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Original Article

Validated TLC-densitometric analysis for determination of carotenoids in fancy carp (*Cyprinus carpio*) serum and the application for pharmacokinetic parameter assessment

Bundit Yuangsoi*, Orapint Jintasataporn, Nontavit Areechon and Prathak Tabthipwon

Department of Aquaculture, Faculty of Fisheries,
Kasetsart University, Chatuchak, Bangkok, 10900 Thailand.

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Abstract

A densitometric Thin-layer Chromatographic (TLC) method of carotenoids such as astaxanthin, lutein, and β-carotene have been established and validated for quantitative determination of carotenoids in fancy carp serum. This study can be used in the evaluation of pharmacokinetic parameters of carotenoids in fancy carp serum. Analyses of carotenoids were performed on TLC glass plates pre-coated with silica gel 60 as the stationary phase. Linear ascending development was carried out in a twin trough glass chamber saturated with mobile phase consisting of petroleum ether-diethyl ether-acetone (75:15:10, *v/v/v*) at a temperature of 25±2°C. TLC scanner was used for spectrodensitometric scanning and analysis in absorbance mode at 450 nm. The system was found to give compact spots for astaxanthin, lutein, and β-carotene (R_f values of 0.21, 0.17 and 0.97, respectively). The method was validated for linearity, precision, accuracy, LOD, LOQ and HORRAT value. The linear regression analysis data of astaxanthin, lutein, and β-carotene for the calibration plots showed a good linear relationship with r^2 = 0.999, 0.998 and 0.998, respectively, in a concentration range of 0.01-6.50 ug/spot with respect to the peak area. Precision (% RSD_r) of astaxanthin, lutein, and β-carotene was 2.93, 3.34, and 2.61, respectively. The limit of detection (LOD) was 0.011, 0.023 and 0.026 μg/spot, respectively. The additionally limit of quantization (LOQ) was 0.036, 0.075 and 0.085 μg/spot, respectively. The percent recoveries of astaxanthin, lutein, and β-carotene spiked to sample blank showed an average of percent recoveries for astaxanthin (0.3-2.0 μg/ml) of 91.70%, for lutein (0.2-3.0 μg/ul) of 90.47%, and for β-carotene (0.1-1.0 μg/ul) of 102.25%. In all carotenoids, the HORRAT values were below the critical value. Therefore, this method enables simple, rapid, economical and precise quantitative determination of carotenoids in fancy carp serum for evaluated pharmacokinetic parameters. It is possible to use the established method for the routine analysis of carotenoids in serum.

Keywords: TLC-densitometric, carotenoids, pharmacokinetic, validation, fancy carp

1. Introduction

Carotenoids are highly conjugated polyprenoids found in a variety of natural sources. They are classified into two major groups, carotenes and xanthophylls. Carotenoids are the main pigments of many aquatic animals. Fish skin colors primarily depend on the presence of chromatophore (xanthophores and erythrophores) containing carotenoids (e.g. astaxanthin, canthaxanthin, lutein, and zeaxanthin). Fish, like other animals, are unable to perform de novo synthesis of carotenoids (Goodwin, 1984), but they can modify alimentary carotenoids and store them in the integument and other tissues. Different species have different carotenoids metabolized and carotenoids storage for instance. Fancy carp can convert lutein and zeaxanthin to astaxanthin (Katayama *et al.*, 1973).

*Corresponding author.
Email address: tubulan@yahoo.com

Pigment is one of the most significant quality criteria of ornamental fish that is dictating their market value (Paripatananont *et al*., 1999). Therefore, the fancy carp color remains consistently vivid; the enhancement of color is utilized in their diet, particularly carotenoids.

