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THESIS

THE USE OF TEA (*Camellia sinensis*), MULBERRY (*Morus alba*) AND CASSAVA (*Manihot esculenta*) LEAVES AS A NATURAL CAROTENOID SOURCE IN FANCY CARP (*Cyprinus carpio*)

BUNDIT YUANGSOI

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Bundit Yuangsoi 2009: The Use of Tea (*Camellia sinensis*), Mulberry (*Morus alba*) and Cassava (*Manihot esculenta*) Leaves as a Natural Carotenoid Source in Fancy Carp *(Cyprinus carpio)*. Doctor of Philosophy (Aquaculture), Major Field: Aquaculture, Department of Aquaculture. Thesis Advisor: Associate Professor Orapint Jintasataporn, Ph.D. 247 pages.

The carotenoids composition in tea, mulberry and cassava leaves consisted mainly of lutein and β - carotene, and indicate that tea and mulberry leaves have similarly proportion of lutein and β-carotene about 1:1. In contrast, cassava leave has a proportion about 2:1. The toxic substance of tannin in tea leaf was 4.60 ± 0.07% and HCN in cassava leaf was 921.37 ± 29.23 mg HCN equivalent/kg. Hence, tea and cassava leaves were readily consumed providing no more than 10 and 60 % in dietary which was considered safe for animal consumption. The developed TLC-densitometric analysis for quantitative determination of serum carotenoid was validated by determination of linearity, % recovery, $\%RSD_r$, LOD, 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. It is possible to use the established method for the routine analysis of serum carotenoids. The bioavailability of carotenoids were studied in fancy carp after fed single dose oral administration. The results showed that, astaxanthin was absorbed more readily than lutein and β-carotene. Fish fed a diet containing lutein or β-carotene including raw materials of tea, mulberry and cassava leaves can increase astaxanthin concentration in serum similarly to fish fed directly with astaxanthin diet. Therefore, fancy carp can convert lutein and β-carotene to astaxanthin. The impact of dietary carotenoid on skin pigmentaion in fancy carp found that fish fed diets supplemented with either a combination of lutein and β-carotene at 25:25 and 50:50 mg/kg diets, as well as lutein alone at 50 mg/kg had serum TC higher than other groups and fish fed diets of them had serum astaxanthin concentrations similar to fish fed with astaxanthin. Pigmentation response of skin redness of three groups of fish were higher than other treatments and the same group still retained their redness skin after discontinued carotenoid diets. Overall fancy carp fed with carotenoid diets are tendency to improve skin pigmentation. The effect of feed processing on stability of total carotenoid (TC) in formulated carotenoids diets found that TC content in diets remained quite stable, without significant loss during processes. As a result, should be added 10 percents of TC from the needed amount to natural carotenoids or natural raw materials source to achieve the target level TC content. The best storage temperature for formulated carotenoids diet were keep under low temperature at 4 °C, storage at this temperature is an important factor in slowing down oxidation metabolism which helps reducing the loss of TC quantities and rancidity value. Additional of BHT at 250 mg/kg helps reducing of oxidation during feed process and storage period at room temperature. In additional, stored carotenoid diets away from sunlight, air and heat can prolong shelf life of diets. The effects of various dietary carotenoid source of carotenoid extraction from tea, mulberry and cassava leave and their raw materials on the growth, skin pigmentation and immune response in fancy carp, for a rearing period of six weeks. In this study, neither growth nor feed conversion efficiency were affected significantly by dietary treatment for all treatments diets. Fancy carp fed with dietary supplementation of tea leave at TC 25 mg/kg tends to increase slightly higher serum TC and astaxanthin content. Pigmentation response of skin redness of this group showed the best effects on skin coloration and profound influence on immune response in term phagocytic activity. Therefore, tea leave is suggested to be an effective alternative natural carotenoid source to synthetic carotenoid and could be reducing costs related to pellet production and adding value to byproduct from agricultural which will therefore promote increased profit for fish farmers.

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THE USE OF TEA (*Camellia sinensis*), MULBERRY (*Morus alba*) AND CASSAVA (*Manihot esculenta*) LEAVES AS A NATURAL CAROTENOID SOURCE IN FANCY CARP (*Cyprinus carpio*)

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 color is primarily dependent on the presence of chromatophore (xanthophores and erythrophores) containing carotenoids (e.g. astaxanthin, canthaxanthin, lutein and zeaxanthin). In plants, carotenoids are C 40 isoprenoid polyene secondary plant compounds that form lipid soluble yellow, orange and red pigments. Examples of carotenoids include the oxygenated xanthophylls--lutein [(3*R*,3'*R*,6'*R*)-β-carotene-3,3'-diol] and the hydrocarbon carotene--β-carotene (β,β-carotene) (Zaripheh and Erdman, 2002). The carotenoids occur generally in the chloroplasts of green tissue but their color is masked by the chlorophylls, organelles where photosynthesis take place. The leaves of virtually all species contain the same main carotenoids, that is β- carotene (usually 25 – 30% of the total), lutein (around 45 %), violaxanthin (15%) and neoxanthin (15%). Small amounts of α-carotene, α and β-cryptoxanthin, zeaxanthin, antheraxanthin and lutein-5, 6-epoxide are also frequently present and lactucaxanthin is a major xanthophylls in a few species (Gross, 1991). There is a considerable variation in the total carotenoids content in leaves of different species and varieties.

In ornamental high-value species need to achieve high levels of skin pigmentation, together with body shape, fin shape and body size, because of the most important quality criteria dictating informing their market value (Paripatananont *et al*., 1999). Among ornamental fish, 'fancy carps' (*Cyprinus carpio*) is growing worldwide due to the increasing number of hobbyists. One of the frequently found problems includes the color of fancy carp becomes pale after being raised for a period of time,

which is not preferred by hobbyists. Thus, making the fancy carps color remains vivid constantly, they need to enhance color utilized in the diet, particularly 'carotenoids'.

Carotenoids, which are lipid soluble pigments, are responsible for skin color of ornamental fish. The yellow, orange and red hues (color) found in fish skin are the result of a group of carotenoid pigment (Simpson *et al*., 1981). Carotenoids are also vital nutrients for healthy growth, metabolism and reproduction. However, carotenoids cannot be synthesized by most animals, including fishes, and must be obtained from dietary sources (Goodwin, 1984; Torrissen *et al*., 1989; Storebakken and No, 1992) but they can modify alimentary carotenoids and store them in the integument and other tissues. Because different species have different carotenoids metabolized and carotenoids storage for instance, fancy carps can convert lutein, zeaxanthin to astaxanthin (Katayama *et al*., 1973). Hirao *et al*., (1963) and Hsu *et al*., (1972) reported that goldfish fed diets rich in lutein and carotene efficiently converted them to astaxanthin and had a significant increase in total amount of carotenoids per individual fish.

Chromatography is the most efficient technique for separation and identification carotenoids. Recently, HPLC has been widely used for analysing 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 but these techniques are taking much time and 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 plants. Modern TLC has advantages in many analyses, including the following: simplicity of operation; the availability of many sensitive and selective reagents for detection and confirmation without interference of the mobile phase; the ability to repeat detection and quantification at any time with changed parameters because fractions representing the entire sample are stored on the plate; in-system calibration for quantitative analysis; and cost effectiveness because many samples can be analyzed on a single plate with low solvent usage. In situ measurement of zone

with a scanning densitometer (TLC-densitometry analyses) is the preferred technique for quantitative TLC. This method is economical as it utilizes smaller amounts of solvents with minimum sample clean up. Also, in a short duration, a large number of samples are simultaneously analyzed. TLC has no limitation on the choice of the mobile phase and unlike HPLC. Direct application of suspensions, dirty or turbid samples are possible. Therefore, TLC is quickly and economically than determined by HPLC and it permits a simultaneous assay of several components in a multicomponent or fish serum.

Nowadays, natural sources (yeast, bacteria, algae, higher plants and crustacean meal) have been used as dietary supplements to enhance the pigmentation of fish and crustacean (Kalinowski *el al.,* 2005). With the raw materials of plant origin, several carotenoids have found their way into fish diets. Such examples are the lutein and β-carotene which are found in large amount in plants (Zaripheh and Erdman, 2002). Therefore production of carotenoids from natural source especially lutein and β-carotene are a potential alternative source of carotenoids to replace the synthetic astaxanthin.

Casava leaves can be harvested six months after planting and can pick 1-2 time, before the final harvest. The leaves can be used as animal feed. Normally, cassava leaves possess 20-25% protein, 6% fat, 14% fiber, 12% humidity, 7% ash and 0.10 phosphorus (dry matter). The leaves contain approximately 560 mg/Kg of xanthophyll. Mulberry is categorized in Moraceae specie, also known as *Morus spp.* It is planted as fodder for silk worms. Mulberries contain 3.50-6.65% moisture, 15.76-24.38% protein, 41.91-54.91% carbohydrate, 2.27-8.97% fat, 7.65-15.70% ash and 10.20-15.30% fiber (dry matter). It is reported by Doira (1978) that hemolymph in young silk worms are dark yellow because of carotenoids they absorbed from mulberry and Bauernfeind (1981) found carotenoid, namely lutein in silk nest. Tea leaves contain 20-35% polyphenols. They also contain 75-78% moisture, 93-96% organic substances including 20-30% protein, 20-35% polyphenol, 20-25% carbohydrate and 8% fat. The mainly carotenoid in tea leave is xanthophyll such as

neoxanthin, violaxanthin, antheraxanthin, zeaxanthin and lutein including carotene is β-carotene (Suzuki and Shioi, 2003). Ravichandran (2002) reported that tea leave consist total carotenoids 360-730 mg/kg. The carotenoids composition of all leaves are similar quantitative. Nevertheless some plants were composed with toxic substances, traditionally the crop of cassava is grown for the roots, which are used as human and animal food and industrially as a source of starch. The main limiting factor to the use of cassava leaves as animal feed is the presence of cyanogenic glucoside, which given rise to hydrocyanic acid (HCN). The lethal dose of hydrocyanic acid was 600 mg HCN equivalent/kg (Okafor *et. al.,* 2002). Including tea leave that have tannin that the levels of tannin above 5% of the diet are often lethal and the levels from 0.5-2.0% can cause depression in growth (Giner-Chavez, 1996).

The production of carotenoids from natural sources is a potential alternative to replacing the synthetic carotenoids. With the introduction of raw materials of plant origin, several carotenoids have found their way into fish diets such example as the xanthophylls-- lutein and hydrocarbon carotene-- β-carotene which found in large amount in plants. The current results indicate that fancy carp were able to utilize lutein and β-carotene from plant leave efficiently. Similar results were obtained for goldfish but by feeding different natural carotenoid sources, such as red yeast, X. *dendrorhous* (Xu *et al*., 2006), Spirulina (Kiriratnikom *et al*., 2005), C. *vulgaris*, H. *pluvialis* and A. *maxima* (Gouveia *et al*., 2003; Gouveia and Rema, 2005), alfala (Yanar *et al*., 2008). Yanar *et al*. (2008) showed that a natural carotenoid source was as effective as a synthetic carotenoid source, on skin pigmentation of goldfish. Natural carotenoids such as *Chlorella vulgaris* had already proved to be a useful, even competitive coloring for inclusion in the diets of laying hens with the purpose of coloring egg yolk (Gouveia *et al*., 1996); in rainbow trout yielding both muscle and skin pigmentation effect (Gouveia *et al*., 1997 and 1998); in gilthead seabream for skin pigmentation (Gouveia *et al*., 2002) and improved skin coloring in goldfish and fancy carp (Gouveia *et al*., 2003). Hence, higher plant is suggested to be an effective alternative natural carotenoid source to synthetic carotenoid for fancy carp pigmentation.

Thailand is an agricultural country. Generally, there are a lot of crop residues from the process. Furthermore, these residues become wastes and have caused pollution. Carotenoids in plant include oxygenated xanthophylls--lutein and hydrocarbon carotene--β-carotene. There is a considerable variation in carotenoids content in leaves varied by season, species, age, harvesting period and storage. This study makes use of local Thai feedstuffs to be the carotenoid sources for color enhancing diet particularly in fancy carps or ornamental fishes' diets. This becomes a method in lowering the production cost of diets and in adding value to raw material and byproducts from agricultural harvest.

OBJECTIVES

Overall objectives

This study makes use of local Thai feedstuffs to be the carotenoid sources for increasing color pigmentation and booting immunity in fancy carp. This becomes a method in lowering the production cost of color enhancing fancy carp diets and in adding value to a byproducts from agricultural harvest.

Specific objectives of this study are to:

1. Investigate the variation in carotenoids composition and toxic substances in local Thai feedstuffs a byproducts from agricultural harvest.
2. Modify of the selected methodology for the most effective conditions and validation of the established method in developing accuracy and repeatability of carotenoids.
3. Evaluate the pharmacokinetic parameters of astaxanthin, lutein and β-carotene including raw material of tea, mulberry and cassava leave in fancy carp after single dose oral administration.
4. Investigate the effectiveness of three carotenoids, namely astaxanthin, lutein, β-carotene including the combination between lutein and β-carotene, as a dietary carotenoids source for improving skin coloration of fancy carp.
5. Determine carotenoids concentration on stability during feed processing and under different storage temperatures including investigate rancidity parameters during storage.
6. Study the effect of natural carotenoids from raw material and carotenoids crude extraction from local Thai feedstuffs on growth performance, color pigmentation and immune response.

LITERATURE REVIEW

1. Cyprinid

The family Cyprinidae consists of the carps, the true minnows, and their relatives (e.g. the barbs). They are commonly called cyprinids or carp and minnow family. It is the largest family of fresh-water fish, with over 2,400 species in about 220 genera. The family belongs to the order Cypriniformes, of whose genera and species the cyprinids make up two-thirds (Howes, 1991)

Natural color mutations of these carp would have occurred across all populations. Historical records indicate that carp with color mutations were found in China. However, the earliest records of carp with distinct colors kept for selective breeding, true Nishikigoi, have been found in Japan. Depictions of carp or 'koi' with different color variations have been found on 18th century Japanese drawings and paintings. The ornamental cultivation of carp originated in the Niigata region of Japan during the Japanese Edo Period (George, 1995).

1.1 Dietary requirement of Koi

Koi, like all living creatures, require certain amounts of nutrients in the diet on a regular basis. Nutrients are necessary for cell growth, energy, food utilization, and organ function. The five important nutrients are amino acids (protein), lipids (fat), carbohydrate, vitamins and minerals (David, 2001).

1.1.1 Amino Acids

Protein is not required in the diet per se. Protein provides the amino acids that are necessary. Protein in body tissues incorporate about 23 amino acids and among these, 10 amino acids must be supplied (essential amino acids) in the diet since fish cannot synthesize them. Amino acids are needed for maintenance, growth, reproduction and replication of tissues. A large proportion of the amino acid consumed by a fish is catabolized for energy. Fish are well adapted to using an excess protein this way. Catabolism of protein leads to the release of ammonia, which up to

70% is excreted across the gills. Protein is the most important component of the diet of fish because protein intake generally determines growth. Koi diets generally contain 32-38% protein.

Natural diets are generally plankton, invertebrates, worms etc. These are generally rich in protein and have a good amino acid balance. All dietary proteins are not identical in their nutritive value. The nutritional value of a protein source is a function of its digestibility and amino acid makeup. A deficiency of indispensable amino acid creates poor utilization of dietary protein and hence growth retardation, poor weight gain, and feed efficiency. In severe cases, deficiency reduces the ability to resist diseases and lowers the effectiveness of the immune response mechanism.

1.1.2 Lipids (Fats)

Lipids (fats) encompass a large variety of compounds. Lipids have many roles: energy supply, structure, precursors to many reactive substances, etc. In the diet or body of fish, lipids are most commonly found as triglycerides, phospholipids and, sometimes, wax esters. Deficiency in essential fatty acid result in general, in reduction of growth and a number of deficiency signs, including depigmentation, fin erosion, cardiac myopathy, fatty infiltration of liver, and possibly a loss of consciousness for a few seconds following an acute stress.

1.1.3 Carbohydrates

The carbohydrate most commonly found in fish feed is starch, a polymer of glucose. Many fish have a poor ability to utilize carbohydrates. Raw starch in grain and other plant products are generally poorly digested by fish. Koi do not process carbohydrates well and as such, carbohydrates constitute only about 30% of the koi's nutritional requirements. Cooking of the starch during pelletizing or extrusion, however, greatly improves its digestibility for fish. However, even if the starch is digestible, fish only appear to be able to utilize a small amount effectively. Carbohydrates only represent a minor source of energy for fish. A certain amount of starch or other carbohydrates (e.g. lactose, hemicellulose) is, nevertheless, required to achieve proper physical characteristic of the feed.

1.1.4 Vitamins

Vitamins can be equated to the spark plugs in an engine. It is the spark that ignites a reaction. Vitamins regulate the metabolism through enzymes. Vitamins are components of the enzyme systems that, acting like spark plugs, energize and regulate the metabolism. The vitamins are generally defined as dietary essential organic compounds, required only in minute amounts, and which play a catalytic role, but no major structural role.

1.1.5 Minerals

Vitamins alone are not enough. As important as vitamins are, they cannot function with out minerals. Although the body can synthesize some vitamins it cannot manufacture a single mineral. Inorganic elements (minerals) are required by fish for various functions in metabolism and osmoregulation. Fish obtain minerals from their diet but also from their environment. Many minerals are required in trace amounts and are present in sufficient quantity in the surrounding water for the fish to absorb through their gills. In freshwater, there is generally sufficient concentration of calcium, sodium, potassium and chloride for the fish to absorb from the surrounding water and cover its requirements

Summary - Chapter 3

2. Chromatophores

Chromatophores are pigment-containing and light-reflecting cells found in amphibians, fish, reptiles, crustaceans, and cephalopods. They are largely responsible for generating skin and eye color in cold-blooded animals and are generated in the neural crest during embryonic development. Mature chromatophores are grouped into subclasses based on their color (more properly "hue") under white light: xanthophores (yellow), erythrophores (red), iridophores (reflective/iridescent), leucophores (white), melanophores (black/brown) and cyanophores (blue) (Fujii, 2000).

Some species can rapidly change color through mechanisms that translocate pigment and reorient reflective plates within chromatophores. This process, often used as a type of camouflage, is called physiological color change. Cephalopods such as

octopus have complex chromatophore organs controlled by muscles to achieve this, while vertebrates such as chameleons generate a similar effect by cell signaling. Such signals can be hormones or neurotransmitters and may be initiated by changes in mood, temperature, stress or visible changes in local environment.

2.1 Pigments

The major pigments that are detectable in the skin of fish is presented (Fujii, 2000)

2.1.1 Melanins

Of the pigments that color the skin of animals, melanins are also the most widely distributed in fish. Brown to black tones generally result from the presence of melanins. They are highly polymerized compounds derived from the amino acid tyrosine. They are synthesized and deposited in the dark organells of the melanophores, which are called the melanosomes. The first steps in the synthesis are the oxidation of tyrosine to dopa and the oxidation of dopa to dopaquinone by tyrosinase.

2.1.2 Carotenoids

The beautiful and flamboyant coloration exhibited by many fish is mostly due to the presence of carotenoid pigments. Theses are polyene pigments that are insoluble in water and they are deposited in carotenoid vesicles in the xanthophores or erythrophores. There are many molecular species, but all of them are derived from the prey or come directly from plant sourced. Zsometimes plant carotenoids are modified to yield pigments that are found only in animals. Carotenoids commomly found in freshwater fish include beta-carotene, lutein, taraxanthin, astaxanthin, tunaxanthin, doradexanthin and zeaxanthin.

2.1.3 Pteridines

Pteridines are compounds with a pteridine nucleus as their

basic skeleton. They are presence in many species of invertebrates. Only recently were several molecular species in this category found in the skins of poikilothermic vertebrates, playing active roles in the generation of bright colors, namely, yellow, red and brown, as do the caroenoids. In contrast to carotenoids, pteridines are soluble in water. The cell organells that contain pteridines are called pterinosomes.

2.1.4 Purines

Some purine bases play an indispensable role in the generation of color in fish. Among the purines, guanine apparently predominates and extraordinarily large amounts of guanine can be found in the silvery belly skin of most species of fish. Being barely soluble in water, purines are usually found in crystalline frome in irridophores and they act efficiently to reflect light.

2.2 Classification of chromatophore

Color-production falls into distinct classes: biochromes, schemochromes. The biochromes include true pigments, such as carotenoids and pteridines. These pigments selectively absorb parts of the visible light spectrum that makes up white light while permitting other wavelengths to reach the eye of the observer. Schemochromes, also known as "structural colors", produce coloration by reflecting some wavelengths (colors) of light and transmitting others, by causing light waves to interfere within the structure or by scattering light which falls upon them (Fox, 1976).

While all chromatophores contain pigments or reflecting structures (except when there has been a genetic mutation resulting in a disorder like albinism), not all pigment containing cells are chromatophores. Haem, for example, is a biochrome responsible for the red appearance of blood. It is primarily found in red blood cells (erythrocytes), which are generated in bone marrow throughout the life of an organism, rather than being formed during embryological development. Therefore erythrocytes are not classified as chromatophores.

2.2.1 Xanthophores and erythrophores

Chromatophores that contain large amounts of yellow pteridine pigments are named xanthophores and those with an excess of red/orange carotenoids termed erythrophores. It was discovered that pteridine and carotenoid containing vesicles are sometimes found within the same cell, and that the overall color depends on the ratio of red and yellow pigments (Matsumoto, 1965). Therefore the distinction between these chromatophore types is essentially arbitrary. The capacity to generate pteridines from guanosine triphosphate is a feature common to most chromatophores, but xanthophores appear to have supplemental biochemical pathways that result in an excess accumulation of yellow pigment. In contrast, carotenoids are metabolised from the diet and transported to erythrophores.

2.2.2 Iridophores and leucophores

Iridophores, sometimes also called guanophores, are pigment cells that reflect light using plates of crystalline chemochromes made from guanine (Morrison, 1995). When illuminated they generate iridescent colors because of the diffraction of light within the stacked plates. Orientation of the schemochrome determines the nature of the color observed. By using biochromes as colored filters, iridophores create an optical effect known as Tyndall or Rayleigh scattering, producing bright blue or green colors (Fujii, 2000).

A related type of chromatophore, the leucophore, is found in some fish, particularly in the tapetum lucidum. Like iridophores, they utilize crystalline purines (often guanine) to reflect light. Unlike iridophores, however, leucophores have more organized crystals which reduce diffraction. Given a source of white light, they produce a white shine. As with xanthophores and erythrophores, in fish the distinction between iridophores and leucophores is not always obvious, but generally iridophores are considered to generate iridescent or metallic colors while leucophores produce reflective white hues.

2.2.3 Melanophores

Melanophores contain eumelanin, a type of melanin, that appears black or dark brown because of its light absorbing qualities. It is packaged in vesicles called melanosomes and distributed throughout the cell. Eumelanin is generated from tyrosine in a series of catalysed chemical reactions. It is a complex chemical containing units of dihydroxyindole and dihydroxyindole-2-carboxylic acid with some pyrrole rings (Ito and Wakamatsu, 2003). The key enzyme in melanin synthesis is tyrosinase. When this protein is defective, no melanin can be generated resulting in certain types of albinism. In some amphibian species there are other pigments packaged alongside eumelanin.

2.2.4 Cyanophores

In 1995 it was demonstrated that the vibrant blue colors in some types of mandarin fish are not generated by schemochromes. Instead, a cyan biochrome of unknown chemical nature is responsible (Fujii, 2000). This pigment, found within vesicles in at least two species of callionymid fish, is highly unusual in the animal kingdom, as all other blue colorings thus far investigated are schemochromatic. Therefore a novel chromatophore type, the cyanophore, was proposed. Although they appear unusual in their taxonomic restriction, there may be cyanophores (as well as further unusual chromatophore types) in other fish and amphibians.

2.3 Pigment translocation

Many species have the ability to translocate the pigment inside chromatophores, resulting in an apparent change in color. This process, known as *physiological color change*, is most widely studied in melanophores, since melanin is the darkest and most visible pigment. In most species with a relatively thin dermis, the dermal melanophores tend to be flat and cover a large surface area. However, in animals with thick dermal layers, such as adult reptiles, dermal melanophores often form three-dimensional units with other chromatophores. These dermal

chromatophore units (DCU) consist of an uppermost xanthophore or erythrophore layer, then an iridophore layer, and finally a basket-like melanophore layer with processes covering the iridophores (Fujii, 2000).

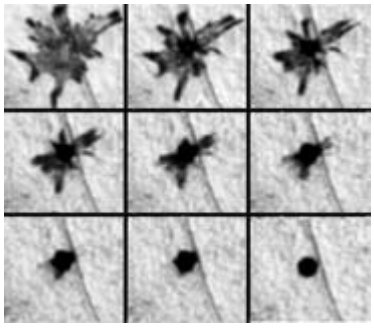


Figure 1 A single zebrafish melanophore imaged by time-lapse photography during pigment aggregation

Source: Logan *et al*. (2006)

The control and mechanics of rapid pigment translocation has been well studied in a number of different species, particularly amphibians and teleost fish (Deacon *et al*., 2003). It has been demonstrated that the process can be under hormonal, neuronal control or both. Neurochemicals that are known to translocate pigment include noradrenaline, through its receptor on the surface on melanophores. The primary hormones involved in regulating translocation appear to be the melanocortins, melatonin and melanin concentrating hormone (MCH), that are produced mainly in the pituitary, pineal gland and hypothalamus respectively. These hormones may also be generated in a paracrine fashion by cells in the skin. At the surface of the melanophore the hormones have been shown to activate specific G-protein coupled receptors that, in turn, transduce the signal into the cell. Melanocortins result in the dispersion of pigment, while melatonin and MCH results in aggregation.

2.4 Physiological color change

Chromatophores evolve from neural crest then distribute through dendrite under dermis more than epidermis. Melanophores, xanthophores, erythrophores and leucophores are dendritic chromatophores. Dispersion and aggregation of colors can be found within chromatophores. However iridophores are considered as immotile cells and colorless, thus categorized as non-dendritic chromatophores. Purines or guanines (reflecting platelets) are found in cytoplasm and the mobilization of chromatophores is called motile activity.

Color changing in fish is known as physiological color changes. It occurs in order to protect fish from hunters or when colors being stored from fed nutrients. Researches found that sympathetic nerve provides better result in color changing compared to endocrine system because chromatophores can move faster. However, endocrine system is controlled by hormones such as melanophore stimulating hormone (MSH) synthesized from pituitary gland melanin concentrating hormone (MCH) synthesized from posterior lobe while epinephrine and norepinephrine are synthesized from chromaffin cells.

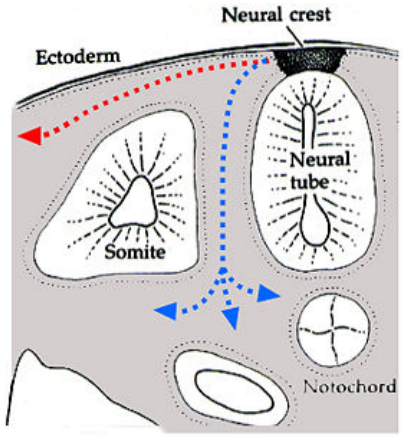


Figure 2 Transverse section of a developing vertebrate trunk showing the dorsolateral (red) and ventromedial (blue) routes of chromatoblast migration.

Source: Kelsh *et al*. (2000)

3. Carotenoids

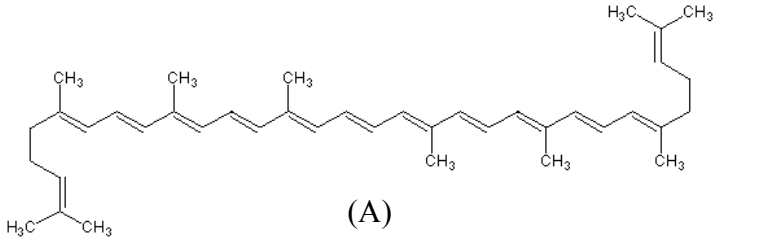
Carotenoids can be found in plants and animals. These color-generating materials are in plastids that are naturally occurring in chromoplasts of plants and some other photosynthetic organisms like algae, some types of fungus and some bacteria.. Though animals cannot produce carotenoids by themselves, they can keep carotenoids as polyunsaturated C40 hydrocarbons which consist of carbon atoms, single bonds along with double bonds. Carotenoids are dissolved in fat thus called lipophore. Carotenoids are tetraterpenes containing 4 connected isoprenes, forming the ring structure. They come in yellow, orange, and red. Light absorption is at 400-600 nm. There are over 600 known carotenoids.

3.1 Carotenoid Chemistry

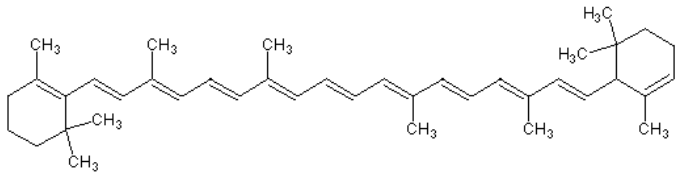
Carotenoids belong to the category of tetraterpenoids (i.e. they contain 40 carbon atoms). Structurally they are in the form of a polyene chain which is sometimes terminated by rings. Chemical structure of carotenoids divided into 2 types

3.1.1 Carotene – molecules of coretene are carbon containing both single and double bonds. Carbon atoms forming a shape of ionone ring which are α-carotene, β-carotene, γ-carotene) and lycopene. The most important one is β-carotene since it could transform to vitamin A.

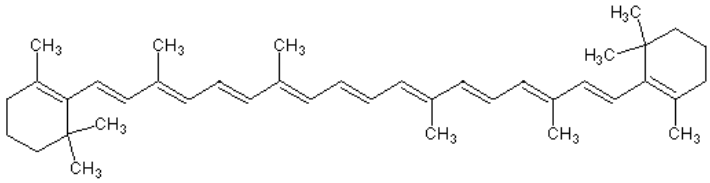
3.1.2 oxycarotenoid or xanthophylls–occurred from oxygen containing hydrocarbons. Xanthophylls are in hydroxyl or keto or esto which work as a function group. They can cause coloring ranges from yellow to orange red. They are found in both plants and animals such as corns, cassava leaves etc. Xanthophylls consist of astaxanthin, canthaxanthin, cryptoxanthin, lutein and zeaxanthin.



(A)



(B)



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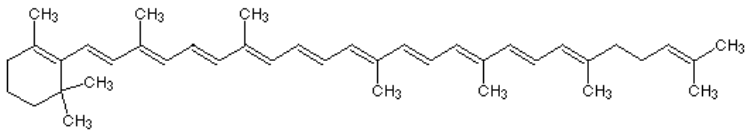
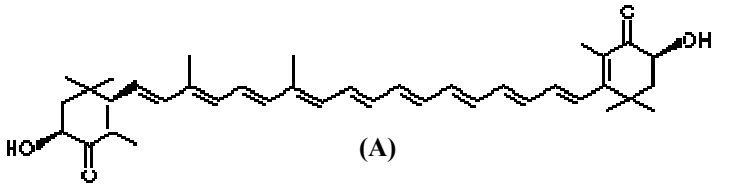
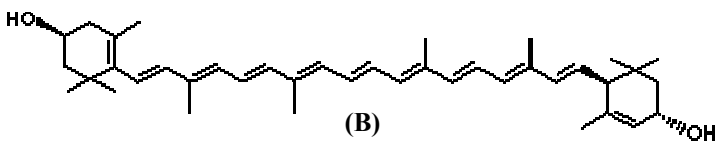


(D)

Figure 3 Chemical structure of carotenoids; A: Lycopene, B: α-carotene, C: β-carotene and D: γ-carotene.



(A)



(B)

Figure 4 Chemical structure of carotenoids; A: astaxanthin and B: lutein

Their color, ranging from pale yellow through bright orange to deep red, is directly linked to their structure. Xanthophylls are often yellow, hence their class name. The double carbon-carbon bonds interact with each other in a process called conjugation, which allows electrons in the molecule to move freely across these areas of the molecule. As the number of double bonds increases, electrons associated with conjugated systems have more room to move, and require less energy to change states. This causes the range of energies of light absorbed by the molecule to decrease. As more frequencies of light are absorbed from the short end of the visible spectrum, the compounds acquire an increasingly red appearance.

3.2 Carotenoid analysis

The extraction techniques, purification process, quantification and identification of carotenoid have been reviewed in detail by several researchers (Davises 1976; De Ritter and Purcell, 1981and Britton *et al*., 2004). There is no single method for extraction owing to the diversity of the biological material. Schiedt and Liaaen-Jensen (1995) provided a detailed description of the precautions to be taken for extraction, isolation, quantification and prevention of artifact formation. Since color is one of distinctive features of the carotenoids, any change or loss in color can be interpreted as a modification to the color absorbing polyene chain. This conjugated double bond chain that is responsible for color is also susceptible to oxidation in the presence of air and is sensitive to damage by light and heat, leading to degradation, isomerization, or artifact formation. Use of inert gases to exclude oxygen, use of antioxidant like BHT, pyrogall or ascorbyl palmitate to protect the carotenoids, low temperatures for extraction and storage, exclusion of sunlight, acid or alkali have been strongly recommended.

Chromatography is the most efficient technique for separation and identification carotenoids. In the past, carotenoids were isolated chromatographically using open column silica or alumina columns. The individual fractions were identified using UV-Vis spectra and quantified using known molar absorptivity ratios. Their purity was evaluated on TLC or paper chromatography (Davies, 1976). Today, HPLC,

particularly reverse-phase is the most popular method for analysing carotenoids in food and biological matrices. Reverse phase HPLC has several advantages over normal-phase methods. The columns use inert hydrocarbons as packing material, which leads to greater stability of the column and reproducibility of results. Carotenoids are well separated because of the differences in hydrophobicity and because there is less degradation and isomerization. In reverse-phase HPLC, the more polar xanthophylls elute before carotene.

Carotenoids can be tentatively identified by comparing retention times (R_f values) on a HPLC column with known standards run under identical conditions and by comparing spectral information. UV-vis spectral have been used for identification, as they are characteristic of a particular carotenoid. Spectral are solvent and concentrationdependent as well and hence can not be used for definitive identification without more information. More definitive identification is possible using, Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) technique but these techniques are taking much time and 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 plants. Modern TLC has advantages in many analyses, including the following: simplicity of operation; the availability of many sensitive and selective reagents for detection and confirmation without interference of the mobile phase; the ability to repeat detection and quantification at any time with changed parameters because fractions representing the entire sample are stored on the plate; in-system calibration for quantitative analysis; and cost effectiveness because many samples can be analyzed on a single plate with low solvent usage. In situ measurement of zone with a scanning densitometer (TLC-densitometry analyses) is the preferred technique for quantitative TLC. This method is economical as it utilizes smaller amounts of solvents with minimum sample clean up. Also, in a short duration, a large number of samples are simultaneously analyzed. TLC has no limitation on the choice of the mobile phase and unlike HPLC. Direct application of suspensions, dirty or turbid samples are possible. Therefore, TLC is quickly and economically than determined

by HPLC and it permits a simultaneous assay of several components in a multicomponent or fish serum.

Quantitative thin-layer chromatography (QTLC) measured by direct photometric scanning has been performed for more than forty years. Despite this long period, this procedure has not achieved the reputation of a very reliable quantitative analytical method. Large standard deviations in measurements have often been mentioned as a reason why TLC was supposedly not acceptable for quantitative work. The most important reasons which have kept TLC from being recognized as a quantitative tool were uncertainties and problems in sample applications, development and scanning. There were many underfined paprameters, factors which contributed to the poor reproducibility of TLC.

Nevertheless, the opposition was unjustified. OTLC can be very powerful: it has a very small systemic error, and a high accuracy anc easily be obtained by using a large number of applications and statistical methods. The significant cause for the poor reproducibility of TLC is the positioning error, in densitometric scanning. This problem can be minimized by using special sample applications, controlled development procedures and sophisticated computer-controlled scanning modes. The real improvement in reproducibility, simplicity and speed of quantitative evaluation, however, is expected with the use of image processing.

In addition, a very intensive development of HPLC nearly arrested interest in basic development in QTLC because the reproducibility of HPLC particularly for routine analysis, was excellent and manufactures found HPLC equipment a good commercial proposition. The probable reason was that HPLC equipment is so sophisticated that improvisation is virtually ruled out. HPLC can be developed to the stage of push-button analysis, so it is also an excellent solution for everyday routine work.

Among the users of chromatography around the world today, however, it is possible to see renewed increasing interest for TLC. Analyts have seen that

sophisticated and specifically oriented methods cannot be properly used if they are not planed according to the results obtained by prescreening using much cheaper, programmable applicators, and development systems, and the use of sophisticated, inexpensive personal computers, hard disks, CCD cameras, and color printers, together open up new possibilities for quantitative TLC.

Thin-layer chromatography today is a dynamically developing modern analytic method. Areas of progress include increase of the spectrum of selectivity, improvement of efficiency, and in certain case simplification of handling. The discussion of bulk sorbents and precoated layers above is not a complete enumeration of all possibilities; e.g., the different carries for the layers (glass plates, aluminum, or plastic sheets) are not shown explicitly. In addition, special plates with very restricted applicability are not discussed.

Focal points of recent and expected future developments in thin-layer chromatography are located in the fields of surface modification and in the improvement of the efficiency of precoated layers. Advances in these areas are preconditions for maintaining and extending the importance of TLC as a qualitative and a quantitative analytical method in chemical laboratories.

Furthermore, a trend in direction of coupling TLC with spectroscopic methods (e.g. FTIR, RAMAN, SERS and MS) is recognizable to enlarge the analytical possibilities. For this purpose tailor-made precoated layers are in preparation.

3.3 Carotenoids in higher plant

Traditionally, carotenoids are thought of as plant pigments. They are indeed found in all green tissues, where they occur in the photosynthetic pigment-protein complexes of the chloroplasts. The yellow carotenoid color is masked by the green of the chlorophylls, which are extracted along with the carotenoids, but is revealed in the autumn leaves of many trees, as the chlorophyll is destroyed and, at the same time, the xanthophylls become esterified with mixtures of fatty acids. With

few exceptions, the leaves (chloroplasts) of all species contain the same collection of main carotenoids, namely β, β-carotene, lutein, vilaxanthin and neoxanthin. Smaller amounts of other compounds, e.g. β-cryptoxanthin, zeaxanthin, antheraxanthin and lutein 5,6-epoxide, can frequently be detected, particularly when appropriate chromatographic fractions are concentrated, and lactucaxanthin is present in some species (Britton, 1995).

Carotenoids are pigments synthesized by photosynthetic microorganisms and plants but not by animals. There are more than 600 carotenoids occurring in nature, of which more than 50 carotenoids are consumed in appreciable quantities in the human diet (Khachik *et al*., 1991). Whereas it is difficult to categorize definitively fruits and vegetables based on carotenoid distribution, Khachik *et al*. (1991) have divided common plant foods into three groups. The carotenoids in the first category of vegetables and fruits contain a wide range of xanthophylls and carotenes; this group includes the green vegetables such as broccoli, green beans, spinach, and peas. The second category contains yellow/red fruits and vegetables with a more unique distribution of carotenoids than the first category, and includes such foods as apricots, carrots, cantaloupe and tomatoes, which contain mostly carotenes. The third category includes squash, oranges, prunes, and peaches; these yellow/orange fruits and vegetables contain xanthophylls esterified with fatty acids, which must be digested before absorption (Wingerath *et al.*, 1995).

3.4 Carotenoids source for animal feed

In aquatic animals farming, there are two types of fodder, macro and micro ingredient. Carotenoids are categorized as micro ingredient which affects to biological function. It also is a strong antioxidant. Carotenoids are organic substance, and are red orange and yellow respectively. They are found in plants and animals though amount of them are varied. However, they are always synthesized in photosynthesis pathway both in algae or zooplankton. Higher organisms, however, are not capable in such synthesis. Carotenoids for aquatic animal are known as

astaxanthin. It helps creating red hue. Another substance is canthaxanthin which intermediates during astaxanthin synthesis process. β-carotene is significant in the way that it is affected by both internal and external factors such as stage of maturation.

3.4.1 Spirulina (also known as, *Spirulian platensis*) is a Blue green algae. It provides high protein (up to 60-90%), high amino acid, 6-14% fat, 10-18% carbohydrate, 3-9% fiber, 6-10% moisture , and 4-5% ash (basis dry matter) as well as some other significant colors-generating materials. It is a source of carotenoids such as β-carotene and zeaxanthin 0.8-1.5% of (dry matter).

3.4.2 Marigold (*Tagetes sp)* is a native Mexico plant. It gives 13.5% protein, 12.2% fat, 19.7% fiber, 48.7% NFE, 0.66% calcium, 0.62 phosphorus, and 5.86 ash (basis with dry matter). Carotenoids found in dried marigold are mostly dihydroxy pigments containing lutein and zeaxanthin.

3.4.3 Casava leaves can be harvested six months after planting. The leaves can be used as animal feed (without affecting fruitage). Good leaves should be green and dry so that they can last 6 months. Cassava leaves, however, contain hydrocyanic so they are dry-stored. Normally, cassava leaves possess 20-25% protein, 6% fat, 14% fiber, 12% humidity, 7% ash and 0.10 phosphorus (dry matter). The leaves contain approximately 560 mg/Kg of xanthophyll.

3.4.4 Leucaena leaves can be maximally mixed with animal feed 4-5% since they contain some toxic known as mimosin which is highly hazardous if over-consumed. Leucaena leucocephala leaves possess 27% protein, 8% fat, 12% fiber, 9.5% moisture, and 10% phosphorus (dry matter). They are capable as color-generating material for carotenoids such as β-carotene 446 mg/Kg , xanthophyll 865 mg/kg. Higher amount of the leaves results in redder yolk egg. Xanthophylls found in Leucaena leaves are as followed:

Green leucaena leucocephala leaves = 583.2 mg/Kg

Dried leucaena leucocephala leaves (sun dried) = 318.2 mg/Kg

Dried leucaena leucocephala leaves (wind dried) = 346.6 mg/Kg

3.4.5 Mulberry is categorized in Moraceae specie, also known as *Morus spp.* It is planted as fodder for silk worms. Mulberries contain 3.50-6.65% moisture, 15.76-24.38% protein, 41.91-54.91% carbohydrate, 2.27-8.97% fat, 7.65-15.70% ash and 10.20-15.30% fiber (dry matter). Nutrition found in mulberry varies from species, ages, and time of harvest. It is reported by Doira (1978) that young silk worms are dark yellow because of carotenoids they absorbed from mulberry. Feeding mulberry to chickens results in redder yolk egg according to the amount of mulberry fed.

3.4.6 Tea leaves contain 20-35% polyphenols. There are not less than 450 organic matters and not less than 15 inorganic matters from green tea leaves. They also contain 75-78% moisture, 93-96% organic substances including 20-30% protein, 20-35% polyphenol, 20-25% carbohydrate and 8% fat. β-carotene and lutein are found in the leaves as well as 1.35-1.76% potassium and 0.5-0.7% calcium.

3.4.7 Shrimp mill are left out from frozen shrimps industry. They include shrimp heads, and tissue-connected husks. Thus gives high protein of approximately 30% though these tissues are hard to digest. Yet they contain calcium, carbonate and chitin. Color-generating substances are 0.88 % of diester astaxanthin (dry matter) and 80 mg/Kg of xanthophylls. Mixing 10% of shrimp mill result in better hue.

3.5 Sources of carotenoids

Aquatic animals cannot generate carotenoid themselves. They acquire it through plants and some animals. After digested, carotenoids are absorbed via intestines along with other nutrition. Carotenoids such as astaxanthin and canthaxanthin are vital. Sources of such carotenoids include:

3.5.1 Natural carotenoids – found in plants mostly yellowish ones such as maize, chili, tomatoes etc. As well as in some livings such as crabs and prawns. Spirulina is another source of carotenoid as well as yeasts e.g., phaffia, rhodozyma. These give approximately 40-95% of astaxanthin.

3.5.2 Synthesized carotenoids – they are made especially for commercial reason e.g., apocarotenoic acid ethyl, astaxanthin and canthaxanthin. They can be divided into 3 types:

3.5.2.1 Carophyll yellow consists of 10% apocarotenoic ester. It is extracted from alfalfa and citrus fruits.

3.5.2.2 Carophyll red consists of 5% apocarotenoic ester and canthaxanthin. It is extracted from chanterekke and shrimps and flamingo feathers.

3.5.2.3 Carophyll orange consists of 10% very small carophyl ester (0.15-0.4 mm diameter). It can be mixed with fodder very well and also provide better stability compared to natural careotenoids.

3.6 Astaxanthin

Astaxanthin provide red hue. It is classified as a xanthophyll, astaxanthin can be found in microalgae, yeast, salmon, trout, krill, shrimp, crayfish, crustaceans, and the feathers of some birds. There are not enough astaxanthin in the nature plus animals cannot produce carotenoids by themselves thus very significant to mix astaxanthin in fodder. Astaxanthin, unlike some carotenoids, does not convert to Vitamin A (retinol) in the human body. Astaxanthin can be accumulated better than xanthophylls (oxygenated carotenoids) such as canthaxanthin, lutein or zeaxanthin. Astaxanthin also contain antioxidant higher than β-carotene as found in the research in vitro. However, it is a powerful antioxidant; it is 10 times more capable than other carotenoids (Miki, 1991).

While astaxanthin is a natural nutritional component, it can be found as a food supplement. The supplement is intended for human, animal, and aquaculture

consumption. The commercial production of astaxanthin comes from both natural and synthetic sources.

3.6.1 Natural sources

The following sources are being used for the commercial production of astaxanthin.

- *Haematococcus pluvialis* (microalge)
- *Xanthophyllomyces dendrorhous*, formerly *Phaffia rhodozyma*
- *Euphausia pacifica* (Pacific krill)
- *Euphausia superba* (Antarctic krill)
- *Pandalus borealis* (shrimp)

3.6.2 Synthetic sources

Today, essentially all commercial astaxanthin for aquaculture is produced synthetically from petrochemical sources, with an annual turnover of over $200 million, and a selling price of ~$2000 per kilo of pure astaxanthin.

3.6.3 Difference between natural and synthetic

Astaxanthin has two chiral centers, at the 3 and 3' positions. Therefore, there are three stereoisomers; (3-R,3'-R), (3-R,3'-S) (meso), and (3-S,3'-S). Synthetic astaxanthin contains a mixture of the three, in approximately 1:2:1 proportions. Naturally occurring astaxanthin varies considerably from one organism to another. The astaxanthin in fish is of whatever stereoisomer the fish ingested.

3.6.4 For animals

Astaxanthin is a reactant of vitamin A synthesis. Thus result in higher survive rate of kurama shrimp and red sea bream plus the increase egg buoyancy. They are good for salmonid fish during their early-feeding period. In rainbow trouts, adding astaxanthin resulted in decreasing of triglyceride and total cholesterol in blood as well as increasing oxidative stress and immunity system.

Astaxanthin are necessary for reproduction of salmonid fish, shrimp eggs and larvae since astaxanthin move fast in ovary to each organ during final stage of eggs. In poultry fodder, it is found that astaxanthin makes better eggs and poultry health. Astaxanthin can increase the percentage of eggs being hatched, decrease salmonella and death rate when yolk sac is inflamed. Astaxanthin can cause effect to terrestrial animal such as in swine; the male pigs fed with astaxanthin have more semen volume and increase oxidative stress immunity.

The primary use of synthetic astaxanthin today is as an animal feed additive to impart coloration, this includes farm-raised salmon and egg yolks. In that, synthetic carotenoid (i.e., colored yellow, red or orange) pigments represent about 15-25% of the cost of production of commercial salmon feed. Today, essentially all commercial astaxanthin for aquaculture is produced synthetically from petrochemical sources.

3.7 Lutein and Zeaxanthin

Lutein and Zeaxanthin are subcategorized of xanthophylls. They give yellow shade and are found in marigolds, green leaves, bacteria etc. Marigold provide high xanthophylls as of 6,000 – 10,000 mg/kg.

Lutein and zeaxanthin are capable as isomers but are not categorized as stereoisomers by lutein. Zeaxanthin are polyisoprenoids containing 40 atoms of carbon shaped as cyclic. Naturally they come in the form of *all-trans* (*all-E*) geometric isomers. Thus caused lutein to possesses 3 chiral while zeaxanthin possesses 2 chiral.

Lutein found in green leafy vegetables such as spinach and kale, lutein is employed by organisms as an antioxidant and for blue light absorption. Lutein is present in the plant as fatty-acid ester, with one or two fatty acids bound to the two hydroxyl-groups. Saponification of lutein esters yields lutein in approximately a 2:1

weight-to-weight conversion. Lutein is also found in egg yolks, animal fats and the corpus luteum.

Lutein is a lipophilic molecule and is generally insoluble in water. The presence of the long chromophore of conjugated double bonds (polyene chain) provides the distinctive light-absorbing properties. The polyene chain is susceptible to oxidative degradation by light or heat and is chemically unstable in acids. The principal natural stereoisomer of lutein is (3R,3'R,6'R)-beta, epsilon-Carotene-3,3'-diol.

3.7.1 As a pigment

This xanthophyll, like its sister compound zeaxanthin, has primarily been used as a natural colorant due to its orange-red color. Lutein absorbs blue light and therefore appears yellow at low concentrations and orange-red at high concentrations.

Lutein was traditionally used in chicken feed to provide the yellow color of broiler chicken skin. Polled consumers viewed yellow chicken skin more favorably than white chicken skin. Such lutein fortification also results in a darker yellow egg yolk. Today the coloring of the egg yolk has become the primary reason for feed fortification. Lutein is not used as a colorant in other foods due to its limited stability, especially in the presence of other dyes.

3.8 Beta-carotene (β-carotene)

The term carotene is used for several related substances having the formula $C_{40}H_{56}$. Carotene is an orange photosynthetic pigment important for photosynthesis. It is responsible for the orange color of the carrot and many other fruits and vegetables. It contributes to photosynthesis by transmitting the light energy it absorbs to chlorophyll.

Chemically, carotene is a terpene, synthesized biochemically from eight isoprene units. As hydrocarbons, carotenes are fat-soluble and insoluble in water.

Beta-carotene is composed of two retinyl groups, and is broken down in the mucosa of the small intestine by beta-carotene dioxygenase to retinal, a form of vitamin A. Carotene can be stored in the liver and converted to vitamin A as needed, thus making it a provitamin A.

The two primary isomers of carotene, α-carotene and β-carotene, differ in the position of double bonds in the cyclic group at the end. β-Carotene is the more common form and can be found in yellow, orange, and green leafy fruits and vegetables. As a rule of thumb, the greater the intensity of the orange color of the fruit or vegetable, the more β-carotene it contains. Carotene protects plant cells against the destructive effects of ultraviolet light. β-Carotene is an anti-oxidant. Carotene is also found in palm oil, corn, and in the milk of Guernsey dairy cows, causing their milk to turn yellow. It is also found in some species of termites.

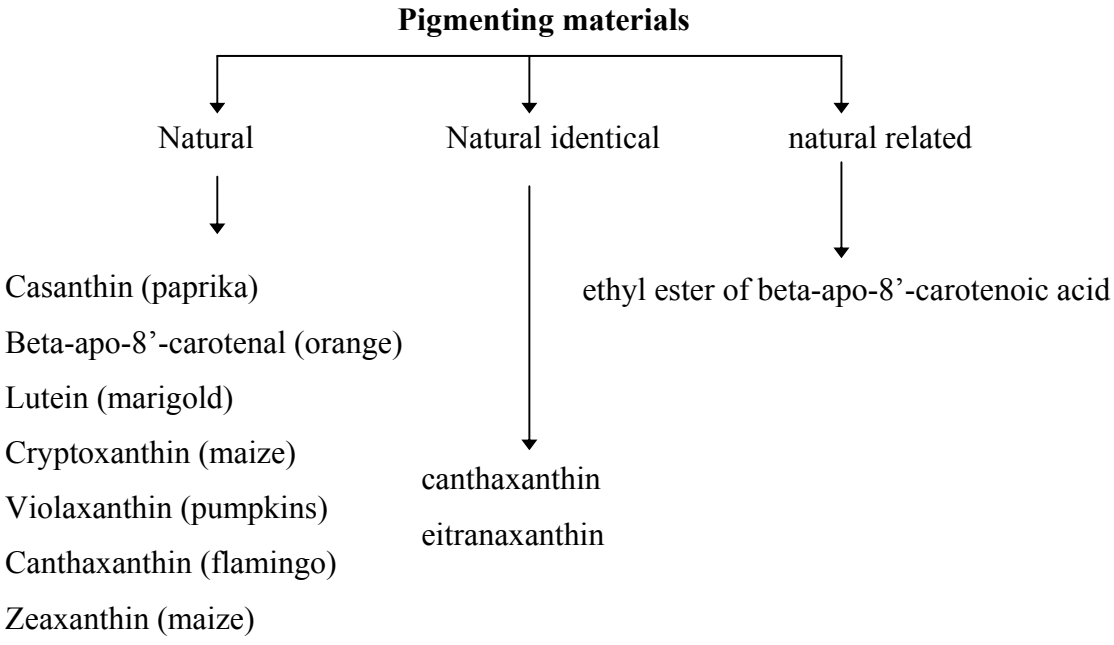


Figure 5 Pigmenting materials use as carotenoids source.

