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Carotenoids Digestibility of Free Astaxanthin and Lutein by Fancy Carp (*Cyprinus carprio*)

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ABSTRACT

The utilization of 500 µg of astaxanthin and lutein was determined in fancy carp. Serum concentration in relation to time curves for astaxanthin and lutein were best fitted to a one-compartment pharmacokinetic model. The results showed that after a single dose, the maximum concentration of free astaxanthin and lutein in the serum (C max) was 54.20 and 78.40 µg.mL^{-1} after 6 and 24 hr, respectively. The volume of distribution was 1,958.80 and 237.20 mL.kg^{-1}, total body clearance was 8.92 and 3.50 mL.hr^{-1}.kg^{-1}, and the areas under the curve were 5,300.00 and 2,082.90 µg.hr.mL^{-1}, respectively. These results indicated that astaxanthin was absorbed more readily than lutein in fancy carp fed on a diet of astaxanthin and lutein. There were significant differences in the fecal-carotenoid contents in the samples collected from carp fed on the two carotenoid diets. The digestibility of astaxanthin and lutein was 81.05 and 70.46%, respectively. Carotenoids leaching from the diets after immersion in water revealed that the percentage loss of astaxanthin and lutein ranged between 0.69–4.68 and 0.82–10.33% of the original amounts, respectively. These values suggested that feed containing carotenoid should be consumed within 15 min in order to avoid loss of immersed carotenoid supplements.

Keywords: digestibility, fancy carp, astaxanthin, lutein, leaching

INTRODUCTION

Currently, in Thailand, fancy carp (*Cyprinus carprio*) are widely exported and generate substantial amounts of money. Fancy carp of the proper size and colors are in high demand and are traded by both Thai and international entrepreneurs (Paripatananont *et al*., 1999). The first-grade and second-grade fish are regularly exported while the rest are traded domestically. However, there is a problem as the fish that are raised locally appear pallid. The colors found on the fish skin are generated by carotenoid which provides yellow, orange and red hues. Since fish cannot synthesize carotenoid themselves, it is therefore essential to feed it to them directly (Goodwin, 1984). Research conducted on the skin of goldfish (*Carassius auratus*) and fancy carp (*Cyprinus carpio*) has indicated that they have similar characteristics; most of the carotenoids in goldfish and fancy carp are zeaxanthin, lutein, α-doradexanthin, β-doradexanthin, astaxanthin, β-carotene and idoxanthin (Goodwin, 1984). Yellow, orange, and red hues found on fish skin are caused by chromatophores which are xanthophores and erythrophores. Research studies on carotenoid utilization have revealed that pharmacokinetic parameters could identify the amount of carotenoid

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being absorbed and that this could be used to consider the relative efficiency of each carotenoid (Gibaldi and Perrier, 1982). However, there have been few studies on the absorption and defecation of carotenoid in fancy carp. Pharmacokinetic research is conducted to quantify the amount of carotenoid circulating in the blood after a single feed has been given and digested. It is also notable that different fish are able to utilize carotenoid with varying levels of efficiency. The present study was undertaken: 1) to evaluate the pharmacokinetic parameters of astaxanthin and lutein in fancy carp after a single dose of oral administration, 2) to study the digestibility of two carotenoid diets fed to fancy carp and 3) to determine the leaching rates associated with the astaxanthin and lutein diets. It was expected that the results of this research would provide sound recommendations for the use of carotenoid in the diet of fancy carp.

MATERIALS AND METHODS

Fish and feed trial

A mixed sex school of fancy carp with a weight (mean ± SD) of 26.93 ± 5.14 g per fish was maintained on a non-pigmented diet for a co-variant period of two weeks prior to being fed with the experimental diets and tested in four replicates. Each treatment was randomly distributed to a separate 20 L aquarium. The diets were designed to achieve a target level of 500 µg for astaxanthin and lutein per fish. Astaxanthin was supplied in a finished product concentration of 10 $g.kg^{-1}$ astaxanthin (BASF, Thailand). Lutein from marigold extract (*Tagetes* spp.) was supplied in a finished product containing 15 $g.kg^{-1}$ (Kemin Industries, Thailand). After administration of a single dose orally, fish were not fed further meals. The feed ingredients were prepared and mixed by a mincer (3 HP Hobart mincer Model 4730). The dry mix was pelletized in a single 30 cm screw from which a feed pellet size of 2–3 mm was obtained. Finally, the feed was dried in a hot air oven at 50 °C for approximately 12 hr.