Chromatography is the most efficient technique for separation and identification of carotenoids. Recently, high performance liquid chromatography (HPLC) has been widely used for analyzing carotenoids in food and biological matrices. The use of mass spectrometry (MS) detection together with HPLC is a powerful tool for the identification of carotenoids, however these techniques are time consuming and coming with high expenditure. Now, Thin-layer Chromatography (TLC) is widely used in laboratories throughout the world for analysis and quality control. TLC has also been widely used to analyze agricultural products and plant materials. Modern TLC has advantages in many analyitical methods, e.g. simplicity of operation; availability of many sensitive and selective reagents for detection and confirmation without interference of the mobile phase; ability to repeat detection and quantification at any time with changed parameters as fractions representing the entire sample are stored on the plate; in-system calibration for quantitative analysis; and cost effectiveness as many samples can be analyzed on a single plate with low solvent usage. In-situ measurements of zone with a scanning densitometer (TLC-densitometry analyses) are the preferred technique for quantitative TLC. This method is economical as it utilizes smaller amounts of solvents with a minimum sample clean up. Also, in a short duration, a large number of samples are simultaneously analyzed. TLC has no limitations in the choice of the mobile phase compared to HPLC. Direct applications of suspensions, dirty or turbid samples are possible. Therefore, TLC is a quick and economical method compared to HPLC, and it permits a simultaneous assay of several components in a multi-component sample or a fish serum.

The bioavailability of a given compound, i.e. the fraction of an administered dose that is absorbed into the circulation, is an important determinant of its efficacy and safety (Gibaldi and Perrier, 1982). There are few studies that have followed the absorption and elimination rates of carotenoids in ornamental fish. Some experiments in salmonids, using radio-labeled compounds, were carried out on carotenoid concentrations in blood after ingestion of single doses or on relative bioavailability of astaxanthin being taken as a reference. Therefore, pharmacokinetic studies dealt with the quantitative aspects of carotenoids uptake and elimination.

This study aims for the modification of the selected methodology for the most effective conditions and validation of the established method in developing accuracy and repeatability. Further, it aims to investigate possible applications with established methods for carotenoids in fish serum in order to study pharmacokinetic parameters.

2. Materials and Methods

2.1 Chemicals

Astaxanthin and β-carotene standard (>95% purity) were purchased from Sigma (USA); lutein standard (>97% purity) was purchased from Chromadex (Canada). All organic solvents were analytical-reagent grade (AR grade). Acetone, hexane, dichrolomethane, petroleum ether, and diethyl ether were purchased from Carlo Erba (Italy).

Carotenoids as a supplement in the experimental diet, astaxanthin, were supplied as a commercial product, containing 10% astaxanthin (BASF, Thailand), lutein from marigolds extracts was supplied as a commercial product containing 15,000 ppm (Kemin Industries, Thailand), and β-carotene used was a Natural Beta carotene 15 mg/capsule (MEGA Lifesciences, Australia).

2.2 Preparation of standard carotenoids solutions

All of the standard carotenoids solutions were prepared under dim light (yellow light). Pure compounds were dissolved in hexane and dichrolomethene (1:1, *v/v*) and the maximum absorbance (l max) was determined at wavelengths ranging between 350 and 600 nm, by using an UV/Vis spectrum and a calculated concentration of the standard solution following Beer-Lambert's Law. Carotenoid standard mixture was stored in brown vials under a nitrogen atmosphere at -20°C. Working standards of each compound were prepared daily to the desired concentration from stock solutions.

2.3 Fish and feed trial

Mixed sexes of fancy carp with an average weight of 26.93±5.14 g/fish were maintained in a 20-liter aquarium tank (triplicate tank per treatment; 3 fish per tank). Fish were fed with a pigment-free diet for two weeks prior to the start of the experiment. The diets were designed to achieve a target level of 500 μg for astaxanthin, lutein, and β-carotene per fish. After administration of a single dose orally, the fish were not fed further meals.

2.4 Sampling procedure

Fish were not fed for three days before receiving a single dose feeding. A control blood sample at 0 hr was taken to measure basal serum astaxanthin, lutein, and β-carotene levels prior to the experiment. Blood was collected from the dorsal vein with 1 ml non-heparinized disposable syringes fitted with 0.55x25 mm disposable needles. Blood sampling occurred at 0.25, 0.5, 1, 3, 6, 12, 24, 36, 48, 72, 96, 120 and 144 hrs after a single dose meal, with three fish sampled at

each sampling time.

All fish sampling were performed under the animal welfare regulations of the Kasersart University Research and Development Institute (KURDI).