3.9 Utilization of carotenoids

3.9.1 Digestion and absorption

Carotenoids are lipid soluble and follow the same absorptive path ways as other dietary lipids. This property has been used as a basis for the carotene absorption test, a screening test for lipid malabsorption. Although efficient digestion and absorption of dietary lipid is a prerequisite for optimum absorption of carotenoids, it is to suggest that carotenoids are simply absorbed of dietary lipid.

Carotenoid absorption involves several steps from the breakdown of the food matrix and release of carotenoids into the lumen of the gastrointestinal tract through their incorporation into lymphatic lipoproteins. This includes mechanical and chemical disruption of the food matrix, dispersion in lipid emulsion particles, solubilization into mixed bile salt micelles, movement across the unstirred water layer adjacent to the microvilli, uptake by the enterocyte, and incorporation into lymphatic lipoproteins. A perturbation at any point along this chain of events will alter carotenoid bioavailability. An overview of carotenoid absorption in the small intestine is depicted in **Figure 6**.

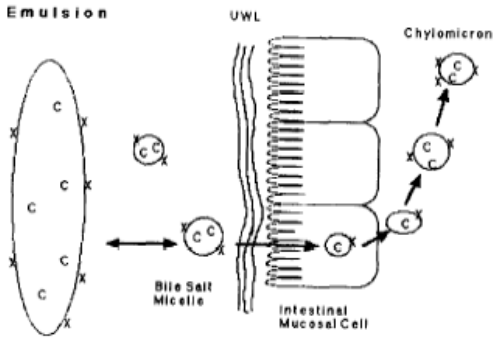


Figure 6 carotenoids from lipid emulsion in the small intestine to bile salt micelles, through the unstirred water layer (UWL) and enterocyte, with incorporation into lymphatic chylomicrons. The nonpolar carotenes (c) are thought to be located in the hydrophobic core of lipid emulsions, bile salt micelles, and chylomicrons, while the polar xanthophylls (X) are more likely surface components.

Source: Furr and Clark (1997)

Before absorption, carotenoids must be released from the food matrix, as they are not free in food but are associated with protein in a variety of plant cell structures (Erdman *et al*., 1993). Once the food is ingested, its mechanical breakdown continues as it is chewed, swallowed, and mixed in the stomach. Gastric hydrolysis of dietary lipids and proteins results in partial release of carotenoids and lipids from the food matrix. The extent of release and the physical-chemical state of the carotenoids in the stomach is not known. Once they are released, however, the lipophilic carotenoids would dissolve in an oily phase of lipid droplets. With mixing, the lipid droplets in the gastric contents become emulsified particles. Thus bile salt and fats are required upon carotenoids absorption process (Simpson and Chichester, 1981).

Shearing forces from normal digestive tract motility bring about the formation of a fine lipid emulsion as the contents of the stomach pass into the duodenum. The emulsion has a triacylglycerol core surrounded by a monomolecular layer of partially digested proteins, polysaccharides and lipids, especially phospholipid and partially ionized fatty acids. The solubility and location of the polar carotenoids (xanthophylls) and the nonpolar carotenoids (carotenes) in emulsions differ. Carotenes are thought to incorporate almost exclusively in the triacylglycerol core of the emulsion, whereas the more polar xanthophylls distribute preferentially at the emulsion surface (Borel *et al.*, 1996). Other lipid soluble nutrients with polar groups such as tocopherol and trans-retinoic acid are also thought to locate at the droplet surface. The significance of location in an emulsion is that the surface components can spontaneously transfer from lipid droplets to mixed micelles, whereas components associated with the emulsion core require digestion of triacylglycerol before transfer (Borel *et al*., 1996). The enzyme best suited to hydrolyze triacylglycerol in emulsions is pancreatic colipase-dependent lipase which is one reason why pancreatic insufficiency decreases plasma carotenoid concentrations (Leo *et al*., 1995).

The products of lipid digestion and minor dietary lipids, including the carotenoids, transfer from the emulsion particle to mixed bile salt micelles. Whereas the mechanism of carotenoid solubilization into mixed micelles is unclear, the presence of bile salt micelles is obligatory, as carotenoid absorption is minimal or

nonexistent when intraluminal bile salts are below the concentration required for aggregation into micelles (Hollander and Ruble, 1978). A major difference between absorption of other dietary lipid and carotenoids is that the carotenoids seem to have an absolute requirement for bile salt micelles, whereas fatty acids, the major product of lipid digestion, can be absorbed in the absence of micelles.

The solubility of carotenoids in mixed micelles is limited and varies with intraluminal concentration of the carotenoid. Canfield *et a1.,* (1990) studied the incorporation of β-carotene into mixed micelles designed to resemble those seen in the lumen of the small intestine. The incorporation of β-carotene into the micelles varied from approximately 4 to 13% with the percent incorporated decreasing with increasing initial concentration of carotenoid. Whereas the solubility of carotenoids differs in emulsions, the polar and nonpolar carotenoids have similar solubility in bile salt miceiles (Borel *et al*., 1996).

Accumulating of carotenoids in fish could be affected from various factors. Apart from mentioned external and internal ones, digestibility, absorption, blood flowing by lipoprotein and absorbing of carotenoids in muscle fiber can affect digestibility. Apparent digestibility coefficient (ADC) of astaxanthin in rainbow trout shows that the percentage of ADC is at 70% which is higher than 35-70% of canthaxanthin. Differences of ADC and pigmenting efficient do not depend only at the type of carotenoid, but also at the geometic isomer of each carotenoids themselves.

Addition of fat in the fodder causes enhanced color change in atlantic salmon (Einen and Skrede, 1998). This goes along with a research conducted by Choubert *et al.* (1991) which say that adding more fat result in improvement of carotenoids digestion in rainbow trout and retention efficiency (Nickell and Bromage, 1998). It can be concluded that using fat affects pigmentation regimes. The area where high absorption of carotenoids is found lies in posterior intestine. Absorption ability depends on variation in feed intake and is varied upon different types of fish whose triacyglycerols are packaged in chylomicron in enterocytes and pumped throughout

the body by metabolized-like chylomicrons. They associate with lipoprotein which acts as limiting factor. Superfluous carotenoids were discarded without affecting accumulated ones. However, digestibility depends on types of carotenoids. It is found that astaxanthin is well digested in the form of esterified compared to free form.

Polar carotenoids (xanthophylls) are around emulsion while non-polar carotenoids (carotenes) are found in emulsion. Emulsion, however, consists of triacylglucerol core where monomolecular layer e.g. digested protein, plysaccharides and lipids particularly phospholipids are found. Specifically in ionized fatty acid, polar carotenoids, carotenoids absorption are highly improved compared to non- polar carotenoids. Such absorption is passive diffusion by concentration gradient. It is also found that adding more fat result in better absorption (Harold and Richard, 1997). Carotenoids found in blood circulation are delivered through lipoprotein. Normally, 75% of plasma carotenoids are delivered via LDL and the rest via VLDL and HDL. Non-polar carotnoids such as α, β-carotene, lycopene are delivered via LDL whilst polar carotenoids such as lutein, astaxanthin are delivered via HDL and LDL (Babin and Vernier, 1989)

3.9.2 Distribution of carotenoids among plasma lipoproteins

Although carotenoid content and relation concentrations in human blood were the hydrocarbon carotenes (for example, α, β-carotene and lycopenes) are transported primarily on LDL plus VLDL, whereas the xanthohylls (lutein, zeaxanthin, β-cryptoxanthin) are distributed approximately equally between HDL and LDL in human serum (Johnson and Russell, 1992). Parker (1996) suggested that the actual content of β-carotene per unit lipid (triacylglycerol plus cholesterol) may be greater in HDL than in LDL. Parker also noted that, although the total surface area of LDL is approximately twice that of HDL in human plasma, the content of xanthophylls (lutein plus zeaxanthin) is greater in HDL than in LDL.

3.9.3 Link between astaxanthin metabolism and fat meatabolism

Two basic premises underlying the description to follow are (i) astaxanthin uptake, transport and delivery are closely associated with fatty acid uptake, transport and delivery and (ii) major features of astaxanthin absorption, metabolism and transport in salmon are similar to the absorption, metabolism and transport of xanthophylls in mammalian systems. There is some solid evidence for

this. Due to their hydrophobic nature, carotenoids are known to be closely associated with fatty acids and transported along with them through the intestine and blood (Parker, 1996). This seems also to be the case for astaxanthin as radioactive studies show that it is associated with all the serum lipoproteins (Asa *et al*., 2000), Which are the main transporters of esterified fatty acids. Furture, it has been found that pigmentation in salmon is quite strongly affected by dietary fat. Higher levels of astaxanthin deposition and retention in rainbow trout (Nickell and Bromage, 1998) and Atlantic salmom (Bjerkeng *et al*., 1997) have been obtained by increasing dietary lipid levels. This association has been observed in humans as well, where adequate dietary fat levels are necessary for optimal carotenoid absorption (Yeum and Russell, 2002). The flow and fate of astaxanthin in Atlantic salmon as shown in **Figure 7**.

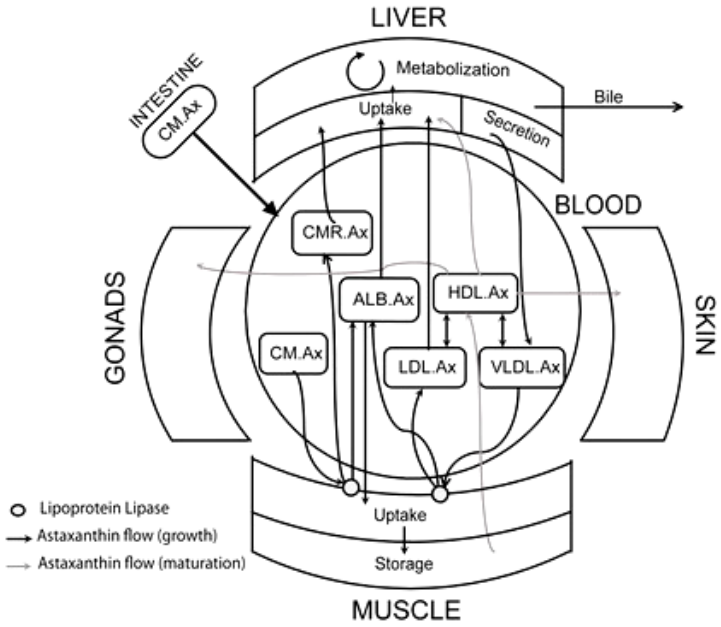


Figure 7 Astaxanthin uptake, transport and deposition. The uptake of astaxanthin from the intestine, transport in the blood and deposition in the muscle by the various lipoproteins in depicted. During sexual maturation/spawning migration (gray lines), the astaxanthin is redistributed from the muscle to the skin and gonads by the HDL.

Source: Hannah *et al.,* (2006)

3.9.4 Uptake and transport into blood

Being fat-soluble, dietary astaxanthin is assumed to be in micellar form in the intestine, together with bile salts, fatty acids, monoglycerides and other fat-soluble vitamins. It is believed to passively diffuse into the intestinal lumen, together with fatty acids, and the uptake seems to be a slow process taking between 18 and 30 hours (Choubert *et al*., 1994). The fatty acids are converted into triacylglycerols (TAG), and the astaxanthin, like other xanthophylls (Zaripheh and Erdman, 2002), is incorporated together with TAGs in lipoprotein spheres called chylomicrons. These are then transported into the blood and due to its polarity, astaxanthin is assumed to be attached to the surface of the chylomicron spheres.

In mammals, transport of chylomicrons from the intestinal lumen into the blood is carried out through lymphatic vessels. While being transported along the blood stream, chylomicrons undergo hydrolysis by lipoprotein lipase (LPL), a triacylglycerol lipase found on the surface of endothelial cells of the tissue capillaries, to yield free fatty acids. The fatty acids and monoglycerols that are derived from chylomicrons in this way are subsequently taken up by the tissues or serum albumin. Changes in the lipid composition of a chylomicron modify the affinity of the associated lipoproteins for its surface, causing the chylomicron to change its apolipoprotein signature. When the chylomicrons have lost about 80% of their initial TAG content, they become small enough to pass through the endothelium in the liver, and in addition their apolipoprotein signature can then be recognized by specific receptors in the liver.

The fate of chylomicron-associated xanthophylls is poorly understood in mammals as well as in fish. It is hypothesized that non-triglyceride components of the chylomicron, including surface molecules such as xanthophylls, may be taken up by extra-hepatic tissues or transferred to other blood lipoproteins. When the chylomicrons reach the liver, however, they still contain a considerable amount of their original carotenoid content. Since fish do not seem to have a lymph system similar to the mammalian one, the chylomicrons are assumed to be transported through the primary blood vessels in the intestine. Aside from this, there is reason to

assume that chylomicron-based astaxanthin transport and delivery in salmon are quite similar to that in mammals, as discussed above. Studies in salmon have provided evidence that astaxanthin is strongly associated with a protein likely to be serum albumin. Albumin is the major transporter of free fatty acids released during lipolysis by LPL to tissues (including liver and muscle). This suggests that albumin may acquire astaxanthin from chylomicrons during lipolysis as well as directly from chylomicrons in the bloodstream.

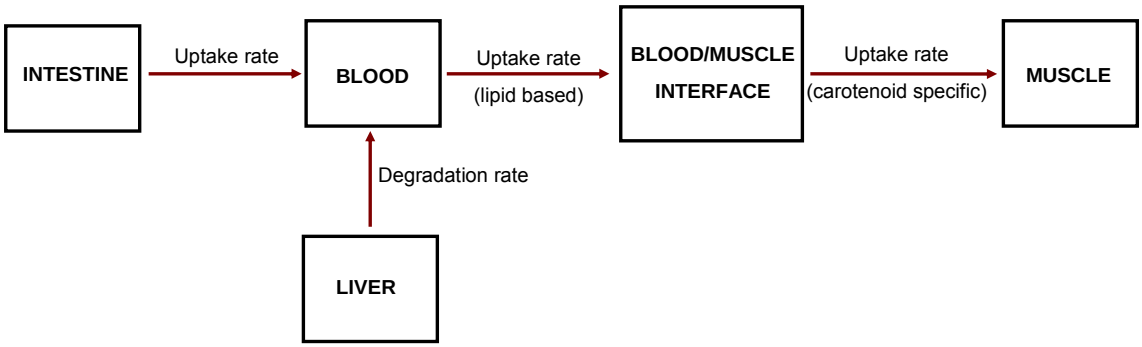


Figure 8 Variation in pigment level due to variation in intestinal uptake likely acocunted for by variation in weight to a substantial degree.

Source: Hannah *et al.,* (2006)

3.9.5 Liver metabolism and excretion

The liver is the main metabolic and excretory organ for Carotenoids (Torrissen and Ingebrigtsen, 1992) and is considered to have the major responsibility for the metabolic loss of astaxanthin. The liver secretes bile into the intestine to aid in lipid digestion as well as in the excretion of metabolic breakdown products, and radioactive labelling studies with canthaxanthin found bile radioactivity levels to be 8 times higher than the level in blood. The astaxanthin metabolites in the bile are secreted into the intestine and re-absorbed. Radiolabeling experiments also indicate that either astaxanthin or its metabolites are excreted by the kidneys of salmon and rainbow trout (Hardy *et al.*, 1990). The liver is therefore either catabolizing astaxanthin to other pigments or to metabolites that no longer have a chromophore. The exact process of astaxanthin metabolism in the liver is unknown, as is the case with β-carotene metabolism.

3.9.6 Transport and deposition in muscle

Despite numerous studies, the mechanism by which free fatty acids enter cells remains poorly understood. Astaxanthin association with LDLs has been observed in salmon (Aas *et al*., 2000) and rainbow trout (Chapman *et al*., 1978), suggesting that astaxanthin-containing LDLs may contribute substantially to the LPL-mediated uptake of astaxanthin by circulating albumin. Astaxanthin is then assumed to be brought to the muscle by circulating albumin. Binding to the muscle cell wall is thought to be non-specific and saturable. After having entered the muscle cell, astaxanthin is deposited in the myotome and binds to actomyosin by weak hydrophobic bonds, forming a complex. The presence of hydroxyl and keto groups at the β-end of the carotenoid increases the binding strength, explaining the higher deposition of astaxanthin compared to other carotenoids in salmon. Metabolites of astaxanthin have also been found in the connective tissues between myotomes.

During VLDL and LDL flow through the blood, some of the TAG found in these lipoproteins is transferred to highdensity lipoprotein packets (HDL) by the cholesteryl ester transfer protein (CETP) (Tyssandier *et al*., 2002). Given that astaxanthin is transported along with the TAGs to the HDLs, this mechanism is likely to explain the observed high levels of HDL associated astaxanthin in immature salmon (Aas *et al*., 2000), where there is no pigment transport out of the muscle. An interesting feature here is that unlike in mammalian systems, Atlantic salmon muscle has been found to express albumin. Carotenoids are highly accumulated around adipose tissue and liver which considered significant organs to accumulate carotenoids. Level of carotenoids in each organ directly affected upon the intake.

3.9.7 Fish pigmentation

Fish pigmentation causes greater value of fish. Amount of color-generated feed affects directly to fish pigmentation (Torrissen, 1995). By this, if carotenoids are at 6-8 mg/kg, human eyes can barely detect the differences thus required a help of tools to compare the color to a fan score. However, more feed intake does not mean more color being accumulated in the skins. There are a number of conditions such as digestion, absorption, blood transport and deposition.

Availability of useable carotenoids can be studied by measuring pigments in fish blood. It is found that canthaxanthin is detected in serum 3 hours after feeding with strongest amount reached at 24 hours after feeding while astaxanthin is detected in a shorter period of time but stronger than canthaxanthin if intake at the same amount. Carotenoids found in blood would decrease in the third day after feeding is executed. Goldfish and carps possess keto-carotenoids such as astaxanthin ester and 4-keto-lutein ester while xanthophyll ester and 4,4'-diketo-3-hydroxy-β-carotene are barely detected. In livers, however, free type carotenoids mostly zeaxanthin, β-carotene and lutein were found but none of keto-carotenoids were identified. In eggs, free type carotenoids were also noticed such as zeaxanthin and lutein while keto-carotenoids i.e. 4-keto-4'-hydroxy- β-carotene, canthaxanthin and astaxanthin (Goodwin, 1984) were rarely sensed. According to Katama *et al*., (1973), goldfish and carps are capable of transforming lutein to astaxanthin. Astaxanthin is in the form of ester-type which consists of lipid thus caused accumulation of staxanthin in the skins and differs from other organs where free type carotenoids were detected.

Oxidation degradation in catabolic pathway of carotenoids occurs at the carotenoids' double bond area. This causes β-carotene which is a reactant of vitamin A synthesis. Carotenoids are known for its property of color changing in fish, they also a reactant of provitamin A and immunity as well. They possess singlet oxygen quenching activity which acts as an antioxidant preventing cell from oxidative damage in immunity.

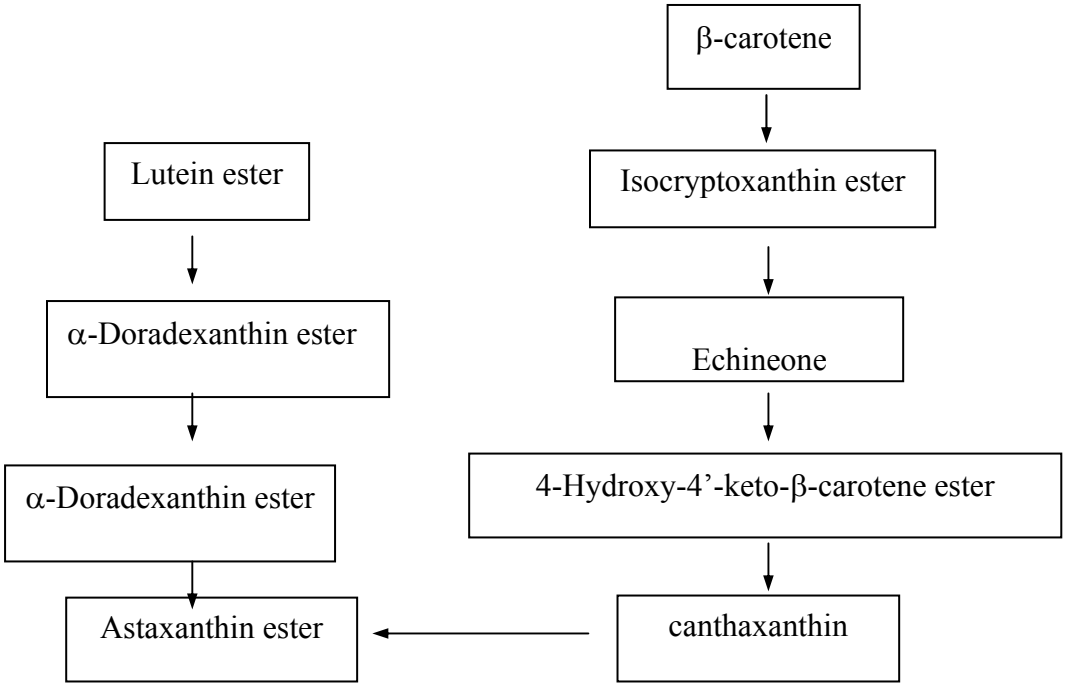


Figure 9 Metabolism of lutein and β-carotene to astaxanthin in goldfish.

Source: Bauernfeind (1981)

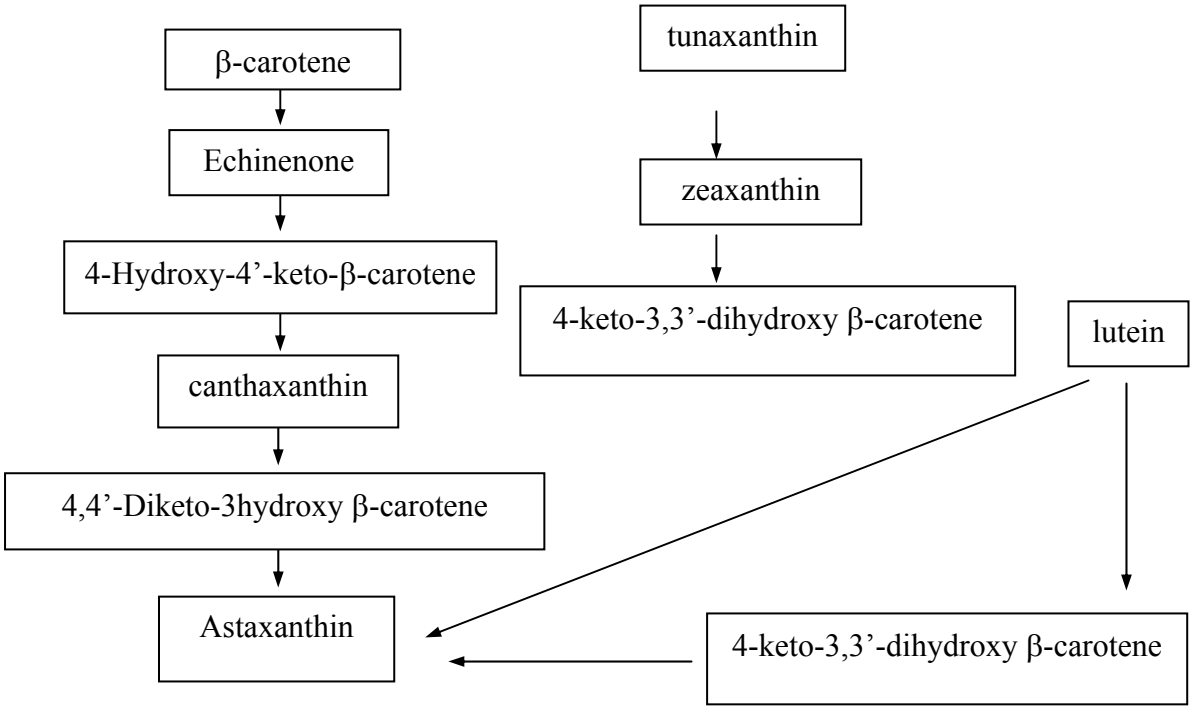


Figure 10 Metabolism of lutein, β-carotene and tunaxanthin to astaxanthin in Goldfish.

Source: Hata and Hata (1972a and 1972b)

Xanthophore in goldfish skin and carps reveals accumulation of lutein. Erythopore consists of astaxanthin. The study of microspectroscopic shows that in one xanthophore cell consists of 11-28 pg luteins while inside a cell of erythophore consist 200-340 pg of astaxanthin. It is also found that xanthophore passes through dermal layer while erythophore passes through outer layer.

3.10 Factors Influencing Carotenoid Composition

The carotenoid composition of foods are affected by factors such as cultivar or variety; part of the plant consumed; stage of maturity; climate or geographic site of production; harvesting and postharvest handling; processing and storage (Gross 1991). A close look at some published values reveals discrepancies that surpass those expected from the effects of these factors, indicating analytic inaccuracy. The analyst must take utmost care to differentiate between natural and analytic variations. Cultivar or varietal differences can be only in terms of the quantitative composition, because essentially the same carotenoids are found in the different varieties. Carotenoids are not evenly distributed in the food itself. There are three factors affecting color changes which are fodder (nutrition), animals, and environment.

3.10.1 Fodder - Raw materials, industrial derivative, and special

preparation can provide carotenoids. The first two are inexpensive yet include high carotenoids. They add better hue to aquatic animals but still give instable in carotenoid amounts. Thus concentrating of these materials offers a solution to the problem. Using canthaxanthin or astaxanthin in fodder would result in 15-30% higher cost. EU allows the usage of 80 mg/kg and 100 mg/kg in astaxanthin. Once fish affected to carotenoids for a couple of weeks, it is found that carotenoids possess ability in liposoluble but oxidation may cause deterioration in carotenoids. Heat can also deteriorate carotenoids for approximately 20%.

3.10.2 Animal related variability – Colors stored in fish skin or

muscles vary e.g., salmon possesses astaxanthin which was kept as esterified. However, astaxanthin was found in muscles. Cerotenoids found in fish skin are 10%

richer than in muscles. It is explainable given the researches from carotenoid functions in phenomena involving light though little studies have been made on precise function of cerotenoids. Still, a number of researches show that colors can prevent cells from visible light by modifying or attenuating the light it absorbed.

3.10.3 Environment – light is considered as primary color response. Chromatophores are affected upon absorbing light e.g., light of 470-530 nm. can cause color variation in cromatophores erythrophore occurred in Nile tilapia. UV can deteriorate colors and leads to less motility in chromatophores.

3.11 Carotenoids action

3.11.1 Immune response

Fish are the first vertebrate to possess immune system similarly to warm-blood animals. Their immune system consists of various cells such as plasma cell, macrophage, lymphocyte basophil, eosinophil and lymphoid organs including frontal kidney and bulge of the cheek which concern in producing immunity for young fish.

Immune response can be categorized into 2 types with non-specific immune response and specific immune response. The first one helps in polymorphonuclear cells and macrophange by phagocytosis and disintegrated by enzyme. Phagocytosis occurs well when a help from antibody and complement are present. The second one disintegrates antigen with a help from macrophage, T-lymphocyte and B- lymphocyte. There are two types of specific immune response including a response in producing antibody to antibody in order to eliminate antigen (humeral immune response) by B-lymphocyte with plasma cell and a response of T-lymphocyte with other cells such as active macrophage and natural killer cell.

Carotenoids have a number of double bond so they possess an ability to increase immunity by quenching singlet oxygen of free radical and other oxidative oxygen species. Thus can be used as antioxidant in a very limit way.

Carotenoids, therefore, can increase the ability of immune system by boosting up host defense function found in animals and human that consumed carotenoids (Bendich, 1989).

Dietary studies examining the role of carotenoids in immunity in fish are still lacking. In fish, phagocytosis has been recognised as an important element in the host's defence against invading microoraganisms (MacArthur and Fletcher, 1985). In the recent research, Thompson *et al*., (1994) found that astaxanthin with vitamin A increased the serum antiprotease activity in rainbow trout, but not growth or other humoral and cellular immune indices. However, Tachibana *et al*. (1997) found spleen lymphocyte proliferation increased in parrot fish larvae after fish fed with β-carotene enhanced rotifers and attributed the high survival. Redder fish in Betta splendens given supplemental carotenoids increased in immune response (to a phytohemagglutination challenge) (Clotfelter *et al*., 2007). Phagocytic rate and phagocytic index in rainbow trout fed with dietary supplemented natural carotenoid sources from red yeast *Phaffia rhodozyma* and marine algae *Dunaliella salina* were significantly higher than those of the control (Amar *et al*., 2006). Similar the report of Amar *et al*. (2001) showed that rainbow trout fed synthetic carotenoid such as β-carotene and astaxanthin elevated humoral factors such as serum complement and lysozyme activity, as well as cellular factors such as phagocytosis. By contrast of Amar *et al*. (2000) reported that the phagocytic activity of isolated head kidney cells was similar at the different level of dietary β-carotene in rainbow trout but indicated enhancement for immune components like serum complement and total plasma immunoglobulin.

3.11.1.1 Non-specific cellular immunity

Phagocytes (primarily polymorphonuclear neutrophils (PMN), and macrophages) are primarily responsible for ingesting particles/pathogens and breaking them down in their phagolysozomes. The main function of PMN is the phagocytosis (engulfing) and subsequent destruction (killing) of foreign materials. The PMN will first migrate (chemotaxis) by chemical gradient toward the foreign materials to be ingested. The primary granules rapidly move to fuse with the

phagosomes, releasing their enzymes which are responsible for digesting the bacterial walls and killing most microorganisms.

Blendich (1989) reviewed the roles of carotenoids in modulating immunological reaction, postulated that carotenoids may enhance immune activity by: (1) quenching excessive reactive oxygen species formed by various immunoactive cells, (2) quenching immunosuppressive peroxides and maintaining membrane fluidity, (3) helping to maintain membrane receptors essential for immune function, and (4) acting in the release of immunomodulatory lipid molecules such as prostaglandins and leukotrienes. These various mechanisms may increase the tumoricidal activity of T-cells, macrophages and/or natural killer cells, as well as enhance traditional antimicrobial immunological function. It was shown that natural killer cell activity in elderly men was enhanced by β-carotene supplementation. It seems that the suppressive effect of carotenoid on the respiratory burst of macrophages is just a way by which carotenoids in vivo protect host cells and tissues from harmful effect of oxygen metabolites overproduced by macrophages and enhance the generation of specific immune responses.

3.11.2 Role as antioxidant

3.11.2.1 Autoxidation

Autoxidation is any oxidation that occurs in open air or in presence of oxygen and/or UV radiation and forms peroxides and hydroperoxides. A classic example of autoxidation is that of simple ethers like diethyl ether, whose peroxides can be dangerously explosive. It can be considered to be a slow, flameless combustion of materials by reaction with oxygen. Autoxidation is important because it is a useful reaction for converting compounds to oxygenated derivatives, and also because it occurs in situations where it is not desired**.**

3.11.2.2 Reactive oxygen species (ROS)

ROS include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in

cell signaling. However, during times of environmental stress ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. They are also generated by exogenous sources such as ionizing radiation

3.11.2.3 Damaging effects

Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases and catalases. Small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, and glutathione also play important roles as cellular antioxidants. Similarly, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals. In contrast, the antioxidant ability of the extracellular space is relatively less--e.g., the most important plasma antioxidant in humans is probably uric acid.

Effects of ROS on cell metabolism have been well documented in a variety of species. These include not only roles in apoptosis (programmed cell death), but also positive effects such as the induction of host defence genes and mobilisation of ion transport systems. This is implicating them more frequently with roles in redox signaling or oxidative signaling. In particular, platelets involved in wound repair and blood homeostasis release ROS to recruit additional platelets to sites of injury. These also provide a link to the adaptive immune system via the recruitment of leukocytes.

Reactive oxygen species are implicated in cellular activity to a variety of inflammatory responses including cardiovascular disease. They may also be involved in hearing impairment via cochlear damage induced by elevated sound levels, ototoxicity of drugs such as cisplatin, and in congenital deafness in both animals and humans. Redox signaling is also implicated in mediation of apoptosis or programmed cell death and ischaemic injury. Specific examples include stroke and heart attack.

Generally, harmful effects of reactive oxygen species on the cell are most often:

- damage of DNA
- oxidations of polydesaturated fatty acids in lipids
- oxidations of amino acids in proteins
- Oxidatively inactivate specific enzymes by oxidation of co-factors

3.11.2.4 Rancidity and antioxidant

This would cause 3 types of chemical reactions which are:

1. Oxidative rancidity: non saturated fatty acid would interact with oxygen in the air so that it is called “oxidation”

2. Hydrolytic rancidity: causes by the fat molecules of digested fat. Free fatty acid comes from the enzyme in the fat together with water. The small molecules in free fatty acid cause bad smell but this type of rancidity can be protected by heat which would kill the enzyme. Water should not be added in fat as well.

3. Ketonic Rancidity: causes with saturated fatty acid which the enzyme reaction comes from yeasts from Ketone which is the smelly substance. Thus, a protection for rancidity is needed in order to get rid of the growing of yeast.

Rancidity reaction occurs because of oxygen which is the auto oxidation. It is drastically destroy fat products. This oxidation reaction occurs from non-saturated fat interact with oxygen in the air. It can be separated into 3 stages.

1. Initiation Stage - non-saturated fatty acid reacts with oxygen which cause free radical

$$RH + O_2 \longrightarrow R^{\bullet}$$

2. Propagation Stage- is a repeated reaction which free particle reaction

interact oxygen, causesn peroxy radical which will interact with hydroperoxide fat acid and free radical, which free radical would interact with oxygen and continue as chain reaction.

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$$

3. Termination Stage- is the allocation stage of free radical in different ways which the allocation could stop the reaction. As a result, low carbon substances such as Aldehyde or Ketone which cause bad small in rancidity are created.

$$\left.\begin{array}{l} R^{\bullet} + R^{\bullet} \\ R^{\bullet} + ROO^{\bullet} \\ ROO^{\bullet} + ROO^{\bullet} \end{array}\right\} \longrightarrow \text{non radical product (malonaldehydes, aldehyde, ketone, hydrocarbon and alcohol)}$$

Oxidation is caused by many factors such as the type of fatty acid, light, temperature, oxygen and enzyme. In order to reduce the cause of rancidity, antioxidant should be added.

Antioxidant is a chemical substance used for adding into fat acid or foods which contain high fat. It helps slowing down the growth of oxidation reaction of auto oxidation in fat. Autooxidation in fat causes aldehyde substance and ketone. Antioxidant helps restricting the growth of free radical from the first stage. It protects the additional of oxidation. There are many types of antioxidant used in chemical substances. The effective types are such as Phenol which contains branch group, is tertiary butyl and the important onces are such as tertiary butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG). Adding antioxidant should be done before storing since once oxidation occurred, antioxidant will not be effective but rather boosting oxidation.

3.11.2.5 Carotenoids as antioxidant

Carotenoids can also used as antioxidant, executing the growth of free radical and oxidation. Thus they play an effective role of eradicating cellular membranes, enzymes and nuclear DNA which ended up in animal's improved health (Burton, 1989).

$$RH + \text{oxidant initiator} = R^{\bullet}$$

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow \text{non radical products}$$

Carotenoids can be served as quenching singlet oxygen in which double bonds act as pro-oxidant.

$$\beta\text{-carotene (other carotenoids)} = \text{formation of inactive product}$$

$$\beta\text{-carotene} + ROO^{\bullet} \longrightarrow \beta\text{-car}^{\bullet}$$

$$\beta\text{-car}^{\bullet} + O_2 \longrightarrow \beta\text{-car-}OO^{\bullet}$$

$$\beta\text{-car}^{\bullet} + ROO^{\bullet} \longrightarrow \text{inactive products}$$

As an antioxidant, astaxanthin surpasses the benefits of others; the antioxidant activities of astaxanthin have been shown to be approximately 10 times greater than those of β-carotene, lutein, zeaxanthin, cantaxanthin, and over 500 times greater than that shown by α-tocoferol. The name 'super vitamin E' has been proposed for astaxanthin (Miki, 1991).

Actually, a fruit- and vegetable- rich diet is recommended to increase the dietary amounts of carotenoids; however, it has been shown that the bioavailability of carotenoids in fruits and vegetables is significantly lower than that of algae-derived supplements (Werman *et al*., 1999). It has also been proved that carotenoid extracts have higher anti-oxidant properties than synthetic ones (Levin *et al*., 1997).

4. Toxic substances

4.1 Tannin

Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins. The astringency from the tannins is what causes the dry and puckery feeling in the mouth following the consumption of red wine, strong tea, or an unripened fruit (McGee, 2004). The term tanning refers to the use of tannins in tanning animal hides into leather; however, the term is widely applied to any large polyphenolic compound containing sufficient hydroxyls and other suitable groups (such as carboxyls) to form strong complexes with proteins and other macromolecules. Tannins have molecular weights ranging from 500 to over 3,000 (Bate-Smith and Swain, 1962). Tannins are incompatible with alkalis, gelatin, heavy metals, iron, lime water, metallic salts, strong oxidizing agents and zinc sulfate.

4.1.1 Tannins: chemical structure

Tannins are one of the many types of secondary compounds found in plant, their are usually subdivided into two groups:

4.1.1.1 Hydrolyzable tannins (HT)

At the center of a hydrolyzable tannin molecule, there is a carbohydrate (usually D-glucose). The hydroxyl groups of the carbohydrate are partially or totally esterified with phenolic groups such as gallic acid (in gallotannins) or ellagic acid (in ellagitannins). Hydrolyzable tannins are hydrolyzed by weak acids or weak bases to produce carbohydrate and phenolic acids.

HTs are molecules with a polyol (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid (-->gallotannins) or ellagic acid (--> ellagitannins). HT are usually present in low amounts in plants.

HT properties:

- hydrolyzed by mild acids or mild bases to yield carbohydrate and phenolicacids
- Under the same conditions, proanthocyanidins (condensed tannins) do not hydrolyze.
- HTs are also hydrolyzed by hot water or enzymes (i.e. tannase).

4.1.1.2 Proanthocyanidins (PA; condensed tannins)

Condensed tannins, also known as proanthocyanidins, are polymers of 2 to 50 (or more) flavonoid units that are joined by carbon-carbon bonds, which are not susceptible to being cleaved by hydrolysis. While hydrolyzable tannins and most condensed tannins are water soluble, some very large condensed tannins are insoluble. PAs are more widely distributed than HTs. They are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis.

- PAs are more often called condensed tannins due to their condensed chemical structure. However, HTs also undergo condensation reaction. The term, condensed tannins, is therefore potentially confusing.
- The term, proanthocyanidins, is derived from the acid catalyzed oxidation reaction that produces red anthocyanidins upon heating PAs in acidic alcohol solutions.
- PAs may contain from 2 to 50 or greater flavonoid units; PA polymers havecomplex structures because the flavonoid units can differ for some substituents and because of the variable sites for interflavan bonds.
- Anthocyanidin pigments are responsible for the wide array of pink, scarlet, red, mauve, violet, and blue colors in flowers, leaves, fruits, fruit juices, and wines. They are also responsible for the astringent taste of fruit and wines.

- PA carbon-carbon bonds are not cleaved by hydrolysis.
- Depending on their chemical structure and degree of polymerization, PAs may or may not be soluble in aqueous organic solvents.

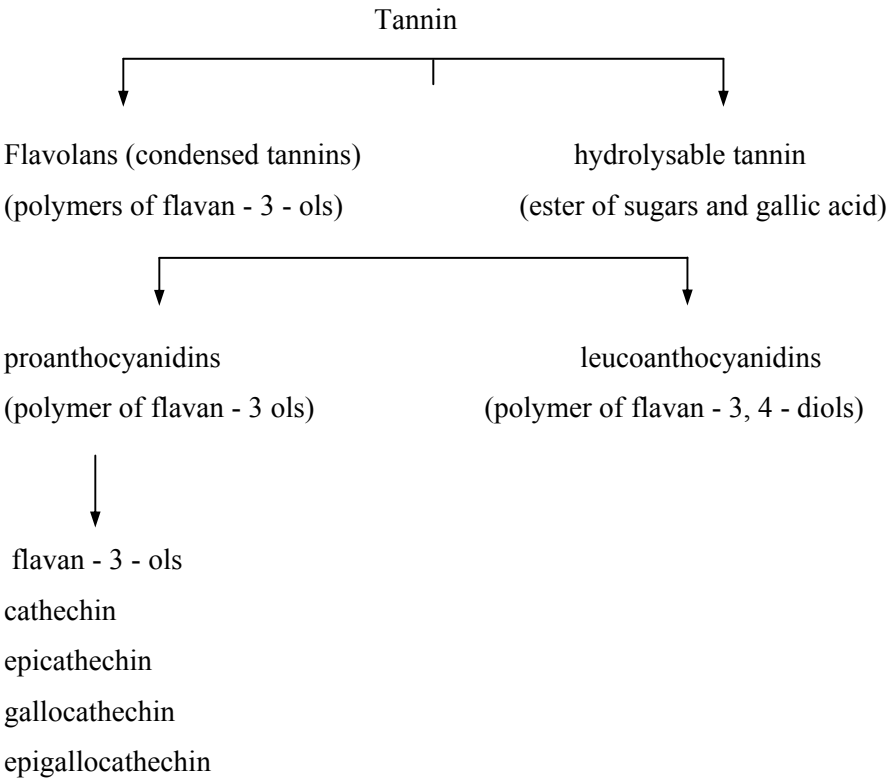


Figure 11 Classification of tannin.

Animals fed diets with a level of tannins under 5% experience (Giner-Chavez, 1996)

- depressed growth rates,
- low protein utilization,
- damage to the mucosal lining of the digestive tract,
- alteration in the excretion of certain cations, and
- increased excretion of proteins and essential amino acids.

In poultry, small quantities of tannins in the diet cause adverse effects

- levels from 0.5 to 2.0% can cause depression in growth and egg production
- levels from 3 to 7% can cause death.

In swine, similar harmful effects of tannins have been found.The addition of additional proteins or amino acids may alleviate the antinutritional effects of tannins. Levels of tannins above 5% of the diet are often lethal. The tannin content appears to be directly related to protein digestibility (Makkar, 1993). Little is known about the effects of tannins on fish.

4.2 Hydrocyanic Acid

Linamarin is a cyanogenic glucoside found in the leaves and roots of plants such as cassava, lima beans, and flax. Upon exposure to enzymes and gut flora in the human intestine, linamarin and its methylated relative lotaustralin can decompose to the toxic chemical hydrogen cyanide; hence food uses of plants that contain significant quantities of linamarin are inhibited by extensive preparation and detoxification requirements. Ingested and absorbed linamarin is rapidly excreted in the urine and the glucoside itself does not appear to be acutely toxic.

The generation of cyanide from linamarin is usually enzymatic and occurs when linamarin is exposed to linamarase, an enzyme normally expressed in the cell walls of cassava plants. Because the resulting cyanide derivatives are volatile, processing methods that induce such exposure are common traditional means of cassava preparation; foodstuffs are usually made from cassava after extended blanching, boiling, or fermentation (Padmaja, 1995).

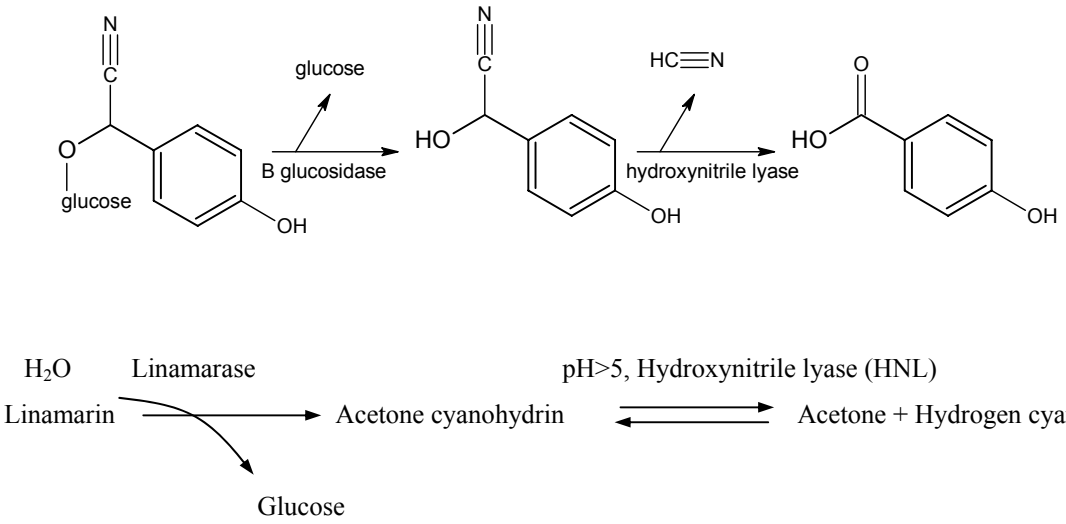


Figure 12 Equation of Cyanogenesis.

Cassava contains two cyanogenic principles named as linamarin and lotaustralin present in all parts of the plant (Nartey, 1981). The normal range of cyanogenic glucoside content in cassava is from 15 to 400 ppm calculated as mg HCN (hydrocyanic acid)/kg fresh weight. But occasionally varieties with very low HCN content (10 mg/kg) or very high HCN content (2000 mg/kg) have been also encountered (Balagopalan *et al*., 1988). Cassava also contains enzymes capable of degrading cyanogenic glucosides. The enzyme linamarase hydrolyses linamarin to hydrocyanic acid.

MATERIALS AND METHODS

Part 1 Carotenoids Analysis and Method Validation

Part 1.1 Monitoring the Occurrence of Carotenoids Composition and Toxic Substances in Local Thai Feedstuffs: Tea, Mulberry and Cassava Leaves

Materials

First, fresh leaves of tea, mulberry and cassava were sun dried for a day and grinded finely, and then their were equally divided and stored in clear plastic bags at the room temperature of 26-28 °C. After being stored for two months, the analysis of their carotenoids contents and toxic substances change were conducted.

Proximate analysis

Feedstuffs were analyzed in triplicate for chemical composition by proximate analysis according to the methods of 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 h; crude protein (N x 6.25) by Kjeldahl method after acid digestion; lipid by petroleum ether extraction in a Soxlet apparatus (**Table 1**).

Table 1 Chemical composition in feedstuffs.

Feedstuffs	Moisture	Dry matter (%)		
		protein	Fat	Ash
Tea	7.50 + 0.70	14.04 + 0.10[b]	2.96 + 0.05	16.15 + 0.05
Mulberry	7.73 ± 0.15	18.81 ± 0.34 [a]	2.60 ± 0.00	14.63 ± 0.18
Cassava	7.23 ± 0.40	18.68 ± 0.13 [a]	2.43 ± 0.35	16.66 ± 0.72
p-value	0.06	0.0001	0.17	0.07

Carotenoids determination

Dried samples of all feedstuffs mentioned above were extracted with acetone, together added BHT (250 ppm) as antioxidant until feedstuffs no color. To grind feedstuff samples with a pestle and mortar. In this regard, the extraction would be more efficient if all samples were moistened with a little water and left for a few minutes before the solvent treatment. After that, added methanolic potassium hydroxide 20% and left in the dark at least two hours. Next, transfer the saponification mixture to a separating funnel. Added hexane, mix and add a similar volume of water. Then, mix with a careful swirling and the two phases were found separated. In this study, only the hyperphase was collected and then determined maximum absorbance wavelength rang over 350- 600 nm. Recorded merely the maximum absorbance value (λ max) and calculated total carotenoids obey Beer-Lambert's Law with extinction coefficiency $E^{1\%}_{1\,cm}$ = 2500 (Britton, 1995).

Total Carotenoids of feedstuffs from hexane extracted were evaporated under a gentle stream of nitrogen gas. Then, redissolved crude carotenoids with hexane and the solution 5 ul flow out and form a spot on the silica layer with the semi-automatic sample application machine in order to conduct the identified and quantified analyses. In this procedure, it would generate narrow spots, which must be sprayed with

nitrogen gas having the 99.995 % purity and flow rate at 4 ul/s (CAMAG, Linomat IV, Switzerland).

Carotenoids composition were identified and quantified by TLC on TLC plated having 10 x 20 cm layer and 0.25 mm thickness (TLC plated silica gel 60, Merck, Germany). In order to identify the correct value of astaxanthin, lutein and β- carotene, it was a must that TLC was always conducted to compare with standards for asxanthin and β-carotene (Sigma); phytochemical reference standard was lutein (Chromadex, Canada).

After applying samples on the TLC plate, then placed plate carefully in the developing chamber, containing petroleum ether - diethyl ether - acetone (75:15:10, *v/v/v*) as eluent. When this developing procedure was finished, the next step was to conduct the quantitative analyze.

Quantitative analysis was carried out in situ by measuring samples and standard zones on layers with a slit-- scanning densitometer compiled from Mantiri *et al*. (1996) and Sherma and Bernard (2003), which used a tungsten lamp at the wavelength 450 nm. (CAMAG TLC Scanner III, CAMAG, Switzerland). Specifically, the total results found in this study must be evaluated with the following calibration function: multi level calibration with linear regression using external standards.

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 determined maximum absorbance (λ max) at wavelength ranges over 350-600 nm. By using UV/Vis spectrum and calculated concentration of standard solution obeying 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 solution.

Toxic Substances

Tannins content

Dried plant material (0.5 g) was extracted with 300 ml of diethyl ether for 20 hrs at room temperature. The residue was boiled for 2 hrs with 100 ml of distilled water, and then allowed to cool, and was filtered. The extract was adjusted to a volume of 100 ml in a volumetric flask. The content of tannins in the extract was determined colorimetrically using Folin–Denis reagent, and by measuring absorbance of the blue complex at 760 nm, using tannic acid solution as a standard solution, as described by Helrich (1990).

HCN Determination

The cyanide content in samples were obtained by alkaline titration method involving the use of 25% NaOH, 6 N NH_4OH solution, 5% potassium iodide and 0.02 N silver nitrate follow by AOAC (1990).

Statistical analysis

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

Part 1.2 Validated TLC-Densitometric Analysis for Determination of Carotenoids in Fancy Carp (*Cyprinus carpio*) Serum

Preparing sample blank

Mixed sexes of fancy carp with an average weight of 26.93 ± 5.14 g/fish were maintained in a 20-litter aquarium tank. Fish were fed with a pigment-free diet for two weeks prior to the start of the experiment. A control blood sample 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 samples (approx. 1 ml/fish) centrifuged at 300 x g, 25°C for 10 min then serum was removed and serum was immediately separated from blood and stored at -20 °C until samples analysis was performed.

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 mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The length of 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. Specifically, the total results found in this study must be evaluated with the following calibration function: multi level calibration with linear regression using external standards.

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 ug/spot.

Validation of the established method

Accuracy (% Recovery)

The accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or as an accepted reference value and the value found. 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 of 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 followed by AOAC (1993). The acceptable values of recovery shown in **Table Appendix A1**.

Precision

Precision is a measure of random errors, and may be expressed as repeatability. 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 and measurement of peak area carried out using three replicates of the same spot (0.01, 0.25, 0.50, 1.25 and 6.25 ug/spot of astaxanthin, lutein and β-carotene) and was

expressed in terms of percent relative standard deviation (%RSD_r). The acceptable values of %RSD_r shown in **Table Appendix A1**.

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.

HORRAT value

The HORRAT value is a useful summary of the precision in analytical chemistry. It overestimates the precision at the extremes, predicting more variability than observed at the high end of the scale and at the low end of the scale. The %RSD_r varies with concentration of analyte when an analyte has lowest amount or concentration, %RSD_r will be high, make the precision of an analytical procedure decrease or unacceptable. Thus, must do the test with the 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.

Acceptable $HORRAT_r$ value for single laboratory validation (SLV) studied, are 0.3-1.3.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is the lowest amount or concentration of an analyte in a sample that can be detected but not necessarity quantitated as an exact value. The LOQ is the

lowest amount or concentration of an analyte in a sample that can be determined quantitatively with suitable precision and accuracy.