Sampling procedure

Fish were not fed for 3 days prior to receiving a single dose feeding. A control blood sample was taken before treatment (0 hr) to measure basal serum astaxanthin and lutein levels. Blood was collected from the caudal vein using 1 mL non-heparinized disposable syringes fitted with 0.55 × 25 mm disposable needles. Blood sampling was administered at 0, 15, 30 min and 1, 3, 6, 12, 24, 36, 48, 72, 96 and 120 hr after single dose feeding, with three fish being sampled at each sampling time. Serum was immediately separated from blood and stored at -20 °C prior to sample analysis.

Chemical composition

The nutrient composition of the diets was analyzed according to AOAC (1990): dry matter after drying in an oven at 105 °C until constant weight, ash content by incineration in a muffle furnace at 600 °C for 6 hr, crude protein content (N × 6.25) by the Kjeldahl method after acid digestion, and crude lipid content by petroleum ether extraction in a Soxhlet apparatus (Table 1).

Carotenoid determination

All experimental diets were extracted with acetone, together with butylated hydroxytoluene (250 $mg.kg^{-1}$) added as an antioxidant, until samples showed no color. Following this, petroleum ether (5 mL) was added and then water was added to the mixture using a separating funnel. Then careful mixing by swirling separated the two phases. In this study, only the upper phase of the extraction of the diets was collected and then determined for maximum absorbance wavelength range over 350–600 nm. The maximum absorbance value (λ max) was measured and total carotenoids calculated from its extinction coefficient (E_{1cm} 2500) by Beer-Lambert's law (Britton, 1995). Serum was vortexed with 1 mL of ethanol for 30 s, then 2

Table 1 Ingredients and proximate composition of experimental diets.

Ingredient ($g.kg^{-1}$)	Experimental diets	
	Astaxanthin	Lutein
Fish meal	300	300
Soybean meal	240	240
Rice bran	240	240
Tapioca starch	50	50
Wheat Flour	50	50
Fish oil	20	20
Lecithin	20	20
Alpha-starch	50	50
Dicalciumphosphate	10	10
Premix	20	20
Total	1000	1000
Nutrient composition by analysis ($g.kg^{-1}$ dry weight basis, mean ± SD)		
Protein	295.86 ± 2.05	
Fat	51.09 ± 0.29	
Moisture	64.93 ± 0.74	
Ash	94.79 ± 0.96	
Free astaxanthin (µg)	479.54 ± 9.39	-
Lutein (µg)	-	488.82 ± 8.66

Premix contained the following diluted in cellulose ($g.kg^{-1}$ mix): vitamin A 6,700,000 IU, vitamin D3 1,350,000 IU, vitamin E 3,000 IU, vitamin K3 33.4 g, vitamin B1 6.7 g, vitamin B2 10 g, vitamin B6 8 g, vitamin B12 13.5 mg, niacin 53 g, pantothenic acid 26.5 g, folic acid 3.3 g, biotin 335 mg, inositol 135 g and vitamin C activity from Stay C-35 105 g.

mL of petroleum ether was added and the mixture was vortexed again for 1 min. The petroleum ether phase was separated by centrifuging at 300×*g* at 25 °C for 10 min (White, 2002).

