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Proximate Composition and Carotenoid Content of Natural Carotenoid Sources and its Colour Enhancement on Marine Ornamental Fish *Amphiprion ocellaris* (Cuveir 1880)

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Abstract: Experimental fish species (*Amphiprion ocellaris)* were cultured for 60 days in five tanks as five groups. The experimental feeds were prepared with four natural carotenoid sources such as Carrot (*Daucus carota*), Marigold petal (*Tagetes erecta*), China rose petal (*Hibiscus rosasinensis*) and Rose petal (*Rosa chinensis*) at a rate of 15g/100g feed. One set of control and four set of experimental groups were maintained for colour enhancement test. All fishes were fed twice a day with one control and four different experimental feeds. At the end of the experiment carotenoid concentration in control averaged 2.687±0.287 mg kg^{-1}, Carrot 7.681±0.462 mg kg^{-1}, Marigold 7.235±0.438 mg kg^{-1}, Hibiscus 5.236± 0.314 mg kg^{-1} and Rose 4.254±0.252 mg kg^{-1}, Pigmentation was the highest in *D. carota* (7.681 mg/kg^{-1}), followed by *T. erecta* (7.235 mg/kg^{-1}), *H. rosasinensis* (7.235 mg/kg^{-1}), *R. chinensis* (4.254 mg/kg^{-1}) and the lowest *(*2.687 mg/kg^{-1}) in control tank. Thin Layer Chromatography was run in which four compounds were identified as spots with maximum *Rf* value in *D. carota Rf* = 0.72, followed by *T. erecta Rf* = 0.68, *Rf* =, *H. rosasinensis* 47 and *R. chinensis Rf* = 22.The results of ANOVA suggested that among days it showed significant value F= 0.0324; P<0.05 and among diets it showed insignificant values F=4.09133; P>0.05. This will be first step for the future studies in this line. From this study it could be concluded that the natural colour enhancer feeds can be prepared at a lower cost using the cheaply available sources.

Key words: *Amphiprion ocellaris* % Carotenoid % Colour Enhancement % Ornamental fish

INTRODUCTION

Ornamental fishes are characterized by a wide diversity of colours and colour patterns and success in the ornamental fish trade is very much dependent on the vibrant colour of the fish. Color is one of the major factors, which determines the price of aquarium fish in the world market [1]. Fish are colored in nature often show faded coloration under intensive culture conditions. Fish like other animals do not synthesize carotenoid and depend on dietary carotenoid content for the coloration. Hence, a direct relationship between carotenoids and pigmentation exists in them [2].

If enhancement of coloration can be done by administrating pigment enriched feed, it will definitely improve the quality and cost of the fish. However, detailed studies on color enrichment in ornamental fish are lacking. Plant sources have also been utilized for inducing pigmentation in fish for example, *spirulina* have been used as a source of carotenoid pigment for rainbow trout and fancy carp [3.4] and marigold petal meal was used for the tiger barb [5].

The commercial value of these fish reflects this requirement; hence, the ornamental fish growers are constantly exploring methods of enhancing skin colouration. However, recent efforts have focused on natural compounds as alternative to synthetic carotenoids as because of concerns about the use of synthetic additives and their high cost. To alleviate this problem the present study was made to evaluate pigmentation quality using the natural carotenoids.

MATERIALS AND METHODS

Fish Rearing and Conditioning: Experimental fish species *A.ocellaris* were collected from marine ornamental fish traders and were acclimated to laboratory condition in an aquarium tank. The fishes were conditioning by treating

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Table 1: Shows Formulation of Experimental Diets (g/100g)

Parameters	Control	A	B	C	D
Ingredients	Soybean(15.22)	Soybean(15.22)	Soybean(15.22)	Soybean(15.22)	Soybean(15.22)
	Fishmeal(15.22)	Fishmeal(15.22)	Fishmeal(15.22)	Fishmeal(15.22)	Fishmeal(15.22)
	Groundnut oilcake(11.28)	Groundnut oilcake(11.28)	Groundnut oilcake(11.28)	Groundnut oilcake(11.28)	Groundnut oilcake(11.28)
	Wheat flour(13.04)	Wheat flour(13.04)	Wheat flour(13.04),	Wheat flour(13.04)	Wheat flour(13.04)
	Tapioca flour(13.04)	Tapioca flour(13.04)	Tapioca flour(13.04),	Tapioca flour(13.04)	Tapioca flour(13.04)
	Rice bran(12.2)	Rice bran(12.2)	Rice bran(12.2)	Rice bran(12.2)	Rice bran(12.2)
	Vitamins (5. 0)	Vitamins(5. 0)	Vitamins(5. 0)	Vitamins(5. 0)	Vitamins (5. 0)
Additives	Nil	Carrot (15)	Marigold(15)	China rose (15)	Rose (15)