In order to estimate the LOD and 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 anlyte in a sample that can be detected but not necessarily quantitated 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, 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 ($s_{y/x}$), there is a normal distribution of the signal at this concentration, the 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.

Linearity

The linearity of a test procedure is its ability (within a given range) to obtain test results proportional to the concentration (amount) of analyte in the sample. Linearity was checked between 0.01, 0.25, 0.50, 1.25 and 6.25 ug/spot of astaxanthin, lutein and ß-carotene. Linear calibration function was calculated and linear regression line; productmoment correlation coefficient equal or higher than 0.995.

Part 2 Bioavailability of Carotenoids

Part 2.1 Bioavailability of Carotenoids; Astaxanthin, Lutein and β-Carotene in Fancy Carp (*Cyprinus carpio*)

Fish and feed trial

Mixed sexes of fancy carp with average weight of 26.93 ± 5.14 g/fish were maintained on a non-pigmented diet for a co-variant period of two weeks prior to feeding the experimental diets, tested in four replicates. Each treatment was randomly distributed to each of 20-liter aquarium tanks. The diets were designed to achieve a target level of 200 μg for astaxanthin, lutein and β-carotene per fish.

Astaxanthin was supplied at a finished product concentration of 10% astaxanthin (BASF, Thailand). Lutein from marigolds extract (*Tagetes* spp.) was supplied in a finished product containing 15000 ppm (Kemin Industries, Thailand). β-carotene was a natural Beta carotene source at 15 mg/capsule (MEGA Lifesciences, Australia). After administration of a single dose orally, fish were not fed further meals.

Sampling procedure

Fish were not fed for 3 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 experiment. Blood was collected from the caudal vein with 1 ml non-heparinized disposable syringes fitted with 0.55x25 mm disposable needles. Blood sampling occurred at 15, 30 min 1, 3, 6, 12, 24, 48, 72, 96 and 120 hr after single dose meal, three fish were sampled at each sampling time. Serum was immediately separated from blood and stored at -20 C until samples analysis was performed.

Carotenoids determination

All experimental diets were extracted with acetone, together with BHT (250 ppm) added as antioxidant, until samples showed no color. After that, petroleum ether was added at 5 ml, mix and add water in a separating funnel. Then, mix with a careful swirling and the two phases were found separated. In this study, only the hyperphase of diets was collected then determined maximum absorbance wavelength range over 350-600 nm. Recorded merely the maximum absorbance value (λ max) and calculated total carotenoids obey Beer-Lambert's Law (Britton, 1995).

Serum was vortexed with 1 ml of ethanol for 30 s, then 2 ml of petroleum ether was added, and the mixture was vortexed for 1 min. The petroleum ether was separated by centrifuging 300 x g, 25°C for 10 min (White *et al*., 2002).

Instrumentation and chromatographic conditions

The resulting hyperphase of experimental diets and serum from petroleum ether extraction were evaporated under a gentle stream of nitrogen gas. This was followed by redissolving crude carotenoids in petroleum ether and the solution 5 ul were spotted on pre-coated silica gel 60 (10 x 20 cm) with 0.25 mm thickness (Merck, Germany) using a CAMAG Linomat IV (Switzerland) sample applicator. A constant application rate of 4 ul/s was employed and spaces between two spots were 14 mm. 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 mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The length of chromatogram run was 70 mm. Densitometric scanning was performed on CAMAG TLC scanner III in the absorbance mode at 450 nm. The source of radiation utilized was a tungsten lamp (compiled from Mantiri *et al*., 1996 and Sherma and Bernard, 2003). In order to identify the correct value of astaxanthin, lutein and β-carotene,

TLC runs were always conducted including authentic standards for astaxanthin and β-carotene (Sigma); lutein (Chromadex, Canada).

Pharmacokinetic parameter analysis

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

Part 2.2 Comparative Pharmacokinetic of Carotenoids from Natural Sources (Tea, Mulberry and Cassava Leave) in Fancy carp (*Cyprinus carpio*)

Fish and feed trial

Mixed sexes of fancy carp with were maintained on a diet without supplemented carotenoid for two weeks prior to feeding the experimental diets, tested in three replicates. Each treatment was randomly distributed to each of 20-liter aquarium tanks. The experimental diets were used plant leave such as tea, mulberry and cassava leave substituting 100 percent of feed ingredients. The diets were designed to achieve a target level of total carotenoid at 25 µg.

Sampling procedure

Fish were not fed for 3 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 experiment. Blood was collected from the caudal vein with 1 ml non-heparinized disposable syringes fitted with 0.55x25 mm disposable needles. Blood sampling occurred at 15, 30 min 1, 6, 12, 24, 48, 72, 96 and 120 hr after single

dose meal, three fish were sampled at each sampling time. Serum was immediately separated from blood and stored at -20 °C until samples analysis was performed.

Carotenoids determination

Dried samples of all feedstuffs (tea, mulberry and cassava leave) were extracted with acetone, together with added BHT (250 mg/kg) as antioxidant, until samples no color. After that, added methanolic potassium hydroxide 20% in extraction from all leaves and left in the dark at least four hours. After that, petroleum ether was added at 5 ml, mix and add water in separating funnel. Then, mix with a careful swirling and the two phases were found separated. In this study, only the hyperphase of plant leave was collected then determined maximum absorbance wavelength range over 350-600 nm. Recorded merely the maximum absorbance value (λ max) and calculated total carotenoids obey Beer-Lambert's Law (Britton, 1995).

Serum was vortexed with 1 ml of ethanol for 30 s, then 2 ml of petroleum ether was added, and the mixture was vortexed for 1 min. The petroleum ether was separated by centrifuging 300 x g, 25°C for 10 min (White *et al*., 2002).

Instrumentation and chromatographic conditions

The resulting hyperphase of all leaves and serum from petroleum ether extraction were evaporated under a gentle stream of nitrogen gas. This was followed by redissolving crude carotenoids in petroleum ether and the solution 5 ul were spotted on pre-coated 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 ul/s was employed and spaces between two spots were 14 mm. 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 mobile phase. The optimized chamber saturation

time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The length of chromatogram run was 70 mm. Densitometric scanning was performed on CAMAG TLC scanner III (CAMAG, Switzerland) in the absorbance mode at 450 nm. The source of radiation utilized was a tungsten lamp (compiled from Mantiri *et al*., 1996 and Sherma and Bernard, 2003). In order to identify the correct value of astaxanthin, lutein and β-carotene, TLC runs were always conducted including authentic standards for astaxanthin and β-carotene (Sigma, USA); lutein (Chromadex, Canada).

Pharmacokinetic parameter analysis

The serum total carotenoids concentration-time curves was best fit to one-compartment pharmacokinetic model. The mean serum total carotenoids concentration-time curves data points of three from each time were then analyzed using pharmacokinetic equation (Evans, 2004)

Part 3 The Impact of Dietary carotenoid (Ataxanthin, Lutein and β-carotene) on Skin Pigmentation of Fancy carp (*Cyprinus carpio*)

Fish, diets and feeding protocol

Mixed sexes of fancy carp, Kohaku (red and white) were maintained on a diet without supplemented carotenoid for a co-variant period of two weeks prior to feeding the nine experimental diets, tested in four replicates. Each replication included two fish with an initial mean wet weight of 48.54 ± 1.44 g/fish, were randomly distributed to each of 36 (20-liter) aquarium tanks.

All diets were designed to contain carotenoids following:

1. Control diet of low total carotenoids at least 5 mg/kg
2. Diet supplemented with astaxanthin at 25 mg/kg
3. Diet supplemented with lutein at 25 mg/kg
4. Diet supplemented with lutein at 50 mg/kg
5. Diet supplemented with β-carotene at 25 mg/kg
6. Diet supplemented with β-carotene at 50 mg/kg
7. Diet supplemented with β-carotene at 75 mg/kg
8. Diet combined with lutein and β-carotene at 25:25 mg/kg
9. Diet combined with lutein and ß-carotene by 50:50 mg/kg

Astaxanthin was supplied at a finished product concentration of 10% astaxanthin (BASF, Thailand). Lutein from marigolds extract (*Tagetes* spp.) was supplied in a finished product containing 15000 mg/kg (Kemin Industries, Thailand). β-carotene was a natural Beta carotene source at 15 mg/capsule (MEGA Lifesciences, Australia).

The daily feed was fed at 2% of body weight and by hand-fed methods twice a day. Total feed was weighed weekly. The duration of the experiment was three weeks

(21 days) with experimental diets containing carotenoids followed by the low carotenoid diet for a week (7 days). Fish were not exposed to natural light, as tanks were covered in black plastic. Water exchange in each aquarium tank were maintained at 80% every three days.

Chemical composition

Nutrient composition of the diets was analysed according to the methods of 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 h; crude protein (N x 6.25) by Kjeldahl method after acid digestion; lipid by petroleum ether extraction in a Soxlet apparatus (as shown in **Table 2**).

Sampling and total carotenoids determination

Blood sampling was carried out weekly over the entire four week experiment. The duration of the experiment was divided two phase, first three weeks (21 days) with experimental diets containing carotenoids followed by the diet without supplemented carotenoid for a week (7 days). Blood was collected from the caudal vein with 1 ml non-heparinized disposable syringes fitted with 0.55 x 25 mm disposable needles. Blood samples (approx. 1 ml/fish) was centrifuged at 300 x g, 25 °C for 10 min 500 ul of the serum was removed and vortexed with 1 ml of ethanol for 30 s, then 2 ml of petroleum ether was added, and the mixture was vortexed for 1 min. The petroleum ether was separated by centrifuging 300 x g, 25 °C for 10 min (White *et al*., 2002)

All experimental diets were extracted with acetone, together with BHT (250 mg/kg) added as antioxidant, until samples showed no color. After that, petroleum ether was added at 5 ml, mix and add water in a separating funnel. Then, mix with a careful swirling and the two phases were found separated. In this study, only the hyperphase of serum and diets were collected and determined for total carotenoids.

Total carotenoid (TC) content in all diets and serum were determined after extraction. Carotenoids are expressed using extinction coefficients ($E^{1\%}_{1\ cm)}$ 2500 and determined by absorbance wavelength at 450 nm. Recorded merely the absorbance value calculated total carotenoids obey Beer-Lambert's Law (Britton, 1995).

Table 2 Ingredients and proximate composition of experimental diets.

Ingredient (Kg)	Experimental diets								
	1	2	3	4	5	6	7	8	9
Fish meal	30	30	30	30	30	30	30	30	30
Soybean meal	24	24	24	24	24	24	24	24	24
Rice bran	24	24	24	24	24	24	24	24	24
Tapioca starch	5	5	5	5	5	5	5	5	5
Wheat Flour	5	5	5	5	5	5	5	5	5
Fish oil	2	2	2	2	2	2	2	2	2
Alpha-starch	5	5	5	5	5	5	5	5	5
Dicalcuim	1	1	1	1	1	1	1	1	1
Premix	2	2	2	2	2	2	2	2	2
Astaxanthin	-	0.025	-	-	-	-	-	-	-
Lutein	-	-	0.17	0.34	-	-	-	0.17	0.34
β-carotene (capsule)*	-	-	-	-	166	333	500	166	333
Lecithin	2	2	2	2	2	2	2	2	2
Total	100	100	100	100	100	100	100	100	100
Proximate composition by analysis (% dry weight on basis)									
Protein	29.54 ± 2.05								
Fat	5.05 ± 0.29								
Fiber	4.81 ± 0.06								
Moist	6.40 ± 0.70								
Ash	9.36 ± 0.96								
Carotenoids compositions by analysis (mg/kg dry weight on basis)									
Total carotenoids	4.45 ± 0.46	29.51± 2.12	27.73 ± 2.73	52.98 ± 2.58	27.01 ± 3.65	57.53 ± 5.51	77.81 ± 6.60	57.43 ± 3.36	109.39 ± 7.84
Astaxanthin	ND.	25.79 ± 0.81	ND.	ND.	ND.	ND.	ND.	ND.	ND.
Lutein	ND.	ND.	23.35 ± 2.92	47.79 ± 2.99	ND.	ND.	ND.	25.53 ± 5.71	46.31 ± 5.71
β-carotene	ND.	ND.	ND.	ND.	24.14 ± 1.51	53.14 ± 3.14	74.49 ± 3.70	28.57 ± 0.57	59.54 ± 1.14

Note: ND means Not detected

Carotenoids composition

The resulting hyperphase of experimental diets and serum from petroleum ether extraction were evaporated under a gentle stream of nitrogen gas. This was followed by redissolving crude carotenoids in petroleum ether and the solution 5 ul were spotted on pre-coated silica gel 60 (10 x 20 cm) with 0.25 mm thickness (Merck, Germany) using a CAMAG Linomat IV (Switzerland) sample applicator. A constant application rate of 4 ul/s was employed and spaces between two spots were 14 mm. 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 mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 $^{\circ}$C). The length of chromatogram run was 70 mm. Densitometric scanning was performed on CAMAG TLC scanner III in the absorbance mode at 450 nm. The source of radiation utilized was a tungsten lamp (compiled from Sherma and Bernard, 2003 and Mantiri *et al*., 1996). In order to identify the correct value of astaxanthin, lutein and β-carotene, TLC runs were always conducted including authentic standards for astaxanthin and β-carotene (Sigma); lutein (Chromadex, Canada).

Colorimetric analysis

Skin color analysis was performed by reflective spectroscopy, with colorimeter (Minolta color reader, CR-10) in accordance with the system CIE L*a*b* (CIELAB) for lightness, redness and yellowness, respectively (Skrede, 1987). From the a* and b* values, the hue (H_{ab}*) was calculated. The hue is expressed as the relationship between the redness and the yellowness of the skin, and is calculated by the equation; H_{ab}* = arctan (b*/a*) (Hunt, 1977). The measurements were performed on red and white position on fish body (left side).

Statistical analysis

Mean value and standard deviation (S.D.) were calculated from the results. One way analysis of variance (ANOVA) was applied for comparison of the mean values, $P \leq 0.05$ was established as significant.

Part 4 Stability of Carotenoid Diets During Feed Processing and Under Different Storage Conditions

Diets

The various of carotenoids source were premixed with rice bran and included in the dry feed mixture at planned concentration. The diets were produced at HOBRAT mincer; model 4730, designed 3 H.P. Dry mix was pelletized in a single screw with a length 30 cm through passed a modified (ratio diameter of input : out put, 3:1 mm). After be feed processed was obtain a feed particle size of 2-3 mm. Feed was dried with hot air 60 °C in oven for approximately 12 hrs. Before bagging, the feed was cooled by stand at room temperature (26-28 °C) for 2 hrs and then their were equally divided in aluminum foil bags. Experimental diets were separated to two groups; firstly, formulated carotenoid diets supplemented carotenoid source with commercial carotenoid. In the last group, carotenoid diets supplemented with carotenoid extraction form tea, mulberry and cassava leaves and raw material of them. All diets were designed to contain carotenoids following:

Groups A

1. Control diet of low total carotenoid (TC) at least 5 mg/kg
2. Diet supplemented with astaxanthin 25 mg/kg
3. Diet supplemented with lutein 25 mg/kg
4. Diet supplemented with β-carotene 25 mg/kg
5. Diet combined with lutein and β-carotene by 25:25 mg/kg
6. Diet combined with lutein and β-carotene by 50:50 mg/kg

Groups B

1. Control diet of low total carotenoid at least 5 mg/kg
2. Diet supplemented with lutein 25 mg/kg (from commercial source)
3. Diet supplemented with β-carotene 25 mg/kg (from commercial source)
4. Diet supplemented with crude TC of tea leave at 25 mg/kg
5. Diet supplemented with crude TC of mulberry leave at 25 mg/kg
6. Diet supplemented with crude TC of cassava leave at 25 mg/kg
7. Diet supplemented with raw material of tea leaves at TC 25 mg/kg
8. Diet supplemented with raw material of mulberry leaves at TC 25 mg/kg
9. Diet supplemented with raw material of cassava leaves at TC 25 mg/kg

Diets were maintained during 2 months under the following conditions:

A. Stored at room temperature of 26-28 °C under natural light exposure.
B. Stored in refrigerator at 4 °C in the dark.

Sampling and analyses

Three samples (approximately 50 g) of each feed were taken from dry mix, after being processed and dryer, respectively, 3 areas in the mash of diets are randomized for sampling collection. The samples were kept at freezer immediately at -10 °C. Analyzed moisture of all samples for investigated dry matter and carotenoids contents.

Carotenoids determination

Three replicates samples of all diets from unit operations were extracted. All experimental diets were extracted with acetone, together with added BHT (250 ppm) as antioxidant, until samples no color. After that, added petroleum ether 5 ml, mix and add water in separating funnel. Then, mix with a careful swirling and the two phases

were found separated. In this study, only the hyperphase of serum and diets were collected and then determined total carotenoid. Total carotenoid (TC) content in all diets were determined after extraction. Carotenoids are expressed using extinction coefficients ($E^{1\%}_{1\ cm}$) 2500 and determined absorbance wavelength rang 450 nm Recorded merely the absorbance value calculated total carotenoids obey Beer-Lambert's Law (Britton, 1995).

Rancidity analysis

Thiobarbituric acid (TBA) content

TBA determination was carried out as follows (Shibata, 1979): TBA Reagent (thiobarbituric acid 1 g in NaOH 0.1 N 75 ml and adjusted to 100 ml with distil water) was prepared, then 1 g of diets sample was weight into a glass-stoppered test tube and 5 ml of water was added and shaked for 2 min. A clear portion was collected, 10 ml of TCA solution, 0.5 ml of 20% EDTA, 1 drop of antioxidant solution and 5 ml of TBA reagent were added. The tube was stoppered and the contents were mixed. Then, the tube was immersed in a boiling water bath for 35 min. A distilled water-TBA reagent blank was also prepared and treated like the sample. After heating, the sample was cooled in tap water for 5 min. A portion of 4 ml was added with chrolofrom 2 ml and centrifuged at 2000 rpm for exactly 10 min. In this study, only the hyperphase was collected and transferred to a cuvette and the optical density of the sample was read against the blank at a wavelength of 535 nm in a spectrophotometer. The optical density value was converted to the moles of malonaldehyde per gram of diet sample by equation.

TBA value = 50 x (A-B)/W

A = OD of sample

B = OD of sample blank

W = sample weight (gram)

Acid value

Acid value was determined by a titration method, as described in AOCS (1971) involving the use of 0.1 N KOH. Acid value represents in milligram of potassium hydroxide (potassium hydroxide, KOH) which is used for the reaction of free fatty acid in 1 gram of sample. The method starts from weighting the diet and adding isopropanal and toluene at the amount of 1:1, the shake them then add indicator (1% of phenolphthalein) 0.5 ml. titrate of 0.1 N KOH shake well until seeing the pink then stop and the color should stay for 30 s.

$$\text{Acid value} = \frac{(A - B) \times N \times 56.1}{W}$$

A = milliliter of KOH standard used for titrate sample

B = milliliter of KOH standard used for titrate sample blank

N = normal of KOH standard

W = sample weight (gram)

Statistical analysis

All data were subjected to a factorial consist two-factor to investigate the interaction between diet type and processing methods (dry mix, after being processed and dryer) for study of feed processing and the interaction between diet type and storage time for study carotenoids stability under different storage temperature and study rancidity during storage. Mean value and standard deviation (S.D.) were calculated from the results. One way analysis of variance (ANOVA) was applied for comparison of the mean values $P \leq 0.05$ was regarded as significant.

Part 5 Potential Use of Natural Carotenoids Source on Growth Performance, Skin Pigmentation and Immune Response in Fancy Carp (*Cyprinus carpio*)

Fish, diets and feeding protocol

Mixed sex of fancy carp were maintained on a non-pigmented diet for a co-variant period of two weeks prior to feeding the seven experimental diets, each treatment was performed in four replicates. Each treatment with an initial mean wet weight of 53.1 ± 3.08 g/fish, were randomly distributed to each of 20-liter aquarium tanks.. The diets using crude extraction and raw material of local feedstuffs were designed to contain total carotenoid (TC) 25 mg/kg, following:

1. Control diet of low TC at least 5 mg/kg
2. Diet supplemented with crude TC extraction of tea leaves
3. Diet supplemented with crude TC extraction of mulberry leaves
4. Diet supplemented with crude TC extraction of cassava leaves
5. Diet supplemented with raw material of tea leaves
6. Diet supplemented with raw material of mulberry leaves
7. Diet supplemented with raw material of cassava leaves

The daily feed was fed at 2% of body weight and by hand-fed methods twice a day. Total feed was weighed weekly. The duration of the experiment was six weeks (45 days) with experimental diets containing carotenoids followed by the diet without supplemented carotenoid diet for two weeks (14 days). Fish were not exposed to natural light, as tanks were covered in black plastic. Water exchange in each aquarium tank were maintained at 80% every three days.

Feed consumption was recorded weekly. Fish from each tank was weighed for measure growth every two weeks throughout the experiment at six weeks that feeding with carotenoid diets and calculated growth performance and feed conversion.

Proximate analysis

Proximate analysis of diets were performed according to the methods of AOAC (1990): dry matter after drying in an oven at 105 ºC until constant weight; ash content by incineration in a muffle furnace at 600 ºC for 6 h; crude protein (N x 6.25) by Kjeldahl method after acid digestion; lipid by petroleum erher extraction in a Soxlet apparatus and crude fiber content. (as shown in **Table 3**).

Sampling and analytical methods

Blood sampling was carried out every two week over at the termination of study at 8 weeks. Blood was collected from the caudal vein with 1 ml non-heparinized disposable syringes fitted with 0.55 x 25 mm disposable needles. Blood samples (approx. 1 ml/fish) was centrifuged at 300 x g, 25 °C for 10 min, 500 ul of the serum was removed and vortexed with 1 ml of ethanol for 30 s, then 2 ml of petroleum ether was added, and the mixture was vortexed for 1 min. The petroleum ether was separated by centrifuging 300 x g, 25 °C for 10 min (White *et al*., 2002).

All experimental diets were extracted with acetone, together with BHT (250 ppm) added as antioxidant, until samples showed no color. After that, petroleum ether was added at 5 ml, mix and add water in a separating funnel. Then, mix with a careful swirling and the two phases were found separated. In this study, only the hyperphase of serum and diets were collected and determined for total carotenoids.

Table 3 Ingredients and proximate composition of experimental diets.

Ingredient (%)	Experimental diets						
	1	2	3	4	5	6	7
Fish meal	30	30	30	30	30	30	30
Soybean meal	24	24	24	24	24	23	24
Rice bran	24	24	24	24	18	17.5	16
Tapioca starch	5	5	5	5	5	5	5
Wheat Flour	5	5	5	5	5	5	5
Fish oil	2	2	2	2	2	2	2
Alpha-starch	5	5	5	5	5	5	5
Dicalcuim	1	1	1	1	1	1	1
Premix	2	2	2	2	2	2	2
Lecithin	2	2	2	2	2	2	2
Crude carotenoid (contained total carotenoid 623.67, 479.21 and 330.64 ppm, using ml unit for 1 kg of diet)							
Tea leaves	-	50	-	-	-	-	-
Mulberry leaves	-	-	60	-	-	-	-
Cassava leaves	-	-	-	85	-	-	-
Raw material							
Tea leaves	-	-	-	-	6	-	-
Mulberry leaves	-	-	-	-	-	7.5	-
Cassava leaves	-	-	-	-	-	-	8
Total	100	100	100	100	100	100	100
Proximate composition by analysis (% dry weight on basis)							
Protein	31.35 ± 0.25	31.64 ± 0.81	31.96 ± 0.13	31.25 ± 0.75	30.79 ± 0.47	31.10 ± 0.53	30.98 ± 0.72
Fat	6.02 ± 1.99	6.40 ± 0.23	6.20 ± 1.45	5.90 ± 0.81	6.40 ± 0.97	6.35 ± 1.21	5.75 ± 2.42
Moisture	7.14 ± 0.43	7.40 ± 0.39	6.92 ± 0.08	6.79 ± 0.43	6.88 ± 0.35	6.51± 0.84	6.89 ± 0.46
Fiber	4.50 ± 0.75	4.67 ± 1.21	4.58 ± 0.64	4.61 ± 0.58	5.15 ± 1.04	5.08 ± 1.35	5.26 ± 2.14
Ash	10.08 ± 0.13	9.67 ± 0.05	9.83 ± 0.16	10.27 ± 0.12	12.19 ± 0.72	12.84 ± 0.61	13.62 ± 0.53

Total Carotenoids determination

Total carotenoid (TC) content in all diets and serum were determined after extraction. Carotenoids are expressed using extinction coefficients ($E^{1\%}_{1\ cm)}$ 2500 and determined by absorbance wavelength at 450 nm. Recorded merely the absorbance value calculated total carotenoids obey Beer-Lambert's Law (Britton, 1995).

Carotenoids composition

The resulting hyperphase of experimental diets and serum from petroleum ether extraction were evaporated under a gentle stream of nitrogen gas. This was followed by redissolving crude carotenoids in petroleum ether and the solution 5 ul were spotted on pre-coated 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. 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 mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The length of chromatogram run was 70 mm. Densitometric scanning was performed on CAMAG TLC scanner III (CAMAG, Switzerland) in the absorbance mode at 450 nm. The source of radiation utilized was a tungsten lamp (compiled from Mantiri *et al*., 1996 and Sherma and Bernard, 2003). In order to identify the correct value of astaxanthin, lutein and β-carotene, TLC runs were always conducted including authentic standards for astaxanthin and β-carotene (Sigma, USA); lutein (Chromadex, Canada).

Colorimetric analysis

Skin color analysis was performed by reflective spectroscopy, with colorimeter (Minolta color reader, CR-10) in accordance with the system CIE L*a*b* (CIELAB) for lightness, redness and yellowness, respectively (Skrede, 1987). The measurements were performed on red and white position on fish body (left side). Skin pigmentation was recorded every two weeks over the end of experiment period.

Phagocytosis

Phagocytic activity (PA, %) was determined before and the end of experiment following the procedure described by Amar *et al*. (2001). Blood was collected from the caudal vein with 1 ml non-heparinized disposable syringes fitted with 0.55 x 25 mm disposable needles. Peripheral blood were diluted 1:2 with RPMI 1640 medium (Sigma Chemical, USA). Meduim supplemented with 0.1 % fetal calf serum (FCS; Gibco, USA) and 1% penicillin/streptomycin (P/S; Sigma). Lymphocyte from peripheral blood was isolated on a percoll has density gradient of 1.077700.1 – 1.0800 g $ml^{-}1$. After adjusting the concentration to 2 x 10^6 cell ml^{-1} with RPMI 1640 medium, 300 µl of the suspension were plated on slide and incubated for 2 h at 20°C for cell attachment. Then, the slides were washed gently three times with the RPMI 1640 medium and 200 µl cell yeast were added at cell count 2 x 10^7 cells ml^{-1} and incubated for 2 h at 20 °C. After final washing to remove unphagocytized cell yeast with medium three times and the slide were stained with Diff-Quick staining solution. From the stained preparation the number of phagocytes with engulfed cell yeast relative to the total number of cells (phagocytic activity, PA %) was determined from 200 cells examined under a light microscope.

Statistical analysis

Mean value and standard deviation (S.D.) were calculated from the results. One way analysis of variance (ANOVA) was applied for comparison of the mean values, $P \leq 0.05$ was established as significant.

RESULTS AND DISCUSSION

Part 1 Carotenoid Analysis and Method Validation

Part 1.1 Monitoring the Occurrence of Carotenoids Composition and Toxic Substances in Local Thai Feedstuffs: Tea, Mulberry and Cassava Leaves

1. Carotenoids composition in feedstuffs

In this study, total carotenoids contents of tea, mulberry and cassava leaves were 623.67 ± 6.51, 479.21 ± 5.16 and 330.64 ± 11.24 mg/kg respectively. UV/Vis spectrum determined maximum absorbance (λ max) at the wavelength range over 350-600 nm of crude extract in all feedstuffs. This effect is illustrated in **Figure 13**, which shows a UV/Vis spectrum of crude hexane extract of tea, mulberry and cassava leaves. All feedstuffs proceded at the maximum absorbance 444 nm (**Table 4**). The contribution at 444 nm an $E^{1\%}_{1cm}$ of 2500 for the total carotenoid is assumed (Schiedt and Liaaen-Jensen, 1995). UV-Vis spectra gave the information on chromophores which consisted of the conjugated double bonds system in carotenoids. Furthermore, UV/Vis spectra could characterize geometrical isomers of carotenoids and UV/Vis spectral data so that it offered a rapid and accurate method for the identification of carotenoids extracted from various natural sources (Takashi *et al.*, 2003).

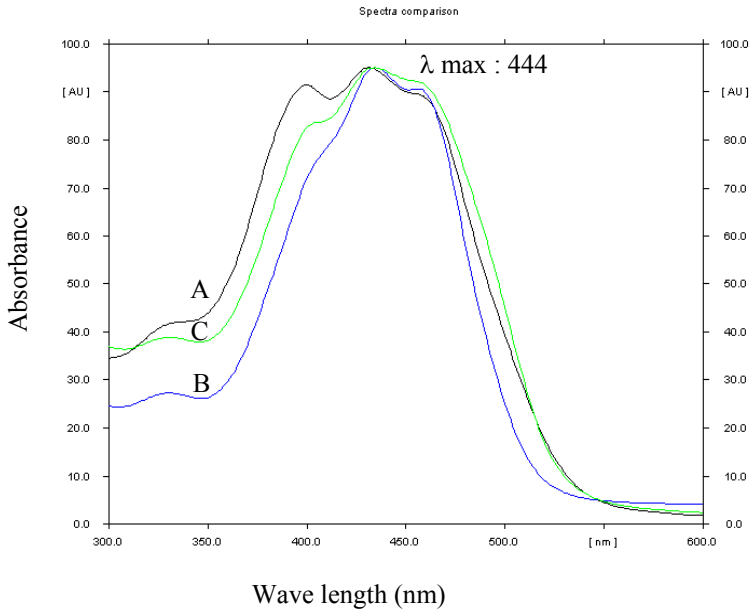


Figure 13 Light absorption spectra of feedstuffs; A: Tea, B: Mulberry and C: Cassava.

Table 4 Maximum absorbance and total carotenoids in feedstuffs.

Feedstuff	Maximum absorbance (λ max) nm.	Total carotenoids (mg/kg)		Percentage of diminish	solvent
		Initial	2 months		
Tea	444	623.67 ± 6.51[a]	502.77 ± 6.92[a]	19.39	hexane
Mulberry	444	479.21 ± 5.16[b]	312.02 ± 2.75[b]	34.89	hexane
Cassava	444	330.64 ± 11.24[c]	273.14 ± 0.89[b]	17.39	hexane
p-value		0.0001	0.0001		

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Hence, the data from this study suggested that the main of carotenoids compositions in tea, mulberry and cassava leaves are xanthophylls because the data related with result of Hendry and Houghton (1996), maximum absorbance in green leaves that contain carotenoids composition like violaxanthin, lutein, zeaxanthin and neoxanthin were 442, 445, 452 and 439 nm respectively. Because the carotenoids

absorb visible or, in a few cases, UV light strongly, they are normally determined quantitatively by spectrophotometry. This can be applied to determine the amount or concentration in a mixture or natural extract.

The quantification of carotenoids compositions in local Thai feedstuffs were identified as lutein, β-carotene and small amount of other pigments on silica gel with Petroleum Ether-Diethyl Ether-Acetone (75:15:10) as eluent. The dominant carotenoids compositions in all raw materials were lutein and β-carotene (**Figure 14** and **Figure 15**). Retention factor (R_f) averaged 0.19, 0.22 and 0.19 identical to lutein standard in tea, mulberry and cassava, respectively. And the least polar zone had an R_f average at 0.98, 0.98 and 0.96 identical to a β-carotene standard. The data of this study related with Muller (1997), reported that lutein and β-carotene (*trans* and *cis* forms) were the predominant carotenoids in all green leaves.

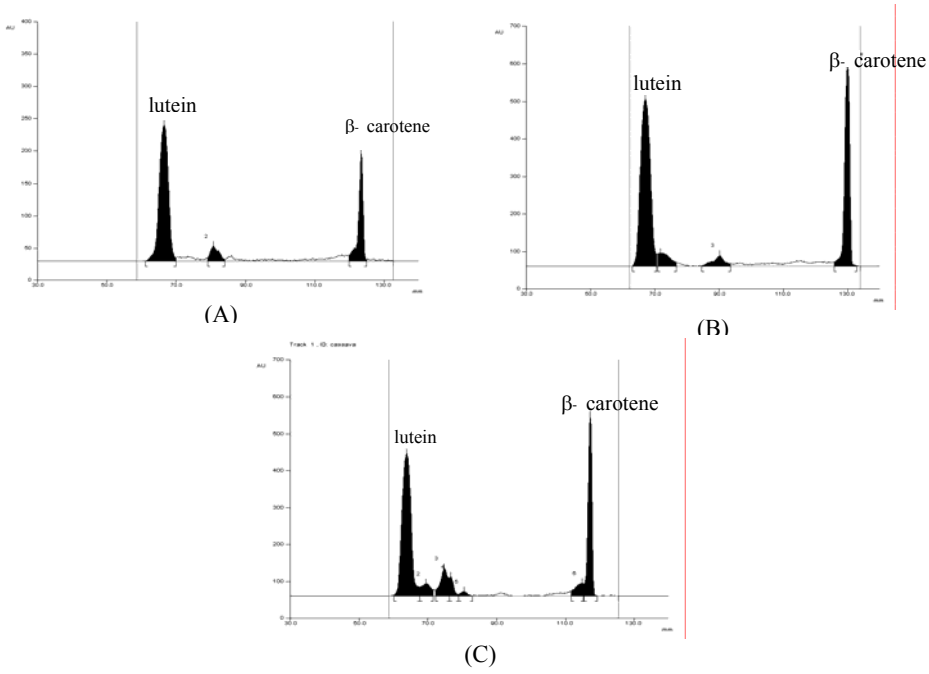


Figure 14 Reflectance densitogram at λ= 450 nm of feedstuffs on silica gel plate developed with Petroleum Ether-Diethyl Ether-Acetone (75:15:10); A: Tea, B: Mulberry and C: Cassava leaves.

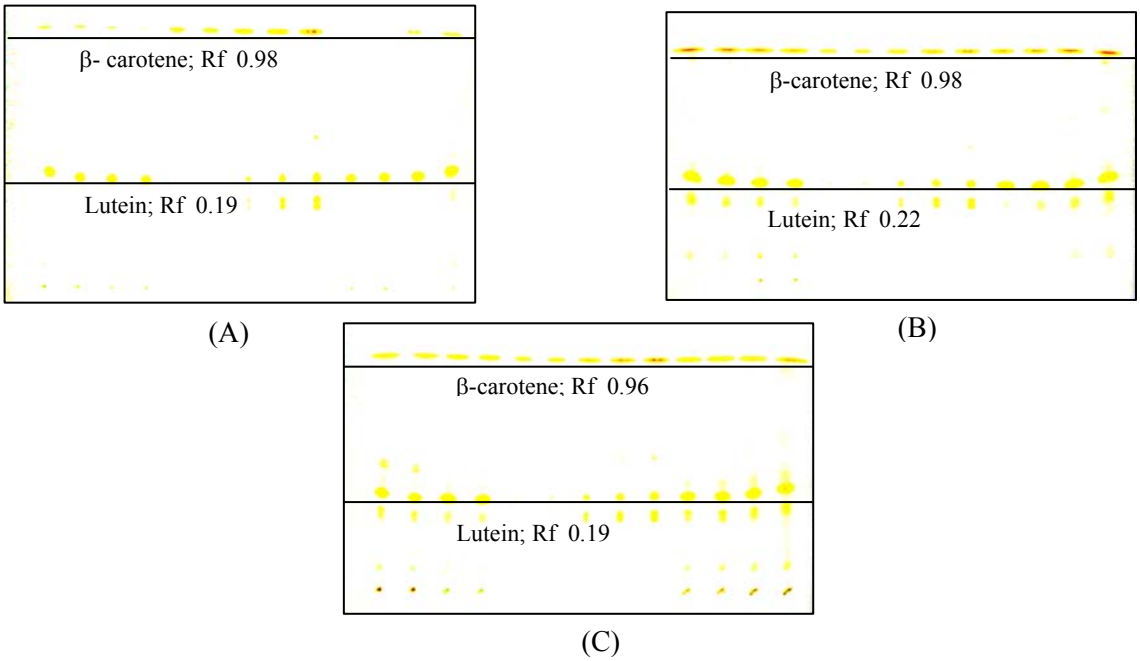


Figure 15 TLC separation of carotenoid in feedstuffs; A: Tea, B: Mulberry and C: Cassava leaves.

The main carotenoids composition in tea, mulberry and cassava leaves showed two pigments; lutein provided a retention factor (R_f) which averaged of 0.20, and β - carotene had R_f average at 0.97. Small amounts of carotenoids that could not be identified had a R_f average at 0.44 in all raw materials which could possibly be zeaxanthin because of this data similar with Olivier *et al*. (2000) reported zeaxanthin had R_f 0.47. The results showed that tea and mulberry leaves comprise the proportion of lutein and β-carotene 0.96: 1.00 and 0.83: 1.00. In contrast, cassava leave comprise the lutein more than β-carotene, so the proportion was 1.84: 1.00 (as shown in **Table 5** and **Figure 16**).

Table 5 Proportion of lutein and β - carotene in feedstuff.

Feedstuff	Total carotenoids (mg/kg)	Percentage of lutein	Rf	Percentage of β - carotene	Rf	Total percentage of unknown	Rf
Tea	623.67 ± 6.51	42.33	0.19	44.10	0.98	13.57	0.12, 0.43
Mulberry	479.21 ± 5.16	43.60	0.22	52.62	0.98	3.78	0.11, 0.48
Cassava	330.64 ± 11.24	60.34	0.19	32.85	0.96	6.81	0.10, 0.42

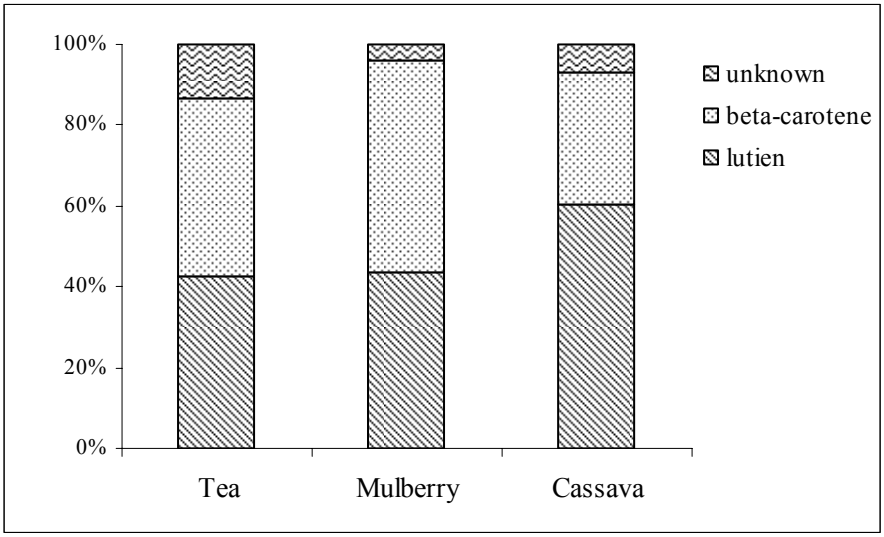


Figure 16 Proportion of lutein and β - carotene in feedstuffs.

In plants, carotenoids are C 40 isoprenoid polyene secondary plant compounds that form lipid soluble yellow, orange and red pigments. Examples of carotenoids include the oxygenated xanthophyll -- lutein [(3*R*,3'*R*,6'*R*)-β-carotene-3,3'-diol] and the hydrocarbon carotene -- β- carotene (β,β-carotene) (Zaripheh and Erdman, 2002). The carotenoids occur generally in the chloroplasts of green tissue but their colour is masked by the chlorophylls, organelles where photosynthesis take place. The leaves of virtually all species contain the same main carotenoids, that is β-carotene (usually 25-30% of the total), lutein (around 45%), violaxanthin (15%) and neoxanthin (15%). Small amounts of α-carotene, α and β-cryptoxanthin, zeaxanthin, antheraxanthin and

lutein-5, 6-epoxide are also frequently present and lactucaxanthin is a major xanthophylls in a few species (Gross, 1991). There is a considerable variation in the total carotenoids content in leaves of different species and varieties.

2. Carotenoids loss after storage

After storing feedstuffs in clear plastic bags at the room temperature of 26-28 °C for two months, it turned out that tea, mulberry and cassava leaves contain percentage of diminish in lutein by 36.43, 32.28 and 49.01, percentage of diminish in β-carotene were 32.54, 30.14 and 15.72 (**Table 6** and **Figure 17**). The data shows stability of lutein was lost more than β-carotene in all feedstuffs because lutein was xanthophylls that comprised of hydroxyl group led to oxidation rapidly and loss more than β-carotene (Goodwin, 1984). The hydrocarbon carotenoids are known as carotenes, while oxygenated derivatives of these hydrocarbons are known as xanthophylls. Due to the system of conjugated double bonds in the molecule, the carotenoids are easily destroyed by oxidative degradation (Gross, 1991). The occurrence of oxidation depends on the presence of oxygen, metals, enzymes, unsaturated lipids, prooxidants, or antioxidants; exposure to light; type and physical state of carotenoid present; severity of the treatment (i.e., destruction of the ultrastructure that protects the carotenoids, increase of surface area, and duration and temperature of heat treatment); packaging material; and storage conditions. (Rodriguez-Amaya, 1997).

Table 6 Separated and identified carotenoids content in feedstuffs after stored under room temperature of 26-28 °C for 2 months.

Feedstuff	Lutein (mg/kg)		Percentage of diminish	β- carotene (mg/kg)		Percentage of diminish
	Initial	2months		Initial	2months	
Tea	264.01 ± 8.66 [a]	167.83 ± 11.68 [a]	36.43	275.01 ± 7.38 [a]	185.52 ± 7.85 [a]	32.54
Mulberry	208.92 ± 17.28 [b]	141.49 ± 8.16 [b]	32.28	252.17 ± 10.39 [a]	176.17 ± 2.58 [a]	30.14
Cassava	199.52 ± 8.39 [b]	101.73 ± 14.86 [c]	49.01	108.61 ± 11.08 [b]	91.54 ± 19.66 [b]	15.72
p-value	0.0059	0.0037		0.0001	0.0033	

Mean with the different letters in same row are significantly different at $p \leq 0.05$

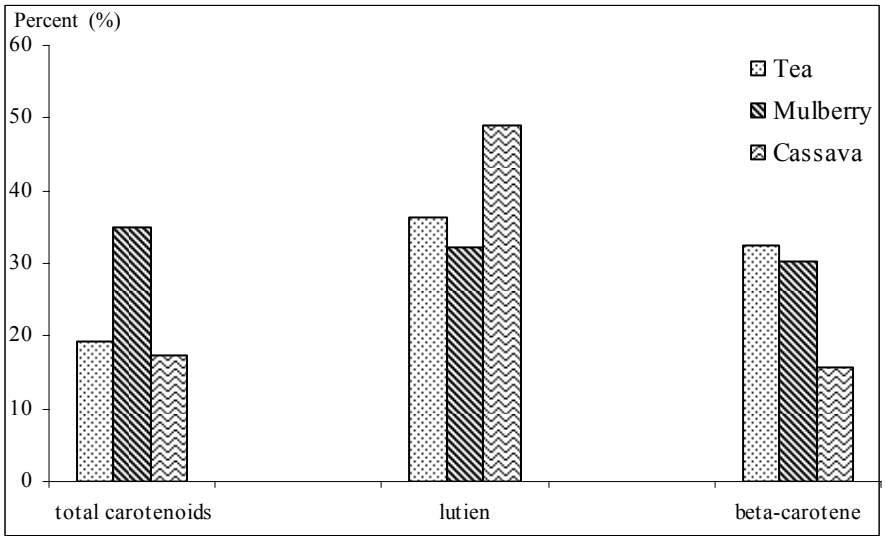


Figure 17 Percentage of diminish carotenoids in feedstuffs for 2 months.

3. Toxic Substances

The toxic substance in tea leaves was tannin. They have been reported to be responsible for decreases in feed intake, growth rate, feed efficiency and protein digestibility (Giner-Chavez, 1996). Therefore, feeds rich in tannins are considered to be of low nutritional value. This study found that initial tannin in tea leaves was 4.60 ± 0.07% and after storing for 2 months it was left only 1.78 ± 0.03%, so, the percentage of diminishing of tannin in tea was 61.30% (**Table 7**). Hence, dried tea leaves were readily consumed providing 10% in formulated diet with tannins contained at 0.48%. Meanwhile, tea leaves that had been stored for two months were able to use 25% more in a formulated diet. Giner-Chavez (1996) reported that levels from 0.5 - 2.0% can cause depression in growth and the levels of tannins above 5% of the diet which are often lethal.

The toxic substance in cassava leaves was hydrocyanic acid; the hydrocyanic acid is stored inside the vacuoles in cytoplasm. The hydrocyanic acid potential varied from 31-630 mg HCN equivalent/kg in the root (fresh weight) and from 540-1450 HCN equivalent/kg in the leaves (fresh weight). This study indicated the level of

HCN in cassava leaves was 921.37 ± 29.23 mg HCN equivalent/kg. Then after two months, the result was 608.87 ± 29.61 HCN equivalent/kg, thus percentage of hydrocyanic diminish was 33.92% (**Table 7**). Therefore dried cassava leaves were readily consumed providing 60% in formulated diet with HCN contained 552.84 mg HCN equivalent/kg while two-months-stored tea leaves were able to use more 90% in formulated diet.

Table 7 Toxic substances and percentage of diminish in feedstuffs after stored under room temperature of 26-28 °C for 2 months.

Feedstuff	Tannin (%)			P-value	Percentage of diminish	Crude carotenoids extraction
	Initial	1 months	2 months			
Tea	4.60 ± 0.07[a]	2.82 ± 0.09[b]	1.78 ± 0.03[c]	0.0001	61.30	0.58 ± 0.12
	HCN (mg HCN equivalent/kg)					
Cassava	921.37 ± 29.23[a]	773.59 ± 29.23[b]	608.87 ± 29.61[c]	0.0001	33.92	156.00 ± 10.39

Mean with the different letters in same row are significantly different at $p \leq 0.05$

The main limiting factor to the use of cassava leaves as animal feed is the presence of HCN. However, several reports indicate that chronic low exposure to HCN can cause neurological, respiratory and hematological disorders. The lethal dose of hydrocyanic acid was 600 mg HCN equivalent/kg. (Okafor *et al*., 2003). This result indicated that both tannin and hydrocyanic acid in dried tea and cassava leaves were readily consumed providing no more than 10 and 60% in dietary which is considered safe for animals. Crude extraction from tea leaves was 0.58 ± 0.12% of tannin and hydrocyanic acid in cassava extracted was 156.00 ± 10.39 mg HCN equivalent/kg. Thus, extractions of carotenoids from tea and cassava leaves were readily consumed and safe for an aquatic animals diet.

Thailand is an agricultural and processed agricultural products country. Generally, there is a lot of crop residues from the process. Furthermore, these residues become waste and have caused pollutions both for human and environment.

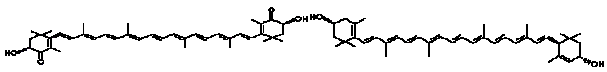
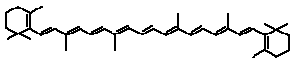
Carotenoids in plant are yellow, orange and red pigments. Examples of carotenoids include oxygenated xanthophylls--lutein and hydrocarbon carotene--β - carotene. There is a considerable variation in carotenoids content in leaves varied by season, species, age, harvesting period and storage. This study investigates the carotenoids composition and toxic substances in tea, mulberry and tea leaves, the byproducts from agricultural harvest. So an extraction of carotenoids from such raw materials is the promising and convenient way to solve this problem. In addition, it provides the exact concentration of carotenoids content. For this reason, crude carotenoids extraction has the height potential to be a new solution for a quicker and easier use. This study makes use of Thai local feedstuffs to be the carotenoid sources for colour enhancing diet particularly in fancy carp or ornamental fishes' diets. This becomes a method in lowering the production cost of diets and in adding value of raw material and byproducts from agricultural harvest.

Part 1.2 Validated TLC-Densitometric Analysis for Determination of Carotenoids in Fancy Carp (*Cyprinus carpio*) Serum

1. Spectra of carotenoids standard

UV/Vis spectrum determined maximum absorbance (λ max) at the wavelength range over 350-600 nm of carotenoid standard: astaxanthin, lutein and β-carotene dissolved in hexane. The data illustrated in **Figure 18**, showed that maximum absorbance in astaxanthin, lutein and β-carotene standard was 468, 444 and 456 nm. Hence, the data from this study related with result of Goodwin (1984) and Britton *et al.* (2004) as displayed in **Table 8.** The maximum absorbance of astaxanthin, lutein and β-carotene in hexane was 470, 440 and 450 nm. Light absorption spectra strongly are normally determined quantitatively by spectrophotometry.

Table 8 Structural formula and physiochemical properties of astaxanthin, lutein and β-carotene.

	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
$E_1^{1\%}$ (n – hexane)	2100	2500	2600

Source: Goodwin (1984) and Britton *et al.* (2004)

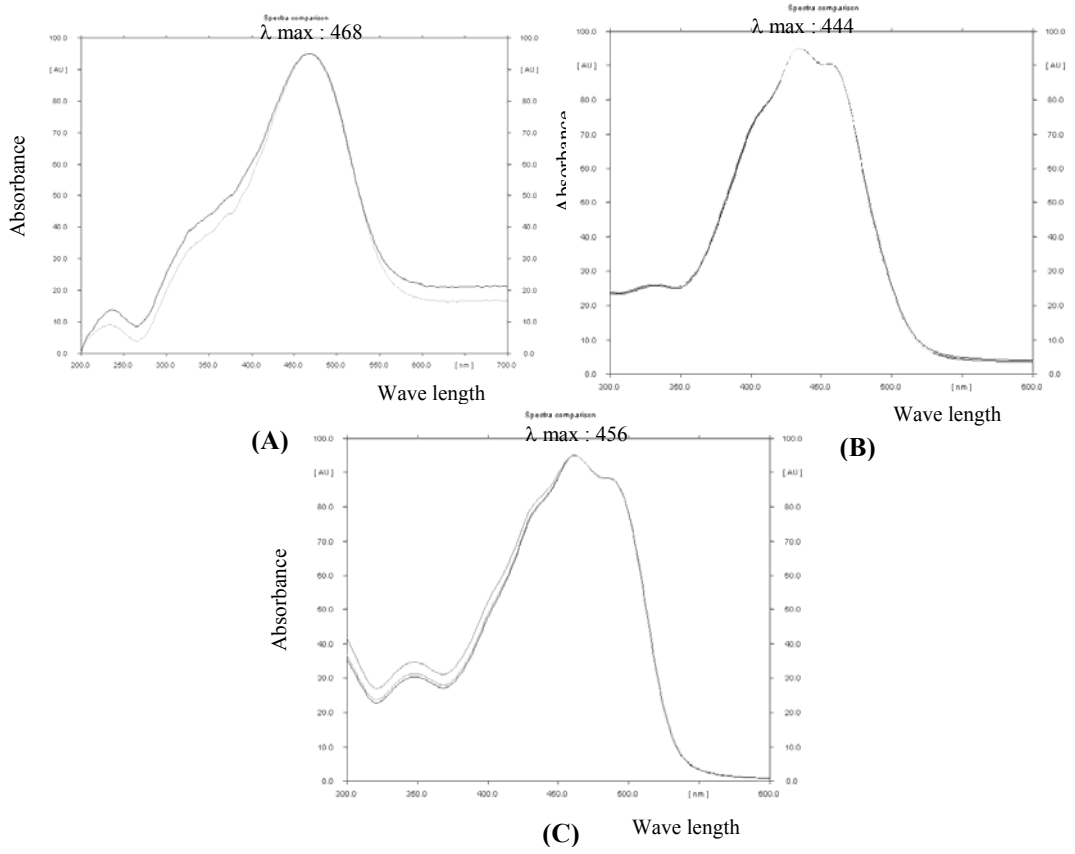


Figure 18 Spectra of carotenoids standard; A: astaxanthin, B: lutein and C: β-carotene.