2.5 Carotenoids determination

Blood was collected from the dorsal vein with 1 ml non-heparinized disposable syringes fitted with 0.55 x 25 mm disposable needles. Blood samples (approx. 1 ml/fish) centrifuged at 300 x g, 25°C for 10 min, 400 µl of the serum was removed and vortexed with 1 ml of ethanol for 30 s, then 2 ml of hexane was added and the mixture was vortexed for 1 min. The hexane was separated by centrifuging 300 x g, 25°C for 10 min (White *et al*., 2002). The resulting hyperphase of serum extracted from hexane was evaporated under a gentle stream of nitrogen gas. Then, the crude carotenoids were redissolved with hexane.

2.6 Instrumentation and chromatographic conditions

The samples were spotted on precoated silica gel 60 (10 x 20 cm) with 0.25 mm thickness (Merck, Germany) using a CAMAG Linomat IV (CAMAG, Switzerland) sample applicator. A constant application rate of 4 µl/s was employed and spaces between two spots were 14 mm. Spots would generate narrow spots, which had to be sprayed with nitrogen gas having a 99.995% purity. The slit dimension was kept at 5 mm x 0.45 mm and 20 mm/s scanning speed was employed. 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 a chromatogram run was 70 mm. Densitometric scanning was performed on a CAMAG TLC scanner III (CAMAG, Switzerland) in the absorbance mode at 450 nm. The source of radiation utilized was a tungsten lamp. The total results found in this study must be evaluated with following calibration function: a multi level calibration with linear regression using external standards.

2.7 Calibration curve of carotenoids

Stock standard solution of carotenoids such as astaxanthin, lutein, and β-carotene were prepared in hexane. Standard solutions were prepared by dilution of the stock solution with hexane to give solutions containing astaxanthin, lutein, and β-carotene in concentration ranges of 0.002-1.25 µg/ul. The concentration of standard solutions was calculated from its extinction coefficient; $E^{1\%}_{1\ cm}$ 2100 in hexane at 470 nm for astaxanthin; $E^{1\%}_{1\ cm}$ 2500 in hexane at 440 nm for lutein and $E^{1\%}_{1\ cm}$ 2600 in hexane at 450 nm for β-carotene. Five microliters from each standard solution was spotted on the TLC plate to obtain a final concentration range of 0.01-6.25 µg/spot.

2.8 Validation of the established method

2.8.1 Precision

Repeatability (within laboratory precision) of a method may be measured by multiple analyses of identical samples at different analytical levels, performed on the same day by a single analyst using the same apparatus and measurement of peak area carried out using 10 replicates of the same spot (0.01, 0.25, 0.50, 1.25 and 6.25 µg/spot of astaxanthin, lutein, and β-carotene) and was expressed in terms of percent relative standard deviation (% RSD_r).

Standard deviation = $Si = [\Sigma(Xi - X)^2/n]^{0.5}$

Relative standard deviation = RSD = Si x 100/X

X = sum of the individual values, Xi, divided by the number of individual values, n.

2.8.2 HORRAT value

The HORRAT value for repeatability is the observed RSD_r divided by the RSD_r value estimated from the Horwizt equation.

HORRAT (r) = RSD_r / Horwizt equation

Horwizt equation for repeatability, $CV_r = 0.66 \times 2^{(1-0.5 \log C)}$

Where C is expressed as a concentration.

2.8.3 Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were considered as 3:1 and 10:1. The corresponding slope and regression standard deviation values were used to establish sensitivity. LOD of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. LOQ of an individual analytical procedure is the lowest amount of analyte in a sample that can be determined quantitatively with suitable precision and accuracy. LOD and LOQ were calculated with the following equation:

LOD = 3 So

LOQ = 10 So

Where So is SD/S, and SD is the standard deviation of the response near LOD or LOQ and S is the slope of the linearity curve near LOD or LOQ.

The LOD was given by the expression 3 $s_{y/x}$/slope, and was based on the assumptions that the standard deviation of the signal of a solution with a concentration near the blank is roughly the standard deviation of y-residuals ($s_{y/x}$), as there is a normal distribution of the signal at this concentration, and a probability of 5% of occurring error type a or b, and that the curve intercepts zero. It was estimated as the arithmetic mean of the different detection limits obtained with the different calibration curves, freshly prepared each day. The LOQ was estimated using the factor 10 instead of 3.