A- Diet with Carrot B- Diet with Marigold C- Diet with China rose D- Diet with Rose

with control diet (no added carotenoid sources) for two weeks to equalize their body carotenoid content, the water exchange and aeration was given sufficiently. Five fiber glass tanks were used and ten fishes were placed in each tank 100L capacity prior administrating experimental diets.

Feed Preparationl: The experimental feeds were prepared with basic ingredients such as wheat flour, rice bran, tapioca flour, soybean, fish meal and groundnut oilcake. They were split into five equal portions. The four natural carotenoid sources such as Carrot (*Daucus carota*), Marigold petal (*Tagetes erecta*), China rose petal (*Hibiscus rosasinensis*) and Rose petal (*Rosa chinensis*) were collected, air dried in the dark room to prevent denature of carotenoids. After dry the sources were powdered and sieved samples in particle size of 0.9 to 1.2mm sieve then stored at -20°C to avoid oxidation of the carotenoids. The natural carotenoid sources were thoroughly mixed with the feeds before pelletization at a rate of approximately 15g/100g (Table 1).

Feeding Experiment: This study was carried out in the indoor system. One set of control and four set of experimental tanks were maintained for colour enhancement test. All fishes were fed twice a day with one control and four different experimental feeds. During the experiment the major physical and chemical parameters were maintained at stable conditions. The experiment was carried out at 60 days. The animals were measured, weighed and analyzed for carotenoid content at every 15 days intervals.

Biochemical Analysis: The biochemical composition of total protein, carbohydrates, lipid and moisture content were estimated for basal diets and carotenoid sources.

Total Protein: The biuret method modified by Raymont *et al*. [6] was employed to estimate the total protein. 25 mg of dried tissue was taken and homogenized in hand homogenizer with 1.0 ml of distilled water. To which 4.0 ml of biuret reagent was added in two instilments of 2.0 ml each and the tissue grinder was cleaned before the content is being transferred to centrifuge tube. After 30 minutes, the sample was centrifuged for 10 minutes the supernatant was collected and the optical density was measured in a spectrophotometer (HITACHI 220S) at a wave length of 540nm against a blank reading. The percentage of protein was calculated by using the formula.

$$\text{Percentage of protein} = \frac{\text{Standard value x optical density}}{\text{Weight of sample}} \times 100$$

Total Carbohydrate: For the estimation of total carbohydrate content, the procedure of Dubois *et al*. [7] was followed. 20 mg of natural carotenoid sources powder and diet ingredients was taken and to this 1.0 ml of the distilled water was followed by 1.0 ml of the 5% phenol solution and 5 ml of concentrated Sulphuric acid one after another. The calorimetric reading was measured in a spectrophotometer (HITACHI 220S) after 30 minutes against a blank at 490 nm.

$$\text{Percentage of carbohydrate} = \frac{\text{Standard value x optical density}}{\text{Weight of sample}} \times 100$$

Total Lipid: The chloroform-methanol extraction procedure of Folch *et al*. [8] was followed. 400 mg of natural carotenoid sources powder and diet ingredients was taken in a test tube to which 5 ml of chloroform: methanol (2:1) mixture was added. The test tube was covered with aluminum foil and allowed to stand for overnight. Then the mixture was filtered and the filtrate was collected in a pre weighted 10 ml beaker which was then dried in an oven. The percentage of lipid was calculated by using the formula.

$$\text{Percentage of lipid} = \frac{\text{Standard value x optical density}}{\text{Weight of sample}} \times 100$$

Water Content: The water content was estimated by subtracting the dry weight of sample from the known wet weight of the sample dried in a hot air oven. The percentage of water content was calculated as follows

$$\text{Percentage of water content} = \frac{\text{Dry weight of the sample}}{\text{Wet weight of the sample}} \times 100$$