2. 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 resolution. 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 19** and **Figure 20**. Well-defined spots were obtained when the chamber was saturated with a mobile phase at room temperature (25 ± 2 ˚C).

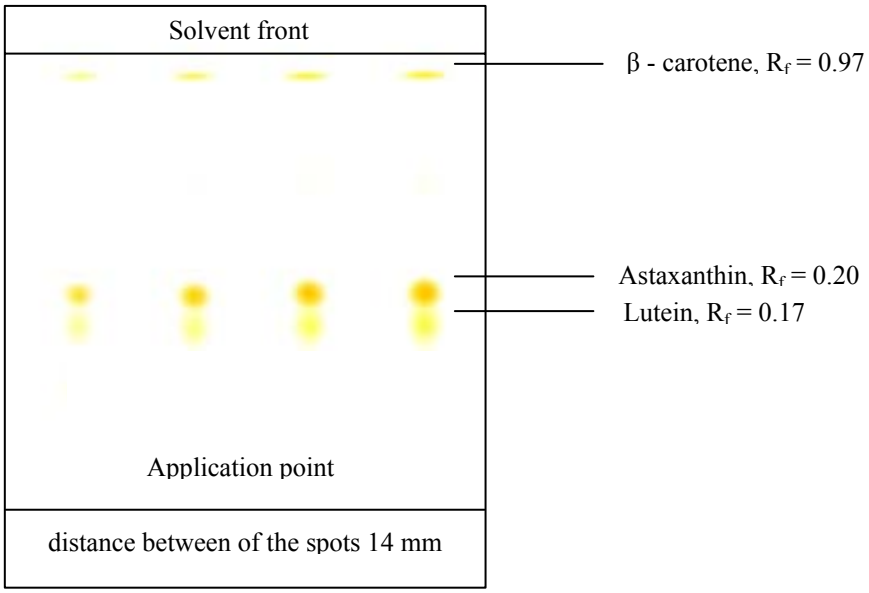


Figure 19 Silica gel TLC plate on which carotenoids standard mixture was separated.

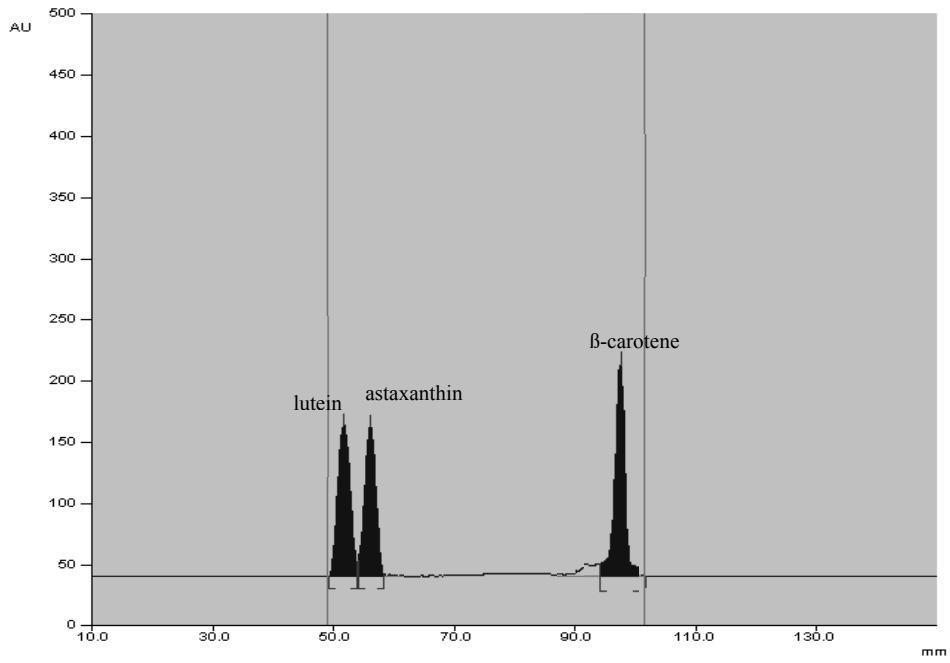


Figure 20 Reflectance densitogram at λ= 450 nm. of carotenoids standard developed with petroleum ether-diethyl ether-acetone (75:15:10, *v/v/v*).

3. 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 ug/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 estimation of astaxanthin, lutein and ß-carotene showed a good linear relationship with r^2 = 0.9982, 0.9980 and 0.9963 in the concentration range 0.01-0.65 ug/spot with respect to peak areas. The acceptable criteria of correlation coefficient (r^2) 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 ß-carotene measurement of peak areas were found to be 1.72, 1.78 and 1.35 % respectively. The measurement of the peak area at three different concentration levels showed low values of $\%RSD_r$ (< 3.7%), which suggested an excellent precision of the method and making it a reliable quantitative tool. (**Table 9**). 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 concentrations of the analyte values that can be detected. Notwithstanding the definitions, it is recommended that limits of detection and quantitation 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 ß-carotene was determined by TLC-densitometric analysis and limit of quantification with signal-to-noise ratio of 3:1 and 10:1 were found to be 0.04, 0.25 and 0.44 µg/spot for LOD and 0.13, 0.82 and 1.45 µg/spot for LOQ, respectively, which indicates the adequate sensitivity of the method.

The percent recoveries of astaxanthin, lutein and ß-carotene spiked to sample blank are shown in **Table 10**. Recovery for astaxanthin (0.3-2.0 µg/ul) the average of percent recoveries was 91.70%, for lutein (0.2-3.0 µg/ul): 90.47 % and for ß-carotene (0.1-1.0 µg/ul): 102.25%. The concentrations covered the range of concern and were close to the quantitation limit. The acceptable limit of recovery for single laboratory validation provided mean recovery 90-108% of analyte concentration 0.1% (AOAC, 1993). The AOAC manual for the peer verified methods program (1993) provided mean recovery 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 ß-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).

In carotenoid analysis, validation of methods has not been strongly advocated, even with the introduction of high-performance liquid chromatography (HPLC), because the emphasis has been on chromatographic separation. In the few papers involving quantification, validation consisted mainly of recovery tests and determination of repeatability (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 carotenoids in fish serum. It is simpler than HPLC and faster because up to nine samples (applied singly with the minimum four standard concentrations) can be analyzed on each plate. Cost of solvent purchase and disposal is very low because no 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.

Table 9 Validation parameters for measurement of carotenoids in serum.

Parameter	Ataxanthin	Lutein	ß-carotene
Correlation coefficient (r^2)	0.9982	0.9980	0.9963
LOD (ug/spot)	0.04	0.25	0.44
LOQ (ug/spot)	0.13	0.82	1.45
Precision (%RSD_r)*	1.72	1.78	1.35
Accuracy (% recovery)	91.70	90.47	102.25
HORRAT(r) value*	0.17	0.22	0.17

Note: * for single laboratory validation

Table 10 Recovery of added carotenoids to sample blank.

Compound	concentration				
	Initial (ug/ul)	Added (ug/ul)	Found (ug/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	

Part 2 Bioavailability of Carotenoids

Part 2.1 Bioavailability of Carotenoids; Astaxanthin, Lutein and β-Carotene in Fancy Carp (*Cyprinus carpio*)

1. Carotenoids diets

The bioavailability of carotenoids in fancy carp was studied by feeding a single dose. The diets were designed to achieve a target level of 200 μg for each of astaxanthin, lutein and β-carotene. Analyzing carotenoids contained in each diet found that the diets have astaxanthin, lutein and β-carotene contained at 173.09 ± 19.00, 187.20 ± 14.79 and 172.54 ± 10.09 μg. The maximum absorbance was 468, 444 and 456 nm, respectively (as shown in **Table 11**). Hence, the data from this study suggest mainly on carotenoids compositions in three diets are astaxanthin, lutein and β-carotene because the data was similar to the results of Goodwin (1984) and Britton *et al*. (2004), the maximum absorbance of astaxanthin, lutein and β-carotene in petroleum ether was 470, 440 and 450 nm.

Table 11 Maximum absorbance and carotenoids concentration in experimental diets.

Experimental diet	Maximum absorbance	Concentration (μg)		
		Astaxanthin	Lutein	β-carotene
Astaxanthin	468	173.09 ± 19.00	ND	ND
Lutein	444	ND	187.20 ± 14.79	ND
β-carotene	456	ND	ND	172.54 ± 10.09

ND = not detected

2. Pharmacokinetic parameters of fancy carp fed with deprived diets

The observed time curves of serum astaxanthin, lutein and β-carotene in fancy carp following a single dose of experimental diets are shown in **Figure 21.** The astaxanthin, lutein and β-carotene concentration in fancy carp were best described by one compartment model with first order absorption, pharmacokinetic parameters displayed in **Table 12**.

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 hr for astaxanthin, lutein and ß-carotene, respectively. Since, then astaxanthin, lutein and ß-carotene were on a gradual decline at 12, 24 and 24 hr after post-dosing.

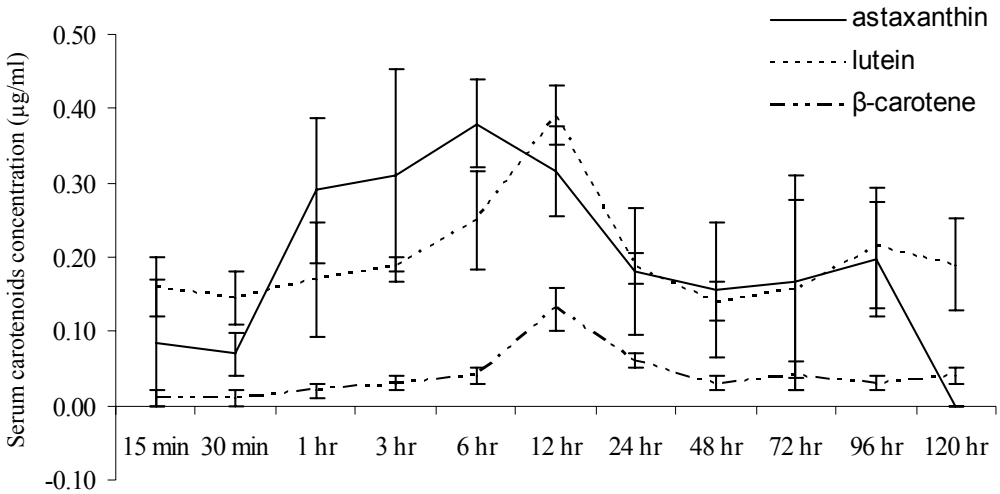


Figure 21 Maximum carotenoids concentration (C max, µg/ml) of fancy carp administration a single dose of astaxanthin, lutein and β-carotene diet.

Fancy carp fed with an astaxanthin diet developed a maximum concentration of astaxanthin (C max) 0.38 µg/ml occurred at 6 hr (T max) while fancy carp fed with a lutein diet contained lutein blood level (C max) 0.39 µg/ml within 12 hr and fancy carp fed with β-carotene diet contained β-carotene in blood level (C max) 0.13 µg/ml

within 12 hr. The volume of distribution (Vd) was 286.11, 129.65 and 80.16 ml/kg, respectively. The data illustrated that free astaxanthin was distributed in the blood circulation system better than lutein and β-carotene. Because the high amount of Vd demonstrates the fact that it can disseminate well in blood circulation. However, the area under the curve (AUC) was 65.36, 64.71 and 21.67 µg-hr/ml. AUC, this indicated the relationship between carotenoid concentration in serum and time. It is close to the amount of carotenoids being absorbed in the blood circulation system. If the area under the curve (AUC) is elevated, it means that carotenoids can be highly absorbed. Bioavailability, expressed as an area under the serum concentration time curve, was highest for fish fed with an astaxanthin diet. Total body clearance (CL) was 9.62, 6.49 and 10.06 ml/hr/kg, indicated that β-carotene was clearance more than astaxanthin and lutein, this clearance was mainly to eradicate carotenoids in anyway. Overall, after fancy carp were fed an astaxanthin diet, they showed better astaxanthin absorption than lutein and β-carotene. When considered together with pharmacokinetic parameter which was Vd and AUC, it proved well dispersed of astaxanthin in blood circulation (Evans, 2004).

Table 12 Pharmacokinetic parameters for astaxanthin lutein and β-carotene derived from serum concentration of fancy carp.

Parameters	Unit	Deprived diets		
		Astaxanthin	Lutein	β-carotene
Vd (area)/kg	ml/kg	286.11	129.65	80.16
CL (area)/kg	ml/hr/kg	9.62	6.49	10.06
AUC (area)	µg-hr/ml	65.36	64.71	21.67
C max (obs)	µg/ml	0.38	0.39	0.13
T max (obs)	hr	6	12	12

Vd (area)/kg : volume of distribution calculations

CL (area)/kg : total body 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

The results of the present study indicated that bioavailability of astaxanthin, lutein and β-carotene in fancy carp following single dose feeding oral administration shows the faster rate of astaxanthin absorption for the fish fed with an astaxanthin diet compared to lutein and β-carotene diet which was similar to the report of Olsen and Bakker (2006). The author related that 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. The absorption of carotenoids depends on disruption of food matrix. These solubility and location of the polar carotenoids (xanthophylls) and the non polar carotenoids (carotene) in emulsions are different. Carotenes are thought to incorporate almost exclusively in the triacylglycerol core of the emulsion, whereas the more polar xanthophylls distribute preferentially at the emulsion surface (Furr and Clark, 1997). It has been shown by several authors, including Yonekura and Nagao (2007) that polar carotenoids (xanthophylls) have higher bioavailability characteristics leading to more transfer across the intestine compared to carotenes. This is related to the polar –OH side chains they possess which are absent in carotenes such as β-carotene, showed the absorption of β-carotene appeared to be very low compared to astaxanthin and lutein.

3. Metabolized serum carotenoids after administration single dose of carotenoids diets

Effect of dietary carotenoids was investigated on metabolism of serum by TLC-densitometric analysis. Fish were fed with diets supplemented with astaxanthin, lutein and β-carotene at 200 μg/fish. After administration of a single dose orally, blood was collected at 15, 30 min 1, 3, 6, 12, 24, 48, 72, 96 and 120 hr. Carotenoids distributed to and metabolized in serum were analyzed and carotenoids derivative are shown in **Figure 22**.

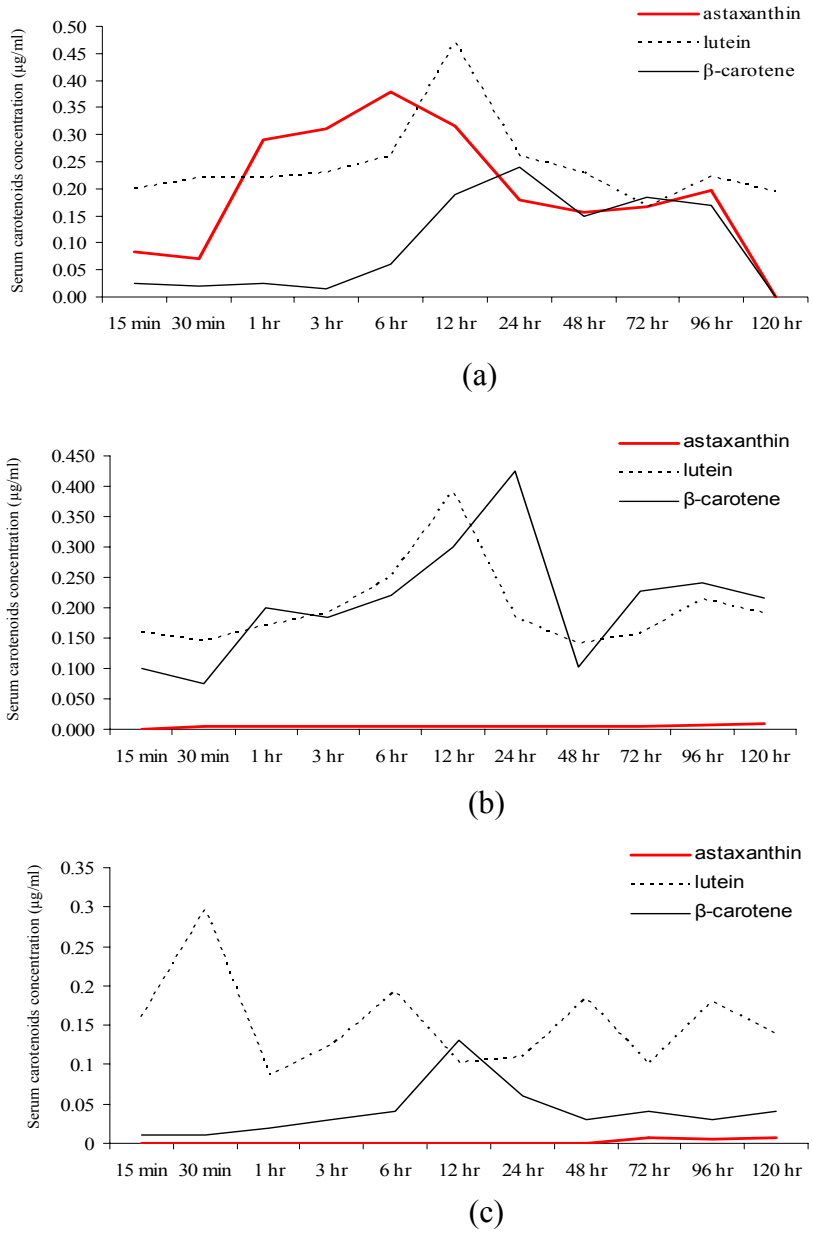


Figure 22 Metabolized serum carotenoids of fancy carp after administration a single dose of astaxanthin (a), lutein (b) and β-carotene diet (c).

In this present study, the data showed that the serum carotenoids of fancy carp after fed with an astaxanthin diet composed of astaxanthin, lutein and β-carotene. Astaxanthin concentration decreased while the time pass, meanwhile lutein and β-carotene concentration was increased in serum. The fancy carp were fed with lutein and β-carotene diets, found serum astaxanthin in 24 and 72 hr after oral administration feeding. Serum astaxanthin concentrations were 0.005 ± 0.02 and

0.007 ± 0.03 μg/ml serum, respectively. Their groups had serum astaxanthin concentration continuing increased serum astaxanthin concentration which can possibly be assumed that lutein and β-carotene were precursor and converted to astaxanthin and made the level of astaxanthin concentration higher in the blood circulation.

Aquatic animal carotenoids have been reviewed by Goodwin (1984); Matsuno and Hiro (1989); Liaaen-Jensen (1991) and Matsuno (2001). Commonly, Carotenoids composition in many fishes are β-carotene, β-cryptoxanthin, tunaxanthins, luteins, zeaxanthins, diatoxanthin, alloxanthin, β-echinenone, canthaxanthin, α-doradexanthin, β-doradexanthin and astaxanthins.

Carotenoid metabolism in animals takes place as a result of enzymes which catalyse three main types of reaction. These main reaction types are (i) the substitution of carotenoid end groups (often β-end groups) by oxygen functions (-OH and -C=O), (ii) the alteration of end groups, e.g. of β to α and (iii) cleavage of the polyene chain to yield apocarotenoids and even the vitamins A (Davies, 1985).

In this study, after fish fed with astaxanthin diet, serum astaxanthin level was decreased because of metabolic conversions. While the time lutein and β-carotene concentration was increased in serum. The results indicated that lutein and β-carotene were carotenoids derivative from reductive metabolite of astaxanthin (**Figure 23**), Schiedt *et al*. (1985) reported that rainbow trout, based on a minute recovery of radioactivity in lutein after feeding of labeled (3S, 3'S)-astaxanthin. An analogous reductive pathway might also be occurring with gilthead seabeam (Gomes *et al*., 2002). Lutein and zeaxanthin are metabolized prior to β-carotene, suggesting the precedence of xanthophylls biochemical conversions (Berticat *et al*., 2000).

By contrast, after fancy carp were fed with lutein and β-carotene diets, found serum astaxanthin in 24 and 72 hr after oral administration feeding. This result suggested that lutein and β-carotene were oxidized either to astaxanthin (**Figure 24**).

The data shows that carotenoids derivative in serum after being fed with lutein, fancy carp can convert lutein to astaxanthin. The results of this present study were similar to other researches, the report of Hirao *et al*. (1963) shows gold fish fed diet rich in lutein and β -carotene efficiently converted it to astaxanthin and the report of Hata and Hata (1972) indicated that gold fish fed with diet containing lutein developed changed the skin color from yellow to orange in 7 days and after 30 days of feeding the skin color progressed to reddish-orange. Katayama *et al.* (1973) and Bauernfeind (1981) reported that gold fish and fancy carp can convert lutein to astaxanthin. In Cypriniformes fish, possible oxidative metabolic pathways from zeaxanthin to astaxanthin via β -carotene triols, and tetrols have been proposed (Tsushima *et al*., 1999). Gold fish fed with lutein and zeaxanthin diets proposed the possible metabolic pathways from lutein and zeaxanthin to astaxanthin (Ohkubo *et al*., 1999) indicated that lutein is converted into β-doradexanthin and leading to the formation of astaxanthin by oxidative pathway (Tanaka *et al*., 1976).

In many animals the most important metabolic products of carotenoids are the retinoids and the metabolic reactions of carotenoids in fish are essentially oxidative pathway. However, all carotenoids compostion in fish serum of this study were decreased while the time passed, fancy carp might to maintain carotenoids level especially xanthophylls. The result indicated that fancy carp might reductive transform these carotenoids into vitamin A (**Figure 23**), thereby decreasing serum carotenoids deposition in blood circulation.

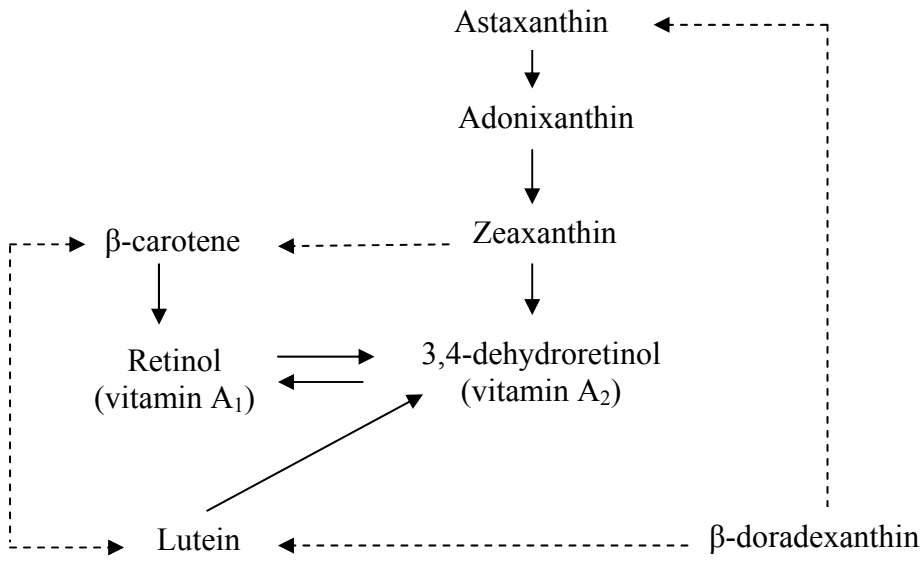


Figure 23 Reductive metabolic pathway of carotenoids as retinoid precursors in fancy carp.

Source: Matsuno (1991)

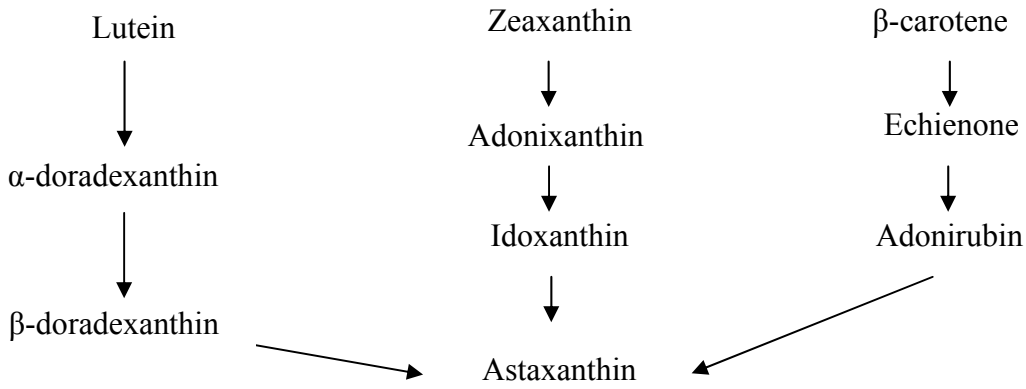


Figure 24 Oxidative metabolic pathway of carotenoids in fancy carp.

Source: Bauernfeind (1981); Hata and Hata (1972a and 1972b) and Matsuno (1991)

All these investigations have been carried out with freshwater fish. This feeding trials were conducted to demonstrate the possibility of the bioconversion of some xanthophylls such as astaxanthin, zeaxanthin, canthaxanthin, lutein and tunaxanthin into 3, 4-dehydroretinol being first transformed into retinol in retinoid-depleted freshwater fishes, marine fish and mammals. Recently, the bioconversion of

astaxanthin into 3, 4-dehydroretinol (vitamin A_2) in mature rainbow trout has been reported by Guillou *et al*. (1989). In the liver xanthophylls such as astaxanthin, zeaxanthin and lutein were directly reductive metabolic pathways and the bioconversion into 3-dehydroretinol (Yamashida *et al*., 1996). Labeled $retinol_1$ and $retinol_2$ were detected only in the liver and ^{3}H-zeaxanthin was largely the predominant precursor of these two vitamin A forms (Guillou *et al*., 1996). Xanthophylls such as canthaxanthin, astxanthin, zeaxanthin, lutein and tunaxanthin that are widely distributed in nature have been found to be converted into retinoid not only in fish but also in mammals like the rat by reductive pathway (Tsushima, 2007). Clearly, the metabolic source of A_2 (3, 4-dehydroretinol) is a xanthophylls containing the 3-hydroxy-β end group (Davies, 1991).

These results suggested that fancy carp were maintained carotenoids level in serum for converted carotenoids to reinoids by reductive pathway because vitamin A is essential for a number of physiological processes, such as regulation of cell differentiation and proliferation; reproductive, stimulated growth and immune response. Vitamin A and its analogs, both in vivo and in vitro, markedly influence the way in which cells differentiate (Olsen, 1994). The retinoid receptors can show both activation and suppression of gene expression, depending on the nature of the heterodimers formed. Genes are activated by retinoids as a result of the binding of an appropriate homo- or heterodimer to a hormone response element in DNA, whereas gene expression seems to be suppressed by the competition between a retinoid receptor and some other transcription factor for the latter's activating partner protein (Mangelsdorf *et al.,* 1994).

The earliest assays for vitamin A were based on the growth response of rats fed a purified diet. The fact that maintenance of normal vision and enhancement of growth are two separate properties of the vitamin A molecule was dramatically demonstrated by the observation that retinoic acid stimulated growth but could not maintain vision (Dowling and Wald, 1960). In vitamin A deficiency, both specific and nonspecific protective mechanisms are impaired: namely, the humoral response to bacterial, parasitic, and viral infections; cell-mediated immunity; mucosal immunity;

natural killer cell activity; and phagocytosis (Olsen, 1994). Thus, the supplementation of vitamin A, will make immune responses generally improve.

Nowadays, natural sources (yeast, bacteria, algae, higher plants and crustacean meal) have been used as dietary supplements to enhance the pigmentation of fish and crustacean (Kalinowski *el al.,* 2005). With the raw materials of plant origin, several carotenoids have found their way into fish diets. Such examples are the lutein and β-carotene which are found in large amount in plants (Zaripheh and Erdman, 2002). Therefore production of carotenoids from natural source especially lutein and β-carotene are a potential alternative source of carotenoids to replace the synthetic astaxanthin.

Part 2.2 Comparative Pharmacokinetic of Carotenoids from Natural Sources (Tea, Mulberry and Cassava Leaves) in Fancy carp (*Cyprinus carpio*)

1. Pharmacokinetic parameters of fancy carp

In this study, total carotenoids contents of tested diets that used raw material of tea, mulberry and cassava leaves as the diet were 24.64 ± 1.27, 23.92 ± 0.12 and 24.65 ± 0.20 mg/kg, respectively. The main carotenoids composition in tea, mulberry and cassava diet showed main carotenoids compositions two pigments; lutein provided 15.59 ± 0.03, 12.29 ± 0.03 and 13.25 ± 0.02 mg/kg and β-carotene had 6.45 ± 0.04, 3.42 ± 0.24 and 5.42 ± 0.03 mg/kg, respectively (**Table 13**).

Table 13 Carotenoids concentration in experimental diets.

Experimental diet	Total carotenoid (μg)	Carotenoids composition (μg)		
		Astaxanthin	Lutein	β-carotene
Tea leaves	24.64 ± 1.27	ND	15.59 ± 0.03	6.45 ± 0.04
Mulberry leaves	23.92 ± 0.12	ND	12.29 ± 0.03	3.42 ± 0.24
Cassava leaves	24.65 ± 0.20	ND	13.25 ± 0.02	5.42 ± 0.03

ND = not detected

The observed time curves of serum total carotenoid in fancy carp after a single dose of experimental diets were best described by one compartment model with first order absorption, pharmacokinetic parameters displayed in **Table 14**.

Table 14 Pharmacokinetic parameters of total carotenoid in fancy carp after single dose administration for tea, mulberry and cassava leaves diets.

Parameters	Unit	Deprived diets		
		Tea leaves	Mulberry leaves	Cassava leaves
Vd (area)/kg	ml/kg	23.40	22.28	23.39
CL (area)/kg	ml/hr/kg	0.24	0.30	0.41
AUC (area)	μg-hr/ml	108.23	79.74	60.58
C max (obs)	μg/ml	0.0022	0.0016	0.0018
T max (obs)	hr	48	72	48

Note: Vd (area)/kg: volume of distribution calculations

CL (area)/kg: total body 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

After a single dose feeding, the results showed that serum total carotenoid of fish fed with tea, mulberry and cassava diet showed a steady rise after feeding. Fancy carp fed with a tea leaves diet developed a maximum concentration of total carotenoids (C max) 0.0022 µg/ml occurred at 48 hr (T max) while fancy carp fed with a mulberry and cassava leaves diets contained serum total carotenoid (C max) 0.0016 and 0.0018 µg/ml within 72 and 48 hr, respectively. The volume of distribution (Vd) was 23.40, 22.28 and 23.39 ml/kg, respectively. The data illustrated that total carotenoid of fish fed with tea leaves diet was distributed in the blood circulation system better than fish fed with mulberry and cassava leaves diet. Because the high amount of Vd demonstrates the fact that it can disseminate well in blood circulation. However, the area under the curve (AUC) was 108.23, 79.74 and 60.58 µg-hr/ml. AUC, indicated the relationship between carotenoid concentration in serum and time. It is close to the amount of carotenoids being absorbed in the blood circulation system. If the area under the curve (AUC) is elevated, it means that carotenoids can be highly absorbed. Utilization, expressed as an area under the serum concentration time curve, was highest for fish fed with a tea leaves diet. Total body clearance (CL) was 0.24, 0.30 and 0.41 ml/hr/kg, indicated that tendency of all groups were clearance of total carotenoid in blood circulation system similarity, this clearance was mainly to eradicate carotenoids in anyway. These results suggested that, total carotenoids were slightly decreased in serum for converted to other carotenoids by metabolic pathway or excretion. Overall, after fancy carp were fed a tea leaves diet and considered together with pharmacokinetic parameter which was Vd and AUC, it proved better dispersed of total carotenoid in blood circulation than fancy carp fed with mulberry and cassava leaves diet.

The results of the present study indicated that utilization of tea, mulberry and cassava leaves diet in fancy carp after single dose feeding oral administration showed that the faster rate of total carotenoid absorption for the fish fed with a tea leaves diet compared to other groups. Because lutein content in tea leaves was higher than mulberry and cassava leaves, it made well distributed in the blood circulation system. The reason of lutein was well absorption because of lutein was polar carotenoids. The absorption of carotenoids upon disruption of food matrix. These solubility and

location of the polar carotenoids (xanthophylls) and the non polar carotenoids (carotene) in emulsions are different. Carotenes are thought to incorporate almost exclusively in the triacylglycerol core of the emulsion, whereas the more polar xanthophylls distribute preferentially at the emulsion surface (Furr and Clark, 1997). It has been shown by several authors, including Yonekura and Nagao (2007) that polar carotenoids (xanthophylls) have higher bioavailability characteristics leading to more transfer across the intestine compared to carotenes.

1. Metabolized serum carotenoids

Fish were fed with diets using tea, mulberry and cassava leaves 100 percent contained total carotenoid at 25 μg. After administration of a single dose orally, blood was collected at 15, 30 min 1, 6, 12, 24, 48, 72, 96 and 120 hr. Carotenoids distributed to and metabolized in serum were analyzed and carotenoids derivative are shown in **Figure 25**.

In this present study, the data shows that after fish fed with a tea, mulberry and cassava leaves, the serum carotenoids of fancy carp while the time passes composed of astaxanthin, lutein and β-carotene. The fancy carp were found serum astaxanthin in early time at 48 hr after oral administration feeding similarly with all fish groups. Serum astaxanthin concentration was 0.0004 ± 0.0001, 0.0004 ± 0.0001 and 0.0005 ± 0.0002 μg/ml serum of fish fed with tea, mulberry and cassava leaves, respectively. Serum astaxanthin level in all group were slightly decreased and maintained in blood circulation throughout the experiment. The data assumed that lutein and β-carotene were precursor and converted to astaxanthin and made the level of astaxanthin concentration higher in the blood circulation. This result suggested that lutein and β-carotene content that composed in plant leaves, fish were oxidized either to astaxanthin. The data shows that carotenoids derivative in serum after being fed with lutein, fancy carp can convert lutein to astaxanthin. The results of this present study are similar to other researches, the report of Hirao *et al*. (1963) and Hsu *et al*. (1972) reported that goldfish fed diets rich in lutein and β-carotene efficiently led to higher levels of astaxanthin due to a conversion and deposition of these substances to

astaxanthin and the report of Hata and Hata (1972a and 1972b) indicated that gold fish fed with diet containing lutein the skin color changed from yellow to orange in 7 days and after 30 days of feeding the skin color progressed to reddish – orange. katayama *et al.* (1973) and Bauernfeind (1981) reported that gold fish and fancy carp can convert lutein to astaxanthin. Gold fish fed with lutein and zeaxanthin diets proposed the possible metabolic pathways from lutein and zeaxanthin to astaxanthin (Ohkubo *et al*., 1999) indicated that lutein is converted into β-doradexanthin and leading to the formation of astaxanthin by oxidative pathway (Tanaka *et al*., 1976).

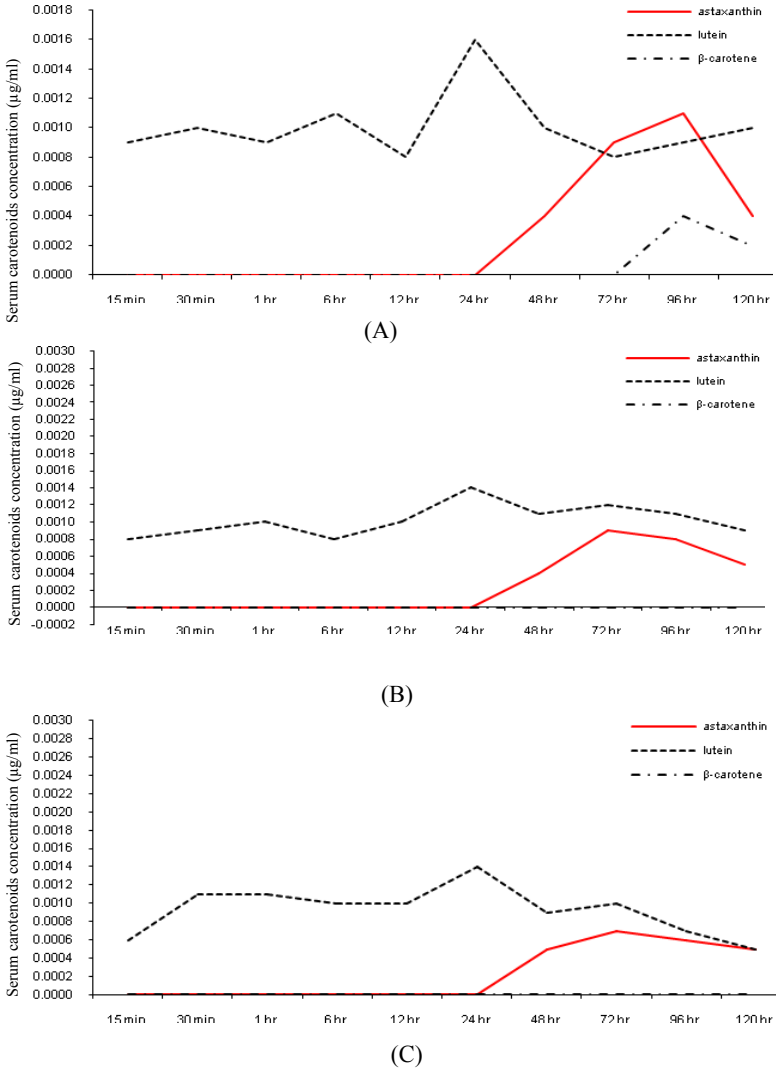


Figure 25 Serum carotenoids composition after administration a single dose of tea leaves (A), mulberry leaves (B) and cassava leaves diet (C).

The production of carotenoids from natural sources is a potential alternative to replacing the synthetic carotenoids. With the introduction of raw materials of plant origin, several carotenoids have found their way into fish diets such example as the xanthophylls-- lutein and hydrocarbon carotene-- β-carotene which found in large amount in plants. The current results indicate that fancy carp were able to utilize lutein and β-carotene from plant leaves efficiently. Similar results were obtained for goldfish but by feeding different natural carotenoid sources, such as red yeast, X. *dendrorhous* (Xu *et al*., 2006), Spirulina (Kiriratnikom *et al*., 2005), C. *vulgaris*, H. *pluvialis* and A. *maxima* (Gouveia *et al*., 2003; Gouveia and Rema, 2005), alfala (Yanar *et al*., 2008). Yanar *et al*. (2008) showed that a natural carotenoid source was as effective as a synthetic carotenoid source, on skin pigmentation of goldfish. Natural carotenoids such as *Chlorella vulgaris* had already proved to be a useful, even competitive coloring for inclusion in the diets of laying hens with the purpose of coloring egg yolk (Gouveia *et al*., 1996); in rainbow trout yielding both muscle and skin pigmentation effect (Gouveia *et al*., 1997 and 1998); in gilthead seabream for skin pigmentation (Gouveia *et al*., 2002) and improved skin coloring in goldfish and fancy carp (Gouveia *et al*., 2003). Hence, higher plant is suggested to be an effective alternative natural carotenoid source to synthetic carotenoid for fancy carp pigmentation. Thailand is an agricultural country. Generally, there are a lot of crop residues from the process. Furthermore, these residues become wastes and have caused pollution. In addition, carotenoids in plant include oxygenated xanthophylls--lutein and hydrocarbon carotene--β-carotene. So, raw material of plant leaves is the promising way to solve this problem. This study makes use of local Thai feedstuffs to be the carotenoid sources for color enhancing diet particularly in fancy carp' diets. This becomes a method in lowering the production cost of diets and in adding value to raw material and byproducts from agricultural harvest.

Part 3 The Impact of Dietary carotenoid (Ataxanthin, Lutein and β-carotene) on Skin Pigmentation of Fancy carp (*Cyprinus carpio*)

1. Serum total carotenoids (serum TC)

The effect of different concentration from carotenoid source (astaxanthin, lutein and β-carotene including lutein plus β-carotene) on serum total carotenoids was determined. Serum total carotenoids in fish fed lutein plus β-carotene at 50:50 mg/kg diet was higher compared to the other treatments ($p \leq 0.05$). This was followed by TC of fish fed with 50 mg/kg lutein diet and 25:25 mg/kg lutein plus β-carotene diet. Values by TC were 7.77 ± 3.27, 7.26 ± 1.92 and 6.24 ± 2.05 µl/ml serum, respectively (as shown in **Table 15** and **Figure 26)**. In fish fed the control diet showed similar a TC values ranging from 0.6-0.7 µl/ml serum throughout the experiment. After discontinuing supplementation of the experimental diet with carotenoid, fish were switched to a non supplemented carotenoid diet of total carotenoid at 4.5 ± 0.5 mg/kg for 7 days. Serum TC concentrations all treatments declined during the fourth week, except control group after discontinuation of the treatment feeding. However, this data showed that serum TC concentration were strongest for the fish fed with 50:50, 25:25 mg/kg lutein plus β-carotene and 50 mg/kg lutein diet groups. The results indicated that, fish fed lutein and combination of lutein plus β-carotene could maintain serum TC level better than the other group.

Table 15 Total serum carotenoids (µg/ml serum) of fancy carp fed with experimental diets over 4 weeks.

Experimental diets	Time			
	Week 1	Week 2	Week 3	Week 4*
1	0.67 ± 0.11^{c}	0.62 ± 0.14^{c}	0.63 ± 0.07^{d}	0.66 ± 0.06^{c}
2	1.48 ± 0.25^{c}	2.85 ± 0.90^{ab}	3.07 ± 0.37^{bcd}	1.60 ± 0.50^{bc}
3	1.56 ± 0.30^{bc}	2.11 ± 0.15^{abc}	3.86 ± 3.89^{abcd}	1.67 ± 0.73^{bc}
4	2.91 ± 0.56^{a}	3.50 ± 0.68^{a}	7.26 ± 1.92^{ab}	3.48 ± 0.52^{a}
5	1.41 ± 0.15^{c}	1.65 ± 0.71^{bc}	2.77 ± 1.85^{cd}	0.89 ± 0.27^{bc}
6	1.06 ± 0.65^{c}	1.64 ± 0.75^{bc}	2.04 ± 1.15^{cd}	0.75 ± 0.11^{bc}
7	2.59 ± 0.33^{ab}	3.05 ± 0.43^{ab}	3.39 ± 0.19^{bcd}	2.04 ± 1.46^{b}
8	3.19 ± 1.03^{a}	3.38 ± 1.27^{a}	6.24 ± 2.05^{abc}	1.32 ± 0.51^{bc}
9	3.52 ± 0.61^{a}	3.62 ± 1.14^{a}	7.77 ± 3.58^{a}	3.97 ± 1.43^{a}
p-value	0.0001	0.0038	0.0142	0.0001

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Note: * discontinued dietary supplementation with carotenoids

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

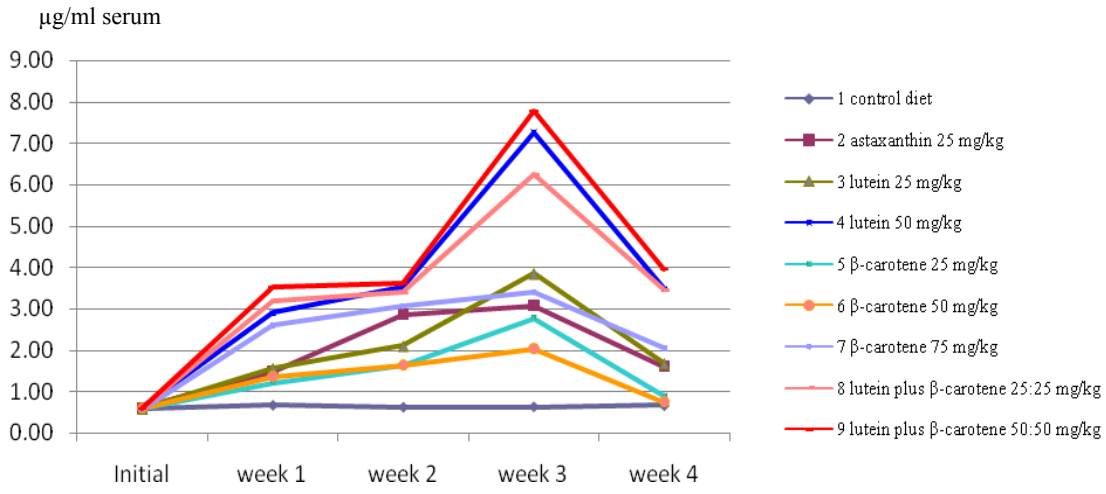


Figure 26 Total carotenoids concentration in serum of fancy carp fed with experimental diets for 4 weeks.

Feed intakes of all treatments (**Table 16**) showed no significant differences ($p \geq 0.05$) but intakes based on carotenoid ingestion did show significant differences ($p \leq 0.05$) among treatments. Serum TC of fish fed with carotenoid-enriched diets for 3 weeks showed increased intakes dependent on dietary supplementation level.

Table 16 Feed Intake (g/fish/day) and total carotenoids intake (μg/fish/day) consumed by fancy carp for 4 week.

Experimental diets	Time			
	Week1	Week2	Week3	Week4*
1	1.39 ± 0.04	0.99 ± 0.05	1.06 ± 0.05	1.06 ± 0.05
2	1.48 ± 0.07	1.10 ± 0.05	1.25 ± 0.00	1.13 ± 0.09
3	1.38 ± 0.14	1.06 ± 0.05	1.39 ± 0.33	1.11 ± 0.12
4	1.39 ± 0.08	1.00 ± 0.09	1.20 ± 0.05	1.07 ± 0.03
5	1.47 ± 0.11	1.04 ± 0.07	1.22 ± 0.02	1.08 ± 0.07
6	1.52 ± 0.07	1.15 ± 0.08	1.22 ± 0.04	1.05 ± 0.09
7	1.51 ± 0.03	1.10 ± 0.05	1.25 ± 0.43	1.01 ± 0.19
8	1.53 ± 0.10	1.08 ± 0.05	1.28 ± 0.25	1.08 ± 0.18
9	1.48 ± 0.12	1.03 ± 0.06	1.16 ± 0.12	1.07 ± 0.06
P-value	0.1602	0.0749	0.6571	0.8958

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Note: * discontinued dietary supplementation with carotenoids

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

Table 16 Continued.

Experimental diets	Time			
	Week1	Week2	Week3	Week4*
Total carotenoids intake (μg/fish/day)				
1	6.18 ± 0.19^{g}	4.40 ± 0.21^{g}	4.73 ± 0.22^{g}	4.73 ± 0.22
2	43.59 ± 2.06^{e}	32.52 ± 1.45^{e}	36.89 ± 0.00^{ef}	5.04 ± 0.40
3	38.25 ±4.03^{ef}	29.35 ± 1.52^{e}	38.42 ± 9.09^{ef}	4.95 ± 0.51
4	73.40 ± 4.39^{d}	52.98 ± 4.88^{d}	63.36 ± 2.41^{cd}	4.78 ± 0.26
5	33.91 ± 2.47^{f}	23.97 ± 1.52^{f}	28.12 ± 0.56^{f}	4.81 ± 0.31
6	70.38 ± 2.59^{d}	49.45 ± 7.39^{d}	51.27 ± 10.28^{de}	4.66 ± 0.37
7	117.53 ± 5.69^{b}	81.21 ± 9.78^{b}	97.42 ± 3.11^{b}	4.47 ± 0.86
8	87.94 ± 5.56^{c}	62.09 ± 5.51^{c}	73.70 ± 4.58^{c}	4.79 ± 0.81
9	161.81 ± 2.81^{a}	112.58 ± 6.42^{a}	127.17 ± 2.93^{a}	4.74 ± 0.28
P-value	0.0001	0.0001	0.0001	0.8923

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Note: * discontinued dietary supplementation with carotenoids

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

2. Serum carotenoids components

Astaxanthin, lutein and β-carotene were indentified and quantified in the blood serum samples by TLC-densitometric analysis as shown in **Table 17**. Briefly, serum astaxanthin in all samples was not detected after a week feeding period with experimental diets. By the second week, astaxanthin was found at 0.5 ± 0.2 μg/ml in serum only in the fish fed with astaxanthin diet at 25 mg/kg. This group showed detectable serum astaxanthin levels until the end of the experiment. At the final third week sampling, serum astaxanthin in fish fed with astaxanthin diet was 1.66 ± 0.18 μg/ml serum. This was seen at the astaxanthin inclusion level ($p \leq 0.05$). In fish fed

the experimental diet supplemented with the combination of lutein and β-carotene at 50:50 and 25:25 mg/kg as well as lutein 50 mg/kg diet there was astaxanthin detectable at 0.9 ± 0.01, 0.7 ± 0.01 and 0.4 ± 0.02 µg/ml serum, respectively (**Figure 27**).

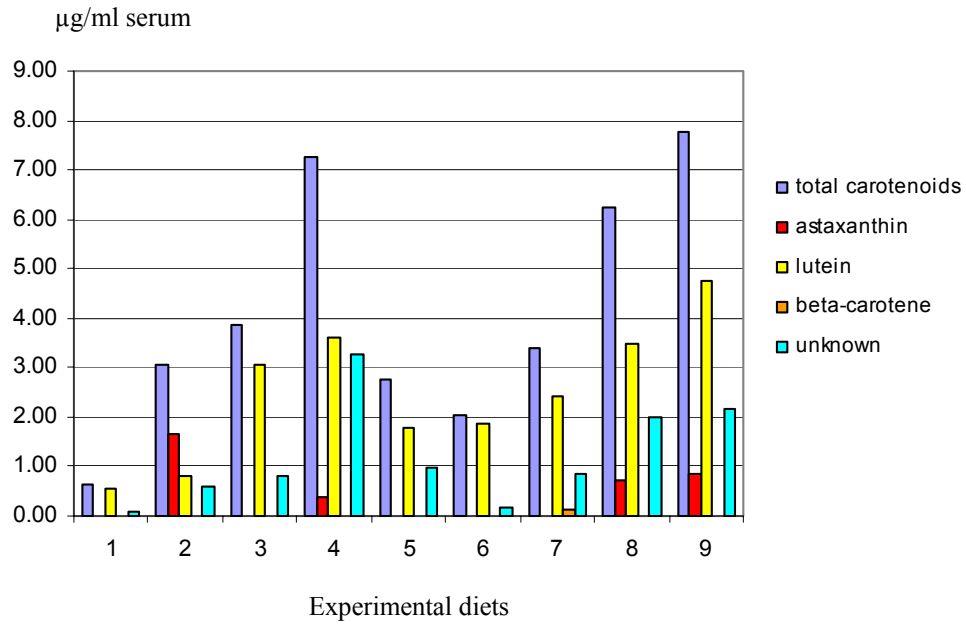


Figure 27 Serum carotenoid components of fancy carp after fed experimental diets over 3 weeks.

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

Serum lutein level was highest and increased following the dietary lutein content. Upon termination of the trial, the same three groups had lutein concentrations of 4.8 ± 0.9, 3.5 ± 0.8 and 3.6 ± 0.20 µg/ml serum. Serum β-carotene levels in serum samples was only found in fish fed with β-carotene 75 mg/kg diet. The analysed value was determined at 0.1 ± 0.01 µg/ml serum. The percentage of carotenoid composition of serum total carotenoids is illustrated in **Figure 28**. The data shows the main carotenoids composition in serum were lutein provided a retention factor (R_f)

which averaged of 0.20, and β-carotene had R_f average at 0.97 (**Figure 29**). Small amounts of astaxanthin that could be identified with R_f average at 0.20.

Table 17 Serum carotenoid components (µg/ml serum) of fancy carp fed with experimental diets over 4 weeks.

Experimental diets	Time			
	Week1	Week2	Week3	Week4*
Astaxanthin				
1	ND	ND ^{b}	ND ^{e}	ND ^{c}
2	ND	0.46 ± 0.18 ^{a}	1.66 ± 0.18 ^{a}	0.84 ± 0.40 ^{a}
3	ND	ND ^{b}	ND ^{e}	ND ^{c}
4	ND	ND ^{b}	0.38 ± 0.02 ^{d}	0.38 ± 0.07 ^{b}
5	ND	ND ^{b}	ND ^{e}	ND ^{c}
6	ND	ND ^{b}	ND ^{e}	ND ^{c}
7	ND	ND ^{b}	ND ^{e}	ND ^{c}
8	ND	ND ^{b}	0.74 ± 0.01 ^{c}	0.26 ± 0.01 ^{b}
9	ND	ND ^{b}	0.86 ± 0.01 ^{b}	0.49 ± 0.01 ^{b}
P-value	-	0.0001	0.0001	0.0001

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Note: * means fish was discontinue enhancement carotenoids diets and ND means Not detected

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

Table 17 Continued.