Instrumentation and chromatographic conditions

The resulting upper phase of the experimental diets and the serum from the petroleum ether extraction were evaporated under a gentle stream of nitrogen gas. This was followed by redissolving the crude carotenoids in petroleum ether and 5 µL of the solution was spotted (10 × 20 cm with 0.25 mm thickness) on pre-coated silica gel 60 (Merck, Germany) using a sample applicator (CAMAG Linomat IV, CAMAG, Switzerland). A constant application rate of 4 $\mu L.s^{-1}$ was employed and the space between two spots was 14 mm. The slit dimension was kept at 5 × 0.45 mm and the scanning speed was 20 $mm.s^{-1}$. The mobile phase consisted of petroleum:ether-diethyl:ether-acetone (75:15:10 v/v/v). Linear ascending development was carried out in a twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The length of the chromatogram runs was established at 70 mm. Densitometric scanning was performed using a CAMAG thin layer chromatogram (TLC) scanner III (CAMAG, Switzerland) in the absorbance mode at 450 nm. The source of radiation utilized was a tungsten lamp (Mantiri *et al*., 1996; Sherma and Bernard, 2003). In order to identify the correct value of astaxanthin and lutein, the TLC runs were always conducted based on authentic standards for astaxanthin

(Sigma, USA) and lutein (Chromadex, Canada). The developed TLC-densitometric analysis for quantitative determination of serum carotenoid was validated by determination of linearity, %recovery, %repeatability relative standard deviation (RSD_r), limit of detection (LOD), limit of quantification (LOQ) and HORRAT(r) value. The data indicated that this method can be successfully used for the analysis of serum carotenoids in fancy carp with good recovery and precision (Yuangsoi *et al*., 2008).

Pharmacokinetic parameter analysis

The astaxanthin and lutein serum concentration time curves best fitted the one-compartment pharmacokinetic model. The mean serum astaxanthin and lutein concentration time curve data points of each of the three replicates were then analyzed using a standard pharmacokinetic equation by Evans (2004).

Digestibility

The carotenoids digestibility of the carotenoid diets was determined by the direct method that involves measuring all the feed consumed by the fish. In the present study, feces were collected for the direct method five hours after fancy carp were fed with the carotenoid diet at a 500 µg dose. Pooled samples of about 1 g of feces (wet weight) were immediately analyzed by the TLC-densitometric analysis method described above.

Leaching rates for carotenoid diets

The leaching rates of the carotenoid diets were tested on 5 g of feed in 250 mL of water at three immersion time intervals of 15, 30 and 60 min involving static water leaching under normal fish culture conditions. To assess the carotenoids leaching rates, experiments were conducted in an environment-controlled room where the temperature was maintained at 25 ± 2 °C. The astaxanthin and lutein concentrations of water soluble carotenoids were measured by UV-Vis spectrometry and then determined for the maximum absorbance wavelength in the range 350–600 nm. The maximum absorbance value (λ max) was measured and astaxanthin calculated with an extinction coefficient of $E_{1\%} = 2100$, while lutein was calculated with an extinction coefficient of $E_{1\%} = 2480$ from Beer-Lambert's law (Britton, 1995). The leached feed and original feed samples were dried in a convection oven at 105 °C for 24 hr and then cooled in a desiccator. Dried feed samples were weighed and analyzed to determine the dry matter retention after leaching and the dry matter of the original samples expressed as percentages.

Statistical analysis

Mean values and standard deviations were calculated from the raw data. Two-group comparisons of the pharmacokinetic parameters of the carotenoids diets were performed with independent *t*-tests. One way analysis of variance was applied for comparison of the mean values, with $P \leq 0.05$ used as the level of significance.

RESULTS AND DISCUSSION

Utilization of carotenoids in fancy carp

The utilization of carotenoids in fancy carp was studied by feeding a school of fish with a single dose of a carotenoids diet at 500 µg. The data showed that the maximum absorbance with the astaxanthin and lutein diets was 468 and 444 nm, respectively. The amount (mean ± SD) of astaxanthin and lutein in the original diets was 479.54 ± 9.39 and 488.82 ± 8.66 µg, respectively. The carotenoid composition and profiles in the diets were identified by TLC-densitometry analysis. The carotenoid composition of the diets showed that lutein and astaxanthin had an average retention factor (R_f) of 0.18 and 0.21, respectively.