Carotenoid Analysis

Spectrophotometer Analysis: The carotenoid content of fish skin was extracted according to the method of Torrissen and Naevdal [9]. Five fish were randomly sampled from each diet treatment per sampling period and used for carotenoid analyses, which were carried out in triplicate. The samples of 200-300 mg skin were collected from both sides between the abdominal and dorsal regions of the fish. These samples were transferred into 10-ml pre weighed glass tubes. After the samples were ground in acetone containing 1.5g of anhydrous sodium sulphate with a homogenizer, the extractions were made up to 10 ml with acetone. The samples were stored for 3 days at 4°C refrigerator and then extracted three or four times until no more colours could be obtained. The solution was centrifuged at 5000 rpm for 5 min and then absorption was measured in a spectrophotometer. A similar method was adapted for total carotenoid analysis of Carrot, Marigold meal Hibiscus and Rose.

Thin Layer Chromatography: For TLC experiment the silica gel plates were used as stationary phase and petroleum/acetone/diethylamide 10:4:1 solvent system used as a mobile phase The Samples were applied on TLC plates and placed in TLC chamber. After 30 min the TLC plates were taken and compounds were identified using iodine vapor.

RESULTS

Proximate Composition and Carotenoid Content of Natural Carotenoid Sources: The carotenoid content from the natural sources were varied from different sources Carrot (1.36%), Marigold (2.30%), Hibiscus (2.19%) and Rose (2.18%). The protein content from the natural carotenoid sources values were 22.5, 12.3, 10.7 and 8.2% of carrot, marigold, hibiscus and rose, respectively. The lipid value was 8.7, 9.3, 6.8 and 12.5% of carrot, marigold, hibiscus and rose, respectively .The carbohydrate content values were ranged between 9.7% in carrot and 6.5% in rose. The moisture content values ranged from 75.54 % in carrots to 65.21% in rose (Table 2).

Proximate Composition from Feed Ingredients: The protein content from the feed ingredients values were 70.5, 60.2, 43.6, 11.5, 2.3 and 12.2% of soybean, fishmeal, groundnut oilcake, wheat flour, tapioca flour and rice bran respectively. The lipid value was 9.8, 5.5, 41.0, 3.6, 76.5 and 1.3% of soybean, fishmeal, groundnut oilcake, wheat flour, tapioca flour and rice bran respectively. The carbohydrate content values were ranged between 62.7% in rice bran and 3.5% fish meal. The moisture content values ranged from 88.45% in tapioca to 45.68% in rice bran (Table 3).

Growth and Survival Rate: All fishes grew normally and no specific signs of disease were observed. All diets were accepted equally well by the fish. When fish were fed with 15 g of *D.carota*, marigold, China rose and Rose petal feed. The maximum growth rate was observed in *D.carota* (6.03± 0.572 mg/g/ wet weight) and minimum growths were observed in control tank (5.5± 0.395mg/g/wet weight) (Table 4).

Total Carotenoid in Fish Tissue: After each 15 days of feeding, the carotenoid content in skin fish fed diets supplemented with increasing pigment levels started to differ from that of fish fed the control diets. In control without carotenoid source supplemented with feed, the animal muscle tissue showed low level of carotenoid content ranged from 1.792 to 2.387mg/kg at the end of experiment. The carotenoid content of the feed with carrot was ranged from 1.765 mg/kg (initial day) to 7.681(60 days) mg/kg in Clown fish. Marigold with the feed, the animal muscle tissue Carotenoid ranged between 1.652 mg/kg (Initial day) and 7.235mg/kg (60 days). The values of hibiscus added with feed, the muscle carotenoid content ranged between 1.567 mg/kg (initial days) and 5.236 mg/kg (60days). Rose source supplemented with the feed, the muscle tissue content was low and the values varied from 1.782 mg/kg (initial day) to 4.254 mg/kg (60 days) (Table 5), (Fig. 1).

Table 2: Shows proximate composition and carotenoid content of natural carotenoid sources

S. No.	Carotenoid sources	Carotenoid content mg/100kg	Proximate composition %			
			Protein	Carbohydrate	Lipid	Moisture
1.	Carrot	1.367± 0.356	22±0.55	9.7±0.18	8.7±0.22	75.54±0.22
2.	Marigold	2.304± 0.345	12. 3±0.35	7.3±0.15	9.3±0.12	68.05±0.36
3.	Hibiscus	2.197± 0.305	15.7±0.28	7.9±0.26	6.8±0.15	71.02±0.54
4.	Rose	2.18±0.139	8.2±0.25	6.5±0.22	12. ±0.12	65.21±0.66

