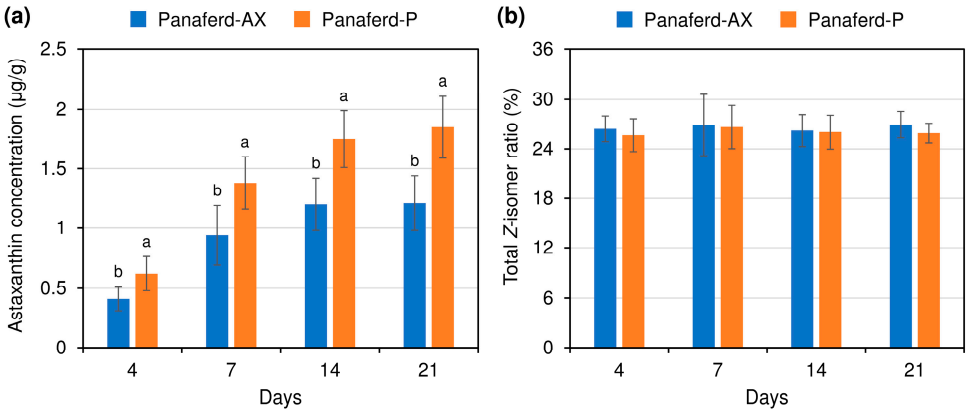

Figure 3. Effects of feeding laying hens with diets containing *P. carotinifaciens* of different particle sizes (Panaferd-AX and Panaferd-P) for 21 days on the (**a**) astaxanthin concentration and (**b**) total *Z*-isomer ratio in egg yolk. Error bars depict the standard deviation (n = 8). Means with different letters within each day are significantly different ($p < 0.05$), whereas means with similar letters are not different.

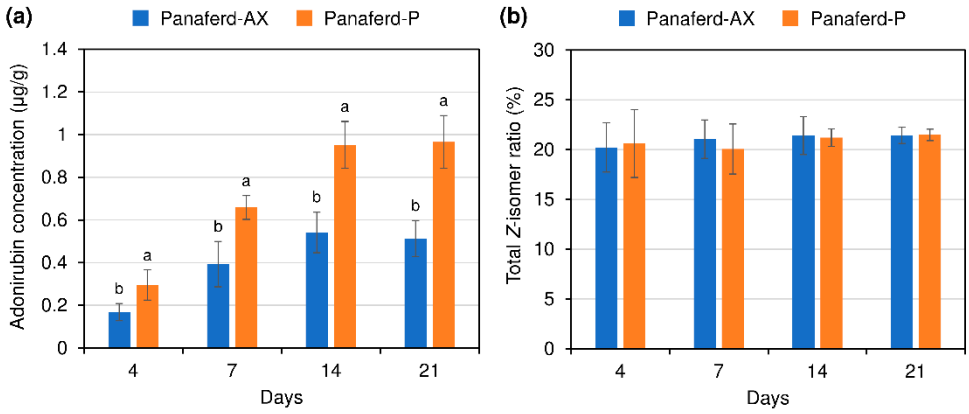

Figure 4. Effects of feeding laying hens with diets containing *P. carotinifaciens* of different particle sizes (Panaferd-AX and Panaferd-P) for 21 days on the (**a**) adonirubin concentration and (**b**) total *Z*-isomer ratio in egg yolk. Error bars depict the standard deviation (n = 8). Means with different letters within each day are significantly different ($p < 0.05$), whereas means with similar letters are not different.

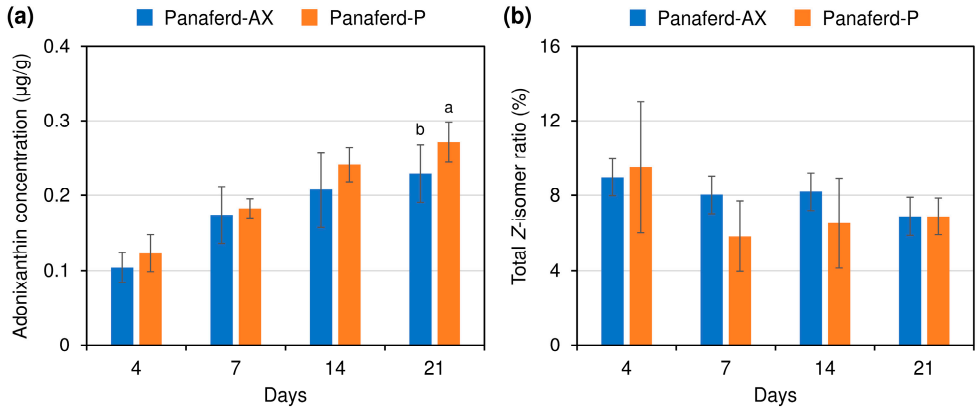

Figure 5. Effects of feeding laying hens with diets containing *P. carotinifaciens* of different particle sizes (Panaferd-AX and Panaferd-P) for 21 days on the (**a**) adonixanthin concentration and (**b**) total *Z*-isomer ratio in egg yolk. Error bars depict the standard deviation (n = 8). Means with different letters within each day are significantly different ($p < 0.05$), whereas means with similar letters are not different.