Experimental diets	Time			
	Week1	Week2	Week3	Week4*
Lutein				
1	0.56 ± 0.03 [f]	0.52 ± 0.08 [d]	0.56 ± 0.10 [e]	0.56 ± 0.05
2	1.15 ± 0.14 [de]	1.45 ± 0.30 [bc]	0.81 ± 0.03 [de]	0.59 ± 0.19
3	1.18 ± 0.07 [de]	1.29 ± 0.16 [cd]	3.04 ± 0.30 [b]	1.49 ± 0.04
4	2.03 ± 0.21 [b]	2.19 ± 0.20 [ab]	3.60 ± 0.15 [b]	2.26 ± 0.20
5	1.00 ± 0.05 [e]	0.94 ± 0.05 [cd]	1.80 ± 0.61 [cd]	0.67 ± 0.29
6	1.28 ± 0.09 [d]	0.77 ± 0.09 [cd]	1.85 ± 0.53 [cde]	0.58 ± 0.28
7	1.84 ± 0.12 [c]	2.29 ± 0.28 [ab]	2.40 ± 0.21 [bc]	1.04 ± 0.50
8	2.56 ± 0.12 [a]	2.63 ± 0.93 [a]	3.49 ± 0.79 [b]	2.20 ± 0.64
9	2.55 ± 0.12 [a]	3.00 ± 0.84 [a]	4.76 ± 0.92 [a]	2.91 ± 0.22
P-value	0.0001	0.0001	0.0001	0.1978
β-carotene				
1	ND	ND	ND [b]	ND [c]
2	ND	ND	ND [b]	ND [c]
3	ND	ND	ND [b]	ND [c]
4	ND	ND	ND [b]	ND [c]
5	ND	ND	ND [b]	ND [c]
6	ND	ND	ND [b]	ND [c]
7	ND	ND	0.13 ± 0.01 [a]	0.13 ± 0.02 [b]
8	ND	ND	ND [b]	0.18 ± 0.02 [ab]
9	ND	ND	ND [b]	0.21 ± 0.12 [a]
P-value	-	-	0.0001	0.0001

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Note: * means fish was discontinue enhancement carotenoids diets and ND means Not detected

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

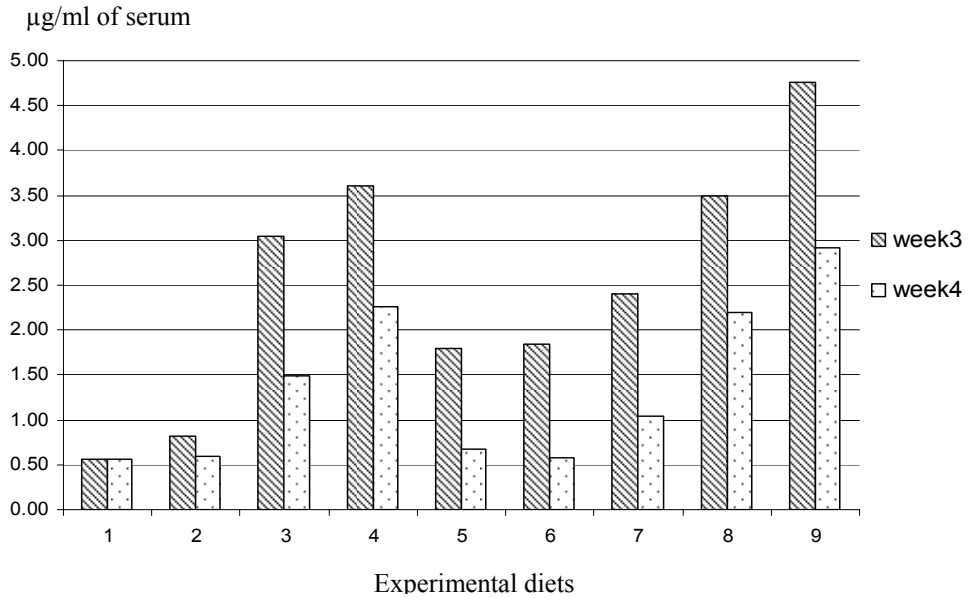


Figure 28 Serum lutein concentration of fancy carp fed with different carotenoids diets for 3 weeks, followed by feeding with the low carotenoid diet over one week (week 4).

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

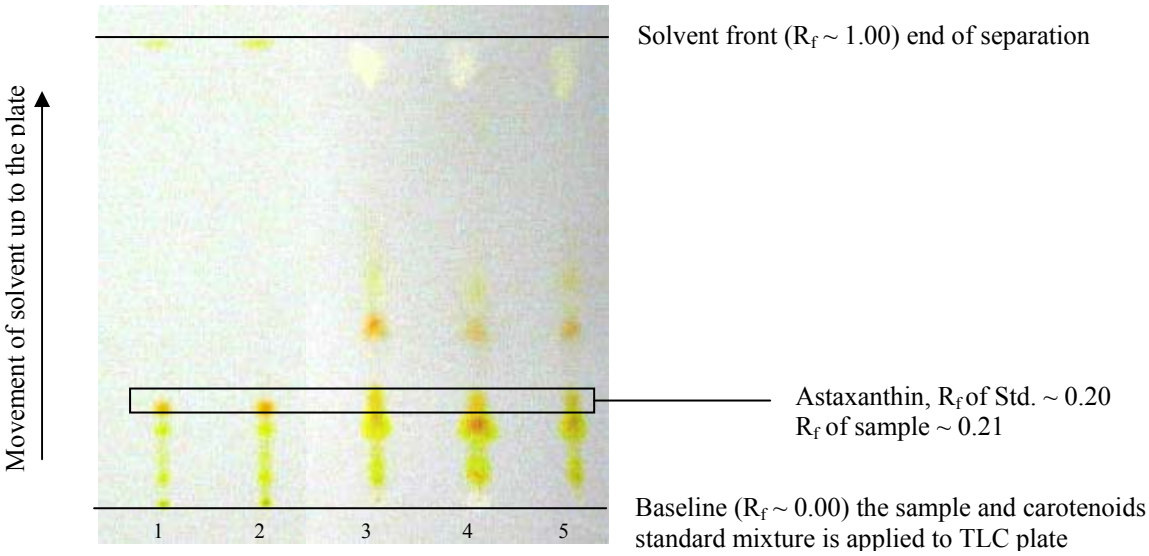


Figure 29 Silica gel TLC on which carotenoids standard mixture and serum extraction of fancy carp fed with lutein plus β-carotene by 50:50 mg/kg diet at the end of experiment were separated, lane 1-2 is carotenoids standard mixture and lane 3-5 is samples; astaxanthin was found at third week.

After three weeks of feeding the experimental diets, fish were fed diet without carotenoid supplemented (4.5 ± 0.5 mg/kg) for one week. The results showed that fish fed with dietary astaxanthin had serum astaxanthin concentration higher than other treatments ($p \leq 0.05$), followed by the experimental group fed the diet with lutein and β-carotene at 50:50, 25:25 mg/kg as well as lutein 50 mg/kg diet. The astaxanthin levels in these diets were 0.8 ± 0.40, 0.5 ± 0.01, 0.3 ± 0.01 and 0.4 ± 0.07 µg/ml serum, respectively. The serum lutein content was similar to the control group in all treatments ($p \geq 0.05$). At the termination of the trial, β-carotene level in serum was found in a group fed the diets containing β-carotene at 75 mg/kg and the diets with 50:50 and 25:25 mg/kg lutein plus β-carotene at 0.1 ± 0.02, 0.2 ± 0.02 and 0.2 ± 0.1 µg/ml serum, respectively.

3. Effect of test diets on skin coloration

Skin color analysis was performed by reflective spectroscopy, with colorimeter (Minolta color reader, CR-10) in accordance with the system CIE L*a*b* for lightness, red and yellow, respectively including hue values. The measurements were performed on red and white positions on the fish bodies (left side). Initial fish were maintained on a non-pigmented diet for two weeks prior to feeding the experimental diets, L, a*, b* and hue values in each experimental groups showed no significant differences ($p \geq 0.05$).

3.1 Red position

Color intensity of fancy carp fed with experimental diets is shown in **Table 18**. Following feeding of experimental diets supplemented with carotenoid over three weeks, the results found that the L values in groups fed diets containing β-carotene at 25, 50 and 75 mg/kg were higher compared to control ($p \leq 0.05$). The higher L value indicated that skin coloration in this position was lightness. In terms of redness (a* value), the highest numerical values were obtained for the groups fed with diets which contained astaxanthin 25, 50:50 and 25:25 mg/kg of lutein plus β-carotene and lutein 50 mg/kg. These groups showed a significant effect on redness skin over that the other treatments ($p \leq 0.05$). The a* values were 28.5 ± 2.5 29.9 ± 1.4, 28.3 ± 0.5 and 28.8 ± 3.9, respectively. Yellowness (b* value) of fancy carp skin for lutein and β-carotene diets were significantly stronger ($p \leq 0.05$) compared to fish fed either with astaxanthin and control diets. The hue values between the different dietary treatments were not significantly different ($p \geq 0.05$), hue values ranged from 0.91-1.00.

Table 18 Body color intensity of fancy carp fed experimental diets over 3 weeks.

Experimental diets	Color parameters			
	L	a	b	Hue
Red color				
Red color prior treatments (data shown on the range)	L = 55.42 – 60.68	a = 17.66 – 24.65	b = 37.27 – 44.95	Hue = 0.91-1.28
1	48.35 ± 3.62^{a}	26.50 ± 1.13^{ab}	35.70 ± 3.39^{c}	0.93 ± 0.04
2	43.30 ± 0.85^{c}	28.54 ± 2.49^{a}	37.30 ± 1.31^{bc}	0.91 ± 0.05
3	45.75 ± 0.91^{abc}	26.92 ± 2.08^{ab}	41.43 ± 1.37^{a}	0.99 ± 0.06
4	46.63 ± 2.74^{ab}	28.83 ± 3.95^{a}	40.13 ± 0.96^{ab}	0.95 ± 0.07
5	46.70 ± 0.86^{a}	26.78 ± 3.72^{ab}	40.15 ± 1.14^{ab}	0.93 ± 0.05
6	46.90 ± 0.80^{a}	26.80 ± 4.12^{b}	40.40 ± 2.22^{ab}	0.98 ± 0.06
7	46.90 ± 1.62^{a}	25.83 ± 3.20^{b}	39.78 ± 1.24^{ab}	1.00 ± 0.07
8	43.55 ± 2.12^{ab}	28.33 ± 0.53^{a}	38.13 ± 2.15^{abc}	0.93 ± 0.03
9	46.30 ± 2.10^{abc}	29.88 ± 1.38^{a}	39.30 ± 3.05^{ab}	0.94 ± 0.04
P-value	0.0228	0.0413	0.0143	0.2240
White color				
White color prior treatments (data shown on the range)	L = 81.85 – 84.60	a = 0.58 – 1.68	b = 6.18 – 9.53	Hue = 1.20-1.56
1	85.75 ± 2.07	1.40 ± 0.29	8.43 ± 1.14^{bc}	1.40 ± 0.05
2	86.30 ± 0.54	2.10 ± 0.83	7.28 ± 0.92^{c}	1.29 ± 0.12
3	84.70 ± 1.54	1.88 ± 0.69	9.95 ± 1.36^{ab}	1.39 ± 0.04
4	86.48 ± 2.08	1.98 ± 0.54	9.12 ± 0.78^{ab}	1.34 ± 0.05
5	4.15 ± 1.67	2.20 ± 0.64	8.13 ± 2.12^{bc}	1.29 ± 0.11
6	84.95 ± 1.95	1.28 ± 0.26	7.90 ± 0.73^{c}	1.41 ± 0.04
7	85.60 ± 0.93	2.18 ± 0.90	8.53 ± 1.38^{bc}	1.31 ± 0.14
8	83.70 ± 0.61	1.85 ± 0.37	10.05 ± 1.24^{ab}	1.38 ± 0.06
9	4.93 ± 0.46	1.45 ± 0.13	11.00 ± 1.25^{a}	1.44 ± 0.02
P-value	0.1079	0.2078	0.0020	0.0871

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

3.2 White position

The intensity of skin color at white position after fish were fed experimental diets, showed that all diets had no apparent influence on skin color intensity of L and a* value ($p \geq 0.05$). The yellowness (b* value) was stronger in a fish fed diet which combined lutein and β-carotene by 50:50 and 25:25 mg/kg including lutein at 25 and 50 mg/kg diet ($p \leq 0.05$). The b* values were 11.0 ± 1.3, 10.3 ± 1.2, 9.9 ± 1.4 and 9.1 ± 0.8, respectively. Meanwhile, hue values of fish skin were no significant differences between diets ($p \geq 0.05$), ranged from 1.29-1.44. The data indicated that white skin changed from white to yellow-white for all treatment groups.

4. Skin fancy carp coloration retention

After fancy carp were fed with carotenoid-supplemented diets for three weeks, the carotenoids were discontinued and fish were fed with the a diet without carotenoid supplemented of 4.5 ± 0.5 mg/kg for a week. The measurements were performed on red and white position on fish body (left side). The results are shown in **Table 19**. The redness (a* value) on red position of all treatment had no influence on skin color intensity ($p \geq 0.05$). The a* values during the final week of discontinued feeding of carotenoid-supplemented diets, fancy carp fed diet lutein plus β-carotene at 50:50 and 25:25 mg/kg as well as lutein 50 mg/kg diet presented retained the red intensity longer than group of astaxanthin. In contrast, the yellowness (b* value) on white position was similar for all experimental groups ($p \geq 0.05$). The data of the hue values showed that fish fed with astaxanthin 25 mg/kg, lutein 50 mg/kg and diet lutein plus β-carotene 25:25 mg/kg had hue value similarly with control group and their groups had hue values lower than the other groups ($P \leq 0.05$). The results indicated that the lower hue value means that fish skin become brightness after fed with diet without supplemented carotenoids for one week. The data found that, yellow-white on white skin areas turned to a whiter as serum lutein was decreased after discontinued feeding of carotenoid-supplemented diets.

Table 19 Body color intensity of fancy carp fed with the low carotenoid supplemented diets over final week (week 4).

Experimental diets	Color parameters			
	L	a	b	Hue
Red color				
1	48.50 ± 3.05^{a}	25.38 ± 1.38	34.18 ± 2.10^{c}	0.93 ± 0.05
2	45.13 ± 2.31^{bc}	26.25 ± 1.41	37.25 ± 0.65^{bc}	0.96 ± 0.03
3	45.88 ± 0.93^{abc}	26.73 ± 1.34	41.00 ± 1.38^{a}	0.99 ± 0.01
4	47.85 ± 2.74^{ab}	28.58 ± 4.10	38.60 ± 1.64^{ab}	0.98 ± 0.03
5	46.95 ± 0.99^{ab}	25.30 ± 1.69	35.48 ± 3.80^{bc}	0.92 ± 0.02
6	47.63 ± 0.38^{ab}	26.65 ± 2.31	41.08 ± 3.61^{a}	0.99 ± 0.02
7	46.28 ± 1.38^{abc}	24.80 ± 0.14	41.23 ± 0.51^{a}	1.00 ± 0.03
8	43.95 ± 1.62^{c}	27.23 ± 3.60	36.35 ± 2.28^{bc}	0.93 ± 0.07
9	45.05 ± 0.22^{bc}	28.40 ± 0.98	39.00 ± 1.21^{ab}	0.94 ± 0.03
P-value	0.0228	0.0908	0.0003	0.0902
White color				
1	86.30 ± 0.85	2.73 ± 0.82^{b}	6.05 ± 2.59	1.09 ± 0.23^{b}
2	87.60 ± 0.58	3.15 ± 0.30^{b}	6.53 ± 1.30	1.11 ± 0.09^{b}
3	85.10 ± 1.77	1.28 ± 0.72^{d}	9.15 ± 1.35	1.42 ± 0.10^{a}
4	85.85 ± 1.58	4.20 ± 0.66^{a}	8.35 ± 1.44	1.10 ± 0.11^{b}
5	86.48 ± 1.11	1.48 ± 0.45^{d}	6.60 ± 0.53	1.35 ± 0.08^{a}
6	86.58 ± 1.83	1.63 ± 0.67^{cd}	6.90 ± 0.41	1.34 ± 0.09^{a}
7	86.00 ± 1.28	1.70 ± 0.66^{cd}	8.25 ± 2.51	1.38 ± 0.10^{a}
8	84.60 ± 0.87	4.20 ± 0.80^{a}	8.73 ± 1.27	1.12 ± 0.07^{b}
9	84.60 ± 2.10	2.63 ± 0.64^{bc}	8.20 ± 1.61	1.26 ± 0.05^{ab}
P-value	0.0924	0.0001	0.0945	0.0005

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Remark: 1 = control diet 2 = astaxanthin 25 mg/kg 3 = lutein 25 mg/kg
4 = lutein 50 mg/kg 5 = β-carotene 25 mg/kg 6 = β-carotene 50 mg/kg
7 = lutein 75 mg/kg 8 = lutein plus β-carotene 25:25 mg/kg
9 = lutein plus β-carotene 50:50 mg/kg

This research ascertains that fancy carp fed with high-carotenoid diets resulted in a high serum total carotenoid (TC) concentration. Fancy carp fed diet combined with lutein and β-carotene by 50:50 mg/kg, which is considered the highest carotenoids victuals, possess highest amount of TC by 7.77 ± 3.28 µl/ml of serum. The result is in line with Amar *et al*. (2004)'s finding which states that rainbow trouts fed with carotenoids victuals (*Phaffia rhodozyma*) at 100 and 200 mg/kg acquire high carotenoids of 200 mg/kg in the serum. Carotenoids found in the serum could be used as an index to define carotenoids utility in vertebrates (Yeum, 2002). Once fishes consume carotenoids, metabolism and absorption are convey carotenoids for temporary accumulation in the liver. Carotenoids are then pushed to blood circulation and accumulated afterward (Yamashita *et al*, 1996).

Fancy carp fed diet combined lutein and β-carotene treatments by 50:50 and 25:25 mg/kg as well as diet supplemented lutein 50 mg/kg possessed detectable astaxanthin in the serum similar to fancy carp fed astaxanthin contained 25 mg/kg treatments which provide astaxanthin 1.66 $\pm$ 0.18 µl/ml of serum. This indicated that fancy carp in such group can convert lutein or β-carotene to astaxanthin. Goodwin (1984) reports that carotenoids found in most of fancy carp' skin is keto-carotenoids i.e., astaxanthin ester and 4-keto-lutein ester. Therefore, in keto-carotenoids synthesis, which is a significant value to red hue pigmentation, directly derives from keto-carotenoids. However, fancy carp fed with lutein- carotenoids and by lutein and β-carotene in a combination, can metabolite these carotenoids to astaxanthin. This is also in line with Katayama *et al.* (1973)'s research which states that each being is capable of changing and accumulating carotenoids differently. They found that fancy carp can alter lutein and zeaxanthin to astaxanthin. The study on skin pigmentation between red position and white position shows that red hue on the body area is related to serum carotenoids these is lie down and accumulation in tissue done by Storebakken *et al*. (1987) and Torrissen (1995) which describe that higher astaxanthin results in better accumulation of carotenoids found in tissues. The accumulation of lutein and β-carotene enhance red intensity and turned white hue to yellow-white hue. This illustrated that types of these carotenoids significantly affect the white hue. The reduction of serum carotenoids with discontinue treatment diet induced yellow-white

hue significantly turned to whiter hue. Choubert *et al*. (1994) which report that after discontinued feeding of carotenoids, the amount of carotenoids found in rainbow trout's blood descend to the lowest point after three days of discontinuer. Consuming carotenoids-added diets will result in fading of yellow hue (Mascio *et al*., 1990). This concludes that fancy carp fed with 50:50 and 25:25 mg/kg of lutein plus β-carotene treatments and added lutein 50 mg/kg treatments can best utilizing carotenoids similar to fish fed with astaxanthin alone at 25 mg/kg. Their can increase astaxanthin concentration accumulated in serm and showed the best effects on skin coloration as redness skin.

Part 4 Stability of Carotenoid Diets During Feed Processing and Under Different Storage Conditions

1. Dry matter and temperature in diets during the production process

A study on the effects of antioxidant; butylated hydroxytoluene (BHT) use at 250 mg/kg in feeds that contain different types and levels of carotenoids, the diets are into two groups of experiment which are group A (commercial carotenoid source) and group B (carotenoid extraction form plant leaves namely tea, mulberry and cassava leaves and raw material of them). As the diets in each group are split into two smaller groups which one without antioxidant and another is contains antioxidant. The study of diets on dry matter in diets during the three production process; dry mixed feeds which are ready to feeds pelleting (dry mix), pelleted feed that are processed through pellet cooker with die surface (after being processed) and drying process of feeds with hot air oven with average temperature of 60 °C for 12 hrs (dryer). The result study of these processes have shown that dry matter values are similar ($p \geq 0.05$). In the process of dry mixed feeds, after being processed and dryer of experimental diets of group A that without antioxidant have dry matter values of 89.80-90.2, 59.83-60.29 and 90.55-91.17%, respectively (shown in **Table 20**). For the diets of group A that with antioxidant have dry matter values of 89.18-90.05, 59.52-60.55 and 90.49-91.00% respectively (shown in **Table 21**).

Table 20 Dry matter in the experimental diets Group A, without antioxidant at the various unit operations.

Experimental diets	Unit operations		
	Dry mix	After being processed	Dryer
	Without antioxidant		
Diet 1: control diet	90.10 ± 0.72	59.86 ± 0.88	90.86 ± 0.67
Diet 2: astaxanthin 25 mg/kg	89.94 ± 0.90	60.20 ± 0.36	90.88 ± 0.66
Diet 3: lutein 25 mg/kg	90.18 ± 0.38	60.29 ± 1.64	90.55 ± 0.74
Diet 4: β-carotene 25 mg/kg	90.21 ± 0.31	60.03 ± 0.30	90.57 ± 0.60
Diet 5: Lutein:β-carotene 25:25 mg/kg	89.80 ± 0.77	59.83 ± 1.70	90.98 ± 0.80
Diet 6: Lutein:β-carotene 50 mg/kg	89.92 ± 0.81	59.94 ± 0.50	91.17 ± 0.74
p-value	0.9704	0.9922	0.8734

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 21 Dry matter in the experimental diets Group A, with antioxidant at the various unit operations.

Experimental diets	Unit operations		
	Dry mix	After being processed	Dryer
	With antioxidant		
Diet 1: control diet	89.18 ± 0.81	59.79 ± 0.94	90.65 ± 0.43
Diet 2: astaxanthin 25 mg/kg	89.46 ± 0.57	59.52 ± 0.72	90.49 ± 0.81
Diet 3: lutein 25 mg/kg	89.84 ± 1.44	59.56 ± 1.23	90.88 ± 0.88
Diet 4: β-carotene 25 mg/kg	89.48 ± 0.18	60.16 ± 0.30	91.00 ± 0.23
Diet 5: Lutein:β-carotene 25:25 mg/kg	90.05 ± 1.58	59.77 ± 0.39	90.92 ± 0.70
Diet 6: Lutein:β-carotene 50 mg/kg	89.24 ± 0.55	60.55 ± 0.60	90.61 ± 0.70
p-value	0.8706	0.5762	0.9174

Mean with the different letters in same column are significantly different at $p \leq 0.05$

The study of diets on dry matter in diets of group B during the three production process; dry mixed feeds, after being processed and dryer in hot air oven. It is found that the diets that without antioxidant have dry matter values of 89.54-90.23, 59.44-60.20 and 90.55-90.86%, respectively (shown in **Table 22**). For those that contain antioxidant have the values of 89.18-90.05, 59.43-60.10 and 90.25-91.00%, orderly (shown in **Table 23**).

Table 22 Dry matter in the experimental diets Group B, without antioxidant at the various unit operations.

Experimental diets	Unit operations		
	Dry mix	After being processed	Dryer
	Without antioxidant		
Diet 1: control diet	90.10 ± 0.72	59.86 ± 0.88	90.86 ± 0.67
Diet 2: lutein 25 mg/kg	90.18 ± 0.38	60.29 ± 1.64	90.55 ± 0.74
Diet 3: β-carotene 25 mg/kg	90.21 ± 0.31	60.03 ± 0.30	90.57 ± 0.60
Diet 4: TC of tea extract 25 mg/kg	90.23 ± 0.52	59.75 ± 0.81	90.73 ± 0.38
Diet 5: TC of mulberry extract 25 mg/kg	90.21 ± 0.31	59.76 ± 1.30	90.87 ± 0.38
Diet 6: TC of cassava extract 25 mg/kg	89.54 ± 1.16	59.76 ± 1.30	90.67 ± 0.85
Diet 7: TC of tea leaves 25 mg/kg	90.09 ± 0.54	60.07 ± 0.98	90.78 ± 0.36
Diet 8: TC of mulberry leaves 25 mg/kg	89.95 ± 0.97	60.20 ± 0.62	90.61 ± 0.62
Diet 9: TC of cassava leaves 25 mg/kg	89.91 ± 0.97	59.44 ± 1.06	90.81 ± 1.15
p-value	0.9618	0.9814	0.9990

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 23 Dry matter in the experimental diets Group B, with antioxidant at the various unit operations.

Experimental diets	Unit operations		
	Dry mix	After being processed	Dryer
	With antioxidant		
Diet 1: control diet	89.18 ± 0.81	59.79 ± 0.94	90.65 ± 0.43
Diet 2: lutein 25 mg/kg	89.84 ± 1.44	59.56 ± 1.23	90.88 ± 0.88
Diet 3: β-carotene 25 mg/kg	89.48 ± 0.18	60.16 ± 0.30	91.00 ± 0.23
Diet 4: TC of tea extract 25 mg/kg	89.19 ± 0.55	60.10 ± 0.58	90.44 ± 0.78
Diet 5: TC of mulberry extract 25 mg/kg	89.46 ± 0.63	59.43 ± 0.70	90.54 ± 0.58
Diet 6: TC of cassava extract 25 mg/kg	89.49 ± 0.81	59.88 ± 0.74	90.33 ± 1.20
Diet 7: TC of tea leaves 25 mg/kg	89.97 ± 0.99	60.06 ± 0.50	90.44 ± 0.88
Diet 8: TC of mulberry leaves 25 mg/kg	89.59 ± 0.73	59.96 ± 0.58	90.25 ± 0.47
Diet 9: TC of cassava leaves 25 mg/kg	90.05 ± 0.71	59.80 ± 0.64	90.81 ± 0.42
p-value	0.8873	0.9405	0.9092

Mean with the different letters in same column are significantly different at $p \leq 0.05$

The temperature of mash feed was studied during production process are separated into 2 processes which were dry mix and after being processed, 3 areas in the mash of diets are randomized for the measurement of temperatures by digital thermometer. It is found that all diets that were passed the two production processes have no different temperatures ($p \geq 0.05$). In the production process of dry mixed and after being processed in diets of group A that without antioxidant have temperature of 31.07-31.80 and 56.13-55.67°C, respectively (**Table 24**). For the ones that contain antioxidant come out with temperature of 31.17-31.80 and 55.77-56.57 °C, orderly (**Table 25**).

Table 24 Temperature in the experimental diets Group A, without antioxidant at the various unit operations.

Experimental diets	Unit operations	
	Dry mix	After being processed
	Without antioxidant	
Diet 1: control diet	31.60 ± 0.40	55.67 ± 0.45
Diet 2: astaxanthin 25 mg/kg	31.80 ± 0.30	56.10 ± 0.52
Diet 3: lutein 25 mg/kg	31.07 ± 0.95	55.87 ± 0.15
Diet 4: β-carotene 25 mg/kg	31.63 ± 0.72	55.90 ± 0.40
Diet 5: Lutein:β-carotene 25:25 mg/kg	31.63 ± 0.40	56.13 ± 0.50
Diet 6: Lutein:β-carotene 50 mg/kg	31.10 ± 0.90	56.17 ± 0.25
p-value	0.6670	0.5976

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 25 Temperature in the experimental diets Group A, with antioxidant at the various unit operations.

Experimental diets	Unit operations	
	Dry mix	After being processed
	With antioxidant	
Diet 1: control diet	31.73 ± 0.72	56.20 ± 0.60
Diet 2: astaxanthin 25 mg/kg	31.80 ± 0.61	56.20 ± 0.60
Diet 3: lutein 25 mg/kg	31.40 ± 0.60	55.77 ± 0.06
Diet 4: β-carotene 25 mg/kg	31.67 ± 0.76	55.87 ± 0.25
Diet 5: Lutein:β-carotene 25:25 mg/kg	31.73 ± 0.64	56.57 ± 0.25
Diet 6: Lutein:β-carotene 50 mg/kg	31.17 ± 1.05	56.07 ± 0.25
p-value	0.8849	0.2358

Mean with the different letters in same column are significantly different at $p \leq 0.05$

For the diets of group B that no antioxidant, in the production process of dry mixed and after being processed with temperature 31.07-31.97 and 55.67-56.17°C, respectively (as shown in **Table 26**). For those that contain antioxidant have the temperature values of 31.20-32.00 a55.77-56.20°C in order (as shown in **Table 27**).

Table 26 Temperature in the experimental diets Group B, without antioxidant at the various unit operations.

Experimental diets	Unit operations	
	Dry mix	After being processed
	Without antioxidant	
Diet 1: control diet	31.60 ± 0.40	55.67 ± 0.45
Diet 2: lutein 25 mg/kg	31.07 ± 0.95	55.87 ± 0.15
Diet 3: β-carotene 25 mg/kg	31.63 ± 0.72	55.90 ± 0.40
Diet 4: TC of tea extract 25 mg/kg	31.97 ± 0.06	55.90 ± 0.20
Diet 5: TC of mulberry extract 25 mg/kg	31.47 ± 0.06	56.17 ± 0.31
Diet 6: TC of cassava extract 25 mg/kg	31.97 ± 0.06	56.17 ± 0.25
Diet 7: TC of tea leaves 25 mg/kg	31.77 ± 0.50	56.00 ± 0.20
Diet 8: TC of mulberry leaves 25 mg/kg	31.37 ± 0.42	56.00 ± 0.52
Diet 9: TC of cassava leaves 25 mg/kg	31.83 ± 0.81	55.93 ± 0.57
p-value	0.5484	0.8187

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 27 Temperature in the experimental diets Group B, with antioxidant at the various unit operations.

Experimental diets	Unit operations	
	Dry mix	After being processed
	With antioxidant	
Diet 1: control diet	31.73 ± 0.72	56.20 ± 0.60
Diet 2: lutein 25 mg/kg	31.40 ± 0.60	55.77 ± 0.06
Diet 3: β-carotene 25 mg/kg	31.67 ± 0.76	55.87 ± 0.25
Diet 4: TC of tea extract 25 mg/kg	31.50 ± 1.21	56.17 ± 0.55
Diet 5: TC of mulberry extract 25 mg/kg	31.40 ± 1.13	55.83 ± 0.78
Diet 6: TC of cassava extract 25 mg/kg	31.63 ± 0.72	56.00 ± 0.30
Diet 7: TC of tea leaves 25 mg/kg	32.00 ± 0.10	56.27 ± 0.45
Diet 8: TC of mulberry leaves 25 mg/kg	31.20 ± 0.61	56.00 ± 0.10
Diet 9: TC of cassava leaves 25 mg/kg	31.37 ± 1.06	55.77 ± 0.35
p-value	0.9748	0.7974

Mean with the different letters in same column are significantly different at $p \leq 0.05$

2. Stability of carotenoid diets during feed processing

A study on the effects of antioxidant (BHT) use at 250 mg/kg diet, the diets in group A that contain different types and levels of carotenoids are divided into two subgroups which without antioxidant and with antioxidant. Study the stability of total carotenoids (TC) in diets being experimented during the three production process; dry mix, after being processed and dryer as shown in **Table 28** and **Table 29**. After all these production processes, the TC in both groups of feeds with and without antioxidant, are significant different ($p \leq 0.05$). This is an effect from different additional amount of carotenoids which TC quantities will directly be changed according to the additional amount in the diets in the experiment. The account of the effects from three production processes until finished stage, it presents that the TC quantities from both subgroups of diets have no differences ($p \geq 0.05$). And the data showed that there was no interaction between diet type and unit operations ($p \geq 0.05$).

This indicates that TC quantities have been well stable in both cases that diets are contained with and without antioxidant from the beginning of the production process until being processed dried feed (as shown in **Table 30**).

Table 28 Total carotenoids content in the experimental diets Group A, without antioxidant at the various unit operations.

Experimental diets	Total carotenoid (mg/kg)
Diet 1: control diet	5.62 ± 0.14d
Diet 2: astaxanthin 25 mg/kg	26.54 ± 2.14c
Diet 3: lutein 25 mg/kg	25.57 ± 2.06c
Diet 4: β-carotene 25 mg/kg	25.71 ± 3.68c
Diet 5: Lutein:β-carotene 25:25 mg/kg	46.80 ± 3.18b
Diet 6: Lutein:β-carotene 50 mg/kg	86.53 ± 4.83a
Unit operations	
Dry mix	37.34
After being processed	35.94
Dryer	35.10
P-value	0.0001
Diet	0.0001
Unit operations	0.1417
Diet* Unit operations	0.9989

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 29 Total carotenoids content in the experimental diets Group A, with antioxidant at the various unit operations.

Experimental diets	Total carotenoid (mg/kg)
Diet 1: control diet	5.85 ± 0.34d
Diet 2: astaxanthin 25 mg/kg	27.18 ± 1.21c
Diet 3: lutein 25 mg/kg	27.21 ± 1.15c
Diet 4: β-carotene 25 mg/kg	27.60 ± 2.65c
Diet 5: Lutein:β-carotene 25:25 mg/kg	47.78 ± 3.01b
Diet 6: Lutein:β-carotene 50 mg/kg	86.48 ± 3.25a
Unit operations	
Dry mix	37.75
After being processed	36.87
Dryer	36.28
P-value	0.0001
Diet	0.0001
Unit operations	0.1770
Diet* Unit operations	0.9991

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 30 Comparing total carotenoids content in the experimental diets of Group A between two subgroups which without and with antioxidant at the various unit operations.

Experimental diets	Total carotenoid (mg/kg)		
	Without antioxidant	With antioxidant	P-value
Diet 1: control diet	5.62 ± 0.14	5.85 ± 0.34	0.57
Diet 2: astaxanthin 25 mg/kg	26.54 ± 2.14	27.18 ± 1.21	0.30
Diet 3: lutein 25 mg/kg	25.57 ± 2.06	27.21 ± 1.15	0.51
Diet 4: β-carotene 25 mg/kg	25.71 ± 3.68	27.60 ± 2.65	0.72
Diet 5: Lutein:β-carotene 25:25 mg/kg	46.80 ± 3.18	47.78 ± 3.01	0.89
Diet 6: Lutein:β-carotene 50 mg/kg	86.53 ± 4.83	86.48 ± 3.25	0.26

Mean with the different letters in same row are significantly different at $p \leq 0.05$

For the diets in group B, the stability of total carotenoids (TC) in the diets being experimented during the three production process; dry mix, after being processed and dryer as shown in **Table 31** and **Table 32**. After all these production processes, the TC in both groups of foods: with and without antioxidant are different ($p \leq 0.05$). This is an effect from different additional amount of carotenoids which TC amount will directly be changed according to the additional amount in the diets in the experiment. The account of the effects from three production processes until finished stage, it presents that the TC quantities from both subgroups of diets have significant differences ($p \leq 0.05$). There was no interaction between diet type and unit operations ($p \geq 0.05$). It is found that the quantities of TC are highest in the production process in dry mix: allies all raw materials for diets. The TC quantities will be reduced after the diets through pellet cooker and passed die surface and dryer processes due to the heat occurred during these processes. And the data found that there was no interaction between diet type and unit operations ($p \geq 0.05$). This indicates that TC quantities have been well stable in both cases that diets are contained with and without antioxidant from the beginning of the production process until being processed dried feed (as shown in **Table 33**). It can be concluded that diets which contains carotenoid extraction from tea leaves, mulberry and cassava leaves including the diets using raw material of them directly are less effective to the resistance of the heat during the feed processing as compared to diets group A which added by commercial carotenoid.

Table 31 Total carotenoids content of experimental diets Group B, without antioxidant at the various unit operations.

Experimental diets	Total carotenoid (mg/kg)
Diet 1: control diet	5.62 ± 0.14b
Diet 2: lutein 25 mg/kg	25.57 ± 2.06a
Diet 3: β-carotene 25 mg/kg	25.71 ± 3.67a
Diet 4: TC of tea extract 25 mg/kg	26.07 ± 3.27a
Diet 5: TC of mulberry extract 25 mg/kg	26.3.5 ± 1.37a
Diet 6: TC of cassava extract 25 mg/kg	26.51 ± 1.29a
Diet 7: TC of tea leaves 25 mg/kg	24.97 ± 2.79a
Diet 8: TC of mulberry leaves 25 mg/kg	25.76 ± 0.77a
Diet 9: TC of cassava leaves 25 mg/kg	25.11 ± 2.79a
Unit operations	
Dry mix	24.82a
After being processed	23.39b
Dryer	22.35b
P-value	0.0001
Diet	0.0001
Unit operations	0.0034
Diet* Unit operations	0.9999

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 32 Total carotenoids content of experimental diets Group B, with antioxidant at the various unit operations.

Experimental diets	Total carotenoid (mg/kg)
Diet 1: control diet	5.85 ± 0.34b
Diet 2: lutein 25 mg/kg	27.42 ± 1.15a
Diet 3: β-carotene 25 mg/kg	27.60 ± 2.65a
Diet 4: TC of tea extract 25 mg/kg	26.71 ± 1.50a
Diet 5: TC of mulberry extract 25 mg/kg	27.42 ± 1.68a
Diet 6: TC of cassava extract 25 mg/kg	26.17 ± 2.03a
Diet 7: TC of tea leaves 25 mg/kg	26.49 ± 2.84a
Diet 8: TC of mulberry leaves 25 mg/kg	25.73 ± 2.52a
Diet 9: TC of cassava leaves 25 mg/kg	25.92 ± 1.74a
Unit operations	
Dry mix	25.08a
After being processed	24.34ab
Dryer	23.61b
P-value	0.0001
Diet	0.0001
Unit operations	0.0439
Diet* Unit operations	0.9996

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 33 Comparing total carotenoids content in the experimental diets of Group B between two subgroups which without and with antioxidant at the various unit operations.

Experimental diets	Total carotenoid (mg/kg)		
	Without antioxidant	Antioxidant	P-value
Diet 1: control diet	5.62 ± 0.14	5.85 ± 0.34	0.57
Diet 2: lutein 25 mg/kg	25.57 ± 2.06	27.42 ± 1.15	0.51
Diet 3: β-carotene 25 mg/kg	25.71 ± 3.67	27.60 ± 2.65	0.72
Diet 4: TC of tea extract 25 mg/kg	26.07 ± 3.27	26.71 ± 1.50	0.98
Diet 5: TC of mulberry extract 25 mg/kg	26.3.5 ± 1.37	27.42 ± 1.68	0.81
Diet 6: TC of cassava extract 25 mg/kg	26.51 ± 1.29	26.17 ± 2.03	0.61
Diet 7: TC of tea leaves 25 mg/kg	24.97 ± 2.79	26.49 ± 2.84	0.42
Diet 8: TC of mulberry leaves 25 mg/kg	25.76 ± 0.77	25.73 ± 2.52	0.12
Diet 9: TC of cassava leaves 25 mg/kg	25.11 ± 2.79	25.92 ± 1.74	0.34

Mean with the different letters in same row are significantly different at $p \leq 0.05$

The experiment of diets that contain different types and levels of carotenoids after all the productions processes until become finish feed found that there is a percentage loss of TC during production process of diets in group A. The loss in the diets starts from after being feed processed which are through from the pellet cooker and passed to die surface. After dryer process, the loss of TC will be reduced more according to the hot air being used in diets contain both with and without antioxidant (BHT). After being feed processed, the percentage loss of TC in diets with and without antioxidant is in the range of 2.07- 5.21 and 2.26- 4.96, respectively and dryer process is from 3.03-7.82 and 2.67-8.47 in order. For diets in group B show similar result which TC level in diets of group A will be changed according to the heat during feed processing. The percentage loss of TC of diets with and without antioxidant after being processed is 2.07-7.65 followed by 2.05-4.96 in order. For after drying with hot air oven, TC quantities are in the range of 3.03-11.89 and 4.84-8.47, respectively. Even antioxidant is added or not in the diets diets in group A before the after being processed to become dried feed, there is no difference of TC quantities.

However, diets in group B which contain natural based products or natural raw materials will have higher chance for the loss of TC if no antioxidant is added before the production process as compared to the group that has antioxidant added.

The results of our factorial total carotenoid (TC) stability trial during feed processing indicate that no interaction was found between diet type and unit operations of feed processing for dry matter (DM) and temperature of mash feed. In a similar study, Booth *et al*. (2002) studied the steam pelleting was unaffected on DM, energy or nitrogen ADCs. Feed processing with mincer machine (soft type pellets) in this study is capable of handling feeds containing from 30% to more than 50% molasses (Harry, 1976), in this study adding an amount of water which in this experiment is about 30 percents. The maximum percentage of molasses is governed by the absorptive qualities of the dry feed ingredients. After this, a rotation auger conveys material to the die and compressed mash feed through perforations in the die forming it into pellets. The heat in the pellet cooker is about 50-60 °C which could cause the diets to become gelatin for improve digestibility. This process involves water, heat, and mechanical stress. Feed additives most sensitive to this process are well known and include vitamin A, K_3 and C, but also carotenoids and feed antibiotics (Gadient, 1986 and 1994).Carotenoids are organic compounds that are potentially sensitive to such treatments. Reported retention values for astaxanthin in extruded feeds range from 86% (Anderson and Sunderland, 2002) depending on processing parameters. Similar stability was obtained by Haaland *et al.* (1993) reported retention values for astaxanthin in extruded feed range from 86% to 94%. Therefore, total carotenoid stability in the final product in this study depended most on processing temperature.

Diets that passed through die surface would still contain 30-40 % of moisture so that the dryer processing by hot air oven needed in order for the moisture to be evaporated. However, using very high temperature reduces nutrient amount in diets as during the process, high temperature is required which caused the diets to receive too much heat for being dry. By using low temperature would cause longer period for this process, but the loss of nutrition in feeds will be less. All part of diets requires

constant heat and wind so that it would help the diets to dry faster and contain the nutrition. Hot air oven is used for this process which it contains 60 °C for 12 hrs, Harry (1976) reported that the optimal of temperature for drying pellets will leave the pellet mill at temperatures as high as 190°F (87.8 °C). Hence, the temperature of dryer in this study which is safe for the loss of nutrition.

The experiment found no differences of the production processes to TC quantities in the stability of the carotenoid supplemented diets (with and without antioxidant), after production processes until becoming finish feed. For the group that contain antioxidant and the experiment of natural carotenoids including natural raw materials added, the TC quantities are reduced after being feed processed and dryer processing respectively. It is because the heat is created during these processes which reduced the quantities of carotenoids in feeds. Heat processes cause considerables loss of carotenoids. Losses can occur by thermal degradation or by feed processing such as pellet cooking or dryer. Feed manufactuers, however, expressed concern about the destruction of vitamins and other feed additives in compound feeds processing. In fact, in the pellet mill system, mash feed encounters moisture, high temperatures, friction and other processing stress factors. This concern prompted various investigations into the extent of losses during the feed process and subsequent pelleting (Broz *et al*, 1997). Pipa and Frank (1989) examined the stability of major vitamins in various feed types pelleted around 120 °C. They found no effects of the process on the recovery of vitamin B1 and E. For vitamin A, losses varied according to the feed type and reached 20% in some cases. For canthaxanthin, also used as carotenoid source for salmonid fish feeds, 18% loss has been reported (Berset, 1987).

Most of the processes employed in feed manufacture are intended to increase the value of feed ingredients. For example, the digestibility may be increased or the palatability improved (Riaz, 2000). On the other hand, micronutrients such as vitamins are more likely to be damaged by the feed manufacturing process. Vitamin bioavailability is affected by the stability of the vitamin and the utilization efficiency (Baker, 1995). Extrusion is the main feed processing technology used in the manufacture of dry feeds. Vitamins and carotenoids are sensitive organic compounds

that can be denatured by water, oxygen, trace minerals, heat and other factors (Wornick, 1968; Schneider, 1986; McGinnis, 1986 and Coelho. 1991). The extrusion process involves water, heat, and mechanical stress, all of which can impact on vitamin and carotenoid stability (Riaz, 2000). Feed processing is a versatile high temperature that may alter the nutritional value of feeds (Camire *et al*., 1990). Among the factors listed above, mainly heat and presence of oxygen are anticipated to influence carotenoid stability during production of dry feed. After mixing, the feed mash is conditioned, a process that involves additional mixing and addition of water and oil, and the temperature is increasing. During pelletized the temperature is furture elevated, and the mash is subjected to strong shear forces. The pellets are dried, normally by using hot air. As a final step in the production for most high energy diets.

This shows that the diets contain tea, mulberry and cassava leaves including these raw materials have lesser durability of TC than in group A which contain commercial carotenoids. The most destructive aspects of the pelleting process have been identified as wet, fat addition and high energy input. Pelleting can affect the natural carotenoid content of feed ingredients as well as the carotenoid added in the form of commercial products. Recently, modern form of carotenoids are usually less affected by the feed processing because they are especially developed for use in animal feeds (Broz *et al.,*1997). As a result, should be added 10 percents of TC from the needed amount to natural carotenoids or natural raw materials to achieve the target level TC. The effect of temperature on the nutritional value of feed processed through conventional processing methods and through minimal processing are discussed. There are several reports on changes that occur in carotenoids during heat processing. Carotenoids have been found to be relatively stable in feeds. In this study found that in diets formulated as fish feed, carotenoid content remained quite stable, without significant loss during the trial period, for all tested diets. Pelleting process of the diets (with a pelleting machine: soft type pellet without steam) didn’t affect carotenoid content of the diets.

3. Stability of carotenoid diets under different storage temperatures

A study on the effects of antioxidant (BHT) use at 250 mg/kg in diets that contain different types and levels of carotenoids, the diets are into two groups of experiment which are group A and B. As the diets in each group are split into two smaller groups which one is without antioxidant and another contains antioxidant. As passed the production processes until turn out to be finished feed, store the diets in aluminum foil bag and seal the top with the bag sealer. Then, separate the bags of both diets groups and keep them at different conditions of temperature which are room temperature at 26-28 °C and keep in cool temperature at 4 °C for 8 weeks. Randomly collected samples of 0, 4 and 8 weeks period, in order to analyze the quantities of TC in the diets. The result of this experiment in diet group A that kept in room temperature and cool temperature shows that TC quantities in diets of both groups are significant different ($p \leq 0.05$) which is an effect from the amount of carotenoids being added into diets, which TC quantities are directly fluctuated to carotenoids quantities (shown in **Table 34** and **Table 35**). Nevertheless, there is no effect in the change of TC quantities in diets from all groups which being kept in different temperatures during trial period and TC content have been well stable in both cases that diets are contained with and without antioxidant (as shown in **Table 36**). It is also shown that the longer time kept for both diets groups which contain antioxidant and none antioxidant under room temperature or cool temperature is no effect to TC quantities.

Table 34 Total carotenoids content of experimental diets of Group A, storage under room temperature for 8 weeks.

Experimental diets	Total carotenoid (mg/kg)	
	Without antioxidant	Antioxidant
Room temperature		
Diet 1: control diet	5.43 ± 0.43d	5.58 ± 0.24d
Diet 2: astaxanthin 25 mg/kg	24.29 ± 2.76c	25.78 ± 2.78c
Diet 3: lutein 25 mg/kg	22.79 ± 1.61c	24.46 ± 2.28c
Diet 4: β-carotene 25 mg/kg	24.01 ± 2.24c	25.14 ± 1.74c
Diet 5: Lutein:β-carotene 25:25 mg/kg	43.99 ± 3.43b	45.43 ± 2.51b
Diet 6: Lutein:β-carotene 50 mg/kg	82.85 ± 3.67a	84.41 ± 2.59a
Storage time		
0 week	35.10	35.10
4 weeks	33.79	35.30
8 weeks	32.79	34.99
P-value	0.0001	0.0001
Diet	0.0001	0.0001
Storage time	0.0597	0.9372
Diet*Storage time	0.9962	0.9997

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 35 Total carotenoids content of experimental diets of Group A, storage under cool temperature at 4 °C for 8 weeks.

Experimental diets	Total carotenoid (mg/kg)	
	Without antioxidant	Antioxidant
Cool temperature at 4 °C		
Diet 1: control diet	5.47 ± 0.16d	5.66 ± 0.14d
Diet 2: astaxanthin 25 mg/kg	25.25 ± 2.69c	26.45 ± 2.21c
Diet 3: lutein 25 mg/kg	23.36 ± 1.92c	25.60 ± 0.94c
Diet 4: β-carotene 25 mg/kg	24.56 ± 1.62c	25.67 ± 2.18c
Diet 5: Lutein:β-carotene 25:25 mg/kg	44.81 ± 3.39b	46.41 ± 2.34b
Diet 6: Lutein:β-carotene 50 mg/kg	83.74 ± 2.31a	85.42 ± 1.77a
Storage time		
0 week	35.10	36.28
4 weeks	34.38	35.79
8 weeks	34.11	35.54
P-value	0.0001	0.0001
Diet	0.0001	0.0001
Storage time	0.4767	0.5644
Diet*Storage time	0.9999	1.000

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 36 Comparing total carotenoids content in the experimental diets of Group A between two subgroups which without and with antioxidant storage under different temperature until trial periods.

Experimental diets	Total carotenoid (mg/kg)		
	Without antioxidant	Antioxidant	P-value
Room temperature			
Diet 1: control diet	5.43 ± 0.43	5.58 ± 0.24	0.35
Diet 2: astaxanthin 25 mg/kg	24.29 ± 2.76	25.78 ± 2.78	0.19
Diet 3: lutein 25 mg/kg	22.79 ± 1.61	24.46 ± 2.28	0.75
Diet 4: β-carotene 25 mg/kg	24.01 ± 2.24	25.14 ± 1.74	0.08
Diet 5: Lutein:β-carotene 25:25 mg/kg	43.99 ± 3.43	45.43 ± 2.51	0.11
Diet 6: Lutein:β-carotene 50 mg/kg	82.85 ± 3.67	84.41 ± 2.59	0.16
Cool temperature at 4 °C			
Diet 1: control diet	5.47 ± 0.16	5.66 ± 0.14	0.42
Diet 2: astaxanthin 25 mg/kg	25.25 ± 2.69	26.45 ± 2.21	0.55
Diet 3: lutein 25 mg/kg	23.36 ± 1.92	25.60 ± 0.94	0.47
Diet 4: β-carotene 25 mg/kg	24.56 ± 1.62	25.67 ± 2.18	0.80
Diet 5: Lutein:β-carotene 25:25 mg/kg	44.81 ± 3.39	46.41 ± 2.34	0.19
Diet 6: Lutein:β-carotene 50 mg/kg	83.74 ± 2.31	85.42 ± 1.77	0.35

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Study the stability of total carotenoids (TC) in the diets of group B being experimented during keep them at different conditions of temperature which are room temperature at 26-28 °C and keep in cool temperature at 4 °C for 8 weeks as the diets in this group are split into two smaller groups which one is without antioxidant and another contains antioxidant. The result of this experiment shows that TC quantities in diets without BHT are significant different ($p \leq 0.05$) which is an effect from the length of storage time under room temperature (shown in **Table 37**). Nevertheless, there is no effect in the change of TC quantities in diets from all groups which adding BHT under room temperature and the both diets group with and without BHT being kept in cool temperature at 4 °C (shown in **Table 38**). This results showed that there is no effect in the change of TC quantities in diets from all groups which being kept in different temperatures during trial period and TC content have been well stable in

both cases that diets are contained with and without antioxidant (as shown in **Table 39**). It is also shown that the longer time kept for diets which contain antioxidant under room temperature and both diets group with and none antioxidant under cool temperature does any effect to TC quantities.

Table 37 Total carotenoids content of experimental diets of Group B, storage under room temperature for 8 weeks.