Table 3: Proximate compositions of basal ingredients of experimental diets Feed

S. No.	Ingredients	Proximate composition %			
		Protein	Carbohydrate	Lipid	Moisture content
1.	Soybean	70.5±0.88	8.2±0.22	9.8±0.21	75.21±0.92
2.	Fishmeal	60.20±0.85	3.5±0.17	5.5±0.20	74.25±0.84
3.	Groundnut oilcake	43.6±0.72	16.2±0.33	41.0±0.21	78.12±0.55
4.	Wheat flour	11.5±0.65	44.2±0.43	3.6±0.21	80.34±0.65
5.	Tapioca flour	2.3±0.22	56.3±0.55	76.5±0.75	88.45±0.74
6.	Rice bran	12.2±0.36	62.7±0.61	1.3±0.12	45.68±0.25

Table 4: Shows Growth rate in A. *ocellaris* fed with different experimental diets for every 15 days interval.

Treatment	Initial Weight of *A.ocellaris* (g)	Weight A *A.ocellaris* fter15th day(g)	Weight After 30th day *A.ocellaris*(g)	Weight After 45th day *A.ocellaris*(g)	Weight After 60th day *A.ocellaris*(g)
Control	5.4±0.321	5.412±0.357	5.43±0.377	5.49±0.381	5.5±0.395
Carrot	5.0±0.298	5.285±0.317	5.46±0.452	5.72±0.523	6.03±0.572
Marigold	4.9±0.214	5.182±0.322	5.28±0.370	5.53±0.422	5.98±0.512
Hibiscus	5.3±0.333	5.320±0.351	5.324±0.382	5.439±0.411	5.62±0.552
Rose	5.2±0.341	5.261±0.381	5.298±0.397	5.442±0.412	5.57±0.526

Table 5: Shows Average values of the total carotenoid concentration (mg kg^{-1}) analyzed in Fresh muscle of the *A.ocellaris*

Treatment	0 day	15th day	30th day	45th day	60th day
Control	1.792 ±0.189	-	-	-	2.687±0.287
Carrot	1.765 ± 0.242	2.532 ± 0.392	4.89 ±0.632	6.739 ±0.428	7.681±0.462
Marigold	1.652 ± 0.289	2.482 ± 0.386	4.366 ±0.368	5.766 ±0. 376	7.235±0.438
Hibiscus	1.567 ± 0.279	2.238 ± 0.372	3.999± 0.425	4.369± 0.325	5.236± 0.314
Rose	1.782 ±0.216	2.48 ±0.146	4.0 ±0.219	4.136 ±0. 237	4.254±0.252

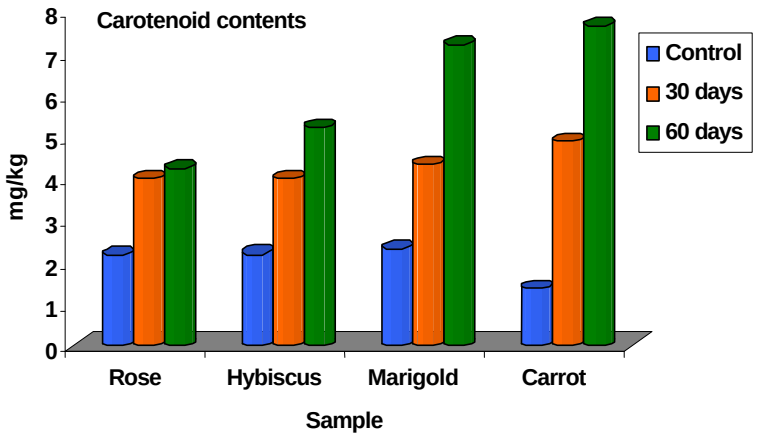

Fig. 1: Carotenoid contents from control and different feeds

Table 6: Shows two ways ANOVA for total carotenoid content of *A. ocellaris* fed with different experimental diets.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	6.602639	3	2.20088	4.09133	0.032451	3.490295
Columns	55.43811	4	13.85953	25.7642	8.2E-06	3.259167
Error	6.455249	12	0.537937			
Total	68.49599	19				

Statistical Analysis: The two way ANOVA was performed to find out the main effects of carotenoid. The analysis of variance (Two way) between the days it shows significant F= 0.0324; P<0.05. And between the diets it shows non significant F=4.09133; P>0.05. (Table 6).