2.8.4 Linearity

Linearity was checked between 0.01, 0.25, 0.50, 1.25 and 6.25 μg/spot of astaxanthin, lutein, and β-carotene. Linear calibration function was calculated and linear regression line was determined, and a product-moment correlation coefficient equal or higher than 0.995.

2.8.5 Recovery

Known quantities of astaxanthin, lutein, and β-carotene standard solutions mixture were spiked at three levels in sample blanks for 10 replicates. The addition of these carotenoids was made at concentration levels, which covered the general range expected in fish serum that were 2.0, 1.0 and 0.3 μg/ml for astaxanthin; 3.0, 1.5 and 0.2 μg/ml for lutein; and 1.0, 0.5, and 0.1 μg/ml for β-carotene, respectively. Samples with the additional carotenoids were extracted using the established procedure described. After chromatography, the concentrations of endogenous carotenoids and endogenous plus added carotenoids in each sample were calculated using standard calibration curves. Subtraction of the endogenous carotenoids gives recovered values of added carotenoids. Percentage recoveries of added carotenoids were calculated following AOAC (1993).

2.9 Pharmacokinetic parameter analysis

The astaxanthin, lutein, and β-carotene concentration-time curves from serum were best fitted to a one-compartment pharmacokinetic model. The mean serum astaxanthin, lutein, and β-carotene concentration-time course data points of three from each time were then analyzed using a pharmacokinetic equation (Evans, 2004)

2.10 Statistical analysis

Mean value and standard deviation (SD) were calculated from the results. One way analysis of variance (ANOVA) was applied for comparison of the mean values $P< 0.05$ was regarded as significant.

3. Results and Discussion

UV/Vis spectrum determined maximum absorbance (l max) at the wavelength range 350-600 nm of carotenoid standard: astaxanthin, lutein, and β-carotene dissolved in hexane. The data illustrated in Figure 1, showed that maximum absorbance in astaxanthin, lutein, and β-carotene standard was 468, 444, and 456 nm, respectively. Hence, the data from this study related with result from Goodwin (1984)

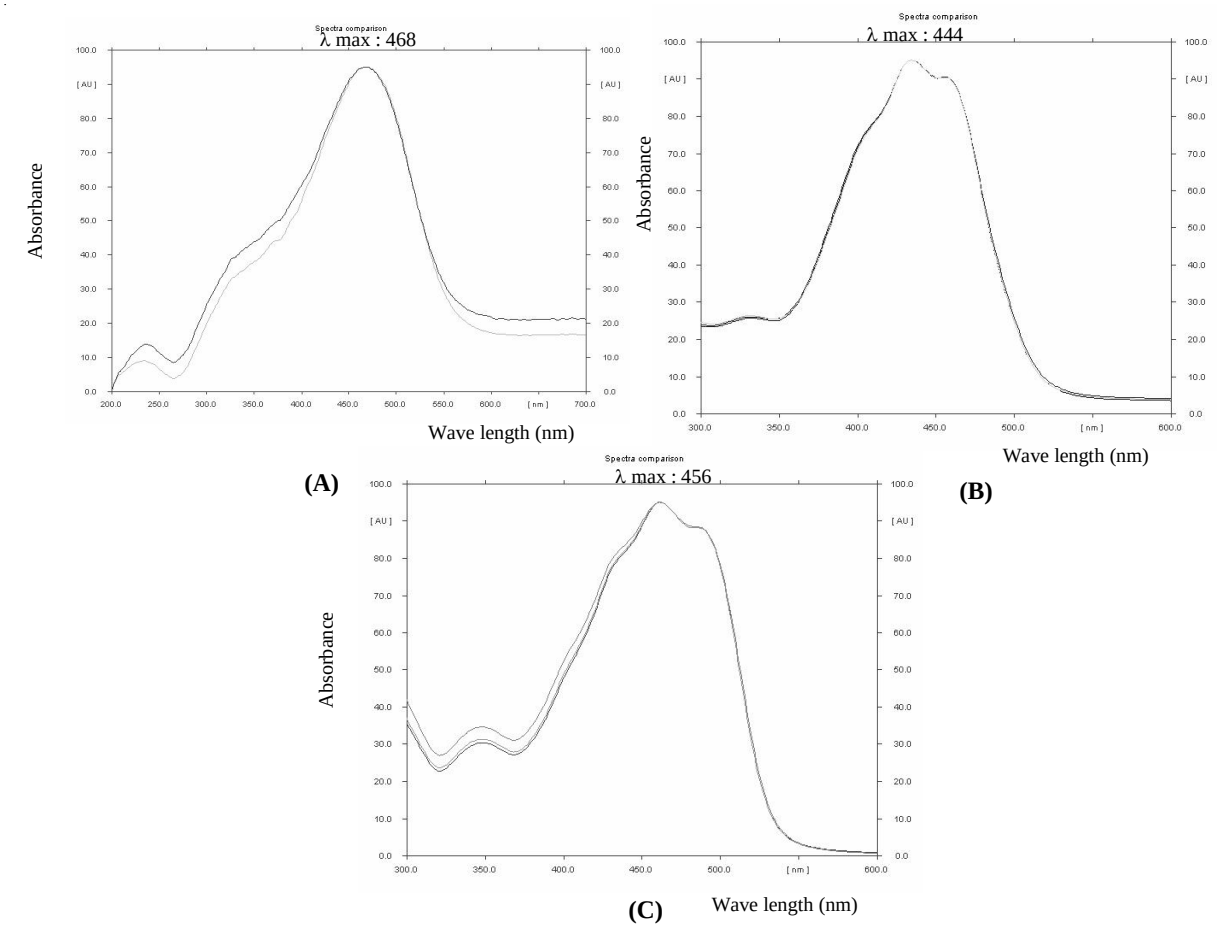
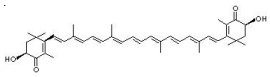
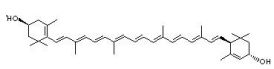
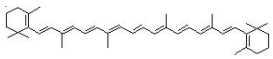