Experimental diets	Total carotenoid (mg/kg)	
	Without antioxidant	Antioxidant
Room temperature		
Diet 1: control diet	5.43 ± 0.43b	5.58 ± 0.24b
Diet 2: lutein 25 mg/kg	22.79 ± 1.61a	42.46 ± 2.29a
Diet 3: β-carotene 25 mg/kg	24.01 ± 2.24a	25.14 ± 2.02a
Diet 4: TC of tea extract 25 mg/kg	23.76 ± 1.75a	24.58 ± 1.77a
Diet 5: TC of mulberry extract 25 mg/kg	24.34 ± 0.62a	25.33 ± 2.55a
Diet 6: TC of cassava extract 25 mg/kg	24.25 ± 1.36a	24.30 ± 1.58a
Diet 7: TC of tea leaves 25 mg/kg	23.11 ± 3.02a	23.92 ± 2.05a
Diet 8: TC of mulberry leaves 25 mg/kg	23.79 ± 2.06a	23.90 ± 1.55a
Diet 9: TC of cassava leaves 25 mg/kg	23.64 ± 2.78a	23.80 ± 2.46a
Storage time		
0 week	22.46a	22.54
4 weeks	21.63ab	22.56
8 weeks	20.95b	22.00
P-value	0.0001	0.0001
Diet	0.0001	0.0001
Storage time	0.0452	0.6240
Diet*Storage time	0.9999	0.9992

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 38 Total carotenoids content of experimental diets of Group B, storage under cool temperature at 4 °C for 8 weeks.

Experimental diets	Total carotenoid (mg/kg)	
	Without antioxidant	Antioxidant
Cool temperature at 4 °C		
Diet 1: control diet	5.47 ± 0.16c	5.66 ± 0.14c
Diet 2: lutein 25 mg/kg	23.36 ± 1.92ab	25.60 ± 0.94a
Diet 3: β-carotene 25 mg/kg	24.56 ± 1.62a	25.67 ± 2.18a
Diet 4: TC of tea extract 25 mg/kg	24.67 ± 1.51ab	25.26 ± 2.08a
Diet 5: TC of mulberry extract 25 mg/kg	24.67 ± 2.07a	26.18 ± 1.54a
Diet 6: TC of cassava extract 25 mg/kg	24.60 ± 1.65a	24.72 ± 1.45ab
Diet 7: TC of tea leaves 25 mg/kg	22.51 ± 2.10b	25.27 ± 1.56a
Diet 8: TC of mulberry leaves 25 mg/kg	23.84 ± 1.02ab	23.16 ± 2.01b
Diet 9: TC of cassava leaves 25 mg/kg	24.24 ± 1.72ab	24.11 ± 2.40ab
Storage time		
0 week	22.46	23.17
4 weeks	21.89	22.77
8 weeks	21.48	22.61
P-value	0.0001	0.0001
Diet	0.0001	0.0001
Storage time	0.1653	0.5588
Diet*Storage time	1.000	1.0000

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 39 Comparing total carotenoids content in the experimental diets of Group B between two subgroups which without and with antioxidant storage under different temperature until trial periods.

Experimental diets	Total carotenoid (mg/kg)		
	Without antioxidant	Antioxidant	P-value
Room temperature			
Diet 1: control diet	5.43 ± 0.43b	5.58 ± 0.24b	0.35
Diet 2: lutein 25 mg/kg	22.79 ± 1.61a	42.46 ± 2.29a	0.75
Diet 3: β-carotene 25 mg/kg	24.01 ± 2.24a	25.14 ± 2.02a	0.08
Diet 4: TC of tea extract 25 mg/kg	23.76 ± 1.75a	24.58 ± 1.77a	0.36
Diet 5: TC of mulberry extract 25 mg/kg	24.34 ± 0.62a	25.33 ± 2.55a	0.32
Diet 6: TC of cassava extract 25 mg/kg	24.25 ± 1.36a	24.30 ± 1.58a	0.75
Diet 7: TC of tea leaves 25 mg/kg	23.11 ± 3.02a	23.92 ± 2.05a	0.25
Diet 8: TC of mulberry leaves 25 mg/kg	23.79 ± 2.06a	23.90 ± 1.55a	0.58
Diet 9: TC of cassava leaves 25 mg/kg	23.64 ± 2.78a	23.80 ± 2.46a	0.81
Cool temperature at 4 °C			
Diet 1: control diet	5.47 ± 0.16c	5.66 ± 0.14c	0.42
Diet 2: lutein 25 mg/kg	23.36 ± 1.92ab	25.60 ± 0.94a	0.47
Diet 3: β-carotene 25 mg/kg	24.56 ± 1.62a	25.67 ± 2.18a	0.80
Diet 4: TC of tea extract 25 mg/kg	24.67 ± 1.51ab	25.26 ± 2.08a	0.19
Diet 5: TC of mulberry extract 25 mg/kg	24.67 ± 2.07a	26.18 ± 1.54a	0.35
Diet 6: TC of cassava extract 25 mg/kg	24.60 ± 1.65a	24.72 ± 1.45ab	0.86
Diet 7: TC of tea leaves 25 mg/kg	22.51 ± 2.10b	25.27 ± 1.56a	0.19
Diet 8: TC of mulberry leaves 25 mg/kg	23.84 ± 1.02ab	23.16 ± 2.01b	0.97
Diet 9: TC of cassava leaves 25 mg/kg	24.24 ± 1.72ab	24.11 ± 2.40ab	0.19

Mean with the different letters in same row are significantly different at $p \leq 0.05$

Perceptively, diets in group A that are mixed with commercial carotenoids source, which divided into 2 subgroups with and without antioxidant (BHT) show no effect on TC quantities, even the diets are kept in either room temperature or cool temperature for 8 weeks through out the experiment period. For diets in group B which contain carotenoids from natural based products or natural raw materials namely tea, mulberry and cassava leaves, TC quantities in diets, with no additional of antioxidant and kept in room temperatures, reduced due to the amount of time the

diets are kept. Moreover, it is found that there is no change of TC quantities within the period of 8 weeks in the diets in the group that contain antioxidant and the group that is kept at 4 °C of cool temperature (with and without antioxidant added) including the diet group that added antioxidant and kept under room temperature. As a result, there is higher level in the percentage loss of TC quantities in diets with the use of natural carotenoids or natural raw materials, keeping the diets without antioxidant added before dried feed process including store these diets at room temperature as compared to the one that is with antioxidant or those that are kept in cool temperature with the degree 4 °C.

After the diets contain different types and levels of carotenoids are kept for 8 weeks at room temperature of 26-28 °C and 4 °C of cool temperature, it shows that the loss of TC quantities in group A (without antioxidant) are higher as compared to the one with antioxidant. As being kept longer at room temperature, 4.79-8.97 and 1.30-2.58 of TC are lost during the 8 weeks period. For the diets that are kept at 4 °C of cool temperature have the value of 2.11-3.8 and 1.09-3.86, respectively. This can be concluded that keeping the carotenoids diets (with and without antioxidant) at cool temperature at 4 °C is more effective in terms of slowing down the loss of TC quantities which has the same result to group B. The loss of TC will be higher for the one without and with antioxidant and kept at room temperature which is in the range of 4.79-10.34 and 2.02-6.47. For the diets that are kept at 4 °C of cool temperature fall in the percentage range of 2.11-5.78 and 1.25-3.86. The overall shows that diets with and without antioxidant that are kept in cool temperature and those with antioxidant that are kept at room temperature are effective in terms of slowing down the quantities of TC. For the 8 weeks experiment, feeds in group A have lesser TC loss than group B which is from natural extraction or natural raw materials. Within this group, if there is no additional antioxidant before feed processing, there will be even more loss of TC. In this work, total carotenoid stability of all formulated carotenoid diets seems to be predominantly affected by storage temperature. Tang and Chen (2002) reported that the stability of freeze-dried carotenoid powder can be substantially improved by storage at low temperature such as 4 °C and revealed that the carotenoid in freeze-dried powder decreased with increasing storage time and temperature. In a similar

study, Chen and Tang (1998) studied the stability of spray-dried carotenoid powder and found that the degradation rate constants (day^{-1}) of the carotenoid decreased with increasing storage temperature or illumination time, whereas the storage temperature was important to a lesser extent in carotenoid stability of biomass of both algae which are *Chlorella vulgaris* and *Haematococcus pluvialis* (Gouveia and Empis, 2003). The data from this study indicated that, the best storage temperature for formulated carotenoids diet were keep under low temperature at 4 °C, storage at refrigerated temperatures is an important factor in slowing down metabolism processes. Addition, adding antioxidant and keep at room temperature which helps reducing the loss of TC quantities.

In this study, extract plant pigments are much more unstable than pigment contained in dry plant incorporated in fish feed diets, which is crucial, because many processing steps e.g. extraction. The use of higher plant as a pigment source of fish feed is very promising in terms of stability of carotenoids because feedstuffs should, as far as possible, be stored for a minimum length of time. Feed are composed of perishable biological material and the maximum permissible storage time is 6 months (Silva and Anderson, 1995), a period during which no great loss of pigment for both microalgae in both fish diets tested, was observed.

4. Rancidity of carotenoid diets under different storage conditions

3.1 Thiobarbituric Acid Test (TBA)

A study on the effects of antioxidant (BHT) use at 250 mg/kg in diets group A that contain different types and levels of carotenoids. The diets are split into two smaller groups which one contains antioxidant and another is without antioxidant. The diets were kept at different conditions of temperature which are room temperature at 26-28 °C and cool temperature at 4 °C for 8 weeks. Randomly collected samples of 0, 4 and 8 weeks period, in order to determine of Thiobarbituric acid test (TBA) value in the experiment, which TBA is popularly used for oxidation measurement in diets. By measuring the intensity of pink color causes by chemical reaction of thiobarbituric

acid and malonaldehyde quantities which are the product in the second stage of rancidity as in milligrams. TBA values in diets with and without antioxidant kept at room temperature were showed in **Table 40**, it is shown that the one without antioxidant has higher TBA value due to the longer period kept. It could be said that the longer the time period, the higher TBA value was significant different ($p \leq 0.05$). Conversely, this does not affect to the group that contain antioxidant ($p \geq 0.05$). Also the same result for the diets that are kept at 4 °C of cool temperature (with and without antioxidant) that has no different TBA due to the longer time kept ($p \geq 0.05$) as shown in **Table 41**. Nevertheless, there is no effect in the TBA value in diets from all groups which being kept in different temperatures during trial period and TBA value have been well stable in both cases that diets are contained with and without antioxidant (as shown in **Table 42**). Additionally, overall of carotenoid diets with and without antioxidant that are kept at 4 °C are more effective than kept at room temperature in terms of slowing down of rancidity. Moreover, the additional of BHT at 250 mg/kg in caroteniods diets affected the slowing rancidity well, even being kept at room temperature.

Table 40 TBA value of experimental diets of Group A, storage under room temperature for 8 weeks.

Experimental diets	TBA (mg/kg)	
	Without antioxidant	Antioxidant
Room temperature		
Diet 1: control diet	5.22 ± 0.25	5.28 ± 0.62
Diet 2: astaxanthin 25 mg/kg	5.43 ± 0.39	5.15 ± 0.63
Diet 3: lutein 25 mg/kg	5.43 ± 0.22	5.33 ± 0.59
Diet 4: β-carotene 25 mg/kg	5.61 ± 0.24	5.19 ± 0.49
Diet 5: Lutein:β-carotene 25:25 mg/kg	5.34 ± 0.26	5.31 ± 0.48
Diet 6: Lutein:β-carotene 50 mg/kg	5.40 ± 0.38	5.31 ± 0.27
Storage time		
0 week	5.12b	5.02
4 weeks	5.44a	5.29
8 weeks	5.65a	5.48
P-value	0.0462	0.9819
Diet	0.2567	0.9831
Storage time	0.0001	0.0891
Diet*Storage time	0.9817	1.0000

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 41 TBA value of experimental diets of Group A, storage under cool temperature at 4 °C room temperature for 8 weeks.

Experimental diets	TBA (mg/kg)	
	Without antioxidant	Antioxidant
Cool temperature at 4 °C		
Diet 1: control diet	5.32 ± 0.28	5.16 ± 0.38
Diet 2: astaxanthin 25 mg/kg	5.41 ± 0.47	5.04 ± 0.57
Diet 3: lutein 25 mg/kg	5.24 ± 0.14	5.26 ± 0.41
Diet 4: β-carotene 25 mg/kg	5.41 ± 0.26	5.04 ± 0.65
Diet 5: Lutein:β-carotene 25:25 mg/kg	5.26 ± 0.36	5.23 ±0.38
Diet 6: Lutein:β-carotene 50 mg/kg	5.31 ± 0.37	5.25 ± 0.26
Storage time		
0 week	5.16	5.02
4 weeks	5.33	5.15
8 weeks	5.48	5.31
P-value	0.8769	0.9902
Diet	0.8769	0.8415
Storage time	0.0505	0.2260
Diet*Storage time	0.9969	1.000

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 42 Comparing TBA value in the experimental diets of Group A between two subgroups which without and with antioxidant storage under different temperature until trial periods.

Experimental diets	TBA		
	Without antioxidant	Antioxidant	P-value
Room temperature			
Diet 1: control diet	5.22 ± 0.25	5.28 ± 0.62	1.00
Diet 2: astaxanthin 25 mg/kg	5.43 ± 0.39	5.15 ± 0.63	0.65
Diet 3: lutein 25 mg/kg	5.43 ± 0.22	5.33 ± 0.59	0.67
Diet 4: β-carotene 25 mg/kg	5.61 ± 0.24	5.19 ± 0.49	0.79
Diet 5: Lutein:β-carotene 25:25 mg/kg	5.34 ± 0.26	5.31 ± 0.48	0.55
Diet 6: Lutein:β-carotene 50 mg/kg	5.40 ± 0.38	5.31 ± 0.27	0.82
Cool temperature at 4 °C			
Diet 1: control diet	5.32 ± 0.28	5.16 ± 0.38	0.32
Diet 2: astaxanthin 25 mg/kg	5.41 ± 0.47	5.04 ± 0.57	0.89
Diet 3: lutein 25 mg/kg	5.24 ± 0.14	5.26 ± 0.41	0.38
Diet 4: β-carotene 25 mg/kg	5.41 ± 0.26	5.04 ± 0.65	0.24
Diet 5: Lutein:β-carotene 25:25 mg/kg	5.26 ± 0.36	5.23 ±0.38	0.37
Diet 6: Lutein:β-carotene 50 mg/kg	5.31 ± 0.37	5.25 ± 0.26	0.91

Mean with the different letters in same row are significantly different at $p \leq 0.05$

The analysis of TBA value in diets group B at 0, 4 and 8 weeks period shows the same TBA value as diets in group A. TBA in diets with and without antioxidant kept at room temperature found that TBA in diets that no antioxidant is higher according to the longer time kept. The longer period kept, the higher TBA ($p \leq 0.05$) (shown in **Table 43**). For the one with antioxidant, the longer period kept does no effect to TBA value ($p \geq 0.05$). Diets kept at 4 °C with and without antioxidant also have the same result which TBA values does not change to become higher due to the longer period kept (shown in **Table 44**). To conclude, keeping carotenoid diets in both group A and B are no effect in the TBA value in both cases that diets are contained with and without antioxidant during trial period (shown in **Table 45**). Therefore, carotenoids diet stored at 4 °C is more effective in slowing down the

rancid than being kept at room temperature. Also, BHT added at 250 mg/kg in caroteniods diets affected the slowing rancidity, even being kept at room temperature.

Table 43 TBA value of experimental diets of Group B, storage under room temperature for 8 weeks.

Experimental diets	TBA	
	Without antioxidant	Antioxidant
Room temperature		
Diet 1: control diet	5.22 ±0.25	5.28 ± 0.62
Diet 2: lutein 25 mg/kg	5.43 ± 0.22	5.33 ± 0.59
Diet 3: β-carotene 25 mg/kg	5.61 ± 0.24	5.19 ± 0.49
Diet 4: TC of tea extract 25 mg/kg	5.42 ± 0.24	5.18 ± 0.71
Diet 5: TC of mulberry extract 25 mg/kg	5.21 ± 0.38	5.28 ± 0.36
Diet 6: TC of cassava extract 25 mg/kg	5.12 ± 0.18	5.28 ± 0.49
Diet 7: TC of tea leaves 25 mg/kg	5.27 ± 0.17	5.35 ± 0.37
Diet 8: TC of mulberry leaves 25 mg/kg	5.38 ± 0.53	5.25 ± 0.40
Diet 9: TC of cassava leaves 25 mg/kg	5.28 ± 0.22	5.20 ± 0.47
Storage time		
0 week	5.08c	4.97b
4 weeks	5.33b	5.26ab
8 weeks	5.56a	5.55a
P-value	0.0261	0.9399
Diet	0.0636	0.9995
Storage time	0.0001	0.0049
Diet*Storage time	1.000	0.9999

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 44 TBA value of experimental diets of Group A, storage under cool temperature at 4 °C room temperature for 8 weeks.

Experimental diets	TBA	
	Without antioxidant	Antioxidant
Cool temperature at 4 °C		
Diet 1: control diet	5.32 ± 0.28	5.16 ± 0.38
Diet 2: lutein 25 mg/kg	5.24 ± 0.14	5.26 ± 0.41
Diet 3: β-carotene 25 mg/kg	5.41 ± 0.26	5.02 ± 0.65
Diet 4: TC of tea extract 25 mg/kg	5.15 ± 0.32	5.14 ± 0.68
Diet 5: TC of mulberry extract 25 mg/kg	5.18 ± 0.27	5.26 ± 0.77
Diet 6: TC of cassava extract 25 mg/kg	5.05 ± 0.19	5.17 ± 0.48
Diet 7: TC of tea leaves 25 mg/kg	5.18 ± 0.29	5.20 ± 0.28
Diet 8: TC of mulberry leaves 25 mg/kg	5.31 ± 0.47	5.12 ± 0.33
Diet 9: TC of cassava leaves 25 mg/kg	5.17 ± 0.19	5.16 ± 0.46
Storage time		
0 week	5.08	4.97
4 weeks	5.25	5.17
8 weeks	5.33	5.36
P-value	0.1517	0.9991
Diet	0.3784	0.9971
Storage time	0.0142	0.0639
Diet*Storage time	0.3482	1.000

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 45 Comparing TBA value in the experimental diets of Group B between two subgroups which without and with antioxidant storage under different temperature until trial periods.

Experimental diets	TBA (mg/kg)		
	Without antioxidant	Antioxidant	P-value
Room temperature			
Diet 1: control diet	5.22 ±0.25	5.28 ± 0.62	1.00
Diet 2: lutein 25 mg/kg	5.43 ± 0.22	5.33 ± 0.59	0.67
Diet 3: β-carotene 25 mg/kg	5.61 ± 0.24	5.19 ± 0.49	0.79
Diet 4: TC of tea extract 25 mg/kg	5.42 ± 0.24	5.18 ± 0.71	0.78
Diet 5: TC of mulberry extract 25 mg/kg	5.21 ± 0.38	5.28 ± 0.36	0.39
Diet 6: TC of cassava extract 25 mg/kg	5.12 ± 0.18	5.28 ± 0.49	0.61
Diet 7: TC of tea leaves 25 mg/kg	5.27 ± 0.17	5.35 ± 0.37	0.90
Diet 8: TC of mulberry leaves 25 mg/kg	5.38 ± 0.53	5.25 ± 0.40	0.44
Diet 9: TC of cassava leaves 25 mg/kg	5.28 ± 0.22	5.20 ± 0.47	0.74
Cool temperature at 4 °C			
Diet 1: control diet	5.32 ± 0.28	5.16 ± 0.38	0.32
Diet 2: lutein 25 mg/kg	5.24 ± 0.14	5.26 ± 0.41	0.38
Diet 3: β-carotene 25 mg/kg	5.41 ± 0.26	5.02 ± 0.65	0.24
Diet 4: TC of tea extract 25 mg/kg	5.15 ± 0.32	5.14 ± 0.68	0.50
Diet 5: TC of mulberry extract 25 mg/kg	5.18 ± 0.27	5.26 ± 0.77	0.85
Diet 6: TC of cassava extract 25 mg/kg	5.05 ± 0.19	5.17 ± 0.48	0.18
Diet 7: TC of tea leaves 25 mg/kg	5.18 ± 0.29	5.20 ± 0.28	0.90
Diet 8: TC of mulberry leaves 25 mg/kg	5.31 ± 0.47	5.12 ± 0.33	0.48
Diet 9: TC of cassava leaves 25 mg/kg	5.17 ± 0.19	5.16 ± 0.46	0.44

Mean with the different letters in same row are significantly different at $p \leq 0.05$

The analysis in comparing TBA value of diets with different types and levels of carotenoids by separated them into 2 groups, which are group A and B. The diets are split into two smaller groups which one contains antioxidant and another is without antioxidant (BHT) at 250 mg/kg, each diets group are stored into foil bags being kept in different temperatures; room temperatures (26-28 °C) and at 4 °C for 8 weeks. Randomly collected samples of 0, 4 and 8 weeks period, TBA value is significant higher ($p \leq 0.05$) in diets with no antioxidant added and are kept at room

temperature. Only this group that TBA value is higher according to the longer time kept. This shows that the longer period kept affect the change of higher TBA value. For diets that are kept at room temperature with antioxidant added and the one that is kept as 4 °C (with and without antioxidant), TBA is not significant different ($p \geq 0.05$) during the whole time of experiment so that the longer time period kept affects no change on TBA value within these groups. By analyze the different types and levels of carotenoids supplemented in both group A and B, found that carotenoids types and levels affect no change to the value of TBA. Hence, from the change of TBA value at 250 mg/kg kept at room temperature or 4 °C, even keeping at 4 °C with no BHT added, slow down the rancid in the secondary oxidation better than without BHT and keep at room temperature. According to the analysis of Papas (1999), in animal feed commonly found TBA value contained no more than 20 milligram/kilogram. The diets in this experiment has quite low TBA value which can be concluded that diets with rancid contain low value.

4.3.2 Acid value

A study on the effects of antioxidant (BHT) use at 250 mg/kg in diets that contain different types and levels of carotenoids, the diets are into two groups of experiment which are group A. As the diets in each group are split into two smaller groups which one contains antioxidant and another is without antioxidant. As passed the dry feed production process to become finish feed, store the diets in aluminum foil bag and seal the top with the bag sealer. Then, separate the bags of both diets groups and keep them at different conditions of temperature which are room temperature at 26-28 °C and cool temperature at 4 °C for 8 weeks. Randomly collected samples of a react with free fatty acid. The acid value would show triglyceride level in oil wasted by enzyme to become free fatty acid. In **Table 46** shows acid value in diets group A that contain antioxidant and none at room temperature that it is significant different ($p \leq 0.05$). The acid value is changed accordingly to the keeping period. The longer time period the diets are kept, the higher value of acid ($p \leq 0.05$). It is also found without antioxidant that the time being kept effects the value of acid value, also there was interaction between diet type and storage time ($p \leq 0.05$). As compared between the diets with and without antioxidant, found that the one with antioxidant has lower

acid value during being kept at room temperature. For the diets (with and without antioxidant) that are kept at 4 °C has different statistical acid value which the longer keeping period, the higher acid value ($p \leq 0.05$). The data showed that the diets without antioxidant has higher acid value than with antioxidant ones (shown in **Table 47**). However, carotenoid diets in both group A and B with and without antioxidant is no effect in the acid value during trial period (shown in **Table 48**). Also, the diets with and without antioxidant added kept at 4 °C are well effective in terms of slowing down the rancid than being kept at room temperature. Overall, the carotenoids diets that contain antioxidant added is more efficient to slow down the rancid than the ones that are without during the study for both keeping condition.

Table 46 Acid value of experimental diets of Group A, storage under room temperature for 8 weeks.

Experimental diets	Acid value (mg/kg)	
	Without antioxidant	Antioxidant
Room temperature		
Diet 1: control diet	32.90 ± 2.12b	28.42 ± 1.48b
Diet 2: astaxanthin 25 mg/kg	30.35 ± 0.98c	30.96 ± 1.16a
Diet 3: lutein 25 mg/kg	34.49 ± 1.45a	31.31 ± 1.79a
Diet 4: β-carotene 25 mg/kg	33.37 ± 0.94ab	31.58 ± 0.82a
Diet 5: Lutein:β-carotene 25:25 mg/kg	34.26 ± 1.19ab	32.11 ± 0.68a
Diet 6: Lutein:β-carotene 50 mg/kg	33.20 ± 1.02ab	28.75 ± 1.72b
Storage time		
0 week	30.26c	29.52b
4 weeks	32.49b	29.84b
8 weeks	36.53a	32.16a
P-value	0.0001	0.0001
Diet	0.0001	0.0001
Storage time	0.0001	0.0001
Diet*Storage time	0.0004	0.3257

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 47 Acid value of experimental diets of Group A, storage under cool temperature at 4 °C room for 8 weeks.

Experimental diets	Acid value (mg/kg)	
	Without antioxidant	Antioxidant
Cool temperature at 4 °C		
Diet 1: control diet	28.49 ± 0.84c	26.60 ± 1.89c
Diet 2: astaxanthin 25 mg/kg	29.41 ± 0.49bc	28.51 ± 1.74bc
Diet 3: lutein 25 mg/kg	31.52 ± 0.68a	28.97 ± 1.06b
Diet 4: β-carotene 25 mg/kg	29.34 ± 1.77bc	31.04 ± 0.87a
Diet 5: Lutein:β-carotene 25:25 mg/kg	30.83 ± 1.95ab	31.28 ± 0.83a
Diet 6: Lutein:β-carotene 50 mg/kg	30.08 ± 1.44abc	28.38 ± 1.83bc
Storage time		
0 week	28.88b	28.19b
4 weeks	30.26a	29.52ab
8 weeks	30.69a	29.96a
P-value	0.0251	0.0040
Diet	0.0042	0.0004
Storage time	0.0049	0.0449
Diet*Storage time	0.9573	0.2936

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 48 Comparing Acid value content in the experimental diets of Group A between two subgroups which without and with antioxidant storage under different temperature until trial periods.

Experimental diets	Acid value (mg/kg)		
	Without antioxidant	Antioxidant	P-value
Room temperature			
Diet 1: control diet	32.90 ± 2.12	28.42 ± 1.48	0.27
Diet 2: astaxanthin 25 mg/kg	30.35 ± 0.98	30.96 ± 1.16	0.92
Diet 3: lutein 25 mg/kg	34.49 ± 1.45	31.31 ± 1.79	0.46
Diet 4: β-carotene 25 mg/kg	33.37 ± 0.94	31.58 ± 0.82	0.06
Diet 5: Lutein:β-carotene 25:25 mg/kg	34.26 ± 1.19	32.11 ± 0.68	0.37
Diet 6: Lutein:β-carotene 50 mg/kg	33.20 ± 1.02	28.75 ± 1.72	0.87
Cool temperature at 4 °C			
Diet 1: control diet	28.49 ± 0.84	26.60 ± 1.89	0.75
Diet 2: astaxanthin 25 mg/kg	29.41 ± 0.49	28.51 ± 1.74	0.47
Diet 3: lutein 25 mg/kg	31.52 ± 0.68	28.97 ± 1.06	0.25
Diet 4: β-carotene 25 mg/kg	29.34 ± 1.77	31.04 ± 0.87	0.61
Diet 5: Lutein:β-carotene 25:25 mg/kg	30.83 ± 1.95	31.28 ± 0.83	0.68
Diet 6: Lutein:β-carotene 50 mg/kg	30.08 ± 1.44	28.38 ± 1.83	0.89

Mean with the different letters in same row are significantly different at $p \leq 0.05$

The compared analysis for acid value in diets group B that are kept at room temperature and at 4 °C at time 0, 4 and 8weeks period, found similar acid value as group A. As experimental period for both diets with and without antioxidant that are kept at room temperature, the acid values are significant different ($p \leq 0.05$) as shown in **Table 49**. The acid values become higher due to the longer keeping period ($p \leq 0.05$). Nevertheless, the diets with antioxidant added has lower acid value than the ones that has no antioxidant added during being kept at room temperature. For the experiment of diets kept at 4 °C for both with and without antioxidant, show the same result value as the previous ones ($p \leq 0.05$) which could be said that the longer the diets are kept, the higher acid value ($p \leq 0.05$) (shown in **Table 50**). The results found that carotenoid diets in both group A and B with and without antioxidant is no effect

in the acid value during trial period (shown in **Table 51**). Apparently, the diets with and without antioxidant have similar values. Though, the value is lesser than the ones that are kept at room temperature. Overall, the diets kept at 4 °C (with and without antioxidant) show more effectiveness in slowing down the rancid than the one kept at room temperature. It is also found that the carotenoids diets in the experiment that added antioxidant during time 0, 4 and 8 weeks period for both keeping conditions (kept at room temperature and at 4 °C) are more effective to slow down of rancid than the ones that only have antioxidant added.

Table 49 Acid value of experimental diets of Group B, storage under room temperature for 8 weeks.

Experimental diets	Acid value (mg/kg)	
	Without antioxidant	Antioxidant
Room temperature		
Diet 1: control diet	32.90 ± 2.12	28.42 ± 1.48d
Diet 2: lutein 25 mg/kg	34.49 ± 1.45	31.31 ± 1.80ab
Diet 3: β-carotene 25 mg/kg	33.37 ± 0.94	31.58 ± 0.82ab
Diet 4: TC of tea extract 25 mg/kg	33.25 ± 1.94	31.55 ± 1.11ab
Diet 5: TC of mulberry extract 25 mg/kg	33.57 ± 2.17	30.69 ± 0.93c
Diet 6: TC of cassava extract 25 mg/kg	32.48 ± 1.28	29.78 ± 1.54c
Diet 7: TC of tea leaves 25 mg/kg	33.27 ± 2.04	31.86 ± 0.97b
Diet 8: TC of mulberry leaves 25 mg/kg	34.11 ± 2.45	32.19 ± 1.28 a
Diet 9: TC of cassava leaves 25 mg/kg	35.04 ± 2.21	32.58 ± 1.31a
Storage time		
0 week	29.03c	28.96c
4 weeks	34.17b	30.98b
8 weeks	37.62a	33.38a
P-value	0.0001	0.0001
Diet	0.3391	0.0001
Storage time	0.0001	0.0001
Diet*Storage time	0.4168	0.0041

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 50 Acid value of experimental diets of Group B, storage under cool temperature at 4 °C room for 8 weeks.

Experimental diets	Acid value (mg/kg)	
	Without antioxidant	Antioxidant
Cool temperature at 4 °C		
Diet 1: control diet	28.49 ± 0.84cd	26.60 ± 1.89d
Diet 2: lutein 25 mg/kg	31.52 ± 0.68a	28.97 ± 1.06bc
Diet 3: β-carotene 25 mg/kg	29.34 ± 1.77bcd	31.04 ± 0.87ab
Diet 4: TC of tea extract 25 mg/kg	29.00 ± 1.02bcd	28.79 ± 1.89c
Diet 5: TC of mulberry extract 25 mg/kg	29.92 ± 0.89b	29.21 ± 1.69abc
Diet 6: TC of cassava extract 25 mg/kg	28.11 ± 1.34d	28.89 ± 2.00c
Diet 7: TC of tea leaves 25 mg/kg	29.62 ± 0.88bc	29.65 ± 0.85abc
Diet 8: TC of mulberry leaves 25 mg/kg	29.25 ± 1.27bcd	30.34 ± 1.65abc
Diet 9: TC of cassava leaves 25 mg/kg	31.80 ± 0.64a	31.19 ± 1.64a
Storage time		
0 week	29.03b	28.96b
4 weeks	28.99b	28.94b
8 weeks	30.99a	30.78a
P-value	0.0001	0.0024
Diet	0.0001	0.0005
Storage time	0.0001	0.0035
Diet*Storage time	0.0235	0.2959

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Table 51 Comparing Acid value content in the experimental diets of Group B between two subgroups which without and with antioxidant storage under different temperature until trial periods.

Experimental diets	Acid value (mg/kg)		
	Without antioxidant	Antioxidant	P-value
Room temperature			
Diet 1: control diet	32.90 ± 2.12	28.42 ± 1.48	0.27
Diet 2: lutein 25 mg/kg	34.49 ± 1.45	31.31 ± 1.80	0.46
Diet 3: β-carotene 25 mg/kg	33.37 ± 0.94	31.58 ± 0.82	0.06
Diet 4: TC of tea extract 25 mg/kg	33.25 ± 1.94	31.55 ± 1.11	0.85
Diet 5: TC of mulberry extract 25 mg/kg	33.57 ± 2.17	30.69 ± 0.93	0.35
Diet 6: TC of cassava extract 25 mg/kg	32.48 ± 1.28	29.78 ± 1.54	0.65
Diet 7: TC of tea leaves 25 mg/kg	33.27 ± 2.04	31.86 ± 0.97	0.68
Diet 8: TC of mulberry leaves 25 mg/kg	34.11 ± 2.45	32.19 ± 1.28	0.22
Diet 9: TC of cassava leaves 25 mg/kg	35.04 ± 2.21	32.58 ± 1.31	0.53
Cool temperature at 4 °C			
Diet 1: control diet	28.49 ± 0.84	26.60 ± 1.89	0.75
Diet 2: lutein 25 mg/kg	31.52 ± 0.68	28.97 ± 1.06	0.25
Diet 3: β-carotene 25 mg/kg	29.34 ± 1.77	31.04 ± 0.87	0.61
Diet 4: TC of tea extract 25 mg/kg	29.00 ± 1.02	28.79 ± 1.89	0.53
Diet 5: TC of mulberry extract 25 mg/kg	29.92 ± 0.89	29.21 ± 1.69	0.33
Diet 6: TC of cassava extract 25 mg/kg	28.11 ± 1.34	28.89 ± 2.00	0.32
Diet 7: TC of tea leaves 25 mg/kg	29.62 ± 0.88	29.65 ± 0.85	0.62
Diet 8: TC of mulberry leaves 25 mg/kg	29.25 ± 1.27	30.34 ± 1.65	0.36
Diet 9: TC of cassava leaves 25 mg/kg	31.80 ± 0.64	31.19 ± 1.64	0.65

Mean with the different letters in same row are significantly different at $p \leq 0.05$

The compared analysis for acid value of diets contain different types and levels of carotenoids, the diets are divided into two groups which are group A and B. The diets added with the of 250 mg/kg. BHT and without are stored into aluminum foil bags and are kept in different temperatures which are at room temperature of 26-28 °C and at 4 °C for 8 weeks. Randomly collected diets samples at time 0, 4 and 8 weeks period, found that acid value significant become higher ($p \leq 0.05$) in both group A and B. It indicates that over time, acid value will become higher according to

the fat in diets causes in hydrolytic rancidity and produces free fatty acid. The report from Papas (1999) reports that fat in diet normally contain not over than 70 milligram/gram so that the diets being kept during 8 weeks in this experiment does not has rancidity yet as it has the acid value of 26.60-35.04 milligram/gram. BHT added at 250 mg/kg being kept at room temperature or at 4 °C limits the growth of hydrolytic rancidity well (Myrna and Precilla,1999).

The result on additional amount of antioxidant (BHT) at 250 mg/kg in fancy carp diets which contain different types and levels of carotenoids, the diets in both group A and B have hydrolytic rancidity. From the analysis for acid value, the feeds in the experiment have higher value according to the longer keeping period. It shows that the additional amount of BHT at 250 mg/kg kept at room temperatures and at 4 °C of cool temperature help limiting the hydrolytic rancidity (Myma and Precilla, 1999). Moreover, the diets kept at 4 °C with no additional of BHT would cause the free fatty acid which lead to rancidity. For oxidative rancidity, the analysis is based on the TBA value in the secondary oxidation of malonaldehyde which TBA value would increase according to the longer keeping time. The change in TBA value shows that BHT added at 250 mg/kg kept at room temperature and at 4 °C or without BHT added with the same cool temperature, have better effect the secondary oxidation than being kept at room temperature without BHT added.

Thus, the additional amount of BHT at 250 mg/kg helps reducing of hydrolytic rancidity and oxidation rancidity. By keeping the carotenoids diets in cool temperatures would also help slowing down the rancidity. The diets need no additional of antioxidant. However, antioxidant added would be convenience with energy saved before being feed processed. It can be kept at normal room temperature but it should be away from sunlight, air and heat which cause rancidity. The experiment result also show that the acid value and TBA in all group diets contain of rancidity value lower than the lethal dose, hence these diets suitable for fish. Too much rancidity would cause free radical or peroxide, hydroperoxide, aldehyde and ketone which these compounds can be found easily in foods with rancidity (Mai and Kinsella,

1979). These compounds would also effect in feed nutrition such as Vitamins, Protein or fats and reduce the digestibility (Cockerell and Holiday, 1975). It slows down the growth performance, reduces the nutrient utilization and may also increase the chance of diseases from feeding animals with diet contained rancidity. In order to slow down the oxidation, it is required to add ethoxyqui, butylated hydroxytoluene (BHT) and butylated hydroxyanisol (BHA) in diets for rancidity protection. It also suggests that the diets contain carotenoids should be kept at low temperature with good storage condition. Avoids the conditions that would cause oxidation by keeping the diets away from air, moist and sunlight or adding antioxidant like BHT to protect the carotenoid during feed process would also help slowing down oxidation which would not affect the change of nutritional in diets. Finally, aquaculture product quality will be the focus of increased attention, notably nutrition attributes and establishment of optimal processing and storage conditions for more effective postharvest utilization. Furthermore, feed in the experiment that are kept at 4 °C with no additional BHT would also help in slowing down the free fatty acid which would cause the of rancid as well.

Part 5 Potential Use of Natural Carotenoids Source on Growth Performance, Skin Pigmentation and Immune Response in Fancy Carp (*Cyprinus carpio*)

1. Carotenoids concentrations of the tested diets

Total carotenoids (TC) concentration including the major carotenoids in the test diets are presented in **Table 52**. The diets were designed to achieve a target level of 25 mg/kg for experimental diet. Three diets formulated with crude carotenoid extraction from tea, mulberry and cassava leaves; and three diets formulated with raw material of them while a diet without supplemented carotenoid served as a control. Analyzing total carotenoids contained in each diet found that the diets have total carotenoid contained at 25.50 ± 3.37, 26.59 ± 2.34, 25.06 ± 2.61, 25.60 ± 0.70, 23.38 ± 4.38 and 24.36 ± 2.71 mg/kg, respectively and non supplemented carotenoid diet as a control was 5.70 ± 0.27 mg/kg on a dry weight basis. The detailed of carotenoids compositions in experimental diets were identified as lutein, β-carotene and small amount of other pigments by TLC-densitometric analysis on silica gel with Petroleum Ether-Diethyl Ether-Acetone (75:15:10, *v/v/v*) as mobile phase. The dominant carotenoids compositions in tested diets were lutein and β-carotene. Retention factor (R_f) averaged 0.19, 0.22 and 0.19 identical to lutein standard in tea, mulberry and cassava leaves, respectively. And the least polar zone had an R_f average at 0.98, 0.98 and 0.96 identical to a β-carotene standard. Lutein content was 20.14 ± 0.79, 18.49 ± 1.45, 15.57 ± 1.42, 11.68 ± 1.16, 10.93 ± 1.51, 11.66 ± 0.99 and 2.58 ± 0.55 mg/kg, respectively, and β-carotene was 10.21 ± 1.19, 9.19 ± 1.05, 10.23 ± 1.00, 9.86 ± 1.14, 7.28 ± 0.78, 9.53 ± 1.57 and 2.28 ± 0.28 mg/kg, respectively.

Table 52 Carotenoids concentration in experimental diets.

Experimental diet	Total carotenoid (mg/kg)	Carotenoids composition (mg/kg)	
		Lutein	β-carotene
Control	5.70 ± 0.27[b]	ND[d]	ND[c]
Crude extraction			
Tea leaves	25.50 ± 3.37[a]	20.14 ± 0.79[a]	10.21 ± 1.19[a]
Mulberry leaves	26.59 ± 2.34[a]	18.49 ± 1.45[a]	9.19 ± 1.05[a]
Cassava leaves	25.06 ± 2.61[a]	15.57 ± 1.42[b]	10.23 ± 1.00[a]
Raw material			
Tea leaves	25.60 ± 0.70[a]	11.68 ± 1.16[c]	9.86 ± 1.14[a]
Mulberry leaves	23.38 ± 4.38[a]	10.93 ± 1.51[c]	7.28 ± 0.78[b]
Cassava leaves	24.36 ± 2.71[a]	11.66 ± 0.99[c]	9.53 ± 1.57[a]
P-value	0.0001	0.0001	0.0001

Mean with the different letters in same column are significantly different at $p \leq 0.05$
Note: ND means Not detected

In this study, the main carotenoids composition in carotenoid enriched diets with crude carotenoid extracted from tea, mulberry and cassava leaves, and raw materials of them showed main carotenoids compositions two pigments; lutein and β - carotene as shown in **Table 52**. Hence, the data from this study related with the results of Muller (1997) reported that lutein and β-carotene were the predominant carotenoids in all green leaves. Similar with Gross (1991) showed that the leaves of virtually all species contain the same main carotenoids, that is β-carotene (usually 25-30% of the total), lutein (around 45%), violaxanthin (15%) and neoxanthin (15%). Small amounts of α-carotene, α and β- cryptoxanthin, zeaxanthin, antheraxanthin and lutein-5, 6-epoxide are also frequently present and lactucaxanthin is a major xanthophylls in a few species.

2. Growth performance

Growth parameters of fish were shown in **Table 53**. Indicate that there were no significant differences in mean final weight, weight gain ($p \geq 0.05$). Nevertheless, the higher final body weight gain was found in fish were feed with the diets supplement with mulberry and cassava leaves including carotenoid extracted from mulberry leaves. In term of feed efficiency, the data showed that there were also no significant differences in feed conversion ratio (FCR) ($p \geq 0.05$) among all groups. All fish grew normally, and no specific signs of disease were observed. No mortality occurred throughout the experiment. Feed intakes of all treatments showed no significant differences ($p \geq 0.05$), and the data of this study showed that total carotenoid intake is increased intake dependent on dietary carotenoids concentration compared with the contaol group that the diet without carotenoid supplemented.

Table 53 Growth performance parameters of fancy carp fed with experimental diets supplemented with carotenoid for rearing period of 6 weeks.

Experimental diets	Rearing parameter					
	WG	ADG	FCR	SR	FI	TC intake
1	26.58 ± 3.75	0.64 ± 0.09	4.28 ± 0.46	100 ± 00	2.68 ± 0.10	0.02 ± 0.001[b]
2	26.37 ± 1.44	0.59 ± 0.08	4.42 ± 0.54	100 ± 00	2.56 ± 0.10	0.08 ± 0.009[a]
3	28.87 ± 2.21	0.69 ± 0.17	4.23 ± 0.83	100 ± 00	2.82 ± 0.18	0.09 ± 0.015[a]
4	22.20 ± 3.53	0.50 ± 0.06	5.63 ± 0.64	100 ± 00	2.78 ± 0.12	0.09 ± 0.005[a]
5	25.95 ± 5.30	0.49 ± 0.18	6.31 ± 2.36	100 ± 00	2.76 ± 0.04	0.09 ± 0.002[a]
6	27.20 ± 3.53	0.68 ± 0.20	4.45 ± 1.17	100 ± 00	2.87 ± 0.35	0.08 ± 0.010[a]
7	27.20 ± 5.40	0.65 ± 0.13	4.53 ± 1.31	100 ± 00	2.81 ± 0.16	0.08 ± 0.005[a]
p-value	0.848	0.280	0.170	-	0.292	0.0005

Mean with the different letters in same colunm are significantly different at $p \leq 0.05$

Note: WG: Weight gain (g) ADG: Average daily gain (g/fish/day)

FCR: Feed conversion ratio (FCR) SR: Survival (%)

FI: Feed intake (g/fish/day) TC intake: Total carotenoid intake (mg/fish/day)

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

In this study, neither growth nor feed conversion efficiency were affected significantly by dietary treatment for all treatments diets. Growth parameters, namely weight gain, feed conversion ratio and survival were similar ($p \geq 0.05$). This agrees with the study of Bell *et al*. (2000), in which no effects of dietary supplement of astaxanthin 70 mg/kg were found on the growth of Atlantic salmon (*Salmo salar*) reared for 22 weeks. Baron *et al*. (2008) fed flame-red dwarf gourami with synthetic astaxanthin, red beet which contains betalain and diet supplemented with black carrots which contains anthocyanin found no difference in growth performance. Amar *et al*. (2001 and 2004) also found that there were no differences in growth and feeding rates among rainbow trout fed astaxanthin and β-carotene including *Dunaliella salina* and *Phaffia rhodozyma* between the treatment groups and the control group. In the study by Gouveia *et al*. (2003), growth and feed efficiency of koi carp and gold fish were not different when fed with diets supplement with C. *vulgaris*, H. *pluvialis*, and *Arthrospira maxima*. Normally growth, feed efficiency and no mortality found in gilthead seabream when fed with diets supplement with H. pluvialis and diets supplemented with synthetic astaxanthin (Gomes *et al*., 2002). In the study by Phromkunthong *et al*. (2007), growth and survival of sex-reversed red tilapia were not different when fed with diets supplement with spirulina used as natural carotenoids source. This agree with the study of Kop and Durmaz, (2007) reported that Cichlids fed with *Porphyridium cruentum* (Rodophyta) as a natural carotenoid source compare with astaxanthin and β-carotene at 50 mg/kg had no statistical difference among all groups in terms of both final body weight and final total length, alike with the report of Harpaz and Padowicz (2007) showed that dwarf cichlid fed with paprika as carotenoid source had no effect on the growth rate and survival. Wang *et al*., (2006) reported that no mortality and growth of characins, an oranamental fish after fed with astaxanthin, β-carotene including combination of astaxanthi and β-carotene (1:1). Similar results had already been obtained by Tejera *et al*. (2007), feeding a diet containing commercial of astaxanthin fed red porgy, the growth rate and the survival of fish were not affected by dietary treatment. Many researches are supported our results that various sources of dietary carotenoid did not affect growth and survival of fancy carp.

3. Serum total carotenoid

The spectrophotometer analysis was made for the serum total carotenoid (TC) change of fancy carp, which were fed on the feed that included different dietary carotenoids. Results on the serum total carotenoid of fancy carp showed that the various dietary carotenoid supplements significantly increased the serum total carotenoid ($p \leq 0.05$) when compared to the serum carotenoid of fish fed control diet (**Table 54**). The increased serum total carotenoid concentration found in the serum after six weeks of feeding demonstrated that fancy carp was able to utilize efficiently the dietary carotenoids.

Table 54 Total serum carotenoids (μg/ml serum) of fancy carp fed with experimental diets over trial period.

Experimental diets	Time			
	Week2	Week4	Week6	Week8*
1	1.11 ± 0.47^{b}	0.95 ± 0.34^{d}	0.93 ± 0.22^{b}	0.86 ± 0.20^{c}
2	4.28 ± 0.97^{a}	5.61 ± 1.65^{bc}	6.89 ± 1.33^{a}	1.30 ± 0.10^{bc}
3	4.60 ± 1.66^{a}	5.47 ± 0.58^{bc}	6.20 ± 4.03^{a}	0.88 ± 0.10^{c}
4	5.12 ± 1.23^{a}	7.58 ± 0.29^{ab}	7.97 ± 0.48^{a}	1.80 ± 0.30^{b}
5	5.15 ± 0.51^{a}	7.98 ± 1.21^{a}	9.31 ± 1.50^{a}	2.74 ± 0.83^{a}
6	4.00 ± 0.45^{a}	4.53 ± 1.22^{c}	7.31 ± 1.66^{a}	1.52 ± 0.34^{b}
7	4.37 ± 0.25^{a}	5.73 ± 1.91^{bc}	7.67 ± 2.20^{a}	1.53 ± 0.41^{bc}
p-value	0.0016	0.0001	0.0027	0.0001

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Note: * discontinue dietary supplementation with carotenoids

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

At the beginning of the experiment, while total carotenoid level in serum of fish was 0.83 ± 0.18 μg/ml, this value rose significantly at the end of the experiment to 6.20-9.31 μg/ml in fish fed diets containing carotenoid. On the other hand, the total carotenoid content of the control group was 0.86-1.11 μg/ml in the same period throughout the experiment. The results are shown in **Figure 30**, at the end of the six weeks experimental period, serum total carotenoids in fish fed with enriched carotenoid diets were higher compared to the control group ($p \leq 0.05$). Serum TC of fish fed with carotenoid enriched diets for 6 weeks showed increased intake dependent on dietary carotenoid concentration compared with the control group. On the other hand, the serum TC concentration of fish fed on carotenoid diets were similar, but the group of fish fed with raw material of tea leaves had a slightly higher serum total carotenoid content than among those groups. Serum total carotenoid was 9.31 ± 1.50 μg/ml.

After six weeks of feeding the experimental diets the fish were fed with a diet without carotenoid contained TC 5.70 ± 0.27 mg/kg for two weeks. All experimental diets groups showed a numerical decrease after discontinuation of the treatment feeding. The results showed that serum total carotenoid concentrations were strongest for the fish fed with tea leaves group. The results indicated that, fish fed with tea leaves could maintain serum total carotenoid level better than the other group ($p \leq 0.05$). In fish fed the diet supplemented with tea leaves was present of serum total carotenoid 2.74 ± 0.83 μg/ml.

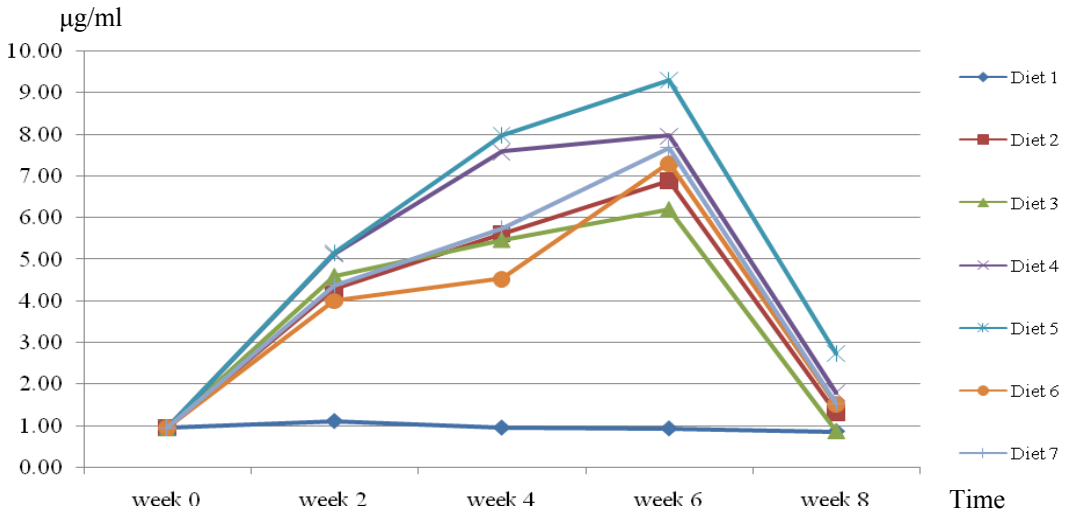


Figure 30 Changes in total carotenoid in serum of fancy carp fed with different diets throughout the experiment period.

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

4. Detailed carotenoid composition

Astaxanthin, lutein and β-carotene were indentified and quantified in the blood serum samples by TLC-densitometric analysis. Data on the detailed serum carotenoid composition of fancy carp fed the various experimental diets for 6 weeks is reported in **Table 55**. After 6 weeks of feeding, carotenoid content in serum of fish fed with carotenoids supplemented diets were found of astaxanthin, lutein and β-carotene. All serum carotenoid content in serum of control fish were significantly lower than the fish fed pigmented diets. Fish fed with diet supplemented with carotenoid enriched diets had higher serum astaxanthin concentration ($p \leq 0.05$) than the control group. The data showed that differences in the total astaxanthin concentration (µg/ml) were found ($p \leq 0.05$) in serum of all fancy carp were fed with pigmented diets but not found in fish fed with basal diet that without carotenoid supplemented. Astaxanthin concentration in the serum were 1.45 ± 0.17, 1.03 ± 0.51, 0.75 ± 0.85, 1.07 ± 0.84, 1.13 ± 0.11 and 1.41 ± 0.15 µg/ml, respectively. Lutein concentration in the serum

was higher ($p \leq 0.05$) for fish fed with diet supplemented with tea leaves carotenoids extraction including raw material of tea and cassava leaves by 3.44 ± 0.33, 3.70 ± 0.41 and 3.32 ± 0.38 μg/ml, respectively. On the other hand, serum β-carotene content did not increase with increasing dietary carotenoid between carotenoid crude extracted from tea, mulberry and cassava leaves, and their raw material. In this study indicated that dietary carotenoid type did not affect serum β-carotene content. β-carotene content in fish were fed with tested diets was 0.27 ± 0.12, 0.32 ± 0.19, 0.17 ± 0.07, 0.13 ± 0.05, 0.13 ± 0.04 and 0.12 ± 0.04 μg/ml, respectively, but not found in fish fed with basal diet that without carotenoid supplemented.