Pharmacokinetic parameters of fancy carp fed on deprived diets

To compare the pharmacokinetic parameters of carotenoids in the serum, samples were collected from fancy carp at 15 and 30 min and 1, 3, 6, 12, 24, 36, 72, 96 and 120 hr after being fed the diets supplemented with 500 µg of astaxanthin and lutein. The fancy carp fed the astaxanthin diet were found to have a maximum serum concentration of astaxanthin (C max) of 54.2 $\mu g.mL^{-1}$ after 6 hr (T max), while the fancy carp fed the lutein-supplemented diet had a C max of 78.4 $\mu g.mL^{-1}$ after 24 hr (T max) indicating that there was faster absorbability in the fancy carp fed the astaxanthin-supplemented diet compared with the ones fed on the lutein diet because the maximum serum concentration was detected in a shorter period.

The volume of carotenoids in the blood system (Vd) for the fancy carp fed on the astaxanthin diet was 1,958.8 $mL.kg^{-1}$, while the fancy carp fed on the lutein diet had a Vd value of 237.2 $mL.kg^{-1}$. The Vd value is related to the area-under-curve values (AUC) (Evans, 2004). The fancy carp fed with astaxanthin had an AUC value of 5,300.00 $\mu g.hr.mL^{-1}$ compared to the lower value of 2,082.90 $\mu g.hr.mL^{-1}$ for the fancy carp fed with lutein. The AUC illustrates the connection between the carotenoids level in the serum over time, which also relates to the quantity of carotenoids absorbed into the body. If the AUC value is high, then carotenoids can be easily absorbed into the body. However, in measuring the excretion of carotenoid levels (CL), it was found that the fancy carp fed with astaxanthin had a CL value of 8.92 $mL.hr^{-1}.kg^{-1}$, while the fancy carp fed with lutein had an excretion value of 3.50 $mL.hr^{-1}.kg^{-1}$. This indicated that astaxanthin in the body system can be better excreted or metabolized, compared to lutein. Excretion helps eliminate carotenoids from the blood system, regardless of the process. Thus, it can be concluded that after receiving the experimental diets, the fancy carp fed on the astaxanthin diet had greater ability to absorb astaxanthin than the ones fed on the lutein diet, due to the fact that the time-maximum concentration (T max) was achieved over a shorter period. This was confirmed by measuring the pharmacokinetic parameters, Vd and AUC, which indicated better distribution in the blood system (Table 2).

The bioavailability of a given compound, that is the fraction of an administered dose which is absorbed into the circulatory system, is an important determinant factor of its efficiency and safety (Gibaldi and Perrier, 1982). However, there are few studies that have examined the absorption and elimination rates of carotenoids in fish. Some experiments in salmonids, using radio-labeled compounds, were carried out on carotenoid concentrations in the blood after ingestion of a

Table 2 Pharmacokinetic parameters for astaxanthin and lutein derived from serum concentration-time data of fancy carp.

Parameter	Unit	Parent diets	
		Astaxanthin (500 µg)	Lutein (500 µg)
Vd	$mL.kg^{-1}$	1958.80	237.20
CL	$mL.hr^{-1}.kg^{-1}$	8.92	3.50
AUC	$\mu g.hr.mL^{-1}$	5300.00	2082.90
C max	$\mu g.mL^{-1}$	54.20	78.40
T max	hr	6.0	24.0

Vd (area).kg^{-1} = Volume of distribution calculations; CL (area).kg^{-1} = Clearance calculations; AUC (area) = Area under the serum concentration time curve; C max (obs) = Maximum observed serum concentrations; T max = Observed time at which C max was achieved.

single dose with the relative bioavailability of astaxanthin being taken as a reference (Aas *et al*., 1999; White *et al*., 2002; Maltby *et al*., 2003; Choubert *et al*., 2005). Maltby *et al*. (2003) reported that Atlantic salmon (*Salmo salar*) fed with a single dose of astaxanthin exhibit better bioavailability by oral administration than by intraperitoneal injection (i.p.) with values of 12 and 8.7%, respectively. The bioavailability of rainbow trout (*Oncorhynchus mykiss*) after being fed a single dose of ^{14}C-keto carotenoids exhibited maximum concentrations of keto-carotenoids in the blood of the fish after16 hr for ^{14}C-astaxanthin and after 20 hr for ^{14}C-canthaxanthin (Choubert *et al*., 2005). This range in time period (16–20 hr) correlated closely with the present study.