3.3. Evaluation of Egg Yolk Pigmentation

The evaluation of the egg yolk pigmentation was performed by examining the appearances (Figure 6), the yolk color fan score, and L*, a*, and b* values of the egg yolk (Figure 7). Feeding hens with diets containing Panaferd-AX and Panaferd-P markedly improved the yolk appearance as well as the color fan score; the score increased by a value of two or more compared to the control group. When comparing Panaferd-AX- and Panaferd-P-containing diets, the latter had a significantly higher yolk color fan score than the former. These results are correlated with the egg yolk concentrations of astaxanthin, adonirubin, and adonixanthin. In this study, when hens were fed with diets containing *P. carotinifaciens* (astaxanthin concentration: 8 mg/kg), the yolk color fan score was over 14. In contrast, when fed with diets rich in lutein such as alfalfa and marigold flower (lutein concentration: approximately 18–30 mg/kg) [2] or diets rich in lycopene such as tomatoes (lycopene concentration: 100 mg/kg) [27], the yolk color fan scores were around 8–12. This suggests that the pigmentation efficiency of astaxanthin in egg yolk is higher than for other carotenoids such as lutein and lycopene. Furthermore, feeding hens with Panaferd-AX and Panaferd-P increased the a* value and decreased the L* and b* values. The appearances of egg yolks showed these trends well (Figure 6); that is, the feeding of the astaxanthin-rich diets contributed to a redness enhancement of the egg yolk.

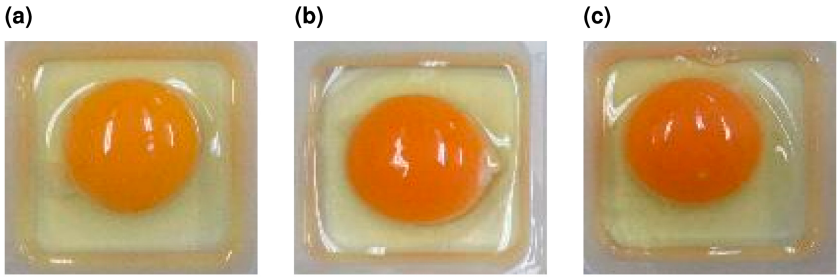

Figure 6. Appearances of egg yolks (**a**) before and after 21 days of feeding hens with diets containing *P. carotinifaciens* powders of different particle sizes: (**b**) Panaferd-AX and (**c**) Panaferd-P.

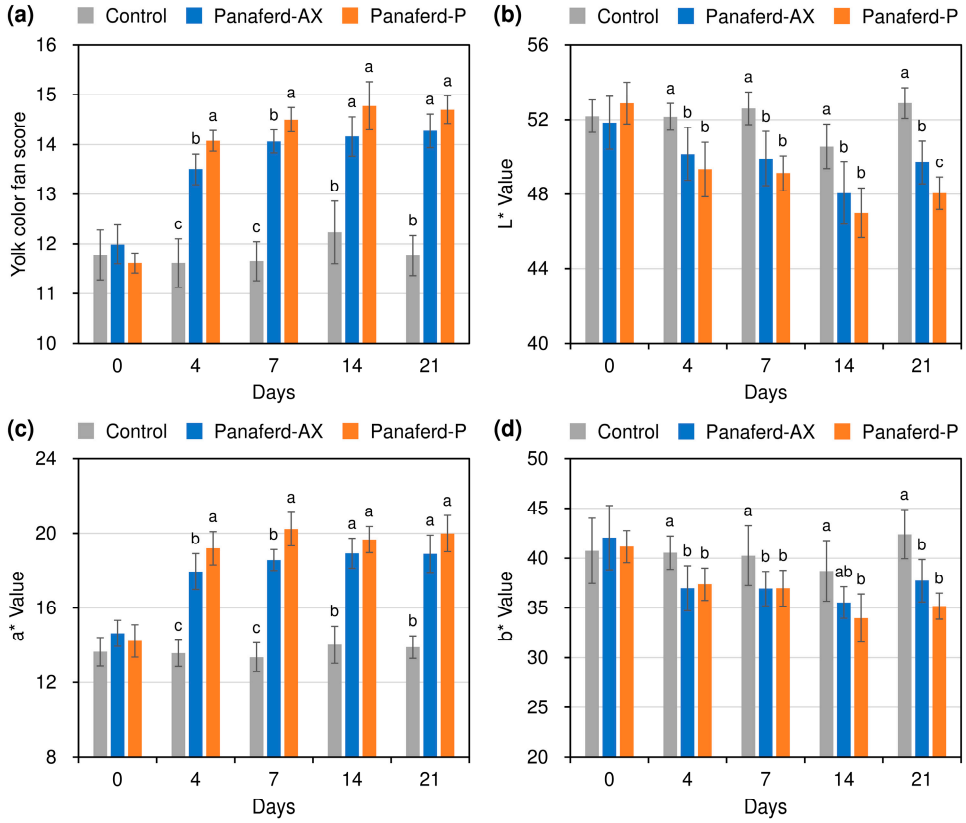

Figure 7. Effects of feeding laying hens with diets containing *P. carotinifaciens* of different particle sizes (Panaferd-AX and Panaferd-P) for 21 days on the (**a**) yolk color fan score and (**b**) L*, (**c**) a*, and (**d**) b* values of egg yolk. Error bars depict the standard deviation (n = 8). Means with different letters within each day are significantly different ($p < 0.05$), whereas means with similar letters are not different.