Table 55 Serum carotenoid components (μg/ml serum) of fancy carp as affected by the different dietary carotenoid sources over trial period.

Experimental diets	Carotenoids components (μg/ml serum)		
	Astaxanthin	Lutein	β-carotene
Fed with carotenoid diets			
1	ND^{c}	0.61 ± 0.08^{c}	ND^{b}
2	1.45 ± 0.17^{a}	3.44 ± 0.33^{a}	0.27 ± 0.12^{a}
3	1.03 ± 0.51^{ab}	2.00 ± 0.6^{b}	0.32 ± 0.19^{a}
4	0.75 ± 0.58^{b}	2.23 ± 0.42^{b}	0.17 ± 0.07^{ab}
5	1.07 ± 0.84^{ab}	3.70 ± 0.41^{a}	0.13 ± 0.05^{ab}
6	1.13 ± 0.11^{ab}	2.50 ± 0.26^{b}	0.13 ± 0.04^{ab}
7	1.41 ± 0.15^{a}	3.32 ± 0.38^{a}	0.12 ± 0.04^{ab}
P-value	0.0001	0.0001	0.0047

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Note: * means fish was discontinue enhancement carotenoids diets and ND means Not detected

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

Table 55 Continued.

Experimental diets	Carotenoids components (μg/ml serum)		
	Astaxanthin	Lutein	β-carotene
Discontued carotenoid diets			
1	ND^{c}	0.59 ± 0.08^{cd}	ND
2	ND^{c}	0.94 ± 0.31^{b}	ND
3	ND^{c}	0.54 ± 0.13^{a}	ND
4	ND^{c}	1.15 ± 0.20^{ab}	ND
5	0.18 ± 0.07^{a}	1.31 ± 0.30^{a}	ND
6	0.13 ± 0.06^{ab}	1.05 ± 0.09^{ab}	ND
7	0.11 ± 0.02^{b}	0.86 ± 0.13^{bc}	ND
P-value	0.0001	0.0002	-

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Note: * means fish was discontinue enhancement carotenoids diets and ND means Not detected

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

Overall, it was found that lutein (range 27.98 to 49.93 % of total carotenoid) was the major carotenoids present in the serum of fancy carp after feeding the experimental diets. Astaxanthin was the second most abundant carotenoid present in the serum of fancy carp comprising 9.41 to 21.04% of the total carotenoids in pigmented diets (**Figure 31**). The data showed that fish were fed with diets supplemented with tea leaves crude extraction, raw material of mulberry and cassava leaves comprise the percentage of serum astaxanthin more than other treatment group.

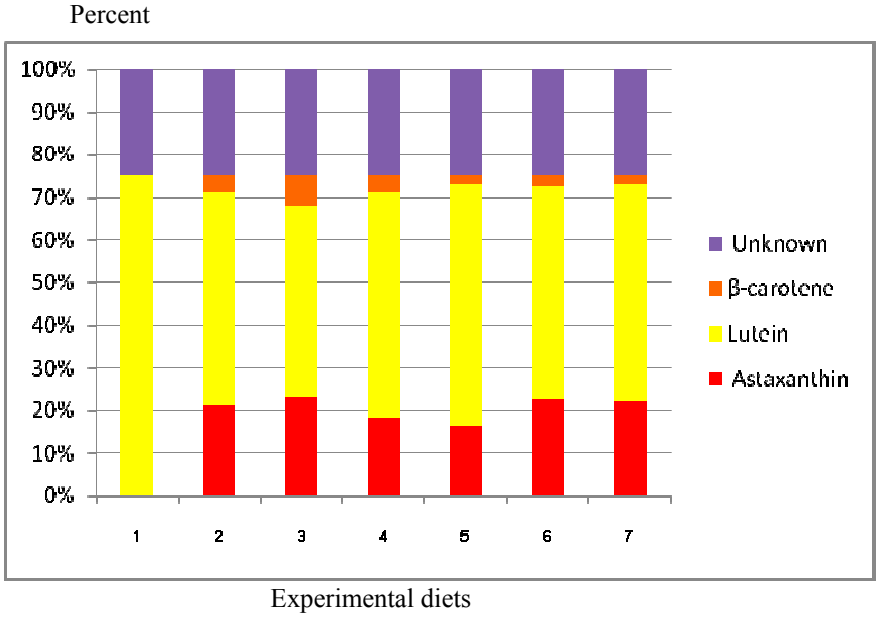


Figure 31 Percentage of serum carotenoid composition in fancy carp as affected by the different dietary carotenoid sources.

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

After discontinuing the experimental diets supplemented with carotenoids, fancy carp were fed with a low carotenoid diet (5.70 ± 0.27 mg/kg) for 2 weeks. Carotenoid dietary supplemented with raw material of tea leaves showed serum astaxanthin higher than other treatments ($p \leq 0.05$), followed by the experimental group fed the diet with mulberry and cassava leaves. The astaxanthin levels in these diets were 0.18 ± 0.07, 0.13 ± 0.06 and 0.11 ± 0.02 µg/ml serum, respectively. The serum lutein of all experimental diets groups showed a numerical decrease after discontinuation of the treatment feeding. The results showed that serum lutein concentrations were strongest for the fish fed with tea leaves group. Overall, the results indicated that, fish fed diet supplemented with raw material of tea leaves could maintain serum TC, astaxanthin and lutein concentration level better than the other group.

In this results showed that various dietary carotenoids supplements increased serum TC content when compared to the pigment content of control group. The highest serum carotenoid content was found in the fish were fed with tea leaves. Plant leaves that was used in this work is a by-product from agriculture, which contained lutein and β-carotene, and the experiment tried to make use of this work is this ingredient. It was observed that is by-product, which was given together with the feed, has an important effect on the color of the skin. The current results indicate that fancy carp were able to utilize lutein and β-carotene from plant leaves efficiently. Similar results were obtained for goldfish but by feeding different natural carotenoid sources, such as red yeast, X. *dendrorhous* (Xu *et al*., 2006), Spirulina (Kiriratnikom *et al*., 2005), C. v*ulgaris*, H. *pluvialis* and A. *maxima* (Gouveia *et al*., 2003; Gouveia and Rema, 2005), alfala (Yanar *et al*., 2008).

Carotenoids are lipid soluble and follow the same absorptive pathways as other dietary lipids. The high lutein content in the serum of fancy carp can also be related to a possible existence of interactions between carotenoids at the intestinal level, which may increase the lutein absorption. However, after six weeks of experimental feeding, the serum carotenoid composition and the dietary carotenoid supply were closely related. This high correlation between plasma carotenoid and dietary carotenoid compositions found in this work for seabream is in accordance with previous results (Choubert *et al*., 1994a and Storebakken and Goswami, 1996). Another type of competition that may occur between carotenoids, is at the level of their blood transport. Carotenoids are transported in the plasma of salmonid fish associated within lipoproteins (Ando *et al*., 1986 and Choubert *et al*., 1994b). In humans, the distribution of carotenoids among different lipoprotein classes has been reported to depend upon the physical properties, presumably the polarity, of the carotenoids: carotenes were found predominantly in the low-density lipoprotein fraction and hydroxyl carotenoids predominantly in the high-density lipoprotein (Furr and Clark, 1997). One may speculate that lipoproteins in the fancy carp might have a greater affinity for lutein than for the other carotenoids, resulting in a faster blood transport and therefore a more efficient deposition.

In this study found that fancy carp could reach its full efficiency in astaxanthin deposition when fish fed with diet supplemented with tea leaves at 25 mg/kg. Serum lutein of fish were fed with the carotenoid diet at this level was significantly higher than other groups ($p \leq 0.05$). The higher lutein in serum indicated that this fish able converted lutein into astaxanthin. Lutein dominates the serum carotenoid in fancy carp, regardless of the carotenoid types in dietary carotenoid. Because the main carotenoids compositions in plant leaves were lutein and β-carotene. From the results of this present study indicated that after fancy carp were fed with plant leaves or crude carotenoid extraction that containing lutein and β-carotene, found astaxanthin level in serum. This result suggested that lutein and β-carotene were oxidized either to astaxanthin. The data shows that fancy carp can convert lutein or β-carotene to astaxanthin. The results of this present study are similar to other researches, the report of Hirao *et al*., (1963) shows gold fish fed diet rich in lutein and β -carotene efficiently converted it to astaxanthin and the report of Hata and Hata (1972) indicated that gold fish fed with diet containing lutein the skin color changed from yellow to orange in 7 days and after 30 days of feeding the skin color progressed to reddish – orange. katayama *et al*., (1973) and Bauernfeind (1981) reported that gold fish and fancy carp can convert lutein to astaxanthin. In Cypriniformes fish, possible oxidative metabolic pathways from zeaxanthin to astaxanthin via β -carotene triols, and tetrols have been proposed (Tsushima *et al*., 1999). Gold fish fed with lutein and zeaxanthin diets proposed the possible metabolic pathways from lutein and zeaxanthin to astaxanthin (Ohkubo *et al*., 1999) indicated that lutein is converted into β-doradexanthin and leading to the formation of astaxanthin by oxidative pathway (Tanaka *et al*., 1976).

Meyers and Chen (1982) classify the aquatic animals into 3 categories by their ability of converting carotenoid into astaxanthin. Type I: salmonoids or seabream type, which cannot oxidize β-ionone of the carotenoid and can only use the oxidized carotenoid. Type II: carp type, which can use and convert lutein, zeaxanthin into astaxanthin and store astaxnthin. Type III: crustacean type, which can convert β-carotene, zeaxanthin, canthaxanthin, and echinenone into astaxnthin. Results from this study show that fancy carp belong to Type II. Dietary carotenoid containing lutein

was convert lutein into astaxanthin and deposit of serum astaxanthin in this fish. Since serum astaxanthin in fish were fed with diet supplemented raw material of plant leaves were not different from those of fish fed in diet supplemented with carotenoid extraction from plant, replacing raw material of dietary in fancy carp diet should be more cost-effective. Since astaxanthin dominated serum carotenoid in this fish and for the highest serum astaxanthin deposition the dietary carotenoid was best at 25 mg/kg, is the most efficient and cost-effective carotenoid formulation.

5. Skin coloration

Skin color assessment was performed by reflectance spectroscopy, in accordance with the system CIE L*a*b* (CIELAB) for lightness, red and yellow respectively (Skrede, 1987). The measurements were performed on red and white positions on the fish bodies (left side).

5.1 Red position

At the end of the six weeks experimental period, color intensity of fancy carp fed with experimental diets is shown in **Table 56**. Following feeding of experimental diets supplemented with carotenoid over six weeks, the results found that the L values had no apparent influence on skin color intensity (L*) of fancy carp ($p \geq 0.05$). Colorings administered in the diet did show a significant effect on redness (a*) and yellow (b*) ($p \leq 0.05$). The group fed the control diet showed a weak redness and yellowness, which differed significantly from values found for group fed the other diets. Although, red tonality was the best in fish fed diet supplemented with tea carotenoid extracted group but this group was similar with the groups fed with diet supplemented with mulberry, cassava carotenoid extracted following the groups fed with raw material of tea and mulberry leaves, red tonality (a*) was 32.76 ± 2.74, 31.04 ± 2.09, 29.78 ± 1.40, 30.94 ± 1.42 and 29.88 ± 1.27, respectively. The results showed that these groups showed the best effect on reddish skin that the other treatments (**Figure 32**). As to yellowness (b*), it was weaker for fish fed the control diet ($p \leq 0.05$) but had significant differences between fish fed the other diets occurred, yellow tonality (b*) of fancy carp fed with cassava carotenoid extracted and

mulberry leaves were higher than for the others but these groups were similar with fish fed with tea and cassava leaves following mulberry and tea carotenoid extracted. The yellowness (b* values) were 40.53 ± 1.40, 41.06 ± 2.92, 40.25 ± 4.37, 38.88 ± 1.86, 37.92 ± 1.63 and 37.08 ± 1.06, respectively.

5.2 White position

The intensity of skin color at white position after fish were fed experimental diets, showed that all diets had no apparent influence on skin color intensity of L* and a* value ($p \geq 0.05$). The yellow tonality (b*) was stronger in a fish fed with carotenoid enriched diets comparing with the control group ($p \leq 0.05$). The b* values were 9.73 ± 0.85, 11.32 ± 2.14, 10.85 ± 1.88, 11.40 ± 1.46, 9.58 ± 0.88 and 10.92 ± 0.82, respectively. The data indicated that white skin of these groups were changed from white hue to yellow-white hue.

Table 56 Body color intensity of fancy carp fed experimental diets over 6 weeks.

Experimental diets	Time		
	L	a	b
Red color			
1	47.88 ± 3.04	21.42 ± 1.61^{c}	35.28 ± 2.89^{c}
2	43.62 ± 1.24	32.76 ± 2.74^{a}	37.08 ± 1.06^{bc}
3	44.40 ± 1.38	31.04 ± 2.09^{ab}	37.92 ± 1.63^{abc}
4	45.37 ± 1.79	29.78 ± 1.40^{ab}	40.53 ± 1.40^{a}
5	45.75 ± 2.82	30.94 ± 1.42^{ab}	40.25 ± 4.37^{ab}
6	45.42 ± 2.25	29.88 ± 1.27^{ab}	41.06 ± 2.92^{a}
7	45.25 ± 0.86	29.12 ± 4.06^{b}	38.88 ± 1.86^{ab}
P-value	0.064	0.0001	0.005

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

Table 56 Continued.

Experimental diets	Time		
	L	a	b
White color			
1	85.72 ± 0.99	1.12 ± 0.48	7.82 ± 0.88[b]
2	85.35 ± 1.70	1.18 ± 0.26	9.73 ± 0.85[a]
3	84.62 ± 0.73	1.28 ± 0.33	11.32 ± 2.14[a]
4	84.55 ± 1.27	1.30 ± 0.33	10.85 ± 1.88[a]
5	85.23 ± 0.96	1.34 ± 0.28	11.40 ± 1.46[a]
6	85.73 ± 1.68	1.12 ± 0.28	9.58 ± 0.88[a]
7	84.13 ± 1.20	1.62 ± 0.66	10.92 ± 0.82[a]
P-value	0.244	0.403	0.001

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

6. Skin fancy carp coloration retention

After fancy carp discontinued enhancement carotenoid diet for two weeks. Fish were fed with a diet without carotenoid supplemented contained TC 5.70 ± 0.27 mg/kg. The measurements were performed on red and white position on fish body (left side), the results are shown in **Table 57**. Once again, skin lightness (L* value) was not influenced positively by the diet. Red tonality (a* value) on red position of all treatment had influence on skin color intensity ($p \leq 0.05$). The a* values during the final week of discontinued feeding of carotenoid-supplemented diets, fancy carp fed with tea leaves and crude carotenoid extraction groups presented retained the red hue longer than other groups. The red tonality (a* value) was 31.82 ± 3.10 and 31.28 ± 2.36, respectively. In contrast, the yellow tonality (b* value) on white position was similar for all experimental groups ($p \geq 0.05$). The data found that, yellow-white hue on white skin areas turned to a whiter hue as serum lutein was decreased after discontinued feeding of carotenoid-supplemented diets (as shown in **Figure 33**).

Table 57 Body color intensity of fancy carp fed with the low carotenoid supplemented diets over final 2 weeks (week 8).

Experimental diets	Time		
	L	a	b
Red color			
1	46.80 ± 3.29	19.20 ± 1.57^{d}	36.98 ± 3.94^{bc}
2	45.80 ± 2.71	31.28 ± 2.36^{ab}	35.38 ± 1.90^{c}
3	47.85 ± 2.27	27.58 ± 2.74^{c}	40.10 ± 1.60^{ab}
4	47.12 ± 2.84	27.50 ± 2.64^{c}	39.87 ± 2.96^{ab}
5	46.77 ± 2.22	31.82 ± 3.10^{a}	41.32 ± 2.81^{a}
6	45.88 ± 3.52	28.35 ± 2.25^{bc}	38.83 ± 3.67^{abc}
7	45.73 ± 0.55	27.84 ± 0.36^{c}	39.03 ± 1.78^{abc}
P-value	0.844	0.0001	0.031
White color			
1	86.23 ± 1.24	1.17 ± 0.40	6.82 ± 0.51
2	85.98 ± 1.36	1.30 ± 0.20	8.08 ± 0.75
3	85.52 ± 0.79	1.35 ± 0.72	7.98 ± 1.33
4	84.92 ± 0.70	1.22 ± 0.84	7.90 ± 1.03
5	85.00 ± 1.63	1.22 ± 0.25	8.36 ± 1.48
6	85.73 ± 1.07	1.23 ± 0.32	7.43 ± 1.01
7	84.27 ± 1.36	1.30 ± 0.35	8.35 ± 1.22
P-value	0.112	0.999	0.270

Mean with the different letters in same column are significantly different at $p \leq 0.05$

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

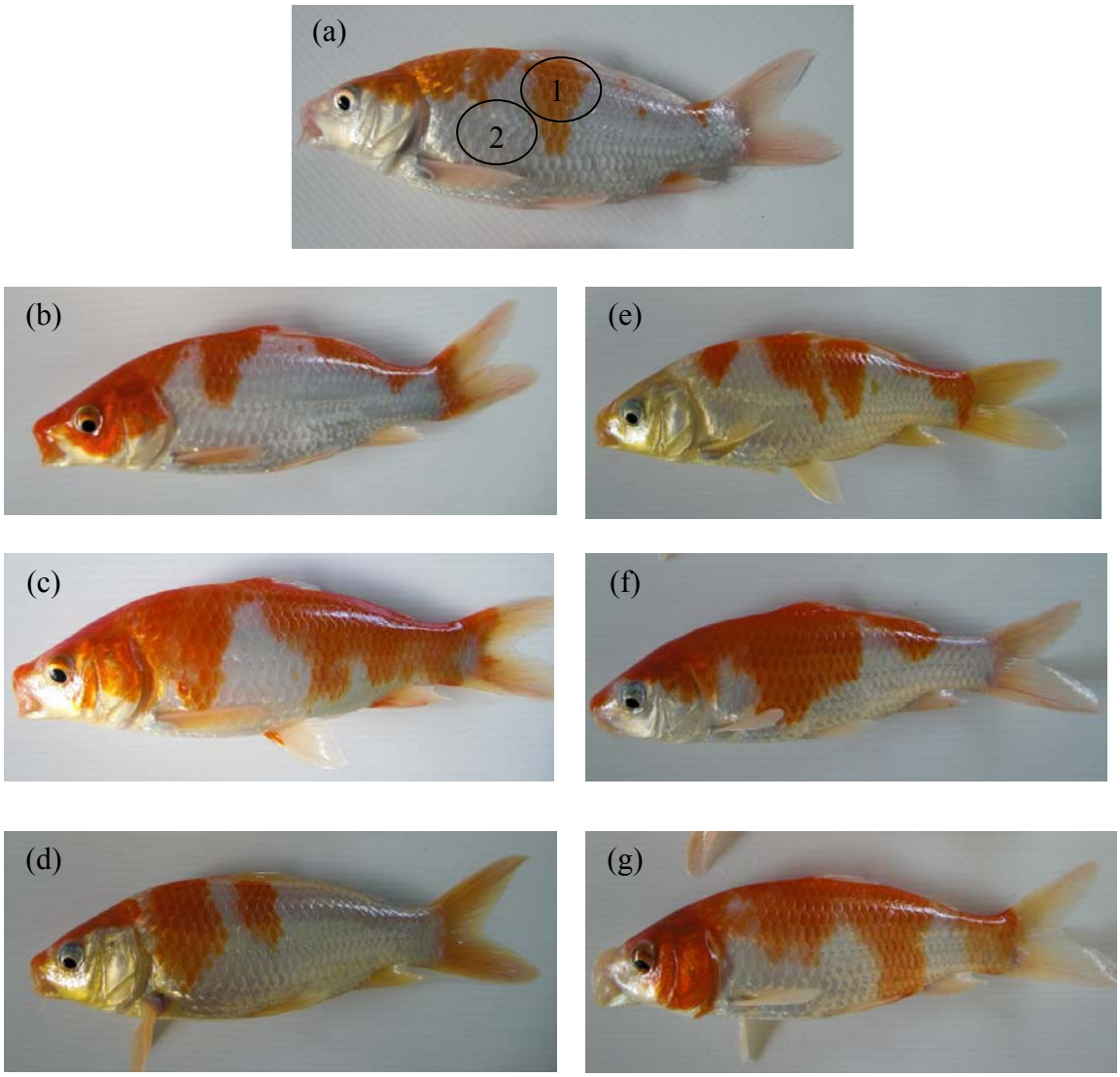


Figure 32 Digital image of fancy carp from each of the experimental diets over 6 weeks: (a) control (also showing sampling areas for color measurement (1) red position and (2) white position where all measurements were taken on the same side for all of the fish used in the experiment), (b) crude tea extraction, (c) crude mulberry extraction, (d) crude cassava extraction, (e) tea leaves, (f) mulberry leaves and (g) cassava leaves.

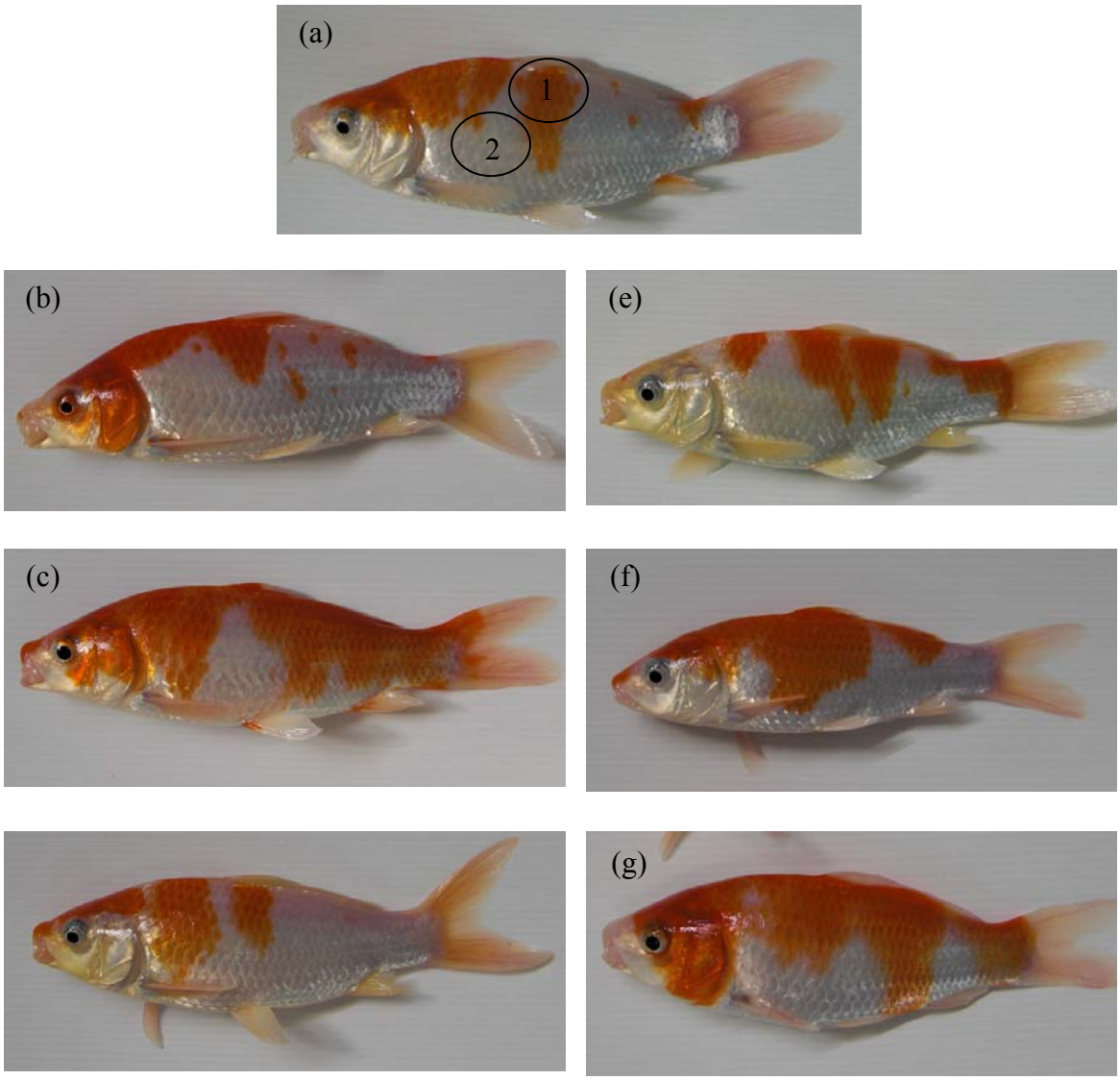


Figure 33 Digital image of fancy carp fed with the low carotenoid diets over final 2 weeks: (a) control (also showing sampling areas for color measurement (1) red position and (2) white position where all measurements were taken on the same side for all of the fish used in the experiment), (b) crude tea extraction, (c) crude mulberry extraction, (d) crude cassava extraction, (e) tea leaves, (f) mulberry leaves and (g) cassava leaves.

According to the results obtained from the experiment, it was observed that the fancy carp responsed to coloration effected by the use of natural carotenoid sources from a by-product namely; tea, mulberry and cassava leaves. Aside from skin redness, yellowness and carotenoid concentration, skin lightness was the only color parameter not influenced positively by feeding with pigmented diet. This is supported by the negative correlation observed between this color variable and skin carotenoid concentration. In salmonids such as Atlantic salmon and Artic charr a negative correlation was also reported between muscle carotenoid concentration and muscle lightness (L*) (Bjerkeng *et al*., 1997; Hatlen *et al*., 1998). Natural carotenoids such as *Chlorella vulgaris* had already proved to be a useful, even competitive coloring for inclusion in the diets of laying hens with the purpose of coloring egg yolk (Gouveia *et al*. 1996); in rainbow trout yielding both muscle and skin pigmentation effect (Gouveia *et al*. 1997 and 1998); in gilthead seabream for skin pigmentation (Gouveia *et al*. 2002) and improved skin coloring in goldfish and fancy carp (Gouveia *et al*. 2003).

In determining the pigmenting efficiencies of these diets in the skin of fancy carp, consideration must be given to how these pigments are converted from precursor pigments and the total content in the skin. Fish, like other animals such as birds (McGraw and Hill, 2001), absorb dietary carotenoids through the intestinal mucosa (Furr and Clark, 1997), transport them through the blood via serum lipoproteins (Bowen *et al*. 2002), metabolically oxidize them to other forms (Katayama *et al*. 1973) and deposit them into specialized skin cells called chromatophores (Chatzifotis *et al*. 2005). Goldfish and Koi carp can convert the lutein and zeaxanthin including β-carotene is precursor of astaxanthin (Katayama *et al*. 1973). Astaxanthin can also be stored directly in these species. Sparse research has been conducted on the biology of fancy carp, so further work is needed to investigate the conversion and total content of carotenoid pigments in the skin.

In ornamental high-value fish species there is a need to achieve high levels of skin pigmentation, together with optimal body shape, fin shape and body size, because of their importance as quality criteria for their market value (Paripatananont

et al. 1999). Coloring and patterns are the characteristics that attract people to the hobby of ornamental fish keeping. The same two factors are also very important in determining the quality and therefore the value of any particular fish. In this investigation the diet containing the tea leaves was the most successful in inducing red coloration in the skin of fancy carp. This was most apparent after six weeks of feeding, when red chromaticity values were significantly higher than those in the control diet. Yanar *et al*. (2008) showed that a natural carotenoid source was as effective as a synthetic carotenoid source, on skin pigmentation of goldfish. Carotenoid pigmentation it is not only influenced by the type of dietary carotenoid supplemented in the diet, but also by other factors such as pigment source, concentration, length of carotenoid feeding and by dietary ingredients present in the diet (Bjerkeng, 2000). Up to date, carotenoid pigmentation studies on fancy carp skin have been focused mainly on the inclusion of the appropriate type of carotenoid and natural source of carotenoid in the diet, for this body skin to acquire the natural red coloration. Hence, higher plant is suggested to be an effective alternative natural carotenoid source to synthetic carotenoid for fancy carp pigmentation. The authors believe they have indicated in this work, that plant leaves that is a by-product after harvested may contribute to enhanced image and quality of fancy carp, especially the natural carotenoids contained with tea leaves.

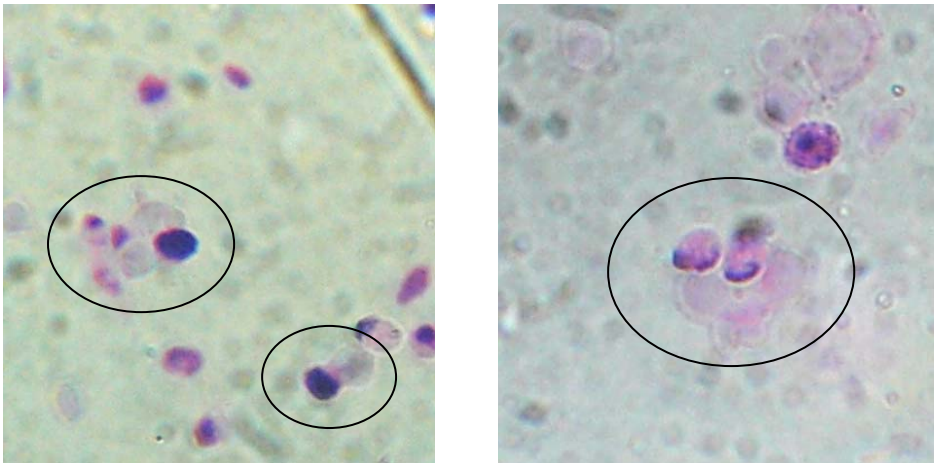
7. Phagocytosis

Phagocytic activity of fish was shown in **Table 58**. The data of this study indicate that phagocytic activity was significantly greater in the in fish fed with carotenoid supplemented groups compared with the control group ($P \leq 0.05$), suggesting that these carotenoids enhanced membrane stability and increased the mechanical ability of phagocytes to engulf foreign particles (**Figure 34**). The phagocytic activity (PA, %) was 11.88 ± 1.89, 15.63 ± 2.19, 16.67 ± 2.47, 18.75 ± 2.78, 20.09 ± 2.93, 18.43 ± 3.04 and 20.95 ± 1.68, respectively. The group of fish fed with raw material of tea and cassava leaves had a higher percentage of PA than other groups (as shown in **Figure 35**).

Table 58 The phagocytic activity (PA, %) of yeast by blood leukocytes from fancy carp fed with various natural carotenoid diet for 6 weeks.

Experimental diet	Phagocytic activity (PA %)
Diet 1: control	11.88 ± 1.89^{c}
Diet 2: supplemented with TC of tea extract	15.63 ± 2.72^{bc}
Diet 3 : supplemented with TC of mulberry extract	16.67 ± 2.45^{ab}
Diet 4: supplemented with TC of cassava extract	18.75 ± 2.78^{ab}
Diet 5: supplemented with tea leaves	20.09 ± 2.93^{a}
Diet 6: supplemented with mulberry leaves	18.43 ± 3.04^{ab}
Diet 7: supplemented with cassava leaves	20.95 ± 1.68^{a}
P-value	0.0013

Mean with the different letters in same column are significantly different at $p \leq 0.05$



Macrophages are attached cell yeast

Macrophages are engulfing cell yeast which ends up in its cytoplasm

Figure 34 Phagocytosis from blood was isolated on a percoll density gradient of 1.0777- 1.0800 g ml-1 after incubated with cell yeast for 2 hrs at 20 °C in fancy carp.

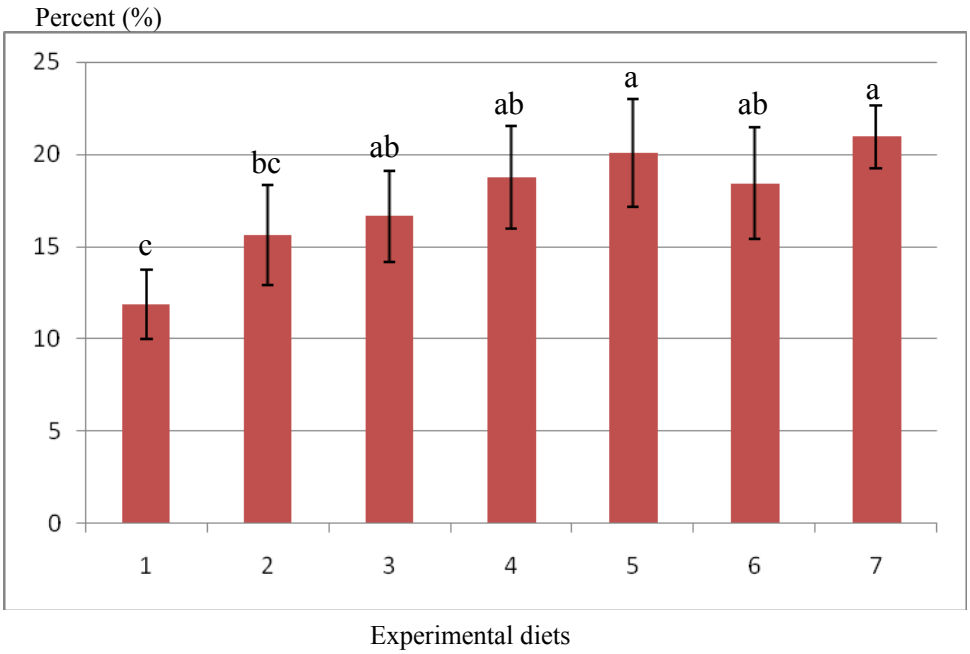


Figure 35 The phagocytic activity (PA, %) of yeast by blood leukocytes from fancy carp fed with various natural carotenoid diet for 6 weeks. Mean values with the different letters are significantly different ($p \leq 0.05$).

Remark: 1 = control diet 2 = TC of tea extract 25 mg/kg 3 = TC of mulberry extract 25 mg/kg
4 = TC of cassava extract 25 mg/kg 5 = TC of tea leaves 25 mg/kg
6 = TC of mulberry leaves 25 mg/kg 7 = TC of cassava leaves 25 mg/kg

In this present study, lymphocyte from peripheral blood was isolated on a percoll has density gradient of 1.077700.1-1.0800 g ml^{-}1. Hence, the leucocyte types of this study assumed similarity with the characteristics of the peripheral blood cell suspensions of Verburg-Van Kemenade *et al*. (1994) reported that leucocyte fraction of density range 1.070-1.080 g ml^{-}1 was obtained by discontinues percoll gradient centrifugation. Fraction contained the main population consisted of neutrophilic granulocytes (80%) and the remaining 20% consisted of basophilic granulocytes, macrophages and lymphocytes. Light microscopic analyses of the phagocytic process in vito indicate that, of the cells studied, macrophages are most efficient phagocytic cells. Phagocytosis analysis in the present study showed that phagocytic activity of lymphocyte cells was greater in the carotenoid supplemented groups compared to the control.

Dietary studies examining the role of carotenoids in immunity in fish are still lacking. In fish, phagocytosis has been recognised as an important element in the host's defence against invading microoraganisms (MacArthur and Fletcher, 1985). In the recent research, Thompson *et al*., (1994) found that astaxanthin with vitamin A increased the serum antiprotease activity in rainbow trout, but not growth or other humoral and cellular immune indices. However, Tachibana *et al*. (1997) found spleen lymphocyte proliferation increased in parrot fish larvae after fish fed with β-carotene enhanced rotifers and attributed the high survival. Redder fish in Betta splendens given supplemental carotenoids increased in immune response (to a phytohemagglutination challenge) (Clotfelter *et al*., 2007). Phagocytic rate and phagocytic index in rainbow trout fed with dietary supplemented natural carotenoid sources from red yeast *Phaffia rhodozyma* and marine algae *Dunaliella salina* were significantly higher than those of the control (Amar *et al*., 2006). Similar the report of Amar *et al*., (2001) showed that rainbow trout fed synthetic carotenoid such as β-carotene and astaxanthin elevated humoral factors such as serum complement and lysozyme activity, as well as cellular factors such as phagocytosis. By contrast of Amar *et al*. (2000) reported that the phagocytic activity of isolated head kidney cells was similar at the different level of dietary β-carotene in rainbow trout but indicated enhancement for immune components like serum complement and total plasma immunoglobulin.

Natural antioxidants, including the carotenoids and vitamin A, E and C, are important to animal health. They function as natural antioxidants to remove harmful free radicals produced through normal cellular activity and from environmental stressor, thereby maintaining the structural integrity of immune cells. Therefore, carotenoids and vitamin A, E and C are important to animal health by enhancing immunity (Chew, 1996). Immunoenhancement by dietary carotenoid manipulation may complement, if not offer an alternative to the use of drugs in aquaculture. In fish, an increasing number of studies have demonstrated the role of various dietary carotenoids in immune responses (Blazer and Wolke, 1984; Kiron *et al*., 1995).

CONCLUSIONS

As main conclusions of this work, one can say that a dietary supplementation of 25 mg/kg of tea leaves for 6 weeks tends to increase skin pigmentation and have a profound influence on immune response of fancy. In the diet is good enough to obtain a natural pigmentation in fancy carp. However, a minimum dose of synthetic carotenoid and crude carotenoid extraction should be used to obtain a desirable for improving skin coloring, their are much more expensive than raw materials of plant leaves that is a by-product from agriculture harvested. Thus, tea leaves is suggested to be an effective alternative natural carotenoid source to synthetic carotenoid for fancy carp pigmentation and boots immune response including using plant leaves could be reducing costs related to pellet production which will therefore promote increased profit for fish farmers.

This part summarizes the results of the study following:

In part 1, This study was to investigate the carotenoids composition and toxic substances in local Thai feedstuffs a byproducts from agricultural harvest namely; tea, mulberry and cassava leaves. Results of the studies showed that feedstuffs consisted mainly of lutein and β- carotene, and indicate that tea and mulberry leaves have similarly proportion of lutein and β-carotene about 1:1. In contrast, cassava leaves comprise the lutein more than β-carotene and has a proportion of lutein and β-carotene about 2:1. For aquatic animal safety, this results suggested that both tannin and hydrocyanic acid in dried tea and cassava leaves could be used not more than 10 and 60%, respectively. This study might be of use for the raw materials as the carotenoid sources for lowering the production cost of the fancy carp and ornamental fishes diet including adding value to agricultural byproducts.

The developed TLC-densitometric analysis was validated by determination of linearity, accuracy (% recovery), precision (%RSD_r), LOD, LOQ and $HORRAT_r$ value for a single laboratory. The data indicated that this method can be successfully

used for the analysis of carotenoids with good recoveries and precision. Thus, this method enables simple, rapid, economically and precise quantitative determination of carotenoids. It is simpler than HPLC and faster because up to nine samples (applied singly with the minimum four standard concentrations) can be analyzed on each plate. Cost of solvent purchase and disposal is very low because no more than 25 ml of mobile phase is required in the chamber trough containing the plate to develop these 13 chromatograms. It is possible to use the established method for the routine analysis of carotenoids.

The study in part 2 was to evaluate the pharmacokinetic parameters of astaxanthin, lutein and β-carotene in fancy carp after single dose oral administration. The bioavailability of carotenoids were administrated in a single dose in fancy carp. In all carotenoid concentration-time curves from serum was best fit to one-compartment pharmacokinetic model. Astaxanthin administration was absorbed into blood circulation better than lutein and β-carotene. The present studies indicate that, fancy carp were fed with lutein and β-carotene diets, found serum astaxanthin in 24 and 72 hr after oral administration feeding. The present studies indicated that fancy carp can convert lutein and β-carotene to astaxanthin, fancy carp can increase astaxanthin concentration accumulated in serum similarly to fish fed with astaxanthin diet directly. Consequently, a carotenoid enhancing diet of fancy carp can be formulated by lutein or β-carotene source.

This study was study pharmacokinetic parameters of raw materials of tea, mulberry and cassava leaves in fancy carp and fish were fed single dose administration. Total carotenoids concentration time curves from serum were best fit to one compartment pharmacokinetic model. It concluded fancy carp were fed with tea, mulberry and cassava leaves; found serum astaxanthin in same time at 48 hr after oral administration feeding. The present studies indicated that, fancy carp can convert lutein and β-carotene that the mainly carotenoids composition in plant leaves to astaxanthin in blood circulation. Hence, this becomes a method in lowering the production cost of diets and in adding value to raw material and byproducts from agricultural harvest. Also, using production of carotenoids from a natural source

especially plant leaves is a potential alternative to replace the synthetic carotenoids as well.

Part 3 was a study on the effect of carotenoid sources on skin pigmentaion in fancy carp. The experiment was investigated by diets supplemented with either astaxanthin, lutein, β-carotene or combinations of lutein and β-carotene. Carotenoid supplementation of fancy carp diets are an efficient way to improve the fancy carp skin pigmentation. In terms of serum total carotenoids (TC), fish fed diets supplemented with either a combination of lutein and β-carotene at 25:25 and 50:50 mg/kg diets, as well as lutein alone at 50 mg/kg had serum TC stronger than other groups, and fish fed diets of them had serum astaxanthin concentrations similar to fish fed with astaxanthin alone at 25 mg/kg. Pigmentation response of skin redness of three groups of fancy carp were higher than other treatments, showed the best effects on skin coloration as measured by redness skin. Furthermore, pigmentation effects were retained after their discontinuation feeding with dietary carotenoid. Therefore, diets supplemented with a combination of lutein plus β-carotene at 25:25 and 50:50 mg/kg, as well as lutein alone at 50 mg/kg are promising sources of dietary supplements for skin pigmentation.

Part 4 was a study on the stability of formulated carotenoid diets. All diets are split into two smaller groups which one contains antioxidant and another is without antioxidant at 250 mg/kg. Firstly, all diets were evaluated in total carotenoid (TC) loss during feed processing. In this study found that carotenoid content in all diets remained quite stable, without significant loss during feed processing. The TC loss in the diets starts from after being feed processed which are through from the pellet cooker and passed to die surface, and after dryer process, heat processes cause considerable loss of carotenoids. As a result, should be added 10 percents of TC from the needed amount to natural carotenoids or natural raw materials to achieve the target level TC. Nevertheless, pelleting process of the diets (with a pelleting machine: soft type pellet without steam) didn’t affect carotenoid content of the diets.

The data of a study on the effect of different storage time of dietary enriched carotenoid indicated that, the best storage temperature for formulated carotenoids diet were keep under low temperature at 4 °C for 8 weeks, storage at refrigerated temperatures is an important factor in slowing down oxidation metabolism which helps reducing the loss of TC quantities and rancidity value. However, antioxidant added would be convenience with energy saved before being feed processed. Thus, the additional of BHT at 250 mg/kg helps reducing of oxidation during feed process and storage time. It can be kept at normal room temperature but it should be away from sunlight, air and heat.

In part 5 that is final part of this study was undertaken to investigate the effects of various dietary carotenoid source on the growth, skin pigmentation and immune response in fancy carp for a rearing period of six weeks. In this study, neither growth nor feed conversion efficiency were affected significantly by dietary treatment for all treatments diets. Growth parameters, namely weight gain, feed conversion ratio and survival were similar. Fish were fed with tea leaves had the highest serum TC, astaxanthin and lutein concentration better than the other group. Tea leaves supplementation of fancy carp diets are an efficient way to improve the carp skin pigmentation. Furthermore, pigmentation effects were retained after their discontinuation feeding with dietary carotenoid. Therefore, diets supplemented with a tea leaves is able to efficiently utilize dietary natural, and deposit astaxanthin to acquire an acceptable dark red skin. Besides, carotenoids are important to animal health by enhancing immunity because of fish were fed with carotenoids diets had the highest phagocytic rate and index better than the control group. Hence, replacing raw material of tea leaves in fancy carp diet should be more efficient and cost-effective carotenoid formulation.

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APPENDIX

Appendix A

Part 1 Carotenoids Analysis and Method Validation

Appendix Table A1 Acceptable recovery and precision (RSD_r) at different concentrations.

Active Ingred. (%)	Unit	Recovery (%)	RSD_r (%)
100	100%	98-102	1.3
>=10	10%	98-102	1.8
>=1	1%	97-103	2.7
>=0.1	0.1 %	95-105	3.7
0.01	100 ppm	90-107	5.3
0.001	10 ppm	80-110	7.3
0.0001	1 ppm	80-110	11
0.00001	100 ppb	80-110	15
0.000001	10 ppb	60-115	21
0.0000001	1 ppb	40-120	30

Source: AOAC manual for the Peer Verified Methods program (1993)

Part 2 Bioavailability of Carotenoids

Appendix Table A2 Serum carotenoids concentration (ug/ml) from astaxanthin, lutein and β-carotene diets after oral administration a single dose at each sampling time.

Time (hr)	Astaxanthin	Lutein	β-carotene
15 min	0.08 ± 0.09 ^{ed}	0.16 ± 0.04	0.01 ± 0.01 ^{c}
30 mim	0.70 ± 0.03 ^{ed}	0.15 ± 0.04	0.01 ± 0.01 ^{c}
1	0.29 ± 0.10 ^{abc}	0.17 ± 0.08	0.02 ± 0.01 ^{c}
3	0.31 ± 0.01 ^{ab}	0.19 ± 0.13	0.03 ± 0.01 ^{c}
6	0.38 ± 0.06 ^{a}	0.25 ± 0.13	0.04 ± 0.01 ^{bc}
12	0.32 ± 0.06 ^{ab}	0.39 ± 0.04	0.13 ± 0.03 ^{a}
24	0.18 ± 0.08 ^{cde}	0.19 ± 0.02	0.06 ± 0.01 ^{b}
48	0.16 ± 0.09 ^{bcde}	0.14 ± 0.01	0.03 ± 0.01 ^{c}
72	0.17 ± 0.14 ^{bcde}	0.16 ± 0.12	0.04 ± 0.02 ^{bc}
96	0.20 ± 0.08 ^{bcd}	0.21 ± 0.08	0.03 ± 0.01 ^{c}
120	ND	0.19 ± 0.06	0.04 ± 0.01 ^{bc}
144	ND	0.13 ± 0.06	0.03 ± 0.02 ^{c}
P-value	0.0002	0.876	0.0001

ND: Not Detected

Part 4 Stability of Carotenoid Diets During Feed Processing and Under Different Storage Conditions

Appendix Table A3 Total carotenoids content in the experimental diets Group A, at the various unit operations.

Experimental Diets	Total carotenoid (mg/kg)	
	Without antioxidant	With antioxidant
Dry mix		
Diet 1: control diet	5.72 ± 0.12	5.99 ± 0.55
Diet 2: astaxanthin 25 ppm	27.44 ± 4.89	27.64 ± 0.18
Diet 3: lutein 25 ppm	27.78 ± 1.84	28.49 ± 1.12
Diet 4: β-carotene 25 ppm	26.74 ± 4.65	28.57 ± 3.55
Diet 5: Lutein:β-carotene 25:25 ppm	47.89 ± 2.93	48.51 ± 3.93
Diet 6: Lutein:β-carotene 50 ppm	88.49 ± 7.95	87.27 ± 2.99
After being processed		
Diet 1: control diet	5.60 ± 0.10	5.86 ± 0.19
Diet 2: astaxanthin 25 ppm	26.60 ± 0.54	27.00 ± 2.63
Diet 3: lutein 25 ppm	25.26 ± 1.87	27.08 ± 1.59
Diet 4: β-carotene 25 ppm	25.34 ± 3.39	27.98 ± 1.50
Diet 5: Lutein:β-carotene 25:25 ppm	46.68 ± 1.88	47.16 ± 2.01
Diet 6: Lutein:β-carotene 50 ppm	86.13 ± 2.25	86.17± 3.76
Dryer		
Diet 1: control diet	5.54 ± 0.19	5.70 ± 0.27
Diet 2: astaxanthin 25 ppm	25.56 ± 0.98	26.91 ± 0.84
Diet 3: lutein 25 ppm	23.67 ± 2.45	26.08 ± 0.75
Diet 4: β-carotene 25 ppm	25.04 ± 2.97	26.25 ± 2.90
Diet 5: Lutein:β-carotene 25:25 ppm	45.85 ± 4.72	46.75 ± 3.08
Diet 6: Lutein:β-carotene 50 ppm	84.97 ± 4.28	86.01 ± 3.00

Appendix Table A4 Total carotenoids loss of experimental diets Group A, at the various unit operations (%).

Experimental diets	Unit operations			
	After being processed	Dryer	After being processed	Dryer
	Without antioxidant		With antioxidant	
Diet 1: control diet	2.07	3.03	2.19	4.84
Diet 2: astaxanthin 25 ppm	3.07	6.85	2.35	2.67
Diet 3: lutein 25 ppm	4.07	7.82	4.96	8.47
Diet 4: β-carotene 25 ppm	5.21	6.37	2.08	8.15
Diet 5: Lutein:β-carotene 25:25 ppm	2.52	4.25	2.79	3.63
Diet 6: Lutein:β-carotene 50 ppm	2.66	3.98	2.26	4.44

Appendix Table A5 Total carotenoids content in the experimental diets Group B, at the various unit operations.

Experimental Diets	Total carotenoid (mg/kg)	
	Without antioxidant	With antioxidant
Dry mix		
Diet 1: control diet	5.72 ± 0.12	5.99 ± 0.55
Diet 2: lutein 25 ppm	27.78 ± 1.84	28.49 ± 1.12
Diet 3: β-carotene 25 ppm	26.74 ± 4.65	28.57 ± 3.55
Diet 4: TC of tea extract 25 ppm	27.15 ± 4.43	27.71 ± 0.74
Diet 5: TC of mulberry extract 25 ppm	27.50 ± 1.67	28.29 ± 1.25
Diet 6: TC of cassava extract 25 ppm	27.98 ± 0.55	26.98 ± 0.85
Diet 7: TC of tea leave 25 ppm	26.54 ± 1.15	27.21 ± 4.28
Diet 8: TC of mulberry leave 25 ppm	27.30 ± 1.29	25.87 ± 0.16
Diet 9: TC of cassava leave 25 ppm	26.67 ± 5.88	26.63 ± 2.67
After being processed		
Diet 1: control diet	5.60 ± 0.10	5.86 ± 0.19
Diet 2: lutein 25 ppm	25.26 ± 1.87	27.08 ± 1.59
Diet 3: β-carotene 25 ppm	25.34 ± 3.39	27.98 ± 1.50
Diet 4: TC of tea extract 25 ppm	26.12 ± 4.12	26.91 ± 0.40
Diet 5: TC of mulberry extract 25 ppm	26.05 ± 2.11	27.37 ± 1.43
Diet 6: TC of cassava extract 25 ppm	26.57 ± 0.86	26.48 ± 2.64
Diet 7: TC of tea leave 25 ppm	24.98 ± 3.16	26.65 ± 3.60
Diet 8: TC of mulberry leave 25 ppm	25.93 ± 0.55	25.27 ± 2.77
Diet 9: TC of cassava leave 25 ppm	24.63 ± 0.28	25.44 ± 0.60
Dryer		
Diet 1: control diet	5.54 ± 0.19	5.70 ± 0.27
Diet 2: lutein 25 ppm	23.67 ± 2.45	26.08 ± 0.75
Diet 3: β-carotene 25 ppm	25.04 ± 2.97	26.25 ± 2.90
Diet 4: TC of tea extract 25 ppm	24.95 ± 1.26	25.50 ± 3.37
Diet 5: TC of mulberry extract 25 ppm	25.50 ± 0.34	26.59 ± 2.34
Diet 6: TC of cassava extract 25 ppm	24.98 ± 2.46	25.07 ± 2.61
Diet 7: TC of tea leave 25 ppm	23.38 ± 4.06	25.60 ± 0.70
Diet 8: TC of mulberry leave 25 ppm	24.05 ± 0.56	26.05 ± 2.10
Diet 9: TC of cassava leave 25 ppm	24.02 ± 2.21	25.69 ± 1.95

Appendix Table A6 Total carotenoids loss of experimental diets Group B, at the various unit operations (%).

Experimental diets	Unit operations			
	After being processed	Dryer	After being processed	Dryer
	Without antioxidant		With antioxidant	
Diet 1: control diet	2.07	3.03	2.19	4.84
Diet 2: lutein 25 ppm	4.04	7.82	4.96	8.47
Diet 3: β-carotene 25 ppm	5.21	6.37	2.08	8.15
Diet 4: TC of tea extract 25 ppm	3.80	8.10	2.90	7.99
Diet 5: TC of mulberry extract 25 ppm	5.30	7.27	3.26	6.03
Diet 6: TC of cassava extract 25 ppm	5.04	10.72	2.84	7.08
Diet 7: TC of tea leave 25 ppm	5.89	11.91	2.05	5.92
Diet 8: TC of mulberry leave 25 ppm	5.02	11.89	2.44	4.59
Diet 9: TC of cassava leave 25 ppm	7.65	9.92	4.57	6.18

Appendix Table A7 Total carotenoids content in the experimental diets Group A, storage under room temperature during the trial period.