The outcome of the present study shows that fancy carp can utilize astaxanthin better than lutein. Similarly, it was noted that Atlantic salmon fed with two types of diets (astaxanthin and lutein, and astaxanthin alone) possessed a higher volume of astaxanthin when compared to fish fed on a lutein diet (Olsen and Baker, 2006). It can be concluded that astaxanthin can be absorbed and accumulated better than lutein.

When comparing the pharmacokinetic parameters of metabolized carotenoids in the serum after feeding on astaxanthin and lutein diets, it was found that fancy carp treated with astaxanthin processed this with reductive metabolization which then produced lutein (C max) of 65.00µg. mL^{-1} after 12 hr (T max). The results show that in fish fed on an astaxanthin diet, the serum astaxanthin levels were decreased because of metabolic conversion. Over time, lutein concentration increased in the serum. Schiedt *et al*. (1985) working with rainbow trout reported results indicated that lutein was indeed carotenoid-derived as a reductive metabolite of astaxanthin based on minute traces of radioactivity in the lutein after feeding labeled (3S, 3'S),astaxanthin. Carotenoid metabolism in animals takes place as a result of enzymes which catalyze three main types of reaction—namely, 1) the substitution of carotenoid end groups (often β-end groups) by oxygen functions (-OH and -C=O); 2) the alteration of end groups, for example, of β to α; and 3) cleavage of the polyene chain to yield apocarotenoids and vitamin A (Davies, 1985). In many animals, the most important metabolic products of carotenoids are the retinoids, and the metabolic reactions of carotenoids in fish are essentially by oxidative pathways (Matsuno, 1991).

There was no significant difference between the amounts of free astaxanthin in the serum from fish fed with either of the diets. In other words, the amount of astaxanthin found in the lutein diet had a C max value of 40.90 µg.mL^{-1} while the fancy carp fed with astaxanthin had a C max value of 54.2 µg.mL^{-1}. However, there was a difference in T max where metabolization occurred after 24 hr for astaxanthin while it occurred in 6 hr for the astaxanthin diet. It can be concluded that the fancy carp fed on the lutein diet could convert the lutein to astaxanthin but required more time to accumulate astaxanthin into the blood system (Table 3). There are reports (Hata and Hata, 1972a, 1972b) showing that goldfish fed on a lutein diet had skin with a more reddish hue and that goldfish possessed the ability to transform lutein into astaxanthin, though their skin would turn from yellow to orange within seven days. Katayama *et al*. (1973) also reported that both goldfish and fancy carp share the ability to convert lutein into astaxanthin.

In the present study, five hours after fancy carp were fed the astaxanthin and lutein diets, feces were collected by a direct method. As shown in Table 4, the digestibility of astaxanthin and lutein was 81.05 and 70.46%, respectively. The digestibility of astaxanthin in this study correlated to the study by Choubert and Storebakken (1996) that used an automatic feces collector and reported 79.1% of ADC astaxanthin in rainbow trout. Digestibility studies on fish can be used to estimate the bio-availability of specific nutrients in their

diet. The collection of fish feces is associated with problems such as contamination of feces with uneaten feed and fish scales, and nutrient leaching from feces into the surrounding water, resulting in a possible overestimation of digestibility (Watanabe *et al*., 1996).

Leaching rates for carotenoid diets

Carotenoid leaching rates from carotenoid diets were calculated from the carotenoid content of the feed before and after immersion in water. The dry matter and percentage of carotenoids lost by leaching in water are shown in Table 5. The carotenoid loss in diets includes astaxanthin and lutein being lost after 15, 30 and 60 min of immersion in static water. The results showed that the rate of carotenoid loss was not significantly different between the different types of diet. Mean dry matter retention ± SD after 15, 30 and 60 min of static-water leaching under normal fish culture conditions was 8.44 ± 0.32, 8.48 ± 0.28 and 8.11 ± 0.15, respectively, for the astaxanthin diet and 8.74 ± 0.26, 8.76 ± 0.38 and 8.28 ± 0.19%, respectively, for the lutein diet; these results were

Table 3 Pharmacokinetic parameters of metabolization for astaxanthin and lutein parent diets derived from serum concentration-time data of fancy carp.