3.4. Evaluation of Other Egg Qualities

The effects of feeding hens with diets containing Panaferd-AX- and Panaferd-P on egg qualities (egg weight, yolk weight, albumen height, and Haugh unit) have been shown in Table 2. There were no significant differences in egg weight, yolk weight, albumen height, and the Haugh unit between the different diets (with and without adding *P. carotinifaciens*). Furthermore, the particle size of the dried *P. carotinifaciens* cell powder did not affect the above-mentioned egg qualities. Yang et al. did not observe significant differences in egg weight, yolk height, and the Haugh unit when feeding 1.3 mg/kg astaxanthin to Hy-Line Brown laying hens [45]. Walker et al. also did not observe significant effects on the yolk weight and the Haugh unit when Hy-Line W-36 hens were fed up to 400 mg/kg of astaxanthin in their diets [6]. Our results are consistent with these previous studies in that they suggest that feeding hens with astaxanthin does not have significant effects on the egg quality other than the carotenoid concentration and egg color.

Table 2. Effects of feeding laying hens with diets containing *P. carotinifaciens* powders of different particle sizes (Panaferd-AX and Panaferd-P) on egg quality ([1] Feeding diets without *P. carotinifaciens*; [2] Calculated as $100 \times \log (H + 7.6 - 1.7 \times W^{0.37})$, where H is the albumen height (mm) and W is the egg weight (g)).

		Diet				
Items	**Days**	**Control** [1]	**Panaferd-AX**	**Panaferd-P**	***SEM***	***p*-Value**
Egg weight (g)	0	57.3	57.7	55.3	4.8	0.576
	4	58.0	55.8	59.2	5.8	0.517
	7	58.8	58.4	59.0	3.6	0.947
	14	60.4	58.6	59.9	3.0	0.503
	21	59.8	59.7	61.0	3.6	0.701
Yolk weight (g)	0	13.8	14.6	13.0	2.6	0.459
	4	14.2	13.7	15.2	2.8	0.578
	7	14.9	14.5	14.6	1.1	0.794
	14	15.5	14.9	15.3	1.0	0.442
	21	16.0	15.7	15.6	0.8	0.626
Albumen height (mm)	0	7.0	7.1	7.9	1.0	0.121
	4	7.7	8.1	7.4	1.6	0.706
	7	7.4	7.7	7.7	1.2	0.885
	14	8.3	7.6	8.5	1.2	0.250
	21	7.1	7.5	6.8	1.3	0.598
Haugh unit [2]	0	84.7	84.3	89.9	10.1	0.468
	4	91.6	93.4	86.2	10.6	0.384
	7	86.6	91.5	89.1	7.5	0.441
	14	94.6	90.4	95.7	6.4	0.240
	21	83.2	87.0	82.8	10.1	0.664

4. Conclusions

Feeding hens with dried *P. carotinifaciens* cell powders (Panaferd-AX and Panaferd-P) increased the concentrations of valuable carotenoids (astaxanthin, adonirubin, and adonixanthin) in their egg yolk and enhanced the egg yolk pigmentation. Their pigmentation efficiencies were improved by the pulverization of the *P. carotinifaciens* powder. Moreover, this study accurately evaluated the total *Z*-isomer ratios of astaxanthin, adonirubin, and adonixanthin in egg yolk for the first time, using normal-phase HPLC with an improved solvent system. The all-*E*-isomers of carotenoids are the most predominant geometric isomers in *P. carotinifaciens*. However, large amounts of astaxanthin and adonirubin *Z*-isomers were present (over 25% and 20% of the total *Z*-isomer ratio, respectively) in the egg yolk. Since several types of carotenoid *Z*-isomers, including astaxanthin, have a higher bioavailability and bioactivity than the all-*E*-isomers, the information produced by this study is valuable for enhancing the nutritional value of hens' egg yolk through feeding hens astaxanthin-rich diets.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-8994/12/6/923/s1, Figure S1: Calculation of Q-ratio as an indicator of the Z-peak intensity, Table S1: Absorption maxima and relative intensities of the Z-peaks for adonirubin, astaxanthin, and adonixanthin isomers.

Author Contributions: Conceptualization, M.H., Y.K., and Y.H.; analysis, M.H., Y.K., and Y.H.; investigation, M.H., Y.K., S.J. and Y.H.; resources, Y.K., K.H., and T.U.; writing—original draft preparation, M.H. and Y.H.; writing—review and editing, Y.K., K.H., T.U., and S.J.; project administration, K.H. and T.U; All authors have read and agreed to the published version of the manuscript.

Funding: This work was partly supported by JSPS KAKENHI Grant Number 19K15779.

Acknowledgments: We thank our laboratory members for their helpful discussions.

Conflicts of Interest: The authors declare no conflict of interest.

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