Figure 1. Spectra of carotenoids standard; A: astaxanthin, B: lutein and C: β-carotene.

Table 1. Structural formula and physiochemical properties of astaxanthin, lutein, and β-carotene (compiled from Goodwin,1984, and Britton *et al.*, 2004).

	Astaxanthin	Lutein	β-carotene
			
Formula	$C_{40}H_{52}O_4$	$C_{40}H_{56}O_2$	$C_{40}H_{56}$
Molecular weight	596.86	568.87	536.90
Melting point	216°C	177-178°C	176-183°C
Absorption (nm)			
n-hexane	470	440	450
$E^{1\%}_{1\ cm}$ (n-hexane)	2100	2500	2600

and Britton *et al.* (2004) as shown in Table 1. The maximum absorbance of astaxanthin, lutein, and β-carotene in hexane was 470, 440, and 450 nm, respectively. Light absorption spectra strongly are normally determined quantitatively by spectrophotometry.

3.1 Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the fancy carp serum. Initially petroleum ether-diethyl ether-acetone in varying ratios was tried. The mobile phase petroleum ether-diethyl ether-acetone (75:15:10, *v/v/v*) gave good resolutions. The system was found to give compact spots for astaxanthin, lutein, and β-carotene (R_f value of 0.20, 0.17 and 0.97) as shown in Figure 2 and Figure 3. Well-defined spots were obtained when the chamber was saturated with a mobile phase at room temperature (25±2°C).

3.2 Validation of the established method

Linearity was evaluated by determining five standard working solutions concentration (0.01, 0.05, 0.25, 1.25 and 6.25 μg/spot) of astaxanthin, lutein, and β-carotene in triplicate. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The developed TLC method for the estimation of astaxanthin, lutein, and β-carotene showed a good linear relationship with r^2 = 0.999, 0.998 and 0.998 in the concentration range 0.01-0.65 μg/spot with respect to peak areas. The acceptable criteria of correlation coefficient (r^2) was equal or higher than 0.995 (AOAC, 1993). Precision evaluates how well a method performs under different conditions of repeated use. It is the degree of agreement between determined values and is generally expressed in terms of standard deviation or coefficients of variation (CVs), also called relative standard deviation (RSD_r). Repeatability (within laboratory precision) of a method may be measured by multiple analyses of identical samples at different analyte levels, performed on the same day by a single analyst using the same apparatus. This present study showed the $\%RSD_r$ for repeatability of astaxanthin, lutein and β-carotene measurement of peak areas to be 2.93, 3.34, and 2.61%, respectively. The measurement of the peak area at five different concentration levels showed low values of $\%RSD_r$ (< 3.7%), which suggested an excellent precision