Parameter	Unit	Lutein metabolized from astaxanthin diet (500 µg)	Astaxanthin metabolized from lutein diet (500 µg)
Vd	$ml.kg^{-1}$	4349.40	810.40
CL	$ml.hr^{-1}.kg^{-1}$	35.94	1.27
AUC	$\mu g.hr.ml^{-1}$	516.80	14631.10
C max	$\mu g.ml^{-1}$	65.00	40.90
T max	hr	12.00	24.00

Vd (area).kg^{-1} = Volume of distribution calculations; CL (area).kg^{-1} = Clearance calculations; AUC (area) = Area under the serum concentration time curve; C max (obs) = Maximum observed serum concentrations; T max = Observed time at which C max was achieved.

Table 4 Carotenoids concentration (mean ± SD) in feces after feeding (oral administration).

Experimental diet	Carotenoids concentration in feces (µg)	Percentage of digestibility
Astaxanthin	48.06 ± 1.20^{b}	81.05^{a}
Lutein	66.41 ± 1.55^{a}	70.46^{b}
P-value	0.0001	0.0001

^{ab} = Different superscript letters in a column indicate a significant difference at $P \leq 0.05$.

Table 5 Carotenoids concentration leached from diets after immersion for 15, 30 and 60 min.

Time	Astaxanthin			Lutein		
(min)	Dry matter (%)	Soluble in water ($\mu g.mL^{-1}$)	% Loss (%)	Dry matter ($\mu g.mL^{-1}$)	Soluble in water	% Loss
15	8.44 ± 0.32	1.76 ± 0.46^{c}	0.69 ± 0.18^{c}	8.79 ± 0.26	1.84 ± 0.26^{c}	0.82 ± 0.12^{c}
30	8.48 ± 0.28	7.27 ± 2.27^{b}	2.88 ± 0.95^{b}	8.76 ± 0.38	9.97 ± 0.43^{b}	4.42 ± 0.24^{b}
60	8.11 ± 0.15	11.88 ± 0.44^{a}	4.68 ± 0.23^{a}	8.28 ± 0.19	23.24 ± 1.72^{a}	10.33 ± 1.01^{a}
P-value	0.165	0.0003	0.0004	0.103	0.0001	0.0001

^{ab} = Different superscript letters in a column indicate a significant difference at $P \leq 0.05$.

not significantly different. The difference in dry-matter retention between the two feeds intensified with increasing levels of leaching time, which was similar to other reports (Leonard *et al*., 2002). The retention or loss of nutrients and the rate at which such losses occur were not evaluated in the present study and are therefore recommended for further investigation.

It was found that astaxanthin and lutein had greater losses of carotenoid ($P \leq 0.05$) based on the immersion times of 15, 30 and 60 min with the percentage loss (mean ± SD) of astaxanthin being 0.69 ± 0.18, 2.88 ± 0.95 and 4.68 ± 0.23, respectively. For the lutein diet based on the same immersion times, the percentage loss (mean ± SD) of lutein was 0.82 ± 0.12, 4.42 ± 0.24 and 10.33 ± 1.01, respectively. These results suggested that longer immersion resulted in greater loss of carotenoid. Therefore, fancy carp would receive less carotenoid due to the loss of carotenoid in water during the time required for the aquatic animals to feed (normally a 15-20 min period, except for slow-consuming species such as shrimps which might possibly take up to 1 hr). Hence, it is essential for keepers to understand the nature of diets to achieve maximum utility for the specimens under their care.

CONCLUSION

The utilization of the carotenoids, astaxanthin and lutein, in fancy carp based on the serum analysis showed that time curves provided the best fit for one compartment of the pharmacokinetic model. Astaxanthin was absorbed better than lutein. It was found that after fancy carp were fed with diets containing lutein, they could convert lutein to astaxanthin. In practice, the results could be utilized to establish a formulated diet for fancy carp or ornamental fish from feedstuffs containing lutein . In addition, diets that contain carotenoid should be consumed within 15 min in order to avoid losses due to immersion. This study also presented a method of developing diets that contain carotenoid and lutein from natural sources, which could replace synthesized sources and consequently lead to cost reductions and more efficient domestication.

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