Thin Layer Chromatography: When performing TLC of the pigment extracts a yellow band with a *R*f = 0•40 was always obtained, identical to reference lutein.TLC of the Astaxanthin (standard) extracts showed a violet colour band with an *Rf* = 0.78% was obtained. In the carrot samples was colour showed and the *Rf* value was 0.72%. In the case of Marigold extracts was showed yellow colour and the *Rf* value was 0.68%. Hibiscus was showed yellow colour and the *Rf* value was showed 47%. The *Rf* value of rose was 22% and the colour was showed yellow.

DISCUSSION

Carotenoids are the primary source of pigmentation in ornamental tropical fish, responsible for various colours like yellow, red and other related colours. Normally, these are obtained through organisms rich in carotenoid content organisms in the aquatic food chain. But commercial feed ingredients such as yellow corn, corn gluten meal and alfalfa are used sources of carotenoids such as zeaxanthin and lutein [10]. Other carotenoid-rich ingredients used are marigold meal (lutein), red pepper (*Capsicum* sp.) extract (capsanthin) and krill or crustacean meals (astaxanthin) [5, 11].

The present study showed that various sources of dietary carotenoid did not affect the growth and survival of fish. This agrees with the study of Bell *et al*. [12], reported in which no effect of dietary supplement of Astaxanthin 70 mg/kg were found on the growth of Atlantic salmon (*Salmo salar*) reared for 22 weeks and also Ezhil *et al*. [13] reported fed with marigold petal 15 g/100 g were increased the growth rate of Red Swordtail (*X.helleri)* reared for 60 days. Sinha *et al.* [14] Studied the growth rate of fishes in the group fed with the China rose petal feed was the highest in terms of weight, with an increased value of carotenoid in skin (4.01µg g^{-1}).

The maximum carotenoid content found in 60 days fed group could be directly related to the enhanced level of carotenoid content in the particular ingredients. In this study the maximum carotenoid content was observed with the feed of Carrot followed by Marigold. It was supported in the earlier Ezhil *et al.* [13] studied the total carotenoids in Red Swordtail Muscle tissue, marigold petal meal was found to be an effective colour enhancer at a cheaper price

The synthetic Astaxanthin was found to be more effective than red pepper and marigold flower although they contained equal amounts of carotenoids. The reason for this may be differences in carotenoid sources. Although salmonids absorb Astaxanthin 10-20 times as much as lutein and zeaxanthin [15] but in the present study the carrot and marigold was absorbed more compare than the others fed such as rose and hibiscus.

The development of manufactured feed could be considered as one of the contributing factors to the tremendous growth of this hobby's widespread popularity over the past 50 years [16]. The increased acceptability of reliance upon manufactured feed for ornamental fish has focused the attention on the nutritional requirements of these beautiful aquatic invertebrates. In the present study the diet was prepared 70.5, 60.20, 43.6, 11.5, 2.3 and 12.2% of protein from the ingredients like soybean, fishmeal, groundnut oilcake, wheat flour, tapioca flour and rice bran. In the present study the proximate composition from the hibiscus the protein value was 15.7%, 6.8% of lipid, carbohydrate content was 7.9% and moisture content was 71.02%. It was supported in the earlier study [14] that the proximate composition from China rose petals had lower moisture (70.5%) and higher protein (17.7%) and lipid (5.25%) contents.

TLC of the pigment extracts a yellow band with an *R*f = 0.40 was always obtained, identical to reference lutein. The absorption spectra in benzene (429, 456 and 488 nm) and chloroform (430, 452 and 482 nm) were also identical the colour of the band was yellow in all the samples except the standard Astaxanthin was violet in colour.

The present study shows among the four different carotenoids were used, carrot was found to be an effective colour enhancer at a cheaper price. The results clearly showed that the maximum carotenoid content was present in the fish fed with carrot in 60 days. For instance, the total carotenoid content in the 60 days fed group was found to be 7.681mg/kg wet weight and for the control group was found to be 2.687±0.287 mg/kg. The results of ANOVA suggested that among days it showed significant value F= 0.0324; P<0.05 and among diets it showed insignificant values F=4.09133; P>0.05. No such work is available and hence this will be first step for the future studies in this line. From the above study it could be concluded that colour enhancer feeds can be prepared at a lower cost using the cheaply available sources.

From the above study it could be concluded that colour enhancer feeds can be prepared at a lower cost using the cheaply available source Carrot (*Daucus carota*),

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