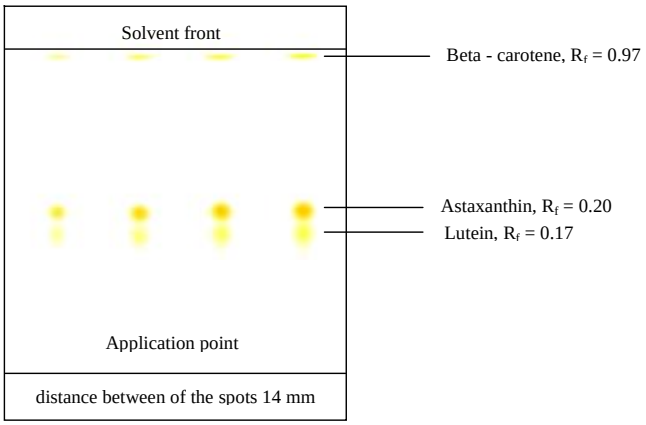


Figure 2. Silica gel TLC plate on which carotenoids standard mixture was separated.

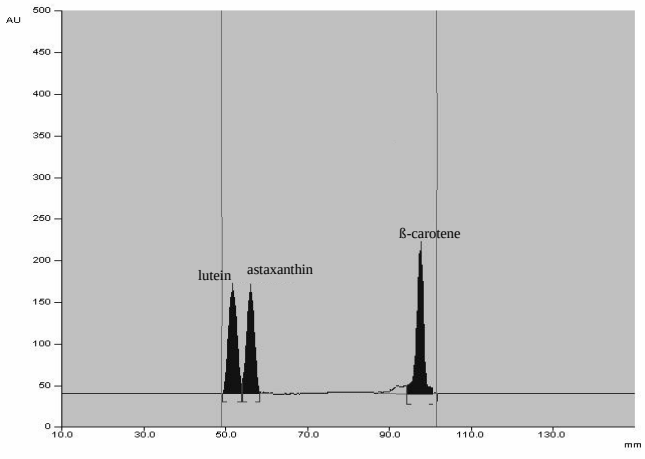


Figure 3. Reflectance densitogram at λ = 450 nm of carotenoids standard developed with petroleum ether-diethyl ether-acetone (75:15:10, *v/v/v*).

Table 2. Validation parameters for measurement of carotenoids in serum.

Parameter	Ataxanthin	Lutein	β-carotene
Correlation coefficient (r^2)	0.999	0.998	0.998
LOD (ug/spot)	0.011	0.023	0.026
LOQ (ug/spot)	0.036	0.075	0.085
Precision (%RSD_r)*	2.93	3.34	2.61
Accuracy (% recovery)	91.70	90.47	102.25
HORRAT(r) value*	0.22	0.26	0.19

Note: * for single laboratory validation

of the method and making it a reliable quantitative tool (Table 2). The AOAC manual for the peer verified methods program (1993) reported that acceptance criteria of %RSD_r should not access 3.7% for analyte concentration at 0.1% in sample (AOAC, 1993).

Where measurements are made at low analyte levels, it is important to know, what are the lowest analyte concentration values that can be detected. Notwithstanding the definitions, it is recommended that limits of detection and quantification should be established in practice from the results of repeated analyses of spiked or endogenous samples. For analysis of major carotenoids, the limits of detection and determination do not have much use. These limits become important when the whole range of carotenoids in a sample is determined, particularly for the minor or trace carotenoids (Delia, 2001). The limit of detection for astaxanthin, lutein, and β-carotene was determined by TLC-densitometric analysis and the limit of quantification with a signal-to-noise ratio of 3:1 and 10:1 were found to be 0.001, 0.023, and 0.026 μg/spot for LOD and 0.036, 0.075 and 0.085 μg/spot for LOQ, respectively, which indicates the adequate sensitivity of the method.

The percent recoveries of astaxanthin, lutein, and β-carotene spiked to sample blank are shown in Table 3. The recovery values for astaxanthin were 0.3-2.0 μg/ul with the average of percent recoveries at 91.70%, for lutein 0.2-3.0 μg/ul with 90.47%, and for β-carotene 0.1-1.0 μg/ul with 102.25%. The concentrations covered the range of concern and were close to the quantification limit. The acceptable limit of recovery for single laboratory validation provided a mean recovery of 90-108% of analyte concentration at 0.1% (AOAC, 1993).

The AOAC manual for the peer verified methods program (1993) provided a mean recovery of 80-110%. The HORRAT value based on experience and for the purpose of exploring the extrapolation of HORRAT values to single laboratory validation (SLV) studies, take as the minimum acceptability one half of the lower limit (0.5 x 0.5 ≈ 0.3) and as the maximum acceptability two thirds of the upper limit (0.67 x 2.0 ≈ 1.3). The HORRAT(r) value of astaxanthin, lutein, and β-carotene was 0.17, 0.22, and 0.17, respectively. In all carotenoid standards, the HORRAT(r) were below the critical value, acceptability HORRAT(r) value are 0.3-1.3 (AOAC, 1993).

3.3 Pharmacokinetic parameters of fancy carp fed with deprived diets

The astaxanthin, lutein, and β-carotene concentration in fancy carp were best described by one compartment model with first order absorption, as the pharmacokinetic parameters are displayed in Table 4.

The results showed that serum astaxanthin, lutein, and β-carotene observed concentration-time curve showed a steady rise after post-dosing, a slow increase to the maximum peak at 6, 12, and 12 hrs for astaxanthin, lutein, and β-carotene, respectively. Since then, astaxanthin, lutein and β-carotene were on a gradual decline at 12, 24, and 24 hrs after post-dosing. Carotenoids compositions in serum are persented in Figure 4.

Fancy carp fed with an astaxanthin diet developed the maximum concentration of astaxanthin (C max) 0.38 μg/ml at 6 hr (T max), while fancy carp fed with a lutein diet contained lutein level in blood (C max) developed 0.39 μg/

Table 3. Recovery of added carotenoids to sample blank.

Compound	concentration				
	Initial (μg/ul)	Added (μg/ul)	Found (μg/ul) mean + SD	Recovery (%)	Average of recovery (%)
Astaxanthin	None	2.0	1.77±0.14	88.44	91.70
		1.0	0.88±0.28	88.22	
		0.3	0.30±0.06	98.44	
Lutein	0.03 ± 0.01	3.0	2.64±0.10	87.96	90.47
		1.5	1.36±0.11	90.61	
		0.2	0.19±0.10	92.85	
β-carotene	0.06 ± 0.01	1.0	0.89±0.12	89.47	102.25
		0.5	0.47±0.02	94.28	
		0.1	0.12±0.01	120.00	

Table 4. Pharmacokinetic parameters for astaxanthin, lutein, and β-carotene derived from serum concentration-time data of fancy carp.

Parameters	Unit	Deprived diets		
		Astaxanthin	Lutein	β-carotene
C max (obs)	μg/ml	0.38	0.39	0.13
T max (obs)	hr	6	12	12
CL (area)/kg	ml/hr/kg	9.62	6.49	10.06

C max (obs): maximum observed serum concentrations
T max: observed time at which C max was achieved
CL (area)/kg: total body clearance calculations

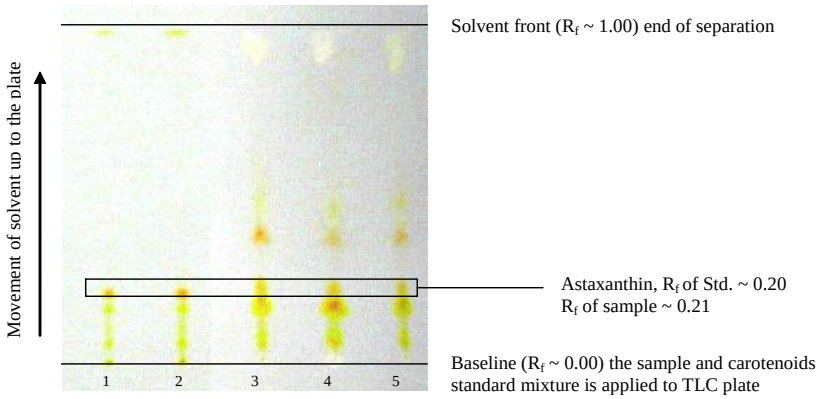


Figure 4. Silica gel TLC plate on which carotenoids standard mixture and serum extraction of fancy carp fed with experimental diet were separated, lane 1-2 are carotenoids standard mixture and lane 3-5 are samples.

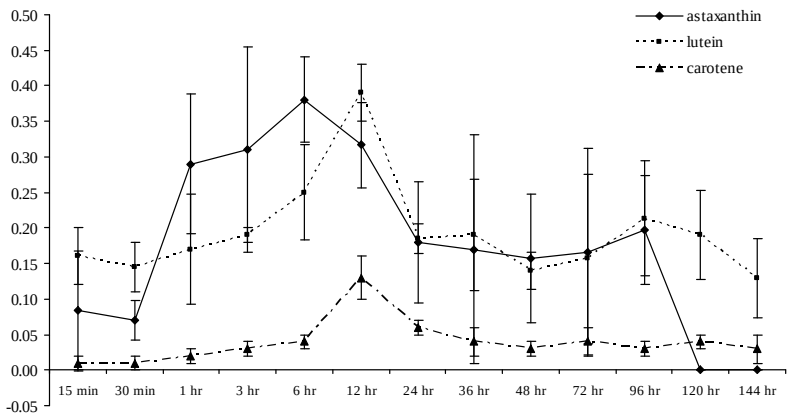


Figure 5. Serum astaxanthin concentration time curve for fancy carp administration with a single dose of astaxanthin, lutein, and β-carotene diet.

ml within 12 hrs, and fancy carp fed with a β-carotene diet contained β-carotene level in blood (C max) developed 0.13 μg/ml within 12 hrs (Figure 5). Total body clearance (CL) was 9.62, 6.49 and 10.06 ml/hr/kg, which indicated that the β-carotene has a higher clearance than astaxanthin and lutein. This clearance was mainly to eradicate carotenoids in anyway whatsoever. Overall, after fancy carp were fed with an astaxanthin diet, they showed better astaxanthin absorption compared to fancy carp fed with lutein and β-carotene. It proved good dispersal of astaxanthin in blood circulation (Evans, 2004).

The results of the present study indicate that utilization of astaxanthin, lutein, and β-carotene in fancy carp following single dose feeding oral administration shows that the faster rate of astaxanthin absorption for the fish fed with an astaxanthin diet compared to fish fed with lutein and β-

carotene diet agree with the report of Olsen and Bakker (2006). The authors, who used Atlantic salmon fed with diets containing astaxanthin and lutein, found that fish given a diet containing astaxanthin had a higher level of astaxanthin in their blood levels than lutein.

In carotenoid analysis, validation of methods has not been strongly advocated, even with the introduction of high-performance liquid chromatography, because the emphasis has been on chromatographic separation. In the few papers involving quantification, validation consisted mainly of recovery tests and determination of repeatability (e.g. Delia, 2001). It has been demonstrated above that validation data for astaxanthin, lutein, and β-carotene quantitative TLC-densitometric method meet the acceptance criteria for accuracy, precision, linearity, and detection and quantification limits set by Michael *et al.* (2002). Further, the validation data is at least as good as values reported regularly in the literature for HPTLC and HPLC analysis of natural extract and nutritional supplements (Sherma and Bernard, 2003).

The described method is suitable for routine determination of carotenoids in fish serum. It is simpler than HPLC and faster because up to nine samples can be analyzed on each plate (applied singly with the minimum four standard concentrations). Costs of solvent purchase and disposal is very low because not more than 25 ml of mobile phase is required in the chamber trough containing the plate to develop these 13 chromatograms, and an additional 25 ml for vapor saturation in the other trough.

4. Conclusion

The developed TLC-densitometric analysis was validated by determination of linearity, accuracy (% recovery), precision (%RSD_r), LOD, LOQ, and HORRAT(r) value for single laboratory. The data indicated that this method can be successfully used for the analysis of carotenoids in fish serum with good recoveries and precision. The method is also applicable to various kinds of serum from fish. Thus, this method enables a simple, rapid, economical, and precise quantitative determination of carotenoids in fancy carp serum for evaluated pharmacokinetic parameter. It is possible to use the established method for the routine analysis of carotenois in fish serum.

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