Experimental Diets	Total carotenoid (mg/kg)	
	Without antioxidant	With antioxidant
Week0		
Diet 1: control diet	5.54 ± 0.19	5.54 ± 0.19
Diet 2: astaxanthin 25 ppm	25.56 ± 0.98	25.56 ± 0.98
Diet 3: lutein 25 ppm	23.67 ± 2.45	23.67 ± 2.45
Diet 4: β-carotene 25 ppm	25.04 ± 2.97	25.04 ± 2.97
Diet 5: Lutein:β-carotene 25:25 ppm	45.85 ± 4.72	45.85 ± 4.72
Diet 6: Lutein:β-carotene 50 ppm	84.97 ± 4.28	84.97 ± 4.28
Week 4		
Diet 1: control diet	5.46 ± 0.38	5.65 ± 0.42
Diet 2: astaxanthin 25 ppm	24.04 ± 3.39	26.22 ± 5.01
Diet 3: lutein 25 ppm	22.88 ± 0.67	24.64 ± 3.33
Diet 4: β-carotene 25 ppm	23.83 ± 1.59	25.36 ± 1.55
Diet 5: Lutein:β-carotene 25:25 ppm	43.70 ± 0.75	45.40 ± 0.81
Diet 6: Lutein:β-carotene 50 ppm	82.85 ± 2.39	84.50 ± 2.24
Week 8		
Diet 1: control diet	5.28 ± 0.73	5.55 ± 0.10
Diet 2: astaxanthin 25 ppm	23.27 ± 3.93	25.55 ± 2.36
Diet 3: lutein 25 ppm	21.83 ± 1.72	25.07 ± 1.07
Diet 4: β-carotene 25 ppm	23.17 ± 2.16	25.03 ± 0.72
Diet 5: Lutein:β-carotene 25:25 ppm	42.45 ± 4.85	45.02 ± 2.33
Diet 6: Lutein:β-carotene 50 ppm	80.74 ± 4.33	83.75 ± 1.25

Appendix Table A8 Total carotenoids content in the experimental diets Group A, storage under 4 °C during the trial period.

Experimental Diets	Total carotenoid (mg/kg)	
	Without antioxidant	With antioxidant
Week0		
Diet 1: control diet	5.54 ± 0.19	5.70 ± 0.27
Diet 2: astaxanthin 25 ppm	25.56 ± 0.98	26.91 ± 0.84
Diet 3: lutein 25 ppm	23.67 ± 2.45	26.08 ± 0.75
Diet 4: β-carotene 25 ppm	25.04 ± 2.97	26.25 ± 2.90
Diet 5: Lutein:β-carotene 25:25 ppm	45.85 ± 4.72	46.75 ± 3.08
Diet 6: Lutein:β-carotene 50 ppm	84.97 ± 4.28	86.01 ± 3.00
Week 4		5.67 ± 0.07
Diet 1: control diet	5.44 ± 0.08	26.45 ± 5.43
Diet 2: astaxanthin 25 ppm	25.18 ± 4.55	25.61 ± 1.11
Diet 3: lutein 25 ppm	23.26 ± 2.19	25.52 ± 2.75
Diet 4: β-carotene 25 ppm	24.45 ± 0.95	46.29 ± 1.79
Diet 5: Lutein:β-carotene 25:25 ppm	44.52 ± 3.60	85.20 ± 1.57
Diet 6: Lutein:β-carotene 50 ppm	83.44 ± 0.55	
Week 8		
Diet 1: control diet	5.41 ± 0.23	5.63 ± 0.09
Diet 2: astaxanthin 25 ppm	25.01 ± 2.56	26.00 ± 0.38
Diet 3: lutein 25 ppm	23.17 ± 1.14	25.12 ± 0.95
Diet 4: β-carotene 25 ppm	24.20 ± 0.97	25.23 ± 0.91
Diet 5: Lutein:β-carotene 25:25 ppm	44.08 ± 1.85	46.19 ± 2.16
Diet 6: Lutein:β-carotene 50 ppm	82.81 ± 2.11	85.07 ± 0.74

Appendix Table A9 Total carotenoids loss of experimental diets Group A, at different time period (%).

Experimental diets	Time			
	4 weeks	8 weeks	4 weeks	8 weeks
	Without antioxidant		With Antioxidant	
Room temperature				
Diet 1: control diet	1.48	4.79	1.92	2.01
Diet 2: astaxanthin 25 ppm	5.94	8.97	2.52	2.56
Diet 3: lutein 25 ppm	3.33	7.74	1.71	2.58
Diet 4: β-carotene 25 ppm	4.80	7.44	1.26	1.30
Diet 5: Lutein:β-carotene 25:25 ppm	4.69	7.43	0.97	1.80
Diet 6: Lutein:β-carotene 50 ppm	2.49	4.98	0.55	1.43
4 degree				
Diet 1: control diet	1.80	2.44	0.50	1.25
Diet 2: astaxanthin 25 ppm	1.51	2.16	1.68	3.37
Diet 3: lutein 25 ppm	1.70	2.11	1.78	3.66
Diet 4: β-carotene 25 ppm	2.35	3.35	2.74	3.86
Diet 5: Lutein:β-carotene 25:25 ppm	2.91	3.87	1.00	1.21
Diet 6: Lutein:β-carotene 50 ppm	1.79	2.54	0.95	1.09

Appendix Table A10 Total carotenoids content in the experimental diets Group B, storage under room temperature during the trial period.

Experimental Diets	Total carotenoid (mg/kg)	
	Without antioxidant	With antioxidant
Week 0		
Diet 1: control diet	5.54 ± 0.19	5.54 ± 0.19
Diet 2: lutein 25 ppm	23.67 ± 2.45	23.67 ± 2.45
Diet 3: β-carotene 25 ppm	25.04 ± 2.97	25.04 ± 2.97
Diet 4: TC of tea extract 25 ppm	24.95 ± 1.26	24.95 ± 1.26
Diet 5: TC of mulberry extract 25 ppm	25.50 ± 0.34	25.50 ± 0.34
Diet 6: TC of cassava extract 25 ppm	24.98 ± 2.46	24.98 ± 2.46
Diet 7: TC of tea leave 25 ppm	23.38 ± 4.06	23.38 ± 4.06
Diet 8: TC of mulberry leave 25 ppm	24.05 ± 0.56	24.05 ± 0.56
Diet 9: TC of cassava leave 25 ppm	25.02 ± 3.86	25.02 ± 3.86
Week 4		
Diet 1: control diet	5.46 ± 0.38	5.65 ± 0.42
Diet 2: lutein 25 ppm	22.88 ± 0.67	24.64 ± 3.33
Diet 3: β-carotene 25 ppm	23.83 ± 1.59	25.36 ± 1.55
Diet 4: TC of tea extract 25 ppm	23.44 ± 0.87	24.77 ± 3.25
Diet 5: TC of mulberry extract 25 ppm	24.67 ± 0.93	25.81 ± 4.50
Diet 6: TC of cassava extract 25 ppm	24.02 ± 0.56	24.53 ± 0.43
Diet 7: TC of tea leave 25 ppm	23.03 ± 2.60	24.61 ± 1.05
Diet 8: TC of mulberry leave 25 ppm	23.72 ± 0.70	23.88 ± 1.52
Diet 9: TC of cassava leave 25 ppm	23.63 ± 2.90	23.60 ± 0.99
Week 8		
Diet 1: control diet	5.28 ± 0.73	5.55 ± 0.10
Diet 2: lutein 25 ppm	21.83 ± 1.72	25.07 ± 1.07
Diet 3: β-carotene 25 ppm	23.17 ± 2.16	25.03 ± 0.72
Diet 4: TC of tea extract 25 ppm	22.87 ± 3.14	24.00 ± 0.81
Diet 5: TC of mulberry extract 25 ppm	22.87 ± 0.59	24.67 ± 2.82
Diet 6: TC of cassava extract 25 ppm	23.75 ± 1.08	23.37 ± 1.85
Diet 7: TC of tea leave 25 ppm	22.92 ± 2.40	23.76 ± 1.05
Diet 8: TC of mulberry leave 25 ppm	23.61 ± 4.92	23.76 ± 2.56
Diet 9: TC of cassava leave 25 ppm	22.26 ± 1.58	22.77 ± 2.55

Appendix Table A11 Total carotenoids content in the experimental diets Group B, storage under 4 °C during the trial period.

Experimental Diets	Total carotenoid (mg/kg)	
	Without antioxidant	With antioxidant
Week 0		
Diet 1: control diet	5.54 ± 0.19	5.70 ± 0.27
Diet 2: lutein 25 ppm	23.67 ± 2.45	26.08 ± 0.75
Diet 3: β-carotene 25 ppm	25.04 ± 2.97	26.25 ± 2.90
Diet 4: TC of tea extract 25 ppm	24.95 ± 1.26	25.50 ± 3.37
Diet 5: TC of mulberry extract 25 ppm	25.50 ± 0.34	26.59 ± 2.34
Diet 6: TC of cassava extract 25 ppm	24.98 ± 2.46	25.07 ± 2.61
Diet 7: TC of tea leave 25 ppm	23.38 ± 4.06	25.60 ± 0.70
Diet 8: TC of mulberry leave 25 ppm	24.05 ± 0.56	23.38 ± 4.38
Diet 9: TC of cassava leave 25 ppm	25.02 ± 3.86	24.36 ± 2.71
Week 4		
Diet 1: control diet	5.44 ± 0.08	5.67 ± 0.07
Diet 2: lutein 25 ppm	23.26 ± 2.16	25.61 ± 1.11
Diet 3: β-carotene 25 ppm	24.45 ± 0.95	25.52 ± 2.75
Diet 4: TC of tea extract 25 ppm	24.23 ± 1.03	25.23 ± 2.59
Diet 5: TC of mulberry extract 25 ppm	24.58 ± 3.01	26.07 ± 1.90
Diet 6: TC of cassava extract 25 ppm	24.58 ± 1.44	24.41 ± 0.61
Diet 7: TC of tea leave 25 ppm	22.56 ± 1.09	25.16 ± 1.79
Diet 8: TC of mulberry leave 25 ppm	23.88 ± 1.02	23.18 ± 0.90
Diet 9: TC of cassava leave 25 ppm	24.10 ± 0.62	24.06 ± 2.52
Week 8		
Diet 1: control diet	5.41 ± 0.23	5.63 ± 0.09
Diet 2: lutein 25 ppm	23.17 ± 1.14	25.12 ± 0.95
Diet 3: β-carotene 25 ppm	24.20 ± 0.97	25.23 ± 0.91
Diet 4: TC of tea extract 25 ppm	23.62 ± 2.25	25.07 ± 0.29
Diet 5: TC of mulberry extract 25 ppm	23.92 ± 2.88	25.88 ± 0.38
Diet 6: TC of cassava extract 25 ppm	24.22 ± 1.06	24.68 ± 1.12
Diet 7: TC of tea leave 25 ppm	21.59 ± 1.16	25.06 ± 2.20
Diet 8: TC of mulberry leave 25 ppm	23.58 ± 1.49	22.92 ± 0.75
Diet 9: TC of cassava leave 25 ppm	23.58 ± 0.70	23.89 ± 1.98

Appendix Table A12 Total carotenoids loss of experimental diets Group B at different time period (%).

Experimental diets	Time			
	4 weeks	8 weeks	4 weeks	8 weeks
	Without antioxidant		With Antioxidant	
Room temperature				
Diet 1: control diet	1.48	4.79	1.78	2.02
Diet 2: lutein 25 ppm	3.33	7.74	1.72	5.58
Diet 3: β-carotene 25 ppm	4.80	7.44	1.26	5.24
Diet 4: TC of tea extract 25 ppm	6.05	8.34	0.71	3.82
Diet 5: TC of mulberry extract 25 ppm	3.28	10.34	1.20	4.42
Diet 6: TC of cassava extract 25 ppm	3.87	4.94	1.81	6.47
Diet 7: TC of tea leave 25 ppm	1.49	1.98	3.45	5.00
Diet 8: TC of mulberry leave 25 ppm	1.39	1.83	6.24	9.52
Diet 9: TC of cassava leave 25 ppm	5.57	7.07	5.70	9.01
4 degree				
Diet 1: control diet	1.80	2.44	0.50	1.25
Diet 2: lutein 25 ppm	1.70	2.11	1.78	3.66
Diet 3: β-carotene 25 ppm	2.35	3.35	2.74	3.86
Diet 4: TC of tea extract 25 ppm	2.88	5.34	1.07	1.69
Diet 5: TC of mulberry extract 25 ppm	3.66	6.22	1.95	2.67
Diet 6: TC of cassava extract 25 ppm	1.60	3.05	2.63	1.53
Diet 7: TC of tea leave 25 ppm	3.50	7.68	1.72	2.09
Diet 8: TC of mulberry leave 25 ppm	0.70	1.98	0.89	1.98
Diet 9: TC of cassava leave 25 ppm	3.70	5.78	1.21	1.90

Appendix Table A13 TBA value in the experimental diets Group A, storage under room temperature during the trial period.

Experimental Diets	TBA (mg/kg)	
	Without antioxidant	With antioxidant
Week0		
Diet 1: control diet	5.03 ± 0.34	5.07 ± 0.71
Diet 2: astaxanthin 25 ppm	5.00 ± 0.55	4.87 ± 0.41
Diet 3: lutein 25 ppm	5.18 ± 0.15	5.12 ± 0.32
Diet 4: β-carotene 25 ppm	5.35 ± 0.43	4.85 ± 1.05
Diet 5: Lutein:β-carotene 25:25 ppm	5.05 ± 0.38	5.14 ± 0.67
Diet 6: Lutein:β-carotene 50 ppm	5.15 ± 0.54	5.07 ± 0.55
Week 4		
Diet 1: control diet	5.25 ± 0.14	5.35 ± 0.85
Diet 2: astaxanthin 25 ppm	5.44 ± 0.45	5.12 ± 0.97
Diet 3: lutein 25 ppm	5.37 ± 0.06	5.37 ± 1.16
Diet 4: β-carotene 25 ppm	5.68 ± 0.15	5.30 ± 0.29
Diet 5: Lutein:β-carotene 25:25 ppm	5.43 ± 0.18	5.35 ± 0.49
Diet 6: Lutein:β-carotene 50 ppm	5.45 ± 0.50	5.24 ± 0.24
Week 8		
Diet 1: control diet	5.39 ± 0.27	5.41 ± 0.33
Diet 2: astaxanthin 25 ppm	5.87 ± 0.16	5.47 ± 0.52
Diet 3: lutein 25 ppm	5.73 ± 0.44	5.51 ± 0.29
Diet 4: β-carotene 25 ppm	5.82 ± 0.15	5.41 ± 0.14
Diet 5: Lutein:β-carotene 25:25 ppm	5.52 ± 0.23	5.44 ± 0.31
Diet 6: Lutein:β-carotene 50 ppm	5.60 ± 0.11	56.1 ± 0.05

Appendix Table A14 TBA value in the experimental diets Group A, storage under 4 °C during the trial period.

Experimental Diets	TBA (mg/kg)	
	Without antioxidant	With antioxidant
Week0		
Diet 1: control diet	5.03 ± 0.34	5.07 ± 0.71
Diet 2: astaxanthin 25 ppm	5.23 ± 0.79	4.87 ± 0.41
Diet 3: lutein 25 ppm	5.18 ± 0.15	5.12 ± 0.32
Diet 4: β-carotene 25 ppm	5.35 ± 0.43	4.85 ± 1.05
Diet 5: Lutein:β-carotene 25:25 ppm	5.05 ± 0.38	5.14 ± 0.67
Diet 6: Lutein:β-carotene 50 ppm	5.15 ± 0.54	5.07 ± 0.55
Week 4		
Diet 1: control diet	5.35 ± 0.33	5.12 ± 0.22
Diet 2: astaxanthin 25 ppm	5.36 ± 0.07	5.01 ± 0.68
Diet 3: lutein 25 ppm	5.24 ± 0.22	5.29 ± 0.46
Diet 4: β-carotene 25 ppm	5.37 ± 0.16	4.91 ± 0.43
Diet 5: Lutein:β-carotene 25:25 ppm	5.30 ± 0.28	5.24 ± 0.20
Diet 6: Lutein:β-carotene 50 ppm	5.38 ± 0.30	5.31 ± 0.10
Week 8		
Diet 1: control diet	5.58 ± 0.19	5.30 ± 0.21
Diet 2: astaxanthin 25 ppm	5.64 ± 0.55	5.24 ± 0.63
Diet 3: lutein 25 ppm	5.30 ± 0.04	5.36 ± 0.44
Diet 4: β-carotene 25 ppm	5.52 ± 0.21	5.31 ± 0.46
Diet 5: Lutein:β-carotene 25:25 ppm	5.42 ± 0.53	5.32 ± 0.29
Diet 6: Lutein:β-carotene 50 ppm	5.42 ± 0.28	5.37 ± 0.13

Appendix Table A15 TBA value in the experimental diets Group B, storage under room temperature during the trial period.

Experimental Diets	TBA (mg/kg)	
	Without antioxidant	With antioxidant
Week 0		
Diet 1: control diet	5.03 ± 0.34	5.07 ± 0.71
Diet 2: lutein 25 ppm	5.18 ± 0.15	5.12 ± 0.32
Diet 3: β-carotene 25 ppm	5.35 ± 0.43	4.85 ± 1.05
Diet 4: TC of tea extract 25 ppm	5.18 ± 0.25	4.89 ± 1.09
Diet 5: TC of mulberry extract 25 ppm	4.95 ± 0.37	4.95 ± 0.20
Diet 6: TC of cassava extract 25 ppm	4.87 ± 0.11	5.09 ± 0.89
Diet 7: TC of tea leave 25 ppm	5.00 ± 0.16	5.03 ± 0.13
Diet 8: TC of mulberry leave 25 ppm	5.12 ± 0.55	4.83 ± 0.14
Diet 9: TC of cassava leave 25 ppm	5.06 ± 0.30	4.90 ± 0.09
Week 4		
Diet 1: control diet	5.25 ± 0.14	5.35 ± 0.85
Diet 2: lutein 25 ppm	5.37 ± 0.06	5.37 ± 1.16
Diet 3: β-carotene 25 ppm	5.68 ± 0.15	5.30 ± 0.29
Diet 4: TC of tea extract 25 ppm	5.42 ± 0.28	5.17 ± 0.87
Diet 5: TC of mulberry extract 25 ppm	5.29 ± 0.68	5.08 ± 0.82
Diet 6: TC of cassava extract 25 ppm	5.02 ± 0.08	5.23 ± 0.29
Diet 7: TC of tea leave 25 ppm	5.31 ± 0.13	5.49 ± 0.81
Diet 8: TC of mulberry leave 25 ppm	5.40 ± 0.43	5.17 ± 0.73
Diet 9: TC of cassava leave 25 ppm	5.26 ± 0.10	5.21 ± 1.01
Week 8		
Diet 1: control diet	5.39 ± 0.27	5.41 ± 0.33
Diet 2: lutein 25 ppm	5.73 ± 0.44	5.51 ± 0.29
Diet 3: β-carotene 25 ppm	5.82 ± 0.15	5.41 ± 0.14
Diet 4: TC of tea extract 25 ppm	5.66 ± 0.21	5.49 ± 0.19
Diet 5: TC of mulberry extract 25 ppm	5.38 ± 0.09	5.81 ± 0.07
Diet 6: TC of cassava extract 25 ppm	5.47 ± 0.36	5.50 ± 0.30
Diet 7: TC of tea leave 25 ppm	5.49 ± 0.23	5.52 ± 0.16
Diet 8: TC of mulberry leave 25 ppm	5.61 ± 0.60	5.75 ± 0.32
Diet 9: TC of cassava leave 25 ppm	5.52 ± 0.28	5.50 ± 0.32

Appendix Table A16 TBA value in the experimental diets Group B, storage under 4 °C during the trial period.

Experimental Diets	TBA (mg/kg)	
	Without antioxidant	With antioxidant
Week 0		
Diet 1: control diet	5.03 ± 0.34	5.07 ± 0.71
Diet 2: lutein 25 ppm	5.18 ± 0.15	5.12 ± 0.32
Diet 3: β-carotene 25 ppm	5.35 ± 0.43	4.85 ± 1.05
Diet 4: TC of tea extract 25 ppm	5.18 ± 0.25	4.89 ± 1.09
Diet 5: TC of mulberry extract 25 ppm	4.95 ± 0.37	4.95 ± 0.20
Diet 6: TC of cassava extract 25 ppm	4.87 ± 0.11	5.09 ± 0.89
Diet 7: TC of tea leave 25 ppm	5.00 ± 0.16	5.03 ± 0.13
Diet 8: TC of mulberry leave 25 ppm	5.12 ± 0.55	4.83 ± 0.14
Diet 9: TC of cassava leave 25 ppm	5.06 ± 0.30	4.90 ± 0.09
Week 4		
Diet 1: control diet	5.35 ± 0.33	5.12 ± 0.22
Diet 2: lutein 25 ppm	5.24 ± 0.22	5.29 ± 0.46
Diet 3: β-carotene 25 ppm	5.37 ± 0.16	4.91 ± 0.43
Diet 4: TC of tea extract 25 ppm	5.53 ± 0.21	5.18 ± 0.74
Diet 5: TC of mulberry extract 25 ppm	5.17 ± 0.30	5.37 ± 1.24
Diet 6: TC of cassava extract 25 ppm	4.95 ± 0.03	5.17 ± 0.17
Diet 7: TC of tea leave 25 ppm	5.22 ± 0.49	5.19 ± 0.23
Diet 8: TC of mulberry leave 25 ppm	5.35 ± 0.22	5.09 ± 0.36
Diet 9: TC of cassava leave 25 ppm	5.14 ± 0.10	5.19 ± 0.87
Week 8		
Diet 1: control diet	5.58 ± 0.19	5.30 ± 0.21
Diet 2: lutein 25 ppm	5.30 ± 0.04	5.36 ± 0.44
Diet 3: β-carotene 25 ppm	5.52 ± 0.21	5.31 ± 0.46
Diet 4: TC of tea extract 25 ppm	4.73 ± 0.51	5.35 ± 0.21
Diet 5: TC of mulberry extract 25 ppm	5.42 ± 0.16	5.46 ± 0.87
Diet 6: TC of cassava extract 25 ppm	5.34 ± 0.45	5.25 ± 0.39
Diet 7: TC of tea leave 25 ppm	5.31 ± 0.22	5.38 ± 0.48
Diet 8: TC of mulberry leave 25 ppm	5.45 ± 0.62	5.43 ± 0.48
Diet 9: TC of cassava leave 25 ppm	5.31 ± 0.18	5.38 ± 0.42

Appendix Table A17 Acid value content in the experimental diets Group A, storage under room temperature during the trial period.

Experimental Diets	Acid value (mg/kg)	
	Without antioxidant	With antioxidant
Week0		
Diet 1: control diet	27.90 ± 1.77	26.21 ± 2.00
Diet 2: astaxanthin 25 ppm	30.05 ± 0.49	30.05 ± 0.55
Diet 3: lutein 25 ppm	31.26 ± 0.56	31.18 ± 0.62
Diet 4: β-carotene 25 ppm	30.11 ± 0.88	31.61 ± 0.22
Diet 5: Lutein:β-carotene 25:25 ppm	31.20 ± 1.44	31.25 ± 0.55
Diet 6: Lutein:β-carotene 50 ppm	31.06 ± 1.06	26.84 ± 2.84
Week 4		
Diet 1: control diet	31.29 ± 2.50	28.13 ± 0.57
Diet 2: astaxanthin 25 ppm	29.53 ± 0.34	30.90 ± 2.62
Diet 3: lutein 25 ppm	34.02 ± 2.89	29.48 ± 2.81
Diet 4: β-carotene 25 ppm	33.88 ± 1.51	31.01 ± 0.78
Diet 5: Lutein:β-carotene 25:25 ppm	33.26 ± 1.40	30.96 ± 0.70
Diet 6: Lutein:β-carotene 50 ppm	32.97 ± 1.25	28.58 ± 1.21
Week 8		
Diet 1: control diet	39.51 ± 2.10	30.92 ± 1.87
Diet 2: astaxanthin 25 ppm	31.48 ± 2.11	31.92 ± 0.32
Diet 3: lutein 25 ppm	38.18 ± 0.90	33.27 ± 1.95
Diet 4: β-carotene 25 ppm	36.12 ± 0.44	32.12 ± 1.47
Diet 5: Lutein:β-carotene 25:25 ppm	38.32 ± 0.74	34.12 ± 0.81
Diet 6: Lutein:β-carotene 50 ppm	35.58 ± 1.06	30.82 ± 1.11

Appendix Table A18 Acid value content in the experimental diets Group A, storage under 4 °C during the trial period.

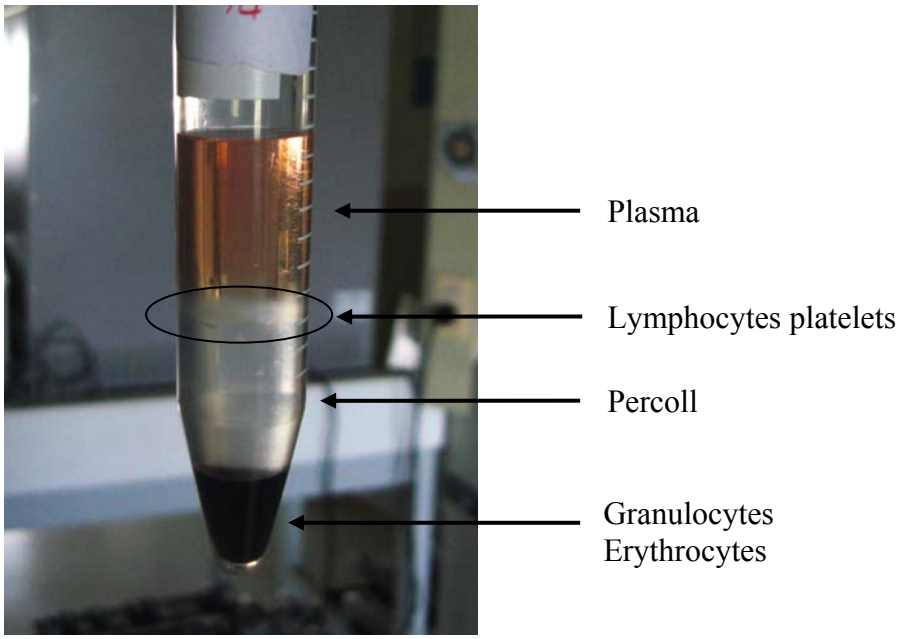
Experimental Diets	Acid value (mg/kg)	
	Without antioxidant	With antioxidant
Week0		
Diet 1: control diet	27.90 ± 1.77	26.21 ± 2.00
Diet 2: astaxanthin 25 ppm	30.05 ± 0.49	30.05 ± 0.55
Diet 3: lutein 25 ppm	31.26 ± 0.56	31.18 ± 0.62
Diet 4: β-carotene 25 ppm	30.11 ± 0.88	31.61 ± 0.22
Diet 5: Lutein:β-carotene 25:25 ppm	31.20 ± 1.44	31.25 ± 0.55
Diet 6: Lutein:β-carotene 50 ppm	31.06 ± 0.76	26.84 ± 2.84
Week 4		
Diet 1: control diet	28.00 ± 0.23	26.35 ± 2.59
Diet 2: astaxanthin 25 ppm	28.33 ± 0.59	26.70 ± 4.03
Diet 3: lutein 25 ppm	30.72 ± 1.14	26.22 ± 1.73
Diet 4: β-carotene 25 ppm	27.99 ± 3.60	30.20 ± 2.15
Diet 5: Lutein:β-carotene 25:25 ppm	29.83 ± 3.40	30.80 ± 1.02
Diet 6: Lutein:β-carotene 50 ppm	28.44 ± 3.35	28.85 ± 3.35
Week 8		
Diet 1: control diet	29.59 ± 0.54	27.55 ± 1.10
Diet 2: astaxanthin 25 ppm	29.85 ± 0.41	28.92 ± 0.64
Diet 3: lutein 25 ppm	32.58 ± 0.33	29.79 ± 0.84
Diet 4: β-carotene 25 ppm	29.91 ± 0.84	31.45 ± 0.24
Diet 5: Lutein:β-carotene 25:25 ppm	31.47 ± 0.98	32.05 ± 0.95
Diet 6: Lutein:β-carotene 50 ppm	30.74 ± 0.21	30.00 ± 1.68

Appendix Table A19 Acid value content in the experimental diets Group B, storage under room temperature during the trial period.

Experimental Diets	Acid value (mg/kg)	
	Without antioxidant	With antioxidant
Week 0		
Diet 1: control diet	27.90 ± 1.77	26.21 ± 2.00
Diet 2: lutein 25 ppm	31.26 ± 0.56	31.18 ± 0.62
Diet 3: β-carotene 25 ppm	30.11 ± 0.88	31.61 ± 0.22
Diet 4: TC of tea extract 25 ppm	28.10 ± 0.60	27.35 ± 1.45
Diet 5: TC of mulberry extract 25 ppm	27.84 ± 1.00	28.46 ± 0.79
Diet 6: TC of cassava extract 25 ppm	28.18 ± 1.42	27.18 ± 2.04
Diet 7: TC of tea leave 25 ppm	28.25 ± 0.32	28.14 ± 0.56
Diet 8: TC of mulberry leave 25 ppm	29.10 ± 1.24	30.25 ± 0.26
Diet 9: TC of cassava leave 25 ppm	30.53 ± 0.29	30.31 ± 0.77
Week 4		
Diet 1: control diet	31.29 ± 2.50	28.13 ± 0.57
Diet 2: lutein 25 ppm	34.02 ± 2.89	29.48 ± 2.81
Diet 3: β-carotene 25 ppm	33.88 ± 1.51	31.01 ± 0.78
Diet 4: TC of tea extract 25 ppm	34.59 ± 1.66	32.02 ± 1.13
Diet 5: TC of mulberry extract 25 ppm	35.76 ± 0.81	30.56 ± 0.81
Diet 6: TC of cassava extract 25 ppm	33.52 ± 1.50	29.64 ± 1.11
Diet 7: TC of tea leave 25 ppm	34.42 ± 1.73	33.11 ± 1.38
Diet 8: TC of mulberry leave 25 ppm	33.83 ± 3.86	32.55 ± 1.86
Diet 9: TC of cassava leave 25 ppm	36.24 ± 4.07	32.31 ± 2.23
Week 8		
Diet 1: control diet	39.51 ± 2.10	30.92 ± 1.87
Diet 2: lutein 25 ppm	38.18 ± 0.90	33.27 ± 1.95
Diet 3: β-carotene 25 ppm	36.12 ± 0.44	31.12 ± 1.47
Diet 4: TC of tea extract 25 ppm	37.06 ± 3.57	35.29 ± 0.75
Diet 5: TC of mulberry extract 25 ppm	37.10 ± 4.72	33.05 ± 1.17
Diet 6: TC of cassava extract 25 ppm	35.72 ± 0.94	32.53 ± 1.48
Diet 7: TC of tea leave 25 ppm	37.14 ± 4.08	34.34 ± 0.97
Diet 8: TC of mulberry leave 25 ppm	39.39 ± 2.26	33.77 ± 1.72
Diet 9: TC of cassava leave 25 ppm	38.35 ± 2.28	35.12 ± 0.92

Appendix Table A20 Acid value content in the experimental diets Group B, storage under 4°C during the trial period.

Experimental Diets	Acid value (mg/kg)	
	Without antioxidant	With antioxidant
Week 0		
Diet 1: control diet	27.90 ± 1.77	26.21 ± 2.00
Diet 2: lutein 25 ppm	31.26 ± 0.56	31.18 ± 0.62
Diet 3: β-carotene 25 ppm	30.11 ± 0.88	31.61 ± 0.22
Diet 4: TC of tea extract 25 ppm	28.10 ± 0.60	27.35 ± 1.45
Diet 5: TC of mulberry extract 25 ppm	27.84 ± 1.00	28.46 ± 0.79
Diet 6: TC of cassava extract 25 ppm	28.18 ± 1.42	27.18 ± 2.04
Diet 7: TC of tea leave 25 ppm	28.25 ± 0.32	28.14 ± 0.56
Diet 8: TC of mulberry leave 25 ppm	29.10 ± 1.24	30.25 ± 0.26
Diet 9: TC of cassava leave 25 ppm	30.53 ± 0.29	30.31 ± 0.77
Week 4		
Diet 1: control diet	28.00 ± 0.23	26.35 ± 2.59
Diet 2: lutein 25 ppm	30.72 ± 1.14	26.22 ± 1.73
Diet 3: β-carotene 25 ppm	27.99 ± 3.60	30.20 ± 2.15
Diet 4: TC of tea extract 25 ppm	28.89 ± 1.66	29.03 ± 2.17
Diet 5: TC of mulberry extract 25 ppm	30.24 ± 1.57	29.32 ± 3.03
Diet 6: TC of cassava extract 25 ppm	27.13 ± 1.64	28.90 ± 1.90
Diet 7: TC of tea leave 25 ppm	29.76 ± 0.90	29.59 ± 1.68
Diet 8: TC of mulberry leave 25 ppm	26.44 ± 1.92	29.32 ± 3.35
Diet 9: TC of cassava leave 25 ppm	31.81 ± 0.75	31.51 ± 3.73
Week 8		
Diet 1: control diet	29.59 ± 0.54	27.55 ± 1.10
Diet 2: lutein 25 ppm	32.58 ± 0.33	29.79 ± 0.84
Diet 3: β-carotene 25 ppm	29.91 ± 0.84	31.45 ± 0.24
Diet 4: TC of tea extract 25 ppm	30.02 ± 0.79	30.58 ± 2.07
Diet 5: TC of mulberry extract 25 ppm	31.67 ± 0.12	30.17 ± 1.26
Diet 6: TC of cassava extract 25 ppm	29.02 ± 0.96	31.46 ± 2.07
Diet 7: TC of tea leave 25 ppm	30.85 ± 1.42	32.00 ± 0.32
Diet 8: TC of mulberry leave 25 ppm	32.22 ± 0.64	32.00 ± 1.35
Diet 9: TC of cassava leave 25 ppm	33.07 ± 0.88	32.02 ± 0.78



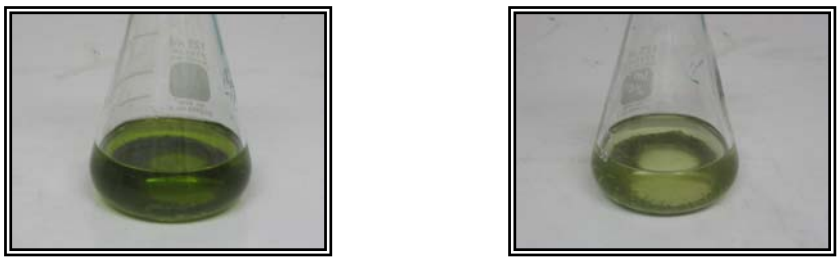
Appendix Figure A1 Separation of fancy carp blood cells in a gradient of percoll.

Appendix B

Materials and Processes for Carotenoids Determination

Extraction

Dried samples of all feedstuffs (tea, mulberry and cassava leave) were extracted with cold acetone, together with added BHT (250 mg/kg) as antioxidant. To grind feedstuff samples with a pestle and mortar until samples no color. In this regard, the extraction would be more efficient if all samples were moistened with a little water and left for a few minutes before the solvent treatment**.**



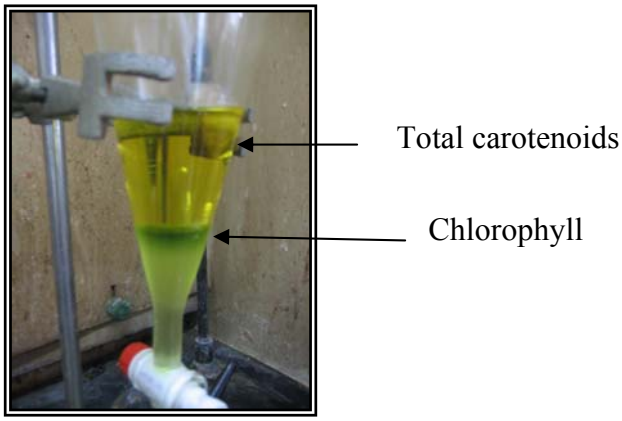
(A) (B)

Appendix Figure B1 Carotenoids extraction; A: Extracted with cold Acetone and B: Extract until no color.

Saponification and Partition

Hydrolysis with alkali (saponification) is used to destroy chlorophyll, destroy neutral fats and oils that can interfere with chromatography, and break down esters of carotenoids. Prepare a concentrated solution of methanolic potassium hydroxide 20%. Add in extraction from all leaves and left in the dark at least four hours. After that, petroleum ether was added at 5 ml, mix and add water in separating funnel. Then, mix with a careful swirling and the two phases were found separated. In this study, only the hyperphase was collected and then determined maximum absorbance wavelength

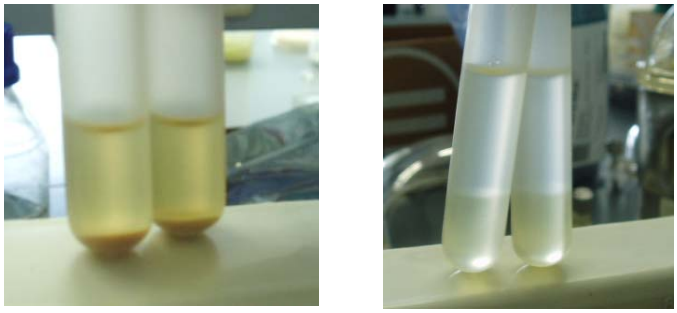
rang over 350 – 600 nm. Recorded merely the maximum absorbance value (λ max) and calculated total carotenoids obey Beer-Lambert's Law.



Appendix Figure B2 Liquid Partition for carotenoids extraction.

Serum carotenoid extraction

Blood was collected from the dorsal vein with 2 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 ul of the serum was removed and vortexed with 1 ml of ethanol for 30 s., then 2 ml of petroleum ether was added, and the mixture was vortexed for 1 min. The petroleum ether was separated by centrifuging 300 x g, 25°C for 10 min.

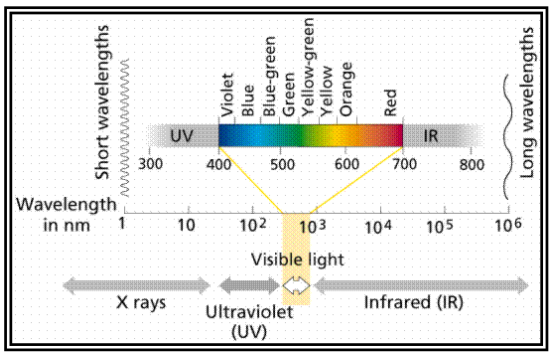


(A) (B)

Appendix Figure B3 Serum carotenoid extraction; A: precipitated protein in serum with ethanol and B: hyperphase of petroleum ether were collected.

UV / Vis Spectrum

At this stage, determine the UV/Vis of the petroleum ether solution. From this can determine the total carotenoid concentration of the total extract, and will get a first indication of the type of carotenoids that may be present. In this study, only the hyperphase of plant leave and serum were collected then determined maximum absorbance wavelength range over 350-600 nm. Recorded merely the maximum absorbance value (λ max) and calculated total carotenoids obey Beer-Lambert's Law.



Appendix Figure B4 Electromagnetic radiation of wavelength.

Calculation of total carotenoids

$$X\ (mg) = (A \times Y \times 1000) / (E_1^{1\%} \times 100)$$

A = Absorbance

Y = volume of total extract (ml)

$E_1^{1\%}$ = Extinction (2500)

Appendix Table B1 Absorption coefficients ($E_1^{1\%}$) of common food carotenoids.

Carotenoid	Solvent	λmax, nm	$E_1^{1\%}$
Antheraxanthin	Ethanol	446	2350
Astaxanthin	Hexane	470	2100
Auroxanthin	Ethanol	400	1850
Bixin	Petroleum ether	456	4200
Canthaxanthin	Petroleum ether	466	2200
Capsanthin	Benzene	483	2072
Capsorubin	Benzene	489	2200
α-Carotene	Petroleum ether	444	2800
	Hexane	445	2710
β-Carotene	Petroleum ether	450	2592
	Ethanol	450	2620
	Chloroform	465	2396
β-Carotene-5,6-epoxide	Hexane	444	2590
β-Carotene-5,6,5′,6′-diepoxide	Hexane	440	2690
δ-Carotene	Petroleum ether	456	3290
γ-Carotene	Petroleum ether	462	3100
	Hexane	462	2760
ζ-Carotene	Hexane	400	2555
Crocetin	Petroleum ether	422	4320
α-Cryptoxanthin/zeinoxanthin	Hexane	445	2636

Appendix Table B1 Continued.

Carotenoid	Solvent	λmax, nm	$E_1^{1\%}$
β-Cryptoxanthin	Petroleum ether Hexane	449, 450	2386, 2460
Echinenone	Petroleum ether	458	2158
Lutein	Ethanol	445	2550
	Diethyl etherDiethyl ether	445, 445	2480, 2600
Lutein-5,6-epoxide	Ethanol Ethanol	441, 441	2400, 2800
Lycopene	Petroleum ether	470	3450
Lycoxanthin	Acetone	474	3080
Mutatochrome	Diethyl ether	428	2260
Neoxanthin	Ethanol	438	2470
	Ethanol	439	2243
Neurosporene	Hexane	440	2918
Phytoene	Petroleum ether	286	1250
Phytofluene	Petroleum ether Hexane	348, 348	1350, 1577
Rubixanthin	Petroleum ether	460	2750
Violaxanthin	Ethanol	440	2550
	Acetone	442	2400
α-Zeacarotene	Petroleum ether	421	2450
	Hexane	421	1850
β-Zeacarotene	Petroleum ether	428	2520
	Hexane	427	1940
Zeaxanthin	Petroleum ether	449	2348
	Ethanol	450	2480
	Ethanol	450	2540
	Acetone	452	2340

Source: Britton (1995).

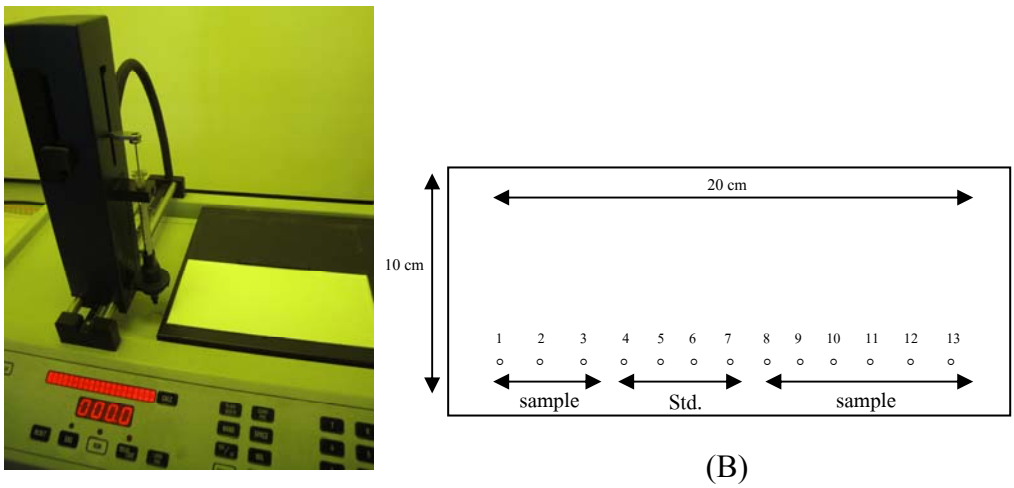
Evaporation

The resulting hyperphase of all leaves and serum from petroleum ether extraction were evaporated under a gentle stream of nitrogen gas.

Appendix Figure B5 Heating block at temperature 40°C with gentle stream of nitrogen gas.

Thin-layer Chromatography (TLC) with densitometric analysis

The resulting hyperphase of all leaves and serum from petroleum ether extraction were evaporated under a gentle stream of nitrogen gas. This was followed by redissolving crude carotenoids in petroleum ether and the solution 5 ul were spotted on pre-coated silica gel 60 (10 x 20 cm) with 0.25 mm thickness (Merck, Germany) using a CAMAG Linomat IV (CAMAG, Switzerland) sample applicator. In this procedure, it would generate narrow spots, which must be sprayed with nitrogen gas having the 99.995 % purity. A constant application rate of 4 ul/s was employed and spaces between two spots were 14 mm. In order to identify the correct value of astaxanthin, lutein and β – carotene, it was a must that TLC was always conducted to compare with reference standard.

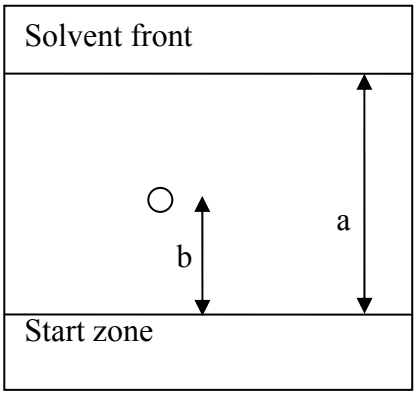


(A) (B)

Appendix Figure B6 Application of the sample; A: semi-automatic sample applicator and B: sample planning; lane 1-3 and 8-13 is the samples, and lane 4-7 is carotenoids standard mixture.

After applying samples on the TLC plate, then placed plate carefully in the developing chamber, containing Petroleum Ether-Diethyl Ether-Acetone (75:15:10; *v/v/v)* as mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2° C). The length of chromatogram run was 70 mm. When this developing procedure was finished, the next step was to conduct the quantitative analyze.

Appendix Figure B7 Developed TLC plate in the developing chamber, containing Petroleum Ether-Diethyl Ether-Acetone (75:15:10; *v/v/v)* as mobile phase.



Appendix Figure B8 R_f: (Related to Front) value**.**

R_f (Retardation Factor or Related to Front) = b/a

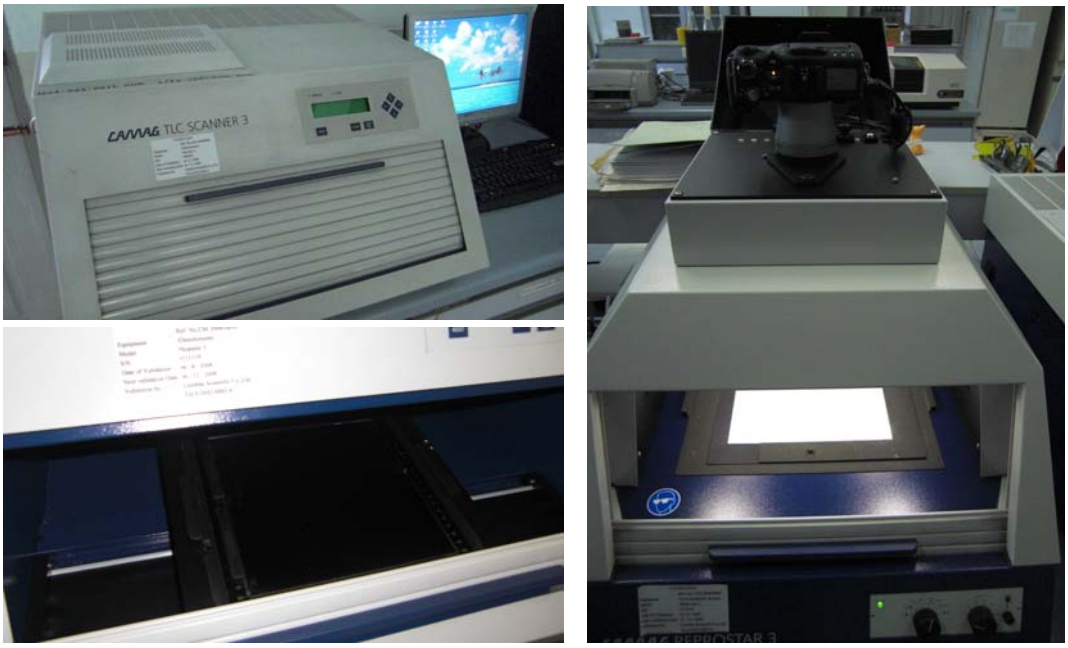
a = migration distance of solvent front (start zone-solvent front distance)

b = migration distance of fraction (start zone- fraction centre distance)

Calculation of quantitative carotenoid

Quantitative analysis was carried out in situ by measuring samples and standard zones on layers with a slit dimension was kept at 5 mm x 0.45 mm and 20 mm/s scanning speed was employed -- scanning densitometer, which used a tungsten

lamp at the wavelength 450 nm. (CAMAG TLC Scanner III, CAMAG, Switzerland). Specifically, the total results found in this study must be evaluated with the following calibration function: multi level calibration with linear regression using external standards.

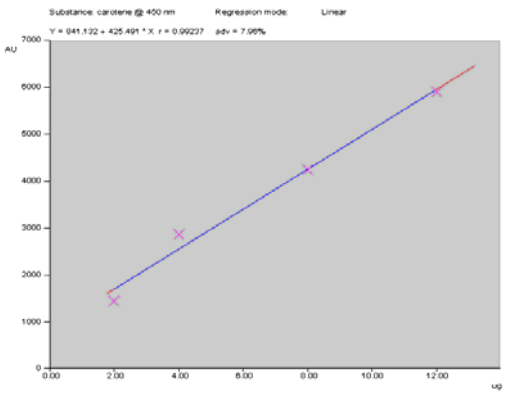


Appendix Figure B9 Fluorodensitometer (TLC scanner) and TLC cabinet with digital camera.

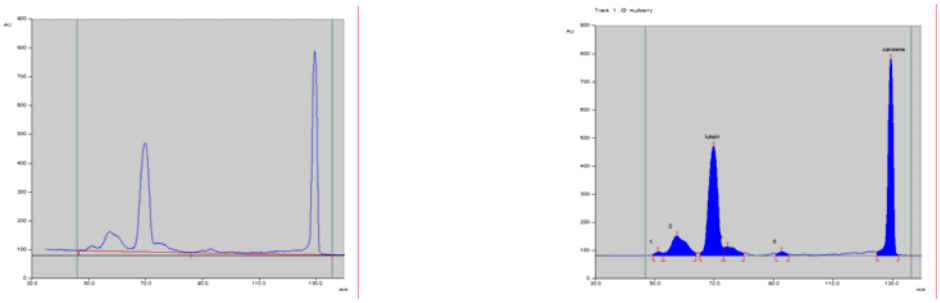
Carotenoids concentration (µg/g)

$$= \frac{\text{Rspl. x CStd. (µg/µl) x V (µl)}}{\text{RStd. x Vap. (µl) x W (g)}}$$

Rspl.: Fluorescence Intensity from peak area in sample
RStd.: Fluorescence Intensity from peak area in carotenoid standard
CStd.: carotenoid standard concentration (µg/µl)
V: final volume of sample (µl)
Vap.: volume of sample applied on TLC plate (µl)
W: weight of sample (g)



Appendix Figure B10 Evaluation supports the following calibration function : multi level calibration with linear regression using external standards



(A) (B)

Appendix Figure B11 The result TLC-densitometric analysis; A: chromatogram from TLC scanner and B: Integrated chromatogram.

PUBLICATONS

1. Validated TLC-Densitometric Analysis for Determination of Carotenoids in Fancy Carp (*Cyprinus carpio*) Serum and the Application for Pharmacokinetic Parameters Assessment
   : Published in ***The Songklanakarin Journal of Science and Technology 30 (6), 693-700, Nov.-Dec. 2008***.

2. The impact of dietary carotenoids (astaxanthin, lutein and β-carotene) on skin pigmentation in fancy carp (*Cyprinus carpio*)
   : Accepted article with revision in ***Aquaculture Nutrition***.

Preparing manuscript for submit in the international journals:

1. Bioavailability of Carotenoids in Fancy Carp (*Cyprinus carpio*): Astaxanthin, Lutein and β-Carotene
2. Comparative Pharmacokinetics of the Feeding of Carotenoids from Natural Sources (Tea, Mulberry and Cassava Leaf) in Fancy carp (*Cyprinus carpio*) diets
3. Potential Use of Natural Carotenoids Source on Growth Performance, Skin Pigmentation and Immune Response in Fancy Carp (*Cyprinus carpio